13TH WORLD RABBIT CONGRESS

2 - 4 OCTOBER 2024

TARRAGONA / SPAIN

PROCEEDINGS

BOOK OF ABSTRACTS

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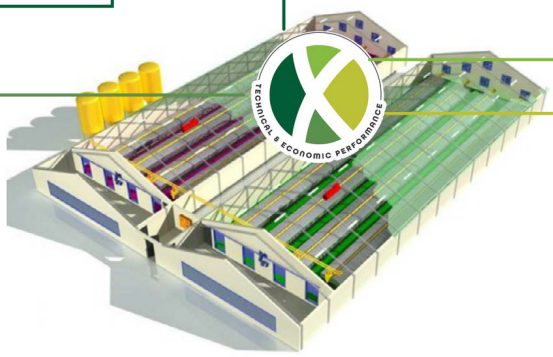

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BIOLOGY & PHYSIOLOGY

CORE GUT MICROBIOTA IN RABBIT: OPPORTUNITIES TO STRENGTHEN THE INTESTINAL BARRIER

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ABSTRACT

The symbiotic relationship between the intestinal microbiota and its host is crucial to the development and functioning of both partners. The microbiota plays a key role in the development and physiology of its host (nutrition, growth, health, and cognition). In turn, the host shapes the microbiota, according to factors that are intrinsic or dependent on its environment. However, the definition of an optimal microbiota that maximises ecosystem services (host benefits) has yet not be established. The symbiotic relationship between the microbiota and its host is based on a complex molecular dialogue at the level of the intestinal epithelium and the underlying mucosal immune system. These interactions condition the establishment of an intestinal barrier, limiting colonisation by microbial pathogens and thereby guaranteeing health. In this review, we propose a 'core' rabbit microbiota definition through a re-analysis of available open-source data. Based on the association between the abundance of bacterial taxa and host traits, we attempt to identify microbiota key species that would likely be involved in growth performance and health. Then, we describe the components of the intestinal barrier and the host-microbiota interaction mechanisms. Finally, we propose early in life nutritional levers to strengthen this intestinal barrier and thereby enhance the health of young rabbits before weaning.

Key words: intestinal epithelium, immune system, feed transition, meta-analysis.

INTRODUCTION

The microbiota is composed of commensal and symbiotic microorganisms that occupy all the different accessible niches in the host's body (Kundu et al., 2017). In rabbits, as in other mammals, next-generation sequencing has facilitated our knowledge of the microbiota taxonomic composition and diversity. According to the results obtained with the latest technologies, the rabbit gastrointestinal microbiota represents the richest and most diverse microbial community inhabiting the rabbit's body (Kundu et al., 2017; Hu et al., 2021). The microbial community varies widely from the stomach to the colon (Cotozzolo et al., 2020; Hu et al., 2021), with the cecum and large intestine showing the highest richness and diversity in bacterial species, while the highest interindividual variability is found in the upper digestive tract. High similarity in alpha and beta diversity is observed between the bacterial communities inhabiting adult rabbit cecum, colon, appendix and hard feces. These results suggest that the lower digestive tract microbiota structures are close (Velasco-Galilea et al., 2018; Hu et al., 2021; Curone et al., 2022). With regard to the taxonomic composition, the most notable differences between feces and cecal content are observed at the genus level, suggesting that hard feces may serve as a relevant proxy for studying the bacterial community composition of the main fermenter, the cecum (Velasco-Galilea et al., 2018).

At the interface between feed and the intestinal epithelium, this microbial community plays a role in the development and health of its host. The intestinal microbiota is involved in the metabolization of dietary nutrients that escape host digestion and substrates derived from host secretions. The gut microbiota produces essential compounds for rabbit nutrition, such

as vitamins (Carabaño et al., 2010), volatile fatty acids (García et al., 2002) and secondary bile acids (Kasbo et al., 2002). In addition, the microbiota plays a role in direct and host-mediated defence against pathogens (Sonnenburg & Bäckhed, 2016). The action of intestinal bacteria on the maturation of the mucosal and systemic immune system has been extensively documented (Pott & Hornef, 2012). All of the microbiota ecosystem services related to host development and health are based on the establishment of a specific host-microbiota dialogue and allows the intestinal barrier functions to be maintained.

By providing favorable life conditions and food, the host in turn shapes its gut microbiota. Ontogenic factors (Beaumont et al., 2022) and the physiological state (Abecia et al., 2007; Savietto et al., 2020) drive the community establishment and its further evolution during adulthood. In addition, host-specific genetic characteristics filter out microbial metacommunities that are able to live in the hindgut (Velasco-Galilea et al., 2022). With regards to extrinsic factors, diet is one of the major levers in the structuring of the gut microbiota (Beaumont et al., 2022). In rabbits, numerous studies have shown that fibre content (Zhu et al., 2015; Li et al., 2023b), and the nature of the fibre (Gómez-Conde et al., 2009; Yang et al., 2020; Liu et al., 2022; Ma et al., 2022; Paës et al., 2022) have an impact on the composition and function of the gut microbiota. Finally, host environment (Velasco-Galilea et al., 2020; Ye et al., 2023) and the presence of littermates (Abecia et al., 2007; Fang et al., 2019) and cage comates (Velasco-Galilea et al., 2022) contribute to further shaping the gut microbiota.

Altogether, the extrinsic and intrinsic capabilities of the host to shape its own intestinal microbiota suggest a high degree of hosting plasticity. This plasticity has thus been identified by research groups as an opportunity to drive the establishment of a particular microbiota that maximizes the ecosystem services rendered to its host mainly in terms of digestive efficiency and health, that in turn impact on growth performances. For instance, Fang et al. (2019) demonstrated that 16% of the variation in weaning weight was attributable to the gut microbiota. Furthermore, moderate correlations have been observed between microbiota composition and growth traits (Velasco-Galilea et al., 2022). The optimal microbial community would be capable of optimising these ecosystem services. However, the specific characteristics of an optimal gut microbiota (diversity, core taxonomic composition and/or function) for an individual or group of individuals remain to be defined.

This review firstly aims to provide insight into the definition of the core cecal microbiota diversity and composition in post weaning rabbits and to identify key taxa that may be associated with improved rabbit health or growth. Given that the symbiotic relationship between the microbiota and its host is based on a fine molecular dialogue at the level of the intestinal epithelium and the underlying mucosal immune system, we secondly review the current understanding of host-microbiota cross-talk in order to promote health in rabbits, with a focus on the intestinal barrier function. Finally, we assess the nutritional strategies aimed at enhancing the host intestinal barrier-microbiota interactions, thereby promoting health.

DEFINITION OF THE CORE RABBIT GUT MICROBIOTA

The first studies investigating the composition and function of the rabbit gut microbiota were based on culture techniques, which were therefore limited to describing the cultivable fraction of the microbiota. This was constrained by the culture medium and conditions available at the time (for a review, see Combes et al., 2013). The use of molecular microbiology techniques has allowed considerable progress in our knowledge of the species inhabiting the rabbit digestive tract, in particular by highlighting taxonomic specificities not observed in other species (undescribed species, dominance of the Firmicutes phylum, Combes et al., 2013). Results presented in the present review focus on the numerous results obtained in the last decade, using sequencing technologies widespread among research groups interested in rabbits. The prevalent techniques combine amplification of a portion of the hypervariable

region of the 16S small subunit ribosomal RNA gene with short-read sequencing. These approaches have the advantage of being relatively simple, cheap and fast. They are based on a number of databases that attempt to be as rich and diverse as possible, and bioinformatics and statistical analysis pipelines are well established. However, these technologies are subject to biases and limitations. These biases include extraction, PCR amplification, sequencing error, bioinformatics analysis, and database for taxonomic affiliation biases. Moreover, 16S RNA gene amplicon short-read sequencing technologies produce compositional data that do not take into account the variable number of copies in the bacterial genome (from 1 to 21 copies, https://rrndb.umms.med.umich.edu/) and exhibit low taxonomic resolution (genus level), which is limited by the short-read lengths (Poretsky et al., 2014). Finally, the last limitation relates to their inability to distinguish between the active and the dead or dormant microbial fractions. Recently, shotgun metagenomics technics (i.e. whole metagenome sequencing) were used in two studies to explore rabbit microbiota (Casto-Rebollo et al., 2023; Zhao et al., 2024). Compared to 16S sequencing, these techniques provide access to bacterial genes, enabling inference of the functional characteristics of the microbiota. However, they only overcome the biases associated with PCR amplification and 16S copy number.

To date, the production of 16S sequence data, combined with its release as part of open science, allows data from different research teams to be re-analysed together to provide a comprehensive description of the rabbit gut microbiota. A literature search over the last 10 years identified 93 studies dealing with the variations in composition of the rabbit gut microbiota in relation to changes in rearing conditions (housing and diet), genetics, growth performance or health status. Of these studies, 70 used 16S RNA gene amplicon sequencing technologies and two used a shotgun metagenomic technology to characterize the rabbit gut microbiota. From these articles, we collected publicly available raw sequencing data (either from NCBI and CNBG repositories or from supplementary data in papers). Overall, concatenation with the available metadata enabled us to collect data from 15 studies, 11 of which included cecal microbiota analyses (Table 1, see GitHub link for detailed procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/).

To define a core microbiota based on data from the literature, we limited our analysis to the 11 studies where cecal microbiota data were available (Table 1). To avoid the variability associated with the establishment of the microbiota and onset of solid feed intake, we only included rabbits older than 49 days (Combes et al., 2011). According to our pipeline using FROGS tools (Escudié et al., 2018) and the Silva 138 taxonomic database (Quast et al., 2013), the average value of the cecal microbiota Shannon index of a grown rabbit (> 48 days of age) was of 5 (Figure 1). Shannon index median values ranged from 4.2 to 5.5. A change in such an index indicates the establishment of a dominance or an increase/decrease of the number of species. These changes may be related to microbiota establishment in the young or unbalanced microbiota in post-weaning rabbit. Indeed, using the same analysis pipeline, the Shannon values were around 3 in 18-day old pups (Paës et al., 2020b, 2022; Beaumont et al., 2022). With the exception of outlier samples in some studies, the Shannon index showed a good overlap between studies suggesting that the diversity was evaluated similarly between studies despite differences in experimental conditions or sample process preparation.

As previously widely described in the literature, the most abundant phyla in the rabbit cecal microbiota are Firmicutes (76.4%), Bacteroidota (16.5%), Verrucomicrobiota (3%), Proteobacteria (1%) and Actinobacteriota (0.9%) (Figure 2).The overwhelming dominance of the Firmicutes phylum is a notable feature of rabbit gut microbiota compared to other farm mammals (cattle & sheep: Szeligowska et al., 2021; pigs: Mach et al., 2015 ; horses: Grimm et al., 2019) or human gut microbiota (Arumugam et al., 2011), but is also strikingly observed in chickens (Allaoua et al., 2022). The mechanisms behind the preferential selection of microbes from the Firmicutes phylum in the rabbit cecum remain to be elucidated, although it

is likely to be related to the specificity of its digestive strategy (hindgut fermentor, herbivorous and caecotrophic species) (Ley et al., 2008). In rabbits, the relative abundance of Firmicutes has been shown to increase with age at the expense of Bacteroidota. Read et al. (2019) have suggested that the ratio of Firmicutes to Bacteroidota would be a suitable index of microbiota maturity, while Chen et al. (2019) evidenced that this ratio steadily decreased in severe intestinal disorder compared to a healthy rabbit. In the re-analysis of data from healthy rabbits, this ratio shows a high variability between studies (from 1.7 to 17.9), according to the metadata. Although there is a confounding effect between age and study, Firmicutes to Bacteroidota ratio may reflect animal age, but this high variability between studies may indicate a low robustness of the absolute value (Figure 3).

Table 1: Studies included in our re-analysis for cecal microbiota composition evaluation

Study	Data accession	16S RNA gene hypervariable regions	Age (days)	Experimental conditions	Number of samples
Read et al. 2019	PRJNA315608	V3-V4	49	Preweaning diet modulation	30
Wang et al. 2019	PRJNA512067	V3-V4	58, 70, 82	Water drinking temperature	82
Paës et al. 2020	PRJNA589727	V3-V4	57	Early access to diet in the nest	40
Cotozolo et al. 2021	PRJNA1069001	V3-V4	110	Digestive segment	14
Dabou et al. 2021	PRJNA645756	V3-V4	77	Insect fats dietary supplementation	24
Feng et al. 2022	PEERJ_13068	V3-V4	76	Environmental enrichment	12
Liu et al. 2022	PRJNA781070	V3-V4	87	Dietary fibre modulation	12
Mora et al. 2022	PRJNA524130	V4-V5	66	Microbiota and growth causal relationship modelling	407
Paës et al. 2022	PRJNA615661	V3-V4	58	Early access to diet in the nest	29
Li et al. 2023	CPN0003860	V3-V4	73	Dietary non-fibrous carbohydrate to neutral detergent fibre ratio	30

Lachnospiraceae (19.6%), Oscillospiraceae (17.9%), Ruminococcaceae (13.3%), Muribaculaceae (7.2%), and Christensenellaceae (6.7 %) were the 5 dominant families found in almost all the studies (Figure 2). Interestingly, among the 288 genera observed, only 36 genera were found in all 11 studies. Oscillospiraceae NK4A214 (13.8%), Ruminococcus (10.4%), Christensenellaceae R-7 (9.2%), Lachnospiraceae NK4A136 (7.6%), and Ruminococcaceae V9D2013 (5%) groups were the 5 most abundant genera (Figure 4). Using whole metagenomic sequencing based techniques, microbiota exploration at the species level has recently been reported (Casto-Rebollo et al., 2023; Zhao et al., 2024). Several species have been reported as the characteristic species of 60 and 90 days old rabbit (Zhao et al., 2024) or associated with selection for resilience (Casto-Rebollo et al., 2023).

In terms of beta diversity evaluated at the genus level on the 771 samples across the 11 studies, cecal community structure seems to cluster according to study and even to research group, but interestingly without strong opposition or outlier samples (Figure 5). Most of the variability of the bacterial community at the genus level could be attributed to the relative

abundance of *Akkermensia*, Christensenellaceae R-7, and Ruminococcaceae NK4A214 groups. With the exception of the study conducted by Mora et al. (2022), all studies employed the 16SrRNA hypervariable V3V4 region for sequencing (Table 1). The observed variability, including the absence of the *Akkermensia* genus in our own studies (Read et al., 2019; Paës et al., 2020b, 2022), can be explained by the differences in the primer V3-V4 sequence with different degenerated nucleotides at different positions (see GitHub link for primer list https://lcauquil.pages.mia.inra.fr/review_wrc2024/).

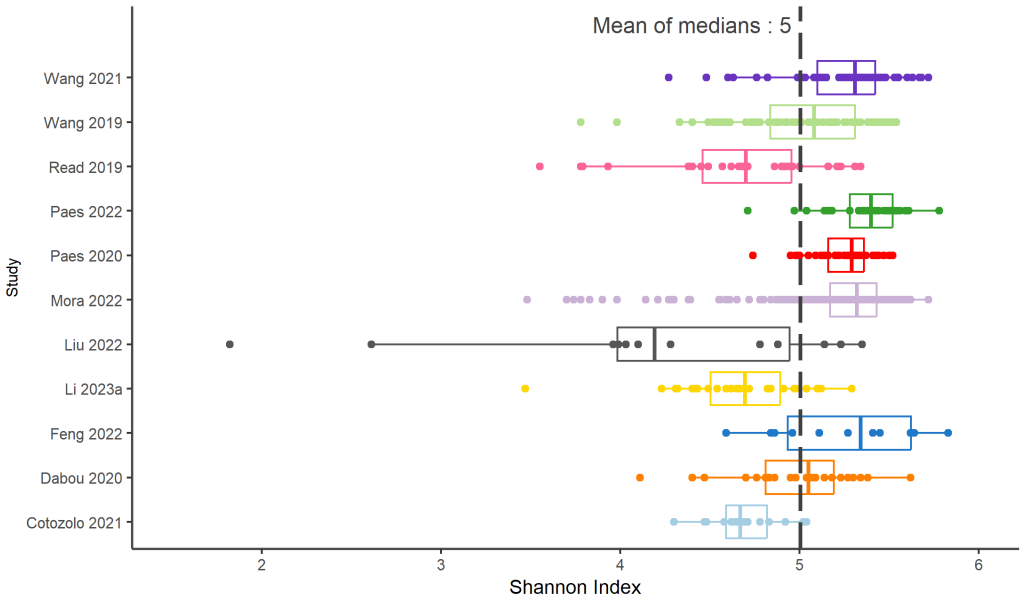

Figure 1: Shannon index calculated in rabbit cecal microbiota from 11 publicly available studies providing 16S RNA gene amplicon data (see github link for detailed procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/)

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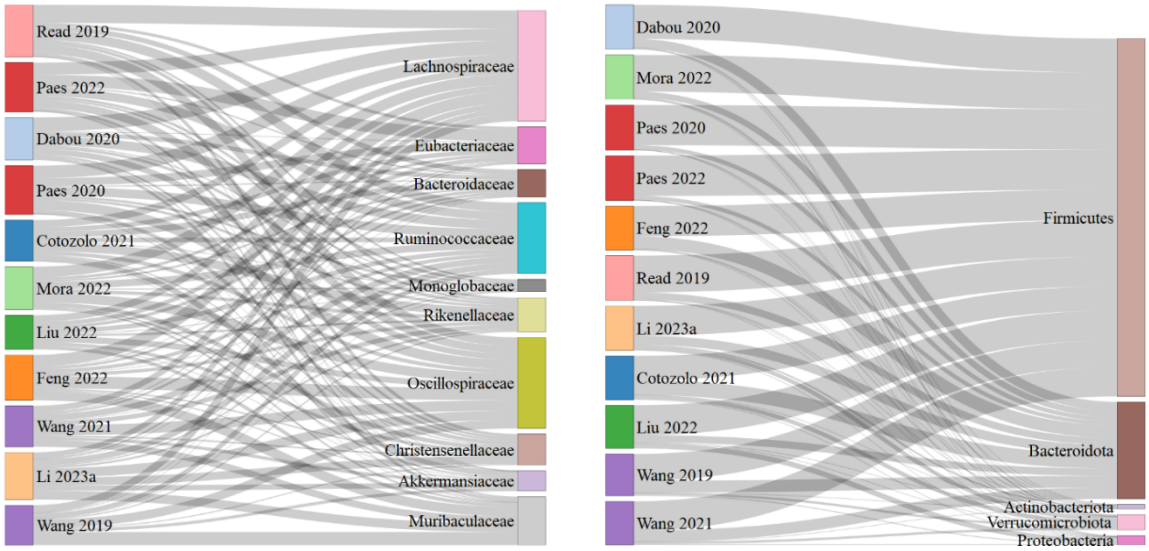

Figure 2: Relative abundances of (A) phyla and (B) top 10 families in rabbit cecal content using 11 freely available studies providing 16S RNA gene amplicon data (see github link for detailed calculation procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/).

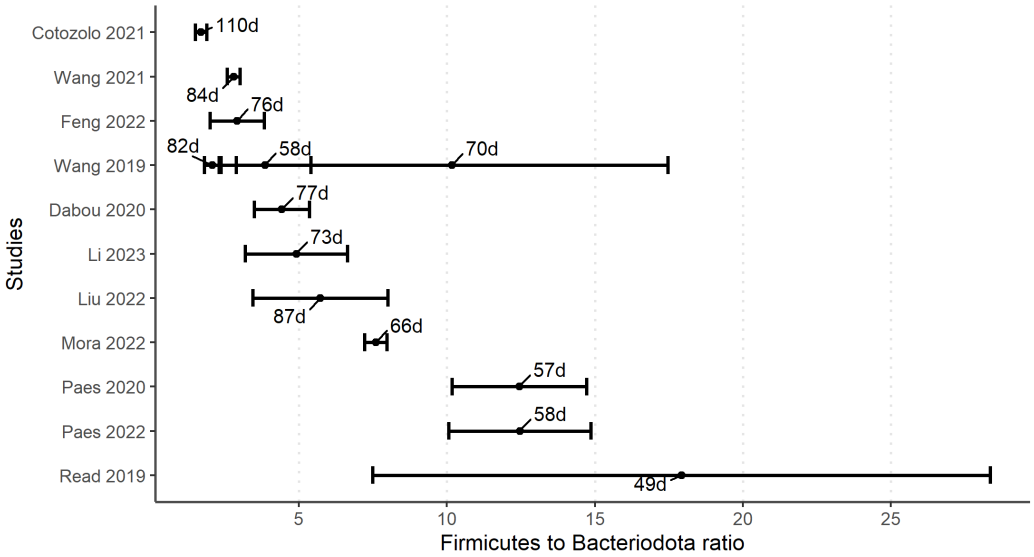

Figure 3: Firmicutes to Bacteroidota ratio calculated in rabbit cecal microbiota from 11 publicly available studies providing 16S RNA gene amplicon data (see GitHub link for detailed calculation procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/)

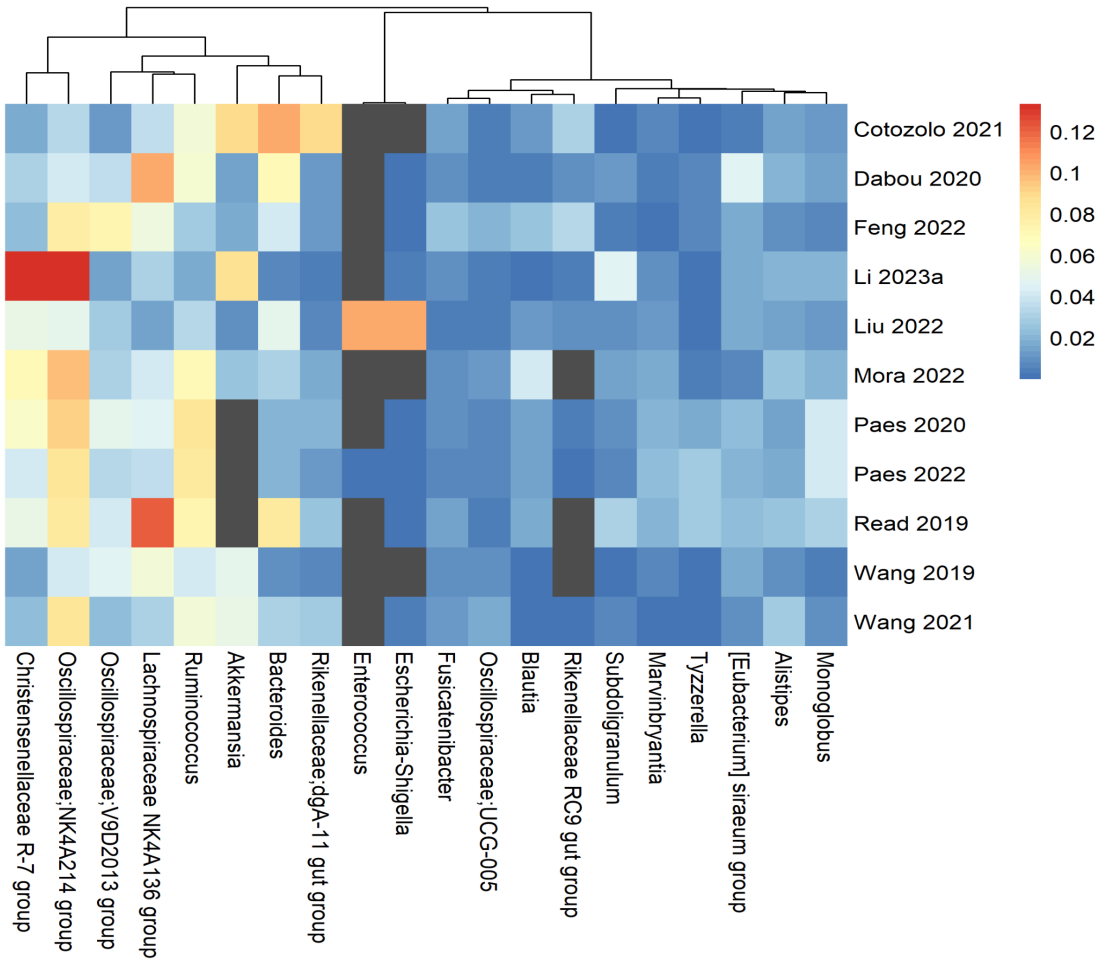

Figure 4: Relative abundances of top twenty genera in rabbit cecal content using 16S RNA gene amplicon data from 11 publicly available studies (see GitHub link for detailed calculation procedure https://lcauquil.pages.mia.inra.fr/review_wrc2024/). The colors represent the relative abundance from low (blue) to high values (red). Grey color indicates the absence of the genus.

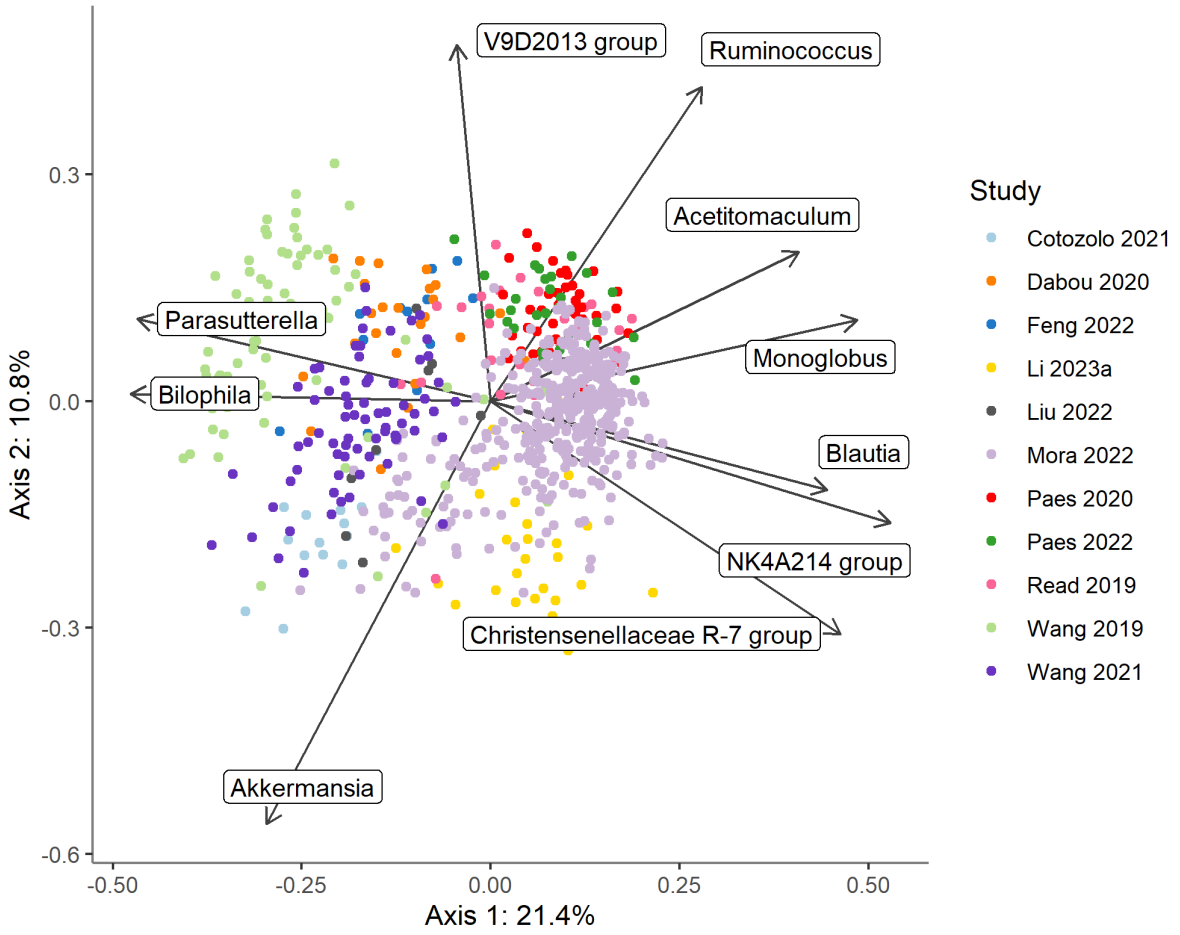

Figure 5: PCoA analysis of cecal bacterial communities at the genus taxonomic level. Dots are colored according to the the study from which 16S RNA gene amplicon data were extracted. (see GitHub link for detailed procedure, https://lcauquil.pages.mia.inra.fr/review_wrc2024/)

Given the average composition of the cecal microbiota, we wanted to identify the taxa that were associated with improved health or growth in rabbits. Out of the 93 recorded studies, our analysis identifies 29 articles, describing positive or negative associations with microbiota composition. These 29 studies represent a wide range of experimental conditions (age, dietary challenge, drinking water study, use of prebiotics or antibiotics, modification of environmental conditions, healthy *vs.* epizootic rabbit enteropathy (ERE) rabbits). Interestingly, some taxa have a more consistent link to a negative or positive effect in relation to the phenotype measured (Table 2). For example, increased relative abundance of *Bacteroides* in rabbits appears to be detrimental to growth and health (Baüerl et al., 2014; Chen et al., 2019; Velasco-Galilea et al., 2021; Liu et al., 2022; Puón-Peláez et al., 2022). *Bacteroides* is a dominant genus in suckling rabbits, which abundance sharply decreases with the introduction of solid feed and may therefore not be adapted to plant carbohydrate substrates. Health and growth performance are also likely to be positively associated with *Ruminococcus* relative abundance (Baüerl et al., 2014; Ye et al., 2022; Li et al., 2023a; Wu et al., 2023). Conversely, for some taxa the phenotype association is not consistent across studies (Table 3). For instance, *Rikenella* relative abundance is associated with positive effects in three studies (Luo et al., 2020; Wang et al., 2021; Du et al., 2023), but is also associated with ERE compared to healthy rabbits (Baüerl et al., 2014). Regarding cecal

microbiota taxonomic composition, it seems difficult to draw a conclusion on possible key taxa improving health or growth in rabbits. Several explanations can be found: **(i)** First, rabbit bacterial strains inhabiting the cecum are far from being well defined. Indeed, re-analysis of our 11 studies revealed that, at the genus level, 20 to 49 % of sequences were affiliated to "unknown genus". Furthermore, some members (OTU, operational taxonomic unit; or ASV, amplicon sequence variant) of a same genus could show either positive or negative associations with a given phenotype. For example, Velasco-Galilea et al., (2021) reported that *Ruminococcus*, *Butyricicoccus*, and *Bacteroides* genera displayed this inconsistency, suggesting functional and/or physiological taxonomic heterogeneity. Altogether, improving our knowledge of the microbiota at the strain level is essential to discover key gut bacterial species involved in rabbit health and/or growth. **(ii)** Secondly, in many studies the sample size may be too small to detect a significant difference between treatments regarding the size effect and taxa abundance variability (Table 2). Furthermore, the sensitivity of the sequencing technique must also be considered. Paës et al. (2020) demonstrated that the relative quantification of OTUs with low abundance (under 0.5 %) is poorly reproducible. **(iii)** Finally, the beneficial or detrimental role of bacteria may depend on life experience of host and environmental factors. Symbiosis between the microbiota and its host encompasses different forms of relationships (Belkaid & Harrison, 2017). Indeed, the same microorganism can evolve as a mutualist, commensal or parasite, depending on the genetic background, nutritional status or co-infection of its host.

While it appears difficult to identify key species or microbial communities that are beneficial to health or growth phenotype, perturbations in microbiota composition or diversity are often described in association with disease. These changes are commonly referred to as dysbiosis (Sekirov & Finlay, 2009). In rabbits, several studies have shown an association between digestive diseases of unknown aetiology and microbiota dysbiosis (Baüerl et al., 2014; Jin et al., 2018; Chen et al., 2019). In the case of ERE, the microbial diversity is significantly reduced, resulting in a decrease in the relative abundance of *Alistipes* and *Ruminococcus* genera, while that of *Bacteroides*, *Akkermansia*, *Rikenella* and *Clostridium* increased (Baüerl et al., 2014; Jin et al., 2018), as well as that of *Escherichia* and *Lysinibacillus* (Jin et al., 2018). Overall, disruption of gut microbiota leads to the proliferation of opportunistic or pathogenic bacteria occupying commensal bacterial niches with access to previously unavailable nutrients and creating an environment that maximizes their pathogenicity. How specific microbes or microbial communities help to prevent the onset of dysbiosis is far from being fully understood. However, it is clear that the microbiota plays a key role in gut barrier function through two main mechanisms: limiting colonization by opportunistic bacteria and strengthening the epithelial barrier and the gut immune system. The following section ("Components of the gut barrier") defines the gut barrier and then describes what is known about the three components of rabbit gut barrier: microbiota role, intestinal epithelium and mucosal immunity. Then, we review the current understanding of host-microbiota cross-talk in order to promote gut barrier function and thus digestive health in rabbits (chapter "Microbial influence on host gut barrier").

Table 2: Genus level taxa of the gut microbiota associated with growth or health in rabbits.

Taxa[1]	**Associated effects (+: positive in red; - negative in blue), Author, Aim of the study; (segment if not cecum) (number of rabbits involved)**
Akkermansia	(+) Huang 2021, Clostridium butyricum supplementation & health (n=9) (-) Li et al 2023 Ammonia exposure (n=6) (-) Li et al 2023, NFC/NDF growth & health (n=5) (-) Puón-Peláez et al 2022, ERE vs healthy rabbit (n = 12) (-) Chen et al 2019, Deficient fiber diet & health (n = 43) (-) Baüerl et al 2014, ERE (n=30)
Alistipes	(+) Chen et al 2021, Bacitracin supplementation & health (n=8) (-) Li et al 2023, Ammonia exposure (n=6) (-) Wang et al 2021, feeding rhythm & health (n=72); (-) Drouilhet et al 2016, Genetic selection & growth (n=18); (-) Baüerl et al 2014, ERE (n=30)
Bacteroides	(-) Liu 2022, Dietary Fiber & growth (n=6); (-) Velasco-Galilea et al 2021, growth and feeding regime (n=201) (-) Puón-Peláez et al 2022, ERE vs healthy rabbit (n = 12); (-) Chen et al 2019, deficient fiber diet & health (n = 43) (-) Baüerl et al 2014, ERE (n=30)
Blautia	(+) Li et al 2023, Temperature and humidity index & growth (n=6) (-) Fang et al 2019, Weaning weight (feces) (n= 135)
Butyricicoccus	(-) Velasco-Galilea et al 2021, Growth and feeding regime (n=201) (-) Fang et al 2019, Weaning weight (feces) (n= 135)
Christensenellaceae_R-7	(-) Li et al 2023, Ammonia exposure (n=6) (-) Fang et al 2020, ADG (feces), (n=180)
Clostridium	(+) North et al 2019, Quercetin supplementation and sex & growth (n = 12) (-) Puón-Peláez et al 2022, ERE vs healthy rabbit (n = 12)
Clostridium XlVb	(-) Baüerl et al 2014, ERE (n=30) (-) Chen et al 2019, deficient fiber diet & health (n = 43)
Desulfovibrio	(-) Mora et al 2022, Growth in restricted or ad libitum feeding (n=206); (-) Wang et al 2021, feeding rhythm & health (n=72)
Dorea	(+) Ma et al 2022 Oat β-glucan & growth (n=6); (+) Chen et al 2021, Bacitracin supplementation & health (n=8)
Erysipelatoclostridium	(+) Feng et al 2022, Environmental enrichment & behaviour (n=6) (-) Puón-Peláez et al 2022 ERE vs healthy rabbit (n = 12)
Escherichia-Shigella	(+) Ma et al 2022, Oat β-glucan & growth (n=6);

Taxa[1]	**Associated effects (+: positive in red; - negative in blue), Author, Aim of the study; (segment if not cecum) (number of rabbits involved)**
	(+) Ye et al 2022 Clostridium butyricum supplementation & growth (n=6)
Lachnospiraceae_NK4A136	(+) Du et al 2023 Copper supplementation & health (n=4) (+) Li et al 2023 Ammonia exposure (n=6); (+) Li et al 2023 NFC/NDF growth Health (n=5) (-) Li et al 2023 Temperature and humidity index & growth (n=6)
Papillibacter	(+) Chen et al 2022, Chlorogenic acid & growth & health (n= 4) (+) Yasob et al 2021, Heat stress and Moringa oleifera leaf supplementation & health (n=21)
Rikenella	(+) Du et al 2023, Copper supplementation & health (n=4) (+) Wang et al 2021, Feeding rhythm & health (n=72) (+) Luo et al 2021, Bacillus subtilis HH2 supplementation induced colitis model & health (colon) (n=16) (-) Baüerl et al 2014 ERE (n=30)
Ruminococcaceae_NK4A214	(+) Li et al 2023, NFC/NDF growth Health (n=5); (+) Liu et al 2022, Dietary Fiber & growth (n=6) (-) Ye et al 2022, Clostridium butyricum supplementation & growth, (n=6)
Ruminococcaceae_UCG-005	(+) Chen et al 2022, Chlorogenic acid & growth & health (n= 4); (+) Ye et al 2022, Clostridium butyricum supplementation & growth (n=6);
Ruminococcaceae_UCG-013	(+) Li et al 2023, NFC/NDF growth Health (n=5); (+) Kong et al 2022, Enzymolytic soybean meal inclusion & health (n=4); (-) Ye et al 2022, Clostridium butyricum supplementation & growth (n=6)
Ruminococcaceae_V9D2013	(+) Liu et al 2022, Dietary Fiber & growth (n=6) (+) Wang et al 2021, feeding rhythm & health (n=72)
Ruminococcus	(+) Wu et al 2023, Carvacrol supplementation & health (n=4); (+) Li et al 2023 Ammonia exposure (n=6) (+) Ye et al 2022, Clostridium butyricum supplementatin & growth (n=6) (+) Baüerl et al 2014, ERE (n=30)
Subdoligranulum	(+) Li et al 2023 Ammonia exposure (n=6) (+) Li et al 2023 NFC/NDF growth Health (n=5)
Synergistes	(-) Wang et al 2021, feeding rhythm & health (n=72) (-) Puón-Peláez et al 2022 ERE vs healthy rabbit (n = 12)

[1]Only genus level taxa observed to be differentially abundant according to treatment in at least two studies are listed.

COMPONENTS OF THE GUT BARRIER

According to Viggiano et al (2015), the gut barrier is a functional unit, organized as a multi-layer system, made up of two main components: a physical barrier that prevents bacterial translocation, and a functional immune barrier that is able to discriminate between pathogens and commensal microorganisms. From the outer layer to the inner layer, the intestinal barrier is composed of the gut microbiota, the mucus layer, the epithelial cells and the innate and adaptive immune cells forming the gut-associated lymphoid tissue.

Gut microbiota barrier

The commensal gut microbiota acts as a barrier to colonization by pathogenic and opportunistic microbes through several mechanisms (McKenney & Kendall, 2016). **(i)** As a result of the host selection pressure, commensal microbes adapted to the intestinal environment conditions, are ecological gatekeepers in healthy guts. Indeed, commensal microbes compete with pathogens for the nutrients and adhesion sites. For instance, members of the Muribaculaceae, Lachnospiraceae, Rikenellaceae, and Bacteroidaceae families, dominant in the rabbits gut (Figure 2), are able to reduce *Clostridioides difficile* colonization in mice based on competition for mucosal sugars (Pereira et al., 2020). **(ii)** A second mechanism relies on the antimicrobial properties of metabolites produced by commensal bacteria, such as short-chain fatty acids (SCFA) or secondary bile acids, which contribute to colonization resistance. For instance, in rabbits, SCFA limit the effects of *Shigella* infection by reducing its abundance (Rabbani et al., 1999). **(iii)** Commensal bacteria also produce bacteriocins, which are peptides that inhibit the growth or kill other bacteria. In rabbits, fecal *Enterococcus* isolates have been shown to produce enterocins (entP, entB and entA), which inhibit the growth of Gram-positive strains (Simonová & Lauková, 2007; Lengliz et al., 2021). **(iv)** Cell-to-cell signalling, such as quorum sensing systems, can have a profound impact on the microbial community structure. It has been shown that molecules produced by the gut microbiota both stabilize the microbiota and suppress bacterial virulence (McKenney & Kendall, 2016). In rabbits, the impact of quorum sensing systems has received little attention, despite the fact that their gut microbiota produce metabolites such as indole, which play a key role in this chemical communication between bacteria (Kim & Park, 2015). However, there is some evidence to suggest that quorum sensing molecules are involved in bacterial pathogenesis in rabbits (*Clostridium perfringens* type C strain (Vidal et al., 2012); rabbit enteropathogenic *Escherichia coli* (Zhu et al., 2007)).

Gut epithelial barrier

The intestinal epithelium is the first layer of cells exposed to the gut microbiota and its products (Figure 6). Indeed, this monolayer of cells is located at the surface of the mucosa and forms a physical and immunological barrier between the intestinal lumen and the organism (Peterson et Artis, 2014). In rabbits, stem cells located at the bottom of crypts renew epithelial cells within a few days (Grant et Specian, 2001), which is a critical mechanism for maintaining the integrity of the epithelial barrier that is exposed to harmful microbial products. The formation of tight junctions at the apical side of epithelial cells is another key mechanism preventing the entry of microbial compounds into the organism (Figure 6). During their migration to the top of the crypt, epithelial precursors differentiate into the absorptive or the secretory lineage.

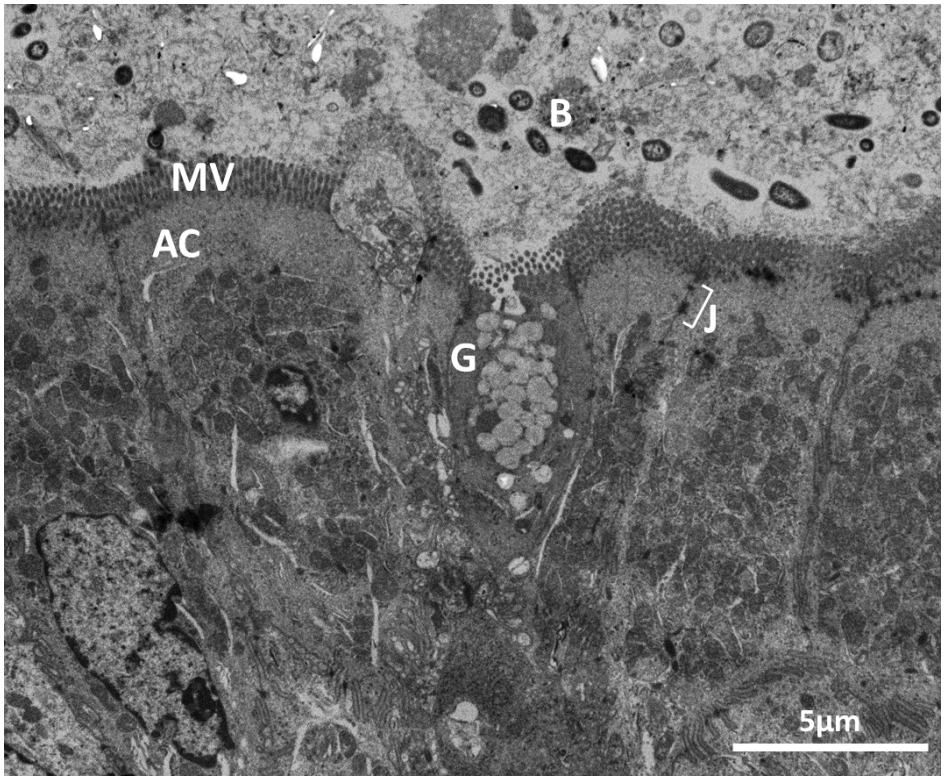

Figure 6: Transmission electron microscopy observation of the rabbit cecal epithelium. AC: absorptive epithelial cell, B: bacteria in the luminal content, G: goblet cell filled with mucin granules, J: cell junctions, MV: microvilli.

The majority of epithelial cells belong to the absorptive lineage and are characterized by the presence of densely packed microvilli decorated with transmembrane mucins (e.g. MUC1, MUC13) that form the glycocalyx, which is both a protective layer for the organism and an attachment site for bacteria (Figure 6) (Pelaseyed & Hansson, 2020). A rare subset of absorptive cells called microfold (M) cells is also present in the rabbit intestinal epithelium associated with lymphoid follicles, as described below (Lelouard et al., 2001). Our recent single-cell RNA-sequencing analysis of the rabbit cecal epithelium also revealed the presence of a rare subset of mature absorptive cells characterized by the expression of the ion channel bestrophin 4 (BEST4) (Malonga et al., 2024). The putative functions of BEST4+ cells (e.g. sensing and responding to changes in luminal pH, hydration of mucus and secretion of antimicrobial peptides) suggest their implication in the cross-talk with the microbiota but this hypothesis remains to be experimentally validated. The most abundant secretory cells are goblet cells, which secrete mucus and play an important role in immune regulation, conferring them the role of gatekeepers of the microbiota (Figure 6) (Birchenough et al., 2015). Paneth cells are a subset of secretory epithelial cells located at the base of crypts and have been identified in the rabbit small intestine (Satoh et al., 1990; Cui et al., 2023). Paneth cells are specialized for the secretion of antimicrobial peptides (e.g. LYZ, REG3G, DEFB1), but other types of epithelial cells may play this role in the large intestine. Finally, tuft cells are a rare subset of epithelial secretory cells that play an important role in mouse and human intestinal immune response triggered by the microbiota (Silverman et al., 2024), but to our knowledge their presence has not been demonstrated in rabbits.

A reliable model to study the rabbit intestinal epithelium has long been lacking due to the absence of epithelial cell lines in this species. Recently, organoid models have been developed to culture the rabbit intestinal epithelium *in vitro* (Mussard et al., 2020; Kardia et al., 2021). In this culture system, stem cells isolated from the rabbit intestine produce 3D-structures formed by a single layer of polarized epithelial cells composed of the major lineages found *in vivo*, such as absorptive and goblet cells. Moreover, rabbit organoid cells

can be dissociated and seeded on cell culture inserts (2D monolayers) in order to facilitate the access to the apical side, where interactions between the epithelium and the microbiota occur.

Gut immune barrier

Besides the intestinal epithelium, the immune system plays a key defence role in the gut. Indeed, the intestinal mucosa is associated with a dense lymphoid tissue known as the gut-associated lymphoid tissue (GALT). All immune cells are derived from pluripotent cells of the bone marrow, which derive myeloid lineage cells (granulocytes, macrophages, mastocytes and dendritic cells) and lymphoid lineage cells (B and T lymphocytes) that migrate towards primary lymphoid organs to mature before homing to secondary lymphoid organs such as the GALT. In the GALT, the immune cells are gathered in inductive and effector immune sites and act in cooperation to recognize, capture, neutralize and destroy pathogens.

The inductive sites are organized lymphoid tissue, ranging from simple follicles mainly composed mainly of B lymphocytes to specialized aggregates of lymphoid follicles such as Peyer's patches (PP). In rabbits, two to ten PP can be found in the small intestine, increasing in size and number from the duodenum to the ileum (Fortun-Lamothe & Boullier, 2007). PP are covered by the follicle-associated epithelium (FAE), containing M cells. M cells take up antigens from the gut lumen and present them to underlying cells, mainly dendritic cells and macrophages, which are able to recognize pathogen-associated molecular patterns (PAMP) on the surface of pathogens through pattern recognition receptors (PRR). In rabbits, a very large PP called the *sacculus rotundus* is located in the terminal ileum. Another unique feature of the rabbit immune system is the *vermiform appendix*, located at the caudal end of the cecum (Tizard, 2023) (Figure 7). Usually considered as a secondary lymphoid organ in other species, the appendix is a primary lymphoid organ in rabbits. *Sacculus rotundus* and *vermiform appendix* represent more than 50 % of the lymphoid tissue in rabbit gut (Arrazuria et al., 2018). These structures are thought to play a major role in inducing immune response to the gut microbiota in rabbits.

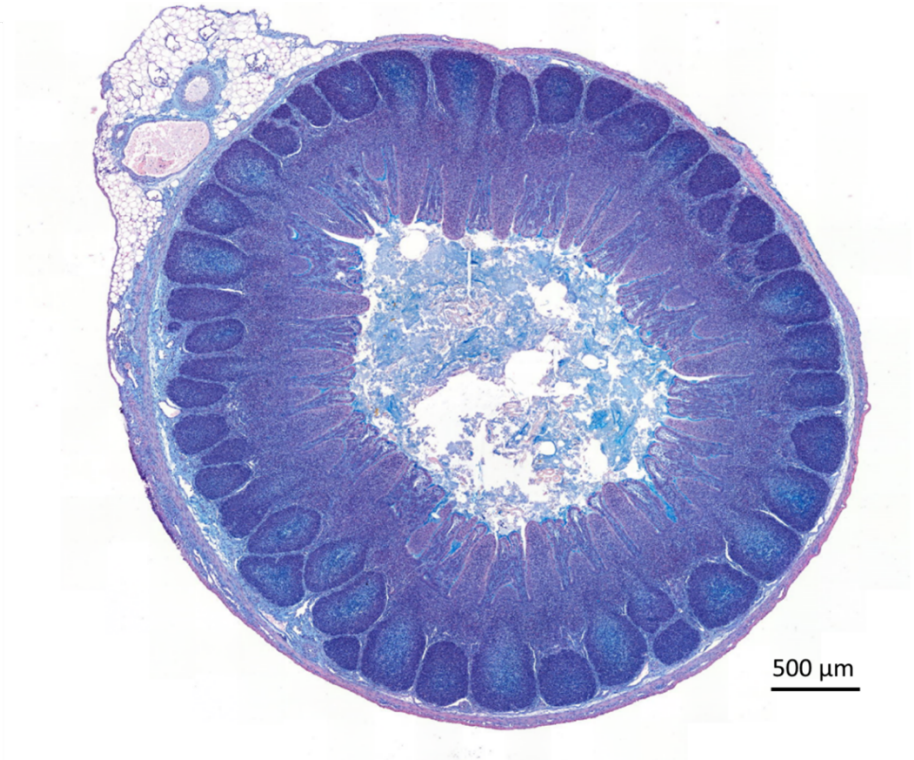

Figure 7: Histological observation of the rabbit vermiform appendix. The tissue section was stained with Alcian blue and periodic acid-Shiff. Large lymphoid follicles are seen around the appendix circumference.

The detection of external aggressions by innate immune cells at inductive sites leads to the phagocytosis of pathogens and the production of mediator molecules, cytokines and chemokines, which relay inflammatory messages and engage immune responses at the effector sites. The effector immune sites are diffusely located in the epithelium (intra-epithelial lymphocytes) and in the underlying *lamina propria* (Wagner et al., 2022). A recent single cell analysis of the rabbit cecum revealed the diversity of cells of haematopoietic origin (CD45+) in the *lamina propria,* including dendritic cells (DC), macrophages, B and T lymphocytes, basophils and natural killer cells (Knudsen et al., 2022). Macrophages and DC are also referred to as antigen presenting cells (APC) because they will present microbial products to B and T lymphocytes (Drouet-Viard et Fortun-Lamothe, 2010). Lymphocytes exert a specialized response targeted towards specific antigens recognized thanks to their cell receptor (B or T cell receptor) acquired during their maturation in primary lymphoid organs. This recognition activates two types of response. The humoral mediated response, characterized by the release of antibodies by B lymphocytes, also known as immunoglobulins (Ig). The predominant immunoglobulin produced in the gut is secretory IgA (70 to 90%), which are transported through epithelial transcytosis to the gut lumen, where they cross-link microorganisms to keep them away from of the epithelium. The cellular mediated response is mediated by CD8+ cytotoxic and CD4+ regulatory T lymphocytes. The coordinated action of these cells is critical for the balance between regulatory and inflammatory responses in order to tolerate commensal microorganisms while defending the host against pathogens.

MICROBIAL INFLUENCE ON HOST GUT BARRIER

The gut microbiota plays a crucial role in regulating the host intestinal barrier mainly through the release of metabolites and the recognition of microbial molecular patterns (Hertli & Zimmermann, 2022).

Microbiota-derived metabolites tune the gut barrier function

The release of metabolites by gut bacteria is a central mechanism mediating the action of the gut microbiota on host cells. These metabolites can be produced from substrates available in the gut lumen (i.e. derived from the diet, from host secretions or from microbial products). In rabbits, as in other species, the main metabolites produced by gut bacteria are the SCFA acetate, butyrate and propionate which are mainly produced by fibre fermentation. These SCFA are generally considered to be beneficial for health as they can i) inhibit the growth of pathogens, ii) serve as energy substrates in host cells, iii) enhance the intestinal barrier function (Ghosh et al., 2021). Other minor SCFA derived from amino acid fermentation can also be detected in the rabbit gut contents (e.g. valerate, isobutyrate, isovalerate and isocaproate) (Li et al., 2022), but their effects on host cells remain poorly characterized. Bacterial deamination of amino acids also releases ammonia, which is present in high concentrations (mM) in the rabbit gut (Beaumont et al., 2022).

A recent untargeted shotgun metagenomic study, predicting the microbial origin of metabolites revealed that the rabbit cecal microbiota is capable of producing a large diversity of metabolites (Casto-Rebollo et al., 2023). Numerous rabbit cecal metabolites were derived from amino acids such as tyrosine (e.g. 3-(4-hydroxyphenyl)lactate), tryptophan (e.g. indole, indole-lactate, indole-propionate), phenylalanine (e.g. phenyl-lactate, 3-hydroxyphenylacetate), lysine (e.g. 5-aminovalerate) and histidine (e.g. imidazole propionate). Among these, indole derivatives are well characterized for their ability to enhance gut barrier function (Ghosh et al., 2021). Rabbit microbiota also produce metabolites derived from plant components (e.g. equol) or from benzoate metabolism (e.g. 3-phenylpropionate, 3-(4-hydroxyphenyl)propionate, 3-(3-hydroxyphenyl)propionate). These compounds are considered to have anti-inflammatory effects and protect against oxidative stress (Ghosh et al., 2021). The rabbit gut microbiota is also capable of metabolizing host-secreted bile acids into secondary bile acids (e.g. glycodeoxycholate, chenodeoxycholate,

deoxycholate, ursodeoxycholate) (Kasbo et al., 2002; Casto-Rebollo et al., 2023; Zhao et al., 2024). Secondary bile acids have antimicrobial properties and regulate the barrier function (Ghosh et al., 2021). In suckling rabbits, the gut microbiota are also known to produce metabolites derived from choline (e.g. trimethylamine) (Beaumont et al., 2020, 2022), but the consequences for the gut barrier function are not well defined. Other metabolic intermediates of microbial fermentations can be detected in rabbits cecal contents such as ethanol, succinate and formate (Beaumont et al., 2020, 2022). It is important to note that most of these metabolites found in the rabbit gut lack absolute quantification, which would be required to test their effects on rabbit intestinal cells at physiological concentrations.

The effects of microbiota-derived metabolites on the gut barrier have mostly been characterized in human, rodent and porcine cells, while only a few studies have evaluated their effects in rabbit intestinal cells. The global effect of metabolites produced by the gut microbiota before or after the introduction of solid food was evaluated by treating rabbit cecal organoids with sterile supernatants of cecal content from 18- or 25-day old rabbits (Beaumont et al., 2020). The results showed that compounds present in the rabbit cecum after the onset of solid feeding were able to modulate the expression of components of epithelial defence (antimicrobial peptides, toll-like-receptors), which reflected the maturation of the gut barrier observed *in vivo* at the transition from suckling to weaning. Another study showed that serotonin, which can be produced by both the host and the microbiota, increased the expression of the tight junction protein claudin-1 (CLDN1) in rabbit intestinal epithelial cells cultured in vitro (Wang et al., 2021). Despite these findings, additional studies are required to further investigate the effects of microbiota-derived metabolites on rabbit intestinal cells.

Microbiota-derived metabolites also act on intestinal immune cells, either directly or through epithelial-mediated signalling. Although data are lacking in the rabbit species, these aspects have been characterized in humans, rodents and, to a lesser extent, in pigs. SCFA have been thoroughly investigated and butyrate has been shown to be the most potent SCFA to regulate intestinal immune responses. Butyrate enhances the tolerogenic response of macrophages and dendritic cells. For instance, in macrophages, butyrate induces antimicrobial peptide production and reduces the inflammatory response. By stimulating DCs, butyrate subsequently induces the differentiation of naive T cells. Butyrate and propionate also act directly on naive T cells, controlling their differentiation into regulatory T cells (Martin-Gallausiaux et al., 2021, for review). In pigs, *in vitro* studies showed that in peripheral blood mononuclear cells (PBMC) co-cultured with gut epithelial cells and stimulated with lipopolysaccharides (component of Gram-negative bacteria cell wall), NF-κB expression was strongly downregulated by acetate and propionate supplementation, demonstrating a protective effect of these SCFA on acute inflammation (Andrani et al., 2023). Although less well documented, SCFA such as acetate also appear to influence B cell maturation (Martin-Gallausiaux et al., 2021; for review) and support the expansion of innate lymphoid cells (Sepahi et al., 2021). Tryptophan-derived metabolites and secondary bile acids have also been shown to affect DCs, macrophages and regulatory T lymphocytes, in particular by inhibiting NF-κB pathway and downstream inflammatory response (Hosseinkhani et al., 2021). However, the specific effects of microbial metabolites on rabbit immune cells remain to be explored, notably by using primary culture of immune cells isolated from the rabbit gut.

Modulation of the gut barrier function via recognition of microbial molecular patterns and direct cell contact

Besides metabolites, the gut microbiota is able to modulate the host gut barrier function through direct contact, or through the recognition of microbial/ PAMP by PRR, including toll-like receptors (TLR) (in rabbits: Chen et al., 2014; De Vos et al., 2022). The TLRs expressed on the epithelial cell surface (TLR 1, 2, 4, 5 and 6) mainly recognize components of the bacteria cell wall, such as peptidoglycan, lipoprotein, and lipopolysaccharide in the case of

Gram-negative bacteria. TLR recognition of bacterial polysaccharides usually elicits strong antibody responses. However, this bacterial-host recognition in commensal interactions is not well defined. The large structural diversity of bacterial polysaccharides, due to the variety of monosaccharide composition, glycosidic linkages, and conformation of the polymers might likely contribute to induce, suppress or modulate the immune response (Comstock & Kasper, 2006).

In addition to recognising bacterial surface polysaccharides, the protein Amuc_1100 expressed on the outer membrane of *Akkermansia muciniphila* has been shown to signal via TLR2 and reduce colitis (Wang et al., 2020). *Akkermensia* genus has been observed in the rabbit gut in several studies (Figure 4), but its abundance has been alternatively associated with positive or negative effects (Table 2). These discrepancies among studies underlines that the same microbe may act as a mutualist, commensal or pathobiont depending on its environment.

Bacteroides upregulates fucosylation in the host epithelium, thereby modulating the barrier function. *Bacteroides* cleaves host fucose residues, which can then be internalized and catabolized by the bacteria for energy or for incorporation into capsular polysaccharides or bacterial glycoproteins. This host-bacteria interaction probably has significant consequences for the success of *Bacteroides* in the gut (Comstock & Kasper, 2006). In rabbits, the abundance of *Bacteroides* is particularly high in young animals before weaning (Combes et al., 2014; Read et al., 2019; Paës et al., 2020b, 2022). The rabbit would therefore be an interesting model for studying this positive host-microbiota interaction related to the maturation of the gut barrier function.

Segmented filamentous bacteria (SFB), belonging to the Clostridiaceae family, have been shown to attach directly to intestinal epithelial cells in several species, including rabbits (Heczko et al., 2000). SFB harbor a nipple-like appendage that is inserted into the epithelium, particularly in the follicle-associated epithelium of Peyer's patches (Caselli et al., 2010). This attachment does not damage the host cell and plays a crucial role in the postnatal maturation of the mucosal immune system. In rabbits, the presence of SFB has been observed by light and electron microscopy and its presence has been correlated with the absence of rabbit enteropathogenic *Escherichia coli* (EPEC) 0103 ileal colonization (Heczko et al., 2000). Age-related patterns of SFB in rabbits have never been reported. In rodents and humans, colonization occurs at the onset of the weaning process and decreases thereafter (Grant & Specian, 2001). Characterizing the prevalence of SFB in the rabbit gut microbiota could provide insight into their potential role in facilitating the maturation of the gut barrier function and easing the weaning transition.

NUTRITIONAL OPPORTUNITIES TO STRENGTHEN POSITIVE INTESTINAL HOST-MICROBIOTA INTERACTIONS IN EARLY LIFE TO PROMOTE INTESTINAL BARRIER AND HEALTH

Under breeding conditions, rabbits are particularly susceptible to digestive disorders, especially around weaning. "Positive" host-microbiota interactions are essential for the co-maturation of the two symbiotic partners to ensure the development of the barrier function and thus preserve digestive health. Strengthening the gut barrier by stimulating host-microbiota interactions prior to weaning could be an effective lever to maintain digestive health in young rabbits.

Early in life, the microbiota in rabbits, as in other mammals, is characterized by a low microbial diversity at the individual level but greater phylogenetic diversity between individuals (human: Yatsunenko et al., 2012; pig: Mach et al., 2015; calf: Rey et al., 2014; and rabbit: Read et al., 2019). In contrast, after weaning, when solid feed intake is well established, the composition of the microbiota is more homogeneous between individuals,

with less phylogenetic diversity. The postnatal period therefore represents a window of permissiveness for engineering the microbiota architecture, when the richness and diversity of the microbiota are low and the selective pressure exerted by the host on its microbiota is reduced due to an immature mucosal immunity. As it has been widely demonstrated, diet is one of the most effective ways to shape microbiota composition and function (Gómez-Conde et al., 2009; Zhu et al., 2015; Yang et al., 2020; Liu et al., 2022; Ma et al., 2022; Paës et al., 2022; Li et al., 2023b). Several studies have been conducted on innovative preweaning nutritional strategies to modulate microbiota and promote host-microbiota interactions. The following section focuses on results on effects of milk with a focus on milk oligosaccharide components, solid feed intake and coprophagic behavior in the nest, and cessation of milk intake (i.e. weaning), on microbiota and host gut barrier functions.

Milk intake and its prebiotic action through milk oligosaccharides

Milk provides the newborn with essential nutritional components (protein and fat rich milk) and non-nutritional bioactive components. Among the latter, rabbit milk is particularly rich in short-chain fatty acids with antibacterial properties. Several proteins also exert antimicrobial activities such as transferrins, α and β-caseins and immunoglobulins, which are also involved in the passive immunity (Maertens et al., 2006). Recently, other bioactive components have been studied for their immunomodulatory functions and effects on the gut microbiota, namely the milk oligosaccharides (MO). They consist of three to ten monosaccharide units and are composed of five monosaccharides: glucose, galactose, N-acetyl-glucosamine, fucose and sialic acid residues. The combinations of monosaccharides and their linkages result in the formation of linear or branched structures of great diversity. Host enzymes do not have the capacity to digest these molecules, so they turn to gut bacteria, being the first prebiotics available to newborns. *Bacteroides* has been shown to metabolize MO (Marcobal et al., 2011). Bacterial MO consumption results in metabolite production, such as butyrate, an essential energy source for colonic epithelial cells (Walsh et al., 2020). Other studies have shown the potential of neutral fucosylated and non-fucosylated MO to directly enhance intestinal epithelial barrier integrity in a Caco-2-cell line (Boll et al., 2024). Kong et al. (2019) also used a Caco-2-cell line to show the stimulatory effect of fucosylated milk oligosaccharides on glycocalyx development of intestinal epithelial cells. MO structure in itself plays antimicrobial and anti-adhesive roles. They act as decoys for infectious microorganisms by mimicking gut epithelial binding receptors used by pathogens to invade the host gut (Walsh et al., 2020). In particular, the 3'-sialyllactose has been shown to bind the pathogen *Helicobacter pylori* in an intestinal epithelial cell line model (Simon et al., 1997) while 2'-fucosyllactose inhibited adhesion of *Campylobacter jejuni* in a cell line model (Yu et al., 2016). Finally, MO can educate and modulate the host immune system, via direct linkage or indirectly through bacterial metabolites (Ayechu-Muruzabal et al., 2018). It is believed that MO, especially the sialylated types are able to bind to specific lectin receptors present on the surface of numerous immune cells such as dendritic cells and macrophages (Rousseaux et al., 2021).

The MO composition, has been reported in human milk and for a wide range of non-human mammals, highlighting significant variations in concentration and composition between species and lactation stage. However, MO has not been characterized in rabbits. Our recent analysis of MO from 67 female rabbits has shown that sialylated forms predominate (83 %) (Combes et al., 2023). However, the diversity of MO in rabbit milk is much lower than in human milk. A further characterization of MO in rabbits is necessary to elucidate their potential direct and microbiota-mediated role in strengthening the intestinal barrier. This research could lead to the development of health-promoting strategies in rabbits, including i) the use of dietary supplementation with MO as a novel type of prebiotic and as a tool to enhance the barrier function, ii) the optimization of MO composition in milk by genetic selection or dietary modulation, iii) the provision of bacteria used as probiotics (e.g. *Bacteroides* strains) capable of degrading MO.

Introduction of solid food

Under rearing conditions, suckling rabbits have access to solid food from 12-15 days of age (Scapinello et al., 1999) as soon as they are able to leave the nest and reach their mother's feeder. Rabbit pups gradually move from milk, which is highly digestible by the hydrolytic action of the host's enzymes, to a more complex and less digestible solid food, containing in particular indigestible plant polysaccharides. The arrival of these new substrates leads to the creation of new ecological conditions in the gut, allowing the establishment and dominance of new microbial species better adapted to their degradation. The introduction of solid feed thus contributes to a drastic change in the composition and metabolic activity of the gut microbiota (Padilha et al., 1999; Read et al., 2019; Beaumont et al., 2020; Zhao et al., 2024). This maturation in the microbiota composition and metabolic activity are closely linked to the development of the epithelial barrier function, as observed with the altered expression of genes involved in tight junctions, mucin production and in innate immunity (production of cytokines and antimicrobial peptides) (Beaumont et al., 2020). In particular, it has been shown that the butyrate produced by the bacteria after the introduction of solid food plays a key role in the development of the epithelial barrier *in vitro* (Beaumont et al., 2020). Optimizing the nutritional composition of the feed available before weaning is undoubtedly a relevant way to strengthen the gut barrier function. At the onset of solid feed intake, dietary modulation has been shown to alter the dynamics of microbiota establishment (dietary modulation of energy and crude protein content; Read et al. 2019), favoring the Lachnospiraceae family over the Ruminococcaceae family (dehydrated alfalfa: Mattioli et al., 2019).

Early introduction of solid food (before 12-15 days of age in the nest), while maintaining milk consumption, has been evaluated as a strategy to optimize host-microbiota interactions. In rabbits, the possibility of stimulating early feed intake in the nest was first demonstrated by Kacsala et al. (2018). To optimize this early solid feed intake, we have developed a nutritional substrate in the form of a gel, taking into account the food preferences of suckling rabbits and without altering milk intake (Paës et al., 2020a). This early stimulation of solid food ingestion, significant from 7 days of age, leads to an increased bacterial diversity before weaning and an increased abundance of Lachnospiraceae and Ruminococcaceae at the expense of Bacteroidaceae, the latter being characteristic of the microbiota of exclusively suckling rabbits. These changes in the microbiota were amplified with higher levels of feed intake and were also related to the litter weight (Paës et al., 2020b). These observations reflect an acceleration in the maturation of the microbiota and can be explained by a targeted seeding of the digestive microbiota at the beginning of life, which favours the development of bacteria specialized in the degradation of complex parietal carbohydrates. Early feed intake was also associated with an increase in the cecal levels of 7 amino acids, as well as acetate and butyrate in the intestinal lumen of rabbits, which indicated a change of bacterial metabolism (Paës et al., 2022). This maturation of the gut microbiota induced by early solid food ingestion could therefore improve rabbit health. Indeed, in piglets, changes in the microbiota associated with early feeding have been shown to reduce diarrhoea and improve growth performance (Choudhury et al., 2021). In rabbits, it remains to be determined whether changes in microbiota composition and in the luminal concentrations of metabolites, molecular intermediates in the microbiota-host crosstalk, are sufficient to preserve the health of the young rabbits and help them to cope with weaning.

Coprophagia behavior in the nest

The ingestion of solid substrate early in life also includes coprophagia behavior in the nest, i.e. the ingestion of feces deposited by the mother during nursing (Hudson & Distel, 1982; Kovács et al., 2006; Combes et al., 2014). Coprophagia early in life has a protective effect on rabbit health by increasing survival rate (Combes et al., 2014). This beneficial effect is associated with an acceleration of the implantation dynamics of the microbiota in the cecum, allowing bacteria belonging to the Ruminoccocaceae family to become predominant at the expense of those belonging to the Bacteroidaceae family. Furthermore, coprophagia is also

associated with transcriptomic changes in the gut that reflect immune development in the ileum during the first two weeks after weaning. These changes specifically involve the type I interferon signalling, but also include innate immune responses (antimicrobial peptides, mucin and cytokine secretions) and adaptive immune responses (transcriptional regulation of IgA secretion) (Cauquil et al., 2024). Altogether, these results suggest that coprophagia in the nest may favor early host-microbiota interactions with long-lasting effects after weaning. Further studies should be conducted to better understand how coprophagia drives its effect on the mucosal immune system, thereby strengthening the host gut barrier. For instance, it would be interesting to determine whether specific bacterial strains or metabolites present in maternal feces drive these protective effects of early life coprophagia in rabbits.

Cessation of milk intake

Weaning is commonly defined as the transition from exclusive milk intake to exclusive solid feed intake. In rabbit rearing conditions, the term “weaning” is associated with suckling cessation, and the separation of the pups from the doe, and usually occurs between 28 and 35 days of age. Several studies evidence a lower growth when animals are weaned early, between 21 and 25 days of age, compared to 34-35 days of age (Gallois et al., 2008; Cesari et al., 2009; Kovács et al., 2012), which could be associated with a higher susceptibility to digestive disorders, evidenced by an increased mortality (Cesari et al., 2009). Interestingly, early weaning (i.e. suckling cessation) is not associated with changes in microbiota composition or metabolic activity (Kovács et al., 2012; Beaumont et al., 2022). These observations indicate that solid food ingestion, rather than milk intake cessation, shapes the rabbit gut microbiota. Regarding microbial activity, increased levels of SCFA, namely butyrate (Kovács et al., 2012) and acetate (Gallois et al., 2008), are evidenced in rabbits weaned at 21 days of age compared to rabbits weaned at 35 days of age. Beaumont et al. (2022) found no changes in SCFA but higher levels of the polyphenol-derived metabolite 3-phenypropionate at 25 days of age in rabbits weaned at 18 days of age compared to their suckling counterparts. Altogether, early weaning does not induce considerable changes in the digestive microbial compartment. Conversely, early weaning appears to have a strong effect on host physiology and mucosal development. Gutiérrez et al., (2002) showed that pups that were weaned at 25 days of age had shorter villi and deeper crypts in the jejunum at 35 days of age than their suckling counterparts, which was associated with decreased brush border enzyme activity (lactase, maltase and sucrase) and increased alpha-amylase activity. Beaumont et al., (2022) also evidenced strong gene expression modulations in the cecal epithelium of 25 days old rabbits weaned at 18 days of age compared to their suckling counterparts. Namely, stem cell and proliferation markers were down-regulated in early weaned rabbits, whereas the expression of PIGR, which is involved in immunoglobulin transport, was strongly up-regulated, possibly due to the loss of maternal passive immunity. In both aforementioned studies, most of the changes induced by early weaning were consistent with an enhancement of spontaneous development with age, suggesting that early weaning could accelerate gut mucosal development. However, it is important to consider that disrupting the kinetics of microbiota and mucosal maturation can enhance the susceptibility to diseases later in life (Al Nabhani et al., 2019), as evidenced by the increased mortality associated with early weaning in rabbits (Cesari et al., 2009).

CONCLUSIONS

The reanalysis of 16S amplicon sequencing data from different research groups has allowed us to establish the core microbiota of the post-weaning rabbit cecum in a robust manner, including technical and experimental variability. Nevertheless, this analysis highlights that there is still a considerable lack of knowledge regarding the microbes that inhabit the cecal ecosystem, their functions and taxonomy at the species and strain level. Studies based on the sequencing of all bacterial genes (shotgun metagenomics) are already available. These technologies allow the integration of taxonomic knowledge with the functions present in the ecosystem. The advent of long-read sequencing, applied to both 16S amplicon sequencing

and shotgun metagenomics, will increase taxonomic resolution while reducing taxonomic errors and ambiguity in assigning sequences to specific taxa. It will also allow a better detection of rare species due to increased sensitivity and coverage of sequenced microbial genomes (Eisenhofer et al., 2024). The review of the literature over the past decade has enabled the identification of some key species in the rabbit gut ecosystem that are associated with beneficial or detrimental traits related to health and growth. However, we were unable to reach a consensus between the different studies, which precluded us from defining the optimal rabbit microbiota. The large variability in experimental conditions between studies explains much of this difficulty. Another reason may be that, beyond knowing the species, the activity of the microbiota needs to be further studied, either by inference through metagenomic studies or by characterizing the metabolome. Culture of bacterial strains isolated from the rabbit microbiota will also be instrumental in identifying their role in symbiosis.

The microbiota plays a pivotal role in gut health, and more specifically as a central component of the intestinal barrier. The underlying mechanisms of its role as a barrier itself still remain to be investigated in rabbits. Given the taxonomic specificity of the rabbit microbiota, it would be valuable to validate the generic bacterial gatekeeper mechanisms already demonstrated in other mammalian models. The epithelial component of the intestinal barrier also exhibits remarkable features in rabbits, such as the presence of $BEST4^+$ absorptive epithelial cells, which role remains to be elucidated. The development of *in vitro* intestinal organoid models in rabbits offers promising avenues for elucidating the functional interactions between the epithelium and gut bacteria and their products. Additionally, the immune component of the intestinal barrier remains relatively understudied, due to the lack of suitable tools in rabbits, despite the presence of unique lymphoid tissues in this species. Further investigations are therefore warranted.

A deeper understanding of the symbiotic relationship between the microbiota and its host is essential to strengthen the intestinal barrier and consequently digestive health. Top-down approaches such as metagenomics, metabolomics, transcriptomics and proteomics, which do not require cultures of individual species, have provided genomic and metabolic signatures within a complex community, and together generate hypotheses about existing microbe-microbe and microbe-host interactions. Testing hypotheses, and going from these association studies to proven causality, will require performing genetic engineering studies and applying bottom-up synthetic biology approaches combined with functional assays using models of gut barrier host cells. Genetic engineering, i.e. loss, gain and modulation of functions, provides opportunities to probe the intrinsic molecular host-microbiota interactions in rabbits. It will provide a new source of fundamental knowledge and strategic guidance for understanding the role of the gut microbiota and its metabolites on the intestinal barrier.

We have shown that prior to weaning, modulation of dietary intake during the transition from milk to solid food has a strong influence on the microbiota and, concurrently, the host's health. By understanding the causality in microbe-host interactions, it will thus be possible to engineer the establishment of the microbiota in the young rabbit and strengthen the intestinal barrier. For instance, innovative nutritional strategies before weaning might be proposed, such as (i) dietary prebiotic supplementation that focus on targeted health-promoting bacteria, (ii) probiotic supplementation derived from, and thus specific to, the rabbit gut, which is known to strengthen host intestinal barrier function, (iii) symbiotic supplementation (supply of live bacteria with their substrate) or postbiotics, i.e. beneficial metabolites for the host's intestinal barrier produced by cultivated and lysed bacteria. Altogether, it is evident that the microbiota plays a pivotal role in the digestive health of rabbits. Consequently, to optimize its composition and functional capabilities and to enhance the intestinal barrier function, it is necessary to adopt multidisciplinary approaches that integrate diverse methodologies at various scales. These include *in vitro* cellular culture models, notably cell models that combine epithelial and immune cells and bacteria, and *in situ* validation models.

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HEAT STRESS INFLUENCE ON HAEMATOLOGICAL STATUS AND BODY TEMPERATURE IN TWO MATERNAL RABBIT LINES WITH DIFFERENT LONGEVITIES

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ABSTRACT

The study aimed to assess the impact of heat stress on body temperature and immune response in two maternal rabbit lines. Line LP shows higher longevity than line A. A total of 223 nulliparous does were studied, 99 from line A and 124 from line LP. The two lines were distributed contemporaneously between the heat stress period and the comfort period. Body temperature was measured in the does eyeball using infrared thermography, and blood samples were collected for haematological analysis on the 24 hours following their first parity. Results indicated significantly higher eyeball temperatures during heat stress compared to comfort conditions, with line A showing higher temperatures than line LP. Haematological analysis revealed elevated neutrophil percentages during heat stress, while lymphocyte and monocyte percentages decreased. Additionally, differences between the two lines were observed, with line A exhibiting higher white blood cell counts compared to line LP, especially during heat stress. These findings suggest that heat stress adversely affects body temperature and immune response in rabbits, with line LP demonstrating better resilience in regulating body temperature and immune response compared to line A. A better understanding of heat stress response will help to improve resilience and productivity in rabbit farming, especially in regions vulnerable to heat stress due to climate change.

Key words: heat stress, longevity, resilience, body temperature, haematological parameters.

INTRODUCTION

Rabbit farmers currently demand not only highly productive animals but also more longaeval and resilient animals that cope to environmental challenges, such as rising temperatures due to climate change. Heat stress is one of the most important environmental stressors in rabbits (Ebeid et al., 2023) and it is particularly prevalent in Mediterranean regions, where rabbit farming is concentrated (Cullere and Dalle Zotte, 2018). The high sensitivity of rabbits to heat stress (Giorgi and Lionello, 2007; Marai et al., 2002) has a negative effect on reproductive traits, leading to decreased fertility, embryonic development, litter size, litter weight and milk production (Ebeid et al., 2023). Different biomarkers associated with stress have been proposed as an indirect way to estimate the adaptation of animals to the environment and minimize the impact of stressors. Body temperature and haematological status has been used as biomarkers due to their role in the biological processes that underlie resilience (Ebeid et al., 2023; König and May, 2019; Argente et al., 2019a). Therefore, this study aims to compare body temperature and immune responses to heat stress between two highly productive maternal lines with different longevity.

MATERIALS AND METHODS

Animals and experimental design

A total of 223 nulliparous does from two highly productive commercial maternal lines with different longevity (A and LP) were employed in this experiment. Data was collected into two periods: heat stress period/summer (range 23-28°C) and thermal comfort period/winter (13-21°C). Dataset comprised 99 females from line A (46 in heat stress and 53 in thermal comfort) and 124 females from line LP (55 in heat stress and 69 in thermal comfort). Line A

has a standard longevity whereas line LP has a longer longevity. Line LP was founded using longevity and productivity criteria, specifically from females with an average number of parities of 30, which is six times higher than the average longevity (Sánchez et al., 2008). The females were housed at the farm of the Universitat Politècnica de València (Spain) in individual cages (flat- deck) with an extractable nest box with isolated plastic, and under a photoperiod of 16-h light: 8-h dark. Access to feed was *ad libitum* for the entire experimental period with the same standard commercial diet for all animals.

Data collection

For all does, on the first morning following their first parity, blood samples were collected from the marginal ear vein into a tube with tripotassium ethylenediaminetetraacetic acid (K3-EDTA). Samples were analysed in a veterinary analysis laboratory (CEDIVET) to determine white blood cell count, and the count and percentage of lymphocytes, monocytes, neutrophils, and eosinophils. In addition, before blood sampling, the temperature of the rabbits was taken by infrared thermography of the eyeball with a "Testo Irsoft" thermograph and processed with Irsoft software version 1.7. Two successive thermographs measures were taken from each doe, maintaining the same distance and same conditions. The average maximum temperature of eyeball of both measurements was calculated. If differences between both measurements were higher than 0.5 ºC the measurement was discarded from analysis.

Statistical Analysis

Data were analysed using the following model:

$$y_{ijk} = \mu + L_i + S_j + L*S + e_{ijk}$$

Where y_{ijk} is the observed trait, μ is the general mean, L_i is the line effect (with two levels: line A and line LP), S_j is the effect of season (with two levels: heat stress and comfort), L*S is the interaction term between line and season, and e_{ijk} is the residual term. The traits were analysed using Bayesian methodology in R using brms package (Bürkner, 2017). Inferences were made from the estimated marginal posterior distributions of the differences between the two lines A and LP and heat stress and thermal comfort conditions. The probability (P0) of the difference being greater or lower than zero, probability of relevance (Pr, calculated as the difference being greater or lower than 1/3 standard deviations of the trait) and HPD95% (shortest Bayesian confidence intervals with a 95% of probability) were calculated. Probability thresholds were 95% for P0 and 70% for Pr.

RESULTS AND DISCUSSION

Effect of Heat Stress

In comfortable thermal conditions, the estimated mean of eyeball temperature was 35.43 ºC (Table 1), consistent with findings by Ludwig et al. (2007) who reported similar results (35.6 ± 0.5 ºC) in rabbits under similar conditions. Conversely, during heat stress conditions, the mean eyeball temperature rose to 37.43 ºC, mirroring observations by Jaén-Téllez et al. (2020) in summer-fattened rabbits and De Lima et al. (2013) in a study inducing heat stress (37.03±0.09ºC). Posterior mean of the difference between heat stress and thermal comfort conditions was around 2 ºC, this was a relevant difference with a probability of 100% (Table 1).

Regarding haematological parameters, high neutrophils values (5.83 and 4,38 thousand/microliter (k/uL), data no shown) and low lymphocyte values (2.44 and 3,06 k/uL, data no shown) were observed in both periods, heat and comfort periods, respectively. These values fall outside the reference ranges of neutrophils (2.36±0.64 k/uL) and lymphocytes (6.99±0.74 k/uL) (Argente et al., 2019b). This pattern aligns with gestational changes observed in humans (He and Xia, 2019). Physiological stress during pregnancy and delivery is characterized by neutrophilia, elevated neutrophil counts. Conversely, lymphocyte reduction during gestation prevents foetal rejection and is attributed to increased hormone levels. The high neutrophil and low lymphocyte levels observed indicate significant stress.

Higher white blood cell was found in heat stress conditions with a probability of relevance near 70% in agreement with previous results (Ondruska et al., 2011) (Table 1). Percentage

of neutrophils in white blood cells and neutrophils/lymphocytes ratio were higher in heat stress conditions, while percentage of lymphocytes and monocytes in white blood cells were lower compared to thermal comfort conditions (Table 1). These differences were relevant with a probability of 100%. Neutrophils, being the most abundant type of white blood cells, act as the body's first line of defence. An increase in neutrophil indicates that heat stress has prompted an immune response. Moreover, in accordance with our results, heat stress was found to reduce lymphocytes count in female rabbits (Ferrian et al., 2012).

Table 1: Parameters of the posterior marginal distribution of the estimated difference between heat stress and thermal comfort periods for eye temperature and hemograms parameters.

	Heat stress	Comfort	Difference	sd	HPD95%	P0(%)	Pr (%)
Temperature (ºC)	37.43	35.43	2.00	0.09	[1.83 ; 2.16]	100	100
WBC (k/ul)	8.57	7.81	0.76	0.27	[0.22 ; 1.29]	100	67
Neutrophils (%)	67.73	56.57	11.16	1.43	[8.31 ; 13.88]	100	100
Lymphocytes (%)	29.08	38.94	-9.86	1.37	[-12.64 ; -7.24]	100	100
Monocytes (%)	2.62	3.82	-1.20	0.14	[-1.48 ; -0.91]	100	100
Eosinophils (%)	0.61	0.62	-0.01	0.12	[-0.26 ; 0.22]	55	2
N/L	2.97	1.64	1.33	0.26	[0.80 ; 1.83]	100	100

sd: Standard Deviation, HPD 95%: 95% confidence interval, P0: Probability of positive difference > 0 or negative difference < 0. Pr: Probability of relevance. WBC: White blood cell count. N/L: Neutrophils / Lymphocytes.

Effect of lines A and LP

Regarding differences between lines (Table 2), we noted higher eyeball temperatures in line A compared to line LP (approximately 0.4 ºC), with a 96% of relevance probability. Differences between the lines were more pronounced in thermal comfort compared to heat stress conditions (data no shown). Results suggest that line LP maintains lower body temperatures when faced high temperatures and post-partum processes. It's worth noting that line LP has consistently exhibited greater longevity and resilience compared to line A (El Nagar et al., 2021).

Table 2: Parameters of the posterior marginal distribution of the estimated difference between lines A and B for eye temperature and hemograms parameters.

	A	LP	Difference	sd	HPD95%	P0 (%)	Pr (%)
Temperature (ºC)	36.61	36.24	0.37	0.09	[0.21 ; 0.55]	100	96
WBC (k/ul)	8.65	7.73	0.92	0.28	[0.39 ; 1.48]	100	85
Neutrophils (%)	64.14	60.15	3.98	1.42	[1.26 ; 6.83]	100	68
Lymphocytes (%)	32.32	35.70	-3.37	1.36	[-5.96 ; -0.61]	99	56
Monocytes (%)	3.10	3.34	-0.25	0.14	[-0.54 ; 0.02]	96	27
Eosinophils (%)	0.41	0.81	-0.40	0.12	[-0.64 ; -0.16]	100	83
N/L	2.55	2.07	0.48	0.26	[-0.04 ; 1.00]	96	31

sd: Standard Deviation, HPD 95%: 95% confidence interval, P0: Probability of positive difference > 0 or negative difference < 0. Pr: Probability of relevance. WBC: White blood cell count. N/L: Neutrophils / Lymphocytes

In terms of haematological parameters, we observed higher white blood cell counts in line A with an 85% of relevance probability, while line LP showed a higher percentage of eosinophils (Pr=83%). These differences were more pronounced in heat stress compared to thermal comfort conditions (data no shown). No relevant differences between lines were found for the rest of haematological parameters. Our findings regarding white blood cell counts differ from those reported in a study on two lines selected for litter size variability, where primiparous females from the more homogeneous line (least sensitive to stress) had higher leukocyte counts on the first day postpartum, with no differences in neutrophil and lymphocyte percentages (Beloumi et al., 2020). However, our results align with those of Gunia et al. (2019), who found that animals more susceptible to Pasteurella exhibited higher white blood cell counts, a higher percentage of neutrophils, and a lower proportion of lymphocytes compared to Pasteurella-resistant and control animals. Regarding the higher percentage of eosinophils observed in line LP, eosinophils are described as pleiotropic

multifunctional leukocytes involved in various inflammatory responses and modulation of innate and adaptive immunity (Hogan et al., 2008).

CONCLUSIONS

In heat stress periods, both lines exhibited higher eyeball temperatures compared to thermal comfort periods, indicative of stress. Haematological analysis revealed elevated neutrophils and decreased lymphocytes in heat stress conditions, reflecting an immune response to stress. Interestingly, the more longaeval and resilient line (LP) showed better regulation of body temperature, lower WBC but higher eosinophils compared to line A. A better understanding of heat stress response will aid to improve resilience and productivity in rabbit farming, especially in regions vulnerable to heat stress due to climate change.

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METHIONINE REGULATES THE APOPTOSIS IN HEAT-STRESSED DERMAL PAPILLA CELLS VIA WNT/ B-CATENIN PATHWAY

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ABSTRACT

This experiment aimed to investigate the effects of methionine on the growth state of Rex rabbit dermal papilla cells under heat stress. Primary dermal papilla cells transmitted to 4-5 generations were divided into four treatment groups (3 replicates per group): Control group (37°C, base medium without Met), heat stress group (42°C, base medium without Met), heat stress +30mM Met group (42°C, base medium with 30mM Met), heat stress +60mM Met group (42°C, base medium with 60mM Met). The results showed that Met was involved in regulating the apoptosis of dermal papilla cells in heat-stressed Rex rabbits, and slowed down the accelerated process of apoptosis by heat stress. Meanwhile, Met restored the inhibition of heat stress on dermal papilla cells in the S phase. Which may be mediated by Wnt/β-catenin signaling pathway.

Key words: Heat stress, methionine, cell apoptosis, cell cycle, dermal papilla cells.

INTRODUCTION

Hair follicle dermal papilla cells are an important *in vitro* model to study hair growth. Dermal papilla cells (DPCs) are a specialized mesenchymal component of hair, whose size determines the size of hair follicles (Xin et al., 2018). As a reservoir of pluripotent stem cells, nutrients, and growth factors, DPCs maintain and induce the proliferation and differentiation of epithelial cells, and drive the growth and development of hair follicles and the regeneration cycle through the mesenchymal-epithelial interaction within the ecological niche (Houschyar et al., 2020). Rabbits are more susceptible to heat stress due to the lack of sweat glands, which reduces the density of hair follicles and the quality of fur in rabbits, directly affecting production performance and economic benefits (Yue et al., 2023). Methionine, a sulfur-containing amino acid with antioxidant and anti-inflammatory effects, could initiate translation and convert into [S-adenosylmethionine (SAM)] as an anabolic signal. Dietary supplementation of Met improves the growth performance of broilers under heat stress (Walvekar and Laxman, 2019). Therefore, the purpose of our experiment is to determine whether methionine is involved in the regulation of the growth of heat-stressed hair papilla cells, to furnish a molecular basis for exploring how methionine regulates hair follicles of heat-stressed Rex rabbits, and to provide a reference for nutrients to improve hair follicle development under heat stress.

MATERIALS AND METHODS

Cell culture

Primary dermal papilla cells of Rex rabbit back skin were isolated and cultured by a double enzymatic method (Liu et al., 2020). 1×10^6 cells were seeded in six-well plates in Dulbecco's Modified Eagle Medium (DMEM) containing 10% serum high glucose at 37°C. After the cells had grown to 70%-80% confluence, they were replaced with high-glucose DMEM basal medium (Gibco) without Met and adaptive equalization at 37°C for 24h. They were divided into four groups with three replicates per group. The control and heat stress groups were treated with basal medium without Met, and the heat stress + methionine group was treated with 30mM Met and 60mM Met, respectively. heat-stress samples were placed in a 42°C, 5%CO_2 incubator for half an hour, and then rewarmed in a 37°C incubator. Same thing at the same time, three times in three days. On the third day, the samples were collected after heat

stress. The addition of 30mM Met was selected based on the apoptosis results for subsequent experiments.

Flow cytometry

The cells to be tested are stained and made into a single-cell suspension. The cells stained by fluorescence are irradiated by a laser beam to produce scattered light and excitation fluorescence. Annexin V and PI double staining (BD, America) was used to detect cell apoptosis, and propidium iodide (PI) DNA staining (BD, America) was used to detect cell cycle analysis by flow cytometry. The voltage was adjusted before the device was started.

RT-qPCR

The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was chosen as the reference gene to normalize the mRNA expression of target genes. The relative expression ratio of target genes relative to the reference gene was calculated using the 2-ΔΔCT method (Li et al., 2018).

Table1.Primers used for real-time quantitative PCR

Gene	Primer sequence (5′-3′)		Accession No.
GAPDH	F:GGCTGCTTTTAACTCTGGCAAA	R:CGTGGGTGGAATCATACTGGAA	NM_001082253.1
Wnt10b	F: TGTGCCATCCCTCTTCCTTA	R: GGCTCCACCTCTAACTTCTGC	NM_002711076
β-Catenin	F: TTCTTGGGACTCTTGTTCAGC	R: CACTTGGCACACCATCATCT	XM_051852655.1
LRP5	F: CCTTTACGAGCGGAACCAC	R: GCAGGGTAGAACACGTCCAT	XM_051827847.1
TCF3	F: CGGGAGATAGAGCAGGTGAA	R: GGTAGTCATCGCCGTAGGAG	NM_001171390

Statistical Analysis

All data were analyzed with SAS. A one-way ANOVA model was used to evaluate the mean values between groups. Data were expressed as mean ± standard error and root mean square error (R-MSE). P<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Cell apoptosis

As Figure 1 shows, compared with the control group, heat stress significantly increased the proportion of early apoptosis, late apoptosis, and total apoptosis, and promoted the process of apoptosis. However, methionine supplementation significantly reduced the ratio of early apoptosis, late apoptosis, and total apoptosis.

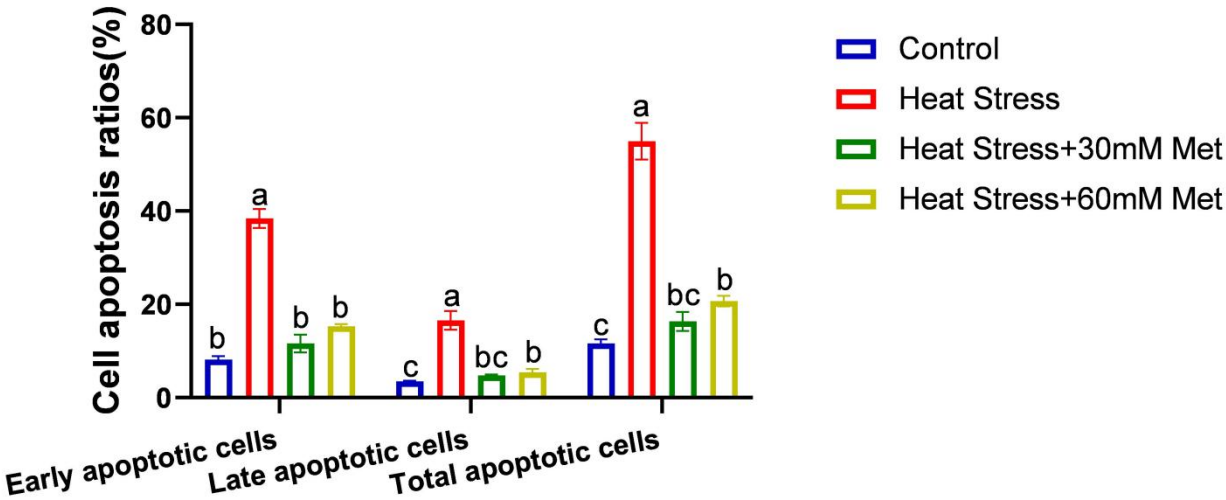

Figure 1. Effect of methionine on apoptosis status of heat-stressed dermal papilla cells. Average values with different superscript letters are statistically significant (p<0.05), the same as below.

Cell cycle

As Figure 2 shows, compared with the control group, the heat stress group significantly increased the proportion of cells in the S phase and decreased the proportion of cells in the G2/M phase, indicating that heat stress caused an increase in DNA replication process and a decrease in the proportion of cells undergoing mitosis. After methionine addition, the proportion of cells in the G0/G1 phase was significantly down-regulated compared with the heat stress group, and the proportion of cells in the G2/M phase was significantly increased, indicating that methionine attenuated G0/G1 phase arrest and enabled more cells to enter the division phase.

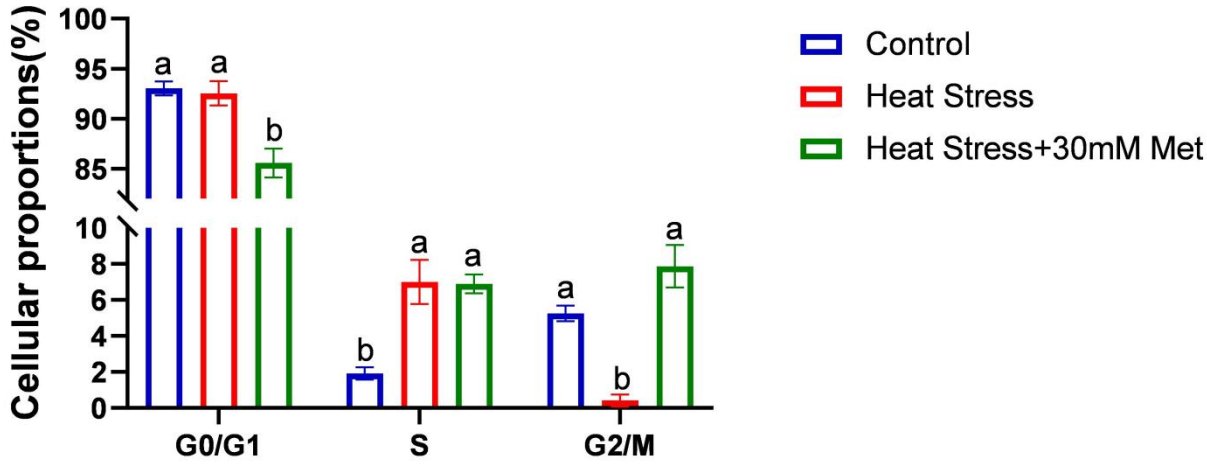

Figure 2. Effect of methionine on cell cycle distribution of heat-stressed dermal papilla cells.

Gene expression

Heat stress significantly increased the gene expression of *Wnt10b*, *β-catenin*, *LRP5*, and *TCF* compared with the control group, which was significantly alleviated by adding methionine (Figure 3). These results suggest that Wnt/β-catenin signaling pathway is involved in the process of methionine regulating hair follicle development in heat stress.

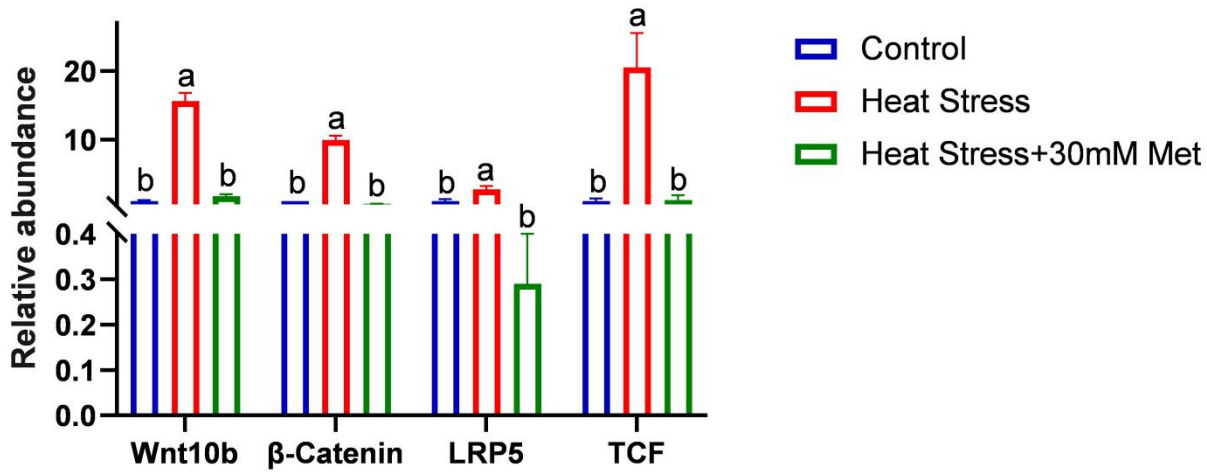

Figure 3. Effect of methionine on the relative quantification of major genes expression.

CONCLUSIONS

Heat stress accelerated the process of cell apoptosis and caused cell arrest in the S phase, which could be alleviated by a methionine supplement. The Wnt/β-catenin signaling pathway is involved in the regulating process.

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ASSESSMENT OF THE EFFICACY OF *MORINGA OLEIFERA* AQUEOUS SEED EXTRACT ON PHYSIOLOGICAL PARAMETERS IN MANAGEMENT OF HEAT STRESS IN FEMALE RABBITS

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ABSTRACT
The present study investigated the efficacy of *Moringa oleifera* aqueous seed extract (MOASE) on biochemical parameters and oxidative stress biomarkers. For this purpose, 28 nulliparous New Zealand white female rabbits aged 6 months old and weighing between 2015.6 and 2322.7 g were randomly assigned to 4 groups according to temperature, relative humidity, temperature and humidity index (THI) and MOASE defined as follows: T0: room temperature (18-24°C), relative humidity (59 ±0.48%), and 23.6 ±1.52 as THI. T1, T2, and T3 had same experimental set -up: 35-36°C, 64 ±0.6%, and 32.5 ±0.7 but additionally co-treated with 100, 200, and 0 mg/kg of body weight (b.w.) MOASE, respectively. The temperature was induced using electrical heaters from 8:00 am to 4:00 pm. At the end of experimental period all animals were slaughtered, blood samples and kidney were collected for analysis of respectively biochemical parameters and oxidative stress biomarkers. Results revealed an increase of creatinine, urea, alanine aminotransferase, aspartate aminotransferase contents and malondialdehyde in animals at T3 compared to those at T0. However, all these parameters decreased in a dose-dependent manner following MOASE administration for each case. A decrease in glucose, blood and kidney protein followed by a decrease in catalase, superoxide dismutase and glutathione peroxidase content for animals at T3 compared to those of T0 were noticed. However, these parameters increased when MOASE was administrated at 200 mg/kg b.w. Conclusively, administration of MOASE at 200 mg/kg b.w mitigated the physiological disturbances occasioned by heat stress in female rabbits.
Keywords: Heat stress, *Moringa oleifera*, Physiology, Rabbit does

INTRODUCTION
Thermal stress is one of the main environmental impediments and occurs when the ambient temperature is under or exceeds the thermoneutral (comfort) zone of the animal (Kumar *et al.*, 2011; Mutwedu *et al.*, 2020). Heat stress is one of the most important stressors in animal production, especially in rabbit farming due to the lack of functional sweat glands (Marai *et al.*, 2002). It has been reported to induce many disturbances in rabbit physiological system. For instance, in an earlier study, there was a decrease in standing and walking behavior while biochemical values including total lipids and cholesterol as well as alanine aminotransaminase and alkaline phosphatase in rabbits submitted to 32.2°C (Jimoh, 2019).
To counter the harmful effects of heat stress, the antioxidant capacity of the animal can be improved by supplementation of vitamin C and E, minerals and trace elements such as zinc, copper, sodium, potassium (Kumar *et al.*, 2011). Additionally, substances with antioxidant activity such as green tea extract (Abshenas *et al.*, 2011), powder of *Zingiber officinale* (Habeeb *et al.*, 2019) and *Moringa oleifera* extract (El-Desoky *et al.*, 2021) can be used.
Moringa oleifera is perennial tree with 5 to 12 m of height belonging to the *Moringaceae* family and is considered as one of the best antioxidant plants throughout the world (Wadhwa, 2013). All parts of this plant including leaves, stembarks, pods and seeds, have been reported to be rich in various bioactive compounds such as alkaloids, saponins, phenols, flavonoids, glycosides, terpenoids and tannins (El-Alfy e*t al.*, 2011) and have been reported to alleviate damages caused by oxidative stress (Chisholm, 2015). However, leaves of

Moringa oleifera have been reported to possess better antioxidant activity (Chumark *et al.*, 2008) and this has led to its overuse compared to other parts of this plant. It is worth exploring the efficacy of other parts of this plant for their role in overcoming oxidative stress in animals. This study was designed to evaluate the efficacy of *Moringa oleifera* aqueous seed extract in attempt to overcome the effects of heat stress on biochemical parameters and oxidative stress biomarkers.

MATERIALS AND METHODS

Animal husbandry

A total of 28 female New Zealand rabbits, aged 6 months and weighing between 2015.6 and 2322.7g were used. Throughout the experimental period, feed and water were provided *ad libitum* and all animals were provided with the basal commercial pelleted ration that met all nutritional requirements of rabbit does according to the National Research Council (NRC, 1977). Before starting the experiment, the animals were weighed and randomly assigned to 4 groups of 7 female rabbits each with comparable body weight and distributed to T0 and T3 (normal and positive control respectively), T1 and T2 (low and high dose of *Moringa oleifera* respectively). Thereafter, during 80 consecutive days, animals were submitted to different room temperatures, relative humidity, temperature humidity index (THI) and of *Moringa oleifera* aqueous seed extract (MOASE) as follows: T0: ambient temperature (18–24°C), 59 ±0.48%, 23.6 ±1.52; T1: 35–36°C, 64 ±0.6%, 32.5 ±0.7, 100 mg/kg b.w. of MOASE; T2: 35–36°C, 64 ±0.6%, 32.5 ±0.7, 200 mg/kg b.w of MOASE; T3: 35–36°C, 64 ±0.6%, 32.5 ±0.7. The heat was induced in each rabbit cage, using electrical heaters (ARMCO; India) from 08:00 h to 16:00 h followed by exposure to the normal air temperature as in the control group from 16.00 h to 08:00 h. During the experimental period, the relative humidity and ambient temperature were recorded twice daily using an automatic thermo-hygrometer (RC dalys, Size: 48x28.6x15.2mm, temperature precision: ±1°C, hygrometry precision: ±5). The selected range of temperature, relative humidity and THI were chosen according to results observed in the pilot study as reported by Mutwedu *et al.* (2020) and was classified as very severe heat stress (Marai *et al.*, 2002). The seeds of *Moringa oleifera* (Lam) aged 2 to 3 years were collected from Masii village of Machakos County in Kenya and were used to obtain the aqueous extract. *Moringa oleifera* aqueous seed extracts (MOASE) were administrated *per os* once a day for 80 days using an endogastric cannula while normal and negative control animals (T0 and T3) orally received 10 ml of distilled water daily.

Oxidative stress biomarkers biochemical analysis

After 80 days of the experimental period, all animals were fasted for 24h and slaughtered. The blood was collected directly by cardiac puncture before sacrificing, put in tubes free from anticoagulant, centrifuged at 3000 rpm for 15 min and supernatant separated as serum and preserved at -20°C for the evaluation of serum content in total cholesterol, albumin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), urea, creatinine, protein and glucose using commercial assay kits.

Immediately after weighing, the right kidney was ground in 0.9% NaCl solution to obtain its 15% homogenates. The resulting homogenate was centrifuged at 4800 rpm for 60 min at 4°C and the aliquots of supernatant kept at -20°C for biochemical estimation of oxidative stress biomarkers. The bovine serum albumin was used as standard in the determination of protein content according to the method described by Lowry *et al.* (1951). Enzymatic activities of catalase (CAT) and reduced glutathione (GSH) as well as the levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were assessed in kidney homogenates using a spectrophotometer (GENESYS 20.0) according to the methods described by Habbu *et al.* (2008), Dimo *et al.* (2006), Kodjo *et al.* (2016) and Sajeeth *et al.* (2011), respectively.

Statistical analysis

All data were submitted to analysis of variance using XL STAT for Windows 10 Software. Results are expressed as mean ± SD, and treatment effects among experimental groups and controls assessed using one-way ANOVA. The differences in mean values were compared using the Tukey HSD post hoc test at 5% significance level.

RESULTS AND DISCUSSION

There was a significant increase (P<0.05) in creatinine, urea, ALAT, ASAT levels and a

decrease (*P*<0.05) in glucose and protein content in animals submitted to heat stress (T1, T2, T3) compared to those of the control group (T0) (Table 3). However, there was an amelioration of these parameters following MOASE administration in a dose dependent manner in heat stressed animals. These results corroborate the findings of Thiruchelvi *et al.* (2012) where 200 mg/kg body weight of the aqueous extract of *Terminalia chebula* was administered following Cadmium toxicity in rats for 21 days. As *Moringa oleifera* extract reduce the levels of raised serum ALAT, ASAT, urea and creatinine and restored the hepatic and renal functions, it has been indicated that the contents of *Moringa oleifera* not only protected the integrity of kidney but also increased its regenerative and reparative capacity (Albrakati, 2017). Additionally, the increase of glucose and protein levels in animals subjected to heat stress and co-treated with MOASE corroborate the findings of Hashem *et al.* (2019) in growing rabbits treated with 250 mg/L of *Moringa oleifera* ethanolic extract in drinking water. The increase in protein content might be associated with the protein intensive metabolism in rabbits' organs following administration of *Moringa* (Melesse *et al.*, 2013).

Table 1: Variation in biochemical parameters administration of escalating doses of MOASE in female rabbits exposed to heat stress.

Parameters	T0	T1	T2	T3	*p*-value
Cholesterol (mg/dl)	136.48±5.27	141.12±8.87	139.14±10.65	136.22±8.46	0.241
Creatinine (mg/dl)	0.64±0.04^{d}	0.75±0.08^{b}	0.72±0.09^{c}	0.86±0.07^{a}	0.007
Urea (mg/dl)	94.41±2.15^{d}	122.15±6.25^{b}	101.92±5.92^{c}	146.09±16.22^{a}	0.038
ALAT (U/L)	44.56±2.08^{d}	53.21±3.37^{b}	48.81±1.08^{c}	59.22±2.38^{a}	0.035
ASAT (U/L)	19.54±1.86^{d}	27.15±1.93^{b}	25.88±1.74^{b}	33.37±2.15^{a}	0.028
Glucose (mmol/L)	7.94±0.18^{a}	7.12±0.88^{a}	7.52±0.71^{a}	4.51±0.42^{b}	<0.001
Total protein (g/L)	78.12±2.27^{a}	71.03±1.33^{c}	74.91±1.25^{b}	60.86±3.54^{d}	0.014
Total albumin (g/dl)	6.92±0.48	7.12±0.67	6.56±0.88	6.81±0.77	0.421

The superscripts a, b, c, d: means that mean values are significantly different at *P* <0.05; T0 control group, T1: 35–36°C+100 mg *MOASE*, T2: 35–36°C+200 mg *MOASE*, T3: 35–36°C. ALAT: alanine aminotransferase; ASAT: aspartate aminotransferase; n=7. Note a significant decrease in creatinine, urea, ALAT and ASAT while an increase in glucose and protein in rabbits submitted to heat stress and receiving doses at 100 mg and 200 mg/kg b.w. of MOASE compared to dose 0 mg/kg b.w. of MOASE

There was a significant decrease (*P*<0.05) in protein content while MDA significantly increased (*P*<0.05) in animals exposed to heat stress compared the control group (T0). However, these parameters improved with the increase in doses of MOASE in the same animals submitted to heat stress without reaching the values of the control group. In addition, the level of CAT, SOD and GHS significantly increased (*P*<0.05) in animals of control group (T0) and those submitted to different doses of MOASE (T1 and T2) compared to animals submitted to heat stress alone (T3). These results are in agreement with findings in mice exposed to Methotrexate and later treated with *Moringa oleifera* leaf extract for 12 consecutive days (Soliman *et al.,* 2020). These antioxidants are used by the biological systems to protect against injuries caused by oxidative damage due to excessive heat (Li *et al*., 2018). The decrease in MDA concentration could be explained by the inhibitory effect of *Moringa oleifera* aqueous seeds extract earlier reported on lipid peroxidation (Soliman *et al.*, 2020). The improvement of these antioxidant enzymes may be as a result of high concentration of phenolic substances such as flavonoids and tannins in *Moringa oleifera,* which are very effective in neutralizing oxygen free radicals (Oloruntola *et al.*, 2018).

Table 2: Variation of oxidative stress biomarkers in the kidneys following administration of escalating doses of MOASE in female rabbits exposed to heat stress.

Parameters	T0	T1	T2	T3	*p*-value
Protein (mg/ml)	13.87±0.23^{a}	8.81±0.37^{c}	10.12±0.33^{b}	5.14±0.69^{d}	0.026
MDA (nmol/mg tissues)	16.37±1.21^{d}	26.04±2.91^{b}	22.16±2.45^{c}	37.43±3.84^{a}	0.029
CAT (UI/mg tissues)	11.23±1.27^{a}	10.77±1.16^{a}	10.82±1.08^{a}	5.97±0.941^{b}	<0.001
SOD (UI/mg tissues)	7.21±0.77^{a}	7.08±0.25^{a}	7.25±0.66^{a}	5.08±0.44^{b}	<0.001
GSH (nmol/mg of tissue wet)	9.37±0.18^{a}	8.81±0.69^{a}	8.93±0.75^{a}	6.01±0.14^{b}	<0.001

The superscripts a, b, c, d: means the mean values are significantly different at *P* <0.05; T0 control group, T1: 35–36°C+100 mg *MOASE*, T2: 35–36°C+200 mg *MOASE*, T3: 35–36°C. CAT: catalase, GSH: reduced glutathione, SOD: superoxide dismutase, MDA: lipid peroxidation; n=7. Note a significant increase in kidney protein, CAT, SOD and GSH while a decrease in MDA in rabbits submitted to heat stress and receiving doses at 100 mg and 200 mg/kg b.w. of MOASE compared to dose 0 mg/kg b.w. of MOASE

CONCLUSION

The administration of 200 mg/kg of body weight of *Moringa oleifera* aqueous seed extract in female rabbits exposed to heat stress (35°C-36°C) partially restored their physiological parameters. This includes the amelioration of biochemical parameters and enzymatic antioxidant biomarkers. The mentioned improvement could be due to a wide diversity of bioactive compounds contained in *Moringa oleifera* which interfered with free radicals produced by heat stress.

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ORAL ADMINISTRATION OF THE FLAVONOID QUERCETIN. A DOSE-RESPONSE APPROACH FOR PRODUCTIVE STUDIES IN THE RABBIT DOE

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ABSTRACT

Rabbit does are subjected to increased oxidative stress caused by the high energy demands of industrial rabbit meat production systems. To overcome this, diet inclusion of antioxidants, such as the flavonoid quercetin, may potentially improve their reproductive efficiency and lifespan. However, there is a lack of information about a safe dose interval of quercetin in the rabbit doe. For this purpose, 30 animals were randomly allocated in 5 experimental groups and each rabbit doe administered 0 (Control), 150 mg/Kg (Group 1), 300 mg/Kg (Group 2), 450 mg/Kg (Group 3) or 600 mg/Kg (Group 4) of quercetin orally once a day for 8 days. At days 0 and 8 health status by means of clinical examination and biochemical parameters were assessed. Our results from the clinical and biochemical analysis show that even though significant differences among days 0 and 8 appeared, none of the biochemical parameters was out of the reference intervals in the rabbit. In conclusion, none of the quercetin doses (150-600 mg/Kg for 8 days) caused a negative effect neither in the clinical nor in the biochemical variables measured, so these doses may be used for studying the potential benefits of quercetin on rabbit does productivity and reproductive parameters.

Key words: rabbit, quercetin, biochemical analysis, clinical examination, dose-response study.

INTRODUCTION

Industrial rabbit meat production requires high energy mobilization in the rabbit doe, as usually gestation and lactation overlap, causing a negative energy balance and the increase of Reactive Oxygen Species (ROS) production, which decreases productive efficiency and shortens their lifespan (Lorenzo et al., 2014). To overcome this situation, antioxidants shall be added to the diet, aiming to improve ROS balance and thus rabbit does lifespan. Quercetin (QUR, 3,5,7,30,40-pentahydroxyflavone) is a potent flavonoid whose antioxidant properties have been proven (Li et al., 2016). However, there is a lack of information regarding a safe oral dosage of QUR for rabbits in the literature. Thus, the objective of this study was to determine the oral dosage administration of the flavonoid QUR in the rabbit doe to assess if it may be used in livestock.

MATERIALS AND METHODS

Experimental design and reagents

A total of 30 rabbit does (4 Kg average weight) were divided into 5 experimental groups of 6 animals each. Groups were allocated according to the dose of QUR (Merck-SIGMA, Ref 337951) administered orally (PO) once a day for 8 days. QUR was mixed with commercial orange juice. According to Manta et al. (2020), just 20 % of the QUR administered orally seems to be absorbed in the gut, thus, each of the rabbit does of each of the 5 experimental groups was administered 0 (Control), 150 mg/Kg (Group 1), 300 mg/Kg (Group 2), 450 mg/Kg (Group 3) or 600 mg/Kg (Group 4) of quercetin orally once a day for 8 days.

Animals

New Zealand White x California rabbit does were allocated in the experimental farm of the Department of Agrarian Production (E.T.S.I.A.A.B. of UPM) in individual cages with free access to water and fed *ad libitum* with a commercial diet (NANTA, Spain). Temperature (20-25ºC), humidity (60-75 %), ventilation and light/darkness (16:8) were controlled by a computed-automated system. All the experimental procedures were approved by the Animal Ethics Committee of the Polytechnic University of Madrid (PROEX 324.6-23) and were in compliance with the Spanish guidelines for the care and use of animals in research (BOE, 53/ 2013), according to European Union Regulation 2010/63/UE.

Clinical examinations

On days 0 and 8 of the study, females were subjected to a veterinary clinical examination to assess their health status. The parameters assessed and the physiological and pathological criteria are defined in the following table:

Table 1. Health parameters assessed by clinical examination

Parameter	Healthy Individuals [5]	Unhealthy Individuals
General Status (GS)	Alert	Apathetic, stressed
Lymph nodes size and texture	Physiological size and texture	Increased or decreased size Harder or softer texture
Mucous membranes aspect	Pale pink	Hyperemic or anemic
CRT (capillary refill time)	< 2 sec	> 2 sec
Heart Rate (HR)	200-300 bpm	Bradycardia: < 200 / min Tachycardia: > 300 / min
Respiratory Rate (RR)	30-60 breaths / min	Bradypnea: < 30 / min Tachypnea: > 60 / min
Abdominal Palpation	Soft and non-discomfort	Hard Intra-abdominal mass

Biochemical analysis

On days 0 and 8 of the study, a blood sample of 2 mL was collected from the marginal ear vein in an EDTA tube and centrifuged at 700 x g for 20 min at 4 ºC. Sequentially, plasma was separated and stored at -80 ºC until biochemical analysis.

Biochemical parameters were measured using an automated chemistry analyzer (VChemy S®, Roche Diagnostics, Switzerland), and included: glucose (GLU), total protein (TP), albumin (ALB), globulin (GLOB), albumin/globulin ratio (A/G), urea (UREA), creatinine (CREA), uric acid (UA), alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total bilirubin (TBIL), amylase (AMY), creatine kinase (CK), total cholesterol (TCHOL), triglycerides (TG), high density lipoproteins (HDL) and low density lipoproteins (LDL).

Statistical Analysis

Statistical analyses were performed using the R environment (v 4.3.1) through RStudio. On Day 0 all animals were considered as a single group as no QUR had been administered yet. Data normality was assessed by Shapiro-Wilk test. Blood biochemical metabolites

concentration as well as HR and RR were compared among all groups on day 8 by one-way ANOVA for normally distributed variables and a post hoc test of Tukey HSD, or Kruskal-Wallis for not normally distributed variables and a post hoc Pairwise Wilcoxon Test. Blood biochemical metabolites concentration was compared at day 8 vs day 0 by T-test for normally distributed variables or Mann-Whitney-Wilcoxon test for not normally distributed variables. The significant level was set at $p < 0.05$ for all tests.

RESULTS AND DISCUSSION

Oral QUR administration at the dosage and intervals performed in this study did not negatively affect the general health status of the rabbit does, as no alterations were found in the characteristics of their lymph nodes, mucous membranes, CRT, HR and abdominal integrity (as examined by abdominal palpation) being all of them within the physiological range (Pastor Meseguer, 2006). However, the RR was slightly above physiological values (30-60/min) in all experimental groups, including the control one (Table 2). This could be attributed to handling the rabbit does for the clinical examination itself and therefore, not to the administration of QUR, as no significant differences among groups or days were found.

Table 2. Mean ± Standard Error of the Mean of the Respiratory Rate assessed in the clinical examinations for each experimental group and sampled day

	Day 0	Day 8				
		Control	Group 1	Group 2	Group 3	Group 4
Respiratory Rate	77.66 ± 1.77	76.67 ± 1.49	78.33 ± 1.37	85.00 ± 1.00	82.00 ± 1.53	84.00 ± 1.63

Blood plasma biochemical profile at day 8, revealed no differences between the experimental groups (Table 3). Interestingly, significant differences between days 0 and 8 were observed for the following parameters: ALB, AST, ALT, UREA, UC, GLUC and LDL (Table 3).

Significant differences observed for the parameters of ALB, AST and UC were between days 0 and 8 in the Control Group and, thus, were not induced by QUR administration. ALB, ALT, UREA, and GLUC levels were significantly different in Group 1 on day 8 compared to day 0, while LDL was significantly different in group 3 at day 8 compared to day 0. No significant differences were observed among Groups 2 or 4 and the other groups or the Control between days 0 and 8. Even though significant differences among days appeared, none of the biochemical parameters was found to be out of the reference intervals in the rabbit (Benson, 1999; Rodríguez et al., 2017), thus, QUR did not seem affect any of those parameters.

CONCLUSIONS

The rabbit doe's blood biochemical profile and health status were not affected by oral QUR administration when administered orally at doses of 150 to 600 mg/Kg for 8 days. Therefore, these doses could be used for long-term studies about QUR effect on rabbit productive and reproductive parameters.

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Table 3. Mean ± Standard Error of the Mean of the blood plasma biochemical metabolites concentrations for each experimental group and sampled day

	Day 0	Day 8				
		Control	Group 1	Group 2	Group 3	Group 4
TP (g/dL)	6.52 ± 0.13	6.52 ± 0.11	6.35 ± 0.18	6.18 ± 0.16	6.42 ± 0.13	6.20 ± 0.11
ALB (g/dL)	4.48 ± 0.11	4.07 ± 0.07 *	4.18 ± 0.08 *	4.28 ± 0.15	4.38 ± 0.11	4.25 ± 0.10
GLOB (g/dL)	2.04 ± 0.18	2.45 ± 0.17	2.17 ± 0.18	1.90 ± 0.30	2.03 ± 0.22	1.95 ± 0.17
AG	2.37 ± 0.23	1.71 ± 0.15	2.01 ± 0.20	2.56 ± 0.40	2.30 ± 0.28	2.28 ± 0.23
TBIL (mg/dL)	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
GGT (U/L)	6.80 ± 0.51	6.67 ± 1.26	6.17 ± 0.48	6.83 ± 0.48	8.00 ± 1.13	7.17 ± 0.75
AST (U/L)	32.70 ± 5.07	16.00 ± 2.59 *	27.83 ± 4.49	36.00 ± 4.86	26.67 ± 5.91	30.00 ± 4.51
ALT (U/L)	41.00 ± 5.65	28.00 ± 5.14	25.67 ± 4.05 *	36.50 ± 4.91	48.33 ± 6.88	29.17 ± 6.65
ALKP (U/L)	31.20 ± 5.19	19.83 ± 1.40	25.17 ± 4.34	37.83 ± 8.77	39.33 ± 10.62	33.00 ± 8.07
AMY (U/L)	288.00 ± 15.11	262.17 ± 23.51	270.17 ± 13.94	324.83 ± 39.77	282.33 ± 28.70	290.67 ± 18.49
CK (U/L)	525.10 ± 53.89	442.83 ± 69.33	547.83 ± 39.39	781.50 ± 185.06	505.50 ± 81.89	553.83 ± 44.51
CREA (mg/dL)	0.94 ± 0.05	1.07 ± 0.07	1.00 ± 0.08	1.02 ± 0.07	0.98 ± 0.08	0.92 ± 0.03
UA (mg/dL)	0.17 ± 0.00	0.18 ± 0.01	0.23 ± 0.06	0.17 ± 0.00	0.18 ± 0.01	0.17 ± 0.00
UREA (mg/dL)	51.20 ± 2.68	44.67 ± 2.99	42.83 ± 2.12 *	46.00 ± 2.34	47.67 ± 1.99	44.33 ± 1.38
UC (mg/dL)	56.10 ± 4.19	42.33 ± 2.46 *	44.33 ± 4.10	46.17 ± 3.48	49.83 ± 3.42	48.67 ± 2.75
GLUC (mg/dL)	136.36 ± 8.33	117.28 ± 2.94	111.97 ± 1.87 *	115.00 ± 4.84	125.80 ± 5.64	122.02 ± 4.05
TCHOL (mg/dL)	60.90 ± 4.80	76.33 ± 8.39	64.67 ± 8.25	67.50 ± 4.26	69.83 ± 5.88	66.67 ± 3.53
TRIG (mg/dL)	51.30 ± 5.17	68.00 ± 11.74	55.67 ± 7.89	72.17 ± 14.37	49.67 ± 5.35	55.33 ± 9.95
HDL (mg/dL)	32.00 ± 1.93	31.33 ± 2.11	27.50 ± 2.57	26.33 ± 2.20	29.50 ± 3.21	29.67 ± 1.71
LDL (mg/dL)	18.70 ± 3.86	31.50 ± 8.92	26.00 ± 6.98	26.83 ± 3.17	30.33 ± 3.70 *	26.17 ± 4.37

Means marked with an asterisk indicate significant differences between day 0 and the marked group at day 8. No significant differences were found among groups at day 8. Abbreviations defined in the section Biochemical analysis.

EFFECT OF HOUSING SYSTEM ON BLOOD AND INTESTINAL TRAITS OF LOCAL RABBIT

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ABSTRACT

This study investigates the impact of housing systems and slaughter age on various welfare indicators in 300 Grigio di Carmagnola (GC) rabbits. Upon weaning at 35 days, the rabbits were evenly distributed among three housing systems: Group housing (G), Single cages (S), and a Mixed pilot system (M). In the mixed system, rabbits were initially housed in groups until puberty and then transitioned to single cages until commercial slaughter at 120 and 150 days. During slaughter, blood and intestinal samples were collected from 20 rabbits per group for analysis of hematological parameters and intestinal histomorphometry. The group housing system, particularly after puberty (80 days), exhibited instances of intra-group conflicts resulting in significant negative consequences. Conversely, the single cage system led to reduced immune competence and increased damage to intestinal villi, especially notable at 120 days ($P<0.05$). Rabbits in the mixed system demonstrated elevated lymphocyte levels ($P=0.05$) and improved absorption rates ($P<0.001$), likely attributable to the transition to single cages at 80 days until commercial slaughter. Based on these initial findings, the mixed pilot system appears to offer a favorable balance between rabbit welfare (utilizing group housing until 80 days) and productivity (switching to single cages until 150 days) for Piedmont's local rabbit production, particularly for cycles exceeding 80 days.

Key words: Local rabbit, Housing system, Slaughter age, Hematic parameters, Intestinal histomorphometry.

INTRODUCTION

For growing rabbits, the new guidelines issued by the Italian Ministry of Health recommend pen and group farming. However, while this breeding system appears feasible for commercial hybrids with fast growth rates and a mean slaughter age of 70-77 days, it may not be suitable for local breeds such as Grigio di Carmagnola rabbits. These local breeds typically have medium to slow growth rates and a mean slaughter age of 120-150 days, and implementing pen and group farming could potentially compromise rabbit welfare by increasing the risk of severe injuries and stress (Rommers et al., 2006). Furthermore, achieving the recommended targets could impose greater financial burdens on farmers (Princz et al., 2008). This economic strain may be more manageable for industrial rabbit producers, whose feed-to-gain ratio is approximately 2.5, compared to breeders of local breeds, which typically have slower to medium growth rates and a feed-to-gain ratio of ≥ 5. Nevertheless, local breeds possess valuable traits such as good production potential and resilience to stressors, making it crucial to preserve these specific adaptive characteristics in the future. Rabbit lymphocytes, in particular, are highly susceptible to the effects of chronic stress (Washington and Hoosier, 2012). Both physiological stresses such as trauma, burns, and surgeries, as well as psychological stress, can induce dysfunction in the intestinal barrier of mammals (Soderholm and Perdue, 2001). Therefore, it is imperative to study and define a suitable balance between rabbit productivity and welfare. The objective of this study was to assess the effects of three housing systems (HS) - Single cage (S), Group colony (G), and Mixed pilot system (M) - as well as two slaughter ages (A: 120-150 days), on the hematological traits and intestinal health of Grigio di Carmagnola rabbits.

MATERIALS AND METHODS

The trial was performed at Department of Veterinary Science facilities, during the period from March to July 2022 and were approved by the Bioethics Committee of the University of Turin (Prot. n. 0245520)

Animals and experimental design

300 weaned rabbits of the Carmagnola breed were randomly allocated into three different housing systems (HS): Traditional single cages (S), Group farming (G), and a Mixed pilot system (M). In the mixed system, rabbits were initially raised in group housing until reaching sexual maturity (80 days old), after which they were transferred to single cages until slaughter. Due to an increase in severe injuries and stress, all rabbits in the group farming (G) system were urgently slaughtered at 100 days old. Consequently, the experimental protocol proceeded with the single cage (S) and mixed system (M) Carmagnola rabbits. At the age of 120 and 150 days, which are the commercial ages for slaughtering Grigio rabbits, the single cage (S) and mixed system (M) rabbits were slaughtered after a 12-hour fasting period. Blood samples (20 per group per age) were collected during slaughter. Following carcass dissection, intestinal samples from the duodenum, jejunum, and ileum, as well as liver and spleen specimens, were excised and preserved in a 10% buffered formalin solution for subsequent morphometric and histopathological analyses, respectively.

Rabbit's blood traits

Complete blood count was performed on EDTA blood samples with an automated laser analyzer (ADVIA®120 Hematology System, Siemens Diagnostics). Automated differentials were checked by microscopic evaluation of blood smears stained with May Grunwald-Giemsa. Main hematic parameters determined were White Blood Cell Count (WBC; cells/µL), Neutrophils (NEUT; %) and Lymphocytes (LYM; %). These parameters were analyzed to gain insights into the immune system and overall health status of the individuals across different age groups.

Rabbit's intestine histomorphometry

The morphometric indices evaluated in this study included villus height (VH), measured from the tip of the villus to the crypt, crypt depth (CD), measured from the base of the villus to the submucosa, and villus width (VW). These analyses were conducted on 10 well-oriented and intact villi and 10 crypts selected from both the duodenum and jejunum. Additionally, the thickness of the muscular layer (MuT) and mucosal weight (MW) were recorded at three standardized points within the gut mucosal and muscular layers per captured field. All measurements were expressed in millimeters (mm). Furthermore, histopathological assessments were performed to evaluate hepatocyte degeneration (LD) and liver inflammation (LIV infl), as well as follicular hyperplasia/depletion in the spleen (SPL Iperl/depl). The overall inflammation of the duodenum (DU_infl), jejunum (JE_infl), and ileum (IL_infl) was histologically assessed, examining inflammatory infiltrates in the mucosa, submucosa, and muscularis layers for each segment. Additionally, the activation of the Gut-Associated Lymphoid Tissue (GALT) in the submucosa was considered, including the number and size of lymphoid structures. Histopathological alterations were categorized as absent (score = 0), mild (score = 1), moderate (score = 2), or severe (score = 3). The total score for each gut segment was calculated by summing the scores for the mucosa and submucosa.

Statistical Analysis

A two-way analysis of variance (ANOVA) was performed to assess the impacts of the housing system (HS: S vs M) and slaughter age (A: 120d vs 150d) on blood and intestinal traits. Subsequently, multiple comparisons of the means were conducted using the Duncan test to calculate the least significant difference. Statistical analyses were performed using R software, specifically Version 3.1.2 (R Core Team, 2014). Significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of housing system on blood parameters of Carmagnola's rabbit

Table 1 presents the results regarding the effect of housing systems on blood traits in local rabbits. Among the parameters analyzed, only the percentage of lymphocytes showed a significant effect of the housing system (HS) with a significance level of P<0.05. The M group exhibited higher values compared to the S group, with percentages of 54.8% and 40.5%, respectively. This observed difference could potentially be attributed to the chronic stress response induced by the single cage (S) system. Rabbit lymphocytes are known to be particularly susceptible to stress (Washington and Hoosier, 2012). Chronic psychological stress can lead to elevated levels of glucocorticoids through the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Lacey et al., 2000). Studies in mice have shown that high levels of glucocorticoids are associated with a 50% reduction in peripheral blood lymphocytes (Garvy, King, and Telford, 1993).

Table 1: Effect of housing system and slaughters age and their interaction on blood traits of Carmagnola's rabbits (n=20)

	Housing system (HS)		Age (A)		SEM		p-value	
	S	M	120	150	HS	A	HS	A
WBC (cells/µL)	11.2	12.1	12.9	10.2	0.604	0.509	0.170	0.521
NEUT* (%)	48.0	34.9	46.7	40.3	2.202	1.731	0.563	0.102
LYM* (%)	40.5[b]	54.7[a]	43.5	49.6	2.311	1.819	0.044	0.084

[a, b]: means with different letters on the same row differ significantly for P≤0.05. *: χ value. WBC: White Blood Cells; NEUT: Neutrophils; LYM: Lymphocytes.

Effect of housing system on intestine histomorphometry of Carmagnola's rabbit

Table 2 presents the intestinal traits of rabbits at commercial slaughter, with the housing system showing a significant effect on villus height (VH) (P<0.05), mucosal weight (MW) (P<0.001), and hepatocyte degeneration (LD) (P<0.05). The M group exhibited higher values for VH (0.32mm vs 0.26mm for S and M groups, respectively), MW (0.40mm vs 0.34mm for S and M groups, respectively), and LD (0.61 vs 0.19 in S and M groups, respectively) compared to S rabbits. This indicates that rabbits in the M group had longer villi (VH), resulting in higher mucosal weight (MW), indicative of enhanced absorption capabilities. These findings suggest potential improvements in growth and nutrient absorption efficiency in the M system. The observed improvement in intestinal morphology in the M group may be linked to enhanced activity of digestive enzymes and improved small intestinal mucosa morphology, as supported by rabbit live and slaughter performance data presented elsewhere (WRC2024). Additionally, the improved intestinal morphology in the M group may contribute to a better intestinal microbial balance, leading to improved feed digestion and absorption. This hypothesis is supported by the fact that the restriction of movement, as highlighted by EFSA (2020), had the highest welfare impact scores for growing rabbits, and the LD value decreased after one month (150 days slaughter) when rabbits adapted to the new system. Chronic stress, as induced by the S housing system, can have a profound effect on the gastrointestinal tract, potentially leading to shorter villi due to inflammation, which in turn reduces the absorption surface. As previously mentioned, MW was lower in the S group, leading to a thicker mucosa. These findings regarding the characteristics of villi in the S group are consistent with their lower immune competence (% LYM) due to chronic stress associated with S housing. Moreover, induced stress can lead to negative changes in gut morphometry or intestinal inflammation, as supported by previous research (Berenjian et al., 2021). Age also showed a significant effect on muscular thickness (MuT) and hepatocyte degeneration (LD) gastrointestinal traits (P<0.05), with younger rabbits exhibiting higher MuT and LD, potentially as a consequence of more inflamed villi.

Table 2: Housing system and slaughter age effect on intestinal traits of Carmagnola's rabbit (n=20)

	Housing system (HS)		Age (A)		SEM		p-value	
	S	M	120	150	HS	A	HS	A
VH (mm)	0.26[b]	0.32[a]	0.29	0.29	0.022	0.324	0.024	0.870
CD (mm)	0.02	0.03	0.03	0.02	0.003	0.003	0.911	0.133
VW (mm)	0.04	0.05	0.04	0.04	0.001	0.001	0.104	0.516
MW (mm)	0.34[B]	0.40[A]	0.37[b]	0.37[b]	0.039	0.026	0.001	0.802
MuT (mm)	0.06	0.06	0.07[a]	0.06[b]	0.064	0.048	0.744	0.042
DU_Infl	1.31	1.48	1.56	1.25	0.243	0.343	0.662	0.411
LIV_Infl	0.31	0.57	0.44	0.45	0.166	0.164	0.125	0.975
LD	0.19[b]	0.61[a]	0.61[b]	0.20[a]	0.172	0.120	0.012	0.016
JE_Infl	1.08	0.82	1.08	0.82	0.446	0.246	0.429	0.426
SPL_Iperpl	0.55	0.62	0.76	0.84	0.046	0.051	0.631	0.523
SPL_Depl	0.69	0.71	0.82	0.79	0.033	0.065	0.415	0.325
IL _Infl	0.61	0.57	0.65	0.61	0.051	0.069	0.539	0.442

[a, b]: means with different letters on the same row differ significantly for P≤0.05. A, B: means with different letters on the same row differ significantly for P≤0.001. VH: villus height; CD: crypt depth; VW: villus width; MW: mucosal weight; MuT: muscular thickness; DU_Infl: duodenum inflammation; LIV_Infl: liver inflammation; LD: liver degeneration; JE_Infl: jejunum inflammation; SPL_Iperpl: spleen hyperplasia; SPL_Depl: spleen depletion; IL_Infl: ileum inflammation.

CONCLUSIONS

In summary, the study reveals that group housing (G) systems lead to conflicts in rabbit's post-puberty, making them unsuitable for medium to slow-growing breeds. Single cage (S) systems, on the other hand, result in decreased immune competence and intestinal damage. Conversely, the mixed pilot system (M) shows better outcomes in lymphocyte level and intestinal health. Given the longer rearing cycle (>80 days) of Grigio di Carmagnola rabbits, these findings are significant and suggest that the mixed pilot system (M) provides a balanced management of the welfare and the physiological needs of rabbits.

ACKNOWLEDGEMENTS

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MATERNAL MELATONIN: EFFECTIVE INTERVENTIONS FOR THE BENEFICIAL DEVELOPMENTAL PROGRAMMING OF HAIR FOLLICLES IN RABBIT OFFSPRING

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ABSTRACT

Maternal melatonin (MT) can pass through the placental barrier and enter the fetal circulation. MT can improve hair follicle (HF) development, which can potentially be enhanced through nutritional intervention strategies during pregnancy. However, little is known about the effect of maternal MT treatment on fetal HF development. In this study, we implanted pregnant rabbits with 10 mg of MT- and non-MT-containing silica gel microcapsules implanted in the dorsum of the neck and gently to prevent abortions, and measured the HF density and extent of HF cell apoptosis in the rabbit neonates. Using a combination of untargeted metabolomics and RNA sequencing, the mechanism by which maternal MT affects offspring HFs was clarified by analyzing the correlations and interactions between differentially expressed genes and metabolites. We found that implanting MT significantly reduced HF cell apoptosis and promoted HF density in neonatal rabbits. Some genes, such as *JUN*, *FOS*, and *NGF*, were downregulated while equol and nicotinamide adenine dinucleotide phosphate (NADP) were significantly upregulated. These genes regulate the apoptosis pathway to decrease the apoptosis rate of HF cells and regulate the MAPK pathway to increase HF density. *JUN* and *FOS* regulate the TNF signaling pathway and upregulate the downstream metabolite estradiol to increase HF density. We propose that maternal treatment with MT promotes beneficial developmental HF programming in offspring and our findings support the possibility of clinical interventions against HF development by targeting MT.

Keywords: Hair follicle, Melatonin, Reprogramming, Offspring, Rex rabbit

INTRODUCTION

Hair folliculogenesis is completed during the embryonic period, and ectodermal–mesodermal interactions are tightly regulated (Schneider, 2009). Fetal development encompasses a programming process that involves the interaction of genetic information and environmental factors, resulting in the formation of highly adaptable phenotypes (Rasmussen, 2022). Thus, improving hair follicle (HF) development is crucial during the fetal period; however, to our knowledge, there is limited research on this topic. Melatonin (MT) is closely associated with the hair growth cycle, hair pigmentation, and melanoma growth control (Amaral and Cipolla-Neto, 2018). Mammalian embryonic development depends on maternal MT supplied through the placenta, which crosses the placental barrier without modification (Sagrillo-Fagundes , 2016). This study aimed to determine whether maternal MT treatment during pregnancy benefits the developmental programming of HFs and investigate the mechanisms underlying the function of maternal MT in offspring HF development.

MATERIALS AND METHODS

Animal management and experimental design

The pregnant rabbits were randomly divided into two groups, the MT treatment (MT group, n = 40) and control group (CR group, n = 39). In the MT group, MT microcapsules were implanted subcutaneously into the rabbits. Microcapsules of the same size containing only silica gel were implanted into the rabbits of the CR group. All rabbits were raised in natural

light with food and water provided *ad libitum*. At birth, seven neonates from various litters of the two groupswere euthanized via cervical dislocation and immediately skinned (following the animal welfare principle of "reduction", seven individuals were able to meet the objectives of this experiment). Next, 1 cm × 1 cm skin samples from the center of the back were collected for RNA sequencing and metabolomics analysis. Another set of skin samples (1 cm diameter) from the back were collected for the Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL) assay.

Statistical analysis

SPSS software (version 20.0) was used to perform statistical analyses regarding HF development. An unpaired Student's *t*-test was used to determine the significance of differences between the MT and CR groups, with significance set at $P<0.05$.

RESULTS AND DISCUSSION

Maternal MT supplementation decreases HF cell apoptosis and promotes HF density in neonatal rabbits

The MT group showed fewer TUNEL-positive apoptotic cells and limited 4,6-diamino-2-phenyl indole (DAPI) staining compared to that in the CR group (Figure 1a).

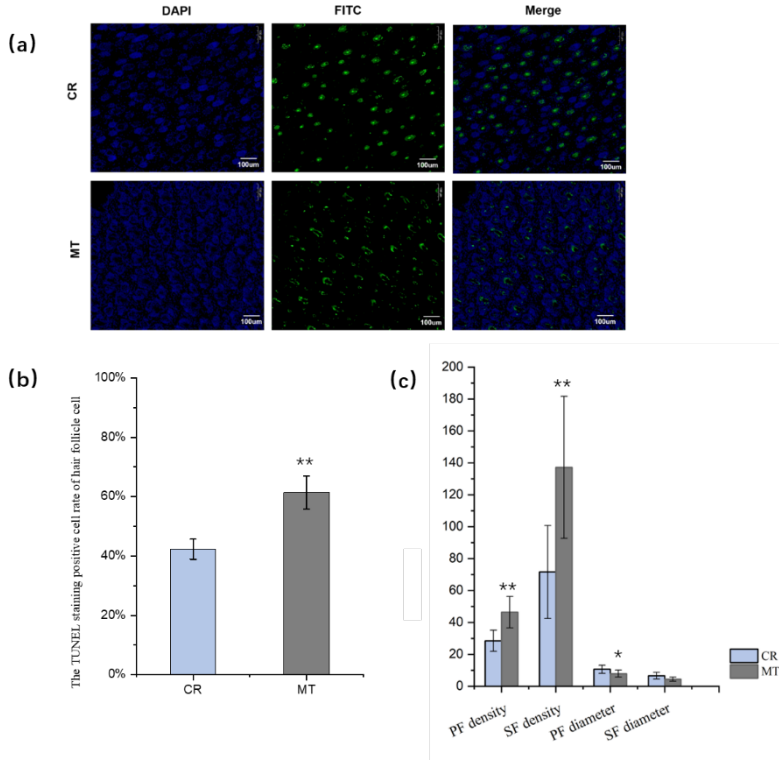

Figure1. (a) Terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling TUNEL staining of rabbit neonate skin samples in the melatonin treat (MT) and control (CR) groups. DAPI: 4,6-diamino-2-phenyl indole ;FITC:Fluorescein Isothiocyanate (b) Comparison of the rate of TUNEL-positive staining in the hair follicle cells of the two groups. (c) Density of primary and secondary follicles of neonatal rabbits in the two groups. (d) Hair diameter of primary and secondary follicles of neonatal rabbits in the two groups.

The apoptosis rate in the MT group was significantly lower than that in the CR group ($P<0.05$, Figure 1b). The MT group showed a higher and lowed degree of hair density and hairiness, respectively (Figure 1c). Other studies suggested that MT could induce HF cell apoptosis. The implantation of MT in cashmere goats was found to enhance the activity of antioxidant enzymes. The use of MT during pregnancy also reduced apoptosis in neonatal rats (Abdollahzade, 2021). In the present study, we showed that MT could reprogram HF development.

Differentially expressed genes and metabolites analysis of neonate skin tissues from the MT and CR groups

In total, 2,532 differentially expressed genes (DEGs) were detected, of which 1,131 were upregulated and 1,401 were downregulated. DEGs were enriched for metabolic pathways related to lipid, amino acid, endocrine-related, nutrient digestion, and absorption pathways. The key genes *FOS*, *JUN*, *NGF*, *FOSL1*, *FGF18*, *DUSP5*, *DUSP1*, *AXIN2*, *IL1A*, *HMGCS2*, *FZD2*, *PDGFRB*, and *RSPO1* were used to elucidate the effect and role of gene expression on offspring HF development after maternal MT implantation. 50 and 43 differentially

expressed metabolites were significantly upregulated and downregulated were identified, respectively. Among of them, pyridoxamine, N-acetylornithine, ursodeoxycholic acid, traumatic acid, dronabinol, pipecolic acid, nicotinic acid, 4-pyridoxic acid, N4-acetylcytidine, 1-(4-methoxyphenyl)-2-propanone, and gamma-glutamylleucine were the key DEMs we screened.

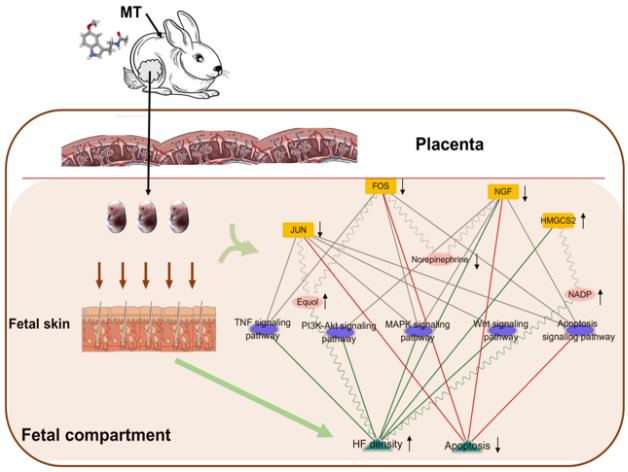

Figure 2. The proposed mechanism of maternal melatonin effective interventions in the developmental programming of hair follicles in rabbit offspring

In summary, maternal treatment with MT promoted HF density and inhibited HF cell apoptosis in neonates. As shown in Figure 2, after implantation during pregnancy, MT crosses the placental barrier and acts on fetal skin to benefit the developmental programming of HFs through inhibition of HF cell apoptosis, and also promoted HF density. This process causes the downregulation of the key genes *JUN*, *FOS*, and *NGF* in the skin. These genes regulate the apoptosis pathway to decrease the apoptosis rate of HF cells and regulate the MAPK pathway to increase HF density. Our study provides evidence that maternal treatment with MT seems to promote developmental HF programming in direct offspring.

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EFFECT OF EARLY MALE RABBIT (ORYCTOLAGUS CUNICULUS) CASTRATION ON BEHAVIOUR, EXTERNAL GENITAL ANATOMY AND WEIGHT GROWTH

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ABSTRACT

Civil society now demands on pet rabbits to be bred in pairs. After puberty, whole rabbits fight and behave in a way that is not appreciated by their owners and that can be life-threatening. If they are sterilised too late, their acquired behaviour may remain (as urine and marking). Our study therefore compared 20 male rabbits neutered early at 40 days of age with 20 of their non-operated brothers. We found that sexual, aggressive, or territorial behaviour had disappeared in the neutered animals. The external genitalia of the neutered rabbits retained their infantile character. The penis almost resembled a vulva, and the scrotum was almost invisible. The weight growth of the rabbits was not affected, male rabbits sterilised early maintained a similar weight gain and average daily gain (ADG) to whole male rabbits, but they were not fed ad libitum.

Key words: Male rabbit, early sterilisation, growth, behaviour, external genitalia.

INTRODUCTION

For ethical concerns, civil society wants pet rabbits to be bred so that they can interact socially with congeners. In Germany, rabbits are sold in pairs. The sex ratio for rabbits is around 50%. Inevitably, reeding rabbits in pairs requires finding a solution to house two males together. Whole adult males fight to the point of mutilation. Sterilisation is one solution (Kalagassi *et al*., 1999). It produces more sociable rabbits. But if it is carried out late, the operation is technically more delicate. In addition, pubescent rabbits develop certain behavioural habits (such as urinating in the air) and late sterilisation does not always correct these undesirable acquired behaviours. Our work therefore involved testing a technique of early sterilisation of males at 40 days. The purpose of our study - validated by the Oniris Veterinary Epidemiological and Clinical Research Ethics Committee - was to assess the impact of this procedure on behaviour, genital organs and weight growth. Another study currently being published will focus on bone growth in the same animals.

MATERIALS AND METHODS

Animals

These were Hycole blanc medium broiler rabbits born on the same day on the same farm. This strain is selected for producing rabbits that are very homogeneous in terms of growth between 35 and 70 days of age, justifying the choice of carrying out the study on this strain of rabbits in order to obtain statistical results with a low standard deviation. The rabbits were 40 days old on the day of the operation. On the same day, they were injected subcutaneously with a transponder enabling them to be identified. The rabbits included in the study weighed between 850 and 950 g at 30 days of age and were clinically healthy, as were their littermates, on the day of inclusion.

The necessary but enough rabbits in the "sterilised" group (test group of 20 subjects) and the "whole" group (control group of 20 subjects) was determined using the Biostatgv website. The 20 surgically sterilised male rabbits were randomly selected from the batch of 40 subjects. All rabbits were vaccinated against myxomatosis and VHD (viral haemorrhagic disease RHDV, RHDV2 strain 2010 and 2016) at 33 days of age.

The 37 rabbits alive at the end of the study were rehabilitated. 22 rabbits found a foster family directly on leaving the study. The remaining 15 were entrusted to a rehabilitation association. The study was validated by an ethics committee.

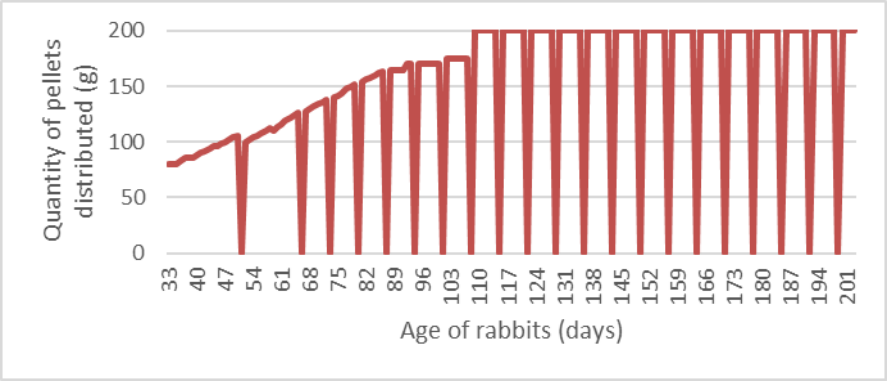
Accommodation

The rabbits were entrusted to a professional breeder who provided a breeding building with mulched concrete hutches (60 cm X 90cm) and daily care for the duration of the study (monitoring, feeding, cage cleaning). The rabbits grew up in pairs of "one whole rabbit - one sterilised rabbit" in the same hutch from 40 days to 70 days of age, then in individual hutches from 71 days to the end of the study at 204 days of age, as a precautionary measure, the whole brother being able to express, at puberty, a very dominant character over his sterilised brother.

Feeding

Drinking water was made available *ad libitum* via pipettes and after cleaning and disinfection of the water circuits. All the rabbits were given the same quantity of the same compound feed intended for growing rabbits six days a week, throughout the study, according to the quantities indicated in Figure 1.

Figure 1: Quantity of pellets distributed according to the age of the rabbits during the study (g)



Anaesthetic protocol

The chosen protocol provides sufficient general anaesthesia for more than 30 minutes for an operation lasting a maximum of 10 minutes.

Premedication was with Glycopyrrolate (atropine was not chosen because of the potential presence of atropinase in rabbits) at 0.1 mg/Kg LW (live weight) administered intramuscularly.

Anaesthesia was achieved using a mixture of ketamine hydrochloride administered at 15 mg/Kg LW and medetomidine hydrochloride at 0.1 mg/Kg LW injected mixed intramuscularly.

Prior to surgery, each sleeping rabbit received a tracer subcutaneous injection of intratesticular lidocaine hydrochloride at a rate of 0.1 ml per purse.

After surgery, each operated rabbit received a subcutaneous injection of meloxicam at 0.5 mg/kg LW to manage any pain.

As soon as the operation is over, the rabbit wakes up under a heat lamp after an injection of atipamezole at 1 mg/Kg live weight (Coquelle, 2022; Boucher and Nouaille, 2013; Wengers, 2012).

Surgical technique

Surgical sterilisation of the young male rabbit involved removal of the testicles via the scrotum. After triple disinfection with alcohol and an aqueous solution of povidone-iodine, which also enabled the hair to be pressed against the skin, a 5 mm incision was made in the scrotal skin, followed by a 3 mm incision in the vaginal skin. Each testicle was removed and the testicular cords and vas deferens were cauterised. The wounds healed within a few days and were not closed.

Control for the study

The farmer observed the animals every day and one or more vets observed and handled them every week. The rabbits were weighed weekly on an Ecorel® scale accurate to 50 g, which was calibrated by a metrologist at the start of the study. Their growth curves were plotted. The average daily gain (ADG) of each rabbit was calculated from 30 to 204 days of age. At the same time, the size of several growth plates and the size of the radius were measured after X-ray for use in a complementary study.

RESULTS AND DISCUSSION

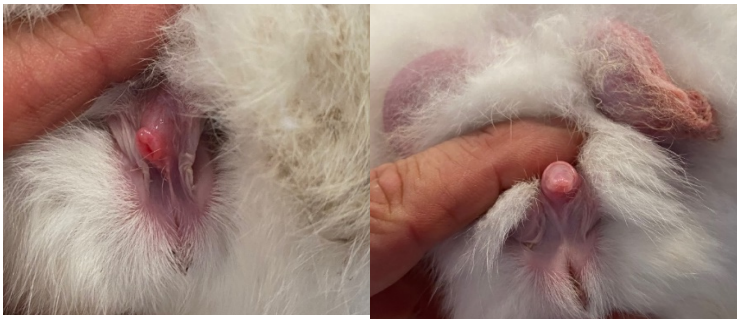
Behaviour

After several authors, we can confirm with this study that sterilisation puts an end to the sexual, territorial (urine marking) and intraspecific aggressive behaviour that usually appears at the time of puberty in male rabbits (Coquelle, 2022; Fortun-Lamothe, 2015). None of our sterilised rabbits showed this type of behaviour, although territorial marking was observed on their whole brothers. Early neutered rabbits do not develop sexual and territorial behaviour as adults and can cohabit harmoniously with their peers (Jehl et al., 1998; Kalagassy et al., 1999).

Anatomy of the external genital tract

Puberty is the period when the genital organs develop under the influence of sex hormones. This results in an increase in the size of the penis, scrotum, and testicles (Coquelle, 2022). The 20 sterilised male rabbits did not develop like their full brothers and retained their infantile character. The penis of an early-neutered rabbit that has become an adult almost resembled a vulva and is 2/3 smaller. The testicles were of course not developed, but the scrotum was almost invisible (Figure 2).

Figure 2: Genitalia of 6-month-old male rabbits. On the left, a male rabbit sterilised at 40 days of age, on the right, a whole male rabbit.



Weight growth and average daily gain

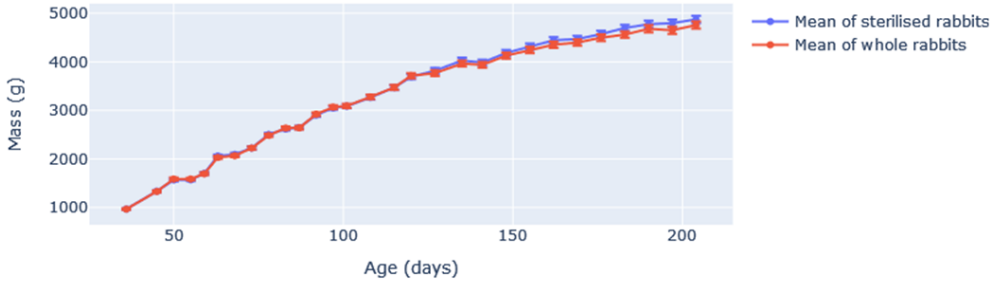
Weighing the rabbits shows that the weight growth of early sterilised and rationed rabbits was similar to that of whole rabbits up to the age of 4 months. For the same quantity of feed distributed per animal, although sterilised rabbits showed slightly high live weight values, the difference was not significant (Figure 3).

Similarly, as other authors have also shown, we found that male rabbits sterilised early maintained a similar weight growth to whole male rabbits (Jehl *et al*., 1998).

Rabbits, like many domestic mammals, show a sigmoid weight curve during their growth, with an initial phase of rapid growth followed by a phase of slower growth before a phase of stabilisation of weight gain. Growth is then complete. Rationing rabbits by providing them with a ration that just meets their growth and maintenance needs prevents them from having access to "ad libitum" feeding, which can encourage obesity (an increase of more than 15% in body fat) in sterilised rabbits. After the end of growth, an individual's live weight can fluctuate mainly according to variations in energy intake and loss. As with domestic

carnivores, it seems important not to allow sterilised animals to eat as much as they want, as their maintenance requirements are lower than those of whole rabbits.
Sterilisation is known to be a major risk factor for weight gain. Courcier's study reported that sterilised rabbits were 5.4 times more likely to be significantly overweight than whole rabbits (Courcier *et al.,* 2012; Georgiev *et al.,* 2011).
Obesity in rabbits can have several serious consequences for their health. These include increased intolerance to exercise and heat, increased risk of heart disease, arterial hypertension, osteoarticular diseases such as osteoarthritis, pododermatitis, impaired caecotrophy and vitamin deficiencies, myiasis and cheyletielosis due to increased difficulty in grooming (Adji *et al.,* 2022).

Figure 3: Evolution of the live weight of sterilized and whole rabbit



CONCLUSION

Early sterilisation of rabbits at around 40 days of age is one way of reducing the risk of surgery and making the operation more effective, as it completely cancels out the rabbit's sexual, territorial, or agonistic behaviour once it has reached adulthood. Castration results in the non-development of external sexual organs such as the penis and scrotum. However, this technique does not affect weight growth in rationed rabbits.

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ESTROGEN PROFILE OF RABBITS BUCKS FED LINSEED AND ALGAE DIETARY SUPPLEMENTATION

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ABSTRACT

The Omega Rabbit project (Ω RABBIT) aims to develop a new quality food product "Ωrabbit meat" assessing the impact of different n-3 fatty acids source (flax derived products or algae as *Padina pavonica*) on rabbit metabolism and in turn on meat quality. Moreover, the research intended to evaluate also the effect of 5% linseed and 5% linseed + 0.2% algae dietary addition on the steroid hormone profile (hormone concentration and receptors) of rabbit bucks. Forty-five New Zealand White male rabbits (6 mo) were divided into 3 dietary groups (15 animals/group): control (CNT) fed with a commercial diet, linseed group (L5%) and linseed-algae group (L5%PP) fed with the commercial diet supplemented with 5% linseed alone and 5% of linseed plus 0.2% of freeze-dried *Padina pavonica* algae, respectively, for 110 days (50 adaptation + 60 experimental). Semen and Blood was collected every week. Semen and blood testosterone was assessed by radioimmunoassay kits, whereas α and β-estrogens receptors (ER) by immune-fluoresce assay. The supplemented diets have an impact in the ER concentrations: L5% and L5%PP groups showed also a higher expression of sperm β ER than CNT. The diets affect also the hormonal profile of rabbit bucks: testosterone plasma concentrations were higher at 14 days (experimental period) of dietary administration in L5%PP group, whereas at 21 days the highest one was recorded for L5%. Low values respect to the CNT were recorded with linseed administration after 21 days, mainly due to the modulation of estrogen receptors. Furthermore, the reduction of testosterone due to linseed administration suggested a competition in steroids hormone. The 17-β etradiol evaluation will also be analyzed to better explain such competitive effects.

Key words: rabbit, linseed, algae, metabolism, estrogens.

INTRODUCTION

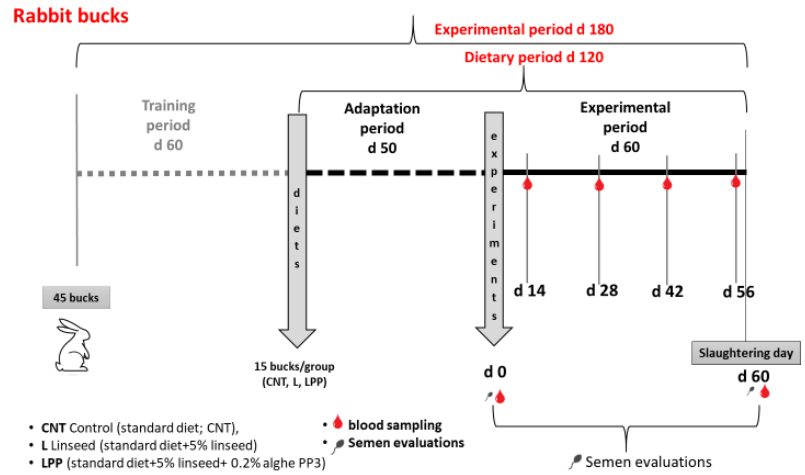
The Omega Rabbit project (Ω RABBIT) aims to develop a new quality food product "Ωrabbit meat" assessed the impact of different n-3 fatty acids source (flax derived products or algae as *Padina pavonica*) on rabbit metabolism and in turn on meat quality. It is well documented that linseed supplementation affects rabbit estrogen profile due to the phytoestrogens administration (Mattioli et al., 2017). Sex steroids are key reproductive system hormones in both sexes. Estrogens have always been considered the female sex steroid hormones and androgens as their male counterparts (Dewaele et al., 2022). However, it has been clear for many decades that the situation is more complex; on the one hand because the synthesis of estrogens is made from androgens, thus implying the presence, at least transitory, of androgens in females, and on the other hand because estrogens are also produced by the testes of mammals where their roles remain to be elucidated. This research aimed to evaluate the effect of 5% linseed and 5% linseed + 0.2% algae dietary addition on the testosterone plasmaconcentrations and α and β-estrogens receptors (ER) expression of rabbit bucks.

MATERIALS AND METHODS

Animals and experimental design

At a rabbit farm affiliated to Perugia University (Az. Agricola Brachino Patrizia), 45 male rabbits of the New Zealand White breed (6 months old) were housed in single cages and divided into 3 dietary groups (15 animals/group): the control group (CNT), was fed with a standard (commercial) diet, the linseed group (L5%) was fed with a standard diet enriched with 5% linseed and the linseed-algae group (L5%PP) was fed with a diet supplemented with freeze-dried 0.2% *Padina pavonica* algae + 5% linseed (Figure 1).

Figure 1 Experimental design of male rabbit



The diets were formulated to be isoenergetic and were supplied by a feed industry for experimental purposes (Martini srl). Ingredients and chemical composition are reported in Table 1. The diets were administered for a total of 110 days (50 adaptation + 60 experimental; Figure 1). Semen and blood was collected every week starting from T1 (7 days of the experimental period) up to T8 (48 days of experimental period). Blood samples were taken from the ear vein in 5 mL EDTA tubes. For the testosterone determination, plasma was separated by centrifugation at 2700 x g for 10 min and stored at -20° C until the next assay. Semen was taken used an artificial vagina as reported by Boiti et al. (2005).

Chemical analyses

Total testosterone was measured in duplicate by commercially available radioimmunoassay kits (Beckman Coulter – IFU-IM1119-01). The intra- and inter-assay coefficients of variation (CVs) were 10.4% and 17.8%, respectively. The lower limit of detection was 0.04 ng/mL.

The sperm of rabbit bucks fed the experimental diets were treated with 10% buffered formalin for 24 h at 4 °C and then washed in water for 1 h ad detailed in Castellini et al. (2022). Specimens were treated overnight at 4 °C with the primary antibodies anti-α or β-estrogens receptors diluted 1:20. After three washes for 10 min in phosphate-buffered saline (PBS), the slides (excluding those treated with conjugated primary antibody) were incubated with goat anti-rabbit antibody Alexa Fluor® 488 conjugate (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA), diluted at 1:100, for 1 h at room temperature. The slides were washed three times with PBS and treated with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Milan, Italy) for 10 min, followed by washing with PBS for 10 min. Finally, the slides were mounted with 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma-Aldrich, Milan, Italy).

Statistical Analysis

The statistical analysis of All the numerical results was carried out with the ANOVA model where the fixed effects evaluated were time, diet and their interaction. For the multiple comparison, the Bonferroni ad hoc test was used, with an alpha value of 5%.

Table 1. Ingredients (%) of the experimental diets for the rabbit bucks

	CNT	L5%	L5%PP
Ingredients			
Bran Wheat	25.07	24.87	24.92
Barley	13.33	13	13
Sunflower seed meal	12	11.67	11.5
Alfalfa	10.83	13	13
Sunflower husks	10	10	10
Beet pulp	7.5	5.67	5.5
Extruded linseed	-	5	5
Full-fat soybean	5	2.95	3.1
Wheat straw	4.17	2	2
Molasses cane	3	3	3
Wheat	2.5	2.5	2.5
Grape pips meal	2.33	1.67	1.67
Soya hulls	0	1.67	1.67
Calcium carbonate	1.6	1.48	1.42
Soybean oil	0.78	-	-
Sodium chloride	0.4	0.4	0.4
Palm oil	0.33	-	-
Carboxymethylcellulose	0.3	0.3	0.3
Oligo-vitamin supplement[1]	0.25	0.25	0.25
Alga PP	-	-	0.2
Lysine HCl	0.16	0.17	0.17
Liquid acidifier[2]	0.15	0.15	0.15
Magnesium oxide	0.1	0.1	0.1
Methionine hydroxyanalog	0.06	0.07	0.07
Liquid choline	0.05	0.05	0.05
Vitamin E 50%	-	0.03	0.03
L Threonine	0.03	0.01	0.01
DL Methionine	0.03	-	-
Chemical composition			
Protein	16.5	16.5	16.5
Lipids	3.62	3.93	3.93
Fiber	17.16	16.79	16.79
Ash	7.99	8.09	8.09
ED rabbit (kcal)	2350.00	2350.00	2350.00

[1]Vitamin Mineral premix composition: Vitamine A, D3, E, K3, B1, B2, B6, B12, Biotin, Niacinamide, Folic acid, Calcium pantothenate.
[2]Liquid acidifier composition: Formic acid 75%

RESULTS AND DISCUSSION

Effect of experimental diets on semen estrogen raptors distribution

Sperm of L5% and L5%PP groups showed higher expression of β ER than CNT but not for the α ER (Figure 2). Furthermore, a different distribution on sperm surface was also recorded: β ER were mainly located in the head, while α ER in the midpiece of sperm, probably due to their different functions in sperm.

Effect of experimental diets on testosterone concentration

Blood testosterone (Figure 3) showed a higher concentration at 14 days of dietary administration in L5%PP group, whereas at 21 days the highest one was recorded for L5%.

Low values respect to the CNT were recorded with linseed administration after 21 days.

Figure 2 α and β estrogen receptors distribution by immune fluorescence on spermatozoa of rabbit fed experimental diets for 60 days

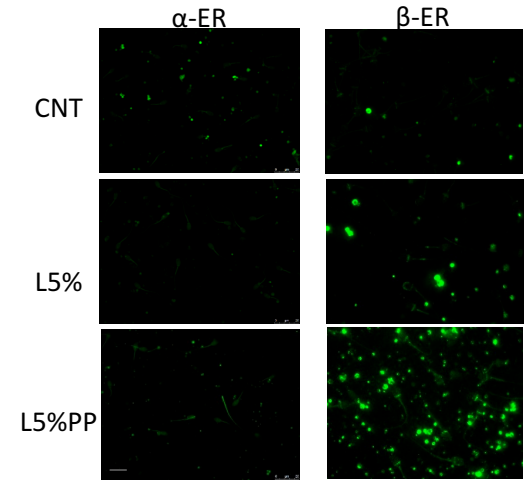
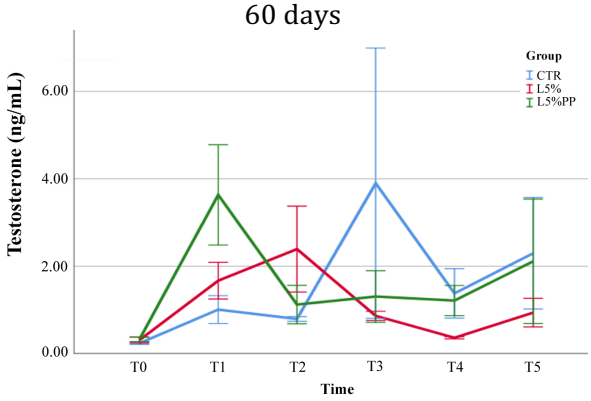


Figure 3 Blood testosterone concentration (ng/mL) of rabbit fed experimental diets for 60 days



Mean ± standard errors

CONCLUSIONS

Dietary administration of 5% flaxseed widely affected the hormone profile of rabbit bucks, mainly due to the modulation of estrogen receptors. Furthermore, the reduction of testosterone due to linseed administration suggested a competition in steroids hormone. The 17-β estradiol evaluation will also be analysed to better explain such competitive effects.

ACKNOWLEDGEMENTS

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CURCUMIN ALLEVIATES OXIDATIVE STRESS AND INJURY IN THE LIVER INDUCED BY ZEARALENONE IN MEAT RABBITS

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ABSTRACT

In this study, we investigated the role of curcumin in alleviating zearalenone-induced oxidative stress and injury in the liver in meat rabbits and the underlying mechanisms. It was revealed that dietary supplementation of zearalenone significantly increased the level of malondialdehyde in the liver and decreased the activity of superoxide dismutase ($P<0.05$), leading to oxidative stress in the liver of meat rabbits. The dietary supplementation of zearalenone significantly increased the activities of glutamic oxalic transaminase and glutamic pyruvic transaminase in the blood of meat rabbits ($P<0.05$), leading to liver injury. Additionally, the inclusion of curcumin ameliorated oxidative stress in the liver of meat rabbits through the Nrf2-ARE signaling pathways, thereby mitigating liver injury. This study offers a new perspective on the application of curcumin and the development of zearalenone antidotes.

Keywords: Curcumin, zearalenone, meat rabbit, liver injury, Nrf2/ARE

INTRODUCTION

Zearalenone (ZEA) is a mycotoxin mainly produced by fungi of the genus Fusarium, which exhibits a wide range of toxicity, including liver toxicity (Wu et al., 2023a). ZEA is widely present in rabbit feed and can induce liver injury by causing oxidative stress and triggering inflammatory responses (Wu et al., 2023b). Curcumin (CUR), the primary active ingredient of turmeric from the Zingiberaceae family, has various biological activities, including anti-oxidation and anti-inflammation (Abd El-Hack et al., 2021). It remains unclear whether CUR can alleviate ZEA-induced liver injury in meat rabbits and its underlying possible mechanism. Therefore, in this study, we aimed to investigate the impact of CUR on the livers of meat rabbits exposed to ZEA-contaminated feed.

MATERIALS AND METHODS

A total of 90 healthy Hyla meat rabbits (half male, half female) were selected and randomly assigned based on gender and average body weight to three groups (30 replicates/30 meat rabbits per group): i) animals in the control group were fed the basal diet; ii) in the ZEA group the basal diet added with 600 μg/kg ZEA; and iii) in the ZEA+CUR group the basal diet added with 600 μg/kg ZEA and 150 mg/kg CUR. The site and cages were fully disinfected prior to the beginning of this study. The meat rabbits were housed individually with free access to feed and water. The pre-study period lasted for 7 days, followed by the formal study period of 35 days. The basal diet was formulated according to the feeding standards recommended by the National Research Council (NRC, 1977) for meat rabbits (16.4% crude protein; 14.9% crude fibre and 3.4% ether extract).

Table 1 Composition and nutrient levels of the basal diet (air-dry basis, %).

Ingredients	Content	Nutritional level	Content
Corn	8.00	Digestible energy, MJ/kg	9.33
Wheat bran	33.00	Crude protein	16.37
Soybean meal	11.00	Ether extract	3.40
Wheat middling	4.00	Crude fibre	14.91
Rice Bran	5.00	Crude ash	12.35
Peatnut vite	15.00	Neutral detergent fibre	42.08
Peanut hull	12.50	Acid detergent fibre	23.97
Artemsia argyi powder	8.00	Acid detergent lignin	5.62
Limestone	1.05	Calcium	1.60
NaCl	0.50	Total Phosphorus	0.80
$Ca(HCO_3)_2$	0.50		
Lysine	0.30		
Methionine	0.15		
Premix	1.00		
Total	100.00		

Blood and liver indicators

At the end of the formal study period, eight rabbits (half male, half female) with weights close to the average body weight were randomly selected from each group after a 12-hour fasting period. Blood and liver indicators were measured using ELISA, following specific kit instructions.

Expression levels of liver-related genes

Total RNA was extracted from liver tissues using the Trizol kit. RNA concentration was determined using NanoDrop Lite. cDNA was reversed transcribed using a reverse transcription kit. The qRT-PCR premix was composed of quantitative primer, cDNA template, and the ChamQ Universal SYBR qPCR Master Mix kit, and qRT-PCR amplifications were then conducted in a Bio-Rad CFX96 real-time contact PCR detection system using GAPDH as the reference gene. The experiment was repeated three times. The expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Data statistics and analysis

Data were reported as average values and analyzed using one-way ANOVA in SPSS 28.0 software, and multiple comparisons were conducted using Duncan's method. P values < 0.05 were indicative of significant differences.

RESULTS

Table 2 shows that, compared with the control group, blood ALT and AST activities were significantly in the ZEA group (P<0.05). Compared with the control group, the ZEA group showed significant decreases in liver SOD activity (P<0.05) and significant increases in the liver MDA levels (P<0.05).

As shown in Figure 1, compared with the control group, liver HO-1 mRNA levels were significantly decreased (P<0.05) in the ZEA group, whereas HSP70 mRNA levels were increased (P<0.05). Compared with the control group, liver Nrf2, GSH-Px, and HSP70 mRNA levels were significantly increased (P<0.05) in the ZEA+CUR group. Compared with the ZEA

group, liver Nrf2, HO-1, and SOD1 mRNA levels (P<0.05) were significantly increased in the ZEA+CUR group, whereas HSP70 mRNA levels were significantly decreased (P<0.05).

Table 2 Effect of CUR on serum and liver antioxidant indicators of meat rabbits fed a ZEA-contaminated diet

Items	Control group	ZEA group	ZEA+CUR group	SEM	p-Value
Serum					
ALT, U/L	21.81[a]	36.91[b]	28.47[a]	1.821	0.001
AST, U/L	12.53[a]	17.65[b]	14.41[ab]	0.860	0.041
ALP, U/L	112.93	119.90	105.25	3.604	0.262
Liver					
TAOC, U/mg prot	31.70	26.82	34.28	1.311	0.055
GSH-Px, U/mg prot	153.86	146.23	167.19	5.313	0.275
SOD, U/mg prot	25.62[b]	19.39[a]	20.76[a]	1.061	0.034
CAT, U/mg prot	36.40	31.86	39.84	1.418	0.064
MDA, U/mg prot	22.52[a]	31.79[b]	23.63[a]	1.620	0.031

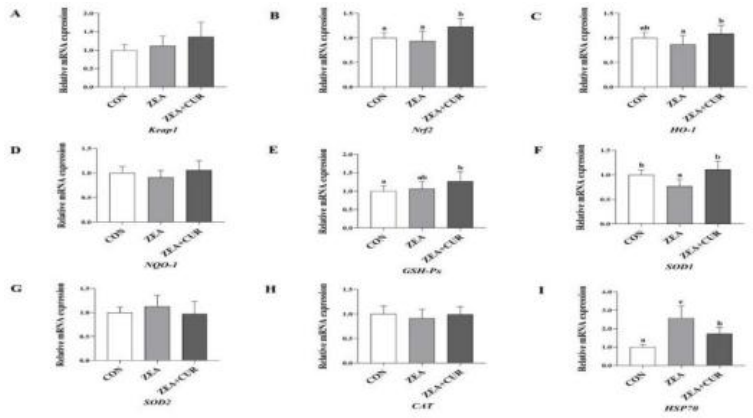

Figure 1: Effects of CUR on the expression levels of liver antioxidant-related genes in meat rabbits fed on a ZEA-contaminated diet. *Keap1* gene (A), *Nrf2* gene (B), *HO-1* gene (C), *NQO-1* gene (D), *GSH-Px* gene (E), *SOD1* gene (F), *SOD2* gene (G), *CAT* gene (H), *HSP70* gene (I). The bar chart shows the mean and standard deviation (n=8). Different letters above the bars indicate statistical differences (P<0.05), while no letters or the same letters indicate no significant differences (P>0.05). *Keap1*, kelch-like ECH-associated protein 1; *Nrf2*, nuclear factor E2-related factor 2; *HO-1*, heme oxygenase-1; *NQO-1*, NAD (P) H: quinone oxidoreductase 1; *GSH-Px*, glutathione peroxidase; *SOD1*, copper and zinc superoxide dismutase; *SOD2*, manganese superoxide dismutase; *CAT*, catalase; *HSP70*, heat shock protein 70; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

DISCUSSION

In this study, ZEA treatment caused significant increases in blood ALT and AST activities in rabbits, thus suggesting liver injury. ZEA treatment led to a significant downregulated of the activity of some antioxidant enzymes and the expression levels of related genes in the liver of rabbits. However, it also resulted in a significant up-regulation of MDA contents and HSP70 mRNA levels, thus suggesting the presence of oxidative stress. The addition of CUR restored the antioxidant enzyme activity and MDA levels of rabbits to levels similar to those

of the control group, but the liver SOD activity and HSP70 mRNA levels were significantly higher than those of the control group, indicating the alleviation of oxidative stress caused by ZEA. Herein, we found that CUR treatment could significantly upregulate liver Nrf2, HO-1, GSH-Px, and SOD1 mRNA levels in the ZEA+CUR group compared to the ZEA group or the control group, suggesting that CUR could increase the expression of downstream antioxidant enzyme genes via the Nrf2-antioxidant response element signaling pathway.

CONCLUSION

CUR could alleviate ZEA-induced oxidative stress response-induced liver injury via the activation of Nrf2-ARE signaling pathways, thus playing a role in liver protection.

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BREEDING & GENETICS

ENHANCING THE SUSTAINABILITY OF RABBIT PRODUCTION FROM THE PERSPECTIVE OF ANIMAL GENETICS

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ABSTRACT

The concept of sustainability, originating from the late 1980s, emphasises the ability to maintain processes over time without compromising future generations' needs. It encompasses social, environmental, and economic dimensions, although controversies persist regarding the latter's inclusion. In the case of rabbit production the economic dimension is paramount to guarantee the future sustainability of the sector given the large number of threads, mainly economical, it is facing. The major challenge for considering the social and environmental planes of sustainability in breeding programs is to properly include these dimensions in the functions defining the relevance of the different traits to be considered during the development of specialised lines. Note however that the key drivers of the current economic sustainability of the sector: prolificacy, feed efficiency and some functional traits like resilience and survivability, are also the most likely levers of the environmental and social components of sustainability. In this context, the development of specialized lines is the most valuable contribution to sustainability by animal geneticists, the maternal lines specialised on producing large amounts of healthy weaned kits and the terminal sire lines specialised on efficiently transforming feed into meat. With regard to the feed efficiency important milestones have been achieved during the last years, many of them related to the fact that kits are raised in collective cages, and under these rearing conditions, tools have been developed to measure feed intake at individual level, as well as to explore the role that one individual imposes on their cage-mates. In spite of the fact that genomic tools have been developed and used to explore the role of genomic regions of different traits of interest, this information is still far from being used in applied breeding programs. In the near future, we could predict that breeding programs for enhancing sustainability will still mainly rely on pedigree records and phenotypic information for prolificacy and feed efficiency; but enriching the list of available phenotypes with additional traits, most likely obtained under automatic recording systems, to explicitly account for the social and environmental planes of the sustainability. In this framework, *omic* tools will perform a valuable role for further digging on the biological basis controlling the major drivers of rabbit production sustainability, and hopefully in the future this information could be directly incorporated into the breeding programs.

Key words: Feed efficiency, Genetic Section, Animal Breeding, New Phenotypes, *Omic* Tools

WHAT DOES SUSTAINABILITY MEAN?

The concept of sustainability might refer to the ability to maintain or support a process continuously over time. This idea is strongly linked to the concept of sustainable development, defined in the late eighties of the last century in “Our Common Future” (World Commission on Environment and Development, 1987), the foundational document of sustainable development. In said document it was stated that sustainable development implies “to satisfy the needs of the present generation without compromising the chance for future generations to satisfy them”. In this context, it was proposed that three dimensions were relevant to grant the sustainability of any system: social, environment and economic. These three plans are however not free of controversy (Purvis et al., 2019), since many

experts consider that the economical dimension should not be contemplated since it is rapidly associated with “economic growth”, which in turn has been associated with many cases of environmental and social deterioration. Nonetheless, in “Our Common Future” the role of the economic dimension was fully recognized, since it makes a call for “a new era of economic growth that is forceful and at the same time socially and environmentally sustainable” (Purvis et al., 2019). In general, we could affirm that proper definitions of sustainability are far from being rigorous since they are based not only on scientific evidence but also on what people find valuable, and this second component includes largely intangible elements difficult to systematise.

Beyond this attempt to formalise the sustainability concept what seems evident and has already been demonstrated, is the impact of livestock production on the environment (Xu et al., 2021). It has been quantified that the impact for producing one Kg of protein from rabbit meat is 51.4 Kg of $CO_{2\ eq.}$ and 0.5 Kg of $N_{eq.}$ (Cesari et al., 2018). These values are higher than those of poultry production, similar to the figures from pig production and much lower than those of beef production.

Thus, we could say that the environmental dimension would be a priority in analysing the elements of rabbit production to enhance its sustainability. But if one considers the economic threats surrounding the rabbit sector, the economic factor is in our opinion the most critical dimension for rabbit production sustainability in the short and even in the long term. Thus, in the case of rabbit production, the above statement could be rephrased as: “a new era of economic survival must be pursued while granting a social and environmental sustainability around it”.

In this context, an appealing strategy for increasing economic efficiency/profitability of any production system consists of maximising the number of output units obtained per input unit provided. But the rabbit production sector has the handicap of being a minority sector strongly influenced by other market estates, which might distort the actual relevance of the different components of the system.

Of course, this discussion about the need of developing a sustainable rabbit production relies on the fact that this sector produces something relevant to society. If this point were to be questioned, i.e. the production of rabbit meat does not merit the associated environmental impact, all arguments below are completely irrelevant and we had better stop rabbit farming.

The objective of this revision is to describe the research initiatives, mainly in the field of animal genetics, that have been conducted in the last few years to enhance the sustainability of the rabbit production sector. We will review the most relevant traits to improve the sector sustainability and how these traits can be identified. We can anticipate that one of these traits is the efficiency of converting feed into meat, and we devote a few sections to review important features of these efficiency traits, like the fact that it is necessary to record the individual feed intake and that kits are raised in groups. Then we review how up-to-date genomic and other *omic* tools are being used for the study of traits directly or indirectly linked to the concept of sustainability. Finishing the revision is a reflection on the characteristics of future breeding programs to further enhance rabbit production sustainability.

RELEVANT TRAITS FOR ENHANCING RABBIT PRODUCTION SUSTAINABILITY

Fortun-Lamothe et al. (2009) published an assessment of the contribution of intense rabbit production to the sustainable development of our society. They did it using a semi-quantitative scale applied to nine indicators and concluded that for some indicators (use of antibiotics, animal biodiversity, or animal welfare) the contribution of the intensive rabbit production system penalises an overall sustainable development while for other indicators (living and working conditions, quality of products or economic profitability) the contribution of the intensive rabbit production is positive. Beyond these semi-quantitative results, what is important about the study by Fortun-Lamothe et al. (2009) is that they classify indicators into the three pillars of sustainability. This helps to organise the contribution of the different traits and features of rabbit production. In this regard the indicators assigned to the economic

plane of sustainability encompass the economic profit of the activity and issues related to this profitability, like economic specialisation or the need for strong investments in facilities. The indicators assigned to the environmental dimension basically include those linked to the impact the activity might have on the ecosystems, which in the case of the rabbit farms is strongly linked to the use of energy, the use of antibiotics and the biodiversity of the populations considered for production. Finally, the indicators assigned to the social dimension of sustainability are somewhat more diverse, in the sense that they might conceal either the perception society has about rabbit production and what we get from it; paradigmatic examples being the concern of society about animal welfare, or the worry about the quality of the products the system is generating. But this dimension also covers issues related to the contribution of the activity to our social development, reviewing to what extent the labour conditions are favourable or assessing whether the activity generates inclusive labour opportunities. In the following sections the different indicators listed above are treated in a rather loose manner, and in many cases, they are openly ignored, being the traits we talk about directly assigned to contribute to the different sustainability planes in an implicit way. For our arguments this informal treatment of the different sustainability indicators is not a relevant limitation because one particular trait might contribute in different ways to the different planes of sustainability.

The most relevant traits defining the profitability of any production sector could be determined by analyses like those used to compute the economic weights at defining selection indexes in breeding programs (Brascamp et al., 1985). This is traditionally done by deriving an economic revenue function with respect to the different traits considered at defining the function. The challenge would be to turn the social and environment planes of sustainability into economical units. Some people might perceive that these two dimensions might lose their identity by following this procedure, but this is not so. Social and environment dimensions of sustainability would become much more evident since they involve economic units that everyone clearly understands, i.e. Euros. So, the point might not necessarily be the development of new traits to explicitly consider environmental or social dimensions of sustainability, but to redefine the rank of already recorded traits to economically account for these sustainability dimensions.

In other species like pigs and cattle there are already examples of derivations of economic weights accounting specifically for the environmental impact expressed as economic units within reviewed revenue functions. Methodologically, they can either rely on complex bio-economic models and stochastic simulations (Ali et al., 2018), or on traditional revenue functions applied to both economic units and emission units, which are latterly weighted at the time of deriving the final economic values (Amer et al., 2018; Alfonso 2019). Regardless of the method, the point is that the environmental dimension of sustainability must be expressed in economic units.

The calculation of economic weights conducted so far in rabbit production (Cartuche et al., 2014), scarce anyhow, did not directly consider the societal or environmental penalties or bonuses for these nuances associated with the different traits. The challenge, as has been indicated, is that it is difficult to express many of the societal or environmental concerns behind the traits' marginal increases in monetary units.

Exclusively from an economical point of view, it seems that the most relevant traits are the does prolificacy and the efficiency in the use of feed by both does and growing kits (Cartuche et al., 2014). Note however that this exclusively economic criteria could sometimes be aligned with aspects of the societal and the environmental dimensions of rabbit production. In this regard, a significant improvement of feed efficiency will have direct and tangible economic benefits for the rabbitries, as feeding costs represent up to 70% total costs, but will also aid in minimizing the use of resources for production. This fact will directly contribute to mitigating the environmental impact of the production and to improve the perception that society might have of our sector; positioning us, as a sector worried about an optimised use of resources. Thus, acting on the efficiency in the use of feed, we will address the three

dimensions of sustainability. Another example could be the survivability of the kits, which is a major component of farm-level efficiency (Gidenne et al., 2017), and is also strongly linked with animals' welfare, a major component of the societal dimension of livestock production. These intuitive arguments can be supported by derivations of economic weights accounting for environmental impact in the pig industry, which also point out that the most relevant traits from a strictly economical point of view are still the most relevant ones also when the environmental component is considered (Alfonso 2019, Ali et al., 2018).

Thus, we could affirm that considering feed efficiency and prolificacy as target traits to be improved is an adequate strategy to enhance rabbit production sustainability. Note, however, that these concepts (feed efficiency and prolificacy), when they are treated as farm-level technical indexes broad enough to cover a wide range of additional elements of the production. For instance, prolificacy at weaning can be improved by reducing mortality during lactation. Similarly, an improvement of feed efficiency during fattening can be achieved through reducing post-weaning mortality. These examples on the consideration of mortality to expand the biological concepts of prolificacy and feed efficiency to technical production indexes is a way to give room for other animal traits in the sustainability definition. These traits are named functional traits (García et al., 2021) and they are characteristics linked with health, fertility and longevity that reflect concepts like resistance to diseases, resilience, robustness, rusticity, or plasticity. Many of these concepts directly reflect the capability of the animal to maintain performance even in harsh or simply diverse environments. In the case of rabbit production three examples of functional traits are the doe's functional longevity (Sánchez et al., 2008, Larzul et al., 2014), the across-parities environmental variability in prolificacy (Blasco et al., 2017; Argente et al., 2019) or the within-litter environmental variability in birth weight (Garreau et al., 2008) and the resistance to different diseases and syndromes (Garreau et al., 2021). Keeping these functional traits at an appropriate level has an enormous impact on production sustainability, since it will not only aid the improvement of the economical revenue of the activity, but it will also help to keep high levels of animal welfare, since all of them are strongly correlated with animals' survivability and different forms of robustness and resilience. Of course, the ways to enhance all these traits with the final aim to improve rabbit production sustainability are as numerous as disciplines within animal science: Nutrition, Reproduction, Pathology and Genetics.

In the current revision we will set the focus on one of the pillars for sustainability, feed efficiency, reviewing aspects directly linked to its genetic determinism, and more particularly to issues and procedures related to the genetic improvement of this type of trait.

FEED EFFICIENCY IN RABBIT PRODUCTION FARMS: EFFICIENT PRODUCTION OF WEANED KIT AND EFFICIENT USE OF FEED FOR GROWTH.

Feed efficiency in rabbit production at farm level could be defined as the ratio between input and output units. In this context, we could distinguish between factors that modify the ratio by allowing either maintaining or even reducing the inputs, while keeping constant or increasing the outputs; and factors promoting an increase of both inputs and outputs, but the increase in outputs being proportionally larger than that in inputs. The wider the level of definition, the larger the number of traits that would determine the efficiency. At whole-farm level, efficiency would depend on both reproductive and post-weaning performances, while efficiency in the fattening sections would only depend on the post-weaning performances. The traditional three way cross (Baselga et al., 2021) used in European countries for rabbit production aims to enhance efficiency at farm level: specialised maternal lines are responsible for granting efficient production of weaned kits, while terminal sire lines would allow for the kits to grow fast with a low feed conversion ratio and acceptable carcass quality.

To the best of our knowledge the efficiency in producing weaned kits has never been explicitly considered in rabbit breeding programs of maternal lines. The strategy has been to increase litter size at weaning, to increase prolificacy while reducing mortality or just to increase survivability, but never explicitly accounting for the input units needed to sustain the

production. It was assumed that the marginal cost of producing an extra kit at weaning would be much lower than the benefit of having such an increase in weaned kits. These strategies to enhance the efficiency at farm level could be understood as scale factors since the produced units are not generated in a more efficient way; on the contrary, the enhanced efficiency would be simply a consequence of the fixed costs being divided by a larger number of output units.

The case of the paternal lines is slightly different since there have been selection processes, directly considering efficiency criteria, using different functions balancing inputs (e.g. feed intake) and output (e.g. growth) units. Note however that the traditional way to improve feed efficiency in terminal sire lines was indirect selection for either post-weaning growth or final weight; the main argument supporting this strategy was the medium-high genetic correlation between growth and feed conversion ratio. In addition, fast growing animals would imply a shorter fattening interval to reach the final weight, which would imply a reduced overall intake and the additional advantage of concentrating post-weaning growth at lower ages, on which better feed conversion efficiency has been documented (Larzul and Rochambeau, 2004; Ramon et al., 2015).

Blasco et al. (2021) concluded in their review about genetic parameters for growth and feed efficiency in terminal sire lines that both heritability of growth and its genetic correlation with feed conversion ratio in rabbits seem to be lower than those reported in other species. These authors argued that these parameter estimates hardly justify considering indirect selection procedures as an efficient way to improve feed efficacy. What is more, the experimentally reported response pointed out generational changes even lower than those expected given the genetic parameter estimates. There is not an evident explanation about the low genetic variation or the low heritability values in rabbits for traits showing much higher parameter values in other species. Perhaps evolutionary studies coupled with up-to-date genomic tools could aid in elucidating this situation that seems to be peculiar for our species and could be aligned with the tolerance of the rabbit to high levels of inbreeding. In this context ideas like those proposed by INRAe team in FeedSeq (Gilbert, 2023) project on using Translational Genomics concepts to study together rabbits and other species would help to shed some light on the above-described biological limitations in many rabbit traits.

SELECTING FEED EFFICIENCY IN GROUP-RAISED ANIMALS

Growing rabbits are usually raised in group cages, where it is not evident how to record the individual intake. Precisely, the difficulty for obtaining these individual intake records is another important reason for not considering feed efficiency as a direct selection criterion for the improvement of terminal sire lines. Nonetheless, some experimental processes of selection for feed efficiency have been conducted by raising animals in individual cages, the most recent was the one by Drouilhet et al. (2016). In this experiment two lines were selected, one to reduce residual feed intake (RFI) (ConsoResidual line) and the other to increase growth under restriction (ADGrestrict line). Near perfect results were reported: ConsoResidual line reduced the intake without penalising growth and ADGrestrict line improved the growth without increasing the intake. In latter reports of the ConsoResidual line (Garreau et al., 2018) slightly different results were presented, the reduction in the feed conversion ratio of the ConsoResidual line (-0.035 units per generation) was accompanied by a reduction in the daily growth rate (-0.277 g/d per generation). This result is compatible with the reported genetic correlations in these lines (Drouilhet et al. 2013), also this antagonistic relationship has been also observed in other species (pigs, poultry, etc).

Garreau et al. (2018) also studied the interaction between rearing conditions (individual *vs* group cages) and the genetic response, discarding the hypothesis of a strong genotype by rearing condition interaction, despite showing some non-significant scale changes in the responses for growth and intake depending on whether they were obtained in group or individual cages.

Several projects focusing on feed efficiency have been conducted at IRTA since 2012. We considered that efficiency should be evaluated and improved in conditions close to commercial ones, thus we have conducted selection processes with animals raised in collective cages. A first, tentative approach for using records from animals raised in collective cages for breeding purposes was made at handling cage-average feed intake records (Piles and Sánchez, 2019) jointly with individual growth information to estimate genetic parameters and to predict breeding values for individual feed efficiency. Genetic parameters estimates were in the range of estimates previously reported by Drouilhet et al. (2013) from records of animals raised in individual cages. Two important finds by Piles and Sánchez (2019) were the limited genetic determinism for feed efficiency when animals were raised under feed restriction, and the negative genetic correlation between growth and residual feed intake, i.e. feed intake beyond the expected needs of the batch. When the residual feed intake was corrected for the genetic expected needs the unfavourable genetic correlation with growth disappeared, but the resulting feed efficiency trait had a nearly null heritability. In parallel to this study, we worked on the design and manufacture of a feeding device allowing us to record individual feed intake of animals raised in group-cages. This electronic feeding device acts as a sensor recording the amount of feed available at every time for the animals and retrieving the identification of the animals at entering the feeder (Sánchez et al. 2024).

This system has been used to genetically improve the feed efficiency of one line selected to reduce the residual feed intake (RFI line), and to hourly (from 6am to 6pm) restrict the animals' access to feed in another line selected for increasing daily growth under restriction (ADGR line). This selection experiment was completed with a third line selected to reduce the cage-average residual feed intake (GRP line). Preliminary results on the response attained in these selection processes are presented in a communication presented to this conference. Very briefly, the RFI line was the one with the clearest response on reducing FCR, and a trend was also observed in the GRP line; both responses on FCR were accompanied by an unfavourable response on daily growth. The ADGR line did not show any response to the selection for growth under feed restriction. To a certain extent, these results were expected given the previously estimated genetic parameters (Piles and Sánchez 2019; Drouilhet et al. 2013).

The applied selection processes fully considered the fact that animals are raised in collective cages, where social interactions between cage mates might play an important role in the determinisms of the feed efficiency and other performance traits. In the case of the GRP line the selection unit is the whole group of animals being raised in the same cage, i.e. we are selecting the combination of genes between cage mates that maximise the cage-average RFI. Thus, we were not only selecting direct genes promoting individual RFI but also genes favourably affecting the RFI of cage-mates. In the case of the RFI and ADGR lines the role of the social interactions is also implicitly considered. Cage mates are close relatives, i.e. full or half sibs; and with this within-cage familiar structure, breeding value predictions from an animal model are predictions of the total breeding value (Muir et al., 2013; Bijma et al., 2011). This is a breeding value that optimally weights the effect of genes affecting the performance of animal itself, and the effects of genes affecting the performance of its cage-mates.

This concern about the role of social interaction effects on growth and feed efficiency has been a common element in the line of projects conducted by our team during the last few years. We have demonstrated that genetic social interaction effects are a major determinant of the growth of rabbits raised under feed restrictions (Piles et al., 2017), not being so relevant for the case of animals fed *ad libitum*. This could be a factor explaining the strong genotype by feeding regimen interactions we have estimated (Piles and Sánchez, 2019). Information provided by the electronic feeders (Piles et al., 2024) as well as by other technological tools such as computer vision systems (Sánchez et al., 2022) for remotely monitoring the growing rabbits is also allowing us to better understand the role that the

interactions between cage-mates might play on determining traits of paramount relevance for sustainable rabbit production.

GENOMIC TOOLS FOR THE STUDY OF KEY SUSTAINABILITY TRAITS.

From an animal breeding perspective, the possibility to densely type the genome of the selection candidates has revolutionised animal breeding schemes in all the major livestock species. Since approximately 2018 the rabbit has had a specific genome-wide panel for genotyping which includes 200,000 Single Nucleotide Polymorphism (SNP). This panel was developed after research on the domestication process of the species (Carneiro et al.,2014); the whole-genome sequence material generated was proposed for use by Affymetrix for developing a commercial genotype panel. This development was promoted and dynamized from RGB-Net COST action (Garreau et al., 2012) that put in common the interest of several research disciplines using or studying the rabbit species: evolutionary and biomedical sciences, and animal production. Since its launch a number of studies have been conducted using the rabbit 200K SNP panel. Only one study has evaluated the value of simulated SNPs panels in applied breeding programs (Mancin et al., 2021). In this study the most convenient scenario seems to be that genotyping the candidates with a low-density panel (600 SNPs) and them imputing their genotypes to high-density (200K SNPs), keeping sires and grand-sires genotyped with the high-density panel, and dams genotyped with a medium-density panel (6K SNPs). Under this scenario the additional response, with respect to a selection based on a pedigree based BLUP prediction, was just 0.011 kits per generation and the need for genotyping investment was expected to be between 10,000 and 15,000 € per year. Beyond this being the most convenient scenario, we believe it is still far from being practically applicable since such a high investment would hardly be supported by the limited gain in prolificacy obtained.

Most studies considering genomic information were aimed at identifying genes and genomic regions underlying traits of interest for the rabbit production sector, among them the major determinant of rabbit production sustainability: feed efficiency and prolificacy. Sánchez et al., (2020) performed a genome-wide association study (GWAS) in rabbits aiming to identify chromosomal regions associated with growth and feed efficiency. Despite the limited power of their design, based on cage-average feed efficiency measurements, interesting associations for feed efficiency were declared in chromosome 16, but no candidate genes were identified. In the case of growth, QTL regions were declared in chromosomes 3 and 5, harbouring genes already described to be associated with growth, such as FTO and NDUFAF6. Garreau et al. (2023) have also reported QTL regions for feed efficiency traits measured in individual cages. They declared one region in chromosome 18 associated to RFI, this region harbours the gene GOT1, a clear candidate because it is involved in the metabolism of amino acids and urea. They also declared one region in chromosome 7 associated with FCR but this region lacks any biologically relevant annotated gen. To the best of our knowledge the only genome-wide association study conducted so far for prolificacy traits is the study by Sosa-Madrid et al. (2020), they reported regions on chromosome 17 associated with several prolificacy traits, like total born alive, number born alive, implanted embryo and ovulation rate. There are several genes in this region that could be considered as candidates for explaining the variation on the prolificacy traits: BMP4, PTDGR, PTGER2, STYX and CDKN3. For total born alive, this region could be said to have a major effect since it explains up to 38% of the genetic variance. The animal material used in this study comprises two lines divergently selected for uterine capacity (Santacreu et al., 2005) and a control unselected population formed from vitrified embryos from the base population common to both selected lines. In addition to this recent GWAS study for prolificacy traits previous attempts to identify genes controlling these types of traits were also conducted following the candidate gene approach, by comparing polymorphisms and expression levels of certain genes of interests between the same lines divergently selected for uterine capacity (Argente et al., 2010; Ballester et al., 2013; Peiró et al., 2008; Merchán et al., 2009).

As it has been already indicated another important set of traits in the definition of rabbit production sustainability are the functional traits. In relation to these traits, and within the framework of the divergent selection experiment for environmental variability of prolificacy (Blasco et al., 2017) several genomic studies have been conducted to bring light to the biological pathways involved in the control of such a complex trait, and those correlated with it, i.e. resilience traits. Casto-Rebollo et al. (2020) using both commercial genome-wide SNP panels and whole-genome sequences of animals divergently selected reported that genes (e.g. DOCK2, HDAC9, ITGB8, and HUNK) and pathways involved in the immune-system play a key role in the determinism of environmental variability, sustaining the strong link between environmental variability, resilience, and stress tolerance.

One important conclusion from all the studies about the identification of genes or genomic regions involved in the control of feed efficiency, prolificacy and other functional traits, is that the majority of the genes or loci that are involved in the control of such traits do not seem to have a major effect; thus, selection methods incorporating specific information about the segregation status of single (or few) genes or locus do not seem to be the most sensible approach for conducting efficient selection programs; there being more suitable models assuming the existence of multiple genes with small effect, as is the case of the traditional infinitesimal model or genomic selection procedures that regardless of the effect of the markers, they just consider the genomic information to refine the expected relationship between relatives into actually observed percentage of genome sharing. One exception to this statement could be the region on chromosome 17 affecting total born alive. It has been hypothesised that the fixation of this region is the reason why half of response in divergent selection process was achieved in the first two, out of ten, generations of selection for uterine capacity (Blasco et al., 2005).

Beyond the genomic studies on the major components of the sustainability reviewed above, significant efforts have been made to identify genomic regions involved in the control of other traits of interest to the rabbit industry or to the rabbit research community. One example is the intramuscular fat (Sosa-Madrid et al., 2020a, 2020b), for this trait QTL regions have been declared in chromosomes 1, 3, 6, 7, 8, 13, 16 and 17; and in some of these regions, functional candidate genes are mapped. The experimental design of these studies provided higher statistical power than studies with homogeneous populations, since they were conducted with animals divergently selected for intramuscular fat.

Similar to the use of genomic tools for the assessment of genomic regions involved in the control of traits directly or indirectly involved in the determination of rabbit production sustainability, in the last five-seven years several studies have been conducted to clarify the role that gut microbiota plays at defining traits linked to sustainability. These activities, in the majority of the cases, are not strictly genetic studies since their aim is to identify microbial taxa involved in the control of traits of interest, nonetheless as most of these activities have been conducted within the framework of different genetic selection programs, and technically they rely on different forms of genomic sequencing (of the bacteria genome), many times they are clustered as within the animal genetic domine. Zeng et al. (2015), Drouilhet et al. (2016), Fang et al. (2020) and Velasco-Galilea et al. (2021) have explored the role of gut microbiota on feed efficiency and growth traits. Similarly, for functional traits the Polytechnic University of Valencia and Miguel Hernández University teams have reported evidence of the role of gut microbiota on across-parity environmental variation of the prolificacy (Casto-Rebollo et al., 2023) and on longevity or resilience (Biada et al., 2021). Also, the influence of gut microbiota on intramuscular fat content has been reported (Martínez-Álvaro et al. 2021). The studies on functional traits, and on the intramuscular fat content, rely on comparisons between populations divergently selected for the traits of interest or with extreme phenotypes; thus, as for the case of the genomic studies, optimal designs were considered. An overall result of these studies is that gut microbiota plays a significant role for most of the traits studied, but the effects tend to be a consequence of the participation of many species. From a traditional animal breeding perspective, a common element around the study of the

gut microbiota has been the estimation of the heritabilities of the different taxa present in the different populations, as well as the genetic correlations with different performance traits (Mora et al., 202). The values of these parameters range from nearly null to moderate-high. Until now, it is not evident how to take advantage of the biological knowledge about the role of the gut microbiota on the traits of interest to speed up the rate of response in breeding programs, in spite of the fact that some of the taxa clearly show inherited variability.

THE FUTURE OF BREEDING PROGRAMS FOR ENHANCING RABBIT PRODUCTION SUSTAINABILITY

The first element that must be addressed to optimise future breeding programs for enhancing rabbit production sustainability is the proper economical evaluation of social and environmental dimensions of the sustainability within the profit functions that are used to derive economic weights to be considered in selection indexes. To do this, multidisciplinary collaboration will be mandatory, requesting the intervention of social science and economy researchers, life cycle analysis experts, scientists on biosystem environmental sustainability and obviously geneticists. The initial output of this revision of the profit functions would be the recalculation of the monetary coefficients, accounting for social and environmental dimensions of sustainability. This might generate an alternative ranking of the traditional traits considered so far in the breeding programs. Another point of revision around the profit functions will be the consideration of new traits to explain the benefits of the rabbitries. These new traits will be those explicitly accounting for specific dimensions of sustainability. One example, in a scenario where important costs might be associated with manure handling, a penalty associated with this handling could be introduced in the profit function, and at the time of deriving the economic weight this new trait “manure production” will be ranked jointly with the traits considered so far. The point is how to generate new phenotype records for these new traits to be included in the profit functions, again the cooperation of experts from different fields will be mandatory: Nutritionists, Physiologists, Ethologists, Pathologists and Clinical Veterinarians. These experts must bring biological knowledge about the new processes to be considered in future breeding programs, but this expertise will have to be weighed by a major need of any breeding program: phenotypic records must be recorded at individual level in the largest number of animals possible. Thus, the intervention of (bio)-technological experts allowing the development of tools for massively recording phenotypes correlated with the new biological processes to track is also needed. In the rabbit production sector, there are already examples under development of such technological tools, like for example electronic feeders to individual record feed intake in cage-raised animals (Sánchez et al., 2024), computer vision systems to monitor activity, behaviour or health and stress status (Sánchez et al., 2022; Jaén-Téllez, J.A. et al.,2020,), the use of NIR prediction equations to generate phenotypes informing about the chemical composition of the body or of the meat (Zomeño et al., 2011), the use of wearable sensors to monitor activity (Piles et al., 2023) or the use of electrical bioimpedance techniques also to predict body chemical composition (Nicodemus et al., 2009). These types of tools can be said to be yet under development and a much stronger commitment by researchers and the sector as a whole would be needed to further develop and improve them, and also to bring new ideas and tools to our sector. There are other examples of technical developments in other sectors that could easily be adapted to rabbit breeding programs: individual NIR-based digestibility assessments in pigs (Derau et al, 2021), individual Nitrogen and Phosphorus excretion (pigs: Saeys et al., 2005, poultry: Cheng et al., 2009). These techniques have the added value of being non-invasive, enhancing in this way the welfare of the animal at the time of data recording.

Beyond the generation of new phenotypes and a proper evaluation of the social and environmental dimensions of sustainability we most likely will see how *omics* tools will continue its application within breeding programs. Most likely, as is currently done, to explore the genetic and non-genetic control of the traits of interest; but hopefully, in the near future, also to help to improve the accuracy when making selection decisions. Nowadays, this is not

certainly the case because genotyping costs are far much higher than the gain in responses that can be achieved. Note however that biotechnological tools and platforms for genotyping and sequencing are evolving very rapidly. Thus, it may happen that in the future we will have genotyping or sequencing platforms that would result economically affordable and easy to use even on the farm; and they could be useful for the improvement of characteristics that may continue to be very difficult to measure, as may be the case with the carcass traits.

CONCLUSIONS

Sustainability is a wide and complex concept linked to three dimensions: social, environmental, and economic. Unfortunately only the last has clearly defined and easy-to-understand units. Efforts must be made on translating the social and environmental dimensions of sustainability to tangible and measurable elements, or alternatively to fully integrate them into the economic dimension, i.e. directly charging Euros per unit of environmental impact. Despite the certain lack of definition of some sustainability planes, we can affirm that prolificacy and feed efficiency seem to be the most relevant traits influencing rabbit production sustainability, but other functional traits, related to resilience and robustness, may also play an important role in the sustainability of rabbit production. Note however, that an updated evaluation of their impact on economic, social, and environmental sustainability is worth doing. In any case, elements determining the sustainability may act either as scale factors or as direct elements of actual production efficacy. The direct consequence of the feed efficiency being a pillar of sustainability is confirmed by the fact that breeding programs develop specialised lines with enhanced capabilities to extract and use resources from the feed. To this aim, traditional selection procedures relying on phenotypic data and pedigree relationships seem to be the way to go. But we cannot fail to consider the opportunities opened by omic tools and up-to-date technologies for automatic data recording to enrich both breeding programs and biological studies of traditional and yet-to-be-developed phenotypes.

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GENETIC PARAMETERS FOR HEALTH AND PRODUCTION TRAITS IN THE GGP OPTIMA RABBIT LINE

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ABSTRACT

Genetic parameters for health and production traits were estimated in the great-grandparental (GGP) OPTIMA line, using the REML methodology applied to a multitrait animal model. Heritability estimates of infectious disease score (IDS), individual birth weight (IBW) and within litter standard deviation of birth weight (STDBW) were low but significantly different from zero (0.07 ± 0.01, 0.08 ± 0.01 and 0.11 ± 0.04, respectively. Heritability estimate of carcass yield was moderate (0.26 ± 0.09). Heritability for number born alive per litter (NBA) was consistent with the literature (0.09 ± 0.02) but heritabilty for standard deviation of number born alive (STDNBA) was not significantly different from zero (0.02 ± 0.05). All phenotypic correlations were close to zero except for the moderate favorable correlation between NBA and STDNBA (-0.21 ± 0.02). In general, genetic correlations were low, meaning a global genetic independence between traits but, due to the limited number of performances, their standard errors were high. The most remarkable values were the significant favorable genetic correlations between IDS and the two production traits IBW and CY (-0.40 ± 0.14 and -0.26 ± 0.17, respectively). It is therefore possible to effectively improve both health, production and ease of handling traits.

Key words: rabbit, genetic parameters, environmental variance, disease resistance, multi-performance.

INTRODUCTION

Livestock farming systems and breeding schemes must evolve and find compromises to meet the demands of multi-performance and societal expectations. The great-grandparental (GGP) OPTIMA female line is selected in this mind. The GGP OPTIMA selection strategy follows the principle of sustainability of breeding by combining: the robustness of breeders, their production performances and ease of handling, the latter being achieved, for example, with a more uniform weight of newborns. The ultimate aim is to pass on these qualities to the offspring, in this case their daughters: the parental females.The objective of the study was to estimate genetic parameters of health and production traits in the GGP OPTIMA line in order to investigate how to combine them in a global breeding objective.

MATERIALS AND METHODS

Animals and data

The study was undertaken in the GGP OPTIMA maternal line (Hypharm, La Corbière, Roussay, France), which is widely used as the mother of parental does in rabbit production. The population is managed in overlapping generations, divided in 21 family groups with 42 bucks and 250 does at each insemination (42 days cycle). Those animals are raised in welfare cages, with antibiotic free feed. Rabbits were identified by tattoo and after weaning at 36 days of age, they were placed in collective cages with 6 individuals each and fed with a commercial pelleted food until 72 days of age. Number born alive (NBA) were recorded in 5655 litters from January 2020 to January 2024. Within doe standard deviation of number born alive (STDNBA) was calculated to assess the variability of litter size for does that reached 3 or more parities (841 animals). Individual birth weights (IBW) and birth dates were recorded in the first three litters of each does. There was no individual identification of rabbits at birth i.e. the young rabbit weights were considered as a repeated trait of the female. Newborn rabbits were weighed within 24 h after birth. In rare cases, some were weighed the

first or the second day after birth. For the study, there were 17,617 rabbits were weighed. Within litter standard deviation of birth weights (STDBW) was calculated to assess the variability of weight at birth in each litter (1684 litters).
Clinical signs of diseases occurring naturally on farms were recorded at the end of the test, at 70 days of age. The most likely cause of death of rabbits that died before the end of the test was also recorded after necropsy. Diseases were the following: 1) digestive disease, which includes diarrhea, bloated abdomen, and any form of digestive symptoms, 2) respiratory diseases and pasteurellosis-like symptoms which includes nasal discharge, lung lesion, eye infection, wry neck, 3) non-specific diseases which include abnormal low weights and other clinical signs of infectious origin. The infectious disease score (IDS) was coded as 1 (absence) or 2 (presence of any sign of the above-mentioned diseases observed at 70 days or at death during the fattening period). More details about this trait are given by Gunia et al. (2015). For the study, there were 19,186 rabbits recorded for health from July 2021 to January 2024.The incidence of infectious disease was 18.5 % of the population (score equal to 2) and 38 % of these rabbits died prior to 70 days weighing. For each dam, the Carcass Yield (CY) was measured on 2 to 4 healthy offspring of the first litter. Rabbits were weighed at the 72 days of age at the breeding farm. Then, they were slaughtered in a commercial slaughterhouse. After 2 hours of chilling, each cold carcass was weighed. Carcass yield (CY) was calculated as chilled carcass weight divided by live weight. Descriptive statistics of traits above mentioned are given in Table 1.

Table 1: Number of records (*N*), means, standard deviations of the means (SD) and coefficients of variation (CD) of infectious disease score, number born alive per litter, individual birth weight, carcass yield, within litter standard deviation of birth weight and within doe standard deviation of number born alive

	N	Mean	SD	CD
Infectious disease score (1,2)	19186	1.19	0.39	32.8
Number born alive	5655	10.6	3.6	33.8
Individual birth weight (g)	17617	65.8	13.7	20.8
Carcass yield (%)	1591	53.6	1.9	3.5
Within litter standard deviation of birth weight	1684	9.83	3.39	34.5
Within doe standard deviation of number born alive	841	2.97	1.33	44.8

Model and statistical analysis
The genetic parameters were estimated by the REML methodology applied to a multitrait animal model, using the ASReml Software (Gilmour *et al.,* 2009). Fixed effects retained for each trait, i.e. with *P*-value < 0.05, are given in Table 2. For IDS and CY, the significant fixed effects included in models were litter size at weaning (6 classes: 1-5, 6, 7, 8, 9, 10), batch (20 classes) and litter parity (2 classes: 1, 2 and more). For NBA the significant fixed effects included in the model were year-Season of kindling (17 classes) and the combined effect parity-lactation status (9 classes: first parity, parity 2, 3, 4, 5 and more, combined with lactating and not-lactating status). For IBW and STDBW the fixed effects were year-season of kindling (17 classes) and the combined effect parity-lactation status (9 classes: first parity, parity 2, 3, combined with lactating and not-lactating status), the covariate litter size at birth, the length of gestation (4 classes: 30, 31, 32, 33 days), age at weighing (4 classes: 0, 1, 2, 3 days between date of birth and date of weighing). The only fixed effect included in the model for STDNBA was the female cohort (20 classes: year and week of birth of the female). The models included a random additive polygenic effect for all traits, a random common litter effect for IDS, IBW and CY, a permanent environmental effect to account for the repeated measurement of NBA on the does.

Table 2: Fixed effects retained in the models for infectious disease score (IDS), number born alive per litter (NBA), individual birth weight (IBW), carcass yield (CY), within litter standard deviation of birth weight (STDBW) and within doe standard deviation of number born alive (STDNBA).

	IDS	NBA	IBW	CY	STDBW	STDNBA
Litter size at weaning	X			X		
Batch	X			X		
Litter parity	X			X		
Year-Season of kindling		X	X		X	
Parity-lactation status		X	X		X	
Litter size at birth			X		X	
Length of gestation			X		X	
Age at weighing			X		X	
Female cohort						X

RESULTS AND DISCUSSION

Heritabilities

Genetic parameters for health and production traits are presented in table 3. To estimate the genetic parameters of IDS, the use of a linear model, rather than a threshold model theoretically more suitable for a binary trait, is justified by Gunia et al. (2018): Several studies have indeed shown that estimates of heritability or breeding values from linear and threshold models are strongly correlated (Ramirez-Valverde et al., 2001). Heritability of IDS was low (0.07 ± 0.01) but signficantly different from 0, in consistance with the literature (Gunia et al. 2015, 2018; Eady et al., 2007). Visually assessed disease syndromes are easy to record and therefore routinely collected to select GGP OPTIMA rabbits for improved non-specific disease resistance. Estimates of heritability for production traits NBA and CY (0.09 ± 0.02 and 0.26 ± 0.09, respectively) were generally consistent with the literature (García and Baselga, 2002; Garreau et al., 2008). Few estimates of genetic parameters for CY have been published and the studies were all conducted on paternal lines (Krogmeier et al., 1994; Larzul et al., 2005; Garreau et al., 2008). To our knowledge, we are publishing here the first results of genetic parameters for carcass yield estimated in a rabbit maternal line. The GGP OPTIMA line would therefore be the first rabbit maternal line selected for this trait, in the same way as pig lines. Heritability estimates of IBW and of STDBW were in the same range (0.08 ± 0.01 and 0.11 ± 0.04, respectively). Garreau et al. (2008) reported similar values of heritability for IBW (0.06 ± 0.01) in the GGP22 maternal line of the HYPHARM breeding company. The same authors reported a very low value of heritability for the within-litter variability of birth weight, evaluated by the log squared estimated residual from a mixed linear model on birth weight, in the same population (0.012 ± 0.004). Nevertheless, a divergent selection for this criterion was succesfully carried out by the authors, highlighting significant differences in the within-litter standard deviation of birth weight, the low value of heritability being explained by the logarithmic transformation which rendered the estimate uninterpretable. The heritability estimate of STDNBA was not significantly different from zero. However, Blasco et al. (2017) reported higher value for heritability of the variance of litter size (0.09) in a successful divergent selection for this trait.

Phenotypic and genetic correlations

All phenotypic correlations were close to zero except for the moderate favorable correlation between NBA and STDNBA (-0.21 ± 0.02). Blasco et al. (2017) reported also a negative phenotypic correlation between litter size and the variance of litter size, but slightly lower (-0.09). Due to a limited number of performances, the standard error of genetic correlation estimates were high, especially for the correlation between STDNBA and the other traits. Therefore, values should be interpreted with caution.

Table 3. Estimates of heritability (diagonal), genetic (above diagonal) and phenotypic (below diagonal) correlations, and their SE (±) for health and production traits

	IDS	NBA	IBW	CY	STDBW	STDNBA
IDS	0.07 ± 0.01	0.02 ± 0.18	-0.40 ± 0.14	-0.26 ± 0.17	-0.22 ± 0.21	-0.16 ± 0.59
NBA	-0.01 ± 0.01	0.09 ± 0.02	0.10 ± 0.18	-0.08 ± 0.24	0.22 ± 0.24	-0.24 ± 0.42
IBW	-0.04 ± 0.01	-0.01 ± 0.01	0.08 ± 0.01	0.09 ± 0.21	-0.09 ± 0.21	-0.01 ± 0.56
CY	-0.08 ± 0.03	-0.04 ± 0.03	0.00 ± 0.02	0.26 ± 0.09	0.23 ± 0.28	0.74 ± 0.89
STDBW	-0.02 ± 0.01	0.03 ± 0.02	-0.02 ± 0.01	0.03 ± 0.03	0.11 ± 0.04	0.84 ± 0.89
STDNBA	-0.01 ± 0.02	-0.21 ± 0.02	0.01 ± 0.02	0.04 ± 0.05	0.00 ± 0.03	0.02 ± 0.05

IDS = infectious disease score; NBA = number born alive; IBW = individual body weight; CY = carcass yield; STDBW = standard deviation of body weight; STDNBA = standard deviation of number born alive

In general, genetic correlations were low, meaning a global genetic independence between traits. The most remarkable values were the significant favorable genetic correlations between IDS and the two production traits IBW and CY (-0.40 ± 0.14 and -0.26 ± 0.17, respectively). Other studies reported low or favorable genetic correlations between IDS and NBA (Gunia at al., 2017) or between IDS and CY or body weight at 70 days (Gunia et al., 2015). In the absence of genetic antagonism, it is therefore possible to effectively improve both health, production and ease of handling traits.

CONCLUSIONS

This study shows that it is possible to improve health, performances and ease of handling traits in the GGP OPTIMA line. With the exception of litter size variability, heritability values are sufficiently high, and, favorable or weak genetic correlations between traits allow significant genetic progress to achieve multi-performance and societal expectations objectives, for sustainability of breeding.

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INFLUENCE OF GENETIC DIVERSITY AT INDIVIDUAL AND GROUP LEVEL ON THE HEALTH OF GROWING RABBITS

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ABSTRACT

This study investigates the impact of genetic diversity at both individual and group levels on the health of 1,038 growing rabbits raised without antibiotics during the post-weaning period. By increasing diversity at the individual level (crossbreeding) and at the group level (cross-fostering), our aim is to reduce the dependance on antibiotics in industrial farming systems. The survival, the visual health score, and the white blood cell counts were monitored. We compared purebred and crossbred rabbits, as well as two cross-fostering strategies: between- and within-genotypes. Results show that crossbreed rabbits exhibit better visual health scores at 64 days, indicating a positive effect of genetic diversity at the individual level. However, increasing genetic diversity at the group level through cross-fostering did not yield favorable outcomes, with more rabbits in the between-genotype strategy classified as diseased. Further investigation into the effects of genetic diversity at the herd level is recommended to understand its potential benefits on the farm health. Overall, this study underscores the complex interplay between genetic diversity and health outcomes in industrial rabbit farming systems.

Key words: Genetic diversity, cross-fostering, white blood cell count, crossbred, health.

INTRODUCTION

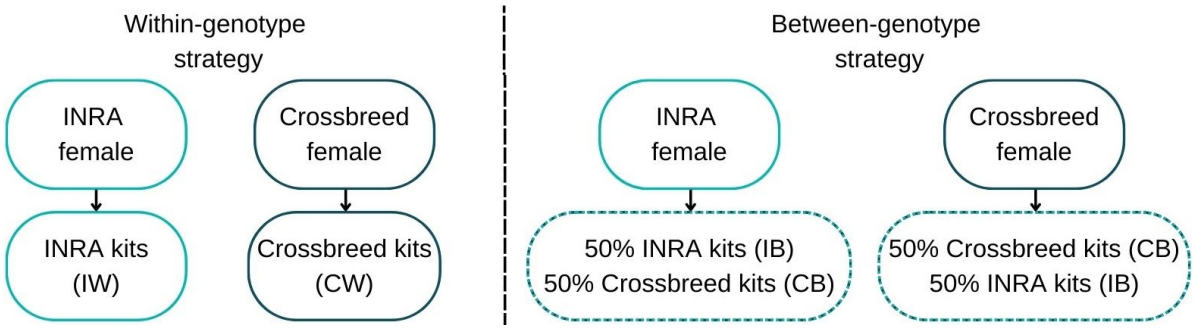
Our study is part of the quest for alternative solutions to reduce the dependence of antibiotics in rabbit farming. Building on the findings of King and Lively (2012), which demonstrate that the genetic diversity of host populations can offer protection against disease or parasites, our study focuses on the benefits of genetic diversity at individual and group levels on rabbit health during the post-weaning period. For the individual-related genetic diversity analysis, we compared purebred and crossbred rabbits. To evaluate genetic diversity at the group level, we compared two cross-fostering strategies: between- and within- genotype. The genetic diversity at the group level was maintained after weaning. Our objective was to evaluate the impact of genetic diversity on health and survival.

MATERIALS AND METHODS

Animals and experimental design

A total 1,038 growing rabbits born in four cohorts with a 42 days interval were monitored between 35 (weaning age) and 64 days of age. They were purebred 481 INRA-1777 kits (hereinafter INRA) and 557 Crossbreed kits (hereinafter Crossbreed) ¾ Fauve-de-Bourgogne and ¼ INRA. The Crossbreed were born from crossbreed ½ Fauve-de-Bourgogne × ½ INRA-1777 does inseminated with semen from purebred Fauve-de-Bourgogne males. The day after birth, rabbit kits were individually identified and two cross-fostering strategies were applied (Figure 1). At each birth cohort, half of the litters were assigned to the between-genotype cross-fostering strategy and the remain half were assigned to the within-genotype cross-fostering strategy. For each strategy, the average litter size at birth of each genotype was respected. We had four experimental groups: **IW**: INRA kits in the within-genotype cross-fostering strategy (n = 242), **CW**: Crossbreed kits in the within-genotype cross-fostering strategy (n = 295), **IB**: INRA kits in the between-genotype cross-fostering strategy (n = 239) and **CB**: Crossbreed kits in the between-genotype cross-fostering strategy (n = 262).

Figure 1: Representation of cross-fostering strategies. **IW**: INRA kits in the within-genotype cross-fostering strategy, **CW**: Crossbreed kits in the within-genotype cross-fostering strategy, **IB**: INRA kits in the between-genotype strategy and **CB**: Crossbreed kits in the between-genotype strategy.



At weaning, the rabbit kits were placed in groups of five to six rabbits in wired cages (L×W×H: 90×46×60 cm). The genetic diversity at the group level (i.e. IW, CW, IB, and CB) was maintained after weaning. Rabbits were raised without antibiotics. They had free access to water and to a commercial feed (STABI-GREEN G, Terrya, Rignac, France) at all times.

Studied Variables

Kits survival. Kits survival from weaning to 64 days of age was monitored daily. Date and cause of death or culling were recorded. Survival data are presented as Kaplan-Meyer survival curves.

Visual health score. Each animal's health score was assessed visually based on the clinical signs of disease at 64 days of age. A total of 952 rabbits were recorded and classified as healthy or sick.

Hematological health score. Between 64 and 67 days of age, blood samples were taken on a representative subgroup of 850 rabbits. The differential count of white blood cells was performed using the MS9-5 Hematology Analyzer (Melet Schloesing Laboratoires). The literature provides reference values for the normal range of white blood cell counts in rabbits. However, they did not correspond to our populations. We therefore defined new normal values adapted to our genotypes. Box and whiskers plots for each white blood cell type were constructed for each genotype for all rabbits having an healthy Visual Health score at 64 days of age. Values falling outside the whiskers (whisker ends being calculated using 1.5 times the interquartile space according to Tukey, 1977), were considered outliers. The minimum and maximum values obtained after excluding outliers were kept as the new normal ranges (Table 1). On the basis of these new threshold values, we classified the rabbits into two classes for each white blood cell population: "normal" if the animal had values within the threshold values for its genotype, or "abnormal" otherwise.

Table 1: Normal white blood cells (× 10^9/L) range from the literature (Fielder, 2022), or estimated from our data on healthy rabbits (visual health scores) at 64 days of age.

Hematological trait	Fielder, 2022	Genotypes	
		INRA	Crossbreed
Total White Blood Cell counts	6.0 - 12.0	3.6 - 12.8	1.9 - 12.3
Lymphocytes	1.6 - 10.6	1.2 - 3.9	0.6 - 4.8
Monocytes	0.05 - 0.5	0.3 - 1.1	0.3 - 0.9
Neutrophils	1.0 - 9.4	1.4 - 7.9	0.0 - 7.3
Eosinophils	0.05 - 0.5	0.0 - 0.2	0.1 - 0.1

Statistical Analysis

The statistical analyses was performed using the R statistical software version 4.2.2 (R CoreTeam, 2023). To analyze survival, a variable called Lifetime was set as the number of days between the weaning date and the date of death or culling. Kit survival was set as the

number of days between the weaning date and the date of death or culling. The model used to produce the Kaplan-Meier estimation of survival probability curves included the growing rabbit's cross-fostering strategy and their genotype. The model was implemented using the R-package {survival}. For the two binary traits: visual health score (healthy 0, sick 1) and hematological score (normal 0, abnormal 1), the data was analyzed using a general linear model. We analyzed the proportion of rabbits classified as 0 or 1 according to the cross-fostering strategy, the genotype and their interaction.

RESULTS AND DISCUSSION

Survival

The overall mortality rate (death or culling) between 35 and 64 days of age was 7.7%, with no significant difference between genotypes or cross-fostering strategies. Digestive syndromes were the main cause of death (66% of cases), followed by respiratory (15% of cases) and others syndromes (19%). Survival curves for the combination of genotype and crossbreeding strategy were statistically significant (P<0.001; Figure 2). The survival curve for the IW group differed from the others. The difference observed is related to the low survival probability observed in the IW group around 45 days of age. The INRA progeny seems to benefit from the between-genotype cross-fostering strategy.

Figure 2: Survival probability curves according to the genetic diversity at the individual (INRA or Crossbreed) or the group (within- and between- cross-fostering strategy) level

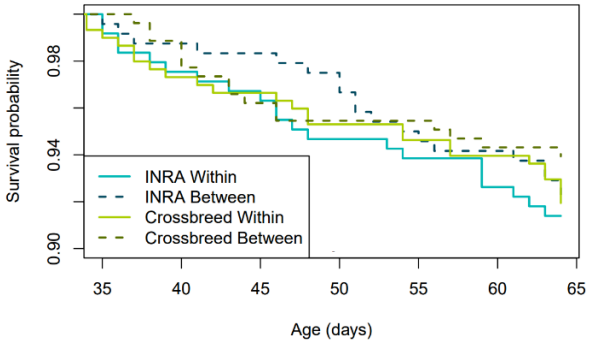
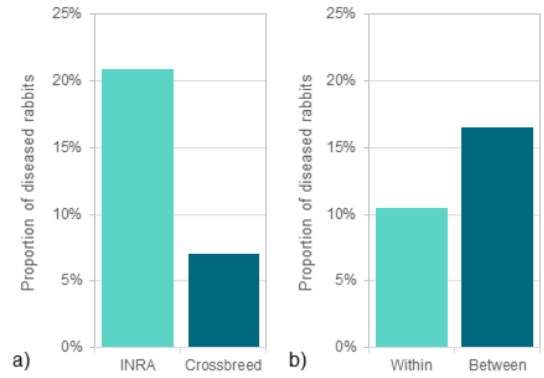


Figure 3. Proportion of rabbits classified as sick on the Visual Health Score according to the genetic diversity at (a) the individual (INRA or Crossbreed) or (b) the group (within- and between- cross-fostering strategy) level



Visual health score

The proportion of sick rabbits at 64 days old (Figure 3) was significantly higher for INRA than for Crossbreed rabbits (20.9% vs. 7.0%, respectively; P<0.001), and significantly higher for the for the between-genotypes compared to the within-genotype cross-fostering strategy (16.5% for *vs*. 10.5%, respectively, P<0.01). No significant interactions between kit genotype and cross-fostering strategy was observed for this trait. Live animals at 64 days old being classified as sick using the visual health score had respiratory syndromes in 95% of the cases.

Hematological health score

The percentage of rabbits with white blood cell populations outside the normal range (here defined: Table 1) is shown in Table 2. Overall, the proportion of rabbits with abnormal values was less than 6% for total white blood cells, lymphocytes, monocytes and neutrophils. For eosinophils, between 11.2% and 26.3% of all rabbits had values outside the normal range. A genotype effect was identified for eosinophils, linked to a high percentage of Crossbreed rabbits in the within-genotype cross-fostering strategy showing eosinophil values outside the normal range compared to other groups (on average, 26.3% *vs*. 13.5%; P<0.05). Interactions between genotype and cross-fostering strategy were also observed for lymphocytes and neutrophils.

Table 2: Effect of genetic diversity at the individual (INRA or Crossbreed genotype) or group (within- or between- cross-fostering strategy) level on the percentage of rabbits with hematological values out of the normal range.

Genotype (G)	INRA		Crossbreed		*P*-values[(1)]		
Cross-fosterig strategy (CS)	Within	Between	Within	Between	G	CS	G×CS
Total white blood cells	2.54	2.51	3.39	1.37	0.90	0.31	0.32
Lymphocytes	0.50	4.02	2.11	0.46	0.30	0.40	<0.01
Monocytes	4.06	4.02	5.08	4.13	0.68	0.71	0.76
Neutrophils	4.56	0.50	2.11	2.29	0.73	0.08	0.03
Eosinophils	11.2[a]	15.1[a]	26.3[b]	14.2[a]	<0.01	0.07	0.04

[(1)]*P*-values of the main effects Genotype (G), Cross-fostering Strategies (CS) and their interaction (G×CS): type II analysis of variance.
[a-b] Percentages having different superscripts differ at $P<0.05$.

Genetic diversity at the individual, group or herd level

We observed a favorable effect of genetic diversity at the individual level on the visual health score, where a higher proportion of Crossbreed rabbits were classified as healthy at 64 days compared to INRA rabbits. This could be explained by the heterosis effect of Crossbreed rabbits. Heterosis has a highly positive effect on health (Blasco et al., 1993). On the opposite, we observed an unfavorable effect of genetic diversity at group level. Rabbits in the within-genotype cross-fostering strategy showed a significantly higher proportion of healthy visual health scores at 64 days compared to rabbits in the between-genotype cross-fostering strategy. Cross-fostering within a single genotype is a common practice in rabbit breeding that tends to increase pre-weaning survival (Heim et al., 2012). Is worth noting that throughout the experimental period (four consecutive cohorts), more than 91% of rabbits (independent on the genotypes and cross-fostering strategies), were classified as health at age 64 days without any antibiotic use. We can hypothesize that individual genetic diversity, and perhaps group genetic diversity may have had a positive effect at herd level. Further studies need to be carried out, for example comparing single-breed herds with herds using a mixture of breeds, to demonstrate the potential benefits of increased genetic diversity at the herd level on health traits.

CONCLUSIONS

We studied the influence of genetic diversity at individual and group level on the health of rabbits during the post-weaning growth period. Crossbreed rabbits showed better visual health scores at 64 days, indicating a positive effect of genetic diversity at the individual level. However, increasing genetic diversity at group level through cross-fostering strategies did not appear to have a favorable effect, with more rabbits in the between-genotype cross-fostering classified as sick for the visual health scores at 64 days. No difference in mortality was observed between groups.

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ENVIRONMENTAL STRESS RESPONSE ASSESSMENT BY INFRARED THERMOGRAPHY IN TWO RABBIT LINES DIVERGENTLY SELECTED FOR LITTER SIZE VARIABILITY

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ABSTRACT

The present research studied the evolution of eyeball temperature measured with infrared thermography in 120 male rabbits belonging to two lines selected divergently for litter size variability. Data were collected before and after sperm extraction, with and without thermal stress, at 3 moments (minutes 0, 1 and 5). The extraction was carried out after measuring the basal temperature at minute 0. The duration of the experiment was 12 weeks. At minute 0, the homogeneous line showed a lower temperature than the heterogeneous line without (36.46ºC vs 36.77ºC; P = 92%) and with thermal stress (37.13ºC vs 37.78ºC; P = 99%). With thermal stress, the heterogeneous line did not respond to the stimulus. Without thermal stress, both lines reacted to the stimulus of extraction, finding differences between minute 0 and 1, but the homogeneous line always remained below the levels of the heterogeneous line. In conclusion, the homogeneous line for litter size variability seems to adapt better to thermal stress conditions than the heterogeneous line.

Key words: Rabbit, Resilience, Stress, Thermography.

INTRODUCTION

Thermal stress is the presence of a high environmental temperature that exceeds the thermal comfort zone of an animal (Kang et al., 2020; Saracila et al., 2020). The Mediterranean climate has hot summers that, affected by climate change, have intensified. Rabbits are very sensitive to high temperatures (Marai et al., 2002). These are homeothermic animals with low thermoregulation capacity due to the absence of sweat glands and the presence of thick fur (Marai et al., 2001; Yağcı et al., 2006). The thermoneutral zone is between 18 and 21ºC (Marai et al., 2001) and the critical temperature at rest between 27 and 28ºC (Oladimeji et al., 2022). When the temperature exceeds 27ºC, the rabbit needs to eliminate excess heat; however, if the temperature continues to increase, it may exceed its dissipation capacity (Oladimeji et al., 2022), increase its body temperature (Bouwknecht et al., 2007) and cause productive and reproductive damage (Ebeid et al., 2023; Liang et al., 2022).

Several tools have been used to measure body temperature. Infrared thermography (ITR) is a simple, effective and non-invasive tool (Unruh et al., 2017) that allows measuring temperature in different anatomical regions, such as the eyeball (Jaén-Tellez et al., 2020; Serrano-Jara et al., 2023).

At the Miguel Hernández University of Elche, two rabbits lines are being selected divergently for litter size variability (Blasco et al., 2017). With a common origin, the females of the homogeneous line (selected to reduce litter size variability) and the heterogeneous line (selected to increase litter size variability) present productive, reproductive and behavioural differences, being more favourable in the homogeneous line (Agea et al., 2021; Argente et al., 2019). However, data related to the males of both lines are not yet available.

The objective of this research is to evaluate the response to a stressful stimulus without and under the effects of thermal stress in males of both lines to determine their ability to adapt to the environment.

MATERIALS AND METHODS

The experimental procedures with animals have been approved by the General Directorate of Agriculture, Livestock and Fisheries of the Generalitat Valenciana with code 2022/VSC/PEA/0226.

Animals and experimental design

The research was carried out on the farm of the Orihuela Higher Polytechnic School of the Miguel Hernández University. The facilities used were a controlled environment, 16:8 photoperiod, *ad libitum* feeding with a commercial feed and water always available.

A total of 120 males belonging to the 17th generation of the heterogeneous and homogeneous lines were used (60 per line). Age ranged between 4.5 and 10 months in both lines. The average weight was 3.53 kg.

Body temperature emissivity was measured using ITR on the eyeball. The images were obtained using a ®FLIR SC660 thermal imaging camera and were processed with the ®ThermaCAM Researcher Pro 2.10 software to obtain the temperature record. The camera was calibrated according to temperature, relative humidity, emissivity, and distance from the subject.

Data collection was carried out for 12 weeks, between June and October, and the measurements were carried out at 3 moments (minutes 0, 1, 5). During sampling the rabbits were restrained for approximately 1 minute. Sperm extraction was carried out between minutes 0 and 1.

From the ambient temperature and relative humidity, the temperature/humidity index (ITH) was calculated: ITH = t − ((0.31 − 0.31 × rh) × (t − 14.4)) where t = average temperature of the house from the time of taking the photographs up to 24 hours before and rh = relative humidity / 100, collected in the same way as the temperature. The weeks in which the data were collected were classified according to the ITH into: with heat stress (ITH > 27.8) and without heat stress (ITH < 27.8) (Marai et al., 2001).

Statistical Analysis

The model used to analyze eyeball temperature included the moment-line-stress effect (12 levels: moments 3; lines 2; stress 2), animal as random effect and its weight as covariate. The Rabbit program developed by the Polytechnic University of Valencia was used.

RESULTS AND DISCUSSION

Thermal comfort

When there was not thermal stress, the homogeneous line showed lower basal temperature than the heterogeneous line (36.46ºC vs 36.77ºC; P = 92%; Figure 1). Both lines showed similar temperature 1 minute and 5 minutes after stressful stimulus.

Both lines react to the stimulus, finding differences between minute 0 and 1 (P = 99% in the homogeneous line, and P = 90% in the heterogeneous line). The results show, on the one hand, that the homogenous line maintains the temperature below the heterogeneous line when at rest but is capable of reacting normally to the stimulus of sperm extraction. On the other hand, the results also indicate that in the absence of thermal stress the response to the stressful stimulus is quickly, as Serrano-Jara et al. (2023) described in a preliminary study.

This effect coincides with the increase in body temperature that occurs due to a greater presence of red blood cells in the bloodstream. This increase is caused by the indirect action of the release of adrenaline and norepinephrine (catecolamines) by the adrenal medulla, in the presence of a stressful stimulus (Axelrod and Reisine, 1984).

Heat stress

At minute 0, temperature of the homogeneous line is lower than in the heterogeneous line (37.13ºC vs 37.78ºC; P = 99%).

The difference between the lines is maintained in the subsequent minutes. At minute 1, the temperature in the homogeneous line is 37.21ºC and 37.84 in the heterogeneous line (P = 99%); At minute 5, the temperature in the homogeneous line is 37.41ºC and 37.92 in the heterogeneous line (P = 97%). A lower temperature in the homogeneous line under thermal stress agrees with the best ability to adapt to the adverse environment that is supported by the higher resilience and productivity than the heterogeneous one (Blasco et al., 2017; Argente et al., 2019; Beloumi et al., 2020; García et al., 2016).

The temperature of the homogeneous line is similar between minutes 0 and 1 (P = 72%), but it increases between minutes 1 and 5 (P = 92%). The heterogeneous line did not react between minutes 0 and 1 (P = 65%), nor between minutes 1 and 5 (P = 72%). No differences were found between minutes 0 and 5 (P = 83%). We can hypostasize that heterogeneous line does not respond to the stimulus caused by sperm extraction because this line is in a state of chronic stress due to the inability to adapt to thermal stress, while the homogenous line presents better resilience.

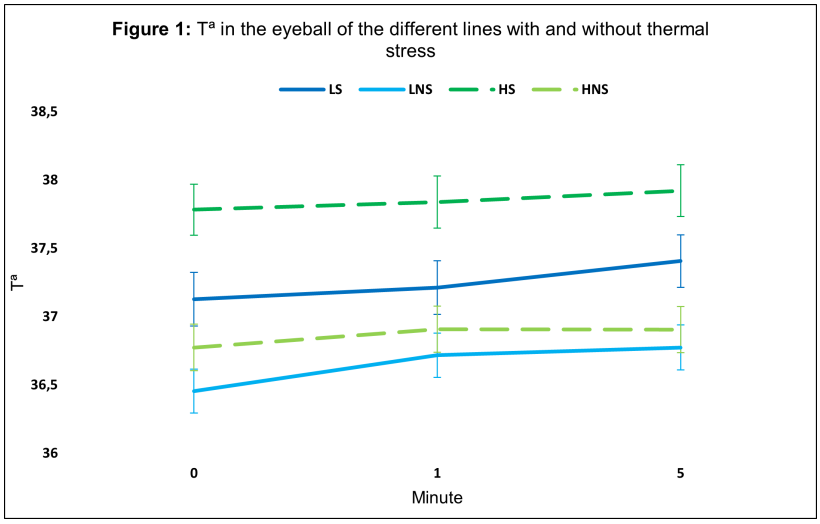

Figure 1: Tª in the eyeball of the different lines with and without thermal stress

LS: homogeneous line stress; LNS: homogeneous lines no stress; HS: heterogeneous stress; HNS; heterogeneous no stress

CONCLUSIONS

In conclusion, the homogeneous line for litter size variability seems to adapt better to thermal stress conditions than the heterogeneous line.

ACKNOWLEDGEMENTS

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EFFECT OF HEAT SHOCK PROTEIN (HSP) PRODUCTION ON GROWTH AND REPRODUCTIVE PERFORMANCE OF HYLA RABBIT IN SOUTH-WESTERN NIGERIA

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ABSTRACT

This study was conducted to evaluate the effects of the gene expressing stress protein, heat shock protein 70 and 90 (Hsp 70, 90) on growth and reproductive performance of Hyla rabbit. A total of twenty four healthy adult rabbits, sixteen females and eight males of average 2.50kg were used in the study. Does were randomly allotted into the four experimental treatments: T_1 (two weeks), T_2 (four weeks), T_3 (six weeks) and T_4 (eight weeks) breeding intervals with four rabbits per treatment in a Completely Randomized Design. Blood was collected into EDTA bottles from all does at three months interval to quantify the Hsp 70 and 90. The experiment lasted for twelve (12) months. The results showed that RT-PCR analysis indicated that both Hsp 70 and Hsp 90 mRNA were expressed in the tested bloods from does 2, 4, 6 and 8 weeks breeding intervals. Hsp 70 and 90 were most adequately expressed in the blood of does in 2 weeks breeding interval, which had a significantly ($p<0.05$) higher expression level than any other tested bloods of other breeding intervals. These results showed that Physiological stress was very high in the does in two weeks breeding interval (T_1) which had adverse effect on growth and reproduction of the does and their kitts in T_1.

Key words: Hyla rabbit, Hsp 70, Hsp 90, RT-PCR, mRNA.

INTRODUCTION

Heat shock proteins (HSPs) are among the most highly expressed cellular proteins across all species (Csermely *et al*., 2008). HSPs protect cells when stressed by elevated temperatures. They account for 1–2% of total protein in unstressed cells. However, when cells are stressed, the fraction of heat shock proteins increases to 4–6% of cellular proteins (Creve *et al*., 2011). It is generally accepted that HSPs protect organisms from the detrimental effects of heat and possibly other stressors including various chemicals, heavy metals, oxidative stress, and desiccation and that stress tolerance depends on the synthesis of HSPs (Kregel, 2002). They act like 'chaperones,' making sure that the cell's proteins are in the right shape and in the right place at the right time. HSPs help new or distorted proteins fold into shape, which is essential for their function. They also shuttle proteins from one compartment to another and transport old proteins to 'garbage disposals' inside the cell. Heat shock proteins are also believed to play a role in the presentation of pieces of proteins (or peptides) on the cell surface to help the immune system recognize diseased cells. Heat-shock proteins are named according to their molecular weight. For example, Hsp60, Hsp70 and Hsp90 which are the most widely studied HSPs refer to families of heat shock proteins on the order of 60, 70 and 90 kDa (kilodaltons) in size respectively (Lahvic *et al*., 2013).

Hyla rabbits have a superiority on high growth rate and prolification rate (Bram *et al*., 2021) and body weight of Hyla's rabbit in the age of 70 days can reach 2 160 g (de la Fuente and Rosell, 2012). They have pure white coats with pink eyes. Hyla breed is the most sort after for meat production in Nigeria today.

The aim of this study was to investigate the effects of different levels of breeding intervals on the secretion of Hsp 70 and 90 by does including the impact of Hsp 70 and 90 production on the growth and reproductive performance of does and kits. Our study will be helpful to clarify

the quantity of both Hsp 70 and 90 secretion that is normal for optimum production in Hyla rabbits.

MATERIALS AND METHODS

Animals and experimental design

A total of twenty four (24) adult rabbits, more than six months old with average body weight of 2500g ± 221 g were used in this study. Females were sixteen (16) and eight (8) males. Does were randomly allotted into the experimental treatments of four (4); T_1 (two weeks), T_2 (four weeks), T_3 (six weeks) and T_4 (eight weeks) breeding intervals with four (4) rabbits per treatment in a Completely Randomized Design (CRD). Bucks were used to service does. All rabbits were fed the same concentrate feed for twelve (12) months of the experiment. All recommended managerial practices were dully observed.

The animals were housed in galvanized battery cage with each hutch measuring 75 X 75 X 75 cm and raised 80 cm from the floor level. The hutches were thoroughly washed and disinfected with morigad disinfectant and allowed to dry for one week before the animals were brought. Water and feed were available always. The rabbits were placed individually in clearly labeled cells. Blood was collected from all does at the interval of three months from main artery of the ear into the Ethylenediamine tetraacetic acid (EDTA) bottles to find out the quantity of Hsp 70 and 90 released into it.

Chemical Analyses

RNA extraction and determination of quality

Total RNA was extracted from female Hyla rabbit blood by TRIzol (Invitrogen, Carlsbad, CA, USA) according to the standard protocol. The RNA samples were treated with DNase I (TaKaRa, Japan) for 4 h. The purity and concentration of total -RNA were quantified by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., San Jose, CA, USA). The blood RNA was assessed by the ratio of the absorbance at 260 and 280 nm of the samples ranged from 1.8 to 2.0. The RNA integrity of the samples was observed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The first stranded cDNA was synthesized by RevertAidTM First Strand cDNA Synthesis Kit (Ferments) following the standard protocol and stored at -20°C.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of target genes

Quantitative real-time RT-PCR was developed to assay mRNA levels of the target genes and the reference genes (bActin) listed in Table 1. The PCR primers were designed according to mRNA sequences in GenBank (Table 1). RT-PCR mixture (20 µL) contained 1 µL of RT reaction mix, 10µL of SYBR® Premix Ex Taq TM (2×) (TaKaRa, China), 0.6µL of 10 µmol/L each of primers and ultra-pure water to 20µL. Reactions were run on a fluorescence iCycler (Bio-Rad). The PCR conditions were as follows: 94°C for 90 s; 43 cycles of 95°C for 15s, annealing temperature of the primers (Table 1) for 20s, 72°C for 15s. Each sample was assayed in duplicate. The threshold cycle (Ct) from RT-PCR was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Changes in the expression of target genes were normalized by the geometric mean of the mRNA measurements of bActin in the same sample.

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription (RT)-PCR was used to amplify the protein-coding regions of rabbit Hsp 70 and Hsp 90 mRNAs and to detect the expression of these mRNA in the rabbit bloods. One µg total RNA was reverse-transcribed to cDNA in a total volume of 20 µL using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Gemany) according to the manufacturer's instructions. The specific primers of bActin (housekeeping gene), Hsp 70, and Hsp 90 genes were designed using Premier Primer 5 based on the sequences of predicted rabbit Hsp 70 (Accession number: XM_283516) and Hsp 90 (Accession number:

XM_283515), as well as bActin (Accession number: NM_236595). All primers used in this study were synthesized by Sangon (Shanghai, China) and presented in Table 1.

Table 1: Sequences of the primers used for real-time PCR and accession number

Gene	GenBank accession no.	Forward primer	Reverse primer
bActin	AM_236595	5′ GGGTGTGATGGTCGGTAT 3′	5′ ACCGTGTTCGATGGGGTA 3′
Ht_HSP70	AM_283516	5′ CGGTGAGCGCAATGTTC 3′	5′ CCAAGTGGGTGTCTCCA 3′
Ht_HSP90	AM_283515	5′ CCAGGAAGAATATGCCGAGT 3′	5′ CACGGAACTCCAACTGACC 3′

b Used to normalize mRNA. (Farcy *et al.,* 2007)

Statistical Analysis

Luciferase data were analyzed using the GLM procedure (SAS Inst. Inc., Cary, NC). For mRNA quantification analysis, n=16. Means were compared using Tukey's analysis. Results are expressed as the mean ± SEM (standard error of the mean). Differences were considered statistically significant with $p<0.05$.

RESULTS

Amplification and blood expression of rabbit Hsp 70 and 90

Nucleotide sequences of 301 bp and 245 bp were obtained for Hsp 70 and Hsp 90 genes, respectively. The pooled RNA was used in this section. The rabbits sampled in this study were of the same breed, from different families, of identical age and comparable body weight, and were raised in the same house using the same feed to describe the distribution of Hsp 70 and Hsp 90 in various bloods would be acceptable. The RT-PCR analysis indicated that both Hsp 70 and Hsp 90 mRNA were expressed in the tested bloods from does 2, 4, 6 and 8 weeks breeding intervals.

Expression level of rabbit Hsp 70 and 90 in different bloods of different breeding intervals

Hsp 70 and Hsp 90 were expressed in bloods of all rabbits from different breeding interval. Hsp 70 and 90 were most adequately expressed ($P<0.05$) in the blood of does in 2 weeks breeding interval, which had a significantly ($P<0.05$) higher expression level than any other tested bloods of other breeding interval. There were no differences in Hsp 70 and 90 expression level among the other treatments ($p>0.05$).

DISCUSSION

Hsp 70 and 90 are proteins that provide antioxidant protection in cells as well as thermotolerance (Lahvic *et al*., 2013). They are produced by cells in response to exposure to stressful conditions. They were first described in relation to heat shock but are now known to also be expressed during other stresses including exposure to cold, UV light and during wound healing or tissue remodeling (Moreira-de-Sousa *et al*., 2018). However, the underlying mechanism of producing HSPs remains unclear. The identification of HSPs receptors might clarify the mechanism of HSPs in various physiological processes. In summary, our study has shown that the rabbit genome encodes Hsp 70 and 90 genes, and these two genes are expressed in a variety of rabbit bloods from different breeding intervals.

CONCLUSIONS

The gene expression of Hsp 70 and 90 occured significantly higher in does that were pregnant and still heavily lactating (two weeks breeding interval). We discovered that Physiological stress was very high in these does because of highest released of Hsp 70 and

90 which had adverse effect on growth and reproductive performance of these does and their kitts. However, further research is required to determine the precise mechanisms of actin of Hsp 70 and 90 in various stressed pathways.

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RELATIONSHIP BETWEEN PROLIFICACY AT FIRST BIRTH AND PERFORMANCES THROUGHOUT THE CAREER OF RABBIT DOES

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ABSTRACT

The aim of this study was to investigate the correlations between born alive (BA) at first kindling (K1) and the productivity criteria of female rabbits throughout their career. Data of 2444 females from 3 farms were studied. Analysis showed that K1 prolificacy had a significant impact on career length ($p<0.01$): respectively 9.5, 9.3, 10.0 and 10.2 BA for females with 1, 2&3, 4 to 9 and 10 and over parities. In addition, BA in K1 had a significant impact on performance throughout the career: does with less than 7 BA in K1 produced in average less born alive rabbits on their all career ($p<0.001$) and weaned rabbits ($p<0.01$), with a higher mortinatality rate ($p<0.001$). However, fertility was not affected ($p=0.197$). Two factors might explain low prolificacy in K1: season of birth ($p<0.001$) and growth before K1 ($p<0.001$). Thus, females born in the summer period had a significantly lower prolificacy (9.3 BA) than females born between January and April (10.4 BA) and between October and December (10.2 BA). In addition, young females must have an adequate growth: average daily gain (ADG) between 14 and 20 weeks old had an impact on BA in K1 ($p<0.001$): 9.8 BA for ADG below 20g/d versus respectively 10.5 and 10.7g/d for ADG between 20 and 24g/d and above 24g/d. Prolificacy in K1 therefore appeared to be a reliable indicator of the future doe productivity. The use of BA in K1 could be an additional culling criterion of young females. This study also showed that young females management impacted their future performances.

Key words: rabbit doe, prolificacy, career, lifespan

INTRODUCTION

In rabbit production, regular renewal of breeding animals is necessary to replace dead and culled females, in order to maintain a constant number of does in the herd and to ensure continuous technical progress. The average renewal rate per batch is 13.9% in France (Itavi, 2022). The renewal of a herd can be achieved by rearing young does from Grand Parent (GP) females, by purchasing Simple Parent (PS) females from multiplier breeders, or by self-renewal. Whatever the method of renewal, nulliparous females must be raised in a specific way (animal management, feeding program...) (Gidenne, 2015). However, due to building and equipment constraints, there is a diversity of managing methods of young females, which combined with the individual variability of the animals, leads to early culling (respectively around 8.5% and 7.5% for parities 1 and 2) and mortality (respectively around 7% and 8.5% of parities 1 and 2) in breeding flocks (Rosell & De La Fuente (2009)). Thus, this loss of animals represents a poor return on investment.

Among causes of loss of female rabbits, Lopez et al. (2017) described those with culling origin only, as they had too little data for mortality. The three main causes of culling are: health problems, which are difficult to predict ; body condition, which would require individual monitoring of does; followed by poor performance, which is often revealed after several kindlings (K) whereas it could be detected earlier. Indeed, authors indicate that females with low prolificacy in K1 and 2 have a lower career length than other females. However, this work was carried out using culling data only. The aim of this study is therefore to show the impact of prolificacy in K1 on performance and career length of does, with data from dead and culled females.

MATERIALS AND METHODS

A database was built with information from 3 farms representing 3 to 6 years of production, i.e. 2444 females having at least one kindling. These farms differed according to the criteria

presented in Table 1. For each of these farms, various productivity data were collected: total born, born alive (BA), mortinatality rate and weaned rabbits, for K1, K2, K3 and for all kindlings; average artificial insemination (AI) success rate; career length; and season of birth. Moreover, analyse of BA in K1 effect was done thanks to 3 categories of BA: "low" (< 7 BA), “medium” (7 to 11 BA) or a "high" (> 11 BA) prolificacy. In addition, and only for Farm 3, data about the young females breeding period (weight and average daily gain) were recorded. Statistical analyses were carried out using R software (version 3.6.0). Data were analysed using a linear mixed-effects model with farm in random effect and maximum parity or BA1 categories or season in fixed effect.

Table 1: Description of farms used in the database.

	Farm 1	Farm 2	Farm 3	Total
Genetics	A	A	B	-
Type of renewal	GP	GP	SP	-
Culling if negative AI1	Yes	No	Yes	-
Number of studied does	543	609	1292	2444
Distribution according to parity				
1	108 (20%)	114 (19%)	287 (22%)	509 (21%)
2&3	159 (29%)	148 (24%)	304 (24%)	611 (25%)
4 to 9	188 (35%)	217 (36%)	475 (37%)	880 (36%)
10 and more	88 (16%)	130 (21%)	226 (17%)	444 (18%)

RESULTS AND DISCUSSION

Relationship between prolificacy and career length

Analysis of the database showed that born alive in K1 (BA1) had a significant impact on the career length of does ($p<0.001$): respectively 9.5, 9.3, 10.0 and 10.2 BA1 for females with 1, 2&3, 4 to 9, and 10 and more parities (Figure 1).

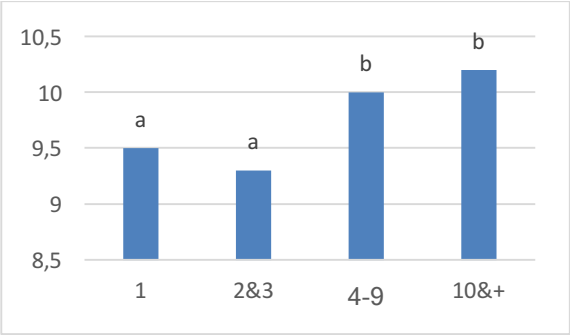

Figure 1: Relation between parity at culling or death and Born Alive in 1st kindling

These differences of BA1 from one doe to another could be explained by variable female receptivity: Theau-Clément (2007) cited that the most prolific does are also the most receptive to AI, which could be explained by more intense ovulatory activity, and therefore better fertility. This potential explanation cannot be verified for primiparous rabbits in our database, as not all farms keep their females with negative palpation after the first AI.
Huneau-Salaun et al. (2015) indicated that age at first AI, genetics, type of renewal and use of hepatoprotectors have an effect on the prolificacy of rabbits does in general, whatever their parity. These factors are unlikely given the diversity of management on the 3 studied farms (different genetics, renewal, feed…) and the number of years of production.
The relationship between prolificacy at K1 and career length might be due to the rearing and managing conditions of young females from their early age: according to Pascual et al.

(2016), females with a birth weight lower than 57g would produce smaller litter size for their first 2 kindlings (about 10.45 BA, versus 11.30 BA for does over 57g). Furthermore, females reared in smaller litters would be heavier at first AI, and then more prolific at K1 (Rommers et al., 2003).

Relationship between prolificacy at K1 and performances of rabbit does

When we compared females according to their prolificacy (low, medium or high) at K1, there was a significant effect of BA1 category on average maximum parity, born alive, mortinatality rate and weaned over the doe's entire career. But the average AI success rate was not significantly different (Table 2). Finally, rabbits does with less than 7 BA1 had a significantly shorter career, with a lower production of live rabbits and weaned, in relation with a higher mortinatality rate.

Table 2: Effect of the category of born alive in kindling 1 on doe performances and career length

	Low (< 7 BA1)	Medium (7-11 BA1)	High (> 11 BA1)	Effect of BA1 category (p-value)
Average born alive in K1	*3.6*	*9.5*	*12.9*	
Average maximum parity	4.3[b]	5.4[a]	5.4[a]	< 0.001
Average success rate at AI (%)	89.3	88.3	88.0	0.197
Average born alive	7.0[c]	10.3[b]	12.2[a]	<0.001
Average mortinatality rate (%)	20.8[c]	5.6[b]	4.3[a]	<0.001
Average weaned	8.1[b]	8.6[a]	8.6[a]	<0.01

Our results confirmed those of Lopez et al. (2017) and completed them by taking into account culled and dead does. These authors indicated that the career length was lower for does producing during parities 1 and 2 the fewest total born (6.71 versus 7.65 for other females) and weaned (5.96 versus 7.35 for other females).

Thus, these results highlighted a relationship between K1 prolificacy and the overall performance of female rabbits. This is why this criterion seems important to be considered in the breeding flock management. Furthermore, in order to detect these unproductive females as quickly as possible, we must understand the origin of this low prolificacy in K1.

Studied factors which could explain a low prolificacy in K1

For the 3 studied farms, season of birth of young females had an effect on BA1 ($p<0.001$): respectively 10.4, 9.3, and 10.2 for the periods January-April, May-September and October-December (Figure 2). Season of birth also had a significant impact on career length, but only for farms 1 ($p<0.001$) and 2 ($p<0.001$) and had no effect for farm 3 ($p=0.37$) (Figure 3). Two factors differed between these groups of farms: genetics and renewal method.

Lopez et al. (2017) publication also indicated an effect of the season on the longevity of female rabbits. For example, breeding does introduced into the herd in the autumn (August-November), and therefore born in the summer (May-August), had a shorter career length (243 days, versus 272 days on average for does born during the rest of the year). This finding seems to confirm that birth of young females during the summer season results in lower prolificity in K1.

The higher ambient temperatures which are characteristic of the summer season induces an under-consumption of feed by pregnant rabbits (Szendro et al., 2018), which could be assumed to lead to a nutritional deficiency of both the female and her in-utero foetuses. And several studies showed that nutritional deficiency of the foetuses during gestation has negative consequences on the future reproductive performance of these foetuses in adulthood (Pascual et al., 2016). Thus, a specific feeding management of GP females during the summer period could be a way to reduce the negative impact of high temperatures on performances.

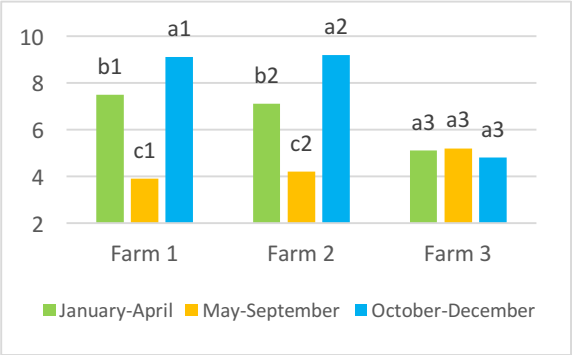

Figure 2: Effect of birth season on BA1.

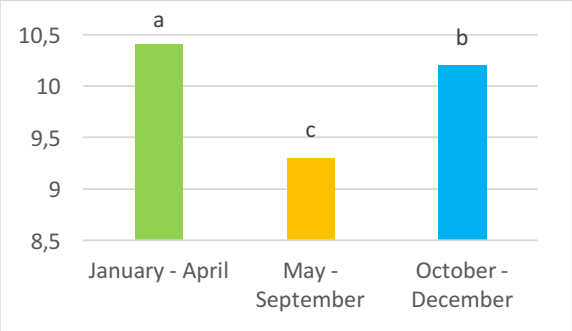

Figure 3: Effect of birth season on career length according to the studied farm **.**

Growth of young females, particularly between 14 and 20 weeks of age, also had an impact on BA1 ($p<0.001$): 9.8 BA1 for Average Daily Gain (ADG) below 20g/d versus 10.5 and 10.7g/d for ADG between 20 and 24g/d and ADG above 24g/d respectively. However, contrary to our study, which showed no effect of weight on BA1 categories, Rommers et al (2003) demonstrated an effect of live weight at first AI (minimum 4 kg) on litter size at K1.

CONCLUSIONS

The level of productivity of breeding females is a key factor in the technical and economic success of a rabbit farm. This productivity is influenced by the renewal and the management of young females, which represent an investment that is expected to produce does with a minimum productivity and longevity. Any cause leading to early sorting (culling or death) of female rabbits is therefore a major economic loss for farms. So it is important to be able to detect low-productivity females at an early stage. This study showed that there is a relationship between BA1, career length and productivity. These under-performances can be due to the young female management. It therefore seems possible to identify early on females predisposed to low productivity or to a short career, thanks to K1 prolificacy. On the other hand, in order to limit these low-productivity primiparous does, it is recommended to pay particular attention to the management of GP females during the summer period, and to monitor the growth of PS females, particularly between 14-20 weeks old.

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PLASMA ORGANIC ACIDS AT DELIVERY IN TWO LINES DIVERGENTLY SELECTED FOR LITTER SIZE VARIABILITY

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ABSTRACT

A divergent selection experiment for litter size variability in rabbits was carried out at the Miguel Hernández University of Elche for sixteen generations of selection. Low litter size variability has been associated with the female's ability to coping with adverse environmental conditions without affecting her welfare. Delivery is a stressful and energy demanding status for female in which supply of oxygen may become limited. Under anaerobic conditions, energy is obtained from the Cori cycle or lactic acid cycle. The objective of this study is to assess the glucose level and plasmatic organic acids profile at the first delivery in the homogeneous and the heterogeneous lines for litter size. A blood sample was taken at the first delivery in 40 females (20 per line) of the sixteenth generation of selection, and the number of live born and dead kits was recorded. Bayesian methodology was used for statistical analysis. The heterogeneous line had a higher concentration of lactic acid and a lower concentration of citrate acid than the homogeneous line at first delivery. These findings could be related to the differences between lines in both the response to stress and the mobilization of body reserves reported in previous studies. In conclusion, the selection for litter size variability shows a correlated response on organic acids profile, and plasma lactic acid concentration can be a biomarker of the stress in situations of high energy demand, such as the delivery.

Key words: citrate acid, environmental sensitivity, lactic acid, litter size variability, stress.

INTRODUCTION

A divergent selection experiment for litter size variability is being carried out at the Miguel Hernández University of Elche. In previous studies, we have found that selection has had successful (Blasco et al., 2017). Besides, females from the heterogeneous line have a higher stress response and a lower disease resistance than those from the homogeneous line (Argente et al., 2019). Moreover, body reserves at delivery were lower in the females from the heterogeneous line, thus this line could cope worse to stressful situations with high energy demand for the animal (García et al., 2019; Agea et al., 2020). These results are consistent with a larger mortality at parity of females and a greater litter mortality at birth and at weaning found in the heterogeneous line (Agea et al., 2019; Argente et al., 2019).

In normoxic condition, it is well known that cells acquire energy from an efficient process such as the Kreb's cycle linked to the generation of adenosine triphosphate via oxidative phosphorylation (Pearce and Pearce, 2013), and shift to anaerobic glycolysis using the lactic acid cycle in response to stress (Dokmanovic et al., 2017) and inflammatory stimuli (MacIver et al., 2013). The citric acid and the lactic acid are the main starting substrates of the Kreb's and the lactic acid cycles, respectively. Our working hypothesis is that under stress the metabolic pathways for supplying energy to cells will differ between the heterogeneous and the homogeneous lines of our experiment.

The objective of this study is to assess the glucose level and plasma organic acids profile at the first delivery in the heterogeneous and the homogeneous lines for litter size.

MATERIALS AND METHODS

Animals and experimental design

Rabbits used in this study come from the 16th generation of a divergent selection experiment for litter size variability, see more details about selection procedure in Blasco et al. (2017). All animals were reared in the farm of the Universidad Miguel Hernández de Elche (Spain). The rabbits were fed a standard commercial diet (Cunilactal®, Nanta S.A.de Heus Nutrición Animal, Las Palas, Spain). Food and water were provided *ad libitum*. Females were housed in individual cages under a constant photoperiod of 16 h continuous light: 8 h continuous darkness, and with controlled ventilation throughout the experiment. Females were first mated at 18 weeks of age and then every 12 days after parturition. The kits were reared by their dams until weaning (30 days of age). Adoptions were not performed.

At the first delivery, the total number of newborn (TNB), live born (NL) and dead kits (ND) was recorder in 20 females of the homogeneous line and 20 females of the heterogeneous line for litter size and, additionally, one blood sample from the ear vein was taken in the same females. Samples were collected into tubes containing K3-EDTA. All samples were immediately centrifuged at 4000 rpm for 15 min, and plasma was stored at -80 ºC until required for organic acids and glucose analyses.

All experimental procedures involving animals were approved by the Miguel Hernández University of Elche Research Ethics Committee (Reference number 2023-VSC-PEA-0079), in accordance with Council Directives 98/58/EC and 2010/63/EU.

Chemical Analyses

A 200 µL of plasma was taken from each sample and put in an Eppendorf tube of 5 ml. A 150 µL of distilled water was added to each one. After shaking the mixture for a few seconds, the sample was incubated at 37 °C for 30 min with an agitation rate of 1200 rpm. Following this, a 650 µL of acetonitrile was added. The sample was incubated again at 37 °C for 30 min with an agitation rate of 1200 rpm. Afterwards, the sample was centrifuged at 16000× g rpm for 3 min at 4 °C. Ultimately, the supernatant was carefully collected, filtered, and transferred to sample vials for LC-MS (Liquid chromatography–mass spectrometry) analysis.

Statistical Analysis

The model used to analyse TNB, NL, ND, glucose and organic acids profile included the effects of season (spring, summer and autumn) and line (homogeneous and heterogeneous line). All analyses were performed using Bayesian methodology. Bounded uniform priors were used for all effects. Residuals were a priori normally distributed with mean **0** and variance $\mathbf{I}\sigma_e^2$. The priors for the variance were also bounded uniform. Marginal posterior distributions of the differences between lines were estimated for all unknowns using Gibbs sampling with the program Rabbit developed by the Institute for Animal Science and Technology (Valencia, Spain). The following parameters were obtained: the median of the difference (D), the highest posterior density region at 95% (HPD95%) and the probability of the difference being higher than zero when D > 0 or lower than zero when D < 0 (P).

RESULTS AND DISCUSSION

Table 1 shows the features of the marginal posterior distribution of the differences at the first delivery between the heterogeneous and the homogeneous lines. In agreement with our previous findings (Blasco et al., 2017; Argente et al., 2017), the heterogeneous line showed a lower litter size than homogenous one, as a consequence of a higher number of dead kits at birth (1.30 dead kits in heterogeneous line vs. 0.59 dead kits in homogeneous line, P=90%).

Table 1: Features of the marginal posterior distribution of the differences between the heterogeneous and homogeneous lines for the total number of newborn (TNB), live born (NL) and dead kits (ND), glucose and organic acids profile.

	Heterogenous line		Homogenous line				
	Mean	SD	Mean	SD	D	HPD95%	P
Rabbits, no.	20		20				
TNB	7.51	0.53	7.88	0.52	-0.41	-1.81, 1.08	70
NL	6.20	0.58	7.30	0.59	-1.09	-2.75, 0.52	91
ND	1.30	0.42	0.59	0.41	0.69	-0.39, 1.91	90
Pyruvic acid, ng/ml x10^3	21.89	0.47	22.19	0.47	-0.29	-1.61, 0.95	68
Citric acid, ng/ml x10^3	43.14	3.25	54.07	3.22	-10.9	-19.7, -1.66	99
Lactic acid, ng/ml x10^3	360.8	31.8	242.8	32.1	117.4	30.5, 204.5	99
Acetic acid, ng/ml x10^3	0.49	0.32	0.13	0.31	0.36	-0.52, 1.23	79
Fumaric acid, ng/ml x10^3	0.09	0.06	0.10	0.06	-0.01	-0.19, 0.15	56
Glucose, ng/ml x10^3	193.5	9.82	201.9	9.92	-8.29	-37.20, 16.31	72

SD: standard deviation. D: median of the difference between heterogeneous and homogeneous lines. HPD95%: highest posterior density region at 95%. P: probability of the difference being > 0 when D> 0, and probability of the difference being < 0 when D < 0.

We found a higher plasma concentration of lactic acid and a lower plasma concentration of citric acid in the heterogeneous line than the homogeneous line (+117.4 x 10^3 ng/ml and -10.9 x 10^3 ng/ml, respectively). Pyruvate is produced as a result of glycolysis gets shunted into two main metabolic pathways. In aerobic conditions, it enters the Kreb's cycle or citric acid cycle, and a series of reactions occur to form ATP and NADH, which goes on to the process of oxidative phosphorylation which produces the majority of ATP in cell. However, in anaerobic conditions, pyruvate gets into the Cori cycle or lactic acid cycle and it is converted to lactic acid in order to produce ATP in cell (Foucher and Tubben, 2023). Under stress or an intense physical exertion such as delivery, the female's demand for energy increases (García et al., 2019) and the supple of oxygen may become limited (Joyner and Casey, 2015). In response, the body may rely more on anaerobic metabolism, leading to increased production of lactic acid. It should be noted that a reduction of oxygen concentration at delivery can increase in perinatal losses (Derrick et al., 2012); and elevated levels of lactic acid in blood can have profound hemodynamic consequences and can lead to death of individual (Gillies et al., 2019). A lower oxygen concentration and a higher level of lactic acid in the heterogeneous line maintained over time would agree to its higher litter mortality at birth and larger culling rate reported by Argente et al. (2019).

CONCLUSIONS

The selection for litter size variability shows a correlated response on organic acids profile, and plasma lactic acid concentration can be a biomarker of the stress in situations of high energy demand, such as the delivery.

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META-ANALYSIS OF ASSOCIATION BETWEEN *IGF-2* GENE POLYMORPHISMS AND BODY WEIGHT OF RABBITS

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ABSTRACT

The Insulin-like growth factor 2 (IGF-2) plays a key role in the regulation of cell proliferation, growth, differentiation and survival. However, the relationship between polymorphic variants of this gene and the body weight of rabbits under different genetic models has not been established. Thus, the objective of this study was to evaluate relationship between IGF-2 gene polymorphisms and body weight of rabbits. The Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) guideline was used to systematically review relevant literatures. A total of 211 studies were identified from databases and screened against pre-determined eligibility criteria. Each study was subjected to Hardy-Weinberg Equilibrium (HWE) test and six genetic models consisting of co-dominant, dominant, recessive and over-dominant models were fitted using OpenMeta® Analyst Software. The Hedges' g method was utilized for computing standardized mean differences (SMDs) to assess both the effect size of individual studies and the overall effect size. Heterogeneity measures were inferred from Q statistic, Tau-squared (τ^2), H-squared (H^2) and I-squared (I^2) values and publication bias was assessed by Egger's regression asymmetry test. There were significant association ($P<0.05$) of IGF-2 gene with body weight of rabbits under all the genetic models evaluated. A simple relationship between these genotypes for body weight of rabbits could be stated as Del/Del > A/Del >A/A. Furthermore, this could infer the superiority of the Del allele over the A allele (Del>A) for body weight of rabbits. These findings implied that the *IGF-2* gene could be a potential candidate gene for marker assisted selection of rabbits for improved body weight.

Key words: IGF-2 gene, rabbit, polymorphism, systematic review, meta-analysis.

INTRODUCTION

Insulin-like growth factor 2 (IGF-2) is a protein hormone responsible for controlling various cellular processes including cell proliferation, growth and differentiation (Bergman et al., 2012). The insulin-like growth factor 2 works together with growth hormone and growth hormone receptor as a complex to regulate the metabolic procedures of growth and development in animals (Alvino et al., 2011). The process of growth and development regulation in animals is accomplished by the activation of insulin-like growth factor 2 which is triggered by the binding of the growth hormone to the growth hormone receptor (Herrington and Carter-Su, 2001). Various authors have reported the association of single nucleotide polymorphisms (SNPs) in IGF-2 encoding gene with growth performance traits of rabbits (Fontanesi et al., 2012; Abdel-Kafy et al., 2014; Ramadan et al., 2020). In addition, three genotypic variants (A/A, A/Del and Del/Del) have been identified in rabbits. However, there have been contrasting reports on the relationship between these variants. In particular, lack of replication and consistency have been found to be an issue in most candidate gene association studies (Pasche and Yi, 2010; Watanabe, 2010). Meta-analysis solves this problem by analyzing variation in the results of different studies by identifying inter-study heterogeneity (Lee, 2015). An elucidation of such association could be crucial for marker-assisted selection in rabbit genetic improvement programmes. Therefore, the objective of this study was to evaluate the relationship between IGF-2 gene and body weight of rabbits using meta-analysis.

MATERIALS AND METHODS

Database and search strategy

We conducted a comprehensive database search for relevant articles following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline (Moher et

al., 2009). The title and abstract of each article identified through database search was screened and relevant articles were selected for full-text assessment using the pre-determined eligibility criteria. A total of 211 articles were identified and retrieved from databases. After removal of duplicates, only 9 studies met the pre-determined inclusion criteria and were included in the meta-analysis of association between *IGF-2* gene and body weight of rabbits. The PRISMA flow diagram is presented in Figure 1. Data were extracted from relevant articles following the Data extraction for complex meta-analysis (DECiMAL) guide described by Pedder et al. (2016). Each study was subjected to HWE test using the gene calculator software freely available online: https://gene-calc.pl/hardy-weinberg-page.

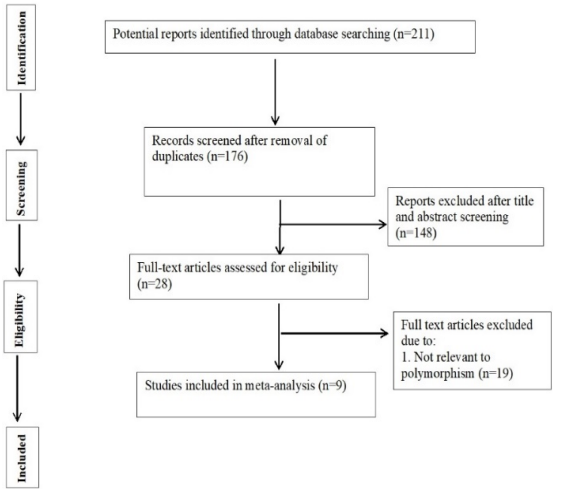

Figure 1: PRISMA flow diagram for selection of studies for meta-analysis of association of *IGF-2* gene with body weight of rabbits

Data analysis

Data were analyzed using OpenMeta Analyst Software (Wallace et al., 2012). The random effects model was used to fit six genetic models under the assumption of co-dominance (3 genetic models: A/A vs A/Del; A/A vs Del/Del and A/Del vs Del/Del), recessive (1 genetic model: AA vs A/Del+Del/Del), dominance (1 genetic model: AA+A/Del vs Del/Del) and over-dominance (1 genetic model: A/Del vs AA+Del/Del) to evaluate the association between IGF-2 gene and body weight of rabbit. The Hedges g method was used to calculate standardized mean differences (SMDs). Heterogeneity tests were also conducted using the Q statistic, Tau-squared (τ^2), H-squared (H^2) and I-squared (I^2). Furthermore, Publication bias was assessed by Egger's regression asymmetry test.

RESULTS AND DISCUSSION

Heterogeneity tests

Table 1 presents the heterogeneity test of six genetic models for the meta-analysis of the association between *IGF-2* gene and body weight of rabbits. The Chi-square test for all genetic models were significant (P<0.05). Furthermore, the I^2 values were high for all genetic models fitted (I^2 >63%, ranged from 63.4% to 92.0%) and were thus considered heterogeneous. The heterogeneity test is a measure of how the effect size varies from one study to another and to assess if the overall effect size is in conformity with normal distribution. Thus, high heterogeneity measures obtained under all genetic models fitted could be interpreted that effect size reported by different studies are due to systematic differences and not solely due to sampling error between studies included in the meta-analysis (Hedges and Olkin, 1985).

Table 1: Heterogeneity test of six genetic models for the meta-analysis of the association between *IGF-2* gene and body weight of rabbits (n=9)

Category	Genetic model	τ^2	H^2	I^2	Chi-square (Q statistic)	P value
Co-dominant	A/A vs A/Del	0.075	3.666	72.7	27.764	<0.001
Co-dominant	A/A vs Del/Del	0.381	12.534	92.0	87.552	<0.001
Co-dominant	A/Del vs Del/Del	0.075	5.150	80.6	39.651	<0.001
Recessive	A/A vs A/Del+Del/Del	0.155	7.212	86.1	55.053	<0.001
Dominant	A/A+A/Del vs Del/Del	0.128	9.083	89.0	69.266	<0.001
Over-dominant	A/Del vs AA+Del/Del	0.020	3.155	63.4	9.320	0.0316

τ^2=Tau-squared; H^2 =H-squared; I^2=I-squared

Overall effect size estimation

Table 2 presents the overall effect size estimates of the six genetic models for the meta-analysis of the association between IGF-2 gene and body weight of rabbits. There were

significant associations (P<0.05) of *IGF-2* gene with body weight of rabbits under all the genetic models fitted. For example, the negative significant (P<0.05) overall effect size under the co-dominant model (A/A vs A/Del) indicated that the body weight of rabbits with A/Del genotype was significantly higher (P<0.05) than the body weight rabbit of AA genotype. Similar trends were also observed under other co-dominant models (A/A vs Del/Del and A/Del vs Del/Del) where rabbits with Del/Del genotype had higher body weight compared to rabbits with AA and A/Del genotypes, respectively. Thus, a simple relationship between these genotypes could be stated as Del/Del > A/Del >A/A. This could infer the superiority of the Del allele over the A allele (Del>A) for body weight of rabbits. Furthermore, under the recessive model (A/A vs A/Del+Del/Del), the overall effect size was significant and negative which indicated that an average of A/Del + Del/Del genotypes had superior body weight compared to the AA genotype. Similarly, the Del/Del outperformed an average of AA and A/Del genotypes in terms of body weight under the dominant model (AA+A/Del vs Del/Del). Furthermore, under the over-dominant model, an average of AA and Del/Del genotypes had higher body weight compared with the A/Del genotype. The superiority of Del/Del genotype over A/Del and A/A genotype is in agreement with the findings of Fontanesi et al. (2012) that reported that the Del/Del genotype had the heaviest finishing weight followed by the A/Del genotype while A/A genotype had the least. Furthermore, Ramadan et al. (2020) also reported that Sinai Gabali rabbits of Del/Del genotype had the highest significant (P<0.05) body weight compared with the A/Del and AA genotype at 4, 8 and 12 weeks of age.
However, Abdel-Kafy et al. (2014) reported no significant difference (P>0.05) in the body weight of A/A, A/Del and Del/Del genotypes of APRI rabbit line at 5, 6 and 8 weeks of age. However, the authors reported that Del/Del polymorphic variants had the highest significant (P<0.05) body weight compared with the AA and A/Del variants at 10 and 12 weeks of age.

Table 2: Overall effect size estimates of the six genetic models for the meta-analysis of the association between *IGF-2* gene and body weight of rabbits (n=9)

Category	Genetic model	Effect size (SMD±SE)	95% CI	95% PI	P value
Co-dominant	AA vs A/Del	-0.238±0.1075	-0.449 to 0.027	-0.934 to 0.458	0.027
Co-dominant	AA vs Del/Del	-0.613±0.2144	-1.033 to 0.192	-2.157 to 0.932	0.004
Co-dominant	A/Del vs Del/Del	-0.36±0.1016	-0.562 to 0.164	-1.052 to 0.326	<0.001
Recessive	AA vs A/Del+Del/Del	-0.38±0.1415	-0.666 to 0.112	-1.377 to 0.599	0.006
Dominant	AA+A/Del vs Del/Del	-0.420±0.1268	-0.669 to 0.172	-1.319 to 0.479	<0.001
Over-dominant	A/Del vs AA+Del/Del	-0.158±0.0427	-0.242 to 0.074	-0.308 to-0.008	<0.001

SMD- Standardized Mean difference; SE-Standard error; CI- Confidence interval; PI -Prediction interval

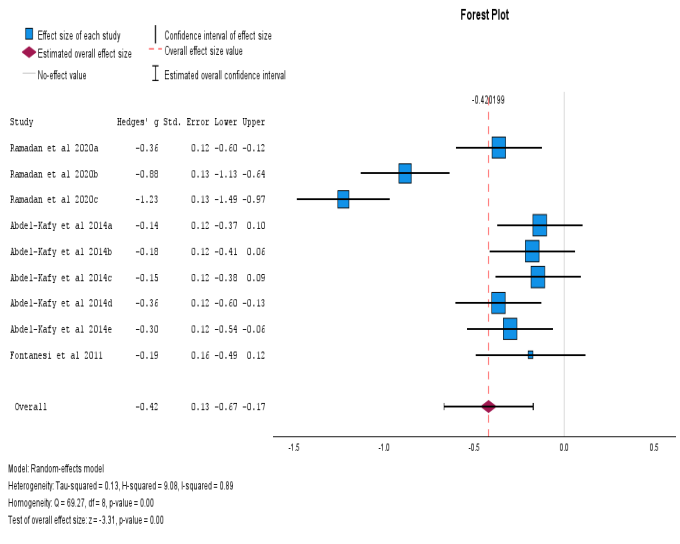

Figure 2: The forest plot of heterogeneity test for the meta-analysis of the association between *IGF-2* gene and body weight of rabbits under a dominant genetic model (AA + A/Del vs Del/Del)

Figure 2 presents the forest plot of heterogeneity test for the meta-analysis of the association between *IGF-2* gene and body weight of rabbits under the dominant genetic model (AA + A/Del vs Del/Del). The overall effect sizes were significant (P<0.05) indicating the existence of significant association between *IGF-2* gene with body weight of rabbits. Effect sizes from most of the studies included in the meta-analysis under this genetic model did not cross the vertical line (the null value) which indicated that the null value lies within the 95% confidence interval of the effect size from each study. Therefore, the study results included in the meta-analysis under this model are

different from the null value and there were statistically significant differences in the body weight among the groups being compared under each genetic model.

Egger's regression test for the assessment of publication bias

Table 3 present the Egger's regression test for six genetic models for the assessment of publication bias for the random effects meta-analysis of association between *IGF-2* gene and body weight of rabbits. The Egger's regression-based tests were not significant (P>0.05) under all six genetic models evaluated suggesting that the number of studies utilized for the meta-analysis were adequate. Furthermore, there were no theoretically missing study under any of the genetic models which indicated that there were no publication bias in the assessment of association between *IGF-2* gene and body weight of rabbits.

Table 3: Egger's regression test for six genetic models for the assessment of publication bias for the random effects meta-analysis of association between *IGF-2* gene and body weight of rabbits

Category	Genetic model	Regression coefficient SE	95% CI	P value	Number of missing studies
Co-dominant	AA vs A/Del	0.214±1.2259	-2.685 to 3.112	0.867	0
Co-dominant	AA vs Del/Del	0.993±0.7136	-0.468 to 3.518	0.138	0
Co-dominant	A/Del vs Del/Del	-0.279±1.4187	-3.634 to 3.076	0.850	0
Recessive	AA vs A/Del+Del/Del	0.550±1.7026	-3.476 to 4.576	0.756	0
Dominant	AA+A/Del vs Del/Del	-0.024±0.619	-2.853 to 2.804	0.881	0
Over-dominant	A/Del vs AA+Del/Del	5.785±2.7899	-0.812 to 12.382	0.077	0

SE- Standard error; CI- Confidence interval

CONCLUSIONS

Single nucleotide polymorphisms in *IGF-2* gene are significantly associated with the body weight of rabbits across various genetic models. The Del/Del genotype had the highest body weight followed by A/Del and A/A genotypes. This suggests that the presence of the Del allele confers a body weight advantage over the A allele. These findings implied that the *IGF-2* gene could be a potential candidate gene for marker assisted selection of rabbits for improved body weight.

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POPULATION GENOMIC ANALYSES DESCRIBE UNIQUE GENETIC FEATURES OF SEVERAL RABBIT BREEDS AND LINES

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ABSTRACT

The rabbit is globally bred for various purposes, including meat, fiber, and fur production and many breeds have been developed, including fancy breeds. The aim of this study was to analyse high density SNP genotyping data from 14 rabbit breeds to detect signatures of selection in the rabbit genome, which could explain the genetic diversity of these genetic resources. Combining a single marker and haplotype-based approaches such as F_{ST}, XP-EHH, and |iHS|, we identified signatures of selection that covered about half of the rabbit genome. Many regions consistently detected in several breeds and with the combination of more than one approach harbour genes associated with pigmentation processes and morphological traits, previously recognized in rabbits and other species (e.g., *ASIP*, *MC1R*, *TYR*, and *LIPH*). The results of this study contribute to a improve the knowledge on the genetic architecture of fancy and meat rabbit breeds and understand the genetic factors that contribute to the genetic diversity of these animal genetic resources.

Key words: Genetic resource, genetic variability, signature of selection, SNP.

INTRODUCTION

The rabbit serves as a versatile livestock species raised for the production of meat, fiber, and fur. This species is also relevant as animal model and companion animal. Many breeds and lines have been therefore developed, having as main objectives the specialization and differentiation of the genetic stocks, which also defined their specific characteristics mainly based on exterior traits (such as coat color, hair structure, body size and shape, ear size and position, and skull structure) and/or performance and production traits. The genetic features of all these genetic resources have been shaped by various genetic events, including genetic drift, bottleneck, isolation, introgression, migration, and crossbreeding. Many of these breeds and lines remain largely untapped and uncharacterized. The sequencing of the rabbit genome, as well as the availability of a high-throughput single nucleotide polymorphism (SNP) genotyping platform, have facilitated genome-wide analyses in this species, including the identification of genomic regions influencing economically relevant traits in meat rabbits (Bovo et al., 2021) Other studies have contributed to explaining the variability of external traits (Fontanesi et al., 2014a). In this study, using single marker and a haplotype-based approaches, we analysed SNP array datasets obtained from several fancy and meat rabbit breeds or lines to identify signature of selection in the rabbit genome which can characterize the investigated rabbit genetic resources.

MATERIALS AND METHODS

Animals and genotyping

Biological specimens, comprising hair roots or buccal swaps, were collected from a cohort of 712 rabbits from Three meat breeds (Italian White, *n* = 256; Italian Spotted, *n* = 93; Italian Silver, *n* = 20) and all fancy breeds (Belgian Hare, *n* = 24; Burgundy Fawn, *n* = 6; Champagne d'Argent, *n* = 19; Checkered Giant, *n* = 79; Coloured Dwarf, *n* = 20; Dwarf Lop, *n* = 20; Ermine, *n* = 20; Giant Grey, *n* = 27; Giant White, *n* = 20; Rex, *n* = 19; Rhinelander, *n* = 28; and Thuringian, *n* = 9). These animals were officially registered by the National Rabbit Breeders Association (ANCI or were from a genetic nucleous under selection for production performances. Genomic DNA was extracted using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). Subsequently, genotyping was

performed utilizing the Affymetrix Axiom OrcunSNP Array (Affymetrix Inc., Santa Clara, CA, USA), which encompasses 199,692 DNA markers. Data quality checks were conducted through the Axiom Analysis Suite and PLINK v.1.9 (Chang et al., 2015), resulting in the exclusion of samples and DNA markers with a call rate below 0.90. Following filtration, a dataset of 702 animals and 139,922 SNPs was used in the subsequent analyses. The SNP dataset for all investigated rabbits was then subjected to phasing using SHAPEIT2, with default parameters (Delaneau et al., 2012).

Signatures of selection detection

The identification of signatures of selection was performed using both single marker and haplotype-based approaches. In the single marker approach, F_{ST} analyses were conducted using the Hudson estimator, chosen for its independence from sample size considerations (Bhatia et al., 2013). Signatures of selection were computed within 350 kb sliding genome windows, with a step size of 100 kb. A total of 7,951 genome windows were calculated, following the testing of windows with varying sizes as outlined by Rubin et al. (2010). Windows containing fewer than 3 SNPs were excluded from the analyses. Using information derived from the F_{ST} approach, two distinct methods were applied to identify signatures of selection. The first method compared one breed against all others, while the second approach grouped certain breeds based on shared characteristics such as coat colour/colour patterns, body size, and use/specialization (fancy vs meat; Silver breeds vs all other breeds; Giant breeds vs all other breeds; Dwarf breeds vs all other breeds; Spotted breeds vs all other breeds; Albino breeds vs all other breeds). Following Rubin et al. (2010), putative signatures of selection were identified from genome windows situated at the extreme lower end of the distributions (99.98th percentile of the distribution), resulting in the identification of 14 genome windows for each comparison.

For the haplotypes-based approach, the rehh R package V 2.04 (Gautier et al., 2017) was used on the phased SNPs to derive integrated haplotype scores (iHS; Voight et al., 2006) for each breed and across-populations, along with extended haplotype homozygosity (XP-EHH; Sabeti et al., 2007) values for both breed comparisons and groups of breed comparisons. Signatures of selection were identified based on absolute values for haplotypes that passed the threshold of the top 98.00th percentile of the empirical distribution, requiring at least three consecutive SNPs within a 350 kb window. Genomic regions exhibiting signatures of selection from the F_{ST}, iHS, or XPEHH methods were annotated using *Bedtools v.2.17*(https://bedtools.readthedocs.io/) by retrieving annotated protein-coding genes from the OryCun2.0 NCBI's GFF file (https://www.ncbi.nlm.nih.gov/).

Table 1. Summary of methods and approaches used for signatures of selection detection.

Approach	Methods	The first group of comparison	The second group of comparison
One breed approach	F_{ST}, XP-EHH	The analysed breed	The remaining rabbit breeds are considered a unique population
Groups of breeds	F_{ST}, XP-EHH	Rabbit breeds share the same features as a unique population	The remaining rabbit breeds are considered a unique population
One breed approach	|iHS|	The analysed breed	NA
Groups of breeds	|iHS|	Rabbit breeds share the same features as a unique population	NA

RESULTS AND DISCUSSION

In total, 309 distinct genome regions under signatures of selection were identified from the F_{ST} analyses. Multiple genomic regions harboring genes associated with coat colour identified. These regions encompassed several genes already shown to affect coat coulor in rabbits or in other species, and including *ASIP, MC1R*, *MITF*, *OCA2* and *TYR.* Additional signatures of selection were detected in genomic regions harboring genes linked to (i) coat structure, such as *LIPH*, and (ii) body size, including (*COL2A1*, *LCORL*, and *GRK5*).

The top 20 |iHS| windows were distributed across the following rabbit chromosomes (OCU): OCU1, OCU2, OCU4, OCU7, OCU9, OCU12, OCU15, and OCU16. These windows encompass multiple genes involved in growth processes and body morphology. Notably, the highest |iHS| value, specifically identified in the commercial meat line and in the group of rabbit breeds/lines, highlighted a window on OCU2 containing the *LCORL* gene, which is well known to affect stature and body size in mammals (e.g. Pryce et al., 2011). Another significant genomic region, identified with the |iHS| method in the Giant breeds, was localised on OCU4 and encompassed the *HMGA2* gene, which is involved in several basic biological processes, including mesenchymal differentiation, adipogenesis, and post-natal myogenesis. The genomic region encompassing this gene was also evidenced in the Dwarf breeds.

The results from the XP-EHH analyses partially overlapped and complemented the |iHS| outcomes. For instance, the *HMGA2* gene region, previously identified in the |iHS| results, was detected also in the fancy Giant breeds. Other genomic regions, encompassing numerous genes associated with growth, body size, and development (e.g., *COL11A2*, *NCAPG2*, *SEMA4D*, *TGFBR2,* and *ZFAT*), emerged from within-breed comparisons and analyses based on groups of breeds using this method but were not evident with the |iHS| method.

Considering all breeds and all applied methods (single marker and haplotype-based approaches), signatures of selection were identified in approximately 1.8 Gb (65 %) of the rabbit genome (Figure 1). From this picture, it emerged that a large fraction of the rabbit genome experienced some relevant modifications in the process of breed constitution (considering that we analysed only a few breeds over the several tens or hundreds that have been constituted). Despite the interesting results obtained in our study, it is crucial to acknowledge certain limitations. The differentiation of genomic regions among rabbit breeds may not always directly imply selection due to the complex history of breed development. While annotated gene functions can aid interpretation, the incompleteness of our knowledge about certain genes introduces uncertainties, particularly in the context of complex traits influenced by multiple genes. Furthermore, the integration of whole-genome sequencing (WGS) data with other 'omics' datasets, such as transcriptomics and proteomics, holds promise for gaining a more profound understanding of the molecular mechanisms underlying selection signatures in the rabbit genome.

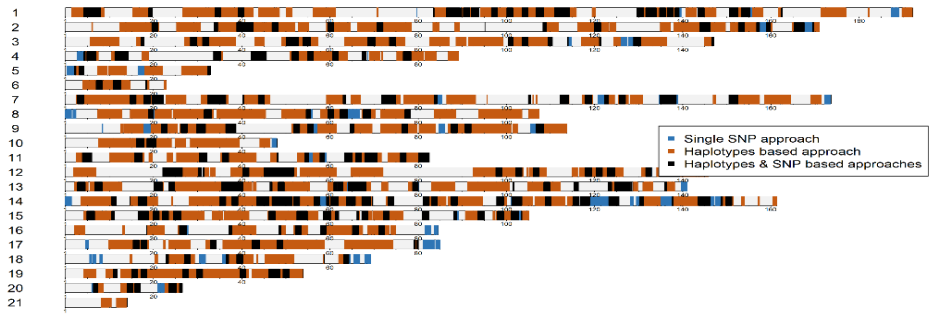

Fig.1. An overview of the genomic regions, distributed in all rabbit autosomes, covered by signatures of selection, identified in all analysed breeds by single marker and haplotype-based approaches.

CONCLUSIONS

This study demonstrates the valuable utility of high-throughput genotyping data in unraveling the population genomic features of several rabbit breeds. Notably, the use of different methods to detect signatures of selection highlighted important genomic regions that could not emerge using just one or another approach. Including other approaches would be pontentially possible to identify additional signatures of selection. Many other rabbit breeds remain to be investigated at the genome level. Their full characterization may identify other important hints able to explain the large phenotypic diversity in this domesticated species.

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TRANSCRIPTOMIC ANALYSIS IN THE LIVER OF TWO RABBIT LINES DIVERGENTLY SELECTED FOR INTRAMUSCULAR FAT CONTENT

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ABSTRACT

The intramuscular fat (IMF) content is an important indicator of meat quality, affecting the sensory properties of meat. IMF is a complex trait with polygenic nature, meaning that it is controlled by multiple genes, each one with a small effect. In this study, the aim was to identify differentially expressed (DE) genes in the liver of two rabbit lines divergently selected for IMF (high and low lines), and to study those that modulate fat deposition. We used a total of 48 rabbits (balanced between sex and line) belonging to the ninth generation of selection for IMF. Liver samples from these animals were used to determine the gene expression levels by 3′ RNA-Seq. This method involves the amplification of the 3' end of the messenger RNA using a combination of polyT primers and random primers. Sequencing of the library was performed on the Illumina NovaSeq X platform with 150 bp paired-end chemistry. A differential expression analysis using a negative binomial model was performed, followed by a functional analysis of the DE genes detected. Our results indicated a total of 551 DE genes between the divergent lines, of which 235 were up-regulated and 316 down-regulated in the high line. Among the DE genes, we detected candidate genes associated with lipolysis, lipogenesis, adipogenesis, fatty acid beta oxidation, fatty acid biosynthesis, lipid transport, or bile acid secretion (e.g., *ALOXE3, ANKRD1, ARSI, BACH2, CPT1B, CROT, CUBN, CYP4A6, ELOVL6, FABP4, FITM1, GCKR, GGT1, HADHB, NR4A1, NR4A2, PLIN2, PPP1R3B, SLC44A4, SLC51A, SLC51B*), among others. Likewise, we detected an overrepresentation of 38 functional annotation terms including 28 pathways, 8 biological processes and 2 molecular functions, among which the peroxisome proliferator-activated receptor signaling (PPAR), fatty acid elongation, fatty acid degradation, biosynthesis of unsaturated fatty acids, arachidonic and retinol metabolism were detected. The liver gene expression study revealed valuable information about candidate genes for IMF deposition in rabbits, but also about bioprocesses and pathways associated with lipid and carbohydrate metabolism.

Key words: hepatic lipid metabolism, fat deposition, liver gene expression, 3′ Tag-Seq, rabbits.

INTRODUCTION

The IMF content and fatty acid (FA) composition are important indicators of meat quality in livestock, including rabbits. IMF content influences the sensory properties of meat such as tenderness, juiciness, and flavour (Wood et al., 2008), and it can be modified by genetic selection. A divergent selection experiment for IMF in the *Longissimus thoracis et lumborum* muscle was performed in rabbits at the Universitat Politècnica de València for 10 generations. The experiment was successful, obtaining a direct response to selection in the 10th generation of 3.8 SD of the trait. The FA composition of both muscle and liver was modified after selection (Zubiri-Gaitán et al., 2022). In addition, a correlated response was observed in plasma metabolome (Zubiri-Gaitán et al., 2023).

Several authors have shown that IMF is a complex trait with polygenic nature (Sosa-Madrid et al., 2020), meaning that it can be affected by the expression levels of multiple genes in different lipogenic tissues (e.g., fat, liver), with complex metabolic interactions. Among those

tissues, the liver has special interest because it is one of the main sites for *de novo* lipogenesis and it is the main lipogenic site in growing rabbits (Gondret et al., 1997). Previous experiments in the aforementioned divergent lines found a greater liver size in the high line, which was related to its greater lipogenic activity (Martínez-Álvaro et al., 2017), together with changes in the liver fatty acid composition (Zubiri-Gaitán et al., 2023).

Our hypothesis is that the different IMF deposition in the divergent rabbit lines could be associated with the gene expression levels in the liver. To test this hypothesis, we measured the gene expression profile in liver samples from the two lines. The objective of this work was to identify differentially expressed (DE) genes between the two divergent lines for IMF, which could be related to their variation in IMF content.

MATERIALS AND METHODS

Animals and experimental design

In this experiment 24 rabbits from the high line (12 males and 12 females) and 24 from the low line (12 males and 12 females) from the 9th generation of selection were used. The animals were slaughtered at 9 weeks of age after 4 hours of fasting by exsanguination after electrical stunning. The liver samples were immediately removed and aliquoted in 2mL cryogenic tubes with 500 µl of RNA later (QIAGEN). The samples were then stored at -80°C until the analysis.

The liver samples were processed by the biotechnology company Seqplexing. Total RNA was isolated from the liver samples using the MagMAX mirVana Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations. Library preparation was performed using the 3' mRNA amplification methodology with a combination of polyT primers and random primers. The quality of the library was evaluated using the QIAxcel Advanced System (Qiagen). Sequencing of the library at 10× of depth was performed on the Illumina NovaSeq X platform with 150 bp paired-end reads (average of 5 million reads/sample), which was finally used for the quantitative study of gene expression levels in liver.

Bioinformatics Analysis

The raw sequences (FASTQ files) were processed using an *in-house* bioinformatics pipeline developed by the Seqplexing company with default parameters. Briefly, the methodology consisted of: (1) quality control with FastQC v0.12.1 software (Andrews, 2010) and trimming with Cutadapt v1.2.0 tool (Martin, 2011), (2) UMI (Unique Molecular Identifier) processing using umi-tools v0.2.3 software (Smith et al., 2017), (3) alignment against the reference genome "UM_NZW_1. 0 (RefSeq GCF_009806435.1)" (*Oryctolagus cuniculus*) using the STAR v2.7.11a software (Dobin et al., 2013), and (4) expression quantification using the HTSeq-count v2.0.5 program (Anders et al., 2015), in order to calculate the counts of each gene.

Statistical Analysis

The counts matrix obtained was used for differential expression analysis using the DESeq2 v1.42.0 R package (Love et al., 2014). A filter of genes was applied, eliminating those that did not have at least 10 counts per gene, and 18943 genes were finally retained. Count normalization was performed using the median of ratios method implemented in the DESeq2 package. The differences between lines in gene expression were estimated applying a negative binomial model including sex (2 levels) and line (2 levels) as fixed effects. The Wald test was used to identify DE genes between lines. Raw P-values were corrected for multiple testing using the Benjamini and Hochberg method. All genes with an absolute fold change (FC) of at least 1.5 ($|\log_2 FC| \geq 0.58$) and a Padj ≤ 0.05, were selected as DE genes. In addition, the list of DE genes was submitted to the ClueGO v2.5.10 plug-in in Cytoscape v3.10.1 software (Bindea et al., 2009; Shannon et al., 2003) for gene ontology (GO) and pathways analysis.

RESULTS AND DISCUSSION

After quality control, a mean of 30.24 million of 150 bp paired-reads per sample, and an average of 92.82% (ranging from 91.37% to 93.88%) of uniquely mapped reads were generated. Previous studies have shown that 3' RNA sequencing provides an alternative to whole transcript analysis with similar levels of reproducibility that the standard RNA-Seq method; moreover the 3′ method is useful in the detection of short transcripts (Ma et al., 2019). 3′ RNA-Seq is superior to standard RNA-Seq in cases of sparse data, which relies on enriching for 3′ ends of the transcript and its use is considered as a proxy for expression of the whole gene (McClure et al., 2023).

Figure 1: Liver 3′ RNA-Seq DE genes between high and low lines for IMF. Dashed lines indicate the values of the threshold.

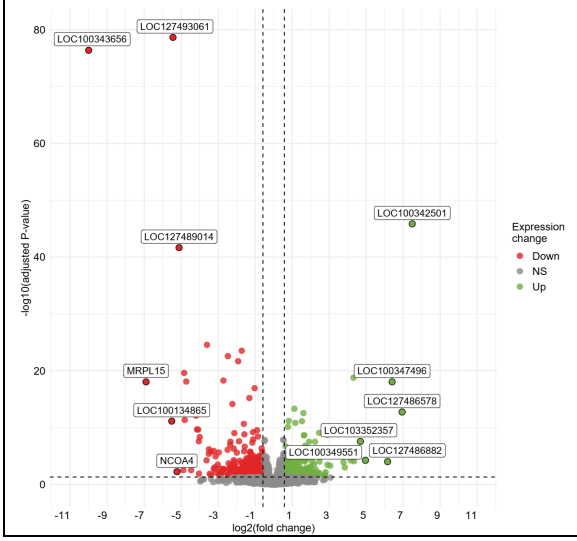
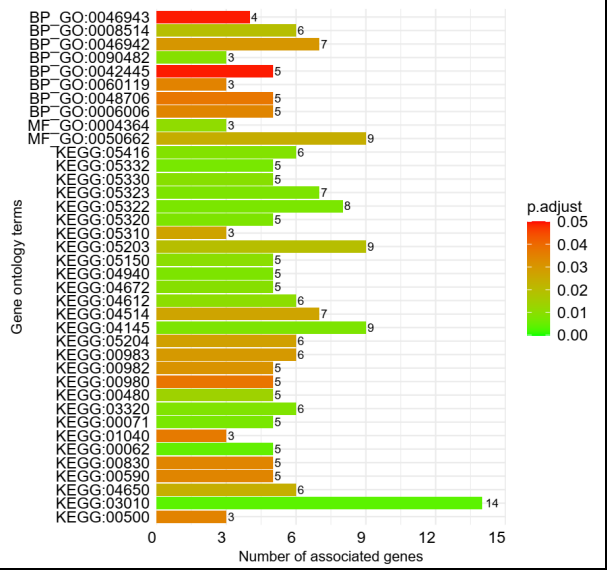


Figure 2: Functional analysis of the DE genes between high and low lines for IMF.



Differential gene expression analysis identified a total of 551 DE genes (absolute FC of 1.5 and Padj < 0.05) between the two divergent lines (**Figure 1**). Likewise, our results indicated that the expression levels of the DE genes in liver were correlated either positively (maximum 0.74) or negatively (maximum 0.74) with IMF content. The volcano plot displays pairwise comparison (H vs L), highlighting 235 up-regulated genes (green points) and 316 down-regulated genes (red points), and the remaining were the genes that did not pass the threshold (grey points).

The DE genes with the highest expression change (absolute FC ≥ 25.76 ~ $|\log_2 FC| \geq 4.69$) between lines were 12, which are shown in Figure 1. We also identified 43 candidate genes with functional annotation for lipid and/or carbohydrate metabolism, fat deposition, or transporters, of which one of the most relevant was the perilipin 2 (*PLIN2*), which is a promising candidate gene for IMF deposition. Our results suggested that the expression of *PLIN2* gene was decreased in the high line (FC = 2.55, Padj = 0.004). The protein level of PLIN2 plays a key role in governing lipid droplet dynamics and their relationship to mitochondria (Xu et al., 2019). PLIN2 is also known as a member of gatekeepers of intracellular lipolysis. Furthermore, in a previous GWAS for FA composition performed in our lines, *PLIN2* was highlighted as one of the functional candidate genes (Laghouaouta et al., 2020). The ELOVL fatty acid elongase 6 (*ELOVL6*) is an interesting lipogenic gene, which showed increased expression (FC = 1.75, Padj = 0.01) in the high line compared to the low line. The ELOVL family plays essential roles in lipid metabolism and cellular functions (Wang et al., 2023). Regarding transporter molecules, it was found that several members of the solute carrier (SLC) superfamily (like *SLC16A11, SLC25A20, SLC27A4*), were differentially expressed. The SLC superfamily comprises genes that encode membrane-bound transporters, which play essential roles in transporting an array of substrates (e.g., amino

acids, glucose, FAs and lipids, acetyl coenzyme A, and vitamins) across cellular membranes (He et al., 2009).

Function analysis with the list of 551 DE genes showed an overrepresentation of 38 terms, including 28 pathways, 8 biological processes and 2 molecular functions (**Figure 2**), among which the KEGG pathways of the ribosome (KEGG:03010), peroxisome proliferator-activated receptor signalling (KEGG:03320) and fatty acid degradation (KEGG:00071) were overrepresented. It is worth noting that PPARs are nuclear receptors that function as ligand-activated transcription factors, which regulate the fatty acid metabolism and modulate gene expression of target genes (Wagner and Wagner, 2020).

CONCLUSIONS

The study revealed a total of 551 DE genes between the lines divergently selected (high and low) for IMF. Among the DE genes, we identified a certain of candidate genes related to lipolysis, lipogenesis, and other bioprocesses and pathways associated with fat deposition and lipid and carbohydrate metabolism.

ACKNOWLEDGEMENTS

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IDENTIFICATION OF KEY GENES AND LNCRNAS UNDERLYING INTRAMUSCULAR FAT DEPOSITION IN RABBITS BY RNA SEQUENCING

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ABSTRACT

Intramuscular fat (IMF) is one of the main factors affecting the meat quality and taste in rabbit. In this study, the meat quality traits at different growth stages in Hycole and Rex rabbits were measured. By comparing the measurements of the performance index in longissimus dorsi (LD) muscles, we found the IMF content increased with the increase of growth in both breeds, while the IMF contents was higher in Rex rabbits than that in Hycole rabbits at the same age. Differentially expressed (DE) mRNAs and DE lncRNAs were identified in LD muscles by whole transcriptome sequencing, and lncRNA-mRNA regulatory network was constructed. The results showed that the number of DE mRNAs/lncRNAs in the late growth period was significantly higher than that in the pre-growth period, and there were significant differences in the expression patterns of LD muscles at different developmental stages. Furthermore, STEM analysis showed that 17 lncRNA-mRNA target gene pairs related to IMF deposition were finally anchored, including 12 mRNAs related to IMF deposition such as *PDGFD* and *PDLIM1*.The study provides insights into the regulatory mechanism of adipose development and sheds light on the genetic breeding for meat quality traits in domestic rabbits.

Key words: rabbit, transcriptome sequencing, mRNA, lncRNA, intramuscular fat.

INTRODUCTION

Intramuscular fat (IMF) is one of the essential traits in meat quality, which influences the tenderness, juiciness and flavor (Hocquette *et al*., 2010), and it contributes to the improvement of sensory quality of meat to meet the requirements of consumers. It was found that the age of the livestock species at slaughter was significantly correlated with intramuscular fat content (Bosch *et al*., 2012). The rabbit has been shown to be an excellent animal model for other livestock species, the Hycole rabbit (HR) is a common meat rabbit breed that has been frequently used for the study of intramuscular fat deposition (Luo *et al*., 2023). Whereas Rex rabbit (RR) is often used to study fur development in rabbits (Zhao *et al*., 2017), it is valuable to study the genetic differences in IMF deposition between rex rabbits and meat rabbits.

With the rapid development of high-throughput sequencing, researchers have found that mRNAs are closely related to IMF deposition in animals. And lncRNAs are important non-coding RNAs (ncRNAs) that regulate fat accumulation, including the process of adipocyte proliferation, differentiation and hypertrophy (Zhang *et al*., 2019). There may be potential non-coding RNAs involved in the regulation of meat quality at different developmental stages in different rabbit breeds (Dou *et al*., 2023).

In the study, we comprehensively compared the differences in the meat traits of the longest dorsal muscle between two rabbit breeds at different developmental stages, and whole transcriptome sequencing was performed to reveal the molecular mechanisms underyling IMF deposition, and the results will provide new insights into the subsequent studies on the genetic breeding of rabbits and the regulatory mechanism of fat development.

MATERIALS AND METHODS

Materials

Female Hycole and Rex rabbits at the age of 35 (weaned period, n=6), 75 (mid-growth period, n=6) and 150 (late-growth period, n=6) days were used in this study. A portion of the muscle tissue was used for histological analysis (n=3). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Northwest A & F University (approval no. DK2024050).

Methods

(1) The meat quality traits were detected according to the standard methods indicated by Hernández *et al*. (2006). An analysis of variance was performed on meat traits.

(2) Fresh LD muscle samples collected from rabbits were analyzed for oil red O staining. Three regions were randomly selected in each sample to measure the area of the lipid droplets.

(3) RNA was extracted from LD muscles, and the concentration and integrity were determined using the Nanodrop and Agilent 5400 Bioanalyzer systems. RNA purity was determined using agarose gel electrophoresis. Only samples with an RNA integrity score of >7.5 were sequenced.

(4) Bioinformatic analysis of DE mRNA and lncRNA and their interactions. Firstly, the differential expression of mRNA and lncRNA between groups at different stages was analyzed. Then, the interaction between lncRNA and mRNA in the LD muscle of Hycole and Rex rabbits was revealed by constructing lncRNA regulatory networks. Finally, the mRNAs related to intramuscular fat deposition were determined through STEM analysis and combined with the lncRNA-mRNA regulatory network results to anchor to specific mRNAs.

RESULTS AND DISCUSSION

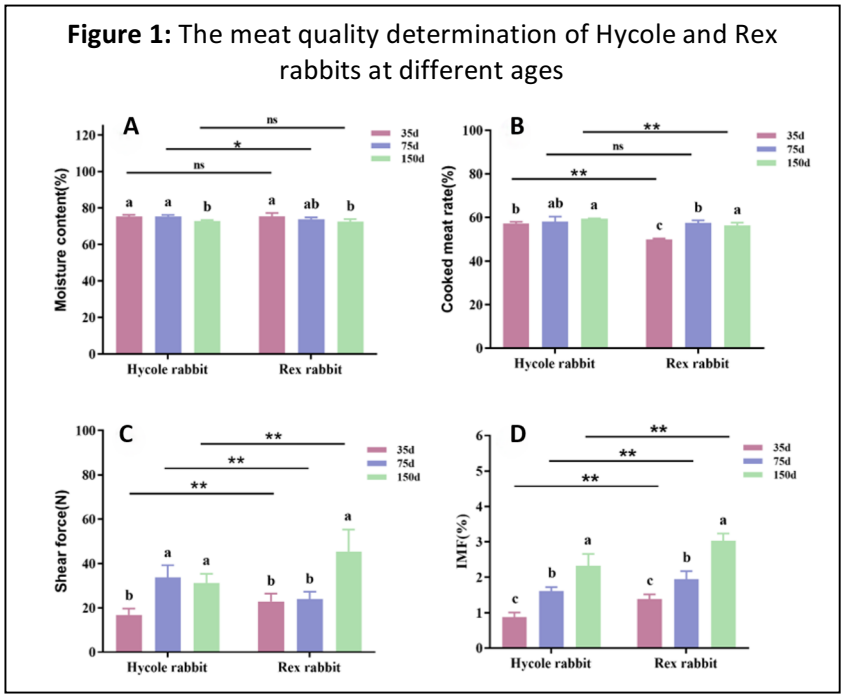


Figure 1: The meat quality determination of Hycole and Rex rabbits at different ages

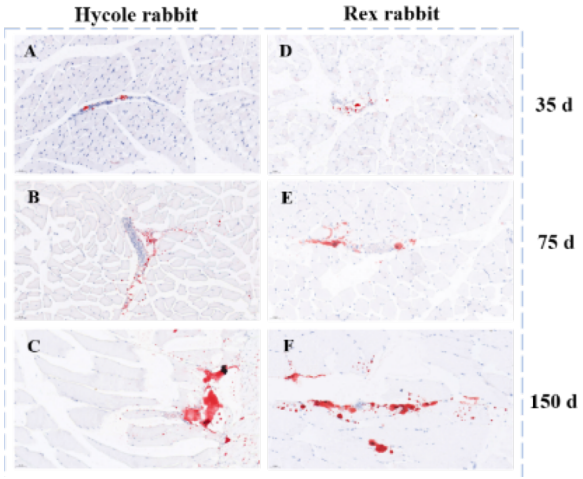
Meat quality measurements in Hycole and Rex Rabbits

The meat quality of Hycole and Rex rabbits at different ages are shown in Figure 1. The IMF content increased significantly with the increase of growth ($p<0.05$). And the IMF content was highly significant greater in all Rex rabbits than that in Hycole rabbits at the same age ($p<0.01$).

Histological analysis of Longissimus dorsi Muscles

As it is shown in Figure 2, the relative total area of lipid droplets also increased with the increase of age. Moreover, the lipid droplet area of rex rabbits was higher than that of meat rabbits in each growth stage. These results were similar to the increase of IMF content of LD muscle at different stages in the two rabbit breeds.

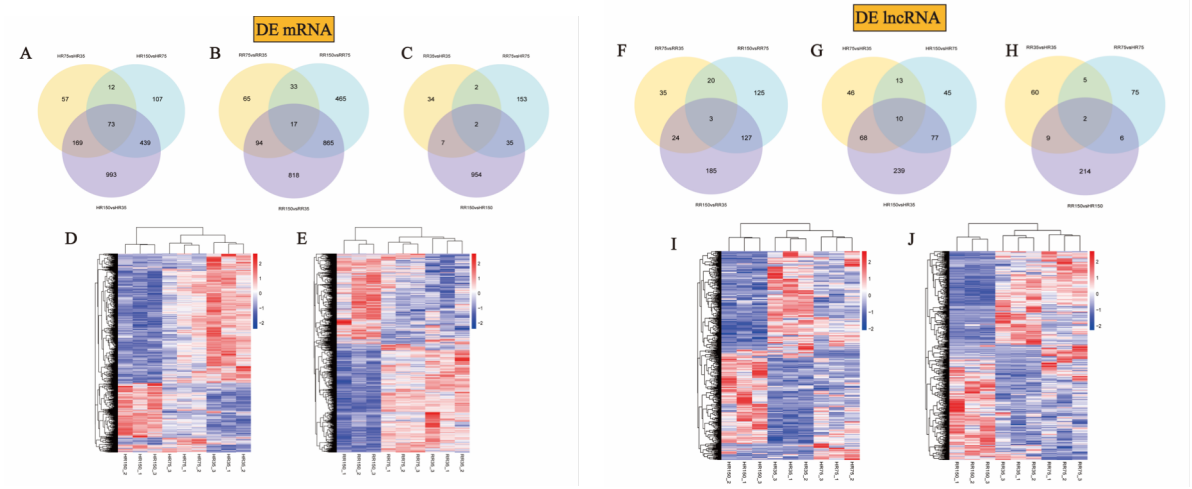
Figure 2: The Histological analysis of rabbit at different stages



Differentially Expressed mRNAs/lncRNAs in the LD Muscles of Hycole and Rex rabbits

The number of DE mRNAs/lncRNAs in the late growth period was significantly higher than the number of DE mRNAs/lncRNAs in the pre-growth period during intramuscular fat deposition in Hycole and Rex rabbits (Figure 3). The cluster analysis showed that DE mRNAs/lncRNAs at different stages were gathered into two major clusters, with one cluster for samples from 35 and 75 days and another cluster for samples from 150 days, suggesting that there were significant differences in the expression patterns of LD muscles at different developmental stages. However, the amount of DE mRNA/lncRNAs between different breeds was significantly less than the amount of DE mRNA/lncRNAs at different growth stages. This suggests that the effect of growth stage on intramuscular fat deposition may be greater.

Figure 3: Expression profiles and bioinformatic analysis of DE mRNAs and lncRNAs



STEM analysis of lncRNA-mRNA

Short time series analysis showed that 638 DE mRNAs in cluster A (profile0, profile2, profile3) were continuously down-regulated, and 273 DE mRNAs in cluster B (profile12, profile13) were continuously up-regulated in Hycole rabbits (Figure 4).

While in Rex rabbits, 523 DE mRNAs in cluster C (profile0, profile2, profile3) were continuously down-regulated, and 191 DE mRNAs in cluster D (profile13) were continuously up-regulated (Figure 4). Among them, 192 down-regulated and 24 up-regulated DE mRNA were found in Rex and Hycole rabbits. This suggests that these differential mRNAs may act as repressors and activators respectively to regulate IMF deposition. The results of STEM analysis were combined with the lncRNA-mRNA regulatory network results, and finally anchored to 17 lncRNA-mRNA target gene pairs related to IMF deposition, including 12 mRNA related to IMF deposition such as *PDGFD* and *PDLIM1* genes.

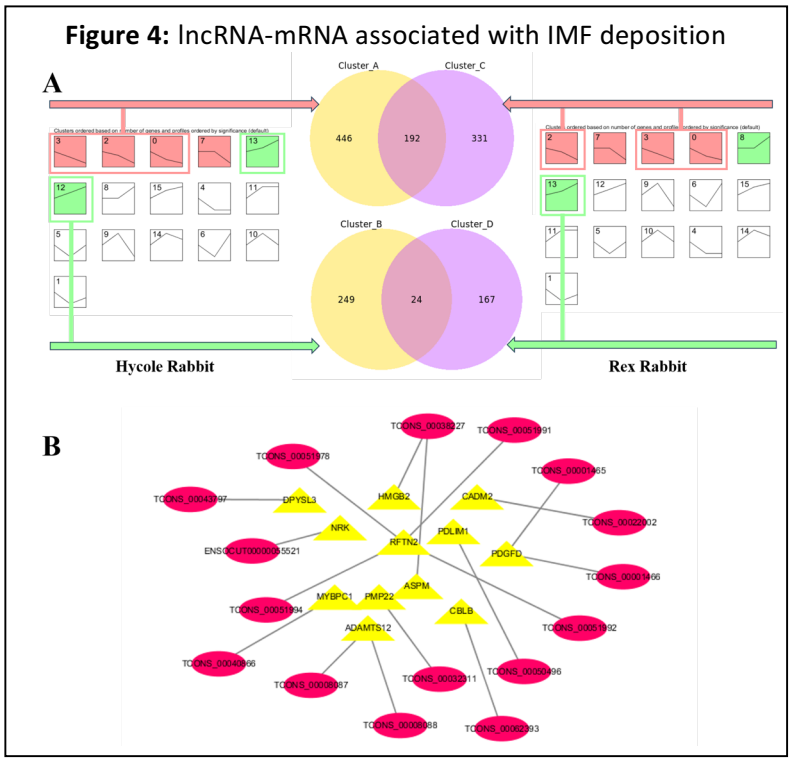


Figure 4: lncRNA-mRNA associated with IMF deposition

CONCLUSIONS

In the study, we compared the phenotypic differences of meat quality traits in rabbits, and performed whole transcriptome analyses, we conluded that the IMF content increased with age in both rabbit breeds, while the IMF content of Rex rabbits was greater than that of Hycole rabbits at the same developmental period. And we finally anchored 17 lncRNA-mRNA target gene pairs related to IMF deposition, involving *PDGFD* and *PDLIM1* genes which potentially contribute to the adipose development.

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EXPLORING MORTALITY AND CULLING CAUSES OF BREEDING DOES THROUGH GUT MICROBIOTA ANALYSIS

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ABSTRACT

Mortality and culling are major concerns in rabbit farming due to their impact on production and economic losses. This study investigates the relationship between gut microbiota composition and mortality/culling cause in female rabbits. The study included 44 female does with recorded causes of culling and mortality. The females were divided into 3 groups; Culled for reproductive reasons (CR), Culled for health reasons (CH) and Deceased (D). Soft fecal samples were collected from all does after the last parity before death or culling and subjected to DNA extraction and 16S rRNA gene sequencing. After bioinformatic analyses the core microbiota of each culling/mortality group was identified and Partial least square discriminant analyses (PLS-DA) was used to assess its association with gut microbiota. The results revealed distinct core microbiotas among the culling and mortality groups. Group D had 22% of its core microbiota not shared with the other groups, followed by CR with 16%, and CH with 13%. Moreover, the PLS-DA final model discriminated between the three groups and the prediction accuracies were: 96% for D, 84% for CR group, and 88% for CH. The group D (Deceased) exhibited the most unique core microbiota and the highest prediction accuracy in discriminant analyses. We hypothesize that alterations in gut microbiota composition, potentially leading to dysbiosis, may contribute to the distinct classification of animals into group D. The initial findings of this study highlight the importance of understanding the role of gut microbiota in animal culling and mortality. Knowledge of microbiota or specific taxa within it, that are associated with adverse outcomes, can contribute to improved animal health, productivity, and welfare.

Key words: Mortality, Culling, Microbiota.

INTRODUCTION

Mortality and culling in rabbit farming are of great importance when considering both production and economic aspects (Larzul et al., 2014). In Spain, adult female rabbits faced a monthly mortality risk of 3.2%, accompanied by a culling risk of between 5.5 and 7% (Rosell and González, 2009). The main causes of mortality in female rabbits are attributed to health problems, especially pathological processes within the respiratory tract, and digestive complications such as enteritis-diarrhea and mucoid enteropathy (Rosell, 2016). In addition, low productivity, which translates into impaired reproductive performance or infertility in females serves as the main reason for culling (Rosell, 2016). Recognizing and understanding the causes of mortality and culling is important from a health, economic and welfare perspective: (1) It can improve the longevity and productivity of the females and their litters (2) It can reduce economic losses in commercial farms, (3) It can improve animal health and welfare (Rosell 2016).

In rabbits, gut microbiota is often associated with various aspects of health, including the immune system, disease, and mortality (Fortun Lamothe and Boullier, 2007). A recent study by Funosas et al. (2021) hypothesized a link between the gut microbiota in rabbits and their life expectancy. However, the relationship between microbiota and the causes of culling and mortality in rabbits remains unexplored. In this study, our aim is to investigate and compare the gut microbiota of females rabbits that were sampled prior to their death or culling, together with the specific causes that were attributed to their death or culling. To our knowledge, no studies have specifically addressed this topic. If the microbiota, or specific taxa within it, were found to vary in relation to the future cause of culling or death, the findings would have significant potential for early detection of animals at risk of mortality or

culling. Furthermore, such knowledge could facilitate selection against microbiota associated with adverse outcomes, thereby contributing to improved animal health, productivity, and welfare.

MATERIALS AND METHODS

Animals

A total of 44 female does were used in this experiment. The does were from maternal lines that are selected for litter size at weaning in farms of the Universitat Politècnica de València. For each doe, the cause of culling or mortality was recorded. All the animals were reared under the same conditions and were allowed to reach the maximum number of parities. They were all housed in individual cages (flat-deck) with an extractable nest box with isolated plastic, and under a photoperiod of 16-h light: 8-h dark and controlled temperature and ventilation. Access to feed was ad libitum throughout the experimental period and the diet was similar for all animals, consisting only of commercial feed.

The recorded mortality and culling causes for each female were organized in three categories: (1) Culled for reproductive reasons or infertility (CR): Does with six failed attempts of mating; A failed attempt is defined as mating occurring without successful pregnancy or mating refusal (n= 17). (2) Culled for health reasons (CH): This category encompasses does culled due to presenting symptoms indicative of various diseases, such as respiratory issues, digestive, mammary gland inflammation (mammitis), eye inflammation (conjunctivitis), abscesses and others. (n= 12). (3) Deceased (D): Does that died during their productive life (n= 15).

Sample collection, sequencing and Bioinformatic analysis sequencing

Samples of soft faeces were collected from the anus of the does during the second week after their last parity. They were immediately frozen at -72 ºC until DNA extraction. Bacterial genomic DNA was isolated from the frozen fecal samples, and the bacterial 16S rRNA gene was sequenced using Illumina MiSeq platform. Primary processing of sequencing reads was carried out on the raw reads, starting with a quality filtering performed using Cutadapt. After, the paired-end Miseq Illumina reads (2*300 bp) were processed in R (R Core Team, 2021), using the DADA2 pipeline for Illumina-sequenced fastq (Callahan et al., 2016). The results were identifying amplicon sequence variants (ASVs) and constructing the ASVs table. After, the final table was imported into QIIME2 software, version 2021.11 (Bolyen et al., 2019). A trained Naive Bayes SILVA classifier was used for taxonomic annotation.

Statistical analyses

All the statistical analyses were performed in R (R Core Team, 2021). To identify the core microbiota, microbial taxa were considered part of the core if they had an occurrence of at least 80% across all samples and a minimum relative abundance of 0.1%. These criteria were used to determine the core microbiota of the entire dataset, and the core microbiota specific to each group of mortality and culling reasons.

Microbiota data are compositional (Greenacre et al., 2021). To account for this, the data were transformed using the centred log-ratio (CLR). Prior to the transformation, the variables that were not present in at least 20% of samples were removed. Subsequently, a count was added to all datasets to deal with the remaining zeros. The CLR transformation were then calculated:

$$CLR(\mathrm{j}|\mu) = log\left(\frac{x_\mathrm{j}}{\mu}\right) = \log\left(x_\mathrm{j}\right) - \log\left(\mu\right) \qquad (1)$$

With μ defined as the geometric mean:

$$\mu = \left(\prod_{i=1}^{n} x_i\right)^{(1/n)} \qquad (2)$$

Samples were collected from animals that either died or were culled at different ages, ranging from 26 to 172 weeks of age. Given the dynamic nature of the microbiota, which

undergoes substantial changes with age (Biagi et al., 2010), we accounted for this age-related effect by correcting the transformed CLR table.

Partial Least Square Discriminant Analyses (PLS-DA)

PLS-DA was used to identify the relevant ASVs that could classify the rabbits between the mortality and culling causes groups. PLS-DA was performed using the 'mixOmics' R package (Lê Cao et al, 2011). A PLS-DA model with 10 components was fitted to each CLR-transformed dataset. Then, an iterative process was performed until each model achieved the highest classification performance. Variable were selected using the variable important prediction (VIP), i.e. the influence of the variables on the model projection and classification for the number of previously selected components. The variables selected for the final PLS-DA were those with a VIP greater than 1 (Galindo-Prieto et al., 2014). To check the robustness of PLS-DA, a confusion matrix and a permutation test were computed for both comparisons.

RESULTS AND DISCUSSION

Core microbiota analysis

The core microbiota using the criteria of 80% occurrence and 0.1% minimum relative abundance yielded a core microbiota of 130 ASVs, and when collapsed to the genus level, the core microbiota was represented by 31 genera. Additionally, we determined the core microbiota of each cause of the mortality and culling: Culled for reproductive reasons (CR), Culled for health reasons (CH) and Deceased (D) (figure 1A). These results indicate an important proportion of the core microbiota that is unique to each group, with group D having 22 % of their core that is not shared with the rest of the groups, followed by CR (16%) and finally CH (13%).

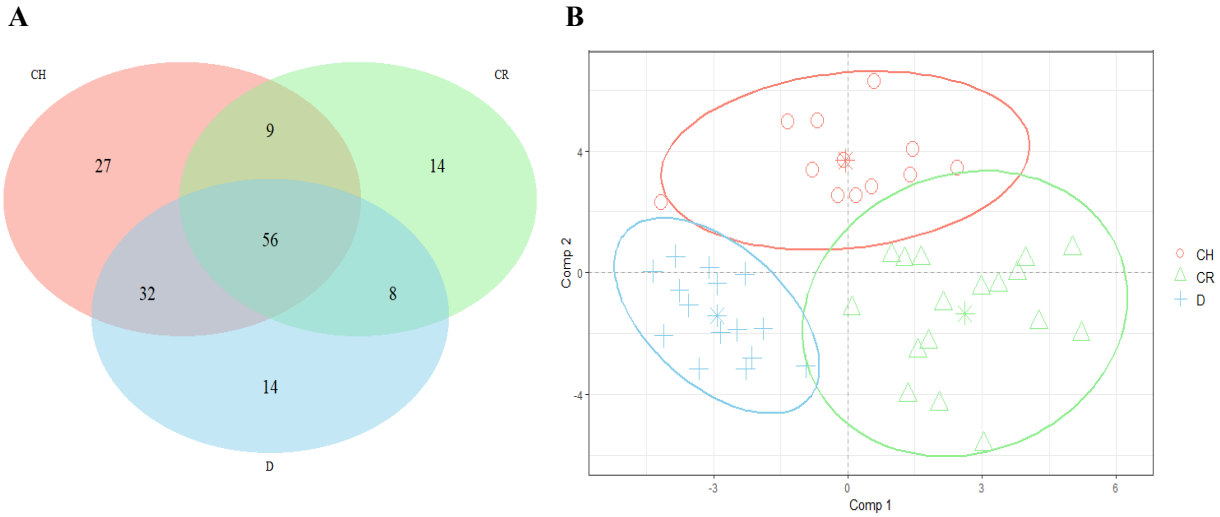

Figure 1: A. Venn diagram of the core microbiota of each of mortality and culling causes. **B**. Individual plots of final partial least squares discriminant analysis (PLS-DA) models

Partial least squares discriminant analyses

After the centred log-ratio (CLR) transformation, the final PLS-DA models identified 59 ASVs that could discriminate between the three groups CR, D and DH (figure 1B). The final 59 ASVs identified in were collapsed at the genus level, which allowed taxonomic information about each ASV. The result of the confusion matrix showed a predictive performance (true positives) of 84% for the CR group, 88% for CH and 96% for D. The predictive performance of the permutation tests was 38%, 30%, and 33% for CR, CH, and D respectively. Indicating that the taxa identified by the PLS-DA do not randomly discriminate between groups.

In agreement with the core microbiota analysis, the group with the highest predictive accuracy was identified as group D, which consisted of females that had died. We

hypothesize that the distinct classification of animals into group D (Deceased), and to a lesser extent, group CH (culled for health reasons), may be due to alterations and potential dysbiosis in their gut microbiota. Research in humans has shown that critically ill patients often have dysbiosis of the intestinal microbiota (Wei et al., 2021). Additionally, in rabbits, indices reflecting dysbiosis, such as the Ruminococcaceae/Enterobacteriaceae index, have been suggested to be potentially associated with life expectancy (Funosas et al., 2021). Further investigations will be conducted to assess evidence of dysbiosis and to compare Ruminococcaceae/Enterobacteriaceae index between the groups studied (CH, CR and D) and a control group.

CONCLUSIONS

Some microbial taxa profile could be associated to different causes of culling and mortality in rabbits. These preliminary results highlight the importance of understanding the role of the gut microbiota in the causes of culling and mortality in female rabbits. Further investigation of the relationship between gut microbiota composition and health outcomes in rabbits is warranted and offers promising avenues for improving the welfare, productivity, and sustainability of rabbit farming practices.

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GENOMIC REGIONS ASSOCIATED WITH THE COMPOSITION OF THE RABBIT CAECAL MICROBIOTA

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ABSTRACT

The aim of our study was to identify genomic regions influencing the composition of the rabbit caecal microbiota, to better understand its genetic determinism. The ribosomal 16S DNA was extracted from the microbial community of the caecum and the ribosomal 16S gene was then sequenced in 525 rabbits originated either from a line selected on feed efficiency or a control non-selected line. After rarefaction and filtration of the sequences, 859 OTUs (operational taxonomic units) were identified. The rabbits were genotyped with the Affimetrix Axiom Rabbit 200K chip. After quality control 514 rabbits and 103,072 SNPs were retained for association study analyses. Eighty-one QTLs were identified for 69 OTUs abundance, with a total of 492 significant SNPs ($p < 0.05$ genome-wide, corrected by the Bonferroni method). Most QTLs were associated with the abundance of a single OTU. Eight QTLs were associated with 2 or 3 OTUs.

Key words: rabbit, feed efficiency, ceacal microbiota, genomics, GWAS.

INTRODUCTION

The rabbit digestive tract hosts a complex and diverse microbial community that plays a key role in numerous physiological functions such as digestion and immunity (Combes et al., 2011). It has been shown to contribute to feed efficiency and growth traits in rabbits (Velasco et al., 2021). Knowledge of the genetic determinism of microbiota composition would help incorporating this information into selection schemes. The aim of this study was to search for chromosomal regions (QTL) associated with microbiota composition, using association studies.

MATERIALS AND METHODS

Animals and data

Animals were derived from the INRAE 1001 line and were raised at the INRAE rabbit experimental facility (IE GenPhySE. (2023). Sheep and rabbits experimental facility. INRAE. https://doi.org/10.17180/ftvh-x393), in compliance with animal experimentation regulations (APAFiS approval number #18416). Details of the protocol are described in Garreau et al. (2019). Briefly, two lines were used in this study: the G10 line, selected for 10 generations for reduced residual consumption, and the G0 line produced from frozen embryos of the ancestral population of the selected line. A total of 490 G10 rabbits and 410 G0 rabbits were tested, in 3 batches separated by 42 days. Each new born rabbit was adopted 48 hours after birth. In each batch, half of the rabbits of each line were raised by G0 does and the other half by G10 does. At weaning (32 days), 456 rabbits were placed in individual rearing cages and 144 in individual digestibility cages. All animals were fed ad libitum with the same commercial pelleted feed until the end of the fattening period (63 days).

Determination of microbiota composition

Caecal content samples were collected from 451 rabbits raised in individual cages and 139 rabbits reared in individual digestibility cages, just after slaughter at 63 days, then stored at -80°C until processed. The ZR Soil Microbe DNA MiniPrepTM kit (ZymoResearch, Freiburg, Germany) was used for microbial DNA extraction. Microbial 16S rRNA was characterized by sequencing (MiSeq sequencer, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain). To account for heterogeneities in sequencing depth between samples, the

abundance table was rarefied to 12,228 reads (minimum sequencing depth of samples) using the phyloseq package (McMurdie and Holmes, 2013). Sequences with a degree of similarity higher than 97% were grouped into OTUs (operational taxonomic units). The final OTU abundance table comprised 11,323 OTUs for the 525 animals with 16S sequences, feed intake performances and complete pedigrees. Rare OTUs were filtered according to two criteria: if absent in more than 10% of samples, and with an average relative abundance (sum of OTU abundances in samples divided by the total number of reads in the dataset) of less than 0.01%. A total of 859 OTUs were finally retained. A Box-Cox transformation was applied to normalize the OTU abundance distributions. The FROGS affiliation software (Galaxy version 3.2.2, Escudié et al., 2018) and the SILVA 138.1 16S pintail100 database (version 138.1, Quast et al., 2013) were used to predict the OTU taxonomy. Taxonomic affiliations were performed with the Blastn+ algorithm (Camacho et al., 2009). When the percentage of assignation was less than 90%, the phylogenetic groups were considered unknown.

Animal genotyping

Animal DNA was extracted from ear biopsies previously collected and stored at -20°C. Rabbits were genotyped using the Affimetrix Axiom Rabbit 200K chip (Santa Clara, CA, USA) at the Centro Nacional de Genotipado laboratory (CeGen, Santiago de Compostela, Spain). The OryCun2.0 assembly of the rabbit genome (Carneiro et al., 2014) was used as a reference for SNP positions on the genome. Quality control was performed on the genotypes using PLINK software (version 1.07, Purcell et al., 2007). All SNPs with allele frequency lower than 5% and call rate lower than 5% were deleted. Animals with less than 95% validated genotypes were removed from the analysis. Finally, after eliminating SNPs according to the Hardy-Weinberg equilibrium principle and those not mapped to autosomes, 514 rabbits and 103,072 SNPs were retained.

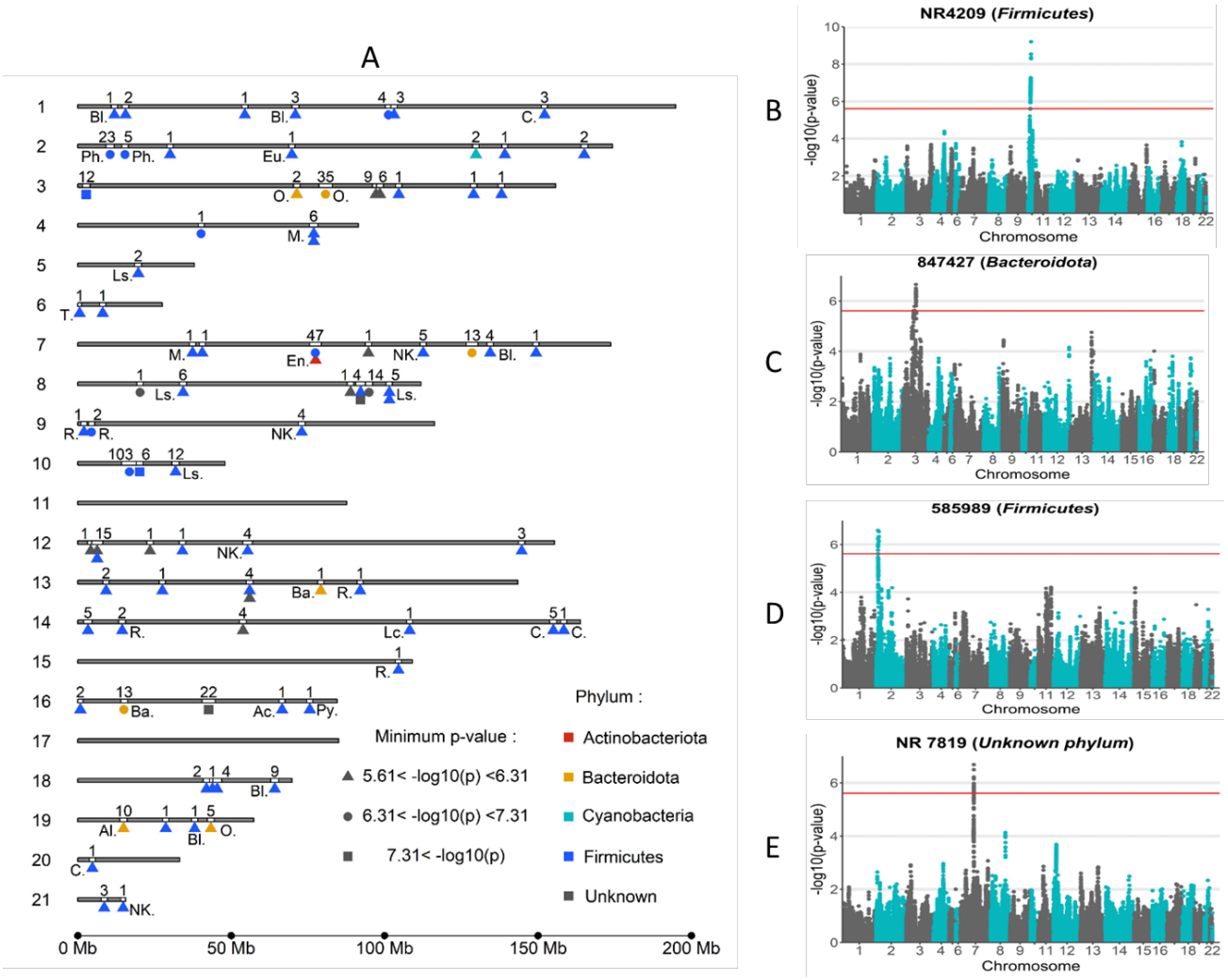
Genomic association analysis

An analysis of variance was applied to test the fixed effects affecting the rarefied and transformed abundance values of each OTU. The same set of fixed effects was retained for all OTUs. The effects retained were significant ($p < 0.05$) for at least 10% of OTUs: combined effect of batch and sequencing plate (72% of OTUs), line of the fostering doe (37%), cage type (31%), and litter size at weaning (10%). The genome-wide association study was carried out on each of OTUs using GEMMA software (version 0.94.1, Zhou and Stephens, 2012). The following univariate animal mixed model was applied to estimate the effects of the SNP: $\boldsymbol{y} = \beta_0 + \boldsymbol{Wa} + \boldsymbol{u} + \boldsymbol{\epsilon}$ with $u \sim \boldsymbol{N}(\mathbf{0}, \sigma_u^2\, \mathbf{G})$ and $\boldsymbol{\epsilon} \sim \boldsymbol{N}(\mathbf{0}, \sigma_e^2 \mathbf{I_n})$, where $\boldsymbol{y}$ is the vector of adjusted OTU abundances, β_0 is the intercept, $\boldsymbol{W}$ is the vector of marker genotypes, $\boldsymbol{a}$ is the marker effect, $\boldsymbol{u}$ is the additive polygenic random effect and $\boldsymbol{\epsilon}$ is the residual random effect. σ_u^2 and σ_e^2 are the polygenic and residual variances. **G** is the centered genomic relatedness matrix calculated with GEMMA software (Zhou and Stephens, 2012). To account for multiple testing for 103,072 SNP markers, the significance level of p-values was corrected by the Bonferroni method using SimpleM software (Gao et al., 2010) to estimate the equivalent number of independent tests, equal to 20,551. The adjusted threshold for a p-value=0.05 was then: $-\log 10\left(\frac{0.05}{20\,551}\right) = 5.61.$ A QTL region was defined by one or multiple significant SNPs distant from less than 1 Mb, plus the surrounding 1 Mb region.

RESULTS AND DISCUSSION

Association studies revealed 492 significant SNPs for 69 OTUs. Out of these, 31 OTUs were affiliated to 16 known and distinct bacterial genera. The genera most frequently associated with significant SNPs were *Ruminococcus* (4 OTUs), *Blautia* (4 OTUs) and the *Lachnospiraceae* NK4A136 group (4 OTUs). Significant SNPs were distributed over 19 of the 21 automosomes (Figure 1). No QTL were detected on chromosomes 11 and 17 (OCU11 and OCU17).

Figure 1. Map of QTL detected for caecal microbiota composition and four Manhattan plots for OTUs having more than 23 significant SNPs



A: QTL regions: white bands corresponding to significant SNPs locations ± 1 Mb (-log10 (p-value) > 5.61).
Number of significant SNPs: labels above the QTL.
Phylum affiliation: color symbols below the QTL region. One symbol represents one or several OTUs sharing an identical phylum affiliation.
Genus affiliation: abbreviations below the QTL region: Ac. Acetatifactor; Al. Alistipes; Ba. Bacteroides; Bl. Blautia; C. Christensenellaceae R-7 group; En. Enterorhabdus; Eu. Eubacterium ruminantium group; Lc. Lachnoclostridium; Ls. Lachnospiraceae NK4A136 group; M. Marvinbryantia; NK. NK4A214 group; O. Odoribacter; Ph. Phascolarctobacterium; Py. Pygmaiobacter; R. Ruminococcus; T. Tyzzerella.
Manhattan plots: (B) OTU NR4209; (C) OTU 847427, (D) OTU 585989, (E) OTU NR7819. Red line: genome wide significance threshold.

Significant signals were distributed across 81 genomic regions with uneven numbers of SNPs: 34 regions included a single significant SNP, while one region on OCU10 included 103 significant markers. Similarly, the number of significant SNPs per OTU varied: 19 OTUs had only one significant SNP, while a maximum of 107 SNPs were significantly associated with a single OTU belonging to the phylum *Firmicutes* (NR4209, Figure 1B).
Fifty OTUs were associated with a single QTL, 18 OTUs with two QTLs and one OTU with four QTLs. The OTU 317315 (*Clostridia vadinBB60 group*) was associated with the highest number of QTLs: two located on OCU2 (29.0-31.0 and 163.9-166.2 Mb) and two on OCU3 (128.0-130.0 and 137.1-139.1 Mb). Seven genomic regions were associated with the abundance of two OTUs. These regions were located on 7 chromosomes: OCU4, OCU7, OCU8, OCU10, OCU12, OCU13 and OCU18. An eighth region on OCU7 was associated

with the abundance of three OTUs belonging to the *Enterorhabdus* genus (NR4488) or to unknown genera (NR7819, NR6518).
Four OTUs had more than 23 significant SNPs, representing almost half of all significant markers (196 SNPs) (Figure 1). These OTUs were mainly assigned to the predominant phyla of the rabbit caecal microbiota: 3 to *Firmicutes* and 1 to *Bacteroidota*. Only two of these OTUs are assigned to known genera: *Odoribacter* and *Phascolarctobacterium*.

CONCLUSIONS

The identification of 81 QTLs documents the genetic determinism of rabbit caecal microbiota. The functional link between these genomic regions and the associated OTUs remains to be elucidated in order to assess whether the microbiota information is relevant for breeding schemes.

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GENETIC LINE RESTORATION AS A STRATEGY FOR SOLVING PROBLEMS IN RABBIT PATERNAL LINES.

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ABSTRACT

A total of 134 rabbit females and their litters were used for this experiment. This study aimed to determine if establishing a new genetic line by introducing robust genetics could address reproductive, productive, and health limitations in a paternal line. The animals belonged to two genetic lines, the R line and the RFLP line. The R line was selected for a high growth rate during the fattening period, and the RFLP line, which was obtained by backcrossing a long-living-productive maternal line (LP line), with the RF line which was founded through a high-intensity of selection of elite animals from the R line. Female fertility and mortality were recorded from the first artificial insemination to the third parturition, and immune parameters were controlled from the first artificial insemination to the first parturition. Individual kit weight and intake were controlled from 18 days of age to weaning and immune parameters were controlled at weaning. Furthermore, a growing trial was performed in three batches with 253 animals (R= 125 animals from 26 litters; RFLP=128 animals from 27 litters), 20 animals of which were used for a digestibility trial. RFLP kits were heavier than R kits at 18 and 28 dpp, and their intake was higher (+15.11%, +17.03%, +41.39%, respectively; $P<0.05$). During the growing period, no significant differences were found between the genetic lines for body weight, average daily gain, feed intake and food conversion ratio. However, mortality and sanitary risk index were higher for R animals (+11.59 pp and + 13.23 pp, respectively; $P<0.05$). RFLP females' survival was higher than R females across the three cycles, and their fertility was significantly higher during the second cycle. In general, R females showed lower lymphocyte count than RFLP. Our findings suggest that the strategies outlined in this study for reestablishing paternal rabbit lines prove effective in improving fertility, health, and disease resistance without compromising growth rate, thereby contributing to future genetic advancements in rabbit farming.

Key words: immunology, genetics, rabbit.

INTRODUCTION

Genetic selection has suffered a gradual intensification over the past century, focusing on enhancing desirable traits, which resulted in remarkable improvements in various species (Hill, 2008). While the benefits of enhanced production efficiency are evident, animals in a population selectively bred for this trait may also face predisposition for various behavioural, physiological, and immunological issues (Rauw et al., 1998).

In paternal rabbit lines, selection for growth rate has improved average daily gain from 0.45 to 1.23 g/d per generation (Piles and Blasco, 2003). However, selection for this criterion has led to reproductive issues such as low libido and sperm count (Pascual et al., 2004), gestational, perinatal and lactation losses (Vicente et al., 2012; Juárez et al., 2021). In addition, innate and adaptative immune cell count appears to be negatively impacted by the selection for productivity criteria (García-Quirós et al., 2014).

This study aimed to assess how to mitigate the negative effects of selecting for growth rate in a paternal rabbit line by creating a population through backcrossing a long-living maternal line with high growth rate animals. The focus was on resource acquisition and allocation in this newly formed line and its impact on reproductive performance, growth rate and immune status.

MATERIALS AND METHODS

The experimental procedure was approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València (UPV; code: 015/VSC/PEA/00061) and carried out following the recommendations of the European Group on Rabbit Nutrition (Fernández-Carmona et al., 2005) and the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes.

Animals and Experimental Design

The animals belonged to two different genetic lines. The R line was selected for growth rate during the fattening period for 37 generations (Estany et al., 1992). The RFLP line was founded by backcrossing the LP line, a long-living robust maternal line, with the RF line (paternal) generating animals with 7/8 RF and 1/8 LP.

The rabbit females were fed *ad libitum* with two commercial diets throughout the experiment. Until the first parturition, the females were fed a diet for growing rabbits and then a diet for reproductive rabbit females. The animals were artificially inseminated (AI) at 20 weeks of age. At parturition, the number of kits per litter was standardised to 6. Kits' weight was controlled at birth, 18 dpp and weaning. The litters were weaned at 28 dpp. The mortality of the females and kits was recorded daily. Females' traits were recorded from first IA to 3rd parity. Blood samples from rabbit females were collected at first AI, first parturition, and first weaning. In addition, blood samples were collected from three kits per litter at first weaning. These last were mixed and processed as a single sample. In addition, 253 weaned rabbits (28 days of life) from the R and RFLP genetic lines were used for a growing trial, 20 of which were used for a digestibility trial, according to Pérez et al. (1995).

Chemical analysis

The individual faeces of growing rabbits were analysed to determine their dry matter (DM; 934.01), ashes (942.05), crude protein (CP; 990.03, Dumas method, CN628 Elemental Analyzer, LECO, St. Joseph, USA) and gross energy content using an adiabatic bomb calorimeter (Gallenkamp Autobomb, Loughborough, RU). Immune parameters were determined using a CytoFLEX S flow cytometer (Beckman Coulter, Indianapolis, IN, USA).

Statistical Analysis

Performance traits of growing rabbits (BW, ADG, DFI and FCR) and leukocyte percentage of females were analysed using a MIXED model by SAS (Statistical Analysis System) for repeated measures, considering the lack of data homoscedasticity. Apparent faecal digestibility coefficient data of growing rabbits and leukocyte percentage of kits were analysed using a GLM model from SAS, including the genetic type as a fixed effect. The effect of genetic type on mortality and morbidity was analysed using logistic regression by the GENMOD procedure of SAS, considering a binomial distribution (logit scale). All survival rates were calculated in relation to the initial number of does. Fertility percentages were analysed using a chi-square test to determine the effect of the genetic type by a CATMOD procedure of SAS (2002). Significant differences were declared at $P<0.05$.

RESULTS AND DISCUSSION

Kit weight at 18 and 28 dpp, and feed intake were significantly higher for RFLP kits (+15.11%, +17.03% for kit weight and +41.39% for feed intake; $P<0.05$). Despite 1/8 LP maternal origin in RFLP animals, potentially affecting growth, no significant differences were observed between genetic types in body weight at 63 days or overall ADG, DFI, and FCR. Growing rabbits from the R line showed lower percentages of CD25+ cells than the RFLP line (-0.8 percentage points; $p<0.05$). Despite having lower leukocyte counts than other genetic types, this doesn't necessarily indicate a compromised immune state (García-Quirós et al., 2014; Moreno-Grúa et al., 2023). However, when compared to other genetic types, such as LP, R line animals are considered less robust (García-Quirós et al., 2014).

Table 1. Effect of the genetic type on growing performance, health traits, digestive utilisation, and immune cell of growing rabbits.

	R		RFLP		P-value
No. of litters	48		58		
Kit weight (g):					
18 dpp	272.3[a]	±9.34	316.8[b]	±8.6	0.0002
28 dpp	470.6[a]	± 16.0	558.2[b]	±14.7	<0.0001
Kit intake 18 to 28 dpp (g DM/d)	3.66[a]	± 0.41	5.57[b]	±0.37	0.0009
No. of animals	125		128		
ADG 28 to 63	49.7	±0.52	49.1	±0.47	0.4000
DFI 28 to 63	92.8	±2.48	95.4	±2.56	0.4364
FCR 28 to 63	1.97	±0.02	1.97	±0.02	0.9251
Body weight at 63	2321	±24.1	2319	±22	0.9369
Mortality 28-63 dpp (%)	28.00[b]		16.41[a]		0.020
Morbidity 28-63 dpp (%)	3.20		1.56		0.176
SRI 28-63 dpp	31.20[b]		17.97[a]		0.022
No. of animals	10		10		
Faecal coefficients:					
Dry Matter	53.6[a]	±0.5	54.9[b]	±0.5	0.0490
Crude Protein	66.5	±0.9	66.7	±0.9	0.8803
Gross Energy	53.4	±0.4	54.7	±0.4	0.0535
No. of litters	19		18		
Immune parameters (%)					
T-lymphocytes	15.50		20.54		0.096
$CD4^+$	9.882		13.76		0.057
$CD25^+$	0.736		1.502		0.007

[a,b] Means not sharing letter were significantly different at P<0.05. dpp: days postpartum; DM: dry matter; ADG: Average Daily Gain; DFI: Dry matter feed intake; FCR: Feed conversion ratio; SRI: Sanitary Risk Index.

Regarding fertility percentages, there is a significant decrease in fertility observed in R females, while RFLP females maintain consistent reproductive effort and productive longevity, as seen in prior studies (Savietto et al., 2015; Peixoto-Gonçalves et al., 2023a). When comparing immune parameters, R females showed lower total lymphocyte percentage than RFLP and reduced CD4+ percentage and CD4+/CD8+ ratio. A lower CD4+/CD8+ ratio suggests a weakened immune system recovery (Tinago et al., 2014). The variation in leukocyte population may contribute to a significant decrease in survival percentage among R line females, notably lower than RFLP by the second parturition.

Table 2. Effect of the genetic line on fertility, survival, and immune cell percentage of rabbit females.

	R	RFLP	SEM	P-value
N. of animals	65	69		
Fertility (%):				
1^{st} cycle	86.7	85.0		0.0412
2^{nd} cycle	70.9[a]	83.3[b]		0.0053
3^{rd} cycle	67.4	75.0		0.4003
Survival (%):				
1^{st} cycle	73.8[a]	84.1[b]		0.0412
2^{nd} cycle	47.7[a]	73.9[b]		0.0053
3^{rd} cycle	36.9[a]	58.0[b]		0.4003
Immune parameters (%)				
No. of observations	40	45		
Total lymphocytes	33.47[a]	38.05[b]	1.598	0.040
$CD4^+$	24.26[a]	27.67[b]	0.922	0.009
Monocytes	5.535[b]	4.636[a]	0.277	0.021
Granulocytes	32.26[b]	28.03[a]	1.285	0.030
$CD4^+/CD8^+$	3.313[a]	4.385[b]	0.243	0.002

[a,b] Means not sharing letter were significantly different at P<0.05; SEM: standard error of means.

CONCLUSIONS

In conclusion, reestablishing paternal rabbit lines with the integrated strategies outlined in this study proves effective in achieving optimal growth rates, fertility traits, and rabbit health. The findings emphasise the importance of a balanced approach, incorporating resilient genetics and considering a broader spectrum of health-related traits alongside productivity criteria for the sustainable improvement of paternal rabbit lines.

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PRELIMINARY ESTIMATES OF THE RESPONSE TO SELECTION FOR FEED EFFICIENCY IN THREE LINES OF MEAT RABBITS

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ABSTRACT

Three lines were selected to increase their post-weaning feed efficiency using different selection strategies, all of them based on records of group-housed rabbits. The RFI line was selected to reduce residual feed consumption measured individually with electronic feeders (**EF**); the GRP line to reduce the group's residual feed consumption; and the ADGR line to increase growth under feed restriction, which was carried out by limiting the feed supply to the period between 06:00 and 18:00 h though EFs.

The results showed that, compared to a control population established from vitrified embryos six generations ago, the RFI and GRP lines presented a decrease in weight at the end of fattening (58 days old) of between 15 and 25 g per generation. This decrease was mainly attributed to a reduced daily growth of -0.82 and -0.40 g/day/generation for RFI line (measured with conventional and EF, respectively) and -0.57 and -0.37 g/day/generation for GRP line. However, these differences were attenuated when adjusted to a constant live body weight (BW) of 2 kg. The evaluation of feed intake was only carried out in animals fed using EF, observing that at 2 kg BW, the RFI and GRP lines consumed less feed than the control population, with reductions of -2.05 and -1.53 g/ day/generation, respectively, although only the first one was statistically significant. This suggests an improvement in feed conversion ratio of -0.04 points per generation in the RFI line. We conclude that genetic selection to improve feed efficiency using EFs is a viable strategy, which can contribute to substantially improve the sustainability and profitability of rabbit production.

Key words: Feed efficiency, Genetic selection, Electronic Feeders, Response, Group.

INTRODUCTION

Generally, in all livestock species, and particularly in the rabbit, the improvement of feed efficiency (**FE**) during the growth and reproduction stages is a fundamental element of the economic and environmental sustainability of livestock production (Cartuche et al., 2014, Hegarty et al., 2007). In this sense, the different disciplines of animal production are studying strategies to improve it. We relay on genetic selection to improve the FE of animals raised in groups. For about 10 years, we have been developing a series of projects that aim to propose selection models and strategies that, optimally, allow genetic improvement of FE in rabbits. An important milestone has been the availability of an electronic feeder (**EF**) to monitor individual feed intake and control feed supply in group-housed growing rabbits (eFeederRab) (Sánchez et al., 2018). This device has been used in a selection process to increase FE in two of our experimental lines (ADGR and RFI) (Sánchez et al., 2019). In a third line (GRP), the residual feed intake of the group was used as selection criterion. After 6 generations of selection, we are evaluating the responses both in feeding efficiency and in other correlated traits that may have been affected by the selection process.

The objective of this work is precisely to show some preliminary results regarding the response achieved in growth and feed efficiency.

MATERIALS AND METHODS

Animals and experimental design

The present study considers the first two batches of contemporary comparison of animals from the three lines selected by different criteria (ADGR, RFI and GRP), all of them trying to improve feed efficiency. In total there were 899 individuals, distributed similarly between the two batches. The does from the first batch were artificially inseminated on 06-23-2023 and the second batch on 08-25-2023. In previous communications (Sánchez et al., 2019, Sánchez et al., 2023), we have already described the three selected populations, that represent, together with a control population (X), the animal material used in this work. Briefly: line ADGR was selected for daily growth under time-based feed restriction. Line RFI was selected for individual residual feed consumption. Finally, line GRP was selected for group's residual feed consumption, the selection unit being all the animals in a cage. The X line was created in 2022 from vitrified embryos collected in 2016-2017 from the base population of the three selected lines. This base population came from animals belonging to the Caldes line after 3 years of no selection. The selection process of the three lines started in 2017 and has continued for 6 generations. The X animals used in this study are grandchildren or great-grandchildren of the animals born from vitrified embryos, thus avoiding the potential impacts of the vitrification and conservation processes on growth and FE (García-Dominguez et al., 2020).

All animals were monitored during the fattening period between 35 and 58 d of age. A total of 340 of them were fed using the EF (eFeederRab) in 6-kits cages (0.17 m^2/kit). The remaining 559 kits were fed using conventional feeders (**CF**) in cages of 4 kits (0.12 m^2/kit). The analyzed traits for kits in both types of cages were the body weight at the beginning and end of the fattening period (**BW35** and **BW58**, respectively) and the average daily gain (**ADG**); for kits in cages with EFs, the average daily feed intake (**ADFI**) and the average daily feed conversion ratio (**FCR**) in the same period were also analyzed.

Statistical Analysis

The model to study BW35 and BW58 did not consider any covariate. However, ADG, ADFI and FCR were analyzed adjusting or not at 2 Kg of live body weight (**BW**). With this correction, the model represents the standard procedure in Spain, where animals are slaughtered when they reach 2 kg BW. The model with no covariate represents the situation in which animals are slaughtered when they reach a fixed age (58 days in this case).

RESULTS AND DISCUSSION

Regarding the changes in BW across lines (Table 1), the greatest differences were observed at 58 days of life. A lower BW of the GRP and RFI lines was observed with both EF and CF. The differences between GRP and RFI were not significant when using EF, but were significant when using the CF. In terms of response (differences with respect to X line divided by the number of generations), these differences in CF represented a decrease of 15 and 26 g/gen in the GRP and RFI lines, respectively. With EF the decreases were of 24 and 26 g/gen. In relative terms, these generational reductions represent between 0.8% and 1.3% of the BW58 of the X line under each of the feeding systems. The BW58 in the ADGR line did not change with either two feeding systems. Kits from RFI line had a lower BW35 than those from the other two lines and the control population. The difference with respect to X were 83 and 106 g in EF and CF, respectively, these two values must be understood as replicates of the same analysis since the type of feeder did not affect BW35. It represents 1.6% of the average of BW35 in the X line. At 58 days of age, GRP and RFI lines showed significantly less growth than X line, either in animals fed using EF or in animals fed using CF (Table 1). In terms of response, these differences amounted to -0.82 and -0.57 g/d per generation for the GRP and RFI lines respectively, when animals were fed using EF and -0.40 and -0.37 g/d per generation when they were fed using CF. These responses represent about 0.8-1.7% of the control line averages in their respective feeding systems. When growth differences were studied at equal final weight (2 kg), the RFI line did not grow significantly less than the control population under either feeding system. On the contrary, the GRP line did grow significantly less than the control population (-2.0 g/d in total, -0.33 g/d per generation) when

both were fed with the EF, but not when both were fed using the CF. The ADGR line showed no significant differences in growth relative to the control population under any of the feeding systems, neither when compared at constant ages or weights. The highest growth of the ADGR line relative to the other two selected populations seems clear.

Table 1: LSMeans (s.e.) for weight and growth traits of the lines under the two feeding systems

Lines	Electronic				Conventional			
	BW35[1]	BW58[1]	ADG[1]	ADG@2Kg[1]	BW35[1]	BW58[1]	ADG[1]	ADG@2Kg[1]
ADGR	943(16)^{a}	1927(24)^{a}	44.7(0.6)^{a}	46.2(0.4)^{a}	1009(12)^{a}	2155(19)^{a}	52.1(0.4)^{a}	49.2(0.3)^{a}
GRP	903(16)^{a}	1805(25)^{b}	41.0(0.7)^{b}	45.0(0.4)^{b}	976(13)^{a}	2049(20)^{b}	48.8(0.5)^{b}	47.9(0.3)^{b}
RFI	856(16)^{b}	1789(25)^{b}	42.5(0.7)^{b}	46.9(0.4)^{a}	907(14)^{b}	1984(21)^{c}	49.0(0.5)^{b}	49.2(0.3)^{a}
X	939(16)^{a}	1948(25)^{a}	45.9(0.7)^{a}	47.0(0.4)^{a}	1013(21)^{a}	2139(32)^{a}	51.2(0.7)^{a}	48.6(0.4)^{ab}

Values within columns that do not share a superscript are significantly different (p-value ≤ 0.05).
[1]BW35: Body weight at 35d. BW58: Body weight at 58 days. ADG: Average Daily Gain. ADG@2Kg: Average Daily Gain at 2kg weight.

At 58 days of age the differences in ADFI between the GRP and RFI lines and X were significant (Table 2) implying a reduction in consumption of 3.01 and 2.62 g/d per generation, respectively. When these differences were calculated at 2 kg BW they were reduced to 2.05 and 1.53 g/d and only the first one represents a significant difference relative to X. At either constant age or constant weight, the ADGR line showed a numerically intermediate ADFI between X and the lines selected to reduce residual feed intake. However, only the contrasts involving the ADGR line relative to the GRP and RFI lines were those significantly different from zero when compared at constant ages. At constant weight, only those relative to the GRP line reached statistical significance, but it must be noted that the difference in ADFI at constant weight between the ADGR and RFI lines reached a notorious magnitude (6.4 g/d).

Table 2: LSMeans (s.e.) for feed intake and feed conversion ratio of the lines

Lines	Electronic			
	ADFI[1]	ADFI@2Kg[1]	FCR[1]	FCR@2Kg[1]
ADGR	138.1(2.8)^{a}	141.0(2.6)^{ac}	3.16(0.08)^{a}	3.10(0.08)^{a}
GRP	123.7(2.9)^{b}	131.5(2.9)^{b}	3.08(0.08)^{a}	2.91(0.08)^{bc}
RFI	126.1(2.9)^{b}	134.6(2.9)^{bc}	3.01(0.08)^{a}	2.83(0.08)^{b}
X	141.8(2.9)^{a}	143.8(2.7)^{a}	3.10(0.08)^{a}	3.05(0.08)^{ac}

Values within columns that do not share a superscript are significantly different (p-value < 0.05).
[1]ADFI: Average Daily Feed Intake; ADFI@2Kg: Average Daily Feed Intake at 2Kg of body weight; FCR: Feed conversion ratio; FCR@2Kg: Feed conversion ratio at 2Kg of body weight.

The differences in ADFI and ADG between the lines led to some significant differences in FCR when the lines were compared at 2 kg BW, but not when compared at a constant age, although in both situations the trend is similar. At 2 kg BW, the GRP and RFI lines had the lowest FCR, with the differences between X and the RFI line being significantly different from zero. In this case, the response was estimated at approximately 0.04 FCR units per generation, 1.2% per generation of the FCR at 2 kg BW of the control line.
Of the three selection methods proposed to improve FE, those generating a significant response in the desired direction are those that involve reducing residual feed intake, whether the criterion is recorded on an individual basis using the electronic feeder (RFI line), or it is recorded as a cage average and the unit of selection is all the group (GRP line). In both cases, the response in FE seems to be achieved through a clear reduction in ADFI, which seems to be accompanied by a reduction in ADG in the case of the GRP line, and in

the BW of the animals, even from 35 days, in the case of the RFI line. Garreau et al. (2019) reported similar results despite the selection was performed with individually housed rabbits. All these results point to efficiency responses that, by reducing final weight and growth, could have a limited impact on rabbit production. This is to be expected, however, as reducing residual feed intake does not guarantee zero correlated responses in other growth and weight traits. Despite this commercial limitation of the proposed selection processes, the generated material could be of some interest as research material to evaluate other efficiency-related aspects of rabbit production, such as the interaction between different diet types and the efficiency level of the animals.

CONCLUSIONS

The electronic feeding system developed by our team seems to be useful for genetic selection in schemes that aim to reduce FCR. The best selection criterion, with a commercial objective in mind, may not be any of those considered so far in our lines. In this sense, a bi-character index that gives positive weight to growth and negative weight to consumption could give rise to a greater response than that observed in our experiment. In future works, in addition to confirm the preliminary response results described here, we will estimate the genetic correlations between growth and feed intake in animals fed with the eFeederRab, which will allow us to precisely define and evaluate the bi-character index mentioned above.

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THE EVALUATION FOR CARCASS PRODUCTION FROM DIFFERENT GENOTYPES OF COMMERCIAL RABBITS IN THE TROPICAL CONDITION

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ABSTRACT

This study aimed to evaluate carcass production from different genotypes of commercial rabbits in the tropical condition. The 39 14-weeks-old bucks comprised 11, 12, and 16 heads, respectively, for New Zealand White, Hyla, and Hycole used in the study. The average body weight was 2.10 ± 0.18. The slaughtering and carcass dissenctions were carried out following procedures of World Rabbit Science Association. Parameters measured were pre-slaughter weight, hot and cold carcass weight, fat weight, reference weight, carcass percentage, four primal cut percentages (fore part, loin, abdominal, fore leg, and rump + hind), and meat bone ratio. Genotype had significant ($P<0.05$) effects on the carcass, fore part, abominal, fore leg, rump+hind percentage.

Key Words: Carcass, Genotypes, Commercial rabbit

INTRODUCTION

Rabbit (*Oryctolagus cuniculus*) is one of the livestock that has potential as a source of animal protein. The protein content in rabbit meat is 18.7% and has a low-fat content of 6.2% compared to beef fat (18.3%) and lamb (17.5%) (Murti et al., 2020). High protein and low fat make the quality of rabbit meat a healthy meat that encourages the development of rabbit livestock as meat-producing livestock (Margatama et al., 2023). Carcass production and quality are influenced by rabbit breed (Tumova et al., 2014). Therefore, the evaluation of carcass production in rabbits is an effective tool for improvement in commercial rabbits (Szendro et al., 2004).

According to figures compiled by the Food and Agriculture Organization (FAO) of the United Nations (UN), China is by far the largest producer rabbit meat followed by Italy and South Korea. It has only been in recents years that rabbit production has become popular in Asia. In general, rabbit production and consumption are similar (Mc Nitt *et al*., 2013). Most tropical countries permit the breeding of rabbits. However, production performance in this kind of environment ought to be as good as it is in temperate climates, like those found in Europe and China. The majority of rabbits bred in developing countries are produced in backyard or small-scale settings (Lebas, 2005). Common rabbit breeds used as commercial cuts are New Zealand White, Hyla, and Hycole. The advantages of the three rabbit breeds are fast growth and high productivity, making them suitable for commercial meat production. This study aims to evaluate carcass production from different genotypes of commercial rabbits.

MATERIALS AND METHODS

The materials used were thirtynine 14-weeks-old bucks consist of three breed, eleven, twelve, and sixteen heads, respectively, for New Zealand White, Hyla, and Hycole. The average body weight was 2.10 ± 0.18. Rabbits were raised in close house system the average daytime temperature is 26ºC and the average nighttime temperature is 22ºC. Rabbits were fed pelleted diet contained 16% of crude protein, 12% of moisture, 2% of crude fat, 14% of crude fiber, and 0.5% calcium min. Rabbits are given water adlibitum with automatic nipple.

Slaughtering Procedure

The slaughtering and carcass dissenctions were carried out following procedures of Rabbits Science Association (Blasco *et al*., 1993). The rabbits were slaughtered after 12 hours of fasting. Rabbits were cut to sever 3 channels, namely jugular veins, digestive tract and respiratory tract. Each carcass was skinned, digestive and urogenital organs are removed after slaughtering and 8 hours of aging in a showcase at 4ºC. Parameters measured include pre-slaughter weight (kg), hot carcass weight (kg), four primal cut weights (shoulder, ribs, loin and rump), internal organs weight (liver, lungs, kidneys, hearts, stomach and intestines).

Statistical Analysis

The general linear model (GLM) procedure of Statistical Analysis System (SAS) OnDemand for Academics (SAS, 2021) was used to analyzed the data with the linear model as follow:

$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$

Where,

Y_{ij} = observations of dependent variable

μ = general mean in the population

α_i = effect of the i^{th} genotype (NZW, Hyla and Hycole)

ε_{ij} = random error associated with each record

The distribution mapping of carcass characteristic was performed by using Canonical discriminant methods.

RESULTS AND DISCUSSION

Table 1 presents the means and SE of the parameters. Those parameters identified differences between the three breeds. The mean and SE values of the carcass traits of the NZW, Hyla, and Hycole. The mean pre-slaughter weight of NZW was 2.05±0.16 kg, 2.11±0.06 kg for hyla, and 2.08±0.26 kg for hycole. The hot carcass weight of NZW was 1.25±0.12 kg, 1.24±0.06 kg for Hyla, and 1.20±0.16 kg for Hycole. The mean values obtained for pre-slaughter weights in this study were, however higher than those obtained by Rotimi et al. (2021), who reported 1.20±0.02 kg for buck rabbits. NZW rabbits had the highest carcass percentage. The NZW carcass percentage was 52.87±1.55 kg which was lower compared to Rajendran et al. (2020), who studied the carcass percentage of 51.44±0.56 kg. The mean values of various cut-up parts such as the fore part, loin, fore leg, and rump+hind percentage were 24.50±1.88, 10.12±1.68, 17.74±1.05, 14.43±1.37, 37.82±2.04 respectively.

Table 1. Carcass characteristic of rabbits

Parameters	NZW	Hyla	Hycole
Pre-slaughter weight (kg)	2.05±0.16	2.11±0.06	2.08±0.26
Hot carcass weight (kg)	1.25±0.12	1.24±0.06	1.20±0.16
Cold carcass weight (kg)	1.10±0.11	1.07±0.06	1.04±0.14
Fat weight (kg)	0.03±0.01	0.02±0.02	0.02±0.01
Reference weight (kg)	0.99±0.10	0.95±0.05	0.94±0.14
Carcass percentage (%)	$52.87±1.55^{a}$	$50.39±2.10^{b}$	$49.30±2.52^{b}$
Fore part percentage (%)	$24.50±1.88^{a}$	$21.93±1.56^{b}$	$26.75±1.68^{c}$
Loin percentage (%)	10.12±1.68	9.23±0.60	9.76±1.18
Abdominal percentage (%)	$17.74±1.05^{ab}$	$17.23±1.21^{bc}$	$16.11±2.18^{c}$
Fore leg percentage (%)	$14.43±1.37^{a}$	$14.44±1.75^{b}$	$16.35±2.04^{b}$
Rump+hind percentage (%)	37.82±2.04	36.59±4.61	37.60±2.77
Meat bone ratio	7.44±1.39	7.61±1.16	7.84±1.69

Genotype had no significant effects ($P>0.05$) on the pre-slaughter weight, hot carcass weight, cold carcass weight, fat weight, reference weight, loin percentage, and meat-bone ratio. Whereas genotype had significant ($P<0.05$) effects on the carcass, forepart, abdominal, foreleg, and rump+hind percentage. According to Fernandez and Fraga (1996), the age, breed, and feeding circumstances may have an impact on the variations in pre-slaughter and hot carcass weights. Important information about the makeup and distribution of the meat

cuts inside the carcass may be gleaned from the variations in the percentages of the carcass and cut points. Comprehending these percentages can aid in the development of strategies for effective carcass utilization in the meat business, as well as in the optimization of meat processing and utilization.

Figure 1 shows that different genotypes were scattered and not clustered. Based on the scattering diagram, the three genotypes had greater differences in carcass characteristics.

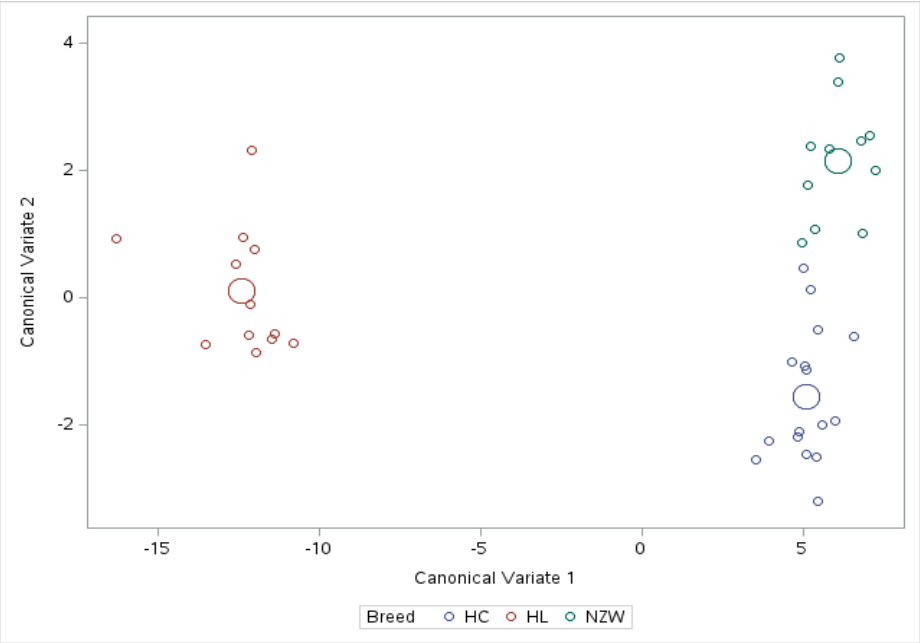

Figure 1: *Scattering diagram for carcass characteristic of NZW, Hyla and Hycole rabbits*

CONCLUSIONS

Hyla, Hycole, and NZW raised in tropical condition were showing the different characteristics of carcass. Carcass percentage, Fore part percentage, Abdominal percentage, and Fore leg percentage showed the greatest contribution as distinguishing factors between genotypes.

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EUROPEAN NETWORK ON LIVESTOCK PHENOMICS (EU-LI-PHE): HOW TO INCLUDE THE RABBIT INTO THE ANIMAL PHENOTYPING SPACE

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ABSTRACT

Phenomics applied to the livestock production sector has one major aim: to systematically describe the animal phenome, referred to as the physical and molecular traits of an animal. EU-LI-PHE constitutes a Europe-centred multidisciplinary, interconnected and inclusive community of experts aiming to boost scientific collaboration, catalyse developments, and transfer livestock phenomics concepts and applications to improve the sustainability and competitiveness of the European livestock industry. The rabbit needs to be included in this context to develop innovations that should benefit rabbit breeding and selection as well as husbandry practices. EU-LI-PHE is focused on i) phenotyping technologies and infrastructures for applications in livestock phenomics, ii) novel approaches and methods for genome to phenome integration in livestock species, iii) computational resources and data analysis methods needed for this big data discipline, iv) the regulatory framework and the societal vision for livestock phenomics and v) the development of a training environment for the benefit of the next generation of researchers in this field. It is expected that EU-LI-PHE will become a reference network on animal phenotyping not only in Europe but also worldwide, contributing to the development of novel solutions for the benefit of the livestock production systems, including the rabbit production sector.

Key words: Animal breeding, Big data, Network, Phenomics, Precision livestock farming

INTRODUCTION

Phenomics is defined as the ensemble of methodologies and technologies for the acquisition, analysis, and exploitation of high-dimensional phenotypic data on an animal-wide scale (Houle et al., 2010). Phenomics is emerging as a major new technical discipline in applied biology, including animal husbandry and breeding. This discipline applied to the livestock production system has one major aim: to systematically describe the phenome, referred to as the physical and molecular traits of an animal.

In the animal production sector, the availability of accurate and specific phenotype data can inform new breeding objectives and related breeding and selection programmes and provide novel essential information for the farmer's daily activities and choices needed to improve reproduction strategies, disease control and welfare of the animals. Therefore, phenomics applied to animal breeding and husbandry is considered an essential innovation to support the sustainability of all animal production systems.

Many different phenotypes can be measured and collected from the animals. Phenotypes can be classified differently according to the level in which they are measured, the type of information that is recorded, the temporal acquisition of the information and the objective of the collected parameters.

Because of the broad heterogeneity in phenotype classes, which also depend on the species, a wide array of scientific approaches and technologies can be used to capture and manage phenotypic information. For example, phenomics can benefit from the development and application of automatic sampling or non-invasive methods to obtain repeated sampling and images, records or continuous data collection (including photographs, videos, sounds, movement traces, etc.) from a part of an animal, the whole individual or a population at different stages, or in derived animal products to describe final external phenotypes with high resolution and in real-time. To capture internal phenotypes, phenomics can also use sequence-based and functional omics technologies to detect and quantify molecular phenotypes (e.g. DNA methylation, RNA transcripts, proteins, metabolites, microbiota, glycomics, etc.).

The broad spectrum of phenotypes and the multiplicity of ways that they can be captured will inevitably produce very large quantities of heterogeneous and complex data, placing phenomics firmly in the realm of data science and "big data". An interesting overlap exists between the development of phenotyping technologies for animal breeding and precision livestock farming (PLF) purposes.

Innovations in these contexts are also needed for the sustainability of the rabbit production sector with the aim to improve both breeding and husbandry practices, considering the peculiarity of the rabbit. New phenotypes and novel methods to measure phenotypic traits should be identified and then applied mainly to support the development and implementation of rabbit selection programs that could speed up the genetic progress for key traits including disease resistance and feed efficiency. Novel monitoring systems and proxy phenotypes to evaluate welfare of the animals can also benefit the rabbit production industry.

The development and the application of phenomics in all livestock species, including the rabbit, clearly requires multi-disciplinary and multi-actor approaches to bring together different expertise, resources, and expectations.

As livestock phenomics requires experts in many fields, with a critical mass of knowledge and technical expertise, we developed a European-based network that has been funded by the European Union. The network is a COST Action (European Network on Livestock Phenomics – EU-LI-PHE - https://www.cost.eu/actions/CA22112/; COST – European Cooperation in Science & Technology, 2023) that has been designed to address four main challenges and structured across activities in four working groups (WGs). This COST Action is funded till 2027.

STRUCTURE AND MISSION OF EU-LI-PHE

The development and application of livestock phenomics tools, methods and the data analytics approaches needed to best leverage high-resolution phenotypic information require substantial investments in terms of time, human resources, and capital investments. The

main challenges in livestock phenomics for research and innovation that are largely unaddressed or, at least for some aspects, only partially addressed, are grouped into four main areas, which reflect the structure in WGs of EU-LI-PHE:

WG1 (Phenotyping technologies), which has the following aims: i) to provide an overview of current phenotyping technologies and infrastructures that can be used for applications in livestock phenomics; and ii) to define a roadmap of the research needs to capture high-dimensional phenotypic information on an animal-wide scale.
WG2 (Genome to phenome integration), which has the following aims: i) to provide an overview of the links between genome/epigenome variation and phenotypic variation at multiple levels in the main livestock species; ii) to identify synergies with related initiatives on functional analyses of livestock genomes; and to iii) to identify knowledge gaps and research needs and provide a road map with a clear trajectory to new applications.
WG3 (Computational resources and methodologies for data analyses), which has the following aims: i) to provide an overview of the computational models, methods and tools available and current and future needs for development of applications in the context of livestock phenomics; and ii) to identify the needed synergies and developments required in terms of cyberinfrastructures and computational capabilities.
WG4 (Economic impact, regulations, policies, and society), which has the following aims: i) to provide an overview on the potential technological and economic impact of livestock phenomics; ii) to summarize the regulatory frameworks around this discipline and evaluate access to information and data generated; and iii) to analyse societal perceptions of livestock phenomics.
In addition to the four main technical areas that provide the to create the scientific backbone of the project, an additional working group, **WG5 (Stakeholder engagement, communication, and dissemination)** is part of the founding pillars of EU-LI-PHE. WG5 links all the activities carried out in WG1-4 as follows: i) to ensure a continuous engagement of the stakeholders; ii) to ensure productive and efficient communications; and iii) to ensure publication of reviews, reports, surveys and establishment of a website and an active social media presence.

EXPECTED OUTCOMES

The specific research and coordination objectives of EU-LI-PHE are focused on: (i) advancing state-of-the-art of high-throughput technologies and protocols required for deep phenotyping to describe phenotypic information at multiple levels in farmed animals; (ii) providing cross-disciplinary knowledge to develop new standards in phenotyping technologies, phenome data descriptors, phenotype ontologies, databases, data structures, storage and sharing; (iii) evaluating available software and bioinformatic tools and defining methods for effective data mining, processing, summarising, integration and visualization of genome/epigenome to phenome data in livestock; (iv) exploring integrative dynamic responses and adaptations of animal phenomes to variable environmental factors; (v) exploring novel data integration and fusion approaches in-cluding omics and sensor data, images, videos and animal movement and sound data for generation and visualisation of complex system models of livestock populations to facilitate prediction of interventions and outcomes; (vi) investigating and proposing new applications for genomic selection and precision livestock farming; (vii) exploring the regulatory landscape around livestock phenomics, including ownership of the data, open access data policies and intellectual property rights; and (viii) analysing stakeholder opinions and societal perceptions of innovations in this field for the reduction of negative impacts on the animals and on the environment.
EU-LI-PHE has specific capacity-building objectives to foster knowledge exchange by: (i) providing well-trained young researchers and professionals in livestock phenomics and related disciplines that complement and complete the background and knowledge needed for the alignment of scientific progress and industry demands; (ii) fostering the exploration and implementation of new training routes and methodologies, with the aim of widening career

prospects for highly specialised researchers who can accumulate integrated skills on different disciplines around big data production and analysis, with an interdisciplinary vision; (iii) stimulating new ideas and innovative methodologies in an open innovation framework to address new opportunities generated by livestock phenomics approaches with a comprehensive strategy of communication and dissemination to attract parallel and synergistic research fields and to benefit the whole scientific community, the relevant industrial sectors and all stakeholders, including policy and decision makers; and (iv) fostering the involvement and collaboration of teams from less research-intensive countries across Europe, promoting their inclusiveness, through the sharing of new knowledge around a network of opportunities focused on livestock phenomics.

CONCLUSIONS

It is expected that EU-LI-PHE will become a reference network on animal phenotyping not only in Europe but also worldwide, contributing to the development of novel solutions for the benefit of the animal production sectors. It is also important to engage the rabbit scientific community to create novel opportunities to boost the sustainability of the rabbit industry.

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INNATE-IMMUNITY GENE EXPRESSION BASED ON *PGR* POLYMORPHISM OF RABBITS IN RELATION TO LITTER SIZE

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ABSTRACT

Aim of present study was to evaluate the effect of different rabbit *PGR* genotype on selected production and reproduction traits (total number of liveborn kits, stillborn kits, pre-weaning, and post-weaning mortality) and analysed expression of selected genes of innate immunity. Rabbit does were of local crossbreed line of New Zealand white x Californian x rabbit of Nitra from three different *PGR* genotype groups (*AA* = 70; *AG* = 70 and *GG* = 70). At the age 77 days total 90 experimental (*AA* = 30; *AG* = 30 and *GG* = 30) growing rabbits from three *PGR* genotypes were slaughtered for sampling of liver for RT2 PCR. The normalized relative expression of selected innate immunity genes (*CD14, CD1A, CD1D, CD209, CD28, IL1A, TLR2, TRAF6*) was determined. Liveborn kits per litter in the group of does with *GG* genotype were significantly higher (P<0.05; 9.98), when compared to genotype *AG* (9.43). Groups of *AA* and *AG* genotypes had significantly (P<0.05) higher number of stillborn kits per litter (0.62 and 0.5 respectively) compared with genotype *GG* (0.34). Genotype *GG* showed higher values (P<0.05) of expression for *CD14, CD1D*, and *TRAF6* gene markers; while *AA* genotype presented higher (P<0.05) values for *CD1A*, *CD209*, and *CD28* when compared with other genotypes. Based on the results of the evaluation of performance parameters, such as vitality and expression of innate immunity genes, we proposed *PGR* as a potential candidate gene in relation to better vitality and more efficient production and reproductive traits of rabbits.

Key words: selection, rabbit breeding, reproductive traits

INTRODUCTION

The present major challenges in the animal breeding are find and identify potential DNA markers for higher vitality and disease resistance and its application in animal selection program (Nicholas, 2005). The innate immune system is an ancient and universal animals' form of defense and has an essential role in protection against infections; nevertheless, it is the first line of host resistance against pathogenic microorganisms. Innate immunity is composed of several components, which include physical barriers, anatomical barriers, phagocyte and epithelial cell enzymes, serum proteins, lectins, antimicrobial peptides, cell receptors, cytokine-releasing cells, and inflammatory mediators. Rabbits with less adaptable genotypes have a predisposition to more sensitivity to diseases and stress, and show a higher degree of variability in litter size (Argente et al. 2014; García et al. 2012). Peiró et al. (2008) described the single nucleotide polymorphism (SNP) in rabbit progesterone receptor gene (*PGR)* promoter, and they found an association between implanted embryos, different litter size and early embryo survival. In our previous study (Ondruška et al. 2020), we found out association between SNP in rabbit gene and different milk production in the first 21 day of lactation. According to our results allele *G* of *PGR* gene can be a potential DNA marker for the rabbit milk production and reduction pre-weaning mortality. Peiró et al. (2007) observed more advanced embryonic stage of development at 48 h in the rabbit population with high litter size. Earlier, the application of stabilizing selection of rabbits on low variability of liveborn kits in the litter leads to better vitality of kits before weaning, and the reproduction performance of the selected females (Ondruska et al. 2021). Aim of present study was to evaluate the effect of different rabbit *PGR* genotype on selected production and reproduction traits (total number of liveborn kits, stillborn kits, pre-weaning, and post-weaning mortality) and analysed expression of selected genes of innate immunity, defence response to bacteria and viruses in relation to different *PGR* genotypes. The contribution of our work consists in

identifying how much individual *PGR* genotypes affect the expression of innate immunity genes.

MATERIALS AND METHODS

Animals, diets and experimental design

The treatment of the animals was approved by institutional ethical committee in accordance with the ethical guidelines presented in Slovak Animal Protection Regulation (RD 377/12).
The trial was performed at the experimental rabbit farm SK U 18021 at the National Agricultural and Food Centre, Nitra. The animals were housed in standard enriched cages and were fed a commercial diet. For the evaluation of reproductive, productive, and growing traits a total number of 210 clinically healthy does and their litters were used. Rabbit does were of local crossbreed line of New Zealand white x Californian x rabbit of Nitra from three different *PGR* genotype groups (*AA* = 70; *AG* = 70 and *GG* = 70). The growing trial period lasted from 1 to 77 days of age. During the experiment, the daily health and mortality of the animals was monitored by a veterinarian.

Molecular Analyses and Genotyping

The buccal swabs for DNA isolation were collected from all analyzed rabbits at the beginning of the trial. For the amplification of *PGR* promoter fragment (558 bp) in this segment were synthesized specific primers according to Peiró et al. (2008) and using gene sequence (GenBank, X06623). For the detection of polymorphisms, PCR-RFLP method with restriction enzyme *Eco31I* was used. The restriction fragments of three different *PGR* genotypes (*AA*=558 bp, *GG*=416+142 bp and *AG*=558+416+142 bp) were obtained.

Evaluation of gene expression

At the age 77 days total 90 experimental (*AA*=30; *AG*=30 and *GG*=30) growing rabbits (both sex equally distributed) from three *PGR* genotypes were slaughtered for sampling of the tissue (liver) for RT2 PCR. Samples were obtained directly after slaughter and flash frozen in liquid nitrogen and storage at -80 °C. Sample preparation and analysis were performed according to the methodological instructions for the RT2PCR kit used in this experiment. RNA was isolated from deep-frozen crushed tissues (liver) with a manual RNA isolation kit (RNeasy Plus Mini Kit). The quality, purity and concentration of RNA were verified by electrophoresis (BIOSTEP, Germany) and spectrophotometry (DS-11 DeNovix, USA). Samples were diluted to the same concentration (ca. 200 ng) and RNA was subsequently transcribed into cDNA using a kit (RT^2 First strand Kit). cDNA samples were applied to a plate (Custom RT2 PCR Array, Qiagen) and analysed by real-time PCR (CFX 96 Real-Time PCR System, Biorad, USA), as a reference gene to normalize gene expression have been used housekeeping gene beta actin (ACTB). Normalized relative expression of selected innate immunity genes (*CD14, CD1a, CD1D, CD209, CD28, IL1A, TLR2, TRAF6*) was evaluated.

Statistical Analysis

All statistical analysis were performed with SPSS 23.0 (SPSS software for Windows, release 23.0., Inc., Chicago, IL, USA). Data on gene expression were analysed with one-way analysis of variance (ANOVA) with genotypes and animal sex as fixed effects. Data were reported as means and pooled SEM. Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

After the application of statistical model, no significant ($P>0.05$) differences were notice based on animal sex, then data are presented and discussed for genotype's effect. In Table 1, data on productive and reproductive traits in different rabbit *PGR* genotypes are reported. Liveborn kits per litter in the group of does with *GG* genotype were significantly higher ($P<0.05$; 9.98), when compared to genotype *AG* (9.43). In the groups of *AA* and *AG* genotypes had significantly ($P<0.05$) higher number of stillborn kits per litter (0.62 and 0.5 respectively) compared with genotype *GG* (0.34). No statistically differences were observed between groups for the number of weaned rabbits. The lowest pre-weaning mortality (42 days old), but not statistically significant, was recorded in the group with the *GG* genotype (12.07%). Peiró et al. (2010) evaluated the association of SNP in the rabbit progesterone

receptor gene with progesterone receptor expression and found the *GG* genotype showed less *PR-B* and *PR-A* expression than the *AA* genotype in the oviduct and uterus. Wang et al. (2009) recorded different genotypes in different breeds of goats in the *PGR* gene. Based on their results indicated, that the *PGR* gene is either a major gene that influences the prolificacy in Jining Gray goats or a molecular marker in close linkage with such a gene. Authors also discussed about the polymorphism of the progesterone receptor gene and its relation to the litter size of Jining Gray goats. Argente et al. (1997) performed an experiment to select animals for uterine size, monitoring the litter size of animals with large and small uterine capacity. They found a correlation between litter size and uterine capacity. Peiró et al. (2008) observed 589 females selected for uterine size for ten generations. They monitored *PGR* gene as a possible candidate gene affecting litter differences and related factors (embryo number and survivability, developmental stage). Ramadan et al. (2020) recorded significantly higher litter size at birth and weaning in heterozygous *AG* genotype in Gabali rabbits. Kotsyubenko et al. (2017) after genotyped 60 individuals of Californian rabbits found the higher frequency of allele *G* (0.35) was in the high litter size rabbits compare with low litter-size rabbits (0.2). El-Aksher et al. (2016) claim that rabbit *PGR* polymorphism in addition to reproductive traits, was also associated with body weight. El-Aksher et al. (2017) genotyped three rabbit breeds (Moshtohor line, V-line, and Gabali) and recorded association the high litter traits with the *GG* genotype in Moshtohor line. Also, our results confirm better reproductive properties *GG* genotype (9.98 liveborn kits/litter) compared with *AG*.

Table 1: Productive and reproductive traits in different rabbit *PGR* genotypes.

Parameter	Female genotype			SEM	p-value[+]
	AA	*AG*	*GG*		
Rabbit does n°	70	70	70		
Liveborn kits/litter (n)	9.88[ab]	9.43[a]	9.98[b]	0.037	*
Stillborn kits/litter (n)	0.62[a]	0.5[a]	0.34[b]	0.018	*
Weaned rabbits (n)	6.92	7.01	7.03	0.008	NS
Pre-weaning mortality (%)	13.78	12.28	12.07	0.120	NS
Post-weaning mortality (%)	16.60	14.07	15.02	0.164	NS

[+]Different letters on the same row means significance at P<0.05

Using RT2-PCR we analysed and evaluated 90 rabbit biological samples of different *PGR* genotypes (*AA, AG, GG*) together. The normalized relative expression of selected innate immunity genes was determined by the ratio of the normalized gene expression. In Table 2, we recorded a statistically significant (P≤0.05) increased expression of rabbit innate immunity gene *CD209* in relation to the expression of samples at *PGR* genotype *AA* (2.78) vs. genotype *AG* (0.94). We found even more significant expression in the *CD1D* and *CD28* genes, with both genes achieving significantly higher (P≤0.01) genotype expression of the *AA* genotype compared to the *AG* genotype and, in the case of the *CD28* gene, also compared with the *GG* genotype (P≤0.05). *CD1D* gene showed the highest values for genotype *GG* (2.11), which were significantly higher (P≤0.05) compared to other two genotypes.

Table 2 Normalized relative expression of innate immunity genes

Gene	Genotype			SE	P-Value
	AA	*AG*	*GG*		
CD14	1.24a	1.18a	1.69b	0.133	0.01623
CD1A	5.32a	1.43b	0.51c	0.719	0.01863
CD1D	1.71a	0.97b	2.11c	0.203	0.00043
CD209	2.78a	0.94b	1.14b	0.299	0.00239
CD28	2.40a	1.25b	1.19b	0.174	0.00022
IL1A	0.89	1.00	0.60	0.194	0.40417
TRL2	1.16	1.26	1.40	0.123	0.36364
TRAF6	1.15a	1.18a	1.63b	0.108	0.00282

TYK2	1.32	1.16	1.17	0.074	0.17379

CONCLUSIONS

In defined populations of rabbits, we performed a complete evaluation of selected production and reproductive indicators and evaluated mortality during the fattening period. Based on our results, it appears that the different PGR genotypes may have effect on expression of innate immunity genes. We recorded the genotype *GG* influenced upregulation of *CD14, CD1D, TRAF6* and downregulation of *CD1A* and *CD28* genes. Based on the results of the evaluation of performance parameters, vitality and expression of innate immunity genes evaluated genotypes, we proposed *PGR* as a potential candidate gene in relation to better vitality and more efficient production and reproductive traits of rabbits.

ACKNOWLEDGEMENTS

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P2-BG1-03

Identification and Differential Expression of mRNA in Summer Testicular Tissue of Rex Rabbits

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Abstract: Previous molecular genetic studies of physiology and spermatogenesis of Rex rabbit testes have focused primarily on a limited number of genes and proteins. To identify additional genes that may play important roles in spermatogenesis regulation, RNA seq technology was used to catalog global gene expression profiles in testes of Rex rabbit with summer versus spring in Rex rabbit testes. We found 4 differential expressed genes for spermatogenesis, which down-regulation significant genes(P≤0.01) in summer versus spring in Rex rabbit testes.KEGG showed that two biological pathways (Ribosome pathway and RAP1 signaling pathway) were also associated with spermatogenesis.

Key Words: Rex rabbit, Summer, Testes, transcriptome profiles,Differentiation

INTRODUCTION

With global warming, the body's exposure to extremely high temperatures will lead to the occurrence of heat stress (Liu et al., 2022a). Heat stress will lead to changes in the number, density and malformation rate of male sperm, resulting in decreased reproductive performance of male animals, and even infertility (Rahman, et al.,2018). Rabbits are homothermal animal with fewer functional sweat glands. At high temperatures, the respiratory rate increases, feed intake decreases, and metabolism disorders in vivo decrease, resulting in decreased production performance (Liu et al.,2022b). When heat stress occurs in animals, mitochondrial apoptosis, germ cell death receptor apoptosis, AMPK and other pathways are affected, thus affecting spermatogenesis and development (Durairajanayagamet al.,2015; Ebeid et al.,2023). In order to further understand the molecular mechanism of heat stress in domestic rabbits, this study used RNA-Seq technology to screen the differential expression pathways of sperm in different seasons, explore the effects of heat stress on the spermatogenesis of male rabbits, and provide theoretical basis for the breeding of heat resistance traits in male rabbits.

MATERIALS AND METHODS

Sample collection and preparation

Six healthy seven-month male Rex rabbit (three male Rex rabbit raised in spring and three male Rex rabbit raised in summer) were selected for sample collection from the Rex rabbit breeding farm of Sichuan in Dayi county, Chengdu city. The testes tissues were quickly collected and immediately placed in liquid nitrogen for the preservation of total RNA.Experiments were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in March 2017) and approved by the Institutional Animal Care and Use Committee in Sichuan Academy of Grassland Sciences, under permit No. CKY-B20230312. Animals were allowed access to feed and water ad libitum under same normal conditions and were humanely sacrificed as necessary to ameliorate suffering.

Extraction of total RNA

Total RNA from the sample was extracted using Trizol reagent®(Invitrogen,USA) kit, the operation steps were carried out according to the operation instructions, and the total RNA were sent to Beijing Novogene company for sequencing.

Library generation and sequencing

Beads with Oligo (dT) were used to isolate poly (A) mRNA from total RNA. The isolated mRNA was fragmented followed by first-strand cDNA synthesis using random hexamer-primers. The second-strand cDNA was synthesized using buffer, dNTPs, RNaseH and DNA polymeraseI. The short cDNA fragments were purified using QiaQuick PCR extraction kit (Qiagen, USA). The fragment ends were repaired and A tailed followed by ligation to

sequencing adaptors. Suitable size fragments were selected following agarose gel electrophoresis and used as templates for PCR amplification. Sequencing of the library was performed using Illumina HiSeq™2000.

Differential expression analysis

Cuffdiff (v2.1.1) provides statistical routines for determining differential expression in digital transcript or gene expression data using a model based on the negative binomial distribution (Trapnell et al., 2010).For biological replicates, transcripts or genes with an P-adjust <0.0 5 were assigned as differential expressed. For non-biological replicates, P-adjust < 0.05 and the absolute value of $\log_2^{\text{Fold change}} < 1$ were set as the threshold for significantly differential expression.

GO and KEGG enrichment analysis

Gene Ontology (GO) enrichment analysis of differential expressed genes were implemented by the GO seq R package, in which gene length bias was corrected. GO terms with corrected *P* value less than 0.05 were considered significantly enriched by differential expressed genes.We used KOBAS software to test the statistical enrichment of differential expression genes in KEGG pathways(http://www.genome.jp/kegg/).

RESULTS AND DISCUSSION

Differentially expressed genes in summer versus spring in Rex rabbit testes

We found 4 differential significant(P≤0.01) down- regulation genes (Table 1) in summer versus spring in Rex rabbit testes.

Table 1 Differentially expressed genes in summer versus spring in Rex rabbit testes

Gene ID	Gene name	Summer	Spring	$\log_2^{\text{fold change}}$	P-value	P-adjusted
ENSOCUG00000028165	Testis expressed 47 (TEX47)	26.944	1915.094	-6.151	1.167×10^{-90}	3.291×10^{-86}
Novel03868	Novel03868	184.763	6064.304	-5.037	1.532×10^{-82}	2.159×10^{-78}
ENSOCUG00000016148	LOC100352202 (uncharacterized protein C6orf201 homolog)	82.988	3045.940	-5.198	1.154×10^{-79}	1.084×10^{-75}
Novel01629	Novel01629	4.853	777.481	-7.324	1.214×10^{-78}	8.557×10^{-75}

Functional classification of the differentially expressed genes

The known genes were also annotated through GO classification analysis and grouped into 3 categories (biological process, 33.33%; cellular component 60%; molecular function, 6.67%) based on their putative functions.

KEGG pathway analysis

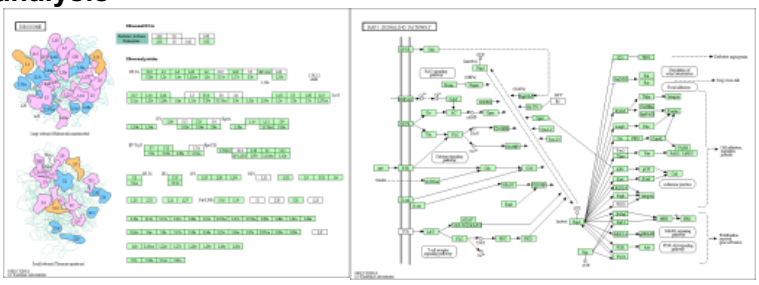

Fig1 Significantly enriched KEGG pathway metabolic pathway map

KEGG showed that two biological pathways (Ribosome pathway and *RAP1* signaling pathway) were associated with spermatogenesis. A total of 145 and 173 different mRNA were found in these two pathways(Figure 1).Yang et al. (2022) found that the marker genes of Sertoli cells and Leydig cells were primarily enriched in the ribosome pathway and related to protein molecular function, which indicates their potential roles in protein synthesis, including many regulatory factors.Rap1 has recently been implicated in the proper differentiation of testicular germ cells and spermatogenesis. Several recent studies have shown the involvement of Rap1in mammalian spermatogenesis (Yang et al.2004a; 2013b).

CONCLUSIONS

We found 4 differential expressed genes for spermatogenesis, which down-regulation significant genes(P≤0.01) in summer versus spring in Rex rabbit testes. KEGG showed that two biological pathways (Ribosome and RAP1 signaling pathway) were also associated with spermatogenesis.

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P2-BG1-06

COMPARATIVE TRANSCRIPTOME PROFILING OF SKELETAL MUSCLE FROM FUJIAN WHITE RABBIT (*ORYCTOLAGUS CUNICULUS*) AT DIFFERENT GROWTH STAGES USING RNA-SEQ

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ABSTRACT

The mechanisms underlying the regulation of skeletal muscle growth and development in rabbits remain unclear. This study aimed to identify candidate genes related to skeletal muscle growth in rabbits and explore their potential regulatory mechanisms. RNA sequencing was performed to compare skeletal muscle transcriptome in Fujian white rabbits at five growth stages with 25 rabbits. A total of 9737 differentially expressed genes (DEGs) acquired from the five groups. KEGG analysis showed that 8249 genes were enriched in 1148 pathways, of which 67 were significantly enriched, mainly in the hypertrophic cardiomyopathy, PPAR signaling pathway, and MAPK signaling pathway. Many of DEGs were well known to be related to growth of skeletal muscle in Fujian white rabbits, such as *MYF5, MYOM2, PGAM2, MSTN*, *MYF6.* These results provide a molecular regulatory mechanism of muscle growth and development in Fujian white rabbits and serve as a theoretical basis for improving the meat performance and growth of meat rabbit breeds.

Key words: skeletal muscle, transcriptome, gene expression, rabbit, growth stage.

INTRODUCTION

Fujian white rabbits are commonly raised in Fujian Province and are native rabbits with a high economic value for meat. However, poor growth performance and low meat production have restricted economic benefits for enterprises related to Fujian white rabbits. In this study, RNA-sequencing (RNA-seq) technology and bioinformatics tools were used to identify major DEGs and their expression pathways in different growth stages of Fujian white rabbits. Candidate genes and key pathways involved in the developmental growth stages of Fujian white rabbits were identified through a comprehensive analysis of DEGs with expression levels that reflected the growth pattern of muscles in Fujian white rabbits. Our findings are useful for understanding the molecular mechanisms regulating the development of skeletal muscle and the pattern of rabbit growth and provide a basis for the subsequent improvement in Chinese native rabbit growth performance.

MATERIALS AND METHODS

Animals and issue Collection

The experimental rabbits used in this study were Fujian White rabbits maintained under a unified management system in the Wuping County Wudong Chatouling Fujian White rabbit ecological breeding farm (Fujian, China). There were 25 rabbits in total, with 5 growth stages (E20 and E26 represent embryos of 20 and 26 days; B1, B30 and B60 represent kids of 1, 30 and 60 days after birth, respectively) and 5 rabbits in each growth stage. The longissimus dorsi were collected and immediately frozen in liquid nitrogen for RNA-seq. Then the other longissimus dorsi muscles of rabbits after birth (1, 30, and 60 days) were fixed in 4% paraformaldehyde for muscle fiber characteristics.

Paraffin sections, staining, and analysis

The longissimus dorsi muscles were embedded in paraffin using a conventional method and these blocks were continuously cut into 5-μm sections. TUNEL and immunofluorescence double staining protocols were used for the sections.

RNA-seq
RNA-seq was performed at Shanghai Majorbio Bio-pharm Technology Co.,Ltd.

Differential expression analysis and Functional enrichment
Differential expression analysis was performed using DESeq2. KEGG pathway analyses were performed using KOBAS (http://kobas.cbi.pku.edu.cn/home.do).

Gene Expression Analysis by qPCR
The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of genes.

Data statistical analyses
Datas were analyzed using a one-way analysis of variance (ANOVA) in SPSS version 21.0.

RESULTS AND DISCUSSION

Muscle Fiber Characteristics of rabbit skeletal at different developmental stages
Double fluorescence sections of the longissimus dorsi at three stages after birth were made for muscle characteristics statistics (Table 1).There were two types (slow-twitch type I and fast-twitch type II) of muscle fibers, mainly type II which accounted for 89.11% at birth, 98.47% at 30 days of age, and 95.74% at 60 days of age. The densities of muscle fiber types I and II decreased significantly with age and the single area of muscle fibers increased significantly.

Table 1: Changes of the muscle fibers in longissimus dorsi of Fujian white rabbits after birth

type	Item	B1	B30	B60	*P*-Value
Ⅰ	Density (N/mm^2)	382.4±56.58	35.43±4.07	26.91±4.20	＜0.001
	single area (10^{-3} mm^2)	0.20±0.02	0.49±0.04	0.98±0.09	＜0.001
	Percentage (%)	10.89±2.78	1.53±0.20	4.26±0.43	0.002
Ⅱ	Density (N/mm^2)	3915.40±466.64	726.69±51.65	394.52±49.12	＜0.001
	single area (10^{-3} mm^2)	0.17±0.02	1.15±0.07	2.06±0.26	＜0.001
	Percentage (%)	89.11±2.78	98.47±0.20	95.74±0.43	0.002

Sequencing Data and DEGs
There were 1,315,508,354 clean reads in total obtained from 25 rabbits, with an average of 52,620,334 clean reads per sample, ranging from 57,143,694 to 43,117,346. The GC content and Q30 were 57.60-54.61% and 95.55-94.44%, respectively. Statistical analysis of DEGs in the longissimus dorsi of Fujian white rabbits at the five stages was shown in Figures 1. There were 9737 genes significantly differentially expressed, with 1372 up-regulated and 1012 down-regulated DEGs in E20 vs. E26, 706 up-regulated and 763 down-regulated in E26 vs. B1, 1808 up-regulated and 2425 down-regulated in B1 vs. B30, and 55 up-regulated and 108 down-regulated in B30 vs. B60. The highest number of DEGs was observed in B1 vs. B30, followed by E20 vs. E26, which means that muscles in rabbits may grow rapidly in the two stages.

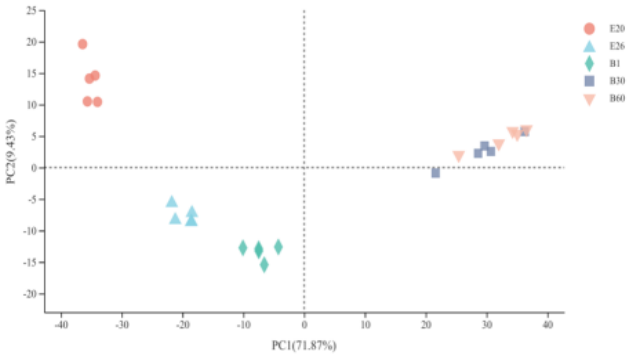

Figure 1: Principal component analysis (PCA) of gene expression of longissimus dorsi in Fujian White rabbits at five different stages

KEGG Analysis

There were 2384 DEGs of Fujian white rabbits were enriched in 331 pathways, of which 26 pathways were significantly enriched, mainly in hypertrophic cardiomyopathy, cardiac muscle contraction, arrhythmogenic right ventricular cardiomyopathy, basal cell carcinoma, adrenergic signaling in cardiomyocytes, and Cushing syndrome in E20 vs. E26. The result was a little similar to the study (Kuang et al., 2019), there were 5320 DEGs between 18 and 26 days of embryonic age in Qixing rabbits obtained and significantly enriched in the cancer, cytoskeleton regulatory, PI3K-AKT signaling, and RAP1 signaling pathways. Compared with the postnatal period, there were more different genes at different days of embryo age. In E26 vs. B1, mainly in the PPAR, cell cycle, fatty acid degradation signaling pathway. In B1 vs. B30, mainly MAPK, cGMP-PKG, and oxytocin signaling pathways. There was no significant difference between the two stages (B30 vs. B60).

qPCR analysis

Five DEGs associated with muscle development were selected for qPCR verification, and the expression levels of myogenic factor 5 (*MYF5*), Myomesin-2 (*MYOM2*), Phosphoglycerate mutase 2 (*PGAM2*), Myostatin (*MSTN*), and Myogenic factor 6 (*MYF6*) in the longissimus dorsi were consistent with the transcriptome sequencing results.

CONCLUSIONS

Transcriptome data at five developmental stages from the fetus to kids were profiled using RNA-Seq technology, which will be helpful for further understanding the molecular sequences and functions of genes related to skeletal muscle growth in Fujian White rabbits at different stages. Differences were observed in the expression of genes, including highly expressed genes, pathways at the growth stages. These findings provide valuable resources for biological research on skeletal muscle growth-related genes in Fujian white rabbits.

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P3-BG2-01

Development and Application of a Whole-Genome Breeding Chip for Meat Rabbits

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Abstract: This research developed a novel 10K liquid-phase breeding chip for meat rabbits that has been created utilising precise positioning sequencing genotyping technology based on target region genomic sequences. This chip can incorporate SNP molecular markers ranging from 1 to 10,350, linked to essential economic traits of meat rabbits, including growth, slaughter, and meat quality characteristics. It serves various purposes such as genetic diversity assessment, germplasm resource identification, kinship determination, whole-genome association analysis of economic traits, and whole-genome selection breeding in meat rabbits. Noteworthy features of this chip include high throughput, extensive interval coverage, high locus detection rate, and flexibility, thereby supporting the molecular breeding of meat rabbits effectively.
Key Words: meat rabbit, SNPs, genetic breeding, liquid-phase gene chip

INTRODUCTION

Compared to other livestock species, rabbit breeding has been limited due to the reliance on traditional methods, resulting in prolonged breeding cycles and higher costs. Fortunately, genomic selection(GS) (Meuwissen et al, 2001)has emerged as a game-changer in the livestock breeding industry(Tan et al., 2017). Leveraging high-density markers across the entire genome, this approach has proven effective in selecting complex quantitative traits and has become a cornerstone in modern breeding practices(Wang et al., 2023). Developing high-throughput and cost-effective SNP genotyping technology is crucial for implementing whole-genome selection breeding in non-model organisms(Ellegren, 2014). Liquid-phase chip technology offers an efficient and adaptable genotyping solution for non-model organisms, characterised by a flexible probe pool design, affordability, and high throughput(Huber et al., 2001).

MATERIALS AND METHODS

Reference population construction and phenotype data collection

The study conducted experiments on Kangda V meat rabbits that were co-bred by Shandong Agricultural University and Qingdao Kangda Rabbit Industry Development Co., Ltd. A reference population of 1,548 meat rabbits was created, and low-depth whole-genome sequencing was performed. The study collected and determined 48 phenotypic traits.

Genome data processing

Ear tissue DNA samples from meat rabbits were extracted using the magnetic bead method. Sequencing library construction was carried out in three batches by three companies, resulting in a total of 4967GB of data with an average sequencing depth of 1.7388X per sample. The data was then processed using Fastp, and a reference sequence index was constructed using BWA software. The sequencing data was aligned to the Kangda meat rabbit reference genome sequence assembled by the research group. SNP calling and genotype filling of the reference population was done using BaseVar+STITCH software(Wang et al., 2022). The genotype data was filtered using Plink/1.9(Purcell et al., 2007) following these criteria: 1) individual detection rate and SNP detection rate less than 0.9; 2) Minor allele frequency (MAF) less than 0.05; 3) SNP Hardy-Weinberg equilibrium test

P value of 1x10−6. After quality control, a total of 1,512 samples with 20,125,019 high-quality SNPs were obtained.

SNP marker selection

The rabbit genome was analyzed using Plink/1.9. A sliding window of 1800 was used to select SNPs. If the LD(Meuwissen et al., 2001) was greater than 0.8, one of the SNPs from each pair was removed, and the window was shifted forward by 500 SNPs.

Phenotypic Data Processing

The measured growth traits, slaughter traits, meat quality traits, and blood component indicators were subjected to normality tests, outliers and missing values were removed, and descriptive statistical analysis was conducted.

Selection of SNP Marker Sites

Whole Genome Association Analysis

Phenotypic data for 48 important economic traits were subjected to whole genome association analysis using the single-trait mixed linear model (MML) in the GMAT software(Wang et al., 2020). To reduce the influence of batches, batch, and gender were set as fixed effects, and the first three principal components from PCA were set as covariance effects to identify loci significantly associated with growth, slaughter, meat quality, and blood routine traits. Selected SNPs from a subset of the rabbit genetic background were evenly distributed across chromosomes.

Design of Locus Probes

The upstream and downstream sequences of the selected SNPs were assessed to design probes for loci that met the criteria, while SNPs that could not be designed with high-quality liquid probes were excluded to obtain a probe pool.

RESULTS AND DISCUSSION

Screening of 10K SNP Markers in Meat Rabbits

A genome-wide association study (GWAS) analysis was conducted using a single-trait mixed linear model. The analysis identified a total of 4,075 SNP loci that showed significant associations with various traits, including growth and development, slaughter, meat quality, and blood components. The Bonferroni method was used for multiple corrections. In the slaughter traits, 241 significant SNPs were found to be related to loin weight, while 47 significant SNPs were related to foreleg weight (Figure 1).

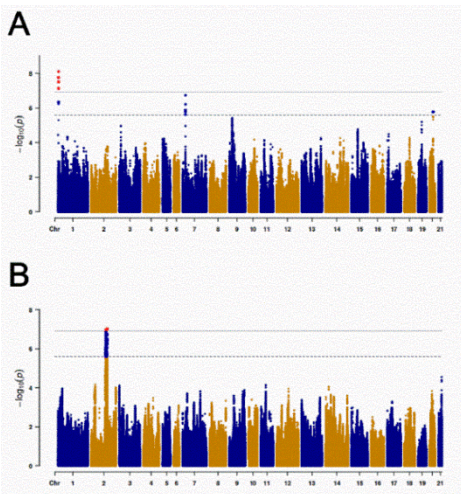

Figure 1: Manhattan plot of traits (A) back loin weight; (B) fore-leg weight. The solid threshold line indicates the genome-wide significance level (−log10(0.05/404,774)), and the dashed threshold line represents the potential genome-wide significance level (−log10(1/404,774)) after Bonferroni correction.

Design and Preparation of Liquid Chip Probes

Based on the probe design principles, the 25bp sequences upstream and downstream of the mentioned sites were evaluated. The standards were set as follows: GC content of 40%-60%, annealing temperature of 55-65^{o}C, up to 4 consecutive bases, and a maximum of 3 variant sites per probe. The final probe pool consisted of 10,350 high-quality SNPs, which were used for the whole-genome breeding chip of meat rabbits (Figure 2).

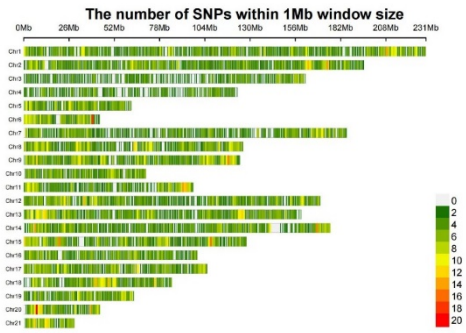

Figure 2: Distribution map of SNP collection on different chromosomes. Different colors represent the number of SNPs in the 1 Mb window.

Liquid-phase Gene Chip Detection

This liquid-phase chip has several advantages, including: (1) It allows for the screening of large rabbit population characteristic sites, with excellent population polymorphism at SNP sites and site information that is both universal and effective. (2) This tool can pinpoint sites that have a significant correlation with important economic traits. This demonstrates that these sites have functional relevance to economic traits. (3) Sites are selected based on the principle of uniform distribution in the genome, with an average spacing of 244.36 kb to ensure the accuracy of breeding value assessment (Figure 3).

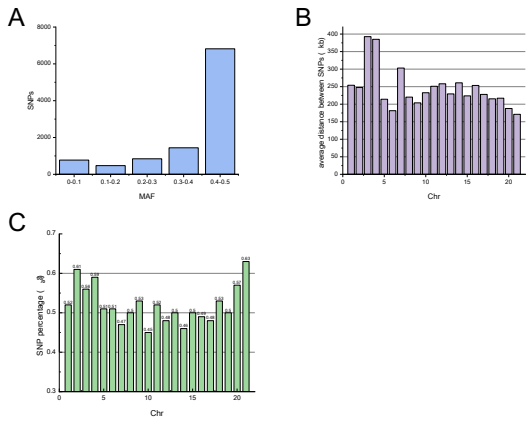

Figure 3: (A) The distribution of minor allele frequency (MAF) in the SNP collection on the chip. (B) The average spacing of SNPs on different chromosomes. (C) The proportion of SNPs on different chromosomes

DISCUSSION

This experiment aims to design and develop a breeding chip for the rapid growth and selection of superior meat rabbits, addressing the lack of breeding chips in the process of breeding superior meat rabbits and compensating for the shortcomings of traditional selective breeding techniques. It provides a low-cost, high-throughput genetic typing technology for the breeding of superior meat rabbits, enabling precise and efficient breeding. This advancement promotes the upgrade of industrial selective breeding technologies to a new stage of whole-genome selection breeding in the rabbit industry, facilitating the healthy and sustainable development of the meat rabbit industry. Additionally, it can serve as a reference for the design and development of breeding chips for other functional pet rabbits.

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P3-BG2-04

Analysis of chromaticity values of yellow angora rabbit hair colour

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Abstract:In a study on the fur color of yellow angora rabbits, 120 yellow angora rabbits were selected, and a TS7020 spectrophotometer was used to measure the fur color of different body parts of the rabbits at various ages. The results showed that the chromaticity of different body parts of yellow angora rabbits changed significantly with age. At 14 days of age, the b* values of the head > back > hind legs > abdomen ($P < 0.01$). The abdomen showed the greatest variation in L*, a*, and b* compared to the other four parts, while the difference between the left hind leg and the right hind leg was not significant ($P > 0.05$). There was a positive correlation between the yellowness of the head and the back of yellow angora rabbits, with rp=0.571** ($p < 0.01$). There was a negative correlation between the L* values and a* values of each part ($P < 0.01$). The L* value of the head showed a highly significant relationship with the b* value ($rp = 0.884$, $p < 0.01$), indicating that the more yellow the fur color of the head, the shinier the surface appearance. **Keywords**: Rabbit Hair Color, colorimeter, phenotypic correlation

INTRODUCTION

In mammals, melatonin controls changes in animal coat color phenotype, is a key hormonal signal and drives changes in multiple neuroendocrine pathways. The light source affects the activity of the melatonin secretion pathway, so that its neurosecretory system can accurately sense changes in the length of light exposure, which results in rhythmic changes in animal coat color at different stages (Bechtold and Loudon, 2007). Genetic factors are known to be decisive for avian skin color and also directly determine color differences between different parts of the body (Janky et al., 1985; Toyomizu et al., 2001). Similarly, the coat color of angora rabbits (colored angora rabbits and colored otter rabbits) is mainly determined by genetic factors, which have a certain effect on the color differences between different parts. Colored angora rabbits have not formed a system from selection and breeding to breeding preservation, and the direct purchase of breeding rabbits in farms has resulted in slow growth of rabbits, miscellaneous breeds, and chaotic management of different coat colors, and no attention is paid to the purification of coat colors and selection and breeding of coat qualities in actual production, and there is no formation of stable coat color strains and a standardized management system (Yang et al, 2019) .At present, the animal hair color, skin color and meat color assessment are mainly: naked eye observation method, colorimetric card comparison method and colorimeter determination(Yang and Chen, 2004; Fu et al., 2006) and so on. Naked-eye observation method and colorimetric card colorimetry requires operators to have certain professional knowledge, is more subjective, and usually requires the collaboration of many people. The colorimeter can be the original CIE stimulus value through the mathematical relationship converted into an easy to understand color values, such as L *, a *, b * table color system, so that objective quantitative indicators can be obtained. Using a colorimeter to determine the color of wool, the wool surface light reflection through the red, green and blue filters, and by the photodetector to measure the three readings, that is, triple stimulus value X (red), Y (green) and Z (blue) values. The triple

stimulus values provide information about the color of the wool under specific illumination viewing conditions, standard light source and field of view (Lv , 2003). Age has a significant effect on the yellowness (b*) of the animal's wool, and the skin yellowness (b*) of the pectoral muscles increased significantly with age, and the yellowness increased with age (Adamski et al., 2016). Other researchers have also found that skin yellowness (b*) increases with age (Połtowicz, 2012; Symeon et al., 2012). In addition, colorimeter determination has the advantages of high data accuracy, objectivity and ease of operation. (Xu et al., 2020)

MATERIALS AND METHODS

The experimental animals were angora rabbits from Shandong Mengyin Yida Rabbit Industry Co., Ltd. A total of 150 yellow-colored offspring with purebred maternal and paternal genotypes from the same batch were selected and reared in cages under uniform feeding conditions during the study. The rabbits were provided with free access to water and feed, and rational lighting was maintained throughout the breeding process. The source of experimental data was reliable, and dedicated personnel were responsible for data collection.

The colorimeter was used to measure the fur color of the head, back, left hind leg, right hind leg, and abdomen of yellow-colored angora rabbits. Prior to measurement, the rabbit fur was gently combed, and the colorimeter was placed lightly on each part of the rabbit. Three measurements were taken at fixed points on each part, and the data was recorded. The chromaticity values of the fur color of the various parts of the angora rabbits were measured at the ages of 14 days, 45 days, 3 months, and 6 months. The same experimental personnel were responsible for data collection at each time poin.

RESULTS AND DISCUSSION

Table 1:The fur chromaticity values of different body parts of the rabbits at 14 days of age

Site	L*	a*	b*
Head	53.05 ± 1.04^{A}	9.88 ± 0.69^{A}	25.51 ± 1.05^{A}
Back	56.88 ± 0.79^{B}	10.42 ± 0.66^{B}	25.05 ± 0.82^{A}
Belly	87.34 ± 1.53^{A}	0.61 ± 0.45^{B}	8.41 ± 1.14^{C}
Left hind leg	66.90 ± 0.78^{B}	6.44 ± 2.29^{B}	21.66 ± 3.70^{B}
Right hind leg	67.17 ± 0.77^{B}	5.66 ± 2.20^{B}	20.60 ± 2.91^{B}

At 14 days of age, the luminance value L* of yellow angora rabbits exhibits the lowest variability. The b* values of different body parts follow the order: head > back > hind legs > abdomen ($P < 0.01$). The variability of L*, a*, and b* values of the abdomen is the highest among the five body parts. Additionally, there is no significant difference between the left hind leg and the right hind leg ($P > 0.05$).

Table 2: The phenotypic correlation of fur chromaticity values across different body parts of rabbits at 14 days of age

Site	L* and a* values	L* and b* values	a* and b* values
Head	-0.863**	0.884**	0.480
Back	-0.724**	-0.074	0.525
Belly	-0.831**	-0.494	0.872**
Left hind leg	-0.903**	-0.830	0.961**

Right hind leg	-0.900**	-0.769	0.944**

The phenotypic correlations between different chromaticity values of the same part in yellow angora rabbits at 14 days of age are presented in Table 8. The L* value was negatively correlated with the a* value of fur color phenotype ($P < 0.01$). The results indicated that fur color with a greener hue appeared brighter. There was a highly significant relationship between the L* value and the b* value of the head ($rp = 0.884$, $p < 0.01$), suggesting that as the fur color of the head became more yellow, the surface appearance became brighter. In contrast, the L* values and the b* value trends were inconsistent in other parts, and there was no significant relationship between them. Only the abdomen and the left and right hind legs showed differences in the a* and b* values, while no significant relationship was observed in other parts.

Table 3: The phenotypic correlation of fur b* values across different body parts of rabbits at 14 days of age

	Head	Back	Belly	Left hind leg	Right hind leg
Head	1	0.571**	0.777	0.268	0.261
Back		1	0.562	0.260	0.283
Belly			1	0.344**	0.288**
Left hind leg				1	0.473**
Right hind leg					1

Table 3 shows the phenotypic correlations of b* values in different parts of yellow angora rabbits. The trend of variation between the left hind leg and the right hind leg is consistent, but the correlation between them is only 0.473. This indicates that the yellowness values are unevenly distributed on the same position of fur in angora rabbits. The fur color of the head is the most prominent trait of interest during on-site selection, with a positive phenotypic correlation between its yellowness and that of the back ($rp=0.571^{**}$, $p<0.01$). However, there were no significant correlations with the yellowness values of the abdomen, left hind leg, and right hind leg.

Table 4: Chromatic values of rabbit hair in different parts of yellow angora rabbits at different ages

Chromatic value	Site	14 days	45 days	3 month	6 month
L*	Head	54.17 ± 1.44^{A}	55.37 ± 3.47^{B}	56.14 ± 2.84^{C}	58.67 ± 2.51^{D}
	Back	57.9 ± 1.94^{A}	69.74 ± 5.33^{B}	74.34 ± 2.50^{C}	82.32 ± 5.64^{D}
	Belly	87.6 ± 1.26	87.65 ± 0.98	88.73 ± 1.53	89.31 ± 2.67
	Left hind leg	67.23 ± 1.35^{A}	74.38 ± 2.31^{B}	80.63 ± 2.24^{C}	$87.31 \pm$

					2.93^{D}
	Right hind leg	68.14 ± 1.14^{A}	75.90 ± 2.66^{B}	81.34 ± 1.02^{C}	87.37 ± 3.61^{D}
	Head	10.67 ± 0.53	10.07 ± 0.52	9.63 ± 0.48	9.27 ± 0.55
	Back	10.49 ± 0.55^{a}	5.00 ± 0.34^{b}	3.57 ± 0.23^{c}	1.55 ± 0.37^{d}
a*	Belly	-0.67 ± 0.44	-0.40 ± 0.21	-0.18 ± 0.20	-0.23±0.51
	Left hind leg	8.81 ± 0.35^{a}	2.86 ± 0.38^{b}	1.77 ± 1.22^{c}	0.38 ± 0.44^{d}
	Right hind leg	8.98 ± 0.29^{a}	3.01 ± 0.26^{b}	1.91 ± 0.96^{c}	0.39 ± 0.30^{d}
	Head	25.55 ± 0.84^{A}	25.16 ± 0.78^{B}	24.79 ± 0.92^{C}	22.89 ± 1.59^{D}
	Back	25.39 ± 2.80^{A}	15.63 ± 3.84^{B}	12.64 ± 3.45^{C}	10.42 ± 2.65^{D}
b*	Belly	8.72 ± 1.87	8.47 ± 1.40	6.66 ± 1.00	5.97 ± 1.69
	Left hind leg	22.45 ± 1.04^{A}	11.03 ± 0.59^{B}	8.74 ± 1.76^{C}	6.53 ± 1.41^{D}
	Right hind leg	22.01 ± 0.97^{A}	11.23 ± 0.72^{B}	8.81 ± 1.17^{C}	6.57 ± 1.03^{D}

By comparison, it was found that the L* values at 14 days, 45 days, 3 months, and 6 months of age all showed a significant increase ($P < 0.01$). Except for the head and abdomen, there were significant differences in the a* values of other parts ($P < 0.05$), while the b* values showed a highly significant decrease ($P < 0.01$). Additionally, the standard deviation of b* values between the left hind leg and the right hind leg differed greatly, indicating a high degree of dispersion of this indicator within the population. The L*, a*, and b* values of the abdomen showed no significant variation with increasing age and therefore cannot be used as a distinguishing feature for different age groups.

DISCUSSION

The results of this study indicate that at 14 days of age, the variability of L*, a*, and b* values in the head, back, left hind leg, and right hind leg of yellow rabbits is relatively low. The order of b* values for each body part is: head > back > hind legs > abdomen ($P < 0.01$). There is a negative correlation between the luminance value L* and the redness value a* of the fur in the head, back, abdomen, left hind leg, and right hind leg ($P < 0.01$). A significant difference exists between the L* and b* values in the head, while other parts show no significant correlation. The a* and b* values of the abdomen, left hind leg, and right hind leg exhibit a highly significant correlation ($P < 0.01$), while the other parts show no significant correlation ($P > 0.05$). With increasing age, the L* value significantly increases across different body parts ($P < 0.01$), while the a* and b* values decrease significantly. A colorimeter can be used to focus on measuring the color of the fur on the head of angora rabbits. The color of the fur on the head can be considered the highest priority phenotype for on-site color determination and selective breeding. Conversely, changes in the abdomen are not significant ($P > 0.05$) and therefore cannot be used as a basis for fur color determination.

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P3-BG2-05

STUDY OF SOME REPRODUCTIVE TRAITS IN HUNGARIAN GIANT RABBIT DOES

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ABSTRACT

The reproductive capacity of the native Hungarian Giant (HG) rabbit does is still less known. There are few studies with more data or considering the environmental effects. We aimed to characterize and assess the performance of our nucleus HG rabbit does mated purebred and extensively between 2020 and 2022 (n=186), according to the season, parity and number of teats. Kindling rate averaged 76% that was higher in autumn (85%) and the highest at the 2nd parity (P<0.05). At kindling, doe live weight was 6808±218 g and the number of live born kits was 10.5±0.63. At lactation week 3, the rabbit does weighed 7107±179 g. At weaning (week 8) the litter size was 7.89±0.48 and it was higher (P=0.007) at the 2nd than more parities. Body weight of the weaned kits averaged 1888±119 g. Among the rabbit does 88% had agouti coat color and 52% were multiparous (≥3). The number of teats was counted in the does and in 594 newborn kits. Compared to the rabbit does (77, 20 and 3%), a positive change (P<0.001) was found because the proportion of the kits with 8, 9 and 10 teat were 56, 31 and 13%, respectively. In summary, our results can serve as a reference for reproductive capacity of this breed. However, more data and detailed analyses with interactions are needed. Less is known on the does' body condition and its changes that can be used to explain the results.

Key words: Fertility, Doe and litter performance, Season, Parity, Number of teats

INTRODUCTION

Earlier we reported the forming of our small Hungarian Giant (HG) rabbit nucleus population for gene conservation and the first results with deep litter and cage housing (Eiben *et al*., 2021). The economic parameters of this native breed are less explored. There are few studies with enough number of rabbits and respecting the environmental effects, and so, reliable results. The productive and some morphological data are continuously collected. The expanding database offers an increasingly better opportunity to characterize our rabbits and study their performance.

This study aimed to investigate the reproductive and rearing traits of the rabbit does naturally mated between 2020 and 2022, according to the season, parity order and number of teats.

MATERIALS AND METHODS

Breeding

Female rabbits were first time mated after 6 months of age and extensively re-mated, i.e. after weaning of their 8 weeks old kits. Rabbit does were moved, the kits stayed together until 9 or 10 weeks of age. At kindling, if possible, from 10-kit larger litters, small newborns having different skin color from those of the adopting does' were fostered. The number of teats was counted at birth in the newborns and at lactation week 3 in the does. In 2020 after summer rest the breeding re-started at the end of August but in 2021 and 2022 two weeks later in September. A total of 186 mating were performed, i.e. 67 in 2020, 61 in 2021 and 58

in 2022. Rabbits having 3 unsuccessful mating or bad condition were excluded from breeding and their data from analysis.

Housing and Feeding

Rabbit does were kept in enriched wire mesh cages (95 x 116 x 70 cm) equipped with a plastic mat and an external nest box (64 x 31 x 38 cm). In the building with natural light through small windows, the daily lighting was 16 hours. Ambient temperature varied between 20-28°C in summer and 8-15°C in winter. Pelleted feed (9.91 MJ/kg digestible energy, 16.5% crude protein, 2.3% crude fat, 15.8% crude fiber), grass hay and drinking water were provided *ad libitum*. In summer, apples or carrot roots were also offered.

Data Collection and Statistical Analysis

Kindling and mortality rates and the distribution of rabbits according to number of teats, were evaluated by Chi-square test. Doe and kit body weights were individually measured or calculated (litter weight/number of kits). The effects of the season, parity order and number of teats on litter size and kit weight were analyzed by ANOVA without interaction (still not enough data) using the Statgraphics 6.0 (1992) statistical software. Total litter size at birth affected (P<0.005) calculated kit weight at weaning, so it was included as a covariate.

RESULTS AND DISCUSSION

Characterization of the rabbit does

At mating, we recorded the coat color, the number of parity and the number of teats. 88% of the rabbit does were agouti, 52% were multiparous (≥3 kindling) and 77% had eight teats (Table 1).

Table 1: Distribution of rabbit does according to coat color, parity order and number of teats

	Coat color				Parity order			Number of teats		
	Agouti	Steel	Black	Yellow	1	2	≥3	8	9	10
Rabbit does (n)	149	11	8	1	42	36	86	110	28	5
(%)	88.2	6.51	4.73	0.59	25.6	21.9	52.4	76.9	19.6	3.50

The average body weight (BW) of the rabbit does (n=106) after kindling was 6808±218 g. It was not affected by the season or parity but rabbits with 9 teats weighed less (P=0.001) than those with 8 teats (Table 2). At lactation week 3, the average BW was 7107±179 g (n=100) and it was lower in spring (P<0.05) and in rabbits with 9 teats than with 8 teats (P<0.05). At lactation week 5, BW was measured only in 2020 (n=18) and it was 7700±476 g on average. More data is needed for a correct assessment of BW. It would also be useful to know the breeding weight and individual weight changes.

Table 2: Body weight (BW) of the rabbit does according to the season, parity and teat number

	Season				Parity order			Teat number		
	Spring	Summer	Autumn	Winter	1	2	≥3	8	9	10
Rabbit does (n)	30	3	40	33	27	29	50	84	19	3
BW after kindling (g)	6679	6511	7148	6895	6528	6981	6915	6781^{b}	6052^{a}	7591^{b}
BW at lactation wk 3 (g)	6861^{a}	6793^{a}	7375^{b}	7401^{b}	6927	7216	7180	7145^{b}	6622^{a}	7555^{ab}

Means with different letters in the same row per trait differ significantly (P<0.05).

Kindling rate

Kindling rate was similar, 80% (47/59) in 2020, 76% (44/58) in 2021 and 73% (38/52) in 2022 resulting in an average of 76% (129/169). Season and parity affected (P<0.05) kindling rate (Table 3). The best result was obtained with rabbits that kindled for the second time (92%; 33/36), In autumn, after summer rest, kindling rate was above average. Doe body weight at mating was not measured that could partly explain the differences found.

Table 3: Kindling rate depending on the season and parity order

	Season				Parity order		
	Spring	Summer	Autumn	Winter	1	2	≥3
Rabbit does (n)	41	9	60	54	42	36	86
Kindling rate (%)	65.9^{ab}	33.3^{a}	85.0^{c}	77.8^{bc}	76.2^{ab}	91.7^{b}	67.4^{a}

Percentages marked with different letters are significantly different (P<0.05).

Litter size and litter weight

The average number of kits born alive was 10.5±0.63 (n=105) and it was affected by each factor (Table 4). At lactation week 3 the average litter weight was 2651±172 g. It was higher (P=0.009) in winter than in autumn. At weaning (week 8), the average litter size was 7.89±0.48 (n=94) and it was higher (P=0.007) in the secondiparous rabbits than in the multiparous does. Kits born in autumn or winter had higher 8 weeks weaning weight than kits born in spring (Table 4).

Table 4: Litter traits depending on the season of birth, does' parity and teat number

	Season				Parity order			Teat number		
	Spring	Summer	Autumn	Winter	1	2	≥3	8	9	10
Litters (n)	29	3	40	33	27	29	49	83	19	3
Born alive per litter (n)	10.3^{b}	12.7^{b}	8.96^{a}	9.19^{ab}	10.7^{ab}	11.1^{b}	9.57^{a}	8.99^{a}	10.2^{ab}	12.2^{b}
Raised per litter (n)	8.29	9.62	7.97	7.98	8.17^{a}	9.33^{b}	7.90^{a}	8.00	7.83	9.57
Litter size at wk 3 (n)	8.15	8.75	7.46	7.65	7.86^{a}	8.84^{b}	7.31^{a}	7.44	7.20	9.38
Litter size at wk 8 (n)	7.84	6.56	7.37	7.33	7.11^{ab}	8.08^{b}	6.62^{a}	6.82	6.53	8.46
Litter weight at wk 3 (g)	2892^{bc}	1760^{a}	2810^{b}	3144^{c}	2475	2898	2580	2551	2351	3052
Kit weight at wk 3 (g)	282.7^{b}	227.9^{a}	394.4^{b}	433.6^{b}	349.2	346.8	383.0	364.1	379.7	335.1
Kit weight at wk 8 (g)	1904^{b}	1298^{a}	2144^{c}	2207^{c}	1830	1866	1967	1777	1762	2125
Kit mortality, 1-8 wk (%)	8.73^{a}	26.9^{b}	8.21^{a}	6.44^{a}	9.79	6.64	9.12	8.15	7.19	4.17

Means with different letters on the same row per trait differ significantly (P<0.05).

Kit weight and mortality

The 1-8-week kit mortality was 6.4-8.7% from autumn to summer (Table 4).

Number of teats in offspring

Compared to the rabbit does (Table 1) a favorable change (P<0.001) was detected in the progeny. The proportion of newborn kits with 8 teats was only 56%, while those with 9 and 10 teat were 31% and 13%, respectively.

Our results confirm that for local rabbit breeds that are generally less selected for reproductive traits as opposed to the hybrids, it is also recommended to consider the effect of the season and parity (Tůma *et al*., 2010; Lazzaroni *et al*., 2012; Dalle-Zotte and Paci, 2013) for the purpose of reliable breed characterization (Belabbas *et al*., 2021) and/or economic utilization (Kowalska and Bielanski, 2011; Savietto *et al*., 2021ab). The number of teats is a useful morphological mark, it helps correct fostering. In meat rabbits, it has an indirect effect on the performance of the rabbit does (Bovo *et al*., 2021). Morphometric characterization, reproductive traits descriptions and breeder's management protocols definition are all important in gene conservation programs determination and rabbit biodiversity protection through reproductive efficiency improvement (Marelli *et al*., 2023).

CONCLUSIONS

Our results can serve as a reference for reproductive performance of the Hungarian Giant rabbit does. However, a larger database and detailed evaluation including interactions.is

suggested for a reliable characterization of this breed. The performance of rabbit does is related to their condition and its changes. It is also advised to evaluate doe weight changes during the breeding cycle to help explaining the results.

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P3-BG2-06

Enhancing Rabbit Production in Mexico: Evaluation of the FESC Line and Crossbreeding

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ABSTRACT

Developed 30 years ago to address Mexico's import restrictions (semen, embryos, meat, etc.), due to its disease-free rabbit status at that point, the FESC genetic line emerged as a valuable domestic alternative. This study evaluated FESC's performance against its parental breeds: New Zealand, California, and Chinchilla. Additionally, crosses between FESC males and these breeds were assessed to understand their hybrid vigor and potential benefits for Mexican rabbit producers. The results are promising. FESC boasts superior fertility 89.44%, increased litter size 9.74 live births, 8.63 weaned rabbits, and impressive weaning weight (667.87 g at 35 days), exceeding values reported by Chino and Gutierrez (2022). Notably, crosses of FESC with the original breeds displayed even greater hybrid advantages. The FESC-Chinchilla cross achieved the highest weaning weight 792.93 g at 35 days, surpassing New Zealand 773.75 g and California 677.34 g. In conclusion, the FESC line demonstrates clear advantages over its parent breeds. Furthermore, the FESC-Chinchilla cross emerges as the most promising option, offering both exceptional weaning weight and high fertility 88.9%. These findings suggest that the FESC line holds significant potential for enhancing the productivity of Mexican rabbit farms.

KEYWORDS: Rabbits, litter size, weaning weight, FESC line.

INTRODUCTION

In the Rabbit Breeding Module of the Center for Agricultural Education of the Faculty of Superior Studies Cuautitlan (FESC), of the National Autonomous University of Mexico (UNAM). Genetic research was carried out to obtain a meat-producing line.

The FESC genetic line is the result of a four-year (1994-1998) breeding program that began with a base population of 221 rabbits (Gallegos, 2011). This foundation lineage consisted of New Zealand White, California, and Chinchilla breeds. Over five selection cycles, researchers focused on improving litter size, birth weight, weaning success, weaning weight, weight at 70 days, and feed conversion (Franco, 2009). Each cycle involved mass selection at 70 days, followed by random mating at 3.5 kg with a 4:1 female-to-male ratio. Progeny were evaluated in a single production cycle. By the fifth cycle, rabbits reached a mature weight of 2.5 kg (Zamora, 1999). By 2000, the program achieved an average weight gain of 300 grams per animal (Boletín UNAM, 2000).

This study aims to compare the performance of FESC rabbits with their parent breeds and offspring from crosses with those breeds. This evaluation will guide future efforts to maintain and improve the FESC line.

MATERIALS AND METHODS

This research was conducted at the Rabbit Breeding Module of the Center for Agricultural Education of the Faculty of Superior Studies Cuautitlan (FESC), of the National Autonomous University of Mexico (UNAM). The study period spanned from July 2022 to September 2023 and involved analyzing data from 388 producer rabbits. All rabbits were housed under identical conditions in multi-purpose Flat-Deck cages (40 cm x 85 cm x 33 cm). A semi-intensive management system was employed, with rabbits fed commercial concentrate and provided water ad libitum. Simple crosses were performed as outlined in Table 1. Fertility, number of live births, birth weight, number of weaned rabbits, and weaning weight were evaluated using analysis of variance (ANOVA) with Minitab statistical software.

Table 1: Crossings

Male	Female	Group	N
New Zealand	New Zelanda	1	92
California	California	2	90
Chinchilla	Chinchilla	3	72
FESC line	FESC line	4	79
New Zealand	FESC line	5	18
California	FESC line	6	18
Chinchilla	FESC line	7	19

Description of the performed crossbreeding

RESULTS AND DISCUSSION

The study analyzed data from 388 female rabbits across the seven groups outlined in Table 1. Fertility was the first parameter evaluated. The FESC line emerged as the clear leader, boasting an impressive 89% fertility rate. Groups 1 (New Zealand breed) and 7 (FESC males x Chinchilla females) followed closely with 88.57% and 88.89% fertility, respectively. Group 2 (California breed) displayed the lowest fertility at 80%, as shown in Figure 1.

These findings hold significant value for rabbit breeding programs, providing valuable insights for optimizing selection strategies.

Moving on to the number of live born rabbits (LBR), Group 4 (FESC line) achieved the highest average, producing an impressive 9.7 LBR per litter ($p < 0.0001$). Group 6 (FESC males x California females) had the lowest LBR at 7.5. However, it's noteworthy that all other groups exceeded 8 LBR per litter, as detailed in Figure 2.

Figure 1: Fertility percentage in evaluated groups.

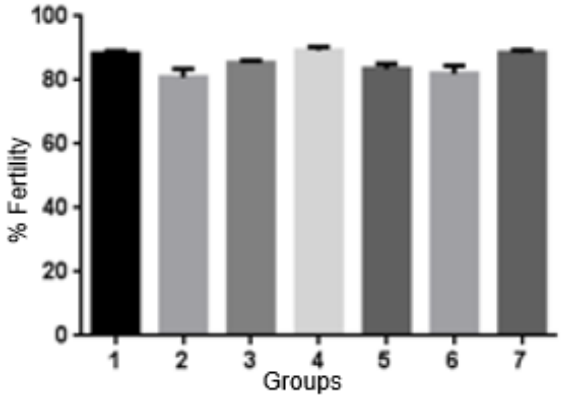


Figure 2: Number of live born rabbits in studied groups. *(P<0.0001).

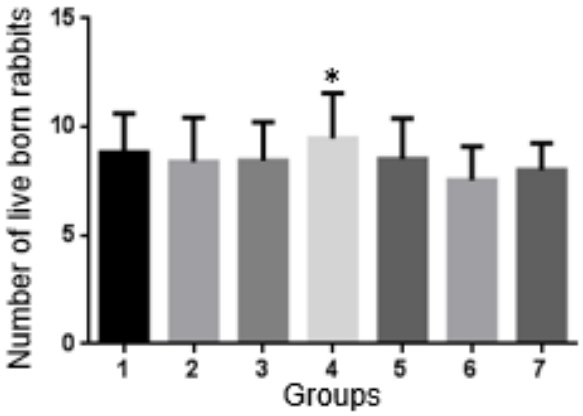
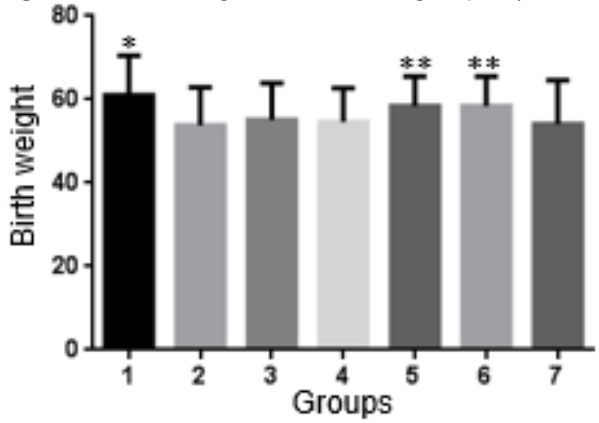


Figure 3: Birth weight in evaluated groups *(P< 0.0001), **(P<0.001).



Birth weight analysis revealed that Group 1 (New Zealand breed) had the highest average weight at 61.02 grams ($p < 0.0001$). Groups 5 and 6 (crosses involving California females) were statistically tied for second place with an average weight of 58.41 grams ($p < 0.001$). Group 3 followed with 55.07 grams, then Group 4 (FESC line) at 54.62 grams. Groups 7 (FESC males x Chinchilla females) and 2 (California breed) had the lowest birth weights at 54.09 grams and 53.85 grams, respectively Figure 3.

Moving on to weaning success, the FESC line Group 4, once again shone, achieving the highest average number of weaned rabbits (WR) at 8.63 ($p < 0.0001$). The New Zealand breed Group 1, followed closely with 7.94 WR. Group 7 (FESC males x Chinchilla females) secured the third position with 7.75 WR, followed by Group 5 (another FESC cross) at 7.5 WR. Groups 3, 2, and 6 achieved weaning averages of 7.41 WR, 7.35 WR, and 7.0 WR, respectively Figure 4.

Figure 4: Number of weaned rabbits per evaluated group *(P<0.0001)

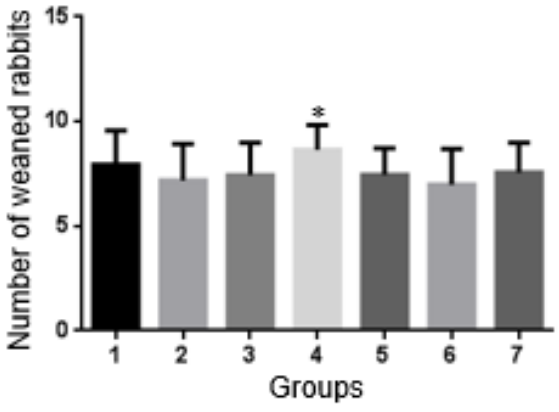
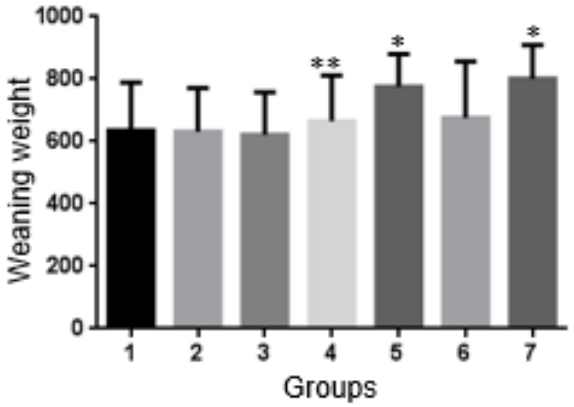


Figure 5: Average weaning weight in the evaluated groups *(P<0.0001), **(P<0.001).



The analysis of weaning weight at 35 days old revealed a fascinating trend. Groups resulting from crosses, Groups 7, 5, and 6, took the spotlight. The cross of FESC line males with Chinchilla females, Group, achieved the highest weaning weight at a remarkable 792.93 g ($p < 0.0001$), as illustrated in Figure 5. This exceptional outcome can be attributed to the hybrid vigor of this cross.

The FESC line itself, Group 4, displayed a very respectable average weaning weight of 667.87 grams ($p < 0.001$), exceeding the values previously reported by Chino and Gutiérrez (2022). Groups 2 (California breed) and 1 (New Zealand breed) followed with averages of 635.14 grams and 635.35 grams, respectively. Group 3 (California x FESC) rounded out the results with an average of 620.17 grams.

This study also explored the potential of crossing FESC line males with the common Mexican meat breeds (New Zealand, California, and Chinchilla). While the sample size is still too small for conclusive results, the FESC-Chinchilla cross emerged as particularly promising. This cross not only achieved the highest weaning weight (792.93 g) but also demonstrated good performance in litter size 7.75 weaned rabbits, birth weight 54.09 g, live born rabbits 8 LBR, and fertility 88.89%. These combined attributes make it a standout candidate for further research.

CONCLUSIONS

This study confirms the continued strength of the FESC line. Compared to its parent breeds (New Zealand, California, and Chinchilla), FESC rabbits exhibit superior performance in fertility, litter size, and weaning success. Notably, the FESC line even surpasses previously reported weaning weights (Chino and Gutiérrez, 2022). These findings solidify FESC as a valuable alternative for Mexican rabbit producers.

Furthermore, the initial evaluation of crosses between FESC males and these same breeds is encouraging. The FESC - Chinchilla cross, demonstrates exceptional potential with its impressive weaning weight and overall productive parameters. While further research is needed to solidify these results, the early signs suggest that incorporating FESC genetics can significantly benefit Mexican rabbit breeding programs.

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P3-BG2-07

Comparative Weaning Performance in Rabbits: FESC Line vs. Parent Breeds

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ABSTRACT

This study aims to explore weight gain during different development stages in litters from four rabbit groups: New Zealand White, California, Chinchilla, and the FESC genetic line. The FESC line was developed 30 years ago as a response to Mexico's import restrictions due to its disease-free rabbit status. These restrictions prohibit the import of live animals, including semen, embryos, and meat. In this study, we analyzed data from a total of 91 litters born between June and December 2023.

The findings of this study are highly promising. At 70 days of age, the FESC line (Group 4) exhibited a significantly higher weight (2.02 ± 0.03 kg) compared to the other three breeds. This result highlights the FESC line as a favorable alternative for rabbit producers in Mexico.

KEY WORDS: rabbits, FESC line, weaning weight, 70 days weight.

INTRODUCTION

According to Baselga (2004), a comprehensive genetic improvement program for meat rabbits is essential to meet the requirements of rabbit meat producers in a specific region or country.

In Mexico, the first significant endeavor to establish such a program took place in 1976 at the Centro Nacional Cunícola de Irapuato, with assistance from the INRA. Currently, the Colegio de Postgraduados and the Facultad de Estudios Superiores Cuautitlán (UNAM, México) are actively involved in the development and selection of rabbit lines (Baselga, 2004).

The FESC line, developed three decades ago, is the result of a targeted breeding program that capitalized on existing rabbit lineage in Mexico. The program aimed to enhance reproductive parameters in a population derived from the New Zealand White, California, and Chinchilla breeds. Through five selection cycles, researchers focused on improving important traits such as litter size (averaging 7.82 live born rabbits per litter), birth weight (68.48 g), weaning weight (846.16 g), and weight at 70 days (2,017.80 g) (Zamora, 1999).

In recent years, we have undertaken a comprehensive reassessment of the FESC line to evaluate its continued effectiveness and identify areas for potential enhancement. Currently, the FESC line demonstrates a fertility rate of 88.7%, resulting in an average litter size of 9 live born rabbits. Weaning success is also noteworthy, with an average of 8.65 rabbits weaned at a weight of 596.54 g (Chino and Gutierrez, 2022).

This study goes beyond evaluating the FESC line alone by assessing its parent breeds (New Zealand, California, and Chinchilla), as well as the offspring resulting from crosses between these breeds and the FESC line. Various parameters were analyzed, including the number of

live and stillborn rabbits, average birth weight, number and weight of weaned rabbits, and weight at 70 days of age.

MATERIALS AND METHODS

The research described in this study was carried out at the Rabbit Breeding Module, located within the Center for Agricultural Education at the Faculty of Higher Studies Cuautitlan (FESC), National Autonomous University of Mexico (UNAM). The study was conducted over a period of six months, from June to December 2023, and involved the evaluation of 91 litters. All rabbits were housed and provided with identical feeding conditions. Simple crosses were performed according to the details outlined in Table 1.

The study examined several variables, including the following:

- Number of live and stillborn rabbits
- Average birth weight
- Number of weaned rabbits
- Average weaning weight
- Number of rabbits at 70 days
- Weight at 70 days

Data analysis was conducted using a linear t-student model, with a fixed effect considered for the genetic group. The SAS program's generalized linear model (GLM) function was employed for this analysis.

Table 1: Crossings

Group	Male	Female
1	New Zealand	New Zealand
2	California	California
3	Chinchilla	Chinchilla
4	FESC line	FESC line

RESULTS AND DISCUSSION

This study involved the analysis of data from 91 litters, categorized into four groups as described in Table 1. The primary focus of the analysis was on the number of live born rabbits (LBR). Group 1 demonstrated the highest average LBR at 9.3, closely followed by Groups 3 and 4 (FESC line) with 8.86 and 8.80 LBR, respectively. Group 2 displayed the lowest average LBR at 8.21 (Table 2).

However, a concerning trend emerged when comparing the current LBR of the FESC line (8.80) to previous reports by Chino and Gutierrez (2022). Over the past two years, the LBR of the FESC line has declined from 9.9 in 2020 to 9.0 in 2021. These findings underscore the need for further investigation and potential improvement strategies.

The rates of stillborn rabbits were found to be statistically insignificant across all groups, consistently remaining at low levels. Group 1 had the highest stillborn rate, but it was merely 0.5%.

Moving on to birth weight, Group 4 (FESC line) achieved the highest average at 59.29 g. This represents a slight improvement over the value reported by Chino and Gutierrez (2022) at 56 g. However, it falls short of the birth weight reported by Zamora in 1999 (68.48 g). Groups 2, 1, and 3 followed with decreasing average birth weights of 58.93 g, 57.27 g, and 55.27 g, respectively.

Regarding the number of weaned rabbits (NWR), Group 1 (New Zealand breed) led with an average of 8.2 weaned rabbits. Group 4 (FESC line) closely followed with 8.16 weaned rabbits, while the remaining groups achieved a maximum of 7 weaned rabbits.

The analysis of average weaning weight revealed positive outcomes for the FESC line (Group 4). The average weight of 780.32 g represents an improvement over previous reports by Chino and Gutierrez (2022) at 645.6 g and Franco (2009) at 735 g. However, it is important to note that this value is 67 g lower than the 848.16 g reported by Zamora in 1999.

Table 2: Mean traits performance values

Group	Observations	Live births	Deadborn	Birth Weight (g)	Number of weaned	weaning weight (g)	rabbits at 70 days	weight at 70 days (kg)
1	19	9.31 ± 0.49[a]	0.31 ± 0.17[a]	57.27 ± 1.91[a]	8.21 ± 0.35[a]	713.81 ± 29.14[ab]	6.68 ± 0.35[a]	1.93 ± 0.04[ab]
2	19	8.21 ± 0.49[a]	0.21 ± 0.17[a]	58.93 ± 1.91[a]	7.63 ± 0.35[a]	753.53 ± 29.14[b]	6.89 ± 0.35[a]	1.83 ± 0.04[b]
3	23	8.86 ± 0.44[a]	0.34 ± 0.15[a]	55.27 ± 1.74[a]	7.34 ± 0.32[a]	676.15 ± 26.49[a]	6.47± 0.32[ab]	1.91 ± 0.03[b]
4	30	8.80 ± 0.39[a]	0.10 ± 0.13[a]	59.29 ± 1.52[a]	8.16 ± 0.28[a]	780.32 ± 23.19[b]	7.36 ± 0.28[b]	2.02 ± 0.03[a]

[a, b,] Means with different literals per characteristic are different (P<0.05)

Our analysis of the number of animals remaining at 70 days of age revealed a significant difference between the groups. Group 4 (FESC line) had the highest average number of animals with 7.36, while Group 3 had the lowest at 6.47. It is important to note that the results were heavily impacted by abnormally high mortality rates, which were beyond the control of the research team. These unforeseen circumstances undoubtedly influenced the outcomes. We plan to continue evaluating these parameters once we have resolved these issues.

When considering the weight at 70 days, Group 4 (FESC line) once again emerged as the leader with an average weight of 2.02 kg. This weight falls within the range reported in previous studies, with Zamora (1999) reporting 2.017 kg and Franco (2009) reporting 1.92 kg. Importantly, the FESC line maintains a statistically significant difference ($p < 0.05$) in weight at 70 days compared to Groups 2 and 3.

CONCLUSIONS

The FESC line consistently showcases its strengths, consistently delivering a high number of live born rabbits and demonstrating superior weight gain at 70 days compared to its parent breeds. This exceptional performance firmly establishes it as a promising option for meat production in Mexico, outperforming traditional breeds.

It is crucial to recognize that factors such as diet and environmental conditions can have a significant impact on these results. Future research should delve into how these elements can further optimize the performance of the FESC line.

Moreover, this study offers valuable insights into the performance of the New Zealand, California, and Chinchilla breeds, serving as a benchmark for future breeding strategies.

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To the Rabbit Breeding Module of the Center for Agricultural Education, Faculty of Higher Studies Cuautitlan (FESC), National Autonomous University of Mexico (UNAM)

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ETHOLOGY & WELFARE

MEASURING ON FARM WELFARE IN RABBITS: A REVIEW WITH EMPHASIS ON ANIMAL BASED INDICATORS

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ABSTRACT

Based on latest definitions, animal welfare has to be referred to a life worth living, as perceived by animals, thanks to positive experiences rather than to the mere absence of negative ones. The measure of on-farm welfare of livestock is crucial to improve farming systems, to identify critical points, and to compare different farming systems in view of welfare labelling protocols. To this purpose, specie-specific protocols are necessary which should use different types of indicators, i.e. resources-based indicators, management-based indicators and, especially, animal-based indicators. These indicators should work under different farming systems and for different animal categories and can be used to assess welfare in the short term or during the productive life of the animal. Last but not least, indicators should be able to measure the affective state of animals as for positive emotions. In this scenario, rabbits are quite unique as little information is available about i) their behavioural needs under farming conditions; ii) the degree of suffering associated with the behavioural restrictions that can occur under farming conditions; iii) the indicators to be used in the very different housing and management conditions in which rabbits can be farmed; and iv) the relationships between emotions and affective states of animals as well as the effect on resiliency of rabbits under different conditions. In this context, the present review aimed at summarising the state of the art and designing a road map for assessing rabbit welfare on farm based on the most recent knowledge and approaches with special emphasis on candidate ABMs for measuring both negative and positive affective states of rabbits. The identification of positive welfare indicators is a big challenge, given the biological and behavioural characteristics of rabbits. Accordingly, a comprehensive and robust assessment of rabbit welfare on farm cannot do without structure- and management-based indicators, which should be included in validated and standardized protocols using a multi-indicator approach.

Key words: welfare consequences, protocols, indicators, positive welfare.

INTRODUCTION

Definitions and measures for animal welfare have been largely discussed and perspectives have been modified during the years. While new challenges have been identified for more animal welfare on farm (Paulovic et al., 2024), there is a common agreement about the fact that welfare is the quality of life as perceived by the animal, which can range from a life worth living, characterized by positive emotions, optimal health and social relationships, to a life no worth living, which is full of negative emotions, illness, pain, and frustration (Mellor et al., 2015; Mellor, 2016; LIFT, 2024).

In this panorama, previous studies (as reviewed by Verga et al., 1997; Trocino and Xiccato, 2006; Szendrő and Mc Nitt, 2012; Szendrő et al., 2019) and EFSA Scientific Opinions (2005; 2020) largely focused on welfare and health of farmed rabbits with special emphasis on welfare consequences, i.e. issues related to negative experiences. In details, EFSA (2020) identified several behavioural restrictions (restriction of movement; resting problems; inability to perform maternal behaviour; inability to express social positive behaviour; inability to

perform gnawing behaviour; abnormal behaviours; fear) and other problems including health issues (prolonged hunger; prolonged thirst; pododermatitis; locomotory disorders; skin lesions; respiratory disorders; gastroenteric disorders; skin disorders; reproductive disorders; mastitis; neonatal disorders; thermal stress). In absence of scientific information, the severity of these welfare consequences for each animal category was scored based on an EKE exercise (Expert Knowledge Elicitation) from 0 (no welfare impairment at all) to 10 (highest possible suffering for a rabbit), with different criteria for behaviour-related (unfulfilled essential behaviour, from high to low motivation; pathological/physiological consequences, and acute stress reaction) and health-related welfare consequences (unfulfilled essential behaviour, e.g. feeding; pain; discomfort).

Nevertheless, while the degree of suffering associated with health issues can be more objective to score, little scientific information is available about the degree of suffering associated with the behavioural restrictions that can occur under farming conditions, as the behavioural needs of rabbits under these conditions have not been fully elucidated (EFSA, 2020). In fact, rabbits are a recently domesticated species compared to other farmed animals and there is less scientific information on their needs and welfare on farm than for other animals.

In this context, EFSA (2012) proposed a common framework for animal welfare risk assessment or animal welfare assessment in practice, which is based on the Welfare Quality project, including four principles and twelve animal-based criteria (Table 1) for which specie-specific and validated indicators should be identified to be used on farms.

Table 1: The principles and animal-based criteria used as guidelines for animal welfare assessment according to the Welfare Quality® project (modified from EFSA, 2012)

Principles	**Criteria**
1. Good feeding	1. Absence of prolonged hunger (i.e. they should have a suitable and appropriate diet) 2. Absence of prolonged thirst (i.e. a sufficient and accessible water supply)
2. Good housing	3. Comfort around resting 4. Thermal comfort (not too hot not too cold) 5. Easy of movement (enough space to move around freely)
3. Good health	6. Absence of injuries (e.g. skin damage and locomotory disorders) 7. Absence of disease (i.e. high standards of hygiene and care) 8. Absence of pain induced by management practice
4. Appropriate behaviour	9. Expression of social behaviours (normal, non-harmful, e.g. grooming) 10. Expression of other behaviours (species-specific behaviours) 11. Good human-animal relationship 12. Positive emotional state (fear, distress, frustration, apathy to be avoided)

A change in the perspective for assessing animal welfare can be identified based on the revision of the Five Domains Model, which initially focused only on the negative impacts on animal welfare: 1) Nutrition (water and food deprivation; malnutrition); 2) Environment (physical and atmospheric challenges); 3) Health (disease, injuries and functional impairment); 4) Behaviour (behavioural and/or interactive movement restrictions); 5) Mental domain (thirst/hunger, anxiety, fear, pain and distress) (Mellor et al., 1994). The revision of the Five Domains Model has highlighted a range of factors generating specific negative or positive responses in the animal per each of the first three domains (Nutrition, Physical Environment and Health) and has redesigned the forth domain (Behavioural Interactions) subdividing it according to the nature of animal interactions with their environment, other non-

human animals, and humans, including consideration of the grading of negative and positive welfare impacts (Mellor et al., 2020).

Whatever the approach for animal welfare assessment, specie-specific protocols are necessary which should use different types of indicators based on resources, management and, especially, animal-based measures (ABMs). In fact, these latter are measurements of physiological or behavioural responses of an animal or an effect of the external environment on it that can be used to assess its welfare (EFSA, 2012).

As for rabbits, there are only three structured examples of protocols including different types of indicators and structured for systematic use on farms. However, to the best of the authors' knowledge, none of these protocols has yet been fully validated considering the very different housing conditions under which rabbits may be farmed. These protocols have been proposed and applied at farm level by private individuals/bodies or are going to be used as part of official veterinary controls.

In Spain, to develop an external welfare certification (Certificado Welfair®, visit https://www.animalwelfair.com/es/certificado-welfair/), the protocols formulated by the Institute of Agrifood Research and Technology (IRTA, Spain) in collaboration with the Basque Institute for Agricultural Research and Development (NEIKER) are based on the Welfare Quality® (Blockhuis et al, 2010) and AWIN® and are differentiated between reproducing does with litters and males (Dalmau et al., 2020) and growing rabbits (Botelho et al., 2020). The protocols define sampling methods and time (first week after parturition, at artificial insemination and after weaning for the breeding sector), besides the scoring systems for each indicator. The scores for each indicator are then weighted and used to calculate an overall welfare score where threshold criteria for welfare assessment are identified.

In France, the farming industry has proposed the EBENE protocol (ITAVI, 2018) for welfare assessment in reproducing does and growing rabbits based on Welfare Quality® principles and criteria. An app (EBENE®) is also available to be used by farmers, technicians and veterinarians. The result of each indicator and the overall result are graphically represented and compared with regional results. The feasibility and repeatability of the protocol have been validated in the field, but improvements are requested (Warin et al., 2021).

In Italy, the Ministry of Health and the National Reference Centre for Animal Welfare (CReNBa) have developed a check list as part of the Classyfarm system available to official veterinarians for assessing rabbit welfare on farms (https://www.classyfarm.it/index.php/what-it) within the framework of official controls related to the annex to Legislative Decree 146/2001 (Implementation of Directive 98/58/EC on the protection of animals kept for farming purposes) and in the “Guidelines for Rabbit Farming” (Circular of the Ministry of Health, 01/09/2021) (Ministero della Salute, 2023). The check list is based on measures and data related to the hazards arising from environmental conditions (management, facilities, equipment and microclimatic conditions) and derived from the detection of the most important ABMs. The end result of the Classyfarm assessment is the classification of farms by an overall welfare score that expresses the level of risk of the farm, besides the identification of legal non-compliances.

Since field data were not yet available and scientific information was not sufficient, EFSA Scientific Opinion, published in 2020, assessed the condition of rabbits kept in different husbandry systems (conventional and alternative) by consulting experts in the field. Specifically, EFSA used an index developed by considering the impact of different farming systems on the behavioural and health welfare consequences in the main rabbit categories obtained through expert consultation (Expert Knowledge Elicitation, EKE).

Overall, based on this brief introduction, information about rabbit welfare and their needs under farming conditions is still scarce; indicators have not been validated under all farming conditions and the use of ABMs is jeopardised; few protocols have been applied in the field and scarce reference data are available about the on-farm welfare of rabbits in Europe, but the EFSA results of the EKE. In this context, the present review aims at summarising the state of the art and designing a road map for assessing rabbit welfare on farm based on the most recent knowledge and approaches with special emphasis on candidate ABMs for measuring both negative and positive affective states of rabbits.

RABBIT BEHAVIOURAL AND WELFARE NEEDS

As with other species, the study of behaviour is crucial for understanding the rabbit needs and assessing its welfare under the different husbandry and housing conditions within the framework of principles identified by EFSA (2012). In the case of rabbits, we must refer to their behaviour in the wild and/or under natural and semi-natural conditions. Domestication of the rabbit is in fact rather recent and has not produced substantial changes in its behavioural repertoire except for the intensity and frequency of certain behaviours, such as the greater daytime activity of the domestic rabbit compared to the wild rabbit (Trocino and Xiccato, 2006; EFSA, 2020; Rödel et al., 2022; Gonzalez-Mariscal et al., 2022). Accordingly, behavioural needs of farmed rabbits are presented hereby with reference to existing knowledge under wild and semi-wild conditions, specifying when known the motivation for the different behaviours with reference both to the principles identified by EFSA (2012) and the Five Domains (Mellors et al., 2020).

Good Feeding and Nutrition

Rabbits are herbivores, they select concentrates, and they are characterised by the physiological mechanism of caecotrophy. Under commercial conditions, diets are formulated to provide the suitable quantity and quality of fibre, besides other nutrients, according to their physiological states and nutritional requirements. In the wild, rabbits spend from 30% to 70% of their activity time outside the burrow to groom, searching for feed and eating, with a variability depending on the age, season and availability of feed. Grazing behaviour is performed mainly during late afternoon and night whereas during daylight rabbits tend to stay in warrens; however, this behavioural pattern can change depending on the presence of predators in their environment (Delibes-Mateos, 2021).

Usually under farming conditions, rabbits are fed *ad libitum* with balanced diets, whereas forage can be added as an environmental enrichment under some farming conditions. Sometimes, specific restriction programs can be implemented to reduce digestive problems in growing rabbits and/or to manage feed intake in young and reproducing does.

Good Housing and Behavioural interactions with the environment

Under wild conditions, rabbit social behaviour, reproduction and survival are based on their capability of creating underground burrows for their housing. These underground areas are fundamental for rabbits to escape predators and to give birth to their blind, deaf and hairless young kits (Delibes-Mateos, 2021), besides keeping them away from adverse climatic conditions. Under farming conditions, burrows/ warrens can play a role in outdoor systems, whereas under conventional conditions different types of cages/pens work.

In the wild, when they have satisfied their nutritional requirements, rabbits spend most of their time resting in group, close to each-other, showing a complex social activity as discussed below (see Appropriate behaviour). Under farming conditions, this implies that suitable surfaces/floors for resting must be provided as for clean space with sufficient dimensions. In fact, rabbits increase self-grooming when kept in dirty soils/litters (Dal Bosco et al., 2002). Moreover, as age increases, growing rabbits reduce the time spent resting with the body stretched in favour of resting with the body crouched (Trocino et al., 2018; Birolo et al., 2020), where the motivations behind this latter behaviour have not been fully elucidated.

Then, for locomotor activities, rabbits usually move on the ground by small jumps; they can use longer jumps to overcome obstacles and reach elevated positions. Their requirements as for the time to be spent in this activity have not yet been defined, whereas time spent moving has always been found to be very limited under farming conditions, when free and easy access to feed and water is provided. Accordingly, space requirements for movement have not yet been set for rabbits under farming conditions. Nevertheless, a movement restriction has been identified as the inability to perform three consecutive steps in a linear direction (EFSA, 2020).

As for relationships between environment/housing and specie-specific behaviours, exploratory activities of rabbits include digging under wild conditions and sniffing their surroundings, often associated with gnawing. This latter behaviour is highly-motivated and has to be satisfied also under farming conditions by the provision of suitable materials.

Other behaviours, which can imply changes/adaptation in the housing systems and could be taken into account in alternative systems with outdoor access, include anti-predator behaviour, i.e., the alert posture on the hind legs and with erect ears, the rapid flight towards a hiding place and immobility that the rabbit uses to confuse and escape predators. In addition, a rabbit can often be found guarding the entrance to the communal burrow and alerting the group about the presence of danger by tapping a paw on the ground. Finally, under natural conditions, rabbits prefer a terrain in which they can easily dig and an environment with abundant vegetation, especially bushes to be used for feeding, but also to hide from predators. These issues can be considered as requirements when designing alternative farming systems with outdoor access. On the other side, under indoor conditions, the usefulness of equipment for hiding also from mates has not been fully proven. In fact, unlike in the wild, domestic rabbits do not hide when disturbed, even when suitable structures are present. The rabbits spend most of their time above the box in the cage than inside (Hansen and Berthelsen, 2000). Reproducing females kept in groups also prefer to use elevated platforms (Huang et al., 2021; Van Damme et al., 2023a, 2023b) or wooden panels to escape aggression (Rommers et al., 2013) rather than PVC pipes. Finally, adult males in individual cages show the least interaction with the box when offered different types of enrichments (Lidford, 1997).

Appropriate Behaviour and Behavioural interactions with other animals

Rabbits are known to be social animals. They form family groups and have a strong territorial behaviour. Thus adults can be aggressive to unknown individuals. The minimum group size is one adult male, one female and her litter, but the social unit may include one to four males and one to nine females. Group size changes depending on the availability of natural resources and the environment (Szendrő et al., 2019; Rödel, 2022). In the group, fights are sporadic because hierarchies are defined early on. Males are usually tolerant towards females and younger rabbits; females sometimes show competition for the choice of the nest site for giving birth and caring for the litter. Hierarchies are separated by sex (von Holst, 2001) and this regulates social relations, access to resources and, therefore, the longevity of the animals (González-Mariscal et al., 2022). To establish these hierarchies, under natural conditions, rabbits fight among them but usually without causing each other serious injuries. In commercial farms, conditions do not allow physical contact among adult rabbits (e.g., reproducing does kept in individual cages) and, when they are kept in groups (e.g., growing and fattening rabbits or reproducing does in collective systems), the conditions under which the group is formed and the social behaviours/activities may substantially differ from what happens under wild conditions. Positive social interactions between adults persist over time between dominant males and females of their groups, which probably helps to establish and maintain social bonds (Von Holst, 1999).

Regarding sexual behaviour, wild rabbits mate almost exclusively in the first hours after parturition and reproductive activity is regulated by the increased number of daylight hours in spring. In commercial farms, rabbits are inseminated 12-18 days after parturition, and less after weaning the litter, to avoid an over-exploitation of the female and a high replacement rate which are associated with fast reproductive rhythms and early mating. Reproductive performance remains high throughout the year with a constant photoperiod of 14-16 hours of light. In commercial farms, the use of artificial insemination prevents the expression of the pre-mating sexual behaviour characteristic of the wild rabbit and still present in domestic rabbits. Nevertheless, the effect of this restriction on the affective state of rabbits is not known.

Reproducing does have specific physiological requirements concerning the maternal behaviour. This latter consists of i) nest construction before parturition; ii) a single, rapid daily nursing session; iii) and the weaning of the litter. Maternal behaviour is controlled by hormonal factors that regulate nest construction, and non-hormonal factors such as the rabbit social position. Both these factors influence litter care and milk production (González-Mariscal et al., 2022). Under natural conditions, 3-4 days before giving birth the doe leaves the communal burrow to prepare the nest in a different site, burrowing into the ground, covering the bottom with plant material and, before giving birth, with her fur torn off from her abdomen and thorax. After parturition, once the first suckling is completed, the doe closes the nest and leaves it to return to her kits only once a day, usually after sunset and for the few minutes (2-5) she needs for lactation. During this short time, thanks to the high protein and energy content of the doe milk, kits ingest the amount of nutrients and energy they need for their rapid growth. The doe definitively opens the nest when the kits reach the age of 18-20 days; at this age, they have already begun to ingest the hard faeces pellets left by the mother in the nest, thus facilitating the start of the microbial colonisation of the caecum. If the doe has been mated immediately after birth, as usual in the wild, milk production decreases abruptly 20 days after birth and around 24-25 days the doe leaves the nest to prepare for the next birth. If the doe is not pregnant, weaning of the litter can take place some days later.

As for other specie-specific behaviours, rabbits perform various comfort activities, including those directed at their own bodies (self-grooming) and those directed at conspecifics (allo-grooming) which can be satisfied only when they are reared in a group. Finally, play behaviour is often registered in weanling kits and in young rabbits up to about 2 months. This behaviour includes active movements such as leaping, frisking together in circles and half-circles and more passive ones such as nuzzling, licking each other gently during resting side by side (Lockley, 1961).

Mental State and Behavioural interactions with humans

Rabbits as prey animals modify their behaviour and activity depending on the presence of predators (Delibes-Mateos, 2021). This means that the rabbit-man relationship is largely affected by how this is managed under farming conditions. Surely, the presence and the contact with conspecifics play a role since solitary animals seem to be more fearful of exploring the surroundings in search of feed whereas rabbits in groups tend to move further away from the scrubland (Villafuerte and Moreno, 1997). Rabbits also interact positively more frequently with conspecifics as juveniles are less stressed and are more active in presence of fearful stimuli such as a predator scent, showing a higher scanning activity (Rödel et al., 2006). A positive effect of the presence of conspecifics on the reduction of the fear level has also been proven in reactivity tests in farmed rabbits kept in individual, bicellular, and collective cages (Trocino et al., 2013).

ON FARM WELFARE ASSESSMENT: ANIMAL-BASED INDICATORS

The assessment of animal welfare based on the above-identified principle/criteria or domain implies the use of indicators, which could be based on animals (ABMs), resources, or management and should be valid (in capturing animal welfare information), feasible (as for

the adaptability to different housing systems and types of environments), and reliable (in providing the same results when the same observer repeats the assessments or when there is agreement between two or more observers after receiving a sufficient training). Based on the most recent outcomes, ABM criteria should be preferred to assess on-farm welfare (EFSA, 2012). In rabbits, there is wide variability and lack of standardization and information with respect to the indicators that can be used under the different farming conditions, besides the scale of measurement, the use or non-use of thresholds, the way in which results are aggregated for the assessment of the welfare situation in the farm and/or for comparison between different situations. In fact, while ABMs have been validated for other species, in the case of the farmed rabbit the scientific literature and field data are scarce (EFSA, 2020). The EFSA Scientific Opinion (2020) proposed possible ABMs for rabbits that could be included in a protocol developed on the basis of the Welfare Quality Project schemes and criteria (De Jong, 2011) (Table 2), which, however, has not been validated and/or applied in the field, yet.

Table 2: Animal-based measures (ABMs) proposed by de Jong et al. (2011) (modified from EFSA, 2020).

	ABMs for reproducing does and growing rabbits
Good feeding	
Absence of prolonged hunger	Body condition score
Absence of prolonged thirst	Resource based measures
Good housing	
Comfort around resting	Fully stretched lying in the pen or at the elevated platform or shelter simultaneous resting in group housing
Thermal comfort	Respiration rate Red ears
Ease of movement	Hopping (number of consecutive hops), jumping, turning, running Number of lame rabbits
Good health	
Absence of injuries	Skin injuries/wounds Pododermatitis (only for reproducing does) Number of toes and ear damage (only for growing rabbits) Trichophagy (only for growing rabbits)
Absence of diseases	Percentage mortality and selection clinical scoring of rabbits, consisting of symptoms listed Technical performance
Absence of pain induced by management procedures	Which mutilations are used (for identification) Presence of tissue growth when using ear marks.
Appropriate behaviour	
Expression of social behaviour	Scoring of injuries and wounds Scoring social behaviour
Expression of other behaviours	Abnormal behaviours Coat condition Kit mortality
Good human-animal relationship	Human approach test
Positive emotional state	Fear for novel objects Description of behaviour of a group Hopping behaviour in young rabbits

More recently, on the basis of the literature published during the last 10 years (2013-2023), different indicators used under different conditions in protocols for assessing the welfare of rabbits on commercial farms have been identified (Paulović et al., 2024) based on the principles identified by the Welfare Quality project and by EFSA for the assessment of animal welfare (Good Behaviour, Good Housing, Good Health, Good feeding) supplemented with the criterion Mental state, according to the Five Domains Model (Table 3). These included both resource-based and ABMs where the welfare consequence intended to be measured have also been specified in Table 3.

Table 3: Indicators used in protocols for assessing the welfare of farmed rabbits (modified from Paulović et al., 2024). Animal Based Indicators (ABMs) are in bold.

Category	Indicator[1]	Description	Welfare consequence[1]
Behaviour	**Abnormal behaviours**	Stereotypic and abnormal behaviour	Inability to perform exploratory or foraging behaviour
	Social behaviour	Agonistic behaviour (grouped as well as individual behaviours such as frequency of displacements), negative, positive social behaviour	Group stress
	Isolated animals	Presence of isolated housed rabbits	Isolation stress
Environment	Cage/pen design	Size, presence of resources (e.g., elevated platform, foot rest) and behaviour related to this (e.g. standing upright, lying fully stretched)	Movement restriction
	Thermal stress	Panting, shivering and climate conditions (e.g., temperature)	Heat/cold stress
	Cleanliness	Clean or dirty body (parts), including wet body (parts)	Resting problem
Health	**Skin lesions, wounds and dermatitis**	On all body parts, including dermatitis, abscesses, dermatomycosis but not on hocks/feet	Soft tissue lesions and integument damage
	Ocular and nasal discharge	Ocular and nasal discharge	Respiratory problems
	Pododermatitis	All degrees of pododermatitis and wet and dirty feet and including heel and middle foot	Soft tissue lesions and integument damage
	Mortality	Total mortality including culls, mortality per category (kits, does)	
Mental state	Animal-human relationship	Includes tests (avoidance distance, touch test), measures such as % or number of animals that can be approached, and aspects of handling by caretakers	Handling stress
Feeding	Water provision	Number of drinkers, cleanliness, water flow, etc.	Prolonged thirst
	Body condition	Includes scoring of body condition, proportions of lean or fat animals	Prolonged hunger
	Feed provision	Feeder space, cleanliness, type of feed, etc.	Prolonged hunger

[1]According to the definitions in EFSA (2022a and 2022b); if the cell is empty, there was no welfare consequence that could be linked to the grouped indicators.

On the other hand, the European Reference Centre for Animal Welfare for Poultry and Other Small Farmed Animals (EURCAW-Poultry SFA, 2023) has previously reviewed indicators used in the different protocols. Based on the available information and an EKE process, EURCAW-Poultry SFA assessed and scored the validity, feasibility, and reliabilityof the different indicators, ranging from 1 (low validity/reliability based on literature or expert opinion; low feasibility, high cost/high execution time/high handling of animals is required) to 3 (high validity/reliability based on literature or expert opinion; high feasibility, low cost equipment is required - e.g. tape measure - or none/low execution time/easy access in all types of facilities/no handling of animals). The following discussion resumes the outcomes of the evaluation by EURCAW-Poultry SFA (2023) with reference to ABMs for the different Domains.

Health

The ABMs related to health are those with the highest validity, feasibility and validity over all animal categories and different protocols (Table 4). Skin alterations or injuries are mostly assessed by visual inspection considering the position, extent and severity of the lesions with different scoring methods. These latter can be more or less simple, based on the need of

facilitating inspections and increasing the feasibility of measurements under commercial and field conditions. Mycosis is assessed as presence/absence, whereas isolation and culture of hair samples have reduced feasibility, despite high validity and reliability. Dermatophytosis must be diagnosed differentially from mite lesions, which are also evaluated as presence/absence.

Table 4: Indicators for assessing Good Health under farming conditions: validity, feasibility and reliability (modified from EURCAW, 2023).

Animal category	Welfare consequence	Animal Based Measures	Validity	Feasibility	Reliability
All	Skin lesions and wounds	Number of animals with wounds on the body and scoring by severity (e.g. size)	XX	XXX	XXX
Growing rabbits	Skin lesions and wounds	Number of animals with skin lesions (abscesses, ulcers etc.) and wounds and severity scoring Number of animals with wounds and scoring by severity Presence of skin lesions (abscesses) and wounds (no scoring) on eyes, ears, body (except ventral part)	XX	XX	XX
All	Skin lesions and wounds	Animals with fallen ears	XXX	XXX	XX
All	Skin problems	Animals with hairless areas	XXX	XXX	XXX
All	Skin problems	Animals with mange	XXX	XXX	XXX
All	Skin problems	Animals with dermatophytosis	XXX	XXX	XXX
All	Skin problems	Hair sampling for the detection of dermatophytes	XXX	X	XXX
Breeding rabbits	Pododermatitis	Number of animals with pododermatitis and severity scoring Number of animals with hyperkeratosis or ulceration (presence/absence)	XXX	XX	XXX
Breeding rabbits	Mastitis	Number of animals with mastitis and scoring by severity (mild/severe) (requires palpation)	XXX	XXX	XXX
All	Mortality	Mortality percentage on farm (period varying according to the protocol)	XXX	XXX	XXX
All	Culling rate	Culling rate on farm (period varying according to the protocol)	XXX	XXX	XXX
All	Respiratory disorders	Animals with nasal discharge (visually)	XX	XXX	XX
All	Respiratory disorders	Nasal swabs for detection of pathogenic respiratory bacteria and quantification	XXX	X	XXX
All	Respiratory disorders	Animals with ocular discharge (visually)	XX	XXX	XXX
All	Respiratory disorders	Animal showing coughing and sneezing (minimum observ. time: 2 min)	XX	XXX	XXX
Breeding rabbits	Gastroenteric disorders	Number of animals with a hard abdomen (Enteropathy)	X	XX	XX
All	Gastroenteric disorders	Number of animals with liquid faeces around the perianal area (diarrhoea)	X	XX	XXX
All	Gastroenteric disorders	Rectal swabs for detection of pathogenic intestinal bacteria and quantification	XXX	X	XXX
All	Locomotory disorders	Number of animals with torticollis (scoring: moderate/severe)	XXX	XXX	XXX
All	Locomotory disorders	Lameness (Gait score)	XXX	XX	XXX

Both pododermatitis and mastitis are evaluated as presence/absence and/or with different scoring that consider the severity and extent of the lesions in reproducing animals and, when possible, take into account the parity order and/or propose aggregations on overall indices referring to entire group in the farm.

The presence of torticollis, often associated with *Pasteurella multocida* middle ear infection and *Encephalitozoon cuniculi* infection, is defined as an abnormal position of the head which causes a vestibular syndrome. It could be assessed in both reproducing females and fattening rabbits considering: no torticollis; a moderate problem, when the animal has a twisted neck but can eat and drink without difficulty; and a severe problem, when the twisted neck makes access to food and water difficult for the animal.

Respiratory disorders are assessed as presence/absence of animals with nasal discharge and/or eye discharge, whereas the presence of coughing and/or sneezing is not the best indicator for these disorders in terms of reliability/repeatability. Gastroenteric disorders can be assessed as animals presenting liquid faeces around the anus and/or considering the presence of pathogenic bacteria isolated from rectal swabs by non-rapid methods.

Mortality and/or culling rates finally represent overall indicators of animal health, being a measure of health problems, inadequate animal management, and overall poor welfare.

Measures can be referred to different production categories and consider the age of the animals (pre-weaning; post-weaning), if aggregating several cycles, and/or referring to average, median and/or minimum values.

Good Feeding and Nutrition

The indicators identified for prolonged hunger are characterized by medium-high validity, feasibility, and reliability (Table 5), where the body condition score (BCS) is a measure of not suitable feeding (unbalanced diet, insufficient quantity of diet) and presence of diseases. An impairment of the body condition has been linked with the onset of diseases such as mastitis, pododermatitis and rhinitis. The BCS can be assessed by visual inspection or by palpation, where visual assessment may reduce the validity and reliability of the indicator, but allows for increased feasibility in terms of time and reduced stress from handling animals. A further indicator, body symmetry, has been recently proposed (Cohen and Ho, 2023) for which information about validity, feasibility and reliability is not yet available.

Table 5: Indicators for assessing Good Feeding under farming conditions: validity, feasibility and reliability (modified from EURCAW, 2023).

Animal category	Welfare consequence	Animal Based Measures	Validity	Feasibility	Reliability
Reproducing does	Prolonged hunger	Body Condition Score assessed with palpation	XXX	XX	XX
Reproducing does	Prolonged hunger	Body Condition Score visually assessed	XX	XX	XX
Growing rabbits	Prolonged hunger	Number of small rabbits: twice as small as the others or very thin animals	XXX	XXX	XXX

Good housing and Behavioural interactions with the Environment

The welfare consequences that have been largely intended to be measured by specific ABMs in the literature firstly refer to behavioural restrictions (resting, movement) and then to physiological alterations of the animal due to inadequate environmental conditions (Table 6).

Generally talking, the corresponding measures have usually low to medium degree of validity, with the exception of animal cleanliness that is a measure recognized as highly valid. This measure is related to possible resting difficulties (associated with physical discomfort, cold stress, injuries, pain) and indirectly measures the hygienic state of the housing or cages, besides the suitability of the facilities (type of flooring) and/or management (density of animals, frequency of cleaning interventions). As for the feasibility and reliability of ABMs intended to measure defects in the design and management of the environment, none of the proposed measures can be considered robust (Table 6).

Table 6: Indicators for assessing Good Housing under farming conditions: validity, feasibility and reliability (modified from EURCAW, 2023).

Animal category	Welfare consequence	Animal Based Measures	Validity	Feasibility	Reliability
Growing rabbits	Resting problem	Lying fully stretched animals	XX	X	X
All	Resting problem	Number of dirty and wet animals (scoring)	XXX	XX	XX
Growing rabbits	Resting problem	Number of animals resting in a group	*Lack of knowledge*		
Reproducing does	Movement restriction	Number of animals performing at least 2 jumps in the same direction or to/from the platform. Number of animals jumping and moving freely (%)	X	XX	X
Growing rabbits	Movement restriction	Number of animals making 1 jump or 2 jumps in different directions (minimum observ. time: 2 min)	X	X	X
All	Movement restriction	Animals in upright position	Not assessed by EURCAW		
Reproducing does	Thermal stress	Panting animals	X	XX	X
All	Thermal stress	Lying fully stretched hyperventilating animals with red ears	XX	X	XX
Reproducing does	Thermal stress	Shivering animals (cold stress)	X	XX	X

Behavioural interactions with other animals and humans and Mental health

The measure of aggression among rabbits in terms of skin lesions of the different categories can be related to lesions when aggression occurs repeatedly and the animals do not have the possibility of retreating or hiding. With respect to the ABMs referring to the Good Behaviour criteria, in most cases they have been proposed to detect abnormal behaviours that may be associated with a condition of stress and frustration, sometimes due to a lack of resources necessary to display species-specific behaviours and/or fear (Table 7).

Generally talking, the validity, feasibility and reliability of these ABMs are usually low or not measurable as they have never been tested under practical conditions (Table 7). The low validity of these ABMs for measuring stress/frustration confirms that information on the affective state (negative or positive) of rabbits is rather scarce.

POSITIVE WELFARE

As described above, the welfare assessment schemes applied up to now for rabbits have always referred to criteria and indicators that could measure the absence of negative experiences in the context of different principles or domains. Nevertheless, as previously introduced, the approach with respect to animal welfare is shifting: providing animals with opportunities for rewarding experiences and situations in which they feel satisfied is recognized a key for their welfare, beyond the alleviation of any suffering. Positive feelings could outweigh negative ones in order to achieve good overall welfare, even if how this could be achieved is still up for debate (Rault et al., 2023). Importantly, the ability of animals to cope with different stimuli and environment for reaching positive emotional states and being resilient under different farming conditions can largely contribute to their welfare status (Rault et al., 2023). Nevertheless, several factors (e.g. genetics, pre-birth and early experiences, etc.) account for individual differences among animals (LIFT, 2024). In other words, the previous (positive or negative) experience of animals can affect their cognitive ability and finally their ability to cope with the environment. Definitively, this is a more comprehensive approach to animal welfare that takes into account both physical and emotional aspects (Turner et al., 2019; Paulovic et al., 2023).

Table 7: Indicators for assessing Behavioural interactions with other animals and humans and Mental state under farming conditions: validity, feasibility and reliability (modified from EURCAW, 2023).

Animal category	Welfare consequence	Animal Based Measures	Validity	Feasibility	Reliability
Reproducing does	Skin lesions and wounds	Number of animals biting other adults or kits (minimum observ. time: 2 min)	XX	X	XX
Growing rabbits	Skin lesions and wounds	Number of animals biting or fighting with conspecifics (minimum observ. time: 2 min)	X	XX	XX
Reproducing does	Inability to perform positive social interaction	Number of allo-grooming events (minimum observ. time: 2 min)	X	XX	XX
Growing rabbits	Inability to perform positive social interaction	Number of allo-grooming events (minimum observ. time: 2 min)	XX	X	XX
All	Inability to perform gnawing behaviour Abnormal behaviour	Number of animals biting or digging the cage for more than 3 sec Animals showing head shaking, swaying, cage gnawing, empty digging, obsessive cleaning (minimum observ. time: 2 min)	XX	X	XX
Reproducing does	Abnormal behaviour	Number of nervous and restless animals (minimum observ. time: 2 min)	X	XX	XX
Growing rabbits	Abnormal behaviour	Number of nervous and restless animals (minimum observ. time: 2 min)	X	X	XX
Reproducing does	Abnormal behaviour	Number of animals performing self-grooming (minimum observ. time: 2 min)	*Lack of knowledge*	XX	XX
Growing rabbits	Abnormal behaviour	Number of animals performing self-grooming (minimum observ. time: 2 min)	*Lack of knowledge*	X	XX
Reproducing does	Fear	Good human-animal relationship: human approach test with a 10 cm stick (for 30 sec)	X	XX	XXX
All	Pain	Squeal loudly and grind the teeth	*Lack of knowledge*		
All	Pain	Assessment of facial expressions (Rabbit Grimace Scale)	XXX	*Lack of knowledge*	

As the reference for the behavioural needs of rabbits are yet those of the wild rabbits, their affective states under farming conditions are also poorly understood. In other words, we do not clearly and fully know the situations that are rewarding and positively stimulate the affective status of rabbits; we have not yet identified indicators of a positive affective status that could help us in evaluating rabbit welfare and/or comparing different farming practices and systems; information about how the cognitive ability of rabbits can be affected by positive or negative experiences, and how this can influence on farm welfare, are not available yet. On the other hand, some information is available in laboratory and companion rabbits (Jirkof et al., 2019; Cohen and Ho, 2023).

Compared to other animals, rabbits are even more sensitive and difficult to deal with respect to this topic. In fact, they are very sensitive animals. As prey animals, they are constantly vigilant and mentally occupied with the potential threat of predators; they can exhibit a range of emotional states in a relatively short period. In general, the study of animal behaviour is considered to be more functional for the assessment of affective state than the measurement of physiological and neuroendocrine variables (Jirkof et al., 2019; Turner et al., 2019). On the

other hand, even behaviours for which a relation with a positive emotional situation is widely recognized (e.g. playing) could in practice give different information both related to positive or non-positive well-being depending on the situation (e.g. different age of the animals, duration and time of expression, context) (Jirkof et al., 2019).

Thus, some behaviours have been identified as possible candidates for measuring a positive affective status and identify positive indicators in rabbits, with special reference to natural behaviours, territorial and hierarchical behaviours, social and exploratory behaviours, and resting behaviours (Table 8). While some of these behaviours are clearly associated with the good status of the animals (e.g., nesting for breeding does, regular eating with occasional drinking and coprophagia for all categories), the validity of others for evaluating positive welfare need to be based on the knowledge of the behavioural requirements of rabbits under farming conditions.

More specifically, investigations would be necessary for the relationship between certain behaviours (with special reference to spontaneous behaviours, playing, movement, social and exploratory behaviour, maternal behaviour, body and ear position, facial expressions, vocalizations) and the positive affective state in different categories of rabbits. The relative importance of these behaviours should evaluate the rabbit response when offering rewarding materials and/or when allowing expressing species-specific behaviours with strong motivation (gnawing materials as environmental enrichment; group resting and allo-grooming; the possibility of moving away from conspecifics; nest construction and access). Indeed, if agency is referred to what animals "want", i.e. motivated behaviours, which could be driven by the associated/expected positive emotions, agency domain and behavioural interactions have been proposed as the framework under which positive animal welfare can be assessed within the Five Domains Model (Littlewood et al., 2023).

Table 8: Putative Candidates behaviours for assessing affective status and positive welfare in rabbits (modified from Cohen and Ho, 2019).

Category	Description
Natural behaviours	Binkying or frolicking (jumping rapidly whilst shaking head and flinging hindlimbs to the side)
	Grooming (self-grooming, allo-grooming, mutual grooming)
	Nocturnal/crepuscular behaviour
	Nesting (for breeding does)
	Regular eating with occasional drinking
	Coprophagia
Territorial and hierarchical behaviours	Scent marking by chinning objects
	Cage guarding*
	Marking territory with urine or faeces (spraying): May be due to frustrated sexual behaviours of entire rabbits*
Social and exploratory behaviours	Foraging
	Investigative behaviour
	Rearing or peri-scoping
	Digging or burrowing
	"Tooth purring" or "teeth chattering": different from tooth grinding (bruxism)
Resting behaviours	Sprawling or stretching out[1]
	Laying down or "flopped" on their side[1]

[1]Could be also negative/neutral

In laboratory rodents as in other species, different tests have been used to assess their biological needs. These tests also referred as apparatus based behavioural test paradigms, including anxiety-related tests, preference tests, strengths of preferences, and cognitive judgment bias tests (Table 9).

Table 9: Main Apparatus Based Behavioural Test Paradigms used in laboratory rodents to assess their affective state (modified from Cohen and Ho, 2019).

Anxiety Related Tests	Elevated plus-maze (EPM) Elevated zero maze (EZM) Black/white box (B/W box or *Dark-light exploration test*) Open field test (OF) Free exploration tests (FET)
Preference Test	Test the preference between two or more items
Strengths of preferences	Test the willingness to pay for the chosen item
Cognitive Judgment Bias	Affective states are measured indirectly testing cognitive abilities

Observations under different conditions (i.e. different apparatus based behavioural test paradigms) will reveal behaviours associated with affective state. In details, in laboratory rodents, the following behaviours have been recorded to evaluate their affective states which could be tested also in rabbits, i.e. spontaneous behaviour, play behaviour, vocalization, facial expression, nest building, burrowing, and grooming. These behaviours have the potential to be used also in the definition of the affective states of rabbits.

These tests can also be used for evaluating any differentiated responses in animals previously offered materials and/or situations that are rewarding with respect to the possibility of expressing species-specific behaviour. Based on the same principle, cognitive tests evaluate the animal response with respect to the ability to make positive/negative judgements on ambiguous stimuli based on their emotional state.

Table 10: Apparatus Based Behavioural Test Paradigms used in welfare studies on farmed rabbits.

Test type	**Tested factors**	**Reference**
Open field test	Presence of gnawing hay blocks	Birolo et al., 2022
	Environmental enrichments; age	Trocino et al., 2019
	Floor type; stocking density; age	Trocino et al., 2018
	Litter size; age	Gümüş et al., 2018
	Pre-natal and post-natal effects of semi-group housing on rabbits behaviour	Buijis and Tuyttens, 2015
	Cages vs pens	Trocino et al., 2014
Novel-Object Test	Gnawing objects	Birolo et al., 2022
	Semi group vs. single housing	Buijis and Tuyttens, 2015
	Environmental enrichments; age	Trocino et al., 2018
Dark-light box test	Litter size; age	Gümüş et al., 2019
Human approach testing	Presence of gnawing objects	Birolo et al., 2022
	Floor type; stocking density; age	Trocino et al., 2018
Preference test	Social contact vs seclusion of does	Dal Bosco et al., 2020
	Cage size	Miko et al., 2012
	Nesting material for does	Farkas et al., 2018
	Floor type; gnawing material	Princz et al., 2008
	Presence of mirrors	Dalle Zotte et al., 2009
	Floor type	Morisse et al., 1999
Tonic immobility	Cages vs pens	Trocino et al., 2014
	Floor type; stocking density; age	Trocino et al., 2018
	Housing system; age	Trocino et al., 2013
Object recognition task	Type of litter; age	Gümüş et al., 2019
Object location task		
Olfactory object recognition task		
Social runway test	Semi group vs. single housing	Buijis and Tuyttens, 2015

Out of the different tests (Table 10), the open-field and the novel-object tests on one side and the human-approach and tonic immobility tests have been largely used to evaluate the level of anxiety or fear of farmed rabbits with respect to a novel environment/object and humans, respectively. Although these tests aim to describe the level of anxiety/fear in animals subjected to different experimental protocols, they can elicit by themselves a state of anxiety and, accordingly, they must be associated with other tests and measurements for providing robust results for comparisons (Jirkof et al., 2019). In fact, Buijis and Tuyttens (2015) hypothesized that the results they obtained with rabbits during the open-field test described more likely the motivation of rabbits to look for conspecifics rather than their fear level towards a new environment. They concluded that this test could not be considered appropriate in terms of (negative) rabbit welfare assessment. On the other side, its potential for evaluating (positive) exploratory behaviours needs further investigation.

In farmed rabbits, several studies also used preference tests for getting information about the most preferred situation out of different cage sizes, nesting material, enrichment types and/or floor type. These tests, however, have not yet been calibrated and validated in rabbits in the perspective of evaluating their welfare on farm via behavioural needs and emotions.

CONCLUSIONS

As the wild rabbits are yet the only reference for behavioural needs, the optimization of rabbit welfare on farm is particularly challenging. This fact is even more impacting in view of the current transition towards collective cage-free housing systems, as foreseen by the European Resolution which followed the Initiative of the European Citizens “End the cage age”. These systems, some major behavioural needs may be challenged, such as social relationships and maternal behaviours of reproducing does, as in farms the conditions under which the group is formed and the social activities or behaviours may differ substantially from those in natural or semi-natural environments.

To date, the assessment of welfare in farmed rabbits under different housing and management conditions has been based on animal-based indicators related to health concerns and behavioural restrictions have been used. In perspective, the assessment of welfare in farmed rabbits should in the future also include positive welfare indicators, considering that animals should be provided with opportunities for positive experiences under the assumption that no pain or suffering is inflicted on an animal. In this context, the identification of positive welfare indicators is even more challenging, given the biological and behavioural characteristics of this species as well as the lack of validated protocols and methods for other species as well. Accordingly, a comprehensive and robust assessment of rabbit welfare on farm cannot do without structure- and management-based indicators, which should be included in validated and standardized protocols using a multi-indicator approach.

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PERFORMANCE OF RABBIT DOES REPRODUCED IN GROUP

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ABSTRACT

The aim of this study was to analyse the productivity and health status of reproducing does raised in a group housing system. In 4 modules of 5 polyvalent cages (20 places in total) roofs were removed, walls were up-extended and 80% of the floor was covered with plastic grid footrests. In addition, the walls between places in the same module were cut out and a trap door was provided which could either be left open to allow does to pass through or closed to avoid it. For six reproductive cycles, 40 females were reproduced in the parks (20 females, 5 females per park; group P) and in individual cages (20 females, one female per cage; group C). All females were inseminated at 11 days post-partum and weaned at 35 days post-partum. In group P, the trap door was kept closed from 7 days prepartum to 7 days postpartum to keep the does in individual housing during peripartum. Does from group P showed, in general, lower fertility rates than those of group C (between 21.1 and 85.0% in group P vs. between 66.7 to 100% in group C), and also lower number of kits born alive per kindling (between 7.26 and 9.86 in group P vs. between 8.06 and 12.5 in group C) and weaned kits per kindling (between 4.72 and 8.45 in group P vs. between 7.79 and 8.50 in group C). At the end of the study, females in group P showed generally poorer health condition, with 40.0% underweight in group P compared to just 6.3% in group C. The observed results indicate that the group housing system under test reduces productivity and negatively affects the health status of the rabbits.

Key words: productivity, health, housing conditions, parks, reproduction in group.

INTRODUCTION

In response to calls from the "End the Cage Age" movement, the European Union plans to review the rearing conditions of livestock that currently is raised in cages. This will happen by 2027, and one possibility in rabbit farms could be to replace current cages with parks. The implementation of new housing systems in rabbit farms may involve a significant investment cost for a sector that is going through a severe crisis, largely generated by high feed prices and the initial impact of new regulations on the reduction of antibiotic use. The "End the Cage Age" movement reflects the concern of a part of the European society that is strongly concerned about animal welfare and health, which assumes that with group reproduction systems the females will benefit from social contact. However, the scientific community has found clear evidence of negative effects when breeding in group (Buijs et al., 2015; Szendro et al., 2013; Van Damme et al., 2023), showing clear increases in aggression in the peripartum period, and reducing both productivity and animal welfare (Zomeño et al., 2018). Moreover, the systems that have been proposed involve a major change in the design of the cage, so their implementation would involve a high-cost investment.

In this study, the objective was testing a park that 1) tries to reduce inversion cost and 2) allows the use of the park for both individual and group reproduction, and for rearing the young rabbits during the fattening period (dual purpose). The effects of breeding in parks on the productivity and health status of does are shown in comparison to breeding in individual cages.

MATERIALS AND METHODS

Animals and experimental design

The study was carried out at IRTA's rabbit experimental farm. Eight modules of five cages each were used (40 cages in total). The cages were 45x96x82 cm (width, depth, height) and

were equipped with a platform of 45x35.5 cm (width, depth). Four of the modules (i.e. 20 cages) were transformed into four parks (five places per park). For this purpose, the roof was removed from each cage and the wire walls were extended up to 43 cm high. Also, 80% of the floor was covered with plastic grid footrest (with plastic strips 1.40 cm wide separated 1.45 cm), leaving free the areas closest to the walls and the area occupied by the caudal area of the animal when it is in the feeder. Finally, an area of 35.5 cm wide x 29 cm high was cut out of the adjoining walls of the same module, enabling the cut-out piece to be used as a door for communication between the places in the same park. When open, the park can be used as a group rearing system and when closed, it is used for individual rearing. The remaining 4 modules (i.e. 20 cages) were left unchanged, with each cage being fitted with a 24.5x37cm plastic grid footrest on the bottom floor and perpendicular to the corridor of the farm.

A total of 40 does of the Prat line (Gómez et al., 2022), selected since 1992 for litter size and commonly used in meat rabbit production farms, were used. The females were inseminated for the first time at 18 weeks of age and were moved to the experimental farm 7 days before parturition, housing 20 females in parks (group P) and 20 in cages (group C). During the study, all females followed the same reproductive management, with insemination at 11 days post parturition and weaning of at 35 days of age. Litters were equalised to 9 or 10 rabbits per lactating female at 3 days post parturition, depending on the reproductive cycle. In the parks, in each cycle, the trap door was closed during the peripartum period (i.e. from 7 days before parturition to 7 days post-partum) and opened during any other time. For six consecutive reproduction cycles the following traits were recorded: fertility; number of born alive and weaned kits; weight of females at parturition, at 7 days post-partum and at weaning; litter weight at birth and individual weaning weights. At 35 days post-partum of the last reproductive cycle the health status of the does was evaluated by the farm veterinarian.

Statistical Analysis

Statistical analyses were performed using R. The effects of the type of housing on the fertility of each cycle and on traits related to the health status of the does at the end of the study were assessed using Fisher's exact test. The effect on the number of kits born alive per kindling was estimated with a mixed model considering the fixed effects of housing type (park or cage), reproductive cycle (RC1 to RC6) and the interaction between them, and the random effect of the doe. The same mixed model, with the addition of the number of kits born alive as a covariate, was used to study the effect of housing on the mean weight of the kits at birth, the weight of the female at kindling and the weight of the female 7 days after kindling. The effect on female weight at weaning, i.e. at 35 days post-partum, was estimated with the same mixed model without covariate, but correcting for the physiological state of the female (pregnant vs. non-pregnant) at the moment of the measurement. Finally, the number of weaned kits per kindling was analysed with a linear fixed effect model, considering the effects of the type of housing, the reproductive cycle and the interaction between them. For this trait, the permanent effect of the doe was not included because in the case of the group P the litters were mixed from 7 days of life.

RESULTS AND DISCUSSION

In general, females housed in parks showed lower values of fertility, number of born alive and number of weaned per kindling (Table 1) than females housed in individual cages. It is important to emphasise that these unfavourable results in the parks imply a reduction between 16.5 and 50.4% in the number of weaned kits per breeding female. The drop in production in group housing systems has already been observed in other studies (Braconnier et al., 2020; Dal Bosco et al., 2019), and is mainly due to increased aggression between the females and from females to the kits in the park.

The effect of type of housing was especially noticeable in the second reproductive cycle: fertility was lower than in other reproductive cycles, as previously observed by other researchers in the second parity of does (Castellini et al., 2010). However, it was especially

reduced in females housed in parks (25%) compared to those in cages (60.0%). Moreover, prolificacy values were clearly lower in females in park (7.26 vs. 12.50 in cage). This value in park breeding females is a clearly abnormal value for the Prat line (9.4 mean between 1992 and 2020; Pascual et al., 2020; 10.3 mean in 2023 - Oscar Perucho, personal communication). Given the anomalous fertility and prolificacy of females in parks in the second cycle, it was decided not to analyse post-parturition traits corresponding to this cycle, i.e. litter and females weaning weights and prolificacy at weaning.

The two types of housing systems assessed differed in three main characteristics: i) the type of floor, ii) the presence of a roof, and iii) the possibility of social interaction between does. The results indicate that the differences observed between the two groups would be mainly due to the last factor, as the variables in which differences are observed are those that can be affected by it. Thus, from the beginning of the study until day 7 post-partum of the first reproductive cycle, the females of both groups were kept in individual places and no differences were observed in fertility or birth weight of the kits at birth. However, in subsequent reproductive cycles, females were inseminated 4 days after the females in P were placed in groups. Thus, females in park were inseminated when they were still establishing their hierarchies (up to six days post regrouping, according to Munari et al., 2020), resulting in lower fertility rates in this group of does than in individually-housed does (with significant differences only in the case of reproductive cycles 2 and 3).

Table 1: Effect of housing type on (re)productive traits of females housed in park (P) or cage (C) during six breeding reproductive cycles (RC1 to RC6).

	RC1		RC2		RC3		RC4		RC5		RC6		p-value[2]
	P	C	P	C	P	C	P	C	P	C	P	C	
n	20	20	19	18	19	18	19	18	19	18	18	17	
F[1]	85.0	85.0	21.1[a]	66.7[b]	52.6[a]	100.0[b]	57.9	72.2	57.9	83.3	61.1	76.5	-
BAK[1]	7.56	8.06	7.26[a]	12.50[b]	9.86	10.04	8.57	9.68	8.90	11.17	9.37	9.02	0.076
BWK1[1]	55.2	55.7	71.3[a]	60.5[b]	64.7	64.5	57.7	63.9	58.8	60.6	66.9	63.0	0.908
BWF1[1]	3.70	3.68	4.01	3.81	3.97	4.04	4.11	4.10	4.00	4.10	4.05	4.26	0.496
BWF7[1]	4.06	3.93	-	-	4.27	4.28	4.17	4.24	4.22	4.27	4.28	4.37	0.673
BWF35[1]	3.85	3.85	-	-	4.23	4.31	4.13	4.27	4.11[a]	4.48[b]	4.37	4.52	0.016
BWK35[1]	723	685	-	-	880	896	808	807	735	782	841	824	0.317
WK[1]	4.72[a]	7.79[b]	-	-	7.7	8.50	6.73	8.45	8.27	8.40	8.45	8.17	0.001

[1]values with different superscripts indicate significant differences within cycle ($p<0.05$); [2] effect of housing type; F: fertility; BAK: number of born alive per kindling; BWK1: average weight per kid at parturition (g); BWF1: weight of lactating females at parturition (kg); BWF7: weight of the lactating females at 7 days post parturition (kg); BWF35:weight of the lactating females at 35 days post partum (kg); BWK 35: weight of the kits at 35 days post partum (g); WK: number kits weaned per kindling.

There are previous studies that indicate that does should be protected from contact with other does during peripartum (Braconnier et al., 2020; Munari et al., 2020; Rommers et al., 2014). The range of days that does must be protected during this peripartum is controversial and has been studied by many authors (Braconnier et al., 2020). On the one hand, short periods of protection during peripartum allow the doe to be isolated from other females for the minimum time, but this increases aggressiveness and reduces productivity. On the other hand, long periods of isolation protect the doe and her productivity but reduce time in group. In this study, a short peripartum period was considered, so that the doe was protected from day 7 before parturition, to avoid aggressive interactions in late gestation, and then moved to group housing at 7 days post-partum trying to minimise the time the does are isolated. This study is part of a project in which a longer peripartum period will be tested in another batch of

does to obtain estimates of the economic and productive impact depending on the time of isolation during peripartum.

The percentage of culled or dead does was not significantly affected by housing type (table 2). However, at the end of the study, females housed in parks showed worse health status than those housed in cages. In the parks, almost half of the females (40.0%) were diagnosed with low weight, and this result agrees with those obtained at 35 days post-partum (table 1).

***Table 2**: Percentage of does culled or dead throughout the cycle and health incidences at the end of the study in park (P; n=15) and cage (C; n=16) housed females.*

	P	C	p-value
Culled or dead (%)[1]	25.0	20.0	0.999
With some health incidence (%)[2]	93.3	62.5	0.105
Mastitis (%)[2]	26.7	6.3	0.291
Low weight (%)[2]	40.0	6.3	0.069
Injury in vulva (%)[2]	13.3	6.3	0.149
Pododermatitis (%)[2]	40.0	31.3	0.894
Mucopurulent nasal discharge (%)[2]	53.3	43.8	0.862

[1] n=20 (group P) and n=20 (group C).
[2] n=15 (group P) and n=16 (group C).

CONCLUSIONS

We can affirm that females housed in parks had worse productive results and presented worse health status after six reproductive cycles than the females housed in cages. This would be, most likely, due to aggressions between them, which clearly represents a worsening of their welfare.

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AGGRESSION AMONG RABBIT DOES IN A PART-TIME GROUP HOUSING SYSTEMS: EFFECTS OF GENOTYPE AND ENVIRONMENTAL ENRICHMENT

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ABSTRACT

To evaluate aggression of reproducing does in a part-time system, 72 crossbred multiparous rabbit does of two genotypes (Grimaud and Hycole) were housed in individual modules of a park system. Then, 18 parks with 4 does were formed from 9 d before kindling until 2 d before kindling and from 12 d after kindling until weaning (33 d after kindling); 9 parks with Grimaud and 9 parks with Hycole does. Within genotype, 3 parks did not have any enrichment; 3 parks contained an elevated platform; the remaining 3 has both a platform and two PVC hiding pipes. Behaviour in all parks was video-recorded for 24 h at −9 d (at first grouping), +12 d (re-grouping), +19 d (nest opening) and +33 d (weaning of litters). The number of bites per 30 min per park was higher (1.22 and 1.79 events) at grouping (9 d before and 12 d after kindling) compared to 0.72 and 0.34 bites at 19 d after kindling and after litter weaning ($P<0.001$). A higher number of bites per 30 min per park was recorded in Hycole does compared to Grimaud at the first grouping (1.54 vs. 0.91; $P<0.001$), whereas differences between the two genotypes were not statistically significant within the other observation days. A higher aggression level ($P<0.001$) was recorded in parks without any enrichment compared to those enriched with platforms or those with both platforms and hiding pipes, with 1.44 bites per observation in the former and 0.86 and 0.76 bites in the latter, respectively. Additionally, significant interactions were recorded between the observation day and the genotype (with Hycole showing more aggression than Grimaud at the first grouping) and between the observation day and the enrichment (enrichments were more effective in reducing aggression at the two grouping times).

Key words: lactating does, collective housing, welfare, aggression, budget time.

INTRODUCTION

Collective systems for reproducing does implies an increase of the total available surface and a mitigation of the movement restriction, the main welfare consequence on farm for reproducing does (EFSA, 2020). Collective housing systems have been initially proposed to allow reproducing does to have contacts with adult conspecific, as under natural conditions (Stauffacher, 1992). Indeed, social requirements on farm are very different compared to natural environment (Rödel, 2022). As a consequence, a high level of aggression is recorded on farm when reproducing does are kept in groups, both in continuous and in part-time group-housing systems (Szendrő et al., 2019; Trocino and Xiccato 2024). Available studies tested different strategies to reduce aggression based on group size, grouping times, lactation management, or provision of environmental enrichments (Bujius et al., 2016; Zomeño et al., 2017, 2018; Van Damme et al., 2022, 2023). Krunt et al. (2022) also found that a proper combination of genotype and housing could reduce aggression and injury in group-housed rabbit does. Thus, the present study aimed at comparing aggression among reproducing does of the two most used commercial genotypes in Italy kept in a part-time housing system equipped or not with different types of structural enrichments, i.e. a platform or a platform and hiding pipes.

MATERIALS AND METHODS

At the 18th day of pregnancy, 72 crossbred multiparous rabbit does (3-5 kindling) (half Grimaud and half Hycole) were moved from a commercial farm to the experimental farm of the University of Padova and housed in individual modules of a park system (0.5 m^2; 92 cm length × 53 cm width) equipped with manual feeders, automatic drinkers, and nests. A total of 18 parks was formed by removing the wire-net walls between adjacent modules to house 4 does, and their litters when present, from −9 until −2 d before kindling, kept individually from −2 until +12 d after kindling, then re-grouped from +12 until +33 d after kindling (weaning). Within the 9 parks of each genotype, three groups were formed based on the environmental enrichment, i.e. 1) parks without enrichment; 2) parks enriched with a platform; and 3) parks enriched with both a platform and two PVC pipes (30 and 50 cm, respectively) for hiding. Litters were standardized to nine kits, controlled lactation was used with nests closed from +2 until +19 d after kindling. The behaviour of does in all parks was video-recorded with infrared cameras for 24 h in four days, i.e., −9 d (at first grouping), +12 d (re-grouping), +19 d (nest opening) and +33 d (weaning of litters). Aggressive interactions among does were recorded as number of bites between does during 30 min/h per 24 h, total observation time of 12 h/per park (experimental unit). The number of bites was submitted to ANOVA by applying a mixed model and the PROC GLIMMIX of SAS (2013), with observation day (OD, −9 d, +12 d, +19 d and +33 d from kindling), genotype (G), enrichment (E) (absence, platform, platform+pipe), observation hour and the interactions OD x G, OD x E, and G x E as fixed effects, the park as a random effect. The Bonferroni t-test was used to compare means.

RESULTS AND DISCUSSION

Significant interactions were recorded between the observation day and the genotype (Figure1a): In details, a higher number of bites per 30 min per park was recorded in Hycole does compared to Grimaud ones at the first grouping (−9 d) (1.54 vs. 0.91 bites; $P<0.001$), whereas differences between the two genotypes were not statistically significant within the other observation days. As for the interaction between the observation day and the enrichment, the differences in the number of bites among rabbit does kept in the differently enriched parks were especially large at the first grouping (−9 d), where the presence of the platform or the platform with the hiding pipes halved the number of bites that were recorded. Then, on the second grouping (+12 d) the reduction of the number of bites was significant only in parks with both enrichments (platform and pipes) compared to not enriched pens, whereas in the parks with only the platform an intermediate result was recorded. Finally, at +19 d, when nests were open and kits were free to go outside around the cage, no difference in bites was recorded among the different types of pens, whereas on the last recordings, when litters were weaned and does remained once again alone, aggression was higher in pens without enrichments compared to the enriched ones without differences for the type of enrichment. As for the main effect of the observation day, aggression among does was higher at the first grouping (−9 d) and at re-grouping (+12 d) compared at opening the nests (+19 d) and at litter weaning (+33 d), i.e. 1.22 and 1.79 vs. 0.72 and 0.34 bites recorded per 30 min per park, respectively ($P<0.001$) (Figure 2a). Then, no difference was recorded between the rabbit does of the two genotypes (Figure 2b), whereas the number of bites observed changed with the environmental enrichments in the parks ($P<0.001$) (Figure 2c). In details, a higher aggression level was recorded in the parks without enrichment compared to those enriched with platforms or those with both platforms and hiding pipes, with 1.44 bites per observation period in the former and 0.86 and 0.76 bites in the latter, respectively. Changes in aggression according to the different stages of the reproduction cycle are consistent with previous observations (Zomeño et al., 2017, 2018) and are dependent on the physiological condition of the reproducing does: hormonal changes related to kindling and the lactation peak account for the major aggression measured when grouping at 12 d after kindling compared to the first grouping. Then, at 19 d after kindling, aggression events were also mitigated by the presence of kits accessing the park and going out of the nests. Finally,

at 33 d after kindling, when litters were removed from the parks, aggression remained low as hierarchy had been already established. Consistently, other authors (Rommers et al., 2011; Andrist et al., 2012) reported a reduction of aggressive behaviors several days after grouping. Nevertheless, under field conditions and over four reproduction cycles in part-time housing systems, Van Damme et al. (2023) confirmed that post-grouping aggression cannot be reduced to acceptable levels by reducing group size (and stocking density) or by providing cage enrichment. On the other side, in small holding conditions and continuous collective systems, Krunt et al. (2022) found that different genotypes and environmental enrichments could be properly combined to reduce aggression.

CONCLUSIONS

The present study confirmed that grouping reproducing does in part-time systems is associated to a high level of aggression which decreases over time. On the other hand, under the present conditions, aggression was mitigated by the presence of environmental enrichments, such as platforms and hiding tubes. With respect to behaviour, a further evaluation on the same recordings should include any observation about possible positive indicators of animal welfare, based on body posture and/or affiliative behaviours, besides results related to the use of the two types of enrichments.

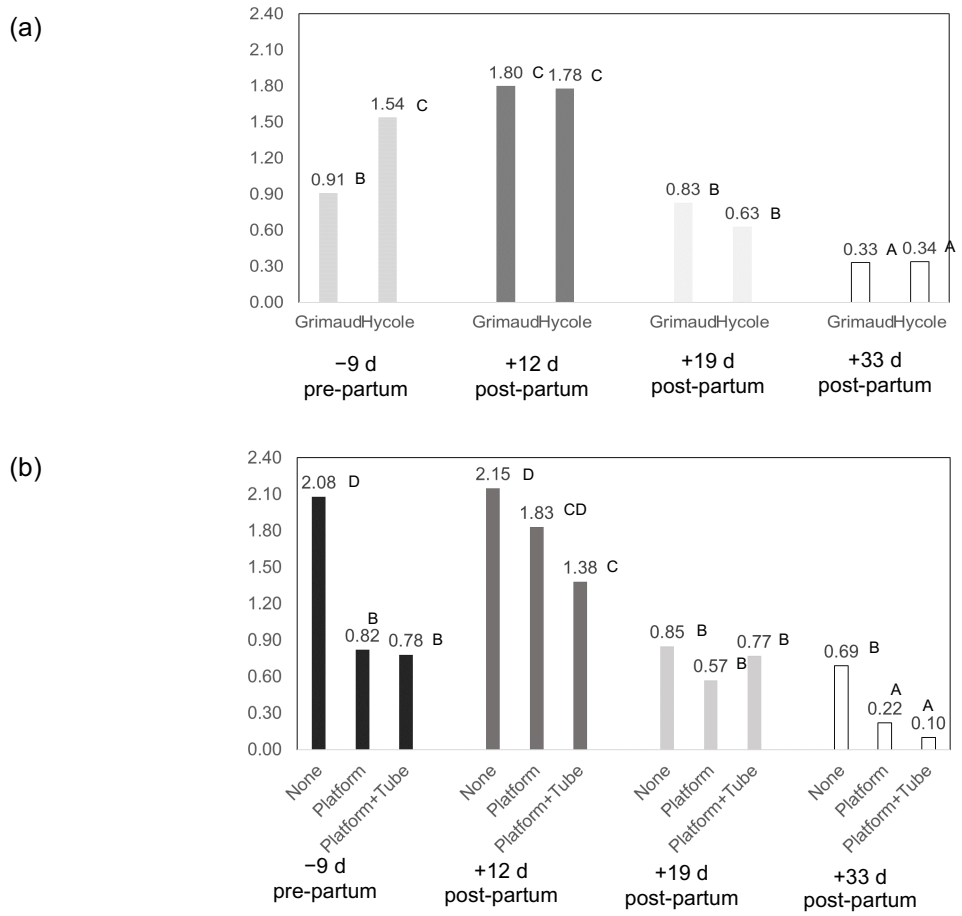

Figure 1: Interaction between: a) observation day and genotype; b) observation day and enrichment on the number of bites per 30 min per park (means) (P<0.001).

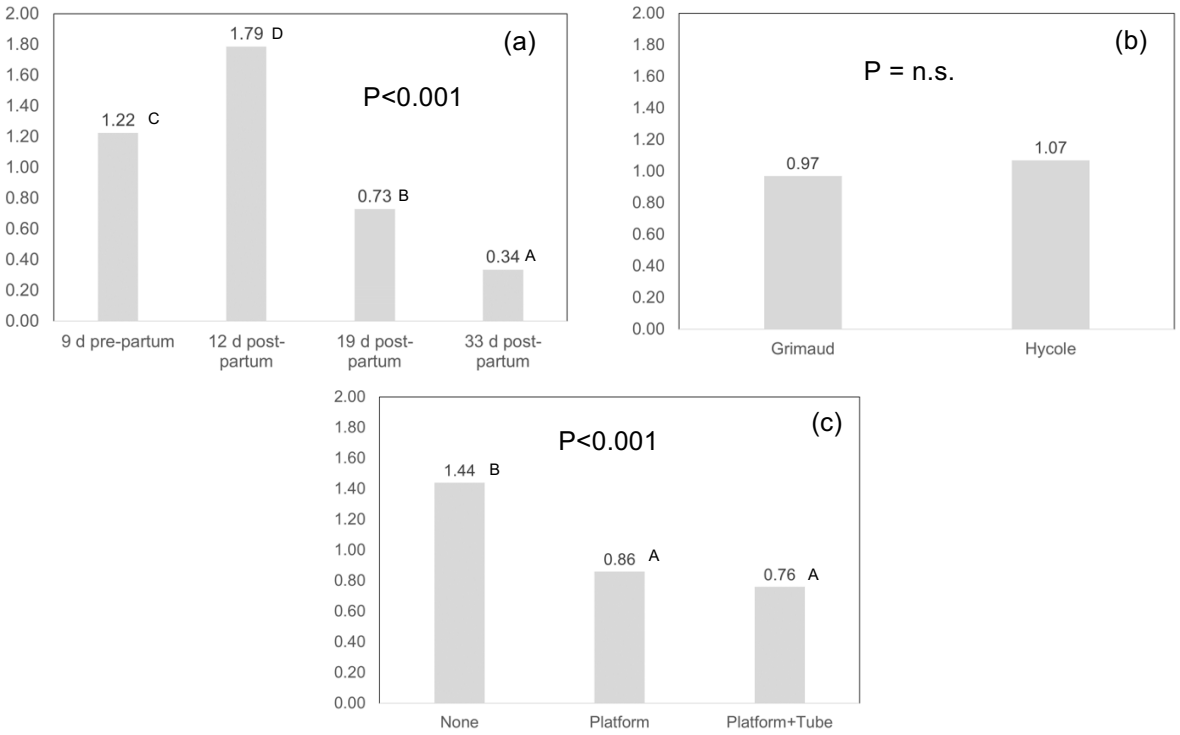

Figure 2: Effect of observation day (a), genotype (Grimaud; Hycole) (b), and enrichment (c) on number of bites per 30 min per park (means).

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GROUP-HOUSING OF YOUNG RABBIT DOES UNTIL FIRST KINDLING: BEHAVIOR, INJURY AND PERFORMANCES ACCORDING TO GROUP SIZE AND MANAGEMENT

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ABSTRACT

An experiment was performed to investigate the effect of housing young does in various group sizes from 11 weeks until a few days before kindling on behavior, injury and performances. 246 rabbit does were split into 6 experimental treatments: individual, 4 does, 8 does, 16 does, 4 does isolated around AI, 4 does with reduced stocking density. No influence of treatments was found on live weight. However, mortality was higher in groups of 16 does vs individual (p-value<0.05) and tended to be higher in groups of 8 does vs individual (p-value<0.1). Injuries were more frequent in all the treatment compared to the individual does (p-value<0.05) without any difference among the treatments. Fertility was severely impaired for all the treatments, except the one with isolated does around AI, with no possible occurrence of pseudo-pregnancy. Rabbit does housed in large groups were more in movements than individual ones (median 10.3% for groups of 16 does vs 2% for individual; p-value<0.05) and movements were more frequent with reduced density. The proportion of does observed in social interaction ranged from 25 to 30%, with no statistical differences between treatments, which highlights the importance to provide does the choice to interact with another does. This study illustrates welfare benefits of grouping young does together (social interactions, more movements), but also an impaired fertility, which is not viable for a commercial farm.

Key words: welfare, social interaction, fertility.

INTRODUCTION

Studies on different housing systems for domestic rabbits showed that group housing of breeding does lead to more aggressive interactions than individual housing (Szendrő *et al.*, 2019). A focus on the behavior of the European wild rabbit (in the wild or under semi-natural conditions) tells us that these wild rabbits live in groups except around parturition when females isolate themselves and look for the best nest sites without disturbances (Rödel *et al.*, 2021). These gregarious animals express many social interactions, thanks to olfactory communication (to recognize each other or to define their living area), auditory / visual communication (to recognize each other, to alert congeners in case of a perceived danger) and tactile communication (to establish hierarchy through aggressive interactions, to ensure group cohesion).

Group housing systems are currently used in commercial farms for growing rabbits, which enables social interactions and many movements (approx. 11% of the farms in France in 2022, ITAVI database). Systems with continuous group housing of does were studied in the literature but have been unsuccessful to be applied in commercial farms (Van Damme *et al.*, 2021). A very high frequency of aggression between females was found, as well as impaired reproductive performances (kits mortality, pseudo-pregnancies) (Van Damme *et al.*, 2021; Huang *et al*., 2021; Laclef *et al*., 2021). Part-time group housing systems for does may contribute to solve these problems by allowing individual parturition as well as social interactions during some part of the reproductive cycles (Szendrő *et al*., 2019). However, aggressive behaviors remain an unsolved problem when regrouping the does after a short period of isolation around parturition, even when hiding places are provided (Van Damme *et al*, 2023). When housing together familiar does, only 2% of severe injuries were observed

before the first parturition, and up to 27% during the reproductive life of the does, which is detrimental to their welfare (Laclef *et al*., 2021).
In France, all young rabbit does are housed in groups until 11 weeks. Then, they are isolated in individual cages for the rest of their reproductive cycle. Raising does individually prevents them from being injured. However, this also prevents positive social interactions such as mutual grooming or resting side by side. Collective housing of rabbit does would also increase the total available space and ease the expression of other specie-specific behaviors such as hops. The aim of this study was to assess the feasibility and the consequences of housing young rabbit does in group before the first parturition.

MATERIALS AND METHODS

Animals and experimental design

246 rabbit does (PS Hyplus optima®, Hypharm) were housed in groups of 5 young females from 5 weeks to 11 weeks at INRAE experimental facilities. The does were feed restricted from 5 to 23 weeks of age (i.e. 1 week before parturition) then fed *ad libitum* until the first kindling. Does had access to fresh water through nipple drinkers. The trial started from week 11, when young females were split into the 6 experimental treatments as described in Table 1. All the females were isolated 4 days prior to parturition, except for "4_d_AI" treatment where does were isolated also 3 weeks before and 1 week after 1st artificial insemination (AI) at 20 week of age.

Table 1: Experimental treatments

Treatment ID	Individual	4_d	8_d	16_d	4_d_AI	4_d_6u
Group size	1	4	8	16	4	4
Housing units number	1	4	8	16	4	6
Density (cm²/doe)	4050	4050	4050	4050	4050	6075
Replication	30	8	4	4	8	8
Total does number	30	32	32	80	32	32

Each housing unit was made of plastic-mesh floor (46cm x 93cm x 90cm, l x w x h) with a platform (46cm x 25cm, l x w) fixed at 35cm above the floor. A nest box (45 × 25, l × w) was located in the front of the unit. For collective housing, adjacent housing units were connected together 4 to 16 housing units via hatches located on the platform and in the nest box. Enrichments were provided with 1 compacted forage block, 1 wood stick, 1 PVC tunnel, 1 steel chain and 1 nest cover for 4 rabbit does (except for the "Individual" treatment: only 1 wood stick due to the lack of space).

Data collection

Live weight of females was controlled each week from the beginning of the trial (11 weeks of age) until 1 week after the 1st AI (20 weeks of age). Live weight was then controlled after kindling, at 24 weeks of age. Does mortality was recorded daily and included dead does and euthanized does by animal keepers (because of injuries or serious health disorders). Fertility ratio was also recorded after the first kindling for all the females.
The presence of injuries was recorded weekly from 11 weeks of age until the 1st AI. A 4 point scale grid was used to evaluate the severity of the injuries: 0 = no damage; 1 = minor injury with scratching on the earn, torn hairs; 2 = moderate injury with torn nail, scratch on the eye or on the back, damaged epidermis, hair torn off by tufts; 3 = serious injury with damaged dermis, raw flesh. The proportion of each score was calculated for each treatment replication, as well as a "mean injury score", that is calculated as follows for each treatment replication:

$$\text{Mean injury score} = \frac{\text{Number of score 1} + 2 * \text{number of score 2} + 3 * \text{number of score 3}}{\text{Total number of scored does}}.$$

Does movements (hops / jumps) and social positive interactions (social contact, mutual grooming and simultaneous use of an enrichment) were assessed by direct observation twice a day (around 10 a.m. and 2 p.m.) and respectively 2 days a week and 4 days a week from the beginning of the trial until the isolation of the does. Mean percentages of movements and of females engaged in social positive interactions were calculated for each

treatment replication, for the whole period (11 to 23 weeks of age). The distribution of the does in the different housing units was also recorded twice a day (around 10 a.m. and 2 p.m.) and 2 days a week from the beginning of the trial until the isolation of the does.

Statistical Analysis

Analyses were performed using the software R version 4.3.3 (R Core Team, 2024). Data were analyzed using non-parametric tests (Kruskal-Wallis and Dunn-Test for pairwise comparisons) as the data distributions were not adequate to run parametric tests. The effect of group size (4_d, 8_d, 16_d), as well as the effect of management practices (isolation around AI: 4_d_AI; increased available space: 4_d_6u) was analyzed regarding live weight, mortality, fertility and injuries. The effect of group size (4_d, 8_d, 16_d) and increased available space (4_d_6u) was analyzed regarding movements and social positive interactions.

RESULTS AND DISCUSSION

Effect of group size and management on performances and injuries

The live weight of the rabbit does was similar between treatments until 21 weeks (median live weight at 21 weeks 4131g; NS).

Mortality was significantly higher in 16_d treatment than in individual treatment (median mortality respectively 18.8% vs 0%; p-value<0.05) and tended to be higher in 8_d treatment than in individual treatment (median mortality respectively 12.5% vs 0%; p-value<0.1).

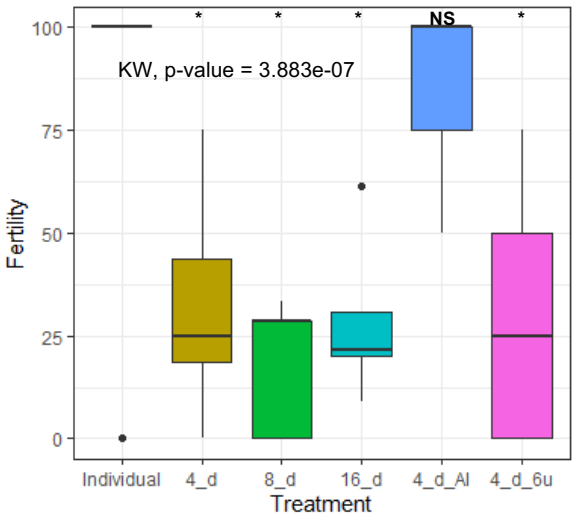

Figure 2a: Fertility ratio according to treatment (horizontal lines = median values for each treatment)

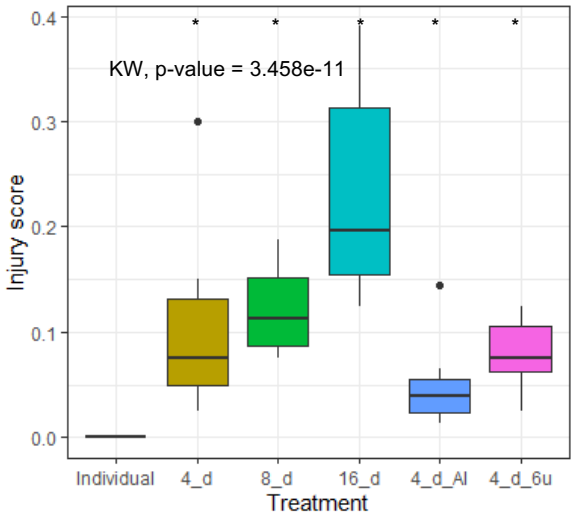

Figure 1b: Injury score according to treatment. (horizontal lines = median values for each treatment)

Except for the does isolated around AI (4_d_AI), fertility was significantly reduced in all treatments compared to individual treatment (median fertility of 25% for 4_does, 28.6% for 8_does, 16% for 16_does vs 100% for individual; p-value<0.05; Figure 1a). Such a result could be explained by pseudo-pregnancies as overlaps were frequently observed around AI, as well as many hairs plucked, probably to prepare nests. A previous study did not found any effect on fertility when housing does by 2 until first mating (Rommers *et al*., 2012). The isolation of the does before and after AI (4_does_AloneAI treatment) prevented these pseudo-pregnancies to occur and enabled a fertility rate similar to those of housed individually (median fertility of 100% for both treatments; NS). Pairwise comparisons indicated no difference between 4_d, 8_d, 16_d and 4_d_6u.

The mean proportion of non-injured does in the group-housed treatments reaches 89.8%, which is consistent with a previous study that highlighted less than 2% of severe scores before the 1st parturition (<0.3% in our study) (Laclef *et al*., 2021). The injury score was

significantly higher in all the treatments compared to the individual treatment (p-value<0.05: Figure 1b), without any significant differences when running pairwise comparisons. A previous study concluded to increased agonistic interactions when the group size was larger (Szendrő *et al.*, 2009).

Effect of group size and management on behaviors

Rabbit does housed in large groups were more in movements (jump, turn around) than individual ones (median 4.5% movements for 4_d, 9.5% for 8_d, 10.3% for 16_d, 5.6% for 4_d_6u vs 1.2% for individual; p-value<0.05) and pairwise comparisons showed more movements with larger group size (p-value<0.05). Movements were also more frequent with reduced density (median 5.6% for 4_d_6u vs 4.5% for 4_d; p-value<0.05). Thus, the additional space enables does to express more hops, a specie-specific behavior that contributes to improve their welfare.

The proportion of does observed in social interaction ranged from 25 to 30%, with no statistical differences between treatments. It highlights the importance to provide does the choice to be alone in a unit, or together with another doe in the same unit. Moreover, higher level of positive social interactions could led to a lower physiological stress response and to a better health status (Rödel and Starkloff, 2014).

CONCLUSIONS

Currently in France, does spent approximately 13% of their life with conspecifics of the same age (from birth to 11 weeks). Raising them in groups until first parturition would increase this figure to 28%, enabling does to interact more with conspecifics, with benefits regarding behavioural needs satisfaction (social positive interactions, more movements). However, this study highlights a very impaired fertility, which is not viable for a commercial farm. Moreover, group-housed does in limited size environments can have negative consequences such as injuries and mortality. Isolation of females 3 weeks before AI and 1 week after is a promising way to enable social interaction while maintaining fertility rate.

ACKNOWLEDGEMENTS

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USE OF THE WINTER GARDEN BY RABBITS

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ABSTRACT

Animal welfare is a very important topic for animal production, including rabbits. A new project for alternative housing system, named Wellap®, has been set with several elements: behavior, feeding, know how on farming and building. The aim of the present trial was to assess the use of the winter garden. 2 pens of 100 weaned rabbits were filmed during the whole period of fattening. A picture was taken every 10 minutes and analyzed by artificial intelligence to define winter garden attendance. Sanitary status was very good in this trial (98.6% of viability). With age, the number of rabbits visiting the winter garden increased significantly, from 8.7% during the period 44-50 days of age to 20.7% during the period 79-84 days of age ($p<0.00001$). Rabbits went out early in the morning and late in the evening. Between 7 a.m. and 8:30 a.m. and from 5:30 p.m. to 7 p.m. close to 25% of the rabbits were outside, whereas, between 10 a.m. and 4 p.m., around 10% of the rabbits were outside ($p<0.00001$). This trial confirms that raising rabbits with access to the outside with pens of 100 rabbits is possible, enabling very good growth performance.

Key words: Animal welfare, animal behavior, alternative housing, pens, fattening rabbits.

INTRODUCTION

Animal welfare is a very important topic for animal production, including rabbits. In 2017, the European parliament questioned the current rabbit farming conditions. The parliament, following the requests of the welfarist NGOs, wants breeders to gradually abandon the cages and replace them with alternative solutions. Studies have been published on fattening rabbits in pens. Housing in pens can sometimes degrade health status when the density is too high and sometimes even alter the growth performance of fattening rabbits, but also allows to develop a more complete behavioral pattern (Maertens et al., 2011; Szendrő and Dalle Zotte, 2011; Trocino et al., 2014).

After 8 trials in ADM experimental farm, the Wellap® concept has been developed in the field since 2 years. In this new building, rabbits have access to outside 24/24. After several bands we demonstrated good growth performance (Guené et al.,2021). These results validate the concept and the building. Thus, the aim of this study, is to collect information regarding attendance at the winter garden.

MATERIALS AND METHODS

Animals and experimental design

The farm, located in Rhône-Alpes-Auvergne in France, has 450 Hycole rabbits does which are inseminated with the PS59 Optima Hypharm buck. The reproduction cycle of this breeding is 49 days long.

The fattening room with winter garden is located in a 120 m² building made of insulated sandwich panels. The building is 6m wide, of which 3m is dedicated to housing the rabbits indoors, 2m to housing the winter garden and 1m for the corridor. The building is made up of 10 pens measuring 2m wide and 5m long (3m indoors and 2m winter garden), enriched with 2 mezzanines of 0.70m² each. The deployed surface area of each pen is therefore 11.4m² including 10m² on the ground. Ventilation is natural, static type, and takes place along the length of the building which is open at the ridge. In winter, 2 small removable hatches are open and allow 24-hour access to the winter garden, while in summer, a large translucent partition is removed and allows natural lighting whatever the season. The structure of the

building has a longitudinal groove under the roof serving as skylights, to create a continuous flow of air.
Each pen contained 100 rabbits weaned at 37 days of age. The duration of the fattening period was 44 days after weaning, the trial took place from the 7th of April to the 21st of May 2023. The allocation of animals in pens was random.
Animals received the same feed program (a commercial post weaning feed followed by a commercial finishing feed), with access to the feed 12h/day. Two gnawing blocks (1 kg Lapety Bloc Fourrage®: 80% of alfalfa and straw) were placed in each pen; 1 inside and 1 outside.

Measures and statistical Analysis

A camera was placed under the roof outside, between two pens among the 10, so as to see the entirety of these two pens. A picture was taken every 10 minutes and analyzed by artificial intelligence. CO2 and NH3 were recorded by a sensor on the camera. Data were represented using a ggplot with R software and were analyzed with a GLM procedure.

RESULTS AND DISCUSSION

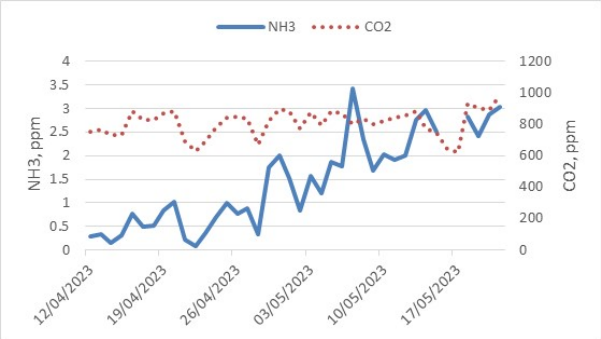

Figure 1: NH_3 and CO_2 levels during the trial

In this trial, the sanitary status was very good, the viability was 98.6% and rabbits weighed 3.123kg at the end of the fattening period (81 days of age).
NH_3 air concentration increased with the age of animals because of the stockage of droppings under the duckboard, from 0.3 at the beginning to 3 ppm at the end of the fattening period. The CO_2 air concentration was quite stable during the fattening period.
These levels are low because there are measured outside, and are thus acceptable.

Winter garden attendance

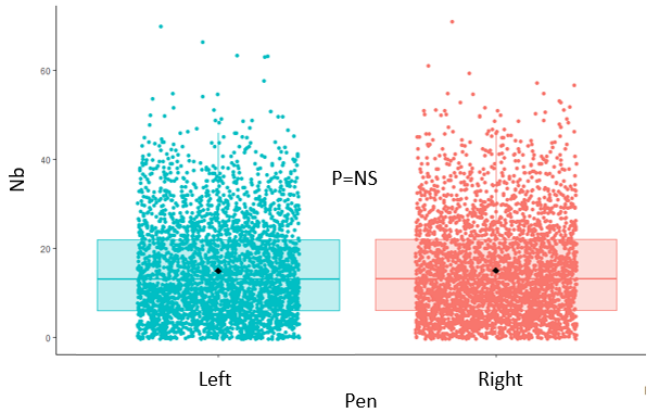

Figure 2: Attendance in the 2 pens

In Fig. 2, each point represent a picture and the number of rabbits that were outside on this picture.

Attendance at the outdoor pen was counted via photos taken by the camera.
The average number of animals visiting the outdoor pen was 15 rabbits/day regardless of the pen studied. There was no difference between the 2 pens studied (Fig 2, P=NS).
Pens are thus comparable.

Pen attendance according to the age of rabbits

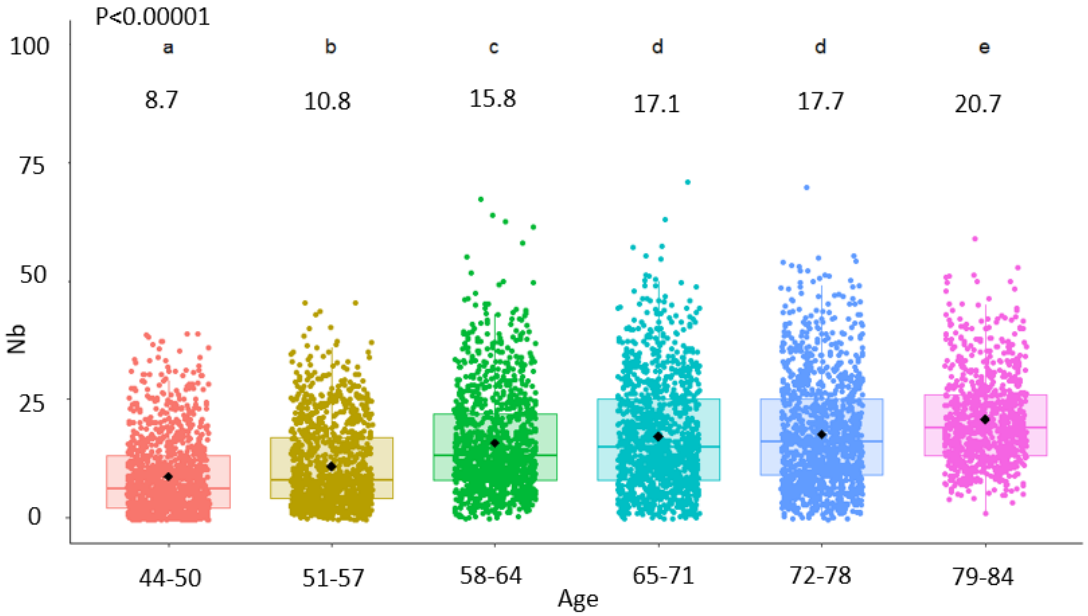

Figure 3: Number of outside rabbits according to the period in the trial.

The number of rabbits visiting the winter garden area increased significantly with the age of the rabbits (Fig 3). This result is consistent with data observed in the study of Guené et al., 2021.

Pen attendance according to time of day

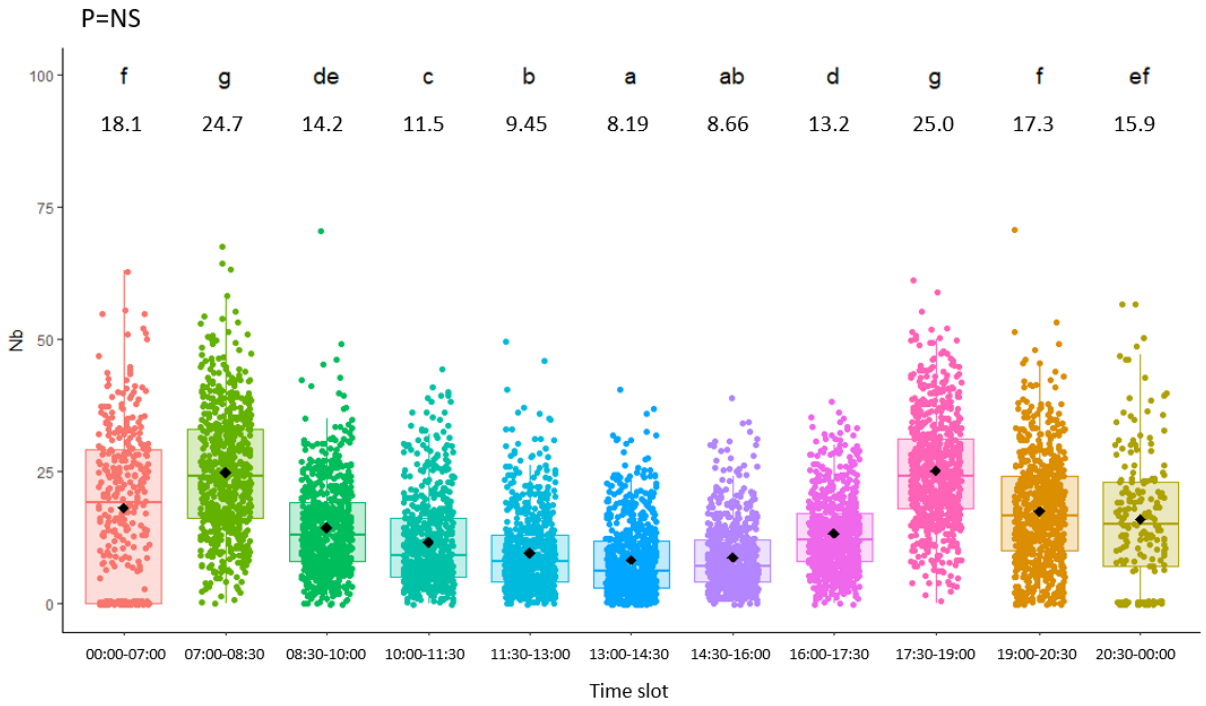

Figure 4: Number of outside rabbits depending on the time of the day.

The rabbits visited the pens in greater numbers at the following times: 7 a.m.-8:30 a.m. and 5:30 p.m.-7 p.m. (p-value <2.2e-16, Fig. 4). On the other hand, at the interval 1-4 p.m., attendance was the lowest.
These results are consistent with data observed in the study of Guené et al., 2021, in which rabbits went outside in the morning and stayed inside in the early afternoon.

CONCLUSIONS

This trial was performed to assess pen attendance. Attendance at the winter garden was not different from one pen to another. With age, use of the winter garden increased. The results of this study confirm previous observations made during in station tests. In view of these observations, rabbits moving from the beginning of cage breeding to a winter garden seem to adapt well since the use of the winter garden increased with age.
Camera with night vision should be very interesting to have an idea of the behavior of the rabbits during the night. It would have been interesting to equip each rabbit with an RFID chip to study whether it is always the same rabbits that come out or not, and thus better understand their behavior. This study could also be repeated in different seasons to study its impact on rabbit behavior

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CAGES VS COURTYARD: EFFECT OF THE HOUSING CONDITIONS ON BEHAVIOUR, STRESS AND PERFORMANCE OF GROWING RABBITS

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ABSTRACT

Abstract

Rabbit welfare is a concern for society. It is strongly influenced by living conditions. We compared the welfare (behaviour and stress), growth and viability of 60 rabbits (♀ Hycole x ♂ PS119 Hypharm), reared in two different housing conditions from 38 (weaning) to 70 days: standard dual-purpose cages (Cage group) versus pen with a winter garden (Courtyard group). The growth was higher in Courtyard group (2.75kg vs 2.46kg at 70 days, $P < 0.01$), the viability was similar for both housing conditions (97% vs 97.5%, NS). The hair corticosterone levels at 70 days tended to be higher in Courtyard group than in Cage group (2.48 vs 1.76 pg/mg hair, $P= 0.08$). In the Courtyard group, rabbits were more active and expressed more exploration behaviours (walking; running; rearing) and more grooming behaviours while in the Cage group, rabbits rested more and had more contact interactions ($P < 0.05$). Access to a courtyard gave the rabbits a richer behavioural repertoire. In conclusion, the present results showed that access to a courtyard could be a good solution to improve rabbit welfare, by diversifying the behavioural repertoire and increasing growth without increasing stress or reducing animal viability.

Key words: Rabbit, Corticosterone, Behaviour, Housing conditions.

INTRODUCTION

The conditions in which animals are reared are of concern to society as a whole, whether professionals (breeders and production sectors), citizens or consumers (IFOP, 2024). The rabbit farming industry (conventional model) is no exception. It has been highly rationalised over the last decades, and progress has been made in all areas of farming (genetics, feed, reproduction, etc.). But it is now being criticised for not allowing animals to fully express their natural behaviour (ANSES, 2018). However, welfare refers not only to behaviour, but also to the stress and health of animals.

Corticosterone is a biological marker of stress and adaptation (Mormède *et al.,* 2007). This molecule is present in all tissues, (body fluids and skin) but as hair is the site of a progressive accumulation, it provides information on chronic animal stress over a period of time.

Rabbit welfare is strongly influenced by living conditions (Fetiveau et al., 2021). The aim of this experiment was to compare i) hair corticosterone values, ii) growth, iii) viability, iv) behaviour, of rabbits reared in standard dual-purpose cages or in floor pens that provide access to an outdoor courtyard.

MATERIALS AND METHODS

Animals and experimental design

The experiment took place in a commercial farm from the 11th of November to the 12th of December 2022. The farm has 450 Hycole does housed in a building comprising two dual-purpose halls with dynamic ventilation, cooling and air extraction and equipped with 820 dual-purpose housing (0.395mx1mx0.3m) with no enrichment (named below as Cage). There is also a 120 m² fattening building with ten 2m x 2m floor pens enriched with two

mezzanines (0.70m^2) and giving a 24-hour access to an 2m x 3m outdoor winter garden (Wellap® system named below as Courtyard (Guené-Grand *et al.,* 2021a, b). Ventilation and lighting are natural. The density is 6 rabbits per dual-purpose housing (650 cm^2/rabbit) or 100 rabbits per pen (1170 cm²/rabbit including indoor pen, outdoor courtyard and mezzanines).
We studied 60 growing rabbits from 15 Hycole does inseminated with PS119 Optima Hypharm semen (4 rabbits per doe). The rabbits were weaned at 38 days of age and distributed in two experimental groups differing in housing conditions: Courtyard group (two rabbits of both sex from each of 15 selected litters = 30 studied rabbits in one pen plus 70 other rabbits), or Cage group (two rabbits from each of the same 15 selected litters per cage plus 4 other rabbits per cage = 30 studied rabbits distributed in 15 cages). Throughout the experiment, rabbits were fed 12 hours a day with a commercial pelleted diet formulated to meet the growing rabbit needs (2300 kcal digestible energy/kg, 14.8% crude protein, 18.9% crude fiber, 2.5% crude fat, 1 ppm Diclazuril ; than 2440 kcal digestible energy/kg, 16.7% crude protein, 16.8% crude fiber, 2.1 % crude fat).

Zootechnical Measures
Rabbits were weighted at 37 (weaning at 38 days of age) and 70 days of age. We selected the rabbits to have the same range of weight at day 37 (1.19±0.10kg vs 1.22±0.07kg in Cage and Courtyard group, respectively; *p*-value 0.55). Mortality was recorded daily. Temperatures were recorded daily in the Courtyard modality.

Corticosterone Dosage
The method was adapted from Davenport *et al.* (2006; see Fillon *et al.,* 2023). At 37 days of age, we shaved the rabbits on the back, near the shoulder on a 5cmx5cm area with a small hair clipper and discarded the hair. The hair samples (250 mg per rabbit) were collected at 70 days by shaving at the same location and stored at -20°C. The hairs were washed twice with isopropanol, then dried and ground to powder. The corticosterone was extracted from 50 mg powder with 1,5 mL methanol. After centrifugation (15 min 13000g) and drying (3H20 at 30°C), the dry pellet was resuspended in 100 µl dilution buffer (Salimetrics® Cortisol). The Elisa immunoassay dosage was performed with Salimetrics® kit (Carlsbad, CA, USA) according to the user manual.

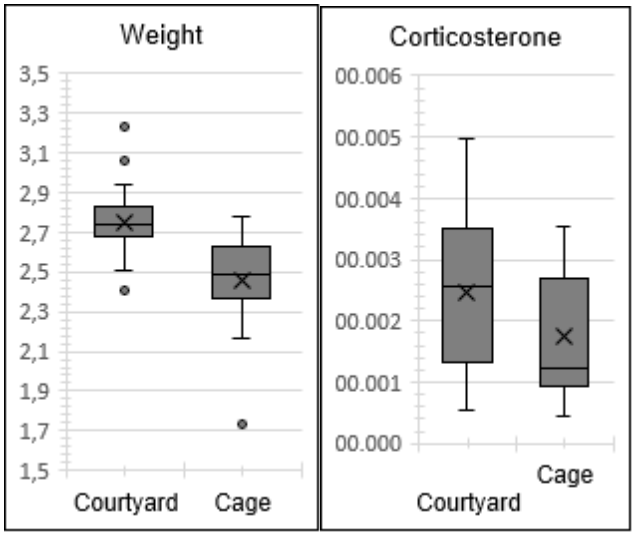
Behavioural Evaluation
The rabbits' behaviour was assessed at 48 and 70 days of age using the scan sampling method (Altmann, 1974) with direct observation during 10 minutes per housing conditions, one time in the morning and one time in the afternoon to obtain 4 different observation sequences per housing conditions For the Courtyard group, the 10 minutes of observation were divided up as follows: 5 minutes for the indoor pen, and 5 minutes for the outdoor courtyard. We shifted to a different rabbit every 2 seconds during ten minutes so as to obtain approximatively the same number of observations despite different number of rabbits (100 rabbits in one pen versus 90 rabbits in 15 cages). The following 18 behaviours were recorded: moving (walking, hopping, running, climbing, jumping), maintenance (eating, resting, gnawing, eating feed blocks, drinking), comfort (grooming, stretching), exploration (rearing, scratching, observing), social interactions (side by side, nose to nose, allogrooming).

Statistical Analysis
The statistical analyses were performed using R software (R Core Team, 2022; https://www.R-project.org/) using Chi square test (viability) or anova housing conditions as fixed effect in the model (weights, corticosterone level, and behavioural data).

RESULTS AND DISCUSSION

The viability was similar in both group (97% vs 97.5% in Courtyard and Cage group; NS) and same as that referenced in French farms (97,1% see Pedro, 2023).

Figure 1: Effect of housing conditions on weight and corticosterone level (pg/mg hair) at day 70

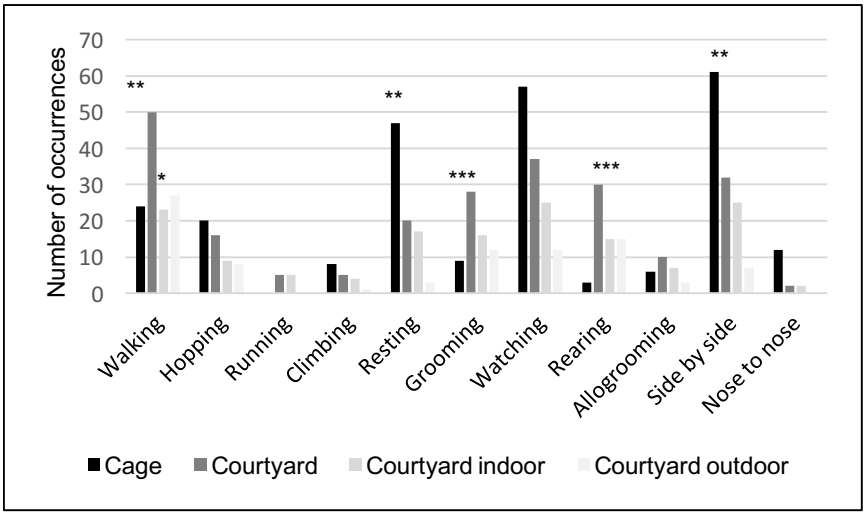


The weight of rabbits at 70 days was higher (+290g) in Courtyard than Cage group (2.75kg vs 2.46kg; $P < 0.01$; Figure 1). This result is consistent with previous results in the same farm (André *et al.,* 2023). In the present study, the rabbits of Courtyard group were $P < 0.01$ exposed to a lower temperature compared to the Cage group building (temperature set between 18 and 20°C in the cage building; 14.7°C on average in the indoor pen for Courtyard group, winter temperatures on average 5.4°C in the outdoor pen) and could have eaten more. $P = 0.08$

The corticosterone levels tended to be lower in the hair of Cage group rabbits than Courtyard group (1.76 vs 2.48 pg/mg hair, $P = 0.08$; Figure 1). This could be explained by a more intense metabolism (thermoregulation, physical activity, faster growth, higher feed intake) in Courtyard group as previously showed in humans (Pontzer et al., 2021). Other studies have shown significant higher hair corticosterone concentration for rabbits raised in pens compared to cages (Trocino *et al.,* 2014, 2022). On the opposite, the lower levels in Cage group could be due to a lack of activity or apathy. Nevertheless, values measured are in the normal range (Fillon *et al.,* 2023).

The jumping, eating feed blocks, stretching and scratching behaviours were never observed during the 10 minutes of behaviour recording. Eating, drinking, gnawing behaviours were rarely observed and were not analysed. The hopping behaviour was discarded from analysis due to a bias in Cage group (reaction to observer). In the Courtyard group, rabbits were more active and expressed more exploration behaviours (walking; running; rearing) and more grooming behaviours (Figure 2).

Figure 2: Effect of housing conditions on behaviour



In the Cage group, rabbits rested more and had more contact interactions (side by side; nose to nose). The more frequent interactions could be the result of the higher density in Cage group compared to Courtyard group. We cannot rule out the possibility that the size of the group

(100 vs. 6) modified the types of interactions. For example, Trocino *et al.* (2022) observed that a larger number of individuals could lead to competition at the feeder. Finally, in the Courtyard group, rabbits walked more outdoor ($P<0.01$) and rest more indoor ($P<0.05$). When resting, rabbits were frequently observed hidden under the mezzanines. Thus, present results showed that in Courtyard system, rabbits could have a spatialized activity with exploration behaviour outside, and resting behaviour inside.

CONCLUSIONS

The present results showed that access to a courtyard could be a good solution to improve rabbit welfare, by diversifying the behavioural repertoire and increasing growth without increasing their stress or reducing animal viability. However, some further studies with more experimental units (more pens, more behavioural data) would be of interest to confirm our findings.

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STUDY OF THE FATTENING RABBIT BEHAVIOR ACCORDING TO FOUR HOUSING SYSTEMS

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ABSTRACT

This study compares the behaviors of 454 fattening rabbits from 32 to 71 days old, in 4 different housing systems: conventional cages (CC), wired pen (WP), floor pens with and without outside access (respectively FPOA and FP). Movies were recorded all along the trial and were analyzed by the scan sampling method at 3 ages (41d, 55d, 69d) and at 3 different hours (9.00 am, 12.00 am and 12.00 pm). Behaviors were grouped in 7 categories and were linked to 6 zones. Regardless of housing systems and periods studied, the main activity is "resting" (66% of animals), mainly followed by "feeding and drinking" (12%), "grooming" (9%) and "moving" (8%). "Resting" varies mostly according to the outside access (70% in FP versus 58% in FPOA; $p<0.05$). For every enrichment (on the top and under PF, in hiding places, on outdoor area), the rest is the main activity ($p<0.05$). « Feeding and drinking » involves respectively 8%, 12%, 12% and 15% of rabbits in CC, WP, FP, and FPOA. This activity decreases with increasing weeks only for floor pens, with a more significant decrease for FPOA ($p<0.05$) due to the decrease of available grass to consume. « Grooming » is expressed by 16% of rabbits in CC (versus in average 7% in other housing systems) and evolves from 10% à 41d to 21% at 69d, which suggests an overexpression of this behavior in CC. « Moving » is equal to 13% in the FPOA (versus 6% in average for the other housing systems) and is significantly higher at 9.00 am (26% versus 8% in average for the other housing systems; $p<0.05$) due to the recent opening of the outside area. « Standing » is rarely observed even though rabbits have more the possibility to do it in pens. For animals with an outside access (FPOA), the outside area is highly used at 9.00 am (61% versus 14% at 12.00 am) all along the fattening period, with a strong diversity of behaviors (gnawing, digging a burrow, …). In summary, floor systems tested allow to satisfy some behavioral needs of rabbits (moving, standing…), which tend to meet the consumer' expectations about animal welfare. But they seem to perform less : there was a higher sanitary sensitivity in FP and FPOA (8% versus 0% pour CC and WP) which impacted their feed intake (-20%). These findings should help to develop a multi-criteria evaluating method more suitable to large group including animal welfare and performances as well as working comfort of breeders.

Key words: rabbit, welfare, housing system, behavior

INTRODUCTION

Animal welfare is an important preoccupation for all European animal productions. In rabbit farms, the improvement of animal welfare includes the study and the implementation of new housing systems, with enrichments of the environment, collective housing with larger or smaller group size, or outdoor access (EURCAW-Poultry-SFA, 2021). The aim is to offer to rabbits the possibility to express more natural behaviors (standing, moving, isolating…) and to maintain their performances. Thus, enriched cages and wired parks with about 30 rabbits were created, but they are made up of wire which is criticized by consumers' associations. That is why floor pens housing systems appeared and allow to house large groups (> 100 rabbits) and allow more various enrichments. In parallel, several surveys show that more than 60% of French consumers would like animals to have an outdoor access (Roguet C., 2017). But only some specific farms offer this possibility to rabbits (organic farms, outdoor breeding systems). In this global context, some studies compared rabbit housing systems with different size, stocking rate, floor type or enrichments. But none evaluates more than 2 housing systems during the same period with large group size in floor pens and with an

outdoor access. This study aims to compare behaviors of fattening rabbits according to 4 housing systems.

MATERIALS AND METHODS

Animals and experimental design

The trial took place in the experimental station of Saint Symphorien (France) at spring 2022. At weaning at 32 days old, 454 rabbits (Hyplus Optima x P69J9) were allocated to 4 groups according to their weight. Water and feed (14.9% crude protein, 3.2% crude fat, 17.6% crude fiber) were distributed ad libitum. Each group was housed in different housing systems : ***1) conventional cage (CC)*** : 28 rabbits housed in 4 cages of 7 rabbits with a surface of 0.42 cm² and a stocking rate of 16.7 rabbits/m² ; ***2) wired pen (WP) :*** 28 rabbits housed in 1 wired park with a surface of 2 m² with 2 platforms (PF) of 0.4 m² each for a stocking rate on the floor of 14 rabbits/m² ; ***3) floor pen without outdoor access (FP)*** : 200 rabbits housed in a pen with plastic slatted floor with a surface of 16 m² and a stocking rate on the floor of 12.5 rabbits /m² ; enrichments used were 2 PF with a total surface of 0.88 m² and hiding places (one niche and one pipe) ; ***4) floor pen with outdoor access (FPOA)*** : 198 rabbits housed in the same pen as FP but with an access from 8 am to 4 pm to an additional enrichment : a 13 m² outdoor area composed of 2 different floor materials (grass and slatted floor).

Video Analysis

Cameras were installed in each housing and videos were recorded every day of the trial. 3 ages (41d, 55d, and 69d) were studied in order to follow the behavior of rabbits all along the trial. Analysis were made on videos of Saturday (low disturbance from Humans) at 3 different times : 9 am, 12 am and 12 pm. Videos were analyzed with the scan sampling method : for each time, 3 video sequences of 10 seconds each within 5 minutes were studied. For the outside access (for FPOAA only), each result of 1 age is the average of 3 different days in order to minimize the impact of weather on the use of the outdoor area. Behaviors of rabbits were grouped according to several categories, inspired by EBENE® (Warin et al., 2017) : feeding (drinking and eating), grooming, resting, moving (short when less than 3 hops, long when greater or equal to 3 hops), standing, interacting socially (positive interactions and aggression) and others behaviors (stretching, rubbing, gnawing non feed material…). These behaviors were linked to 6 zones which were previously defined : feeder, drinker, PF, hiding places, floor and outside area. For each housing, all rabbits were counted and assigned to a behavior and a zone of the housing. Then the number of rabbits for each behavior and each place was related to the total number of rabbits of the housing, resulting in a proportion of animals per activity and per zone. For limiting bias, behavior analyses were done by only one person. Moreover, mortality and environmental parameters (temperature, hygrometry…) were recorded every day. Weighting of all animals and consumed feed were measured once a week.

Statistical Analysis

Statistical analyses were done with R software (version 3.6.0) with Chi² test (threshold of 5%) on an average number of rabbits. When rabbit number was statistically too low, an exact Fisher test was used. Only performances were not statistically analyzed due to the absence of repetitions.

RESULTS AND DISCUSSION

Main activities according to the type of housing system for the studied ages

Independently of housings, studied ages and times, the main activity of rabbit is « resting » (66% of animals), followed by « feeding » (12%), « grooming » (9%), and « moving » (8%). This distribution is close to Huang et al. (2021) observations. Activities « interacting socially », « standing » and « other » represent a minor percentage of rabbits (respectively 1.1%, 0.3% et 1.5%). These figures can be explained by stealth behaviors which are hardly visible with the scan sampling method, which is in accordance with Ferraz et al. (2019).

- **Resting** : this activity is similar between rabbits from CC (68%), WP (67%) and FP (70%) (Figure 1). But for FPOAA, rabbits rest less : 58% ($p<0.05$). Thus, an outdoor access would impact the resting behavior more than stocking rate or spatial organization of inside housings. This observation is in accordance with Fetiveau et al. (2021). Among the 6 zones of housings, resting is mainly applied on the floor (50%) because it is the most available and accessible. Almost one quarter of rabbits rest in the feeding zone (17% near drinkers, 7% near feeders). In FLOA, among rabbits present of the outside area, 15% of them are resting.
- **Feeding** : the percentage of rabbits feeding is respectively 8%, 12%, 12% and 15% in CC, WP, FP and FPOAA. We could expect a higher figure for rabbits in cage, as in the study of Trocino et Xiccato (2006), in which rabbits don't have enrichments and the available space is limited. For FPOA, the outdoor access allows to observe among rabbits which eat, 47% grazing at the expense of feed and drinking water (53%).
- **Grooming** : during our observations, this activity involves 16% of rabbits in CC versus 7% in average in others housings (8% in WP and 6% in FP and FPOAA). Ribikauskas et al. (2010) also measured more grooming in cage in comparison with park (8.5 vs 5.2%).
- **Moving** : for the studied ages and times, this activity is 13% in FPOAA, versus 6% in average for the others housing systems (5% in CC, 6% in WP and 7% in FP). This difference is due to the outdoor area, because the proportion of rabbits moving inside housing systems varies between 5% and 7%, whatever is the housing. Thus, it seems that the outside access would encourage moving behavior, more than the increase of the additional available space.

Activities according to the age and the time of the day

Throughout weeks, there are few significant differences of behaviors. "Feeding" tends to decrease for the 4 housing systems, but it is significantly reduced only for floor pens : as a tendency for FP (15.5% at 41d versus 9.3% at 69d, $p=0.062$), and significantly for FPOAA (19.1% at 41d and 15.6% at 55d vs 8.9% at 69d, $p<0.05$). In CC, grooming evolves from 10% at 41d, 17% at 55d and 21% à 69d. Even though these differences are not significant, we can suppose that in a poor environment as cage, rabbits seem to over-express the grooming behavior. For animals with an outside access (FPOAA), in average 38% of rabbits use the outside area despite the progressive disappearance of available grass : 32%, 46% then 36% for the 3 studied ages. These figures show that rabbits seem to be highly attracted by outdoor, even though there is no feed resource anymore.

There is an effect of time on behaviors : for all 4 housing systems, the frequency of behavior is higher at 9.00 am for "moving", at 12.00 am for "resting" and at 12.00 pm for "feeding". At 9 am, FPOAA counts more walks (26% in average vs 8% for other housing systems ; $p<0.05$) and a high use of the outdoor area (61% vs 14% at 12 am), that we can link with the recent opening of trapdoors. At 12 am and 12 pm, moving is lower than at 9 am (3 to 8% ; $p<0.05$). These values are close to Ribikauskas et al. (2010).

Activities according to the type of enrichment

For every enrichment (on the top and under PF, in hiding places, on outdoor area), "resting" is the main activity and is significantly higher than other activities ($p<0.05$). Under PF, we observe 7 to 10% of rabbits during the day, versus 4% at 12.00 pm. We can explain these figures by a natural need to hide, as written by Trocino et Xiccato (2006), where rabbits seem to not appreciate high ceiling (30 and 40 cm vs 20 cm) or an absence of ceiling. In the grass, even though resting is the main activity, characteristics of this zone give to rabbits the opportunity to express other behaviors (digging burrows…).

Performances

Descriptive technical and sanitary performances are mentioned in the Table 1. No mortality was recorded for CC and WP, whereas it reaches 8% in the 2 floor pens, mainly for digestive causes. This can be explained by a larger number of rabbits per group, which facilitates the dissemination of pathogens between animals. Weights at 71d old also tend to be lower in the floor pens, which can be the consequence of sanitary problems. Reduced feed intake (-20%) can also explain the decrease of performances, in addition with a stable feed conversion

ratio. We have noticed that, per type of housing system, the percentage of rabbits feeding is not in accordance with the measured ADFI. This can be due to the absence of individual follow-up of animals, to a different feeder (linear or circular) and/or to the group size.

Figure 1 : Average distribution of rabbits according to theirs behaviors in global and according to the housing system

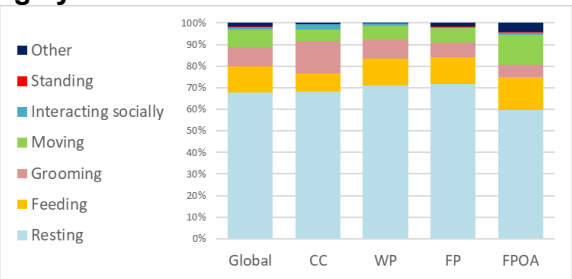

Table 1 : Performances

	CC	WP	FP	FPOAA
Weaning weight 32d (g)	998	1001	1002	1002
Selling weight 71d (g)	3106	3057	2708	2630
Average daily feed intake (ADFI) (g/d)	190	184	147	149
Feed conversion ratio (FCR)	3.52	3.49	3.35	3.57
Mortality (%)	0	0	8	8

CONCLUSIONS

This study allowed to observe and measure simultaneously the diversity of behaviors of rabbits in cages and three alternative housing systems. During the studied periods, the most significant modifications of behaviors were observed in floor pens with an outside access, with a decrease of resting and a raise of moving some time after the trapdoor opening. Others tendencies suggest that the increase of space and the modification of the spatial organization allow the expression of natural behaviors of the rabbit, but in return performances seems to be reduced.

The evaluating method of the rabbit behavior has limits for groups with large number of animals, and need to be adapted in order to obtain a multi-criteria evaluating method which takes into account rabbit welfare as well as performances and working comfort of breeders.

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EFFECT OF TWO STOCKING DENSITIES ON PERFORMANCES OF RABBIT REARED IN GROUND PENS

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ABSTRACT

To meet new societal expectations, an alternative housing system to the cage, called CUNILOFT®, has been developed to allow rabbits to be kept in groups and on the ground, while maintaining good working conditions for the breeder. The objective of this study was to evaluate the influence of stocking density on growing performances and sanitary results. Two consecutive trials were conducted. In these experiments, 1,392 rabbits were housed in ground pens and allotted according to two stocking density : a first group D+ (15.4 rabbits/m² corresponding to an available area of 651cm²/rabbit) made up of 4 collective ground pens with 195 rabbits per pen and a second group D- (12.5 rabbits/m² corresponding to an available area of 801cm²/rabbit) made up of 4 collective ground pens with 153 rabbits per pen. The rabbits were examined for growth performance and sanitary results. Increasing stocking density from 12.5 to 15.4 rabbits/m² impaired daily growth only during the second trial, consequently the final live weight decreased in this trial (+1.7% in trial 1, NS; -5.7% in trial 2, P<0.001). Stocking density did not affect viability results. The percentage of rabbits with wounds due to aggression was almost inexistant in both trials (<0.1%). In conclusion, growth performances of pen-housed rabbits may be affected by a higher stocking density especially in situation when weaning weight is low.

Key words: Ground pen housing, Stocking density, Growth performance, Cuniloft®

INTRODUCTION

Developments in society and the latest scientific advances which aim at better characterizing animal welfare, are leading us to reconsider the cage-rearing system, still commonly used in France for raising rabbits. Regulations will evolve soon as the European Commission has responded positively to a citizens' initiative entitled "End the Cage Age", and is committed to put an end to the use of cages (European Commission 2021), in favor of alternative housing. Some of these have already been the subject of numerous studies enlighting a better expression of natural behaviors (Maertens *et al.*, 2011; Szendrő and Dalle Zotte, 2011; Trocino *et al.*, 2014; Leblatier *et al.*, 2017; Gohier *et al.*, 2023). Thus, to accompany this evolution, some actors in the rabbit sector are developing new housing systems for rabbits.

Taking into account the considerations highlighted by the European Food Safety Authority (EFSA, 2020), namely that to improve the welfare of adult rabbits, it is necessary to increase the space and improve the structure of the housing offered, Mixscience has developed an alternative housing system to the cage. This system was developed to enable rabbits to be raised in collective groups of 100 to 300, depending on the choice of installation, allowing them more movement than in a cage, thus encouraging the expression of their natural behaviors.

Some studies revealed an impact of stocking density on rabbit growth performances in collective pens systems in medium groups from 20 to 27 rabbits (Trocino *et al.*, 2015; Pinheiro *et al.*, 2024). The aim of the present study was to identify how the stocking density of rabbits housed in collective pens in large groups (153 to 195 rabbits) affect their growth performance and sanitary results.

MATERIALS AND METHODS

Animals and housing

The trial was conducted on a commercial breeding during summer period from july to september.

The Cuniloft® system developed by Mixscience, designed to maintain good working conditions for the breeder (working time and ergonomy), consists here of a module of 8 pens within a building. The floor area of each pen was 11.5m², including a 2.1m² burrow for the rabbits to hide in. In each pen, platforms offered the rabbits the possibility of taking refuge above or below. The floor consisted of a slatted floor to allow dejections to be evacuated into a pit scraped daily.

1,392 rabbits from a ♀ Hycole x ♂ Hyplus PS59 crossbreed were divided into two groups at weaning, according to their weight. A first group (D+) made up of 4 collective ground pens with 195 rabbits per pen (i.e. a density of 15.4 rabbits/m² equivalent to an available area of 651cm²/rabbit). A second group (D-) of 4 collective ground pens with 153 rabbits per pen (i.e. a density of 12.5 rabbits/m², equivalent to an available area of 801cm²/rabbit). The rabbits were reared until 71 days.

The trial was carried out in 2 successive bands.

Each pen was weighed two times during a trial: at weaning (37d in first trial and 33d in second trial) and at the end of the fattening period (71d in both trials). Mortality was monitored daily, causes of mortality were also reported. The percentage of rabbits with wounds due to aggression was almost inexistant in both trials (<0.1%).

Feed consumption could not be measured precisely as it was an automatic screw distribution system.

Both groups of rabbits were fed a single fibrous fattening feed throughout the fattening period. Rationing was "hourly" using the Durefix® method: the animals were given an unlimited quantity of feed, distributed at fixed times during the night. The rabbits also had unlimited access to water. Both types of housing presented above offered access to natural light.

Statistical Analysis

Growth performances were processed with a covariate analysis with weaning weight as a covariate using R software (version 3.6.1). The mortality data was compared by a comparison of frequency (Chi²).

RESULTS AND DISCUSSION

Effect of stocking density : 12.5 v. 15.4 rabbits/m²

Final live weight was numerically higher for D- group in trial 1 but the difference is not statistically different (+1.7%, NS). Increasing stocking density from 12.5 to 15.4 rabbits/m² impaired daily growth only in the second trial, consequently the final live weight decreased (-5.7%, P<0.001) (Table 1). It is consistent with the observations made by Trocino *et al.* (2015). The summer period and weaning age (33d) have affected weaning weight of rabbits (average 925g) and then probably their growth during the fattening period assuming that they were less robust at weaning (Combes *et al.*, 2018). The lower viability rate observed in trial 2 may confirm this idea. In this trial, sanitary results were not statistically different between group D- and D+ despite a tendency (p=0.095) (Table 2). Comparatively, in the first trial, growing performances and sanitary results were higher but the effect of stocking density on these responses variables was not significant (p>0.05).

Table 1: Effect of stocking density from weaning until slaughter in growing rabbits reared in collective pens

	Trial 1		Trial 2		Interaction Group*Trial	RCV (%)
	D- 12.5rab./m²	D+ 15.4rab./m²	D- 12.5rab./m²	D+ 15.4rab./m²		
Rabbits, no.	612	780	612	780		
Initial weight (g)	1 161	1 161	926	925		
Live weight 71d (g)	2 589	2 565	2 469	2 380	P<0.05	4.1%
Weight gain (g/d)	41.3	42.0	40.6	38.3	P<0.05	8.5%

RCV, %: residual coefficient of variation

Table 2: Effect of stocking density on sanitary results in growing rabbits reared in collective pens

	D- 12.5rab./m²	D+ 15.4rab./m²	P-value
Trial 1			
Total viability rate (%)	96.9	96.5	NS
Digestive mortality (%)	1.8	2.3	NS
Trial 2			
Total mortality rate (%)	91.7	94.3	T
Digestive mortality (%)	7.0	4.5	T

NS: Not significantly different (P>0.05); T: P<0.10

Unfortunately, the absence of feed consumption measurements makes it impossible to explain consumption dynamics in relation to stocking density. Based on Pinheiro *et al.* (2024) observations it might be assumed that a higher final liveweight observed in D- can be linked to a higher feed intake.

CONCLUSIONS

This study led to the conclusion that under the conditions of our trials, stocking density (12.5 vs 15.4 rabbits/m²) influenced weight growth of pen-reared growing rabbits in one trial. It was not the case in a trial associated with improved growth and viability rates. Sanitary results did not seem to be affected by density.

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EVALUATION OF ENVIRONMENTAL CONDITIONS IN RABBITRIES THROUGH INNOVATIVE MULTIFUNCTIONAL DEVICES

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ABSTRACT

Rabbit housing system and microclimate inside rabbitries has a strong influence on animal growth and health. Inadequate environmental conditions constitute predisposing factors to respiratory diseases. The aim of this work was to investigate the fluctuations of environmental parameters in four fattening rabbit farms with serious respiratory disorders, and one without health problems. The continuous monitoring system has collected data on temperature, humidity, NH_3, CO_2, H_2S, illumination, sound and dust level in the farms and provided valuable information concerning relevant deviations of some environmental parameters, irrespective of the external conditions. Considering the optimal welfare values, the monitoring highlighted that the temperatures were higher than 20°C in three farms respectively for 51%, 89% and 100% of the time, while the relative humidity were below the suggested value of 60% in all the four farms for 30% to 89% of the time. Moreover, the multi-site measurement in the building revealed a heterogeneous distribution of the parameters investigated. The devices used proved to a valuable tool for farmers' decision-making, specifically in efforts to mitigate the impact of environmental conditions on animal health.

Key words: Animal welfare, structures, respiratory diseases, rabbitries indoor environment, ammonia concentration.

INTRODUCTION

Although the microclimatic conditions within farms are recognized as a critical factor for rabbit breeding, the literature on this subject is rather scarce for this species, and not always supported by scientific evidence (Gidenne *et al*., 2017). According to the guidelines EFSA (2020) and the Italian Ministry of Health (2021) for fattening rabbits the optimal temperature range is between 15 and 20 °C and the optimal humidity between 60-70%. Furthermore, the optimal concentration of ammonia (NH_3), according to the same sources, should be kept below 10 ppm while CO_2 has a concentration limit of 1,500 ppm and dust of 5 mg/m^3 in working environments. Regarding lighting conditions for animals, the Ministry of Health (2021) recommends ensuring 20 lux for at least 8 hours per day, with a preference for natural lighting in new buildings. It is essential to note that these recommendations, primarily addressed to other species, may not represent the optimal condition for rabbits, given their burrowing and crepuscular nature, making them inactive during daylight hours (Crowell-Davis, 2021).

To date, there are no established limits for hydrogen sulfide (H_2S) and sound pressure for rabbits. The exploration of environmental micro- and macro-parameters reveals a circadian trend influenced by external climate changes. These changes lead to variations in air extraction speed and quantity, causing fluctuations in temperature (T), relative humidity (RH), air dustiness (PM), and the concentration of harmful gases -primarily CO_2, NH_3, and H_2S- in breeding rooms. Toxic gases may increase as result of the microbial metabolism on feces and urine. The gas levels are maximum at the beginning of the day and may have peaks in areas of the structures where air movement is not optimal or is absent (Ivanova and Hristev, 2022). Particularly, NH_3 is identified as a potent inducer of respiratory pathologies in various animal species. Its effects include ciliary depression and irritation of the respiratory mucosa, not only in farmed animals (Donlon *et al*., 2023; Estevez, 2002; Michiels *et al*., 2015) but also in farm's personnel (Linaker & Smedley, 2002). Airborne particulate matter has the potential

to induce irritation and inflammation in the upper respiratory tract, in addition it can carry microorganisms, contributing to the spread of pathogens (Pearson & Sharples, 1995).
The primary objective of this study was to evaluate the trend and uniformity of some environmental conditions in rabbit farms. Additionally, the research aimed to analyze the daily fluctuations of these conditions, specifically in connection with various management operations.

MATERIALS AND METHODS

Monitored farms

The research was carried out in rabbit farms located in the North East of Italy, a region that hosts over 60% of the national meat rabbit production. Four farms (farm B to E, Table 1) had serious respiratory disorders with a daily mortality rate due to pasteurellosis > 2‰. To serve as a negative control, a commercial farm producing antibiotic-free meat, possessing similar structural characteristics and without health problems, was also included in the study (farm A, Table 1). In all farms the weaning was at 35-40 days and the rabbits were slaughtered at 70-80 days (live weight around 2,7 Kg). The main characteristics of the buildings are reported in Table 1.

Table 1: Main characteristics of the monitored building.

Farm	Size	Cage-type	Heads	Measurement period	Average daily mortality (‰)
A	54x14 m	WRSA	9000	From 20/12/2022 to 07/01/2023	0,91
B	54x7.2 m	Bicellular	4224	From 21/02/2023 to 7/03/2023	2,87
C1	30 x 12 m	WRSA	4000	From 14/03/23 to 20/03/2023	1,39
C2	30 x 12 m	WRSA	4000	From 05/04/23 to 17/04/2023	3,50
D	36.5 x 16.5 m	Pratica	6000	From 27/03/2023 to 03/04/2023	4,5*
E	27.5 x 24.1	Park	6900	From 26/04/2023 to 03/05/2023	3,21

*estimated by the farmer, mortality not annoted

Equipment used and monitoring period

Environmental monitoring was carried out using control units developed by the company IBT System srl (Monza, Italy) in collaboration with the Department of Agricultural and Environmental Sciences of the University of Milan.
The electronics and sensors are inserted in a plastic box measuring 200 x 110 x 60 cm with appropriate openings for the sensors. In particular, on the front there are circular holes with a diameter of 15 mm corresponding to the NH_3 and H_2S sensors and an illuminance sensor. In the lower part there are openings in correspondence with the carbon dioxide and particulate sensors there are the temperature and humidity sensors. On the right side are the power button and the battery charging socket. The device, in fact, can be powered by cable, but has a rechargeable battery with a duration of approximately 20 days.
The device transmits the measurements carried out via radio every 10 minutes to a gateway that receive the data from the devices and sends it to an internet server which stores it and makes it available through a dashboard.
In each building, 6 devices have been placed at the animal height to assess the variation of environmental parameters. The devices have been located in three sections (two lateral and one central) along the passages between cages to monitor the whole building.
Data collection took place between 20/12/2022 and 3/04/2023. The winter-early spring period has been selected as the more critical for respiratory diseases. Each building has been monitored for 7-10 days. In one farm (C, Table 1) data was collected in two different periods of the same cycle.

Statistical Analysis

The statistical processing of the data involved the comparison of the values measured by the devices in the same farm and was carried out using a generalized linear model (SPSS GENLIN procedure) and the significant differences with the Wald test. Furthermore, for each

farm the percentage of time of the exceeding threshold values for the different parameters was calculated.

RESULTS AND DISCUSSION

The mean and median values obtained considering the hourly values of the full set of data collected seems to be in line with the recommendations (Table 2). However, the standard deviation values highlight relevant differences between the farms and within the farm during the day.

The statistical analysis highlighted statistical differences among the 6 devices in the same building for all the farms, confirming the variability of the internal environmental conditions.

Table 2: Mean, median and percentiles values of the parameters monitored in rabbitries (total 1207 hours).

	Mean	Median	Minimum	Maximum	Standard Deviation
T (°C)	18.7	18.8	13.3	25.9	2.8
Relative Humidity (%)	56.0	56.8	32.3	76.4	9.0
CO_2 (ppm)	1,289	1,190	631	3,061	471
H_2S (ppm)	0.0	0.0	0.0	2.2	0.1
NH_3 (ppm)	3.6	2.4	0.6	22.4	3.3
Sound (dbA)	62.8	62.8	54.3	85.0	4.0
$PM_{2.5}$ (µg/m^3)	23.3	12.3	-	148.0	28.5
PM_{10} (µg/m^3)	24.1	12.6	0.2	153.4	29.6
Illuminance (lux)	72.3	0.0	-	4,114.9	209.3

The hours per day in which the condition exceed the thresholds are displayed in Table 3. PM and H_2S are not reported because the values were always below the thresholds.

Temperatures showed significant variation across farms, ranging from 16.1°C to 22.2°C as mean value per farm. Despite differences in rabbit ages among the farms at the time of measurements, the observed temperature setting seemed driven more by farmer's decisions than a careful consideration of the animals' requirements. The observed poor uniformity within the farm suggests potential issues such as non-uniform air distribution and the presence of local air currents. These conditions might contribute to worsening animal health conditions.

Table 3: Percentage of time in which the environmental parameters' threshold detected were exceeded.

Farm	Percentage of the time (%)						
	T < 15°C	T >20°C	UR < 60%	UR >70%	NH_3>10 ppm	CO_2 >1500 ppm	illuminance >20 lux
A	0%	0%	0%	0%	0%	11%	2%
B	19%	0%	64%	0%	5%	25%	45%
C1	0%	100%	30%	10%	30%	86%	6%
C2	0%	89%	89%	0%	0%	16%	5%
D	1%	0%	75%	0%	0%	0%	66%
E	0%	51%	51%	15%	0%	0%	36%

The average values of relative humidity remained below 60%, with limited variability between farms. The identified relative humidity level was lower than the optimal range for extended periods, posing a risk associated with excessive air dryness that may predispose animals to respiratory diseases. Ammonia concentrations consistently remained below 10 ppm on average. In two farms (B and C) NH_3 exceeded 10 ppm for some hours in some days. This is in agreement with the data reported by Calvet *et al*. (2011).

Even if the values of carbon dioxide are acceptable on average, three farms exceeded the threshold values for 11% (farm A), 25% (farm B) and 16 to 86% (farm C). The data obtained are consistent with those reported in other works (Estelles *et al*., 2011; Calvet *et al*., 2011).

Regarding sound pressure, in some studies is reported how chronic exposure to sounds at or above 85 dBA can cause hearing damage in human (NIH, 2020). This is a level recognized also as a general safety threshold for work environment and lab animals, with a

recommendation for these to stay below 65 dB in average (J. Turner *et al*, 2005; OSHA). The values found in this study were never high, with average values that in some farms approached 80 dB only for short times.

In the air quality directive (2008/EC/50), the EU has set two limit values for particulate matter for the protection of human health: the PM_{10} daily mean value may not exceed 50 µg/m3 more than 35 times in a year and the PM_{10} annual mean value may not exceed 40 µg/m3.The measurements indicated very low PM values, lower than 50 µg/m^3 in all farms with the exception of Farm B where higher values were found up to a maximum of 145 µg/m^3, a value which however does not raise health concerns for animals. The results are in line with absolute values of Adell *et al*. (2012).

Illumination was significantly influenced by the presence of windows. Three of the monitored farms had natural light and meet the minimum light duration requirements (>20 lux). Building D exceeded this level for more than 16 hours/day. B and E resulted adequate. Farm A and C, on the other hand, had no windows and a lighting program was not used, therefore the 20 lux limit was exceeded only for 1-1.5 hours per day.

CONCLUSIONS

The surveys provided precious information concerning the variability of the environmental macro- and micro-parameters within the farms examined. This information can be used firstly to improve the improve the environment conditions (temperature, humidity, air quality and sound level) of rabbits raised indoors. The observed environmental conditions measured in the farms with a high incidence of respiratory diseases, may be related to the deviations on the temperature, humidity and some of the measured gases, and particle matter. Additional research is required to correctly link the environmental parameters measured and the respiratory health status of growing rabbits.

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OSHA, Occupational Noise Exposure.

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Welfare assessment of farmed rabbits in Italy: preliminary data of the application of the Classyfarm protocol for official veterinary controls

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ABSTRACT

This study aims to illustrate the results of the first-year application of a welfare assessment protocol for farmed rabbits by official veterinarians in Italy. The protocol judges 33 evaluation elements, divided into 4 Areas (Farm management and Personnel, Facilities and Equipment, Animal Based Measures (ABMs), Major Risks and Warning Systems), as Insufficient, Acceptable and Optimal. The vet inspector could choose 'not applicable' if no judgment could be applied. Two hundred forty-one farms were inspected in 2023. The total number of 'Insufficient', 'Acceptable', 'Optimal' values and 'Not applicable' decisions were counted for each item. These data were combined for each area to provide aggregated results after averaging the number of Acceptable, Insufficient, and Optimal responses. From the overall results, it can be seen that most farms were given a favourable judgement in all four Areas. Farm management and Personnel had the most Acceptable responses (76.5%), Facilities and Equipment had the most Insufficient responses (1.5%) and ABMs had the most Excellent responses (41.4%). Despite the small number of ABMs judged "Insufficient", it was possible to trace them back to an inadequate state of structural and managerial items in 6 cases. Despite some critical points, these results showed promising rabbit farming conditions in Italy and highlighted the need to increase knowledge on rabbit ABMs and their relationship with indirect indicators.

Key words: Welfare assessment, welfare indicators, Classyfarm, Italy, official control.

INTRODUCTION

Europe is the world's second-largest producer of rabbit meat and has about 13.8 million rabbits on farms. Italy, Spain, and France account for 60% of the European production of rabbits (Ismea, 2021). In Europe, no unified and uniform legislation to protect the welfare of rabbits beyond the Council Directive 98/58/EC (Council of the European Union, 1998) has been issued. The Parliament resolution on minimum standards for the protection of rabbits on farm (European Parliament, 2017) tried to set the basis for establishing specific European legislation. In 2021, starting from this resolution and the EFSA's 2020 Scientific Opinion (EFSA, 2020) on the welfare of rabbits in different farming systems, the Italian Ministry of Health updated the already existing guidelines for the protection of farmed rabbits in Italy, first released in 2014 (Italian Ministry of Health, 2021). The ultimate scope was to encourage and accelerate the structural change of commercial farms towards production systems providing better welfare conditions to speed up the process of adopting specific legal requirements and facilitate welfare assessments by official inspectors. Following the revision of the guidelines, a protocol for the assessment of welfare on farms was set up based on direct and indirect indicators. Such protocol, usable for official control by vets and self-assessment of the welfare level of rabbit farms by breeders, is based on the Classyfarm

system. This integrated model, already used for cattle, goats, sheep, pigs, and poultry in Italy, identifies measurable indicators for biosecurity, proper use of drugs, and animal welfare and aims at categorising farms according to their risk for veterinary public health. The welfare assessment protocol aims to highlight legislative non-compliances based on the requirements of the Council Directive 98/58/EC and to target preventive interventions on the main weakness factors of each farm, thereby improving the animals' living conditions. This paper describes the results obtained from official veterinarians' field application of the checklist during the first year of use (March 2023 to January 2024).

MATERIALS AND METHODS

The Classyfarm welfare protocol for official controls

The checklist is based on the analysis of the provisions of the Council Directive 98/58/EC related to hazards arising from environmental conditions (management, facilities, equipment and microclimatic conditions) as well as those derived from the collection of the most important animal-based measures (ABMs) according to the most recent scientific literature. Three risk areas are classified by the Classyfarm information system: Area "Farm management and personnel"; Area "Facilities and equipment"; and Area “Major risks and warning systems". The system conveys the information belonging to the ABMs into a fourth area. Table 1 shows the different evaluation items for each area. Many items, especially those related to ABMs, are based on scoring systems, partially derived and/or adapted from the protocols of Dalmau *et al*. (2020) and Botelho *et al*. (2020). Each evaluation item has two or three detailed answers that could be selected during the inspection corresponding to the following conditions: "Insufficient" in the case of conditions that do not satisfy the welfare needs of the animals and do not allow them to enjoy the five freedoms (FAWC, 1979; EFSA 2020); "Acceptable": in the case of living conditions that, with some exceptions, guarantee the satisfaction of the five freedoms and the welfare needs of all animals present; "Optimal": in the case of particularly favourable conditions that ensure very positive conditions for all animals, clearly better than the required legal standards. The official veterinarian cannot assess a specific item if it is irrelevant on the farm under evaluation by selecting 'not applicable'.

Table 1: The Classyfarm welfare assessment protocol for farmed rabbits; the evaluation items are provided for each area (answers for each item are not shown).

Management and personnel	Facilities and equipment	Animal based measures (ABM)	Major Risks and Warning systems
Number of employees caring for the animals	Absence of harmful cages and equipment for the animals (all groups)	Body condition score (BCS) (breeding animals)	Lighting for inspection
Employee training	Presence of shelters in the outdoor areas for animals kept outside (all groups)	Cleanliness of the animals	Ventilation system alarm
Number of daily inspections of fattening rabbits	Space available for resting and movement (fattening rabbits)	Skin lesions (all groups)	Inspection of automatic and mechanical equipment
Number of daily inspections of breeding rabbits	Nursery (all groups)	Skin mycosis (all groups)	Drug treatment records
Treatment of sick or injured animals	Temperature and humidity (all groups)	Pododermatitis (breeding animals)	Mortality and animal movement records
On-farm culling	Noxious gases and dustiness	Mastitis (breeding animals)	Administration of illicit drugs
Food and daily feed distribution management	Light program for animals (all groups)	Mortality % (0-35 day-old animals)	
Type of feed management		Mortality % (35-60 day-old animals)	
Number of feeders, water supply, and number drinking points (all groups)		Mortality % (>60 day-old animals)	
Hygiene, cleanliness and management of sheds and cages or pens/parks (all groups)			
Biosecurity			

Data collection and aggregation

The raw data from the Classyfarm system and resulting from the online entry of the checklist by official veterinarians at the end of each inspection from March 2023 (start of inspections using the new checklist) to December 2023 were used. Two hundred forty-one farms were inspected during this period: 157 from Northern Italy, 32 from Central Italy, 33 from Southern Italy and 19 from the Islands (Sicily and Sardinia). The assessed farms were 90 mixed/closed cycle farms (breeding and fattening), 75 familiar farms (family rabbit breeding, with a maximum number of 20 nest holes and a maximum of 50 animals older than 30 days), 40 fattening farms, 23 open cycle farms (only breeding), nine breeding centres and four hunting farms. The total number of 'Insufficient', 'Acceptable', 'Optimal' values and 'Not applicable' decisions were counted for each item. To provide aggregated results, these data were combined for each Area after averaging the number of Acceptable, Insufficient, and Optimal responses.

RESULTS AND DISCUSSION

From the overall results per area, it can be seen that most farms were given a positive judgement in all four Areas (Figure 1).

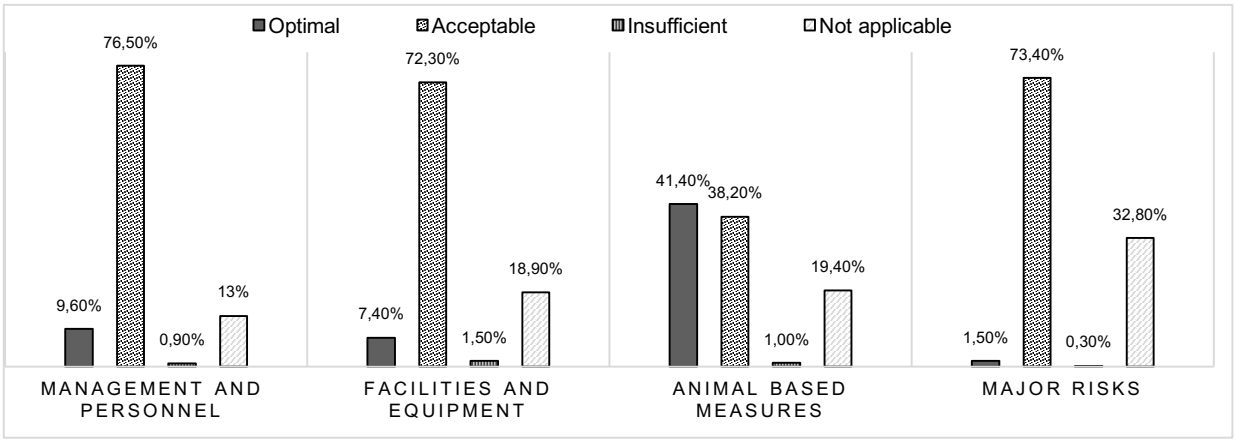

Figure 3: Average percentage of responses divided by each area.

Management and personnel

On average (Figure 1), 76.5% of the farms were rated Acceptable, 9.6% Optimal, and 0.9% Insufficient. Interestingly, 8 out of 241 farms (3.3%) were rated Insufficient for the cleanliness of the facilities and 7 (2.9%) for biosecurity; on the contrary, 65 out of 241 (27%) received an Optimal for the number of employees, 23 (9.5%) for the training of employees and 32 (13.3%) and 30 (12.4%) for daily feed distribution and type of feed management respectively.

Facilities and Equipment

On average (Figure 1), 72.3% of the farms were rated Acceptable, 7.4% Optimal and 1.5% Insufficient. We noticed some items received a remarkable number of both Optimal and Insufficient responses. E.g. for the item “Space available for resting and movement (fattening rabbits)”, 30 (12.4%) farms were considered Optimal and 5 (2.1%) Insufficient; regarding the Absence of harmful housing facilities and buildings for the animals, 15 (6.2%) farms were judged Optimal and 8 (3.3%) insufficient; for Temperature and humidity 28 (11.6%) were evaluated as Optimal and 5 (2.1%) insufficient. However, there are more Acceptable responses than Optimal and Insufficient ones, reflecting the good overall condition of facilities and equipment.

ABMS

On average (Figure 1), 41.5% of the animals (per farm) were rated Optimal, 38.3% Acceptable, and 1.1% Insufficient. Surprisingly, the average number of Optimal exceeds that of Acceptable. This contrasts with the results of the other areas. In fact, when optimal conditions are found for ABM, optimal environmental conditions for the animals should also

be seen. Thus, the acceptable conditions of the farms probably likely allow them to reach, for the ABM considered, superior welfare conditions, even in the absence of environmental conditions considered optimal for the protocol. On the contrary, environmental conditions considered optimal could affect direct parameters that, to date, cannot be assessed on animals due to a lack of scientific knowledge or validated methods. Despite the small number of ABMs judged "Insufficient", it was possible to trace them back to an inadequate state of structural and managerial items in 6 mixed/closed cycle farms. In one case, it was possible to match the degree of animal soiling and the presence of mycosis with the insufficient degree of cage cleanliness, insufficient space, and lack of animal treatment. In another case, "insufficient" pododermatitis coincided with more items judged "insufficient", i.e. biosecurity, cleanliness of cages, harmful materials, absence/presence of infirmary, and animals that did not receive immediate treatment when needed. Mortality items were judged as "insufficient" in 4 farms (1.7%) for animals aged 0-35 days, 3 (1.2%) for animals aged 35-60 days and 7 (2.9%) for animals >60 days. Some interesting cases were registered: i) a high mortality data in all rabbit categories (3 items) coincided with a poorly maintained drug register; ii) a high mortality rate in all rabbit categories coincided with no infirmary and no timely treatment of sick animals; iii) the high mortality rate >60 days coincided with inadequate early care, lack of space, insufficient cleanliness, absence of an infirmary and inadequate temperature and humidity; iv) the high mortality rate > 60 days coincided with inadequate temperature and humidity, gas values and lack of sufficient biosecurity measures.

Major risks and alarm systems

On average (Figure 1), 73.4% of the companies received a judgment of "Acceptable", 1.5% "Optimal", and 0.3% "Insufficient". It is very important to point out that two companies were insufficient due to incorrect recording of drug consumption, which resulted in a penalty, and two others were insufficient due to the lack of an alarm system.

CONCLUSIONS

From this initial analysis of breeding conditions of 241 Italian rabbit farms, the veterinary inspection found a high percentage of facilities being classified as Optimal and Acceptable (above 75% of all cases) in all Areas of the Classyfarm system. However, some critical points emerged concerning the cleanliness of the facilities, the absence of an infirmary, biosecurity, the presence of potentially harmful facilities and the management of drugs. It is important to note that in 6 cases, inadequate ABMs were likely due to unsuitable structural and managerial conditions.

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INFLUENCE OF RESTRICTION VISIT PERIOD AT NEST ON BEHAVIOR AND PERFORMANCE IN RABBITS (*Oryctolagus cuniculus*)

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ABSTRACT

In Venezuela, rabbit production is low owing to scared knowledge on livestock management. Remove young (Y) rabbits of the mother (M) during short times periods among lactation is a common practice in Europe based in its benefits effects. Therefore, in order to evaluate the influence of restriction visit period at nest (N) on behavior activities and performance in M and Y (MY) an assay was carried out. M and its litter rabbits were assigned to treatments (T) at calving. For 28 days (d) MY stay together between 0600 – 0700 hours (h) independently of T. A metallic net was used to divide the cage and restrict the access (R) for the M to the N at 0700 to 0600 h of the next d. A randomly model was used to analyze R. T were = WR: without R; ER: R from 1 to 14 d; LR: R from 14 to 28 d; TR: R from 1 to 28 d. MY were observed a d per week and for one hour (0600, 1000, 1400, 1800 h). ER reached WR in high mean number of Y per litter at weaning ($P<0.05$), and similar than the high total number of Y weaned ($P<0.01$). Y mortality (%) in WR and ER were better ($P<0.01$) than LR and TR. Lower groom activity ($P<0.05$) was observed between M in ER vs. WR. Lactating rabbits in WR prefer entry at the nest at 1400 y 1600 hours. Y of TR upper times licking the cage and in interaction ($P<0.05$) at postweaning. An early restriction visit period at nest for MY interaction induce a metabolic goal or improves in physiology digestion in Y, and its influence welfare and performance parameters positively.

Key words: Rabbits, restriction period, nest, behavior, production

INTRODUCTION

Mother – offspring separation for short periods is a common practice in rabbit production system in industrialized countries in order to improve mainly continuous reproduction cycles (Lebas, 1999). Temporal mother –offspring separation or weaning after three postpartum weeks do not affect negatively survivor of lactating rabbits. Verga *et al*. (2007) determined the reduction of stress and greater homogeneity of the litters as advantages of mother-young separation practice. High offspring mortality and very long time for young rabbits to reach slaughter weight as low pregnancy rate, long postpartum periods and long suckling periods are some zootechnic problems detected in tropical countries as Venezuela (Manzanilla *et al*., 1992). The European Food Society Authority (2005) report that restriction mother visit to the nest application, to guaranty animal welfare, requires precise behavior knowledge of the wild patterns that are retained in rabbits. Some of these patterns are exploring, socializing, feeding, grooming and nursing (Verga *et al*., 2007; Crespo, 2015). Scientific information in rabbit mother-offspring behavior in industrialized or un-industrialized countries is scarce and fragmented (Matics et al., 2004). The rabbit production is considered as a potential alternative to animal production in all countries but especially in un-developed and tropical latitudes (Lebas, 1999) due to high protein meat level and good economical income in rural areas (Nieves, 2007). This assay was design to evaluate the influence of restriction visit period at the nest on the behavior and performance in mother and offspring rabbits during lactation and post-weaning period.

MATERIALS AND METHODS

Localization

The assay was carried out inside a building with comfort temperature (25.5 °C) and humidity (45 %) conditions for rabbits. The building was located at Animal Production Institute inside Campus Maracay of Universidad Central de Venezuela, at 432 meters of altitude and at 67°35'45" W and 10°14'49" N.

Animal management, treatments and variables

Twenty adult female rabbits were mating with two male rabbits during this assay. All animals were from New Zealand x California crossbreed. Fresh potable water and a commercial food (18% crude protein and 15% fiber) were offer *ad libitum* before and during assay. After partum, each mother and its litter were placed within a cage and were assigned to one of four treatments (T) randomly. For 28 days (d) mother and young stay together between 0600 – 0700 hours (h) independently of T. Restriction access (R) was done using a metallic net to divide the cage and block the physic access (non visual) for the mother to the nest during 0700 until 0600 h (23 h/d). T were: WR (n =7): Without R from day 1 to day 35; ER (n =7): Early R from day 1 to day 14; LR (n =7): Late R from day 14 to day 28; TR (n =7): Total R from day 1 to day 28.

The experimental unit to evaluate mothers (mother and its litter) behavior and performance was the cage (30 cm x 35 cm x 70 cm; 3500 cm^2). Animal behavior was evaluated considering activities (ex. grooming, suckling, laying, feeding) of the mother by itself or with its young rabbits and each post weaning young rabbit during one hour at 0600, 1000, 1400 y 1800 hour and during five weeks continuous. MANOVA for repeated time measure was applied. Live weight (gr) was registered each week in order to measure weight variation; as well, mortality was registered. All of these measures were registers while fasting and individually according to mother, offspring or post weaning rabbits. The weaning was applied at 35 day of offspring life. All young rabbits were relocated in pairs per cage (30 cm x 25 cm x 50 cm). The cage was the experimental unit for post weaning growth measure. Fourteen repetitions by each previous treatment were considered for post weaning evaluations. Reproduction variables or cycles were not considered. For performance variables ANOVA and Tuckey test were applied. A ji^2 test was applied for mortality.

RESULT AND DISCUSSION

Mother rabbit behavior

Multivariate analysis showed statistical differences ($P<0.05$) between treatments WR and ER for grooming behavior (9 vs. 5 times observed respectively) only in each evaluation period. Therefore, separation mother – offspring during 23 hours per day and during the first 14 days showed highest stress. Restriction access change the total time of the mother dedicated at different activities like suckling, feeding, drinking or others (Fernandez *et al.*, 2017), but none of them predominate.

Young rabbit post weaning behavior

Results of post weaning interactions between young rabbits (YI) and licking the cage (LC) are at Table 1. YI is associated with socialization behavior and olfactory communication (Fernandez *et al*., 2017), nevertheless, this implicated strong and a learned behavior of the relationships between they and their mother. As can be seen, in TR exacerbated interaction over sociability could be consider as a stereotypic behavior (abnormal behavior, repetitive, invariable, functionless) associated to deprivation (Garner and Mason, 2002).

Table 1 Main effect on activities (n) related to behavior in post weaning young rabbits

	Treatment						
Variable	WR	ER	LR	TR	LSM	R^2	DLSM
YI	1^{b}	2^{ab}	1^{b}	3^{a}	7.99	0.43	1
LC	1^{b}	2^{ab}	2^{ab}	3^{a}	21.93	0.61	1

^{a,b} Media with different letters at the same row are different (P<0.05).; **YI**: Interaction between young rabbits; **LC**: Lick the cage; **LSM**: Least Square Mean, **DLSM**: Difference Least Square Mean

Young rabbits located during pre-weaning in LR and TR had one hour per day to be contact with their mother, and during this time they could interact and specially suckled but is evident that this time was not enough to establish normal post weaning behavior. The barrier, the metallic net restriction to avoid the mother entry inside the nest, for 23 hours per day, exert a strong stressing effect to the young rabbits, changing their normal late behavior. One hour could not compensate de deprivation and compensatory nonassertive activities -LC- are overexpress.

Mother rabbit performance

The litter is the measure of mother productivity during lactating period. The total number of young rabbits per litter (YR/L) (each mother), and total number young rabbits at weaning (TYR) per treatment (all mothers) (Table 2) showed statistical differences (P<0.05) due to effect restriction access period at the nest.

Table 2 Effect of the restriction period to access at the nest on mother performance

	Treatments				LSM
Variable	WR	ER	LR	TR	
YR/L	4.00^{b}	5.17^{a}	4.33^{ab}	4.50^{ab}	1
TYR	25.83^{c}	38.5^{a}	29.67^{bc}	33.00^{ab}	6.12

^{a,b,c} Media with different letters at the same row are different (P<0.05)

YR/L: Average of young rabbit per litter at weaning; **TYR:** Number of total young rabbit at weaning; LSM: Least Square Mean

Limited the access to the mother at the nest with the young rabbits during one hour daily (06:00 to 07:00) during the first 14 days of lactation (ER) or during de late days of lactation or during all of lactation influenced litter survivor positively. It could be due to strong mother – young relationship and intensive suckling during this hour (Matics *et al*., 2004). Rabbit farmers can use this management to improve young rabbits' survival percentage at perinatal period; one of the worse indexes in rabbit tropical systems (Manzanilla *et al*., 1992).

Treatment effect on young rabbit mortality values is shown in Table 3. Place a separating metallic net between the cage and nest at the end of the suckling period (LR) or during all suckling period (TR) are associated to highest mortality values (P<0.01) in this assay.

Table 3 Restriction suckling period effect on young rabbit mortality percentage (%)

	Treatments				X^2 critic
Variable	WR	ER	LT	TR	
Mortality (%)	10.7b	17.8b	32.5a	26.2a	7.8

^{a,b} MDS with different letters at the same row are different (P<0.05).

Similar than Matics *et al.* (2004) reported when the restriction is strong the mother it becomes nervous and raises the crush of young rabbits at entry to the nest.

Young rabbit performance at post weaning

Performance in young rabbits was considered measuring the time in days to reach commercial weight (TCW) and the daily weight gain (DWG) (Table 4). Daily weight gain (DWG) showed differences (P<0.01) due to treatment applied during pre weaning period.

Table 4 Restriction period effect on young rabbits post weaning performance

	Treatment				LSM	P – value
Variable	WR	ER	LR	TR		
DWG (g/d)	19.82[a]	18.23[ab]	16.63[bc]	15.24[c]	2.84	< 0.01
TCW (d)	58	63	70	76	18.20	0.07

[a,b,c] Media with different letters at the same row are different (P<0.01) **TCW (d):** Number of days to reach commercial weight (1,8 kg). **DWG (g/d):** Daily weight gain

The best effect of treatment on DWG was found in WR and ER opposite to TR during pre-weaning period. On the other side, the worst performance appear in TR. Consequently, TR influenced feeding behavior negatively (Fernandez *et al*., 2017). Probably, young rabbits cannot learn feeding behavior from its mothers because of long time separation - 23 hours per day by 28 days-. Stereotypical behaviors (Table 1) showed psychological disturbs (Garner and Mason, 2002). In relation to TCW a tendency (P=0.07) to lower number of days to reached 1.8 kg per rabbit at post weaning was observed in ER and WR. Probably, the variability between young rabbits can not let the statistical differences, but this and the best result in DWG for ER could be explained as a metabolic benefit change and programming during preweaning period (Gandhi et al., 2022).

The findings in this assay show that ER resulted the most convenient way of management for farmers. This management influenced positively the performance of the mother (YR/L, TYR), litter survivor during pre-weaning and the highest weight gain (DWG) during post weaning, without stereotypies. The zootechnic benefit of restriction was demonstrated but reproduction variables must be considered for future research.

CONCLUSIONS

In conclusion, an early restriction visit period at nest for mother and young rabbit interaction induce a metabolic goal or improves in physiology digestion in young rabbits, and its influence zoothecnical and commercial parameters positively and without disorders in behavior.

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CONTROLLED NURSING AS A STRATEGY TO REDUCE MORTALITY OF NEW ZEALAND WHITE KITS

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ABSTRACT

Since mortality in nursing phase is one of the factors that most negatively impacts Brazilian rabbit farming, the objective of this study was to evaluate the controlled nursing as a strategy to improve kit growth and reduce the mortality of New Zealand White kits. Fourty-four New Zealand White does were used, with an average live weight of 3.5 kg and aged between 1 and 2 years. Each nest was standardized with 8 kits, totaling 352 kits in the assay. The experimental design adopted was blocks in time, with two treatments and 22 replicates. Treatments consisted of natural nursing (without human interference) or controlled nursing (does separated from kits for 22 hours and lactation for 2 hours a day (from 08h to 10h am). After birth, the kits remained in constant contact with the female for up to 72 hours, so that they could feed on colostrum (during these days the animals were collective weighed daily). After this period, the does of controlled nursing were kept in the cage next to the nest, while the does in natural nursing remained with the kits throughout the entire experimental period. The experimental period ranged from birth to weaning (0 to 35 days of life) and daily, in the morning, the variables evaluated were: dead kits (mortality rate) and live weight (weight gain). At 15 days of age, two kits per nest (close to medium live weight) were submitted to blood harvesting, through heart puncture, in order to determine biochemical parameters and the complete blood count. Controlled nursing resulted in lower mortality (P= 0.003) than natural nursing, with rates of 12.4% and 22.9%, respectively, denoting that controlled nursing can reduce the mortality of rabbit kits before weaning by almost half. Blood biochemical parameters and blood count did not differ between treatments (P>0.05). In conclusion, controlled nursing reduces the total mortality of New Zealand White kits and does not alter biochemical parameters or blood count, however it requires strict management, with more labor and investments. New strategies and methodologies to reduce rabbit mortality must be developed to ensure animal welfare, good production rates and profits in rabbit farming.

Key words: Blood analyses, Milk, Rabbit farming.

INTRODUCTION

Brazilian rabbit farming is an activity in development, with small production when compared to European and Asian countries (less than 1 thousand farmers, with an estimated herd of 1 million does). The period from the birth to weaning is what leads to the highest mortality rate in rabbit farming (Machado et al., 2021), which is linked to the physiology of the newborn itself, which does not have a fully developed immune system, being in constant transformation, in addition to being more sensitive to environmental weather (Miranda and Castilha, 2020). During the period that the kits are with the female, especially until 15 days of age, it is essential that they feed exclusively on milk (Gidenne et al., 2020), since they still do not consume solid and depend on nutrients from breast milk for their survival and subsequent development (El Nagar et al., 2014).

Besides does breastfeed their offspring one to two times a day, during 3 to 5 minutes (Jiménez and González-Mariscal, 2019), this behavior is linked to the composition of their

milk, which is extremely fatty and protein-rich (Maertens et al., 2006). The frequency of nursing adopted by does should be sufficient to maintain the good and rapid development of the kits. Even so, due to genetic improvement focusing on the selection for large litters (many kits born alive), it is likely that the milk production of females may have changed in quantity over the generations, compromising the maintenance of the litters. Furthermore, blood parameters can indicate the nutritional and health status of the animals, but reference values for lactating rabbits are scarce.

Since mortality in nursing phase is one of the factors that most negatively impacts Brazilian rabbit farming, the objective of this study was to evaluate the controlled nursing as a strategy to improve kit growth and reduce the mortality of New Zealand White kits.

MATERIALS AND METHODS

Animals and experimental design

The experiment was carried out at the State University of Maringá, Brazil, in the Rabbit Farming Sector, in the autumn of 2022 (temperature: 25.4±4.3ºC; relative humidity: 63.7 ±8.1%). All experimental procedures were submitted for approval by the University's Committee for Ethical Conduct in the Use of Animals in Experimentation (CEUA/UEM) (Protocol no. 5542190123). Fourty-four New Zealand White does were used, with an average live weight of 3.5 kg and aged between 1 and 2 years. Each nest was standardized with 8 kits, according to the standardization proposed by Silva et al. (2021), totaling 352 kits in the assay. The experimental design adopted was blocks in time, with two treatments and 22 replicates. Treatments consisted of natural nursing (without human interference) or controlled nursing (does separated from kits for 22 hours and lactation for 2 hours a day (from 08h to 10h am). After birth, the kits remained in constant contact with the female for up to 72 hours, so that they could feed on colostrum (during these days the animals were weighed daily).

The animals were placed in suspended metal cages of 80 x 60 x 45 cm (H x D x L) sized for rabbit production, equipped with semi-automatic galvanized aluminum feeders and automatic nipple drinkers. Wooden nests of 20 x 40 x 25 cm (H x D x L) were used, made with sheets of naval plywood and a screened bottom with aluminum mesh of 3 mm gap. A digital thermometer was used inside the masonry and the light period adopted was natural (around 12.10 h per day). The nests were placed for pregnant females 5 days before the expected birth date and the nesting material (dry pine shavings) was inserted in the nests (about 200g), remaining until 15 days postpartum. The feed was formulated based on alfalfa hay, wheat bran, corn, soybean bran, amino acids, vitamins, minerals and additives, to meet the requirements of growing rabbits (De Blas and Mateos, 2010), pelleted with a 4.5 mm and provided ad libitum, as for water.

Performance, mortality and blood analyses

The experimental period ranged from birth to weaning (0 to 35 days of life) and daily, in the morning, the variables evaluated were: dead kits (mortality rate) and live weight of litter (weight gain). At 15 days of age, two kits per nest (close to medium live weight) were submitted to blood harvesting, through heart puncture. Then, analyzes of glucose, total proteins, triglycerides, total cholesterol, HDL, LDL, albumin and globulines were carried out in plasma by colorimetric method, using commercial kits, following the standard operating procedures, in addition to a complete blood count (red and white blood cells and platelets), in a particular veterinary laboratory.

Statistical Analysis

Analysis of variance (ANOVA) was performed using the “General Linear Models” (GLM) procedure of the statistical software “Statistical Analysis System” (SAS Inst. Inc., Cary, NC, USA). For all analyses, the F test was used, at a significance level (P) of 0.05.

RESULTS AND DISCUSSION

Performance and mortality

Controlled nursing resulted in lower mortality (P= 0.003) than natural nursing (Table 1), with rates of 12.4% and 22.9%, respectively. Zhang et al. (2018) evaluated mortality and DWG of rabbit kits on controlled nursing until 28 days of life and observed that free access to does resulted (P< 0.05) in greater weight gain for litter than controlled nursing, with 28.5 and 26.1g, respectively. Furthermore, the authors found no difference in mortality, with no losses of animals during the experimental period, which also contradicts the results obtained in our work.

Table 1: Performance and mortality rate of New Zealand White kits submitted to natural and controlled nursing for 35 days

	Nursing		SEM[1]	P-value[2]
	Natural	Controlled		
Does, no.	22	22	-	-
Litter size at birth				
Total born	10.2	10.5	-	-
Born alive	8.2	8.6	-	-
Mortality at birth (%)	19.5	18.2		
Litter weight at birth (g)				
Total born	562.9	578.5	-	-
Born alive	448.6	478.2	-	-
Individual weight at birth (g)				
Total born	54.9	55.1	-	-
Born alive	53.5	54.1	-	-
Used in assay (8 kits per doe)	53.6	56.3	1.3	0.319
Litter size at weaning	6.2	7.0	0.4	0.212
Individual weight at weaning (g), 35d	610.9	623.1	18.1	0.742
Weight gain (g/d)	15.9	16.2	0.9	0.456
Total mortality (%)	22.9 a	12.4 b	6.7	0.003

[1] Standard error of mean. [2] Means with different letters on the same row differ significantly (F test).

Szendro et al. (1999) evaluated the separation of does from the litter until weaning, and observed that free access of does to kits maintained a greater weight gain than the controlled treatment, with values of 607g and 573g respectively at 28 days of life. This variation in the weight of the animals among different works may be related to several factors, such as the female's milk production and diet quality, the number of milking per day, beginning of solid food intake, breed, among others (Faria et al., 2004). However, the amount of milk ingested is one of the main factors that affect performance of kits (Maertens et al., 2006), that is often linked to the amount of milk produced by does, what has not been standardized by genetic improvement in Brazil.

Blood analyses

Blood biochemical parameters and blood count (Table 2) did not differ between treatments (P>0.05). Garcia et al. (2021) present control values for glucose, triglycerides and cholesterol parameters in rabbits at 70 days of 163 mg/dL; 115 mg/dL and 108 mg/dL respectively. In the present work the blood collection was performed at 15 days of kits to evaluate de exclusive effect of milk as food. Besides the absence of difference between treatments, the results obtained for glucose in kits differ of adult rabbit due to the low sugar content in milk (specially lactose) and the higher amount of triglycerides and total cholesterol was due to the high amount of fat in milk. The blood count is an important tool for diagnosing and controlling parasites, infectious and chronic diseases. Chaudhuri and Sadhu (1960) mentioned some hemogram values for rabbit kits, with erythrocytes of 4.2 million/cm^3, hematocrit of 32.50% and hemoglobin of 11.0 gm/100 ml of blood, being values close to those found in this work, indicating good health of kits during the assay.

Table 2: Biochemical parameters and blood count of New Zealand White kits at 15 days of life submitted to natural and controlled nursing

	Nursing		SEM[1]	P-value[2]
	Natural	Controlled		
Kits, no.	44	44	-	-
Biochemical parameters				
Glucose (mg/dL)	109.4	99.5	4.0	0.247
Total Proteins (g/dL)	2.9	3.0	0.1	0.583
Albumin (g/dL)	2.2	1.9	0.1	0.145
Globulins (g/dL)	0.7	1.2	0.2	0.172
Triglycerides (mg/dL)	170.0	173.3	16.8	0.812
Cholesterol (mg/dL)	266.5	257.4	20.5	0.495
HDL (mg/dL)	53.4	55.9	7.7	0.301
LDL (mg/dL)	220.4	201.4	14.3	0.552
Blood count				
Erythrocytes (millions/µL)	4.1	4.0	0.3	0.941
Hemoglobin (g/dL)	9.6	9.5	0.8	0.961
Hematocrit (%)	29.2	27.5	3.2	0.792
Platelets (unit/µL)	49,333.3	43,614.4	2,519.2	0.427
Leukocytes (unit/µl)	1225.7	1686.6	431.4	0.126

[1] Standard error of mean. [2] Means with different letters on the same row differ significantly (F test).

CONCLUSIONS

Controlled nursing reduces the total mortality and does not alter biochemical parameters or blood count of New Zealand White kits.

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ASSESSING THE PHYSIOLOGICAL STATE OF RABBITS UNDER HEAT STRESS USING INFRARED THERMOGRAPHY

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ABSTRACT

Heat stress is a significant challenge to both the welfare and productivity of rabbits. This study investigated the effectiveness of infrared thermography (IRT) in assessing the physiological state of rabbits subjected to different temperature regimes. The experiment involved keeping rabbits from two groups in controlled environments for 4 hours, with temperature and humidity indices (TVI): 19.8°C for the control group and 31.8°C for the experimental group. Rabbits' body temperatures were measured throughout the study period using IRT. Additionally, the obtained data were compared with temperature measurements from a thermocouple placed on the inner ear surface and rectal temperature to confirm heat stress. The results indicated significant differences in body temperature between the groups, with rabbits in the heat stress group exhibiting elevated temperatures in all measured areas. Furthermore, a correlation was observed between IRT measurements and thermocouples, suggesting IRT as a potential non-invasive tool for monitoring heat stress in rabbits.
This study highlights the potential of IRT as a valuable non-invasive and objective tool for monitoring the body temperature of rabbits.

Key words: rabbit, heat stress, infrared thermography, physiological state

INTRODUCTION

Among all types of stress, heat stress is a significant problem for the welfare and performance of rabbits. Additionally, globally, heat stress is a prevalent issue in rabbit breeding, causing significant economic losses (Oladimeji *et al.,* 2022). Assessing the physiological state of rabbits under heat stress is crucial for understanding their response to environmental challenges. Heat stress disrupts homeostatic mechanisms (Liang *et al.,* 2022), making it essential for rabbits, being homeothermic animals, to maintain a constant body temperature to carry out their normal activities and vital processes (Yamasaki-Maza *et al.,* 2017).
Traditional methods of measuring temperature in rabbits, such as rectal or ear canal thermometry can be invasive and stressful for the animals. Infrared thermography (IRT) has emerged as a valuable non-invasive tool for monitoring the physiological responses of animals, welfare assessment. (Rekant S. *et al.,* 2016). Therefore, the primary objective of this study was to evaluate the effectiveness of IRT in a military setting compared to traditional methods such as rectal thermometry or thermocouple ear canal thermometry for measuring temperature in rabbits, given its non-invasiveness and potential to reduce animal stress.
This study examines the effectiveness of IRT in assessing the physiological state of rabbits exposed to different temperature regimes.

MATERIALS AND METHODS

Animals and experimental design

The research was conducted at the vivarium of the Institute of Animal Biology National Academy of Sciences. Ten male White Panon rabbits, 60 days old, were divided into two groups: thermoneutral and heat-stress. Rabbits were fed a commercial pelleted, balanced diet (17.55% crude protein and 17.65% crude fiber) ad libitum and provided with water freely. Antibiotics were not administered in water or food. The rabbits were housed in polyvalent

mesh cages measuring 80 cm x 50 cm x 55 cm (length, width, and height, respectively).The control group was kept at an indoor air temperature of 18°C and 60.1% relative humidity. Heat stress conditions for the experimental group were created by raising the holding temperature to 32°C. The experiment lasted 4 hours. A thermohygrometer was used to record the average air temperature and relative humidity every 60 minutes throughout the entire experimental period. The temperature-humidity index (THI) was used to determine the level of heat stress (Marai et al., 2001), where a temperature < 27.8°C indicates no heat stress, 27.8–28.9°C indicates moderate heat stress, 29.0–30.0°C represents average heat stress, and > 30.0°C signifies maximum heat stress. The THI is calculated using the following formula: $THI = t - \left(0.31 - \frac{0.31 \times RH}{100}\right) \times (t - 14.4)$, where t (°C) is the temperature and RH (%) is the relative humidity.
The experiment lasted for 4 hours, during which measurements were taken from the rabbits every 60 minutes. IRT measurements were performed, and the rectal temperature and the temperature of the base of the inner ear were measured using a thermocouple.

Temperature Analyses
A Zeiss DTI 3/35 thermal imager, offering a resolution of 384 x 288 pixels and a 50 Hz frame rate and an measurement accuracy of ±0.05°C was used in this study. Thermogram analysis involved Fiji ImageJ software and the ThermImageJ plugin. We calculated the percentage of each animal's surface area exhibiting elevated temperature across both groups. Furthermore, a thermocouple with a measurement range of -50°C to +50°C and an accuracy of ±0.01°C was employed. To visualize the temperature distribution, we generated thermal maps and corresponding graphs.

Statistical Analysis
The arithmetic and statistical processing of the obtained data will be performed using the open source software "R". Within the framework of the study, the Mann-Whitney U test will be used to compare the indicators between the groups. The intergroup difference in indicators will be considered statistically significant at P≤0.05.

RESULTS AND DISCUSSION

Rabbits are particularly susceptible to dangerously high temperatures, especially when coupled with high humidity (Oladimeji *et al.,* 2022). This vulnerability stems from their inability to sweat and the limited effectiveness of panting as a cooling mechanism.
The microclimate parameters in the control and experimental rooms corresponded to THI of 19.22 and 29.92 respectively (e.g. Table1).

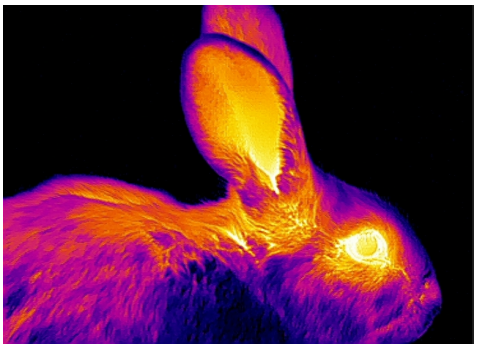
Table 1: Parameters of the experiment

Parameters	Groups	
	Thermoneutral	Heat Stress
Temperature °C	19.8±0.05	31.8±0.11
Humidity %	65.1±0.17	65.1±0.08
THI	19.22±0.22	29.92±0.19

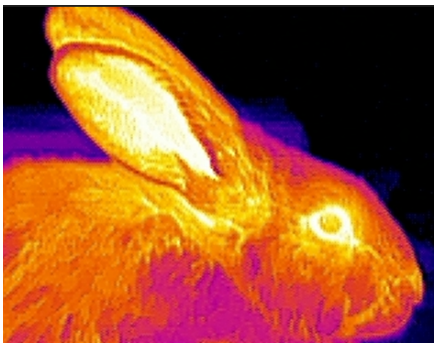
This indicates exposure to average heat stress without prior adaptation for the animals in the experimental group.
Notably, significant increases in rectal and inner ear surface temperatures were observed within the first hour of heat stress. By the fourth hour, rectal temperature reached peak values, with the experimental group exhibiting a significantly higher increase of 2.4% ($P<0.001$) compared to pre-stress levels.
This observed increase in both rectal and inner ear temperatures may be linked to the insulating properties of the rabbits' fur coat, a response to increased metabolic rate, and ultimately, hyperthermia due to impaired thermoregulation. Importantly, the experimental animals abstained from food and water throughout the four-hour stress period. This reduced

feed intake might be a strategy to minimize metabolic heat generation, potentially explaining the observed behavioral adaptations. IRT measurements taken hourly revealed a gradual rise in temperature in the ears, eyes, and nose withers (e.g. Figure 1).

Figure 1: Rabbit head IRT



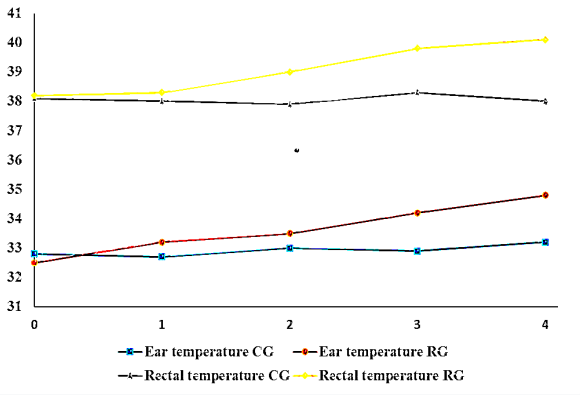
Thermogram of the control group



Thermogram through 4 hours of heat stress

Figure 2: IRT of a rabbit during heat stress and changes in the temperature of physiological points

Thermogram of the rabbit of the experimental group after 4 hours of the experiment



The dynamics of temperature changes of the control and experimental groups was measured using a thermocouple

According to the thermograms, the rabbits' body surface area with elevated temperature (<37°C) increased by 1,81±0.34 % per hour during the temperature stress period ($P<0.05$ for 1 hour and $P < 0.001$ for 4 hours of the experiment). The average body temperature of rabbits, as measured by thermograms during this period, increased by 0.4 ± 0.08 °C per hour. However, the temperature increase across the body surface was uneven. The area of the auricle is a zone where numerous blood vessels allowed to observe changes in blood circulation. Specifically, the larger surface area of the ears reached 38°C within an hour, as observed through thermographic analysis. Additionally, the temperature around the nose and eyes also increased.

IRT diagnostics revealed visually identifiable changes within the first hour of heat stress exposure. However, the temperature measured by a thermocouple placed on the inner ear surface was consistently reliable for diagnosing heat stress until after 3 hours and rectal temperature after 2 hours (e.g. Figure 2). This discrepancy may be attributed to subjective factors, such as the point of application, the density of contact, and the initial rise in the rabbit's body temperature due to the stress of being handled. It is noteworthy that by the end

of the experiment, the entire ear surface displayed a significant temperature increase, demonstrating a strong correlation (e.g. Table 2) with the contact temperature measurements.

Table 2: Correlation coefficients

	Ear temperature	Rectal temperature	IRT
Ear temperature	1		
Rectal temperature	0.962737	1	
IRT	0.971746	0.962309	1

This observation aligns with the established knowledge that, under normal conditions, the inner base of a rabbit's ear exhibits the highest temperature. Under heat stress, as shown in Figure 2, the entire ear surface experiences a remarkable rise in temperature.

CONCLUSIONS

The advantages of thermography over classical methods of measuring temperature in rabbits includes non-invasiveness, reduced stress on the animals, and the ability to monitor temperature changes in real-time over large areas without direct contact. The obtained data correlate well with each other.

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EFFECT OF SUMMER ENVIRONMENTAL TEMPERATURE ON THE BODY TEMPERATURE, BEHAVIOR, AND GROWTH PERFORMANCE OF GROWING RABBITS

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ABSTRACT

In response to the current lack of reliable parameters for assessing the thermal comfort of rabbit housing environments, this study investigates the relationship between environmental temperature and the abdominal temperature, growth performance, and behavior of growing rabbits, aiming to provide reference for the evaluation of rabbit comfort. The study was conducted from July 31 to September 3, 2022, in a vertically ventilated rabbitry with wet curtain fans for cooling and windows closed in Henan Province, China. Data on environmental temperature, feed intake, and weight gain were collected from 93 growing rabbits aged 35 to 70 days, and abdominal temperature and behavior were recorded for three of them. The analysis results indicate that when the environmental temperature exceeds 27°C, there is a tendency for the body temperature of rabbits to increase. With increasing age, daily feed intake and feed conversion ratio gradually increase, and a higher feed conversion ratio implies a decrease in economic benefits. Therefore, it is necessary to plan the slaughter time reasonably in commercial rabbit production. As the environmental temperature rises from 22°C to 30°C, the duration of feed intake and grooming decreases, while the time spent lying down increases. At 47-48 days of age, when the average temperature increases from 25.1°C to 27.7°C, the average body temperature increases by 0.31°C, daily lying time increases from 14.75h/day to 17.51h/day (P<0.01), and daily grooming time decreases from 2.37h/day to 1.59h/day. At 64-68 days of age, grooming time significantly decreases when the average temperature is above 20.3°C. In summary, the body temperature, grooming, and lying behaviors of rabbits have a strong correlation with environmental temperature, making them valuable tools for evaluating rabbit thermal comfort.

Key words: growing rabbits, behavior, body temperature, thermal environment evaluation, environmental regulation parameters.

INTRODUCTION

Given the variability in appropriate thermal environments for domestic rabbits due to factors such as their age and climatic conditions, current environmental control systems in rabbit houses rely on the experience of management personnel, leading to potential issues such as severe temperature fluctuations and heat stress in rabbits (Okab et al., 2008; Cerioli et al., 2011; Guo et al., 2016). The Temperature Humidity Index (THI) formula employed by Maria in 2001 did not base its assessment on actual physiological and growth data of domestic rabbits, resulting in low accuracy in evaluating rabbit housing environments (Marai et al., 2001). In light of this situation, there is a pressing need to develop an environmental evaluation method that utilizes data on the behavior, body temperature, and feed conversion ratio of growing rabbits. This approach would provide more accurate guidance for assessing the comfort of growing rabbits and for the environmental control within rabbit houses (Cerioli et al., 2011; Oladimeji et al., 2022).

MATERIALS AND METHODS

The experiment was conducted from July 31 to September 3, 2022, in a windowed, enclosed rabbit house with wet curtain fans for longitudinal ventilation cooling located in Henan Province, China, and the rabbitry dimensions were 30 meters in length, 10 meters in width,

and 3.5 meters in height. Ninety-three healthy weaned Ipru rabbits, aged 35 days and weighing between 0.86kg and 0.93kg, were selected and housed in the longitudinal front, middle, and rear areas of the rabbit house (three rabbits per cage). Temperature and humidity sensors (recording at 5-minute intervals) and cameras were installed in each area. In each area, one growing rabbit (housed in a single cage) had a thermometer implanted in its peritoneal cavity (accuracy ±0.2°C). The daily feed intake for all rabbits were manually measured (recordings started at the age of 44 days), and weight was recorded weekly (at ages 36, 43, 50, 57, 63, and 70 days). Linear interpolation was used to fill in weekly weight gain data to derive daily weight gain and to construct feed conversion ratio contour maps. Behavioral analysis software CowLog3.0.2 was used to manually annotate behavior videos during two periods of significant temperature fluctuations over consecutive 5 days (ages 45 to 49 days, and 64 to 68 days). Data were organized using Excel 2021 and the Pandas library, with Z-score and linear interpolation methods applied to detect and fill in outliers. The scipy.stats library was used for one-way ANOVA on lying and grooming behaviors across different age.

RESULTS AND DISCUSSION

The impact of environmental temperature on body temperature and growth performance of growing rabbits at different ages

Analysis of the 24-hour body temperature of nocturnal animals and its relationship with environmental temperature is difficult to establish a clear mapping. To reduce the interference of elevated nighttime body temperature on research results and to enrich the temperature data range as much as possible, we chose the data from 11:00 to 17:00, when the temperature rises rapidly, to plot contour maps. The results are shown in Figure 1. As the environmental temperature increases, the body temperature shows an upward trend. The normal body temperature of rabbits is reported to be 38.8~39.1°C (Oladimeji et al., 2022). In Figure 1A, when the environmental temperature exceeds 27°C, there is a tendency for the body temperature to increase. From Figures 1B and 1C, it can be observed that with increasing age, daily feed intake and feed conversion ratio gradually increase. Although feed intake increases, a higher feed conversion ratio implies a decrease in economic benefits. Therefore, it is necessary to plan the slaughter time reasonably in commercial rabbit production.

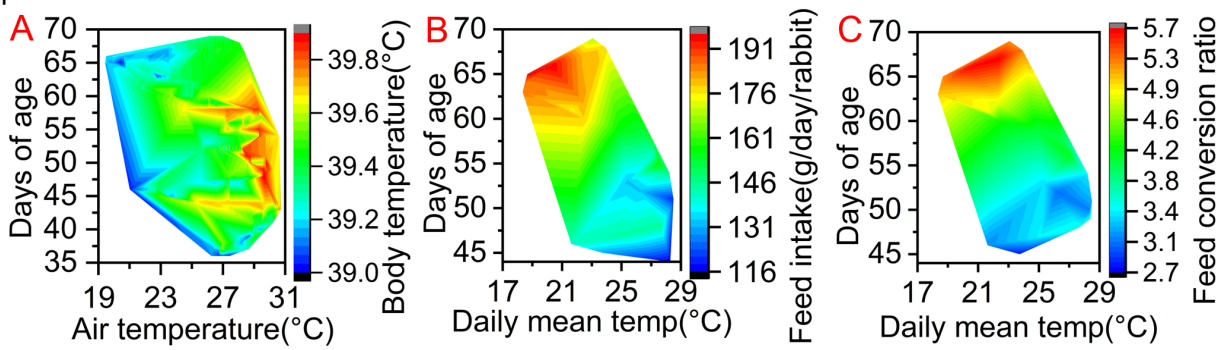

Figure 1: The impact of environmental temperature on peritoneal body temperature and growth performance of growing rabbits at different ages

In Figure A, the temperatures of the body and the environment were collected at 5-minute intervals, while in Figures B and C, the environmental temperatures represent the daily averages.

The impact of environmental temperature on the behavior of growing rabbits at different ages

To further investigate the relationship between the behavior of growing rabbits and environmental temperature, time periods with significant temperature fluctuations (where the peak environmental temperature changes by more than 5°C at different days) were selected for observation (ages 45-49 days and 64-68 days). In Figure 2, as the environmental temperature increases from 22°C (at 45 days of age) to 30°C (at 49 days of age), the

duration of feeding and grooming decreases, while the duration of lying down increases. At 47-48 days of age, when the average environmental temperature increases from 25.1°C to 27.7°C, the average body temperature increases by 0.31°C, and the daily lying time increases from 14.75h/day to 17.51h/day (P<0.01, Figure 2B), while the daily grooming time decreases from 2.37h/day to 1.59h/day (Figure 2C). These results indicate that as the environmental temperature rises, the degree of heat stress in growing rabbits gradually intensifies. Rabbits reduce food intake and activity to decrease heat production, while increasing lying down to increase the body's surface area for heat dissipation. Additionally, the results suggest that growing rabbits may exhibit further signs of heat stress when the daily average temperature exceeds 25.1°C.

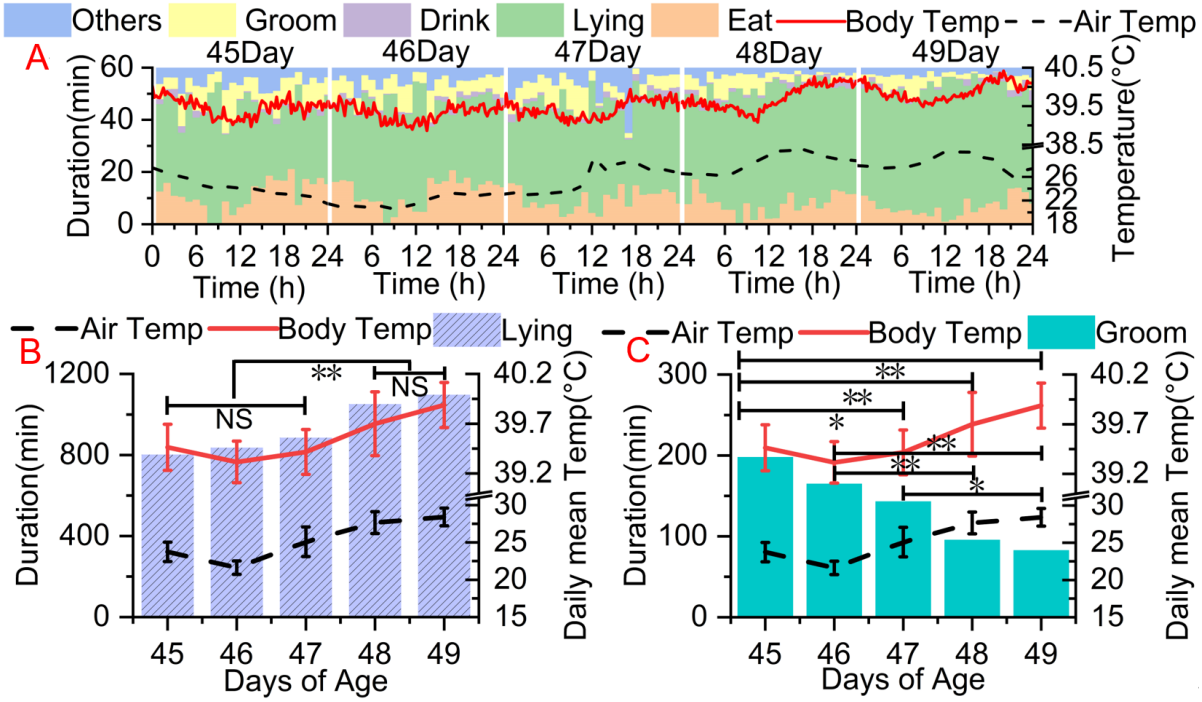

Figure 2: The Effect of Environmental Temperature on the Behavior and Body Temperature of Growing Rabbits Aged 45-49 Days

NS indicates no significant difference, * indicates P<0.05, ** indicates P<0.01; in Figures B and C, the durations of behavior, temperature, and body temperature are represented as daily averages.

From Figure 3, it can be observed that there is no significant difference in the daily lying behavior of growing rabbits aged 64-68 days. However, as the environmental temperature increases, the duration of lying down increases (Figure 3A). At ages 67 and 68 days, the daily grooming duration decreases by 1.51h/day (P<0.05) and 1.55h/day (P<0.01), respectively, compared to age 64 days. There is no significant difference in the daily grooming duration at ages 64, 65, and 66 days (Figure 3B). These results further confirm the strong correlation between environmental temperature and behavior. When the environmental temperature increases, grooming time undergoes significant changes. On the other hand, when the average temperature exceeds 20.3°C (at age 66 days), grooming behavior shows a significant decrease, indicating a potential increase in heat stress. In comparison with the results from Figure 2, where the critical temperature decreased by 4.8°C, this change may be attributed to the gradual enrichment of the rabbit's fur with increasing age, resulting in a gradual decrease in heat tolerance.

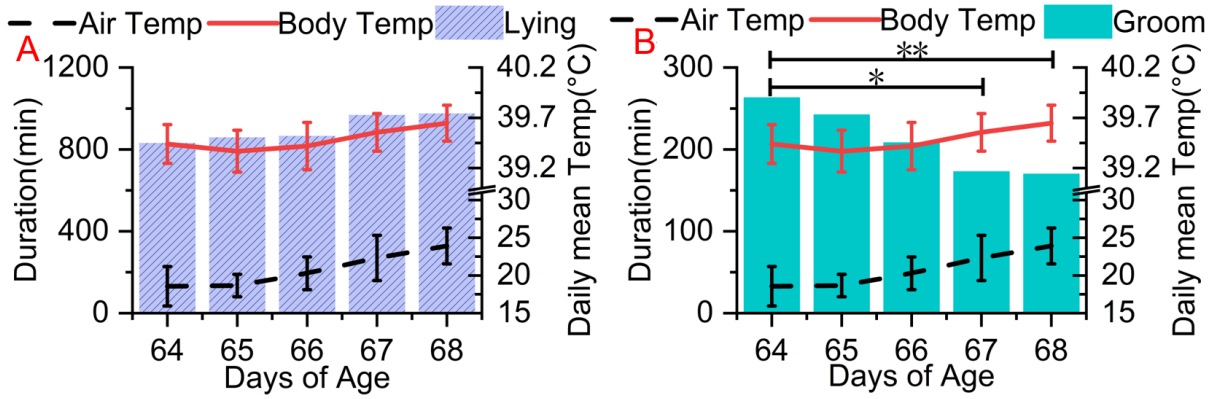

Figure 3: The impact of environmental temperature on behavior and body temperature of growing rabbits aged 64 to 68 days

CONCLUSIONS

This study demonstrates the impact of ambient temperature on the physiological and behavioral responses of growing rabbits. As the ambient temperature increases, there is a decrease in the duration of feeding and grooming, while both lying time and core body temperature increase. These indicators are significantly correlated with ambient temperature and can serve as effective tools for assessing the thermal comfort of domestic rabbits in their environments.

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EFFECT OF TWO STUNNING METHODS ON THE OCURRENCE OF LESIONS IN CALIFORNIA RABBIT CARCASSES

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ABSTRACT

The slaughter of rabbits under welfare conditions is a growing demand from consumers. However, its implementation has been poorly evaluated in rabbit meat production units in Mexico. Better animal welfare conditions enable the acquisition of carcasses with improved quality and fewer lesions. The aim of the study was to relate the occurrence of lesions in rabbit carcasses, with two stunning methods; rabbits' sex; animal weight; and the time transpiring between stunning and slaughter. One hundred and twenty California rabbits weighing 2 kg were used, distributed into 4 treatments with a 2^2 factorial arrangement with the following factors: stunning methods (a1 = concussion, a2 = electric) and animal sex (b1 = male, b2 = female). Descriptive statistics were obtained, treatment ranges were compared using Kruskal-Wallis, and risk factors for carcass lesions were identified through logistic regression analysis. No sex-based differences were found (analysis not detailed here); thus, only the stunning method was evaluated. Rabbits subjected to electric stunning prior to slaughter had fewer lesions in their carcass ($p<0.001$); with such lesions being smaller in size, and mostly located in the limbs. The stunning method was a risk factor in the occurrence of contusions ($p <0.0003$), unlike sex, weight, or the time transpiring between stunning and slaughter. The electric stunning method resulted in fewer lesions on the rabbits' carcasses, which were smaller in size and located in the limbs, compared to the concussion method. It is concluded that the stunning method is a risk factor for the occurrence of lesions in the carcass. Further research is needed to make electrical stunning a common practice in smallholder rabbit farms in Mexico.

Key words: Animal welfare; desensitization; electric stunning; concussion; contusions.

INTRODUCTION

Currently, consumers demand that animal-derived products be sourced under Animal Welfare (AW) conditions. In meat production, there has been a development of slaughter systems that take into account animal welfare (Stoier *et al.*, 2016). A better protection of animals during slaughter contributes to improving the quality of the meat and increases the amount of marketable product, since reducing stress before and during slaughter minimizes physical, sanitary, and biochemical changes (ICF, 2017).

AW must be assessed during *ante mortem* handling, considering the impact on the physical quality of the meat, such as the presence of contusions, which are carcass alterations indicating inadequate pre-slaughter handling (Grandin, 2017). In Mexico, the most commonly used stunning methods are neck dislocation and concussion; however, in other countries, the electric stunning method and its benefits in AW have been studied. Therefore, it is expected to have carcasses with fewer and smaller lesions by applying the electric stunning method compared to the concussion method. The objectives of this study were to compare the effect of two stunning methods, electric stunning and concussion; on the number, size, and location of lesions in California rabbit carcasses, as well as to identify risk factors associated with the presence of lesions.

MATERIALS AND METHODS

Animals and experimental design

The study was carried out on the Montecillo Campus of the Colegio de Postgraduados ("Postgraduate College") using one hundred and twenty 65 ± 5-day-old California rabbits (60 males and 60 females) weighing 2.2 ± 0.2 kg, and without prior fasting. The animals were randomly split into four treatments in a totally random design with a 2^2 factorial arrangement and the following main effects: A: type of stunning (a1 = stunning via concussion, a2 = electric stunning), and B: sex (b1 = male, b2 = female), with 30 repetitions per treatment and each rabbit being an experimental unit. Concussion was achieved by hitting the rabbits with a club on their occipital lobe; electric stunning was done using a Midwest Processing Systems manual electric stunner (model VS200, 120 V and 1 A). Each rabbit received a 600 V, 1 A shock in its occipital lobe for 2 seconds. After being stunned, the rabbits were hung face-down by their hindlimbs and their throats were slit. The time transpiring between stunning and throat-slitting was measured using a chronometer, and contusions were measured with a millimetric ruler.

Assessment of lesions in the carcass

The carcass was divided into two regions: limbs (forelimbs and hindlimbs) and torso (neck, thorax, and loin). Contusions were considered as traumatic lesions involving the rupture of blood vessels, accompanied by blood accumulation but without discontinuity of the skin. The number of lesions, their location, and size were recorded. The extent of the lesions was determined based on the approximate diameter of the affected area, considering three levels: ≤ 0.5 cm; between 0.6 and 1 cm; and > 1 cm. Carcasses with more than one contusion of different extents were assessed by selecting the contusion with the largest extent as criterion for evaluation (Knock and Carroll, 2019).

Statistical Analysis

A logistic-regression analysis was carried out, estimating Odds Ratios (OR) for the presence of lesions in the carcass. For this analysis, the weight of the rabbit and time transpired between stunning and throat-slitting, were transformed into categories: weight was divided into light rabbits (2.0 – 2.2 kg) and heavy rabbits (2.3 – 2.4 kg), while the time was categorized as ≤ 30 seconds or > 30 seconds between stunning and throat-slitting. The presence of carcass lesions was assessed in relation to stunning method, sex, animal weight category, and the time transpired between stunning and slaughter categories. The statistical analyses were carried out using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). The general model used was as follows:

$$P(Y=1/X) = \frac{e^{(\beta_0+\beta_1X_1+\beta_2X_2+\cdots\beta_4X_4)}}{1+e^{(\beta_0+\beta_1X_1+\beta_2X_2+\cdots\beta_4X_4)}}$$

Where:
P = Probability of the Y variable, Y_i, taking the value of Y=1
Y = 1 if lesions occur and Y=0 if they do not occur
β_0, β_1,.., β_4 = Unknown parameters
X_1, ..,X_(4,) = Independent variables (stunning method, sex, weight categories, and the time transpiring between stunning and throat slitting categories).

RESULTS AND DISCUSSION

Location of lesions

Since the statistical analysis revealed no differences based on the rabbits' sex, the only factor evaluated was the effect of the stunning method. Regardless of the stunning method used, 93% of the evaluated carcasses had at least one lesion (Table 1), with differences in lesion location ($p < 0.01$) being found. Valkova *et al.* (2021) found a 1.52% occurrence of traumatic lesions, 0.83 % of which were on the limbs and 0.69 % on the torso, with a

difference between the two of $p < 0.01$. While another study found a 2% mean of carcasses with lesions, the only lesions taken into account were hematomas measuring over 1 cm^2, most of which were found on the hindlimbs, the thoracic muscles, and the inner part of the loin. It is hard to compare the percentages of hematomas reported in the few available studies due to the differences in veterinary-inspection methods and in the classification of carcasses (Petracci *et al.*, 2010).

A larger percentage of lesions was observed on the limbs of animals that were electrically stunned than on their torsos, while animals stunned via concussion to their occipital lobe were found to have more lesions on their torsos than on their limbs. This also explains why stunning via concussion results in a 32.5 % incidence of lesions on both parts of the carcass, compared with a 15.8% occurrence of such lesions when the electric-stunning method is used.

Table 1: Evaluation of lesions on rabbit carcasses with two stunning methods

	Total			Concussion			Electric stunning			
	%	±	SME	%	±	SME	%	±	SME	*P-value*
Carcasses with lesions	93.3		0.61	50 [a]		4.51	43.3 [b]		5.27	0.004
Anatomic location										
Torso	24.2		7.05	16.7[a]		7.8	7.5[b]		8.96	0.019
Limbs	20.8		7.38	0.8[b]		----	20[a]		7.46	<0.001
Both	48.3		4.76	32.5[a]		6.24	15.8[b]		7.89	<0.001
Neither	6.7		9.91	0 [b]		----	6.7 [a]		9.91	0.004
Number of lesions										
Low[c]	54.2		4.22	18.3[b]		8.17	35.8[a]		6.12	<0.001
High[d]	45.8		4.99	31.7[a]		6.6	14.2[b]		8.7	<0.001
Size of lesion										
Small[e]	18.3		8.17	0 [b]		----	18.3 [a]		8.17	<0.001
Medium[f]	10		9.3	4.2[a]		10.67	5.8[a]		10.11	0.545
Large[g]	65		2.79	45.8[a]		4.98	19.2[b]		8.07	<0.001
None	6.7		9.91	0 [b]		----	6.7 [a]		9.91	0.004
Average number of lesions[h]	3.7		0.25	4.82 [a]		0.39	2.67 [b]		0.29	<0.0001

[c]Low = 0 – 3 lesions. [d]High = 4 – 14 lesions. [e]Small ≤ 0.5 cm. [f]Medium = 0.6 – 1.0 cm. [g]Large = > 1.0 cm. [h]The average number of lesions and its SEM are presented in original data. [a, b]. Differences between rows are significant ($p < 0.05$). SEM = Standard Error of the Mean.

Number and size of lesions

The comparison of ranges expressed as categorical values showed differences between stunning methods. In general, there was an average of 3.7 lesions per carcass, with a range of between 0 and 14 for the 120 carcasses. The average number of lesions varied based on the stunning method used, with 4.82 lesions in the case of stunning via concussion, 2.67 lesions in the case of electric stunning (p <0.0001) (Table 1). Since rabbits are easy to handle and hold, making it easy to control external factors that could result in pre-slaughter lesions, the lesions found in the *post-mortem* carcass examinations can be associated with the stunning method used.

All the small lesions occurred with the electric stunning method, with most of them being found on the limbs. These lesions can be associated with the application of electric shocks that might cause muscle contractions that result in damage to the muscle fibers and resultant hemorrhaging in the muscle tissues due to a big increase in intravascular pressure, which in turn could cause the blood capillaries to rupture and bleed (Kranen *et al.*, 2000). Studies carried out on cattle have determined that more small and medium-sized lesions occur

—74.9 % and 19.1 % respectively— than large ones (Knock and Carroll, 2019). This shows that concussion stunning results in larger lesions than electric stunning ($p < 0.01$).

Risk factors in the occurrence of all carcass lesions

The logistic regression showed that the only factor determining carcass lesions was the stunning method used. Concussion stunning is 4.6 times more likely to cause carcass lesions than electric stunning (Table 2). Those studies that evaluate the risk factors associated with carcass lesions do not take stock of the stunning method used.

Table 2: Probability of lesions in California rabbits per causal variable, based on the logistic-regression analysis.

Variable	Category		Parameter	SME (b_i)	RO	CI 95%	*P - Value*
Method	Concussion	(0)	1.53	0.42	4.60	2.01-10.49	0.0003
	Electric	(1)					
Sex	Male	(0)	0.40	0.41	1.49	0.66-3.35	0.34
	Female	(1)					
Weight category	Light rabbits	(0)	-0.19	0.46	0.82	0.33-2.04	0.68
	Heavy rabbits	(1)					
Time category	≤ 30 s	(0)	0.16	0.66	1.17	0.32-4.24	0.81
	> 30 s	(1)					

SME = Standard Mean Error; RF = Risk factor; CI = 95% Confidence Interval. Time category = time transpiring between stunning and throat slitting in seconds.

CONCLUSIONS

Based on the current findings, the use of electrical stunning in rabbits resulted in a better carcass quality. Moreover, this method could enable the slaughtering of rabbits under welfare conditions. The implementation of electrical stunning prior to slaughter in smallholder rabbit farms depends in the development of suitable instruments which require further research.

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VALIDATION OF A FAST SEGMENTING COMPUTER VISION APPROACH TO MONITOR RABBITS

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ABSTRACT

To realize real-time health monitoring of caged rabbits based on computer vision technology in large-scale rabbit farms, it is necessary to segment rabbit images quickly. The paper proposed a semantic segmentation framework for caged rabbits based on deep convolutional neural network approaches using Ghost module and ResNet-18 network. The proposed model was trained, validated and tested on the caged rabbit image dataset. The results show that the proposed framework improves the segmentation frame rate on GPU by 3.8 times compared to U-Net with 99.51% accuracy. The proposed framework reflects better comprehensive performance in segmentation of rabbits, which is more adaptable to the production needs such as real-time inspection of rabbit farms.

Key words: Smart breeding; Deep learning; Semantic Segmentation; Rabbit

INTRODUCTION

China boasts a rich history in rabbit farming. With the continuous improvement in living standards, there has been a shift towards healthier dietary choices, making rabbit meat, known for its high protein and low cholesterol, increasingly popular. According to data from FAOSTAT (2022), the global rabbit meat production was approximately 756,476.01 t, with China contributing 358,152.42 t. Traditional monitoring and assessment methods for commercial rabbit farming heavily rely on subjective visual inspection, providing limited useful information. Furthermore, visual inspection can stress the animals and pose health risks to inspectors. Therefore, adopting advanced automated inspection technology is becoming an inevitable trend in managing rabbit farming.

Monitoring the health of rabbits automatically is crucial throughout their breeding cycle. Various sensors are employed to assess weight, temperature, feed intake, and activity levels, thus evaluating the welfare conditions of animals automatically (Peng *et al.*, 2019; Cuan *et al.*,2020). Computer vision, based on various types of cameras, is widely utilized in livestock and poultry housing for health monitoring. To effectively monitor the growth and health of animals, image segmentation of individual animals or areas of interest within videos or images is necessary for assessing health status through specific organs. Research on livestock (Van Hertem *et al.*, 2013; Salau *et al.*, 2020; Hu *et al.*, 2023) and poultry (Li *et al.*, 2022; Lamping *et al.*, 2022) image segmentation can be categorized into traditional methods based on basic features like color and texture for contour segmentation, and deep learning-based image segmentation.

The complexity of farm equipment, such as cages and water lines, coupled with the high density of rabbit farming, poses significant challenges in capturing rabbit images. Target occlusion, adhesion between subjects, and uneven lighting conditions pose challenges to rabbit image segmentation. Furthermore, the segmentation speeds of common network models fail to meet the real-time inspection requirements. To enhance the segmentation speed while ensuring the performance of rabbit image segmentation and better utilize inspection platforms for monitoring rabbit health conditions on farms, this paper proposes a U-Net-based semantic segmentation framework.

MATERIALS AND METHODS

Image acquisition and dataset construction

Images of rabbits housed in individual cages were captured at Yangguang Rabbit Industry, a commercial rabbit farm with over 30 breeding sheds in Jiyuan City, Henan Province. This study used the SONY CG240C industrial camera model with an exposure time of 23 ms and a highlight of 20 dB. Our research subjects comprised 300 Hyplus rabbits aged between 34 and 1180 days, including pregnant, growing and breeding rabbits. Rabbits were individually housed in galvanized wire cages under controlled light and temperature (18 ~ 24℃) conditions. During data acquisition, three images were continuously captured for each cage position. A 10-second interval followed each acquisition to allow the rabbits to naturally change postures before the next capture. This ensured that each set of images captured different postures in each capture. The original images underwent cropping and resizing to 512x512 pixels, enhancement using the RetinexNet algorithm, and annotation along rabbit contours in COCO format using the open-source software Labelme. The dataset comprises 900 images from individual rabbits, with 810 images used for training and 90 images for validation.

U-Net model

The U-Net structure proposed by Ronneberger *et al.* (2015) is characterized by its distinctive 'U' shape, consisting mainly of a contracting path to capture context information from images and an expansive path for precise localization of the segmented parts. The contracting path includes convolutional layers with 3x3 kernels followed by max pooling for down-sampling, whereas the expansive path comprises convolutional layers with 3x3 kernels and up-sampling convolution of 2x2 kernels, with skip connections bridging corresponding layers of the contracting and expansive paths without convolutional operations.

ResNet-18 network

The ResNet architecture proposed by He *et al.* (2016) introduced residual blocks that utilize shortcut connections, allowing the network to skip certain layers and thereby facilitate faster forward propagation. This design enables signals to be directly transmitted from lower to upper layers, mitigating the problem of network degradation. ResNet-18, composed primarily of residual blocks, includes 18 weight layers.

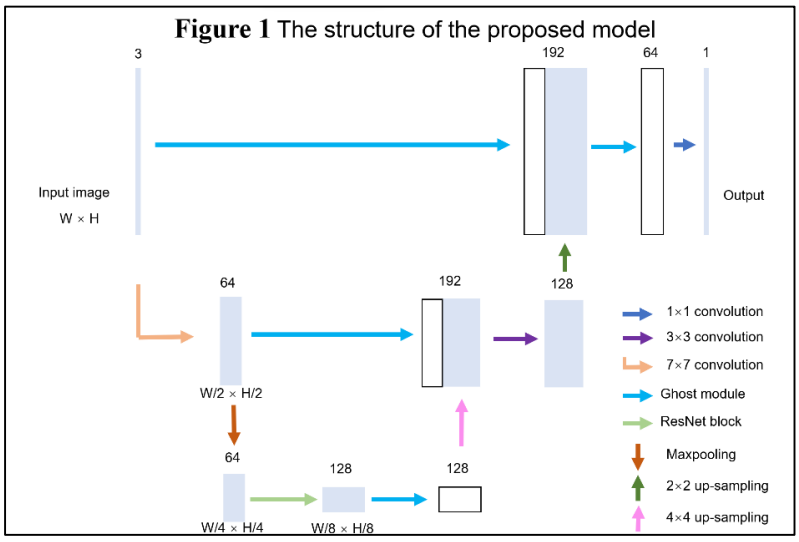
Ghost module

The Ghost Module proposed by Han *et al.* (2020) divides the convolutional process into two distinct parts. The first part performs standard convolutional operations, while the second part consists of a series of simple linear operations that utilize the feature maps generated by the first to produce additional feature maps. This approach effectively reduces the computational load of convolutional operations while maintaining the same level of feature extraction capabilities.

The proposed framework

Considering the unique characteristics of individual rabbit images and the necessity for fast segmentation, modifications and optimizations were implemented on the original U-Net model. These optimizations comprise: (1) reducing the network depth from five to three layers, (2) integrating elements of the ResNet-18 architecture into

Figure 1 The structure of the proposed model



the contracting path to substitute standard 3x3 convolutional layers, and (3) introducing the Ghost Module to replace traditional skip connections and the final two 3x3 convolutions before output. The structure of the proposed framework is depicted in Figure 1.

RESULTS AND DISCUSSION

Evaluation metric

The evaluation metrics for the segmentation framework include Accuracy, Recall, Precision, Dice-score, and the frame rate of image segmentation. These metrics are defined as follows:

$$\text{Accuracy} = \frac{TP+TN}{TP+TN+FP+FN} \quad (1)$$

$$\text{Recall} = \frac{TP}{TP+FN} \quad (2)$$

$$\text{Precision} = \frac{TP}{TP+FP} \quad (3)$$

$$\text{Dice score} = 2 * \frac{\text{Precision} * \text{Recall}}{\text{Precision} + \text{Recall}} \quad (4)$$

Where TP is the number of true positives, TN is the number of true negatives, FP is the number of false positives, and FN is the number of false negatives.

Experimental Results and Analysis

We trained various existing semantic segmentation models alongside the model proposed in this paper to compare their performance. The performance of different networks on rabbit segmentation is shown in Table 1.

The comparison between U-Net and the proposed framework in terms of rabbit segmentation performance clearly shows that our framework underperforms by merely 0.15%, 0.43%, 0.56%, and 0.51% in Accuracy, Recall, Precision, and Dice-score, respectively, yet still maintains high precision. Despite the negligible difference in performance metrics with U-Net, our framework significantly enhances the image segmentation speed on GPU by 3.8 times, respectively. These results underscore that the proposed framework not only retains high accuracy in segmentation but also substantially increases the speed of image segmentation, aligning more closely with the demands of production environments.

The results indicate that while the proposed framework slightly trails behind other networks in terms of Accuracy, Recall, Precision, and Dice score by a narrow margin, it still maintains a high performance score overall. Notably, on a GPU, the proposed framework achieved a segmentation frame rate of 78.92 frames per second (f/s), which is nearly twice as fast as the fastest among the remaining models, the SegNet model. This demonstrates the proposed model's significant advantage in processing speed, emphasizing its potential for real-time applications where rapid image analysis is critical.

Table 1: The comparison of segment models performance

Network	Accuracy（%）	Recall（%）	Precision（%）	Dice-score (%)	frame rate on GPU (frames/second)
U-Net	99.66	99.23	98.47	98.83	20.59
Deeplab V3+	99.73	99.34	98.85	99.09	22.06
SegNet	99.58	98.89	98.25	98.56	39.96
BiseNet	99.65	99.29	98.38	98.82	30.78
Ours	99.51	98.80	97.91	98.32	78.92

CONCLUSIONS

This paper focused on single-cage-bred rabbits at different growth stages and proposed a fast semantic segmentation framework utilizing the Ghost Module and ResNet-18 network. With the proposed framework achieving 99.51% in Accuracy, 98.80% in Recall, 97.91% in Precision, and 98.32% in Dice-score, the segmentation speed on GPU has significantly

increased from 20.59 f/s to 78.92 f/s, respectively. These results demonstrate the model's effectiveness in enhancing the speed of rabbit image segmentation. The proposed image segmentation method serves as an initial step in preprocessing rabbit images. Additional processing is required to extract animal features from the segmented images. Extracting multiple features from segmented images and continuously tracking them enables developing regression methods based on machine learning or deep learning for estimating the liveweight and even detecting dead rabbits. However, this still requires labelled images and classification methods to be developed and validated.

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FARMING SYSTEMS & ECONOMY

DESCRIPTIVE ANALYSIS OF THE ENVIRONMENTAL IMPACT OF INTENSIVE RABBIT PRODUCTION

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ABSTRACT

This descriptive literature review presents some elements allow us to quantify the main contributions of rabbit farming to the global warming, pollution (mostly nitrogen losses, airborne particulate matter), water footprint and biodiversity loss. As the majority of meat rabbits farmed in in the world are raised in indoor cage systems, the majority of studies only cover this production system. A single attempt has been made to quantify the environmental impact of an alternative system, based on rabbits grazing under photovoltaic panels. Although it provides some insights into possible alternatives, the results obtained are not based on real data. Regarding the contribution of rabbit production to global warming, the estimations of greenhouse gas emissions ranged from 3.13 to 3.25 kg of CO_2 eq. per growing rabbit over a 35-day period. No estimates are available for the whole system (all animal categories). Pollution associated with nitrogen losses varied between 40.1 and 59.1 g of N per kg of liveweight gain. Air pollution related to the airborne particulate matter (10 micron) varied from 0.082 to 0.045 mg per m^3, and there was no data available on the water footprint, which is likely to be between those observed for poultry and pig production. For biodiversity loss, there are no studies on the impact of rabbit production on wild life. This communication ends with a brief discussion of the possible alternatives and presents some technical perspectives for the rabbit sector.

Key words: *Oryctolagus cuniculus*, Greenhouse gas, Water use, Air quality, Biodiversity loss.

INTRODUCTION

This brief review in not intended to be an exhaustive compilation on the environmental impacts of intensive animal (rabbit) production. Comprehensive texts on the subject are already available (see Steinfeld *et al.* 2006), and much of the mitigation strategies and policies have been addressed by the Intergovernmental Panel on Climate Change in recent years (https://www.ipcc.ch/srccl/).

As you will see, the literature available on the environmental impact of livestock production is vast and covers many areas, from technical solutions to policy. Ethics, moral philosophy and critiques of the organisation of the global economy and society are also present in the debate about what to do with animal (rabbit) farming. Some argue in favour for its abolition (Reese, 2018), while others clearly demonstrate the importance of farm animals to human societies (Porcher, 2011). Personally, I believe that it is possible to produce the necessary amount of high-quality animal products for every single person on this planet. I also believe that we can do it in an intelligent way. A way that respects the physical and mental integrity of every farmed animal, and properly values the work of both humans and animals involved in our food production chain. A way that preserves nature and guarantees the food sovereignty for the current and future generations.

To the date, I have no clear idea of how to achieve these goals. This brief descriptive analysis of the environmental impact of intensive rabbit production will certainly not solve these problems. What I can offer you here is a simple compendium of what is known about the rabbit industry. But I'll try to give you some thoughts and perspectives on where we should focus our

practices in the coming years. I hope you enjoy reading this work, as much as I have enjoyed writing it.

LITERATURE SEARCHING CRITERIA

The literature search was performed using the Web of Science advanced search query builder (https://www.webofscience.com/wos/woscc/advanced-search). The search period was set to January 1955 and February 2024. All results from the Web of Science Core Collection were checked. The selected editions were: A&HCI, BKCI-SSH, BKCI-S, CCR-EXPANDED, ESCI, IC, CPCI-SSH, CPCI-S, SCI-EXPANDED and SSCI. The query syntax used in the sequential search and the number of documents retrieved (all types) are shown in Table 1.

From these sequential searches, a total of 560 documents (all types) were preselected based on their title. A second screening of all retrieved documents was carried out after reading the abstracts of each book chapter and peer-reviewed research paper. This second screening process resulted in a selection of 422 documents.

All peer-reviewed papers that specifically addressed the environmental impacts of "*rabbit farming*" or "*rabbit production"* were retained for analysis (n=20). Some papers on wild rabbits (n=5) or alternative rabbit production systems (n=7) were also included. From the remaining documents, covering several topics related to the environmental impact of livestock production, the most relevant articles from each decade were carefully examined (n=38).

The aim was to provide an overview of how rabbit production contributes to the environmental problems we face today, such as: greenhouse gas emissions, water footprint, particulate matter emissions or biodiversity loss. The concept of sustainability in rabbit production was briefly discussed.

BRIEF OVERVIEW OF FIVE DECADES OF RESEARCH ON THE RNVIRONMENTAL IMPACTS OF ANIMAL PRODUCTION

This paper is not intended to further assess the environmental impacts of animal (or specifically rabbit) production. Exactly 18 years ago, Steinfeld *et al.* (2006) already produced a comprehensive document on the subject, *Livestock's long shadow: environmental issues and options*. In this book, the authors detailed the global contribution of the livestock sector to land degradation, climate change, water use and pollution, and biodiversity loss. They discussed technical strategies aimed to improve the efficiency of livestock operations and proposed some policy alternatives, such as properly pricing the use of land, water and other natural resources, to shed some light on the livestock's long shadow. Since the publication of this book, the scientific literature on the subject boomed, from 44 to about 364 peer-reviewed scientific papers (including only the articles preselected for this study).

As early as 1978, Hodge highlighted the external costs of livestock production (water pollution, cruelty to animals and nuisance: odour, sewage, pests, etc.) and the negative perception of livestock production by citizens in urban areas. According to Hodge (1978a), the main factors influencing complaints were related to the type of production, the number of animals and the techniques used (or not) to prevent pollution and other nuisances. For instance, intensive dairy and poultry production received most complaints. For pig farming, "*traditional*" farms (up to 250 animals) received no complaints while the so-called "*factory*" farms (over 1 500 pigs) were frequently criticized. In a second study, Hodge (1978b) noted the importance of having clear standards for measuring environmental impacts and argued for the adoption of policies to prevent pollution and nuisance. Since then, the Right-to-Farm Act (https://alec.org/model-policy/right-to-farm-act/), which was intended to protect farmers from nuisance lawsuits, has given "*big agribusiness a free pass to pollute and cause harm to people and our land*", said Diamond *et al*. (2022).

Table 1. Keywords used in the query syntax and number of documents retrieved

Keywords	Searching query syntax *	Documents
Livestock AND		
Environmental impact	(TS=("Livestock production")) AND TS=("Environmental impact")	281
Greenhouse gas	(TS=("Livestock production")) AND TS=("Greenhouse gas")	761
Pollution	(TS=("Livestock production")) AND TS=("Pollution")	436
Pollution AND Waste	((TS=("Livestock production")) AND TS=("Pollution")) AND TS=("Waste")	88
Biodiversity	(TS=("Livestock production")) AND TS=("Biodiversity")	572
Water use	(TS=("Livestock production")) AND TS=("Water use")	144
Water pollution	(TS=("Livestock production")) AND TS=("Water pollution")	58
Air quality	(TS=("Livestock production") AND TS=("Air quality")	67
Rabbit AND		
Environmental impact	(TS=("Rabbit")) AND TS=("Environmental impact")	21
Greenhouse gas	(TS=("Rabbit")) AND TS=("Greenhouse gas")	17
Pollution	(TS=("Rabbit")) AND TS=("Pollution")	140
Pollution AND Waste	((TS=("Rabbit")) AND TS=("Pollution")) AND TS=("Waste")	19
Biodiversity	(TS=("Rabbit")) AND TS=("Biodiversity")	224
Water use	(TS=("Rabbit")) AND TS=("Water use")	5
Water pollution	(TS=("Rabbit")) AND TS=("Water pollution")	4
Air quality	(TS=("Rabbit")) AND TS=("Air quality")	13
Rabbit farming AND		
Environmental impact	(TS=("Rabbit farming")) AND TS=("Environmental impact")	4
Greenhouse gas	(TS=("Rabbit farming")) AND TS=("Greenhouse gas")	3
Pollution	(TS=("Rabbit farming")) AND TS=("Pollution")	1
Pollution AND Waste	((TS=("Rabbit farming ")) AND TS=("Pollution")) AND TS=("Waste")	0
Biodiversity	(TS=("Rabbit farming")) AND TS=("Biodiversity")	0
Water	(TS=("Rabbit farming")) AND TS=("Water")	3
Water use	(TS=("Rabbit farming")) AND TS=("Water use")	0
Water pollution	(TS=("Rabbit farming")) AND TS=("Water pollution")	0
Air quality	TS=("rabbit farming") AND TS=("Air quality")	0
Rabbit production AND		
Environmental impact	(TS=("Rabbit production")) AND TS=("Environmental impact")	4
Greenhouse gas	(TS=("Rabbit production")) AND TS=("Greenhouse gas")	3
Pollution	(TS=("Rabbit production")) AND TS=("Pollution")	1
Pollution AND Waste	((TS=("Rabbit production")) AND TS=("Pollution")) AND TS=("Waste")	0
Biodiversity	(TS=("Rabbit production ")) AND TS=("Biodiversity")	0
Water	(TS=("Rabbit production ")) AND TS=("Water")	10
Water use	(TS=("Rabbit production")) AND TS=("Water use")	0
Water pollution	(TS=("Rabbit farming")) AND TS=("Water pollution")	0
Air quality	(TS=("rabbit production") AND TS=("Air quality")	1

* Boolean expression and field tags (TS for Topic) from https://www.webofscience.com/wos/woscc/advanced-search

In the decade following the works of Hodge only one article was retrieved from the literature. In this work, Conway (1987) introduced and described the properties of agroecosystems - "*an ecological system modified by human beings to produce food, fibre and other agricultural products"*. In his definition, the primary attributes of agroecosystems are: productivity, stability, sustainability and equitability. Together, these four properties define the social value of the agroecosystem. For Conway, productivity is the output of valued products per unit of resource input, stability is the constancy of production in the face of normal fluctuations and cycles in the environment, while sustainability is the ability of an agroecosystem to maintain its productivity in the face of major disturbances (erosion, declining market demand, etc.). Finally, Conway (1987) defined equity as the equitable distribution of the productivity among the human beneficiaries, which in the case of modern agricultural systems seems to favour a reduced number of actors. In Europe, the agriculture policy stated in 1957 under the European Economic Commission has favoured the larger, richer and more specialised farmers through a subsidy system based on production. Pollution, incidents caused by livestock or silage effluents, loss of amenity, recreational and conservation values of the countryside, and the degradation of ancient woodlands, chalk grasslands, herb-rich meadows, heaths, etc. are all side effects of a policy based on productivity alone (Conway,1987).

The nineties were a decade of bright works. Writing about the strategies for sustainable (poultry) production, Stenholm and Waggoner (1991) pointed out to the responsibility of scientists to help the public understand the benefits and risks of all farming practices. They called for a scientific consensus and mentioned that any production system should stand the test of scientific scrutiny, economic analysis, and social acceptance and impact. They also mention that (poultry) scientists should combine their experience and expertise with those of other biological and social sciences. The same being true for information and communication sciences. In itself, this openness to collaboration seems to be the key to achieving a more ambitious set of practices to reduce the impact of livestock production on natural resources.

For the developing nations, sustainability could be achieved by improving the feed quality, the genetic resources and animal health management (Kaasschieter *et al.*,1992). However, this technical improvement should be developed within the small-scale mixed farming systems that predominate in developing countries. Back to Europe, Atkinson and Watson (1996) identified the nitrogen load from animal wastes produced by industrial units of pig, poultry and dairy production as the major environmental problem in British lowland. For these authors, the solution is simple: to develop systems that allow waste to be reuse on primary crop production sites. Similar to what is proposed by the circular bioeconomy or sustainable intensification approaches (Pretty and Bharucha, 2014). In short, Atkinson and Watson (1996) called for holistic farming systems that integrate livestock in a way that reduces the impact on the environment and wildlife (plant and animal) biodiversity.

Two interesting papers close the decade. Waltner-Toes (1996) introduced us to the concept of "*ecosystem health*" while Faye *et al.* (1999) extended this concept to "*agroecosystem health*". For Waltner-Toes (1996), the global agri-food system as it is organised is unsustainable: subsidies for high production in countries with low population densities in farming systems that are heavily dependent on energy and oil-based inputs to function. This author was very critical of using the "*hard system*" view to assess whether or not a production system is sustainable or not. For him, this method runs the risk of considering a production system to be sustainable simply because the current socio-economic context dictates it: for example, "*the jobs of rural people may be considered as less important than the cheapness of urban food"*. To address this problem, he discussed some of the available frameworks before proposing a new one: the '*ecosystem health*'. For him, "*health sciences ask the kind of questions that agroecosystems caretakers should be asking"*. Moreover, the language of health science (screening, diagnosis, risk factors, and fitness) is well understood by the general public, providing *"a rare opportunity for citizens to speak about what they value*

subjectively and what they know objectively". Put simply, the concept of One Health as we know it today (World Health Organization, 2017) is rooted in the ideas discussed by Waltner-Toes (1996) who said: "*definitions of ecosystem health and human community health, although still evolving, emphasizes two components: an element of equilibrium, or balance, and an element of potential, or reserve".* Faye *et al*. (1999) went beyond the definition of "*ecosystem health*" and extended the concept to "*agroecosystem health*". In practice, it is just a matter of scale, where the system boundaries are broadened to include multiple dimensions (biophysical, social, economic) within a complex hierarchy (from the field to the biosphere) where health components (integrity and effectiveness) are present.

The literature reviewed in the period between 2000 and 2010 covered several subjects. It ranged from economic modelling approach to find the best solutions to avoid environmental impacts of different livestock waste-management (Innes, 2000) to ethics in organic livestock production (Lund *et al*., 2003). The concepts of "*land sharing*" (wildlife-friendly farming) and "*land sparing*" (minimising demand for farmland by increasing yields) were also introduced in this period, in a comprehensive work by Green *et al*. (2005). In the same year as the FAO publication *Livestock's long shadow*, Monteny *et al.* (2006) reviewed the greenhouse gas emissions and mitigation strategies for cattle, pig and poultry production. They identified the rumen and the manure from pig and poultry as the main sources of methane (NH_4) emission from livestock. For nitrous oxide (N_2O), the main sources are: nitrogen fertilisers, manure applied to land, and urine deposited by grazing animals. For these authors, technical solutions such as improving feed efficiency or the on-farm biogas production should be used to reduce the greenhouse gas emissions. The era of technical solutions and direct comparisons between organic and industrial systems began. For Bokkers and de Boer (2009), although organic broiler production performed better on economic and social indicators (net farm income, workload, animal health and welfare, and reduced use of antibiotics), the use of the feed efficiency as the unique criteria as a proxy of environmental impact systematically favour the so called "*factory*" farms. This period is concluded by the work of Verburg *et al*. (2009), who analysed the impact of agricultural trade liberalism on land-use related greenhouse gas emissions. For these authors, liberalisation should increase the total greenhouse gas emissions by 6%, due to vegetation clearance for crop production.

The first papers on the environmental impact of rabbit meat production appeared in this decade. Calvet *et al.* (2008) estimated the efficiency of nitrogen use in rabbit production, Fortun-Lamothe *et al*. (2009) evaluated the contribution of intensive rabbit production to sustainable development, and Kaliste *et al*. (2002) and Cambra-López *et al.* (2010) presented the first results on the airborne particulate matter from rabbit farms. Estellés *et al.* (2009) developed a new method to measure greenhouse gas emissions from both rabbit metabolism and manure decomposition, and Estellés *et al.* (2010) measured the daily carbon emissions of fattening rabbits. The following year, Calvet *et al.* (2011) used a different method to characterise the indoor environment and the gas emissions in both commercial and experimental rabbit farms, while Franz *et al.* (2011) quantified the methane emissions from rabbits (and guinea pigs) fed exclusively on hay, observing a daily methane production of 0.2 L per rabbit.

The literature on the environmental impact of rabbit production continued until 2022. In this period, we can cite the work of Adell *et al.* (2012a and 2012b) on the emission, morphology and characteristics of particulate matter in rabbit farms, Estellés *et al.* (2014) and Dinuccio *et al.* (2019), who studied the greenhouse gas emissions from rabbit manure, or Biagini *et al.* (2021) on the potential of feed additives to reduce the ammonia and greenhouse gas emissions from rabbits. Predictions of nutrient flows and the environmental impacts of rabbit farming were studied by Méda *et al.* (2014) using a modelling approach and by Cesari *et al.* (2018), Pascaris *et al.* (2021) and Wang *et al.* (2022) using a life cycle analysis. Theau-Clément *et al*. (2016), using a multi-criteria evaluation method, evaluated the sustainability of two alternative rabbit breeding systems.

As the majority of farmed rabbits are housed indoors in cage, the literature produced on the environmental impact of rabbit farming is limited to this production system. There is only one article that attempted to assess the environmental impact of two alternative rabbit production systems (Pascaris *et al*., 2021). In itself, this work sheds some light on the possible alternatives, but its scientific and social value can be questioned. In fact, the systems studied were based on the prospective work of Lytle *et al.* (2021), who proposed to raise rabbits under solar panels.

The literature reviewed also covered policy. Gerber *et al.* (2010) reviewed several policy instruments (taxes, subsidies, emissions trading, voluntary mitigation efforts) to reduce the greenhouse gas emissions from livestock. Golub *et al.* (2013) studied the impacts of global climate policies on livestock, land-use change, livelihoods, and food security. They found, for example, that fiscal policies targeting only *Annex I* countries (https://unfccc.int/process-and-meetings/what-are-parties-non-party-stakeholders) may result in an expansion of agricultural land in non- *Annex I* nations, leading to an increase in emissions from deforestation.

Consumer behaviour and preferences also play an important role in the policy effectiveness. Schulze *et al.* (2023) showed a strong public preference for a sustainable transformation of livestock production and provided evidence that reducing livestock numbers is an acceptable path from the public's perspective. According to these authors, the greenhouse gas abatement potential of livestock reduction can be huge: it could account for up to 45% of the whole sector, including reductions in emissions from land-use change, fertiliser and pesticide production and use, manure excretion and application, feed processing and transport. These figures appear to contradict those presented by Leroy *et al.* (2022). For these authors, reducing livestock numbers based on the argument that healthy diets are low in red meat and saturated fat seems flawed, and in the context of overall Western lifestyle footprint, a large reduction in meat intake would result in a 2% to 6% reduction on the carbon footprint (*e.g.*, 12 t CO_2-eq. per person annually). Leroy *et al.* (2023) also questioned the claims about the impact of livestock on planetary health. Based on their review, those claims are not justified. However, these authors recognised the importance of understanding that the environmental impact of livestock production depends on the region, ecosystem and practices involved.

GREENHOUSE GAS EMISSIONS & NITROGEN LOSSES IN RABBIT FARMING

The available data on greenhouse gas emissions and nitrogen losses in rabbit production systems are summarised in Table 2. The emissions were classified according to the emission source (farm, animal or manure), the methodology used to estimate (life cycle analysis or modelling) or to measure (gas emission rates, nitrogen balance, and flux or fermentation chambers) these emissions according to the farm type (average Italian or French production system, theoretical farm with or without photovoltaics, commercial or experimental farms).

Farm emissions

Cesari *et al*. (2018) used a life cycle analysis to estimate the total greenhouse gas emissions from a theoretical average Italian rabbit meat production farm. They determined the values of the input variables using data from the literature and considered the feed conversion ratio as the only variable related to production efficiency and environmental impact. After modelling three scenarios of mortality rates (5%, 10% and 20%) in the fattening period (only), the total greenhouse gas emissions were estimated to vary between 3.78 and 4.04 kg of CO_2 eq. per kg of liveweight produced. The authors compared the results with those of other monogastric species (chicken and pig) and concluded that the environmental impact of an average Italian rabbit meat farm is comparable to that of a pig farm. Although this is a positive result, it is possible that some of the postulates used in the analysis may misrepresent the actual environmental impact of rabbit meat production. In their analysis, Cesari *et al*. (2018) excluded the emission from the construction and maintenance of infrastructure (buildings, cages, etc.)

as well as the emission from the use of antibiotics and hormones. Furthermore, emissions related to the manure were downgraded. It was assumed that all manure produced was used in the production of corn on the same farm.

Despite the modelling choices made by Cesari *et al*. (2018), the most interesting result is the high contribution of the feed production to climate change (about 71.2% of the total CO_2 eq. emissions), mainly related to the use of fossil fuels for crop production and the transport of feed ingredients from other countries (soybean meal from Brazil and sugarcane molasses from Thailand). Of the various environmental impacts assessed by Cesari *et al*. (2018), land use change had a negative score. This was related to the use of alfalfa in the diets, a crop that sequester carbon compared to arable crops. A land use of 12.5 m² per kg of liveweight produced was also estimated by the authors.

The only on-farm study presenting actual measurements of greenhouse gas emission from commercial and experimental rabbit farms was conducted by Calvet *et al*. (2009). They measured the real-time emissions of ammonia, carbon dioxide and nitrous oxide in three rabbit farms in eastern Spain (two commercial farms and one experimental farm). Methane emissions were also measured, but could not be determined as readings below 10 ppm are strongly influenced by the air moisture values. All measurements were made with a multi-gas photoacoustic analyser and the emissions of NH_3, CO_2 and N_2O were obtained by calculating the emission rates per animal. Farm emissions included those from the animals and their manure together. In addition, emissions from breeding females and growing rabbits were calculated separately. After converting the results to kg of CO_2 eq. per animal over one year (using the greenhouse gas equivalencies calculator from the U.S.A. Environmental Protection Agency: https://www.epa.gov/energy/greenhouse-gas-equivalencies-calculator), the total greenhouse gas emissions were 164 kg of CO_2 eq. per animal per year. Each female rabbit emitted 134 kg of CO_2 eq. per year, while growing rabbits emitted around 30 kg of CO_2 eq. per animal per year (or about 2.80 kg of CO_2 eq. per animal considering a fattening period of 35 days). Although the values obtained for growing rabbits (rough estimation assuming a fattening period of 35 days) was bellow to that reported by Cesari *et al*. (2018), emissions from other sources, rather than the direct emissions from the animal metabolism and the manure decomposition, were not taken into account by these authors.

The remaining studies assessing the environmental impacts at the farm level (Méda *et al*., 2014; Pascaris *et al*., 2021; Wang *et al*., 2022) present different greenhouse gas emissions values. Using a dynamic modelling, Méda *et al*. (2014) estimated the emissions of ammonia, nitrous oxide, and methane from a typical French rabbit farm with 605 female rabbits. They also considered two manure management systems: slurry or deep pit. The values in the Table 2 are the range of NH_3, N_2O and CH_4 from the two manure management systems. When converted to CO_2 eq., the estimated emissions were 0.498 and 0.536 kg of CO_2 eq. per kg of liveweight produced in the slurry and in the deep pit systems, respectively. These figures are lower than those obtained by Cesari *et al*. (2018), using a life cycle assessment, and Calvet *et al*. (2011), using direct on-farm gas emission measurements. The observed differences between these studies may be related to the methodology used, the assumptions of each model and the system boundaries.

Pascaris *et al*. (2021) compared three systems: grazing rabbits under photovoltaics panel, indoor raised rabbits heated with energy produced on-site from photovoltaic panels, and indoor rabbits heated with energy from fossil fuels. This theoretical photovoltaic system, conceptualized by Lytle *et al*. (2021), cannot be compared to the conventional rabbit production systems present in Europe. In the case of China, Wang *et al*. (2022) estimated the environmental impacts of a Rex rabbit industry chain. In this study, the authors aimed to assess the benefits of a circular industry chain. After modelling two scenarios, one that integrated a biogas power plant for manure treatment and one that outsourced manure treatment without recycling it, a cradle-to-gate life cycle analysis was performed. The global warming potential, expressed in kg of CO_2 eq. was estimated at 30.6 and 44.7 kg of CO_2 eq. for the scenarios with and without biogas manure treatment, respectively. Wang *et al*. (2022) observed a large

contribution of feed cultivation and the electric energy consumption in the feed processing stages on greenhouse gas emissions as well as the other environmental impact categories. In summary, feeding accounted to over 85% of the CO_2 eq. emissions, in a manner consistent with the findings of Cesari *et al*. (2018). Wang *et al*. (2022) also observed that the use of a biogas power plant contributed to a reduction in the global warming potential, as a result of a reduction in the consumption of chemical fertilizers, pesticides and external electric power.

The greenhouse gas emission values vary widely between the studies at the farm level. The differences observed are related to the choice and fine-tuning of the parameters used in the life cycle analysis, to the model assumptions and to the boundaries of the system considered in each study. For example, differences in carcass weight, feeding strategy (adoption of feed restriction or not) and on other parameters such as the reproductive performance of rabbit females (fertility, culling and/or mortality, etc.) will affect the results of a life cycle assessment. In terms of system boundaries, the inclusion or exclusion of a specific treatment of manure to produce part of the cereals used in the rabbit feed also influences the results, as does the inclusion or the exclusion of the slaughter process in the calculations.

Despite its limitations, the life cycle analysis provides information on the different compartments that are the main contributors to greenhouse gas emissions. In the case of rabbit meat production, this method shows that the vast majority of CO_2 eq. emissions are associated with the fossil fuels used in feed production (Cesari *et al*., 2018 and Wang *et al*., 2022). This tool could be further improved by considering direct emissions data from multiple rabbit farms within a single (or multiple) country, as done by Calvet *et al*. (2011), or by using real emissions data from rabbit farms (Estellés *et al*., 2008 and 2014) instead of using standard emissions coefficients. For Goglio *et al*. (2023), this method still lacks accuracy and robustness in addressing sustainability across livestock systems and products. For them, harmonisation is needed.

Modelling approaches are also useful. They help us to understand certain phenomena, but their use should be limited to describing systems that are close to the reality, as done by Méda *et al*. (2014). Analyses based on theoretical systems can also help us to understand future and alternative systems. However, the lack of real data can lead to uncertain estimates, as in the case of Pascaris *et al*. (2021).

The use of real-time greenhouse gas emissions from the rabbit metabolism (respiration, assimilation, excretion, etc.) and from manure decomposition should also be considered. Their use should be preferred when analysing the global warming potential of conventional and alternative rabbit production systems.

Animal emissions

Some authors (Calvet *et al*., 2008; Estellés *et al*., 2009; Estellés *et al*., 2010; Franz *et al*., 2011; Dinuccion *et al*., 2019) have addressed the emissions from the perspective of rabbit metabolism. None of the studies performed have characterized the greenhouse gas emissions of adult animals.

Estellés *et al*. (2009 and 2010), using a flux chamber designed to measure rabbit gas emissions, recorded individual emissions ranging from 1.12 to 2.61 litters of CO_2 per hour. Considering a fattening period of 35 days and a daily CO_2 emission of 47.5 litters per rabbit (assuming an average emission of 1.98 litters of CO_2 per hour for a rabbit of 1.24 kg; Estellés *et al*. 2010), the CO_2 eq. emissions of a single rabbit can reach 3.05 kg of CO_2 eq. in 35 days (because 1 m^3 of CO_2 weights 1.84 kg). This is in line to the estimations from Calvet *et al*. (2008).

Using a flux chamber, Franz *et al*. (2011) measured the CH_4 emission from pygmy rabbits fed on a hay diet. Enteric CH_4 emissions varied from 0.12 to 0.28 litters of CH_4 per day, which is lower compared to other mammalian herbivore species (Clauss *et al*., 2020). After assuming

that 1 m^3 of CH_4 weights 0.72 kg, a single rabbit should produce between 0.003 and 0.007 kg of CH_4 in 35 days, which represents between 0.084 and 0.196 kg of CO_2 eq. Adding up the estimations calculated from the data of Estellés *et al*. (2010) and Franz *et a*l. (2011), a single growing rabbit should produce between 3.13 and 3.25 kg of CO_2 eq. in 35 days.

Table 2. Greenhouse gas (GHG) emissions from rabbit production

GHG emissions	Units	Main emissions	Source	Methodology	Farm type	Reference
3.78 to 4.04	Kg of CO_2 eq. per kg of liveweight	Feed ~ 70.0%	Farm	Life Cycle - estimation	Average Italian - whole system	Cesari *et al.* 2018
199.7	Kg of CO_2 eq. per kg of liveweight	Solar ~ 99.9%	Farm	Life Cycle - estimation	Theoretical - pasture photovoltaic	Pascaris *et al.* 2021
651.6	Kg of CO_2 eq. per kg of liveweight	Feed ~ 69.3%	Farm	Life Cycle - estimation	Theoretical - indoor photovoltaic	Pascaris *et* al. 2021
13 619.9	Kg of CO_2 eq. per kg of liveweight	Energy ~ 96.7%	Farm	Life Cycle - estimation	Theoretical - indoor no photovoltaic	Pascaris *et al*. 2021
30.6 to 44.7	Kg of CO_2 eq. (total emissions)	Feed over 85%	Farm	Life Cycle - estimation	Rex fur production system	Wang *et al*. 2022
11.3 to 13.7	g of NH_3 per kg of liveweight	Not reported	Farm	Modelling - estimation	Average French - whole system	Méda *et al*. 2014
0 to 0.17	g of N_2O per kg of liveweight	Not reported	Farm	Modelling - estimation	Average French - whole system	Méda *et al*. 2014
17.8	g of CH_4 per kg of liveweight	Not reported	Farm	Modelling - estimation	Average French - whole system	Méda *et al*. 2014
38.7 to 65.6	mg of NH_3 per hour and animal	Not reported	Farm	Gas emission rates	Commercial - reproductive does	Calvet *et al*. 2011
4004 to 17 820	mg of CO_2 per hour and animal	Not reported	Farm	Gas emission rates	Commercial - reproductive does	Calvet *et al*. 2011
0 to 20.8	mg of N_2O per hour and animal	Not reported	Farm	Gas emission rates	Commercial - reproductive does	Calvet *et al*. 2011
3.5 to 12.1	mg of NH_3 per hour and animal	Not reported	Farm	Gas emission rates	Commercial - growing rabbits	Calvet *et al*. 2011
1180 to 3880	mg of CO_2 per hour and animal	Not reported	Farm	Gas emission rates	Commercial - growing rabbits	Calvet *et al*. 2011
0 to 2.0	mg of N_2O per hour and animal	Not reported	Farm	Gas emission rates	Commercial - growing rabbits	Calvet *et al*. 2011
1.12 to 1.40	litters of CO_2 per hour	Not reported	Animal	Flux chamber	Experimental - growing rabbits	Estellés *et al*. 2009
1.35 to 2.61	litters of CO_2 per hour	Not reported	Animal	Flux chamber	Experimental - growing rabbits	Estellés *et al*. 2010
0.12 to 0.28	litters of CH_4 per day	Not reported	Animal	Flux chamber	Experimental - pygmy rabbits	Franz *et al*. 2011
40.1 to 42.4	g of N per Kg of liveweight	Not reported	Animal	Nitrogen balance	Experimental - growing rabbits	Calvet *et al*. 2008
57.0 to 59.1	g of N per kg of liveweight	Not reported	Animal	Nitrogen balance	Experimental - growing rabbits	Dinuccio *et al*. 2019
2.95 to 3.26	mg of NH_3 per hour	Not reported	Manure	Flux chamber	Experimental - growing rabbits	Estellés *et al*. 2009
216.9 to 272.4	mg of CO_2 per hour	Not reported	Manure	Flux chamber	Experimental - growing rabbits	Estellés *et al*. 2009
17.8 to 26.4	mg of NH_3 per hour and m^2	Not reported	Manure	Flux chamber	Experimental - growing rabbits	Estellés *et al*. 2014
1.93 to 2.53	mg of N_2O per hour and m^2	Not reported	Manure	Flux chamber	Experimental - growing rabbits	Estellés *et al*. 2014
7.5 to 10.8	mg of CO_2 per hour and m^2	Not reported	Manure	Flux chamber	Experimental - growing rabbits	Estellés *et al*. 2014
16.4 to 18.5	mg of CH_4 per hour and m^2	Not reported	Manure	Flux chamber	Experimental - growing rabbits	Estellés *et al*. 2014
212 to 257	g of CO_2 eq. per kg of manure	Not reported	Manure	Fermentation chamber	Experimental - growing rabbits	Dinuccio *et al*. 2019
2.70	mg of CH_4 per g of manure	Not reported	Manure	Fermentation chamber	Experimental - growing rabbits	Hidayat *et al*. 2021
143.7 to 246.7	g of NH_3 per m^2	Not reported	Manure	Fermentation chamber	Experimental - growing rabbits	Biagini *et al*. 2021
1.04 to 1.60	g of N_2O per m^2	Not reported	Manure	Fermentation chamber	Experimental - growing rabbits	Biagini *et al*. 2021

1779 to 2533	g of CO_2 per m²	Not reported	Manure	Fermentation chamber	Experimental - growing rabbits	Biagini *et al*. 2021
22.8 to 35.2	g of CH_4 m²	Not reported	Manure	Fermentation chamber	Experimental - growing rabbits	Biagini *et al*. 2021
3498 to 4226	g of CO2 eq. m²	Not reported	Manure	Fermentation chamber	Experimental - growing rabbits	Biagini *et al*. 2021

In addition to the greenhouse gas emissions measured at the animal level, nitrogen losses were measured in growing rabbits by Calvet *et a*l. (2008) and Dinuccio *et a*l. (2019). Calvet *et al*. (2008) found excretion values between 40.1 and 42.4 g of N per kg of liveweight while Dinuccio *et al*. (2019) observed higher values, ranging from 57.0 to 59.1 g of N per kg of liveweight. The higher nitrogen excretion found by Dinuccio *et al.* (2019) may be related to the nitrogen content of the rabbit feeds: 28.4 g of N per kg feed compared to 25.9 g of N per kg of feed (on average) on the feeds used by Calvet *et al*. (2008).

Greenhouse gas emissions at the animal level are consistent across the few studies available. As mentioned above, real-time measurements are preferable to averages and proxies. However, further development is needed. There is a lack of information on emissions from adult reproducing animals. There is also a lack of knowledge on the contribution of genetics, nutrition and veterinary practices to greenhouse gas emissions at animal level. Much work is needed in these areas.

Manure emissions

Emissions from rabbit manure were determined by two methods: flux chamber (Estellés *et al*., 2009 and 2014) or fermentation chamber (Dinuccio *et al*., 2019; Hidayat *et al*., 2021; Biagini *et al*., 2021). After placing manure samples in a flux chamber, the NH_3 and CO_2 emissions from fresh manure ranged from 2.95 to 3.26 mg of NH_3 per hour and from 216.9 to 272.4 mg of CO_2 per hour (Estellés *et al*., 2009).

In an attempt to reduce the manure emissions, Estellés *et al*. (2014) added calcium superphosphate powder to the manure pits twice a week at a dose of 50 g per m². Although not statistically significant, the addition of this additive reduced the CO_2 emission from 10.81 to 7.45 g per hour per m² and the N_2O emissions from 2.53 to 1.93 mg per hour per m². On the contrary, CH_4 emissions increased from 16.37 to 18.45 mg per hour per m². When these values were converted to CO_2 eq., the daily emissions per m² were, on average, 680 g of CO_2 eq. with the additive and 978 g of CO_2 eq. without the additive.

Using a different method and metrics, Dinuccio *et al*. (2019) found manure emissions between 212 and 257 g of CO_2 eq. per kg of manure, while Biagini *et al*. (2021) reported daily manure CO_2 eq. emissions between 194 and 235 g per m². These values were lower than those reported by Estellés *et al*. (2014). The different results between Estellés *et al*. (2014) and Biagini *et al*. (2021) may be related to the different methodologies: 24-hours flux chamber measurements versus 18 consecutive days from manure samples placed in a fermentation chamber, respectively.

Hidayat *et al*. (2021) designed a fermentation chamber using PVC tubes. After placing samples of manure from different species (buffalo, chicken, cow, duck, goat and rabbits) they measured the CH_4 emissions for eight consecutive weeks. The aerobic digestion of duck manure produced the highest amount of methane (98.0 mg of CH_4 per g of manure). It was followed by buffalo (21.9 mg of CH_4 per g of manure), cow (20.32 mg of CH_4 per g of manure), chicken (18.0 mg of CH_4 per g of manure) and goat manure (6.01 mg of CH_4 per g of manure). Rabbit manure produced the lowest amount of all measured species, about 2.7 mg CH_4 per g of manure.

Direct measurements of greenhouse gas emissions from rabbit manure are also scarce. Although the results appear to be consistent among the available studies, the influence of the genetics, nutrition or the manure management practices can alter the emission values. Real

data from an array of farms having different genotypes, nutrition strategies and manure treatment practices are required. In spite of this information studies aiming to describe the potential of rabbit manure use in crop and grassland systems should be further developed. Sustainable strategies of rabbit manure recycling should be designed on a whole-farm perspective, as in the case of other species (see Petersen *et al*., 2007; Petersen *et al*., 2013).

PARTICULATE MATTER CONCENTRATION & EMISSIONS

The current data on particulate matter concentrations and emissions from rabbit farms is limited. Adell *et al*. (2012) surveyed two rabbit farms: a fattening facility and a breeding farm, both of which had manure accumulated below the cages for three to four weeks. After 15 days of measurement, the concentration of particulate matter in the fattening facility was 0.082 and 0.012 mg per m^3 of particulate matter of size 10 and 2.5 microns, respectively. In the breeding farm, the air concentrations of particulate matter of sizes 10 and 2.5 microns were 0.048 and 0.012 mg per m^3, respectively. A variety of activities, including floor sweeping, animal handling and the use of pressurised water to clean the cages, were identified as the primary sources of particulate matter emissions (most 10 microns) in the fattening facility. In the breeding farm, sweeping and burning hair were the main activities contributing to particulate matter emissions of both sizes. In addition, the fattening unit appeared to emit more particulate matter outside the farm (on average, 0.52 and 0.02 g per hour of 10- and 2.5-micron particulate matter, respectively) than the breeding unit (on average, 0.33 and 0.06 g per hour of 10- and 2.5-micron particulate matter, respectively). This was due to the very high animal densities in the fattening unit.

In comparison to other species, the overall concentration of particulate matter found in rabbit farms is relatively low. In poultry and pig farms, for example, particulate matter emissions range from 0.05 to 15.3 mg per m^3 for inhalable and from 0.03 to 1.9 mg per m^3 for respirable particulate matter. From a regulatory standpoint, the emissions observed in rabbit houses are below the legal thresholds (Adell *et al*., 2012).

Despite the reduced level of particulate matter found in rabbit rooms, its origin is diverse. This includes fur, skin particles, faeces, urine, bedding, and disinfectants (Kaliste *et al*., 2002). In addition, it can carry ammonia, contain heavy metals, absorb odorants and transport bioaerosols such as bacteria (both gram-positive and gram-negative), fungi, viruses and even bioactive compounds like antibiotic particles (Cambra-López *et al*., 2010). The combination of these factors may potentially increase the health risks for both animals and farmers. The environmental impacts associated with particulate matter emissions from livestock (including reduced visibility, vegetation stress and ecosystem change) represent an additional risk that has not yet been assessed.

Although the studies conducted in rabbit houses resulted in low particulate matter emissions, further investigation is required to better characterise the bioaerosol (bacteria and viruses) and the antibiotic present in these particles. The characterisation of emissions from different farms typologies (purpose, animal density, building dimensions and other technical specifics, etc.) and production systems (outdoor grazing systems, for example) is of great interest. The absence of this information precludes any meaningful evaluation of the environmental impacts associated with rabbit production.

WATER FOOTPRINT

Before diving into the topic of water footprints, it is important to provide some definitions. Due to the different sources and uses of water, the water footprint is colour-coded into three categories: blue, green, and grey water (Mekonnen and Hoekstra, 2012). The blue water footprint is the volume of surface and groundwater required to produce one unit of product. The green water footprint is the volume of rainwater lost to evapotranspiration. The grey water

footprint is the volume of freshwater required to assimilate the pollutant load based on existing ambient water quality standards.

Using data from the Food and Agriculture Organization of the United Nations and from the literature, Mekonnen and Hoekstra (2012) estimated water footprints for several animal products: beef, sheep, goat, pig and chicken meat, eggs, milk, butter, milk powder, cheese and leather. The green, blue and grey water footprints for some of these products, according to the production system, are illustrated in Figure 1.

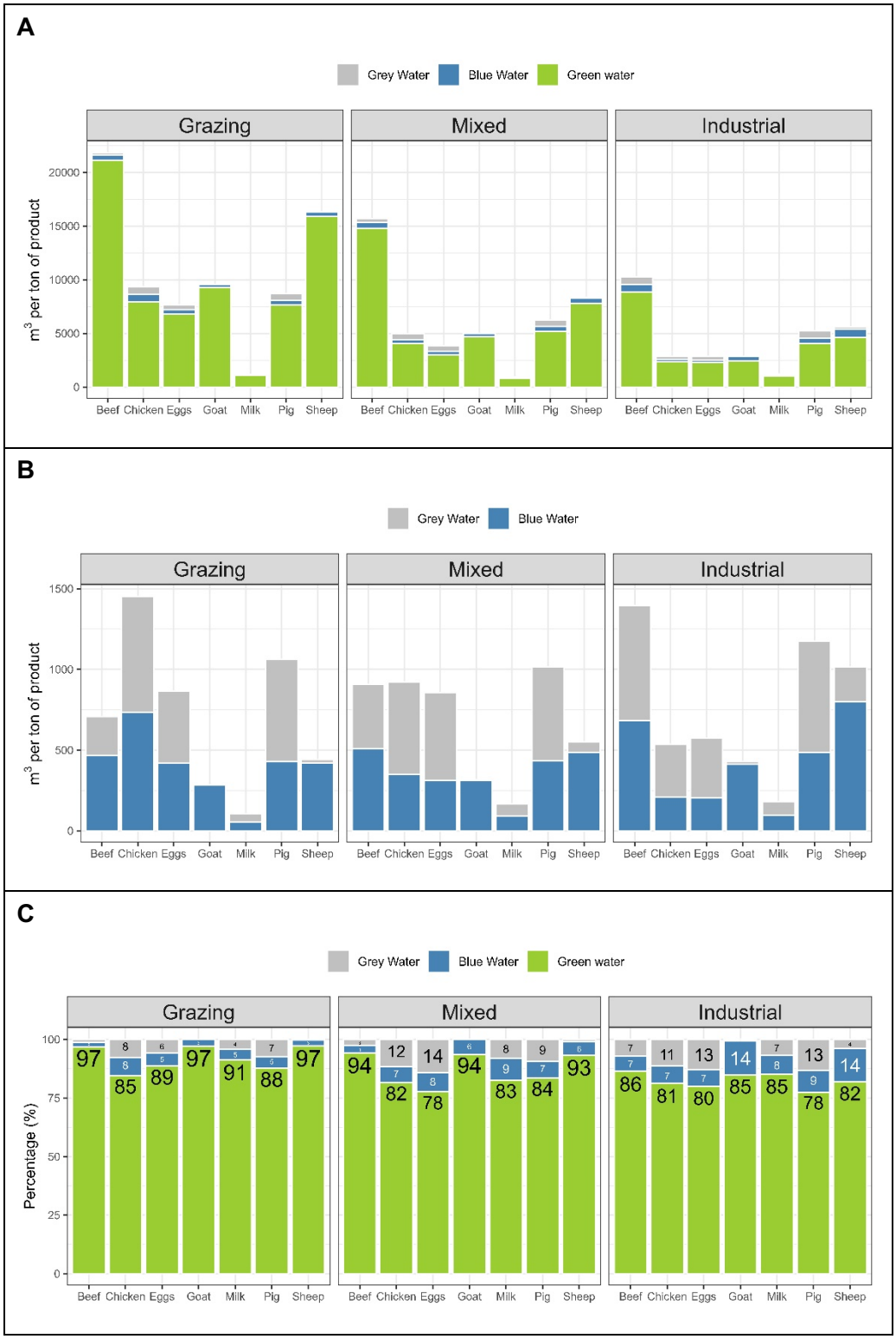

Figure 1: Volume of green, blue and grey water footprint per unit of animal products produced (**A** and **B**) and as a percentage of the total water footprint (**C**) according to the production system (Grazing, Mixed or Industrial). Adapted from Mekonnen and Hoekstra (2012)

At a first sight, the water footprint of grazing systems is higher than that of mixed and industrial systems (all products). The picture changes when the green (rainfall) water footprint is excluded. With the exception of poultry products, the blue and grey water footprints increase from grazing to industrial systems (Figure 1B). In their analysis, Mekonnen and Hoekstra (2012) found that 98% of the water footprint comes from the feed. Drinking water, service water and feed-mixing water only account for 1.1, 0.8 and 0.03% of the total water footprint, respectively. Focus on the blue water footprint in grazing systems, it accounts for 3.6% of the total footprint, being 33% of this consumption related to drinking and service-water use. In contrast, the blue water footprint in industrial systems accounts for 8% of the total water footprint.

The water footprint figures presented by Mekonnen and Hoekstra (2012) are very comprehensive. However, some striking points should be mentioned. Animal waste and water pollution from feed crop production other than nitrogen leakage, were not included in the grey water footprint estimations. In addition to the underestimation of the grey water footprint, this category is often more important in intensive production system. Ran *et al*. (2016) reviewed the methods used to assess water resource use in livestock production. For these authors, the grey water concept is a virtual water proxy of the volume of water required to assimilate pollutants and abate water quality degeneration, and due to the nature of this measure, it should not be added up with green and blue water uses.

In both studies (Mekonnen and Hoekstra, 2012; Ran *et al*., 2016), animal feed production is cited as the main factor contributing to the water use. While Mekonnen and Hoekstra (2012) mention that animal farming based on crop residues, wastes and roughages to feed animals are the ones that puts the lowest pressure on freshwater systems, Ran *et al*. (2016) mention systems where animals are grazed on marginal land that has few alternative uses and little socio-economic values.

Although life cycle assessment studies fail to account for the majority of consumptive green water use, this approach highlights the importance of linking water resource use to local impacts and local water stress (Ran *et al*., 2016). For the rabbit sector, this is the unique approach available. For instance, Cesari *et al*. (2018) estimated that 1.13 m^3 of water eq. is depleted to produce one kilogram of liveweight. These figures, however, are not comparable to the water footprint studies.

Despite the lack of data on the water footprint of meat rabbit production, the common use of crop residues (*e.g.,* cereal straw), by-products (*e.g.,* beet pulp) and coarse materials (*e.g.*, soybean hulls) in the rabbit feed should contribute to placing the water footprint of industrial rabbit meat production somewhere between poultry products and pork. Studies are needed to fill the knowledge gap on water footprint of both industrial and alternative rabbit production systems.

BIODIVERSITY LOSS

Land-use change, habitat degradation, pesticide use, and pollution are among the main factors associated with livestock impact on biodiversity (Green *et al*., 2005; Broom *et al*., 2013). Green *et al.* (2005) proposed two methods to balance the trade-off between food production for a growing human population and biodiversity conservation: land sparing and land sharing. Land sparing focuses on increasing yields on land already used for agriculture, thereby reducing the need to convert intact habitats for food/feed production. The concept of land sharing, on the other hand, includes the maintenance of natural habitat patches, the extensive food production on semi-natural habitats and the adoption of practices that minimise the negative effects of fertilisers and pesticides.

Looking at rangeland ecosystems, Alkemade *et al*. (2013) suggested to increase yields in mixed crop-livestock systems in regions where productivity is still low is the best alternative to save land for nature. However, in regions where technology is advanced and productivity is

already high, no positive effect of increased productivity on biodiversity can be expected (Alkemade *et al*., 2013). In addition, these authors pointed to the risks of technology-driven approaches, as they can lead to the loss of traditional cultures and knowledge, and to the degradation of abandoned land (unless a specific restoration programme is designed).

Although it may seem counterintuitive, efficient livestock production with high biodiversity and good animal welfare is possible (Broom *et al*., 2013). This can be achieved in agroforestry systems, which combine livestock production with native shrubs, trees and edible plants, where animals feed exclusively on plant resources that are not used for human consumption. When properly designed and managed, these complex agroecosystems favour species that act as biocontrols for pests and diseases, farmers find work more satisfying and biodiversity is enhanced (Broom *et al*., 2013). For these authors, with good management, agroforestry systems can replace industrialised, simplified systems and reduce agricultural expansion into protected areas (sharing farmland with wildlife while preserving land for nature; Pearse, 2018).

In the complex debate of the opposing worlds, where some advocate to intensifying food production to save land for nature, while others argue that working with nature in complex agroecosystems is the solution, Kremen and Merenlender (2018) plead for a global landscape management in a matrix that works for biodiversity and for people. For them, a holistic approach is needed to reduce deforestation, which is mainly associated to grow crops to feed farm animals.

Biodiversity and environmental costs of intensive food production practices are well described by Tilman *et al*. (2002). High-density livestock systems, for example, can increase the incidence and promote the emergence of new diseases, often antibiotic-resistant, making the system vulnerable to catastrophic losses of animals to disease (Tilman *et al*., 2002). Pastoral systems, on the contrary, rely on ecosystem services and avoid many of the problems of confinement production. Pastured animals forage on plants growing in a field, and plant growth is favoured by animal wastes. Ruminants and other domestic herbivores are capable of converting low quality roughage into high quality animal products. In summary, well designed and correctly managed grassland-herbivores ecosystems are an efficient and sustainable method of producing high-quality food (Tilman *et al*., 2002).

To my knowledge, there are no studies quantifying the impact of rabbit farming on biodiversity loss. However, the main impact of European rabbit farming on biodiversity may be related to the deforestation to grow protein crops to feed the animals, mainly soya. This commodity and other flagship products are highly controversial and are subject to national regulations. For example, in 2018, the French government adopted a national plan to combat the imported deforestation for unsustainable products (https://www.deforestationimportee.ecologie.gouv.fr/en/sndi/article/sndi).

In the absence of a precise knowledge of the impact of rabbit farming on the biodiversity, several studies have described the contribution of wild rabbits to biodiversity. According to Gálvez-Bravo *et al*. (2009), the occurrence of four lizard species was associated with the presence of rabbit burrows, as they were only found in open pastures where rabbit burrows were present. The intense activity of rabbits around their burrows, the construction of latrines and their grazing behaviour cause a floristic change that promotes a particular heterogeneity in the herbaceous community, contributing to the beta diversity of the ecosystem (Gálvez-Bravo *et al*., 2011). The high quality of the rabbit droppings (high carbon and high nitrogen to phosphorus ratios: 462 and 17.4, respectively) maintained the most diverse plant community when compared to the excretions of other herbivores (European bison, cow, horse and fallow deer) in a mesocosm experiment (Valdéz-Correcher *et al*., 2019).

Wild rabbits also tend to increase habitat complexity and heterogeneity, favouring the abundance and/or richness of plant species through foraging, seed dispersal and soil fertilisation (Delibes-Mateos *et al*., 2008). Rabbits are also an important food source for several species of raptors, such as the Iberian lynx, the Spanish imperial eagle and several small birds of prey and generalist carnivores. Based on the multiple functional roles, and ecosystem

processes and patterns that are provided by the presence of rabbits in the Mediterranean scrubland, Delibes-Mateos *et al*. (2007 and 2008) characterised the European rabbit as a keystone species for this ecosystem in southwestern Europe.

In the light of the benefits of wild rabbits to the biodiversity of several plants and animal species, alternative farming integrating the rabbits as an herbivore in grasslands systems may be of interest. In France, pasture-based and organic rabbit production systems appear to be a promising way of farming (Gidenne *et al*., 2024), with technical results comparable to conventional indoor systems Fetiveau *et al*. (2021, 2023a). This outdoor pasture-based system also favoured the expression of specific behaviours not observed in cage systems, with grazing being the most expressed behaviour (Fetiveau *et al*., 2023b). Although in a prospective phase, the integration rabbits and apple trees, in an agroforestry system (Savietto *et al*., 2023), seem to benefit both plants and animals (Savietto *et al*., 2024). Services of provision (such as food), regulation (diseases control, herbivory, seed dispersal) and support (nutrient cycling, soil formation) naturally emerge in complex agroecosystems[1] integrating domestic plants, animals and wild life.

MULTICRITERIA ANALYSIS

As early as 1987, the United Nations Brundtland Commission (https://www.un.org/en/academic-impact/sustainability) defined sustainability as "*meeting the needs of the present without compromising the ability of future generations to meet their own needs*". Put differently, sustainability is about the use of resources in a rate that is compatible to the replacement of the natural resources in use. In simple words, sustainability is about consumption and understanding that everything is connected: consumption needs products, products use resources and the use of resources impacts the environment. Because everything is connected, sustainability is about systematic thinking in a framework that values economy, equity and the environment in an interconnected and balanced way (see https://www.sustain.ucla.edu/what-is-sustainability/).

In intertwined systems, a multi-criteria analysis is necessary to assess the impact of a production on the three E's (economy, equity and environment). Fortun-Lamothe *et al*. (2009) and Theau-Clément *et al*. (2016) applied this method to evaluate different rabbit breeding practices. After discussing the limitations of the method applied to rabbit meat production, Fortun-Lamothe *et al*. (2009) found a positive result for the economic and social scales: total score of 1 out of 3 points, but a negative contribution to the environmental scale (minus 2 points) due to high use of energy, antibiotics and reduced biodiversity of the system.

Theau-Clément *et al*. (2016) compared three indoor rabbit rearing systems that differed mainly in their reproductive rhythm (intensive, semi-intensive and extensive). After assigning scores to 14 sustainability criteria (five for economic, five for equity and four for environmental), they found that the intensive and extensive systems changed the sustainability profile compared to the semi-intensive system in terms of the economic and equity criteria. The intensive system had a positive effect on two of the five economic criteria and a negative effect on the equity dimension, related to animal welfare, working conditions and product quality. In contrast, the extensive system had a positive effect on the economic criterion related to process efficiency and on the environmental criterion biomass use.

[1] *"Agroecosystem are conceptual constructs, defined in both spatial and functional terms, that are used to describe parts of the biosphere managed primarily for the purpose of producing food, fibre, and other agricultural products; they are made up of people, domesticated plants and animals, biotic and abiotic elements of the underlying soils, drainage networks, as well as interdigitating areas that support natural vegetation and wildlife. Agroecosystems exist because people create them to achieve nutritional and socioeconomic goals; they therefore have socioeconomic and public health, as well as environmental, dimensions"* (Waltner-Toews, 1996).

A BRIEF DISCUSSION AND SOME PERSPECTIVES

This descriptive literature review presents some elements allow us to quantify the main contributions of rabbit farming to the global warming (greenhouse gas emissions), pollution (mostly nitrogen losses, airborne particulate matter), water footprint and biodiversity loss. As the majority of meat rabbits farmed in Europe (and in the world) are raised in indoor cage systems, the available studies only cover this production system. A single attempt has been made to quantify the environmental impact of an alternative system, based on rabbits grazing under photovoltaic panels (Pascaris *et al*., 2021). Although this study provides some insights into possible alternatives, the results obtained should be interpreted with caution as it is not based on real data.

Regarding the contribution of rabbit production to global warming, the estimations of greenhouse gas emissions ranged from 3.13 to 3.25 kg of CO_2 eq. per growing rabbit over a 35-day period (or from 3.78 to 4.04 kg of CO_2 eq. per kg of liveweight produced). Pollution associated with nitrogen losses varied between 40.1 and 59.1 g of N per kg of liveweight produced. Air pollution related to the airborne particulate matter (10 microns) varied from 0.082 to 0.045 mg per m^3, and there was no data available on the water footprint, which is likely to be between those observed for poultry and pig production. For biodiversity loss, there are no studies on the real impact of rabbit production on wild (plant and animals) life. As for greenhouse gas emissions, the latter may be attributed to the land use change for crop production, mainly soybean used for feed production. Wild rabbits, however, can be considered as a keystone species of the Mediterranean scrubland.

In the current context, and this also applies to other livestock species, the "*long shadow*" of animal production is mainly related to the use of chemicals for crop production, to the land use change to produce protein sources for animal feed (Cesari *et al*., 2018) and manure decomposition (Dinuccio *et al*., 2019; Hidayat *et al*., 2021). The studies carried out at the animal level (Estellés *et al*., 2009 and 2010; Franz *et al*., 2011) provide information on the real emissions from the normal metabolism of rabbits (growing animals only). Although this information is of great value, strategies aiming to reduce the greenhouse gas emissions directly related to the natural processes are limited (see Monteny *et al*., 2006; Estellés *et al*., 2014; Biagini *et al*., 2021). Based on the digestive biology of rabbits, the reduced CH_4 emissions from their manure (Hidayat *et al.*, 2021) and the actual feeding practices (use of by-products, lucerne and feed restriction; Gidenne *et al*., 2017), the environmental impact of rabbit meat production is expected to be lower than other livestock species.

The biological fact that living processes lead to the production of greenhouse gases raises the following question: Is life the problem? For some, yes. Animal production should be reduced (Willet *et al*., 2019) to better feed the world and "*save the planet*". More extreme views argue for its abolition (Reese, 2018). For others, it is a question of spatial distribution. But there is no consensus on what should be done. Some will argue that the best policy is to intensify production to save land for nature; others will argue that sharing land with nature is the best option (see Pearse, 2018). Maybe life is the only solution. But how can it be managed in a smart way that allows the production of high-quality food for all? For Kremen and Merenlender (2018), what is needed is an array of nature-friendly food production systems, created and managed in a way that is conducive to the emergence of ecosystem services, as experienced by Broom *et al*. (2013). This view is consistent with the concept of agroecosystems as defined by Conway in 1987: an ecosystem where productivity, stability, sustainability and equity are the key features.

For the rabbit sector (as it currently stands), the alternatives that have been tested in other livestock species, including the integration of crop and livestock, agroforestry and agroecology (Garrett *et al*., 2020), may not be possible. The current socio-economic and political context may limit the evolution of the current food production systems and the development of alternative models. In addition, the rabbit sector, at least in Europe, is facing a steady decline in demand. And this fact would certainly not help us to find alternatives if policies are not implemented to support the evolution required. But "*the show must go on*"!

Together with a team of agronomists, veterinarians, animal scientists and sociologists, we had the opportunity to design (Savietto *et al*., 2023) and test (Savietto *et al*., 2024) an alternative rabbit production system aiming at benefiting from the emergence of interspecific services of combining rabbit and apple production. Although this experiment was a one-shot trail, the first results are promising. We were able to raise rabbits with less concentrated feed (about 28% less pellets compared to the indoor cage system and 5% less compared to a grassland system). In addition, at the end of a 35-day period, the fattened rabbits reached 2.7 kg at 80 days of age, with a reduced use of antibiotics (individual treatment) and only one death (out of 144 rabbits). Since 2021, our team has been producing reference values for pasture-based rabbit production systems, with technical results that are comparable to the indoor cage system (see Fetiveau *et al*., 2021; 2023a), not to mention the clear improvements in terms of animal welfare (Fetiveau et al., 2023b). Other alternatives are also being tested by the French rabbit sector, as presented at the last World Rabbit Congress (see Guené-Grand *et al*., 2021).

Before being adopted, these alternatives should pass the scrutiny of rigorous scientific methods. The social acceptability of the proposed alternatives should also be assessed. But the declining demand for rabbit meat may not help. Despite these challenges, the rabbit sector should face the reality that rabbit farming may become a niche sector. In this sense, production methods that focus on sustainability and animal welfare should be strengthened.

At present, it is not possible to assess the environmental impact of alternative rabbit meat production systems. This is partly because they are still rare or experimental. Secondly, the reference methodologies used for assess greenhouse gas emissions may not be transferable, leading to biased estimates. The lack of harmonisation of current methodologies used to assess the environmental impacts of livestock production, at least for life cycle assessment methodologies (Goglio *et al*., 2023), is an additional limitation.

In this context, the main perspectives of this descriptive review are: (i) in indoor systems, an accurate assessment of the contributions of rabbit meat production to global warming, water footprint, air pollution, land use change and biodiversity loss is needed; (ii) harmonisation of methods and units for reporting results is also needed to allow a direct comparison of the systems studied; (iii) for all the environmental impact parameters, results should be reported in terms of at least two main units, impact per kg of product and impact per area; (iv) impacts should be assessed considering all animal categories, in a whole system analysis; (v) universities, research institutions, farmers, cooperatives and agribusinesses should develop and evaluate alternative systems; (vi) the environmental impact of each alternative tested should be carefully evaluated; (vii) research programmes, and the credit and insurance systems should be redesigned; (viii) policies should be implemented to also pay for the ecosystem services provided by livestock farming and to safeguard food sovereignty and farmers' incomes.

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ORGANIC RABBIT FARMING PERFORMANCES IN FRANCE BASED ON A BENCHMARK WITH GAELA SMARTPHONE APPLICATION

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ABSTRACT

Performances of reproduction were compiled for 11 farms over one year (2021), using the GAELA smartphone app and its associated web service. The herd size averaged 27 does and varied largely among the farms. With 4.6 matings, 2.6 parturitions per female/year were obtained (59.4% fertility rate). The number of new-born and weaned kits per parturition averaged 6.4 and 5.3 respectively. The receptivity rate varied according to the month of mating (P<0.001), with the highest values between January and July (>80%). Fertility also varied according to the month of mating (P<0.001), with the highest values (> 70%) occurred between January and May, and then dropped from June onwards, reaching 21% in October. The number of new-born was below 6.0 kits in winter and over 7.0 in spring and summer. The present study validated the utility of GAELA app and web service to construct a referenced database for organic rabbit farming.

Key words: organic rabbit farming, smartphone application, performances, season

INTRODUCTION

Since January 2022, a new European regulation is applicable for all member countries of the European community (EC) for organic rabbit farming (OJEU 2018; 2020). Organic rabbit farming (ORF) is based on grazing with maximum use of pasture, locally produced food resources, natural breeding and a weaning of 42 days old. In this system, rabbits can be housed in movable cages (**Picture 1**) or in paddocks (**Picture 2**). In France, about 15,000 organic rabbits are produced every year by about 50 farmers, and 20 have more than 30 breeding rabbits. This small supply of organic rabbit meat is not enough to meet consumer demand (Roinsard *et al.*, 2016). Nevertheless, since ORF is only just emerging in Europe, France is leading country with probably over 90% of the whole organic rabbit production.

Picture 1: Movable cages on pasture for organic rabbit farming (© P. Orain)

Picture 2 : Fixed enclosure "paddock" with grazing rabbits (© INRAE, T. Gidenne)

Unlike to industrial systems, there is no performance benchmarking in ORF that impair the development of this emerging sector. Therefore, a database management tool, associated with a smartphone application "GAELA" was developed (Huang *et al.*, 2021) in collaboration with the French association of organic rabbit breeders (AELBF: https://www.facebook.com/aelbf/). GAELA assists the farmer in the daily management of the farm tasks such as: mating, parturitions, weaning, etc.). A GAELA webservice is now available, that allows calculation of performances for the farmer, or calculation of compilated performances for several farms. The present study analysed compiled performances, with GAELA, for a dataset of 11 farms over a

one-year period (2021)

MATERIALS AND METHODS

The smartphone application "GAELA" allows the direct input of information concerning the reproductive parameters of males and females, such mating, parturition and weaning. It includes a calendar and tasks notifications, as well as a data synchronization in a secured data server. The user can access the reproduction performances of each female or male (Huang *et al.*, 2021).

GAELA is associated to a website (webservice), where each registered farmer can access to its own compiled performances of the flock for a chosen time period. The GAELA web service can be used by authorised administrators to calculate performance by compiling the data of several farms (**Figure 1**). Thirty-two farmers were registered in January 2024. For the present study we compiled data for a one-year period (2021) and for 11 farms that recorded regularly their data and with a dataset of more than 20 matings. The analysis was restricted to reproductive performances.

RESULTS AND DISCUSSION

Descriptive analysis of the farms selected

Breeders meanly have 27 females for 5 males (a ratio of 5 to 6 females per male), but ranged from 4 to 80 (**Table 1**). On average in a year, a farm performed 116 matings, resulting in 62 parturitions. The number of weaned kits was 45.2; which suggesting a survival rate of 72% from birth to weaning.

The doe replacement rate averaged 45% with a large range of variation (8 to 30%). For instance, two recent farms have a much higher replacement rate, since many young does have joined the herd (**Table 1**). The culling rate reached 35%, and the average age of doe was 19.5 months. More recent farms having an average age of 10 months.

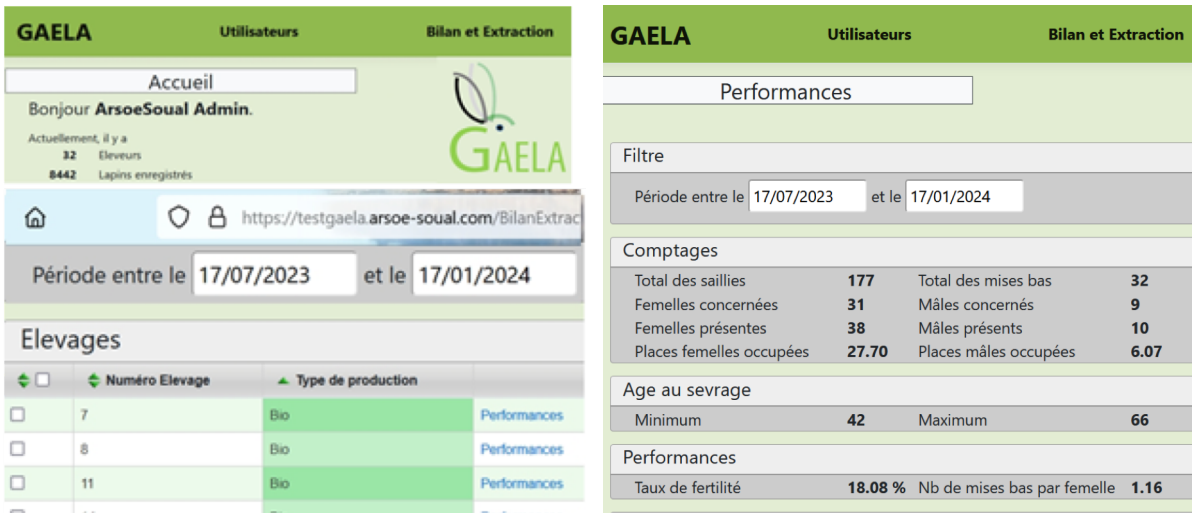

Figure 1: Display of compiled performances for several farms with GAELA website

Doe mortality rate averaged 22% and was null for three recent (and small) farms. This rate is also lower for recent breeders. The average culling age was 27.2 months. In fact, the average age of doe was relatively advanced (199 days) and higher than 174 days reported by Roinsard et al. (2016). Receptivity averaged 81% **(Table 2)**. For one farm, the receptivity was particularly low (36%), since the farmer aimed to ensure a regular reproduction throughout the year, and increased the mating frequency even in periods of low receptivity (winter season). The breeding rhythm was extensive, with 4.6 matings per doe per year, leading to 2.6 parturitions. The fertility rate rounded 60%, and was 7 points lower than previous similar study (Huang *et*

al., 2021). The survival rate at weaning reached 80%, that was better compared to previous studies (74.8%, *Gidenne et al.*, 2020; 69.3%, Huang *et al.,* 2021).

Table 1: Descriptive data of 11 organic rabbit farms in France during year 2021

	Mean	SD	Minimum	Maximum
Reproductive females (mean number/year/farm)	26.6	21.0	4.2	79.8
Reproductive males (mean number/year/farm)	4.9	3.0	1.4	10.1
Matings/year	115.9	101.7	14.0	389.0
Doe replacement rate, %	44.9	29.1	8.0	100.0
Doe culling rate, %	35.3	9.2	22.0	51.0
Doe mortality rate, %	22.2	15.8	0.0	43.0
Doe age (Month)	19.5	6.6	9.8	30.1

Table 2: Reproduction performances of does in 2021 for 11 farms.

	Mean	SD	Minimum	Maximum
Matings/female/year	4.6	2.4	1.2	11.0
Receptivity, %	79.0	18.6	36.0	96.0
Parturition / doe/year	2.6	1.1	0.9	4.8
Fertility rate, %	59.4	19.2	21.0	81.0
Born alive/parturtion	6.4	1.5	3.8	9.8
Weaned /parturition	5.3	1.2	3.9	7.1
Survival rate at weaning, %	80.0	12.0	62.0	95.0
Interval between two parturitions, days	166.3	84.3	77.0	385.0

The study showed that receptivity varied according to the month of mating (P<0.001), with the highest values between January and July (>80%, **Figure 2**). Fertility also varied according to the month of mating (P<0.001), with the highest values (> 70%) between January and May, and then dropped from June onwards, reaching 21% in October. This seasonal variation is classically observed in wild rabbit with high fertility on increasing days length (Boyd and Myhill, 1987). The number of newborn kits was also affected by the season, with the lowest number

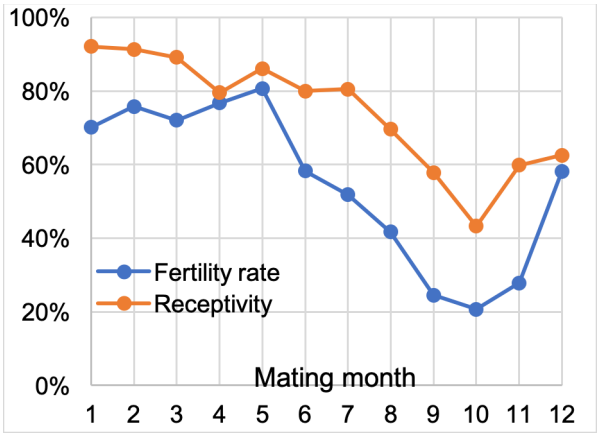

Figure 2: Fertility rate and receptivity according to seasons.

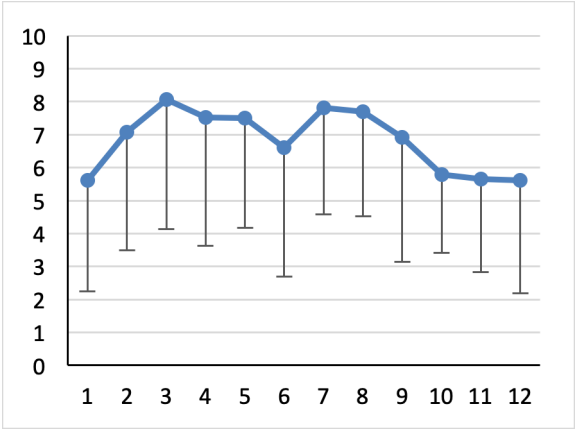

Figure 3: Number of live births by seasons

in winter and the highest in summer (P<0.01, **Figure 3**). The temperature in the nest was probably a main factor, but probably the humidity in the nest should play a role.
The stillbirth rate varied greatly during the year (**Figure 4**), with low values from February to May, and again low in July and August and even in October. The survival rate (**Figure 5**) also evolved with the seasons with low values at the beginning of the winter (November), probably due to a combination of humidity and cold temperatures.

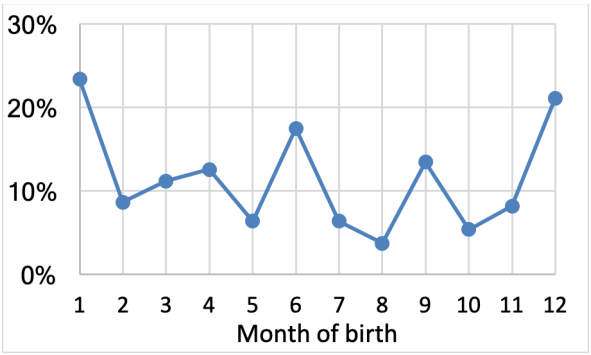

Figure 4: Stillbirth rate according to seasons

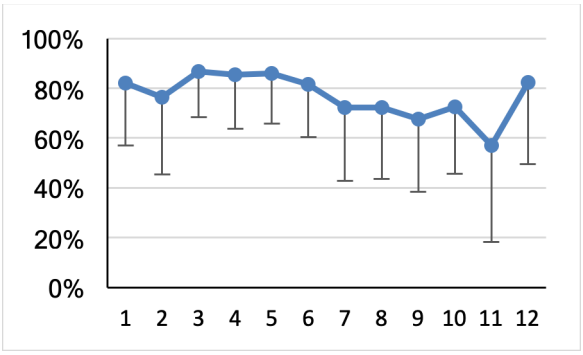

Figure 5: Survival rate according to seasons

CONCLUSION

The present study validated GAELA app and web-service for the construction of performances benchmarking in organic rabbit farming. The result confirmed the existence of a large progress margin in the management of the maternity unit, by improving the survival rate at birth and before weaning (housing management, prophylaxis, etc.). The parturition interval could also be reduced without impairing the survival rate after weaning.

GAELA is building a national reference system for organic rabbit farming, and is evolving with the implementation of sanitary parameters, such vaccines agenda, and death notifications.

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RESEARCH AND DEVELOPMENT OF MECHANICAL FEEDING SYSTEM OF MEAT RABBIT HOUSE BASED ON INTERNET OF THINGS

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ABSTRACT

China is a leading nation in the rabbit breeding industry, annually topping global rankings in both rabbit meat and fur production and exportation. However, feeding has always been one of the more labor-intensive tasks in breeding, with traditional manual feeding methods being time-consuming, labor-intensive, and prone to uneven feed distribution, leading to wastage of feed. With the introduction of "all-in-all-out" industrialized and restricted feeding breeding models, mechanized, automated, and intelligent feeding methods have been widely adopted in rabbit farming. This study aims to address the issues present in traditional feeding methods by designing an Internet of Things (IoT)-based feeding mechanical system for meat rabbit housing, to achieve automated, precise, and energy-efficient management in a factory farming model. The system utilizes a three-tier architecture, including a perception layer (weight sensors, PLC controllers, etc.), a network layer (4G-DTU wireless communication modules, MQTT servers, etc.), and an application layer. Overall testing validated the rationality of the system's design. Feeding performance tests showed that the integrated multiple-feed-box adjustment mechanism achieved an average feeding error of 2.06% and a feed variation coefficient of 1.68%, meeting the design requirements for quantitative feeding of meat rabbits. The research fills a technical gap in suitable technologies for factory farming models, although further optimization of the system structure and user interface remains a direction for future work.

Key words: Internet of things, Precision variable feeding, Electrical control, Intelligent breeding of meat rabbits.

INTRODUCTION

In 2022, China led the global market in rabbit production, contributing 466,000 tons of rabbit meat, or half of the world's supply (Noor et al., 2014). As the industry moves towards industrialization and the all-in-all-out breeding model, challenges such as feed wastage and increased disease risk from traditional free-feeding methods have highlighted the need for more efficient feeding technologies (Cheng et al., 2021). The labor-intensive and imprecise nature of manual feeding necessitates the shift towards mechanized solutions to enhance operational efficiency and tackle these issues. Research into rabbit feeding systems shows a spectrum of practices, from small-scale backyard operations to large-scale industrial setups, with advanced designs in Europe dating back to the 1970s. Technologies adapted from the poultry industry (Ahmed et al., 2021), such as cart-type and auger systems, are in use, with notable examples including France's Chabeauti (Chabeauti, 2020) and Italy's Clerici "Spiraflex" (Spiraflex, 2023). Despite these advancements, existing systems often lack precision and adaptability, especially in larger operations (Zuidhof, 2020). This study advocates for the integration of Internet of Things (IoT) technology to address these limitations and support the rabbit industry's sustainable development (Akhigbe et al., 2021). Recent research into intelligent feeding systems, such as Xu et al.'s (2021) ONEM2M-based model, Kim et al.'s (2016) remote monitoring system, and IoT innovations in aquaculture and poultry (Riansyah et al., 2020), underscores the potential for IoT to enhance feeding efficiency, environmental monitoring, and health prediction through real-time data analysis and control.

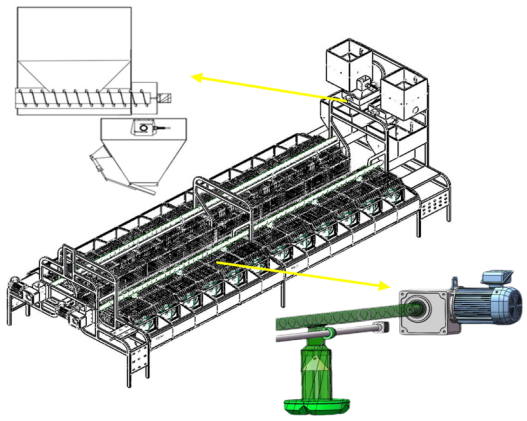
This study aims to design an Internet of Things (IoT)-based feeding system for meat rabbit housing, which will enable automatic adjustment of feed amounts, remote monitoring, and control of feeding times. This innovation is set to advance the digitalization and smart development of the livestock industry, enhancing breeding efficiency, reducing costs, and offering new technological support for the sustainable growth of the rabbit farming sector.

MATERIALS AND METHODS

Systems and experimental design

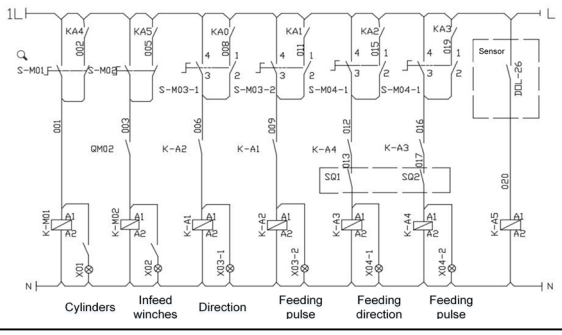
Reflecting on meat rabbits' behavioral and management needs, an auger feeding mechanism was selected for precise feed delivery. This informed the selection of electrical components and the development of a meat rabbit feeding system architecture, integrating a lower-level automatic feeding mechanism and an upper-level feed management system for efficient feed distribution.

Figure 1: Overall structure of mechanical feeding system for meat rabbits



A precision feeding system was designed for meat rabbit enclosures, incorporating the quantitative feed weighing device and the adjustable multi-feed box mechanism to meet the nutritional needs of rabbits based on age and breeding cycle, as shown in Figure 1. This system measures feed accurately, stores it in a hopper, and then distributes it to individual boxes, adjusting the amount based on the rabbits' breeding status. The system features an auger conveyor and weighing unit for transporting and measuring feed, controlled by a stepper motor and monitored by a weight sensor to ensure precise feed allocation. An electromagnetic valve releases the feed after reaching the target weight. The adjustable mechanism allows precise control over feed volume by altering a cone's height within a quantitative cup, facilitating restricted feeding in industrial breeding.

Figure 2: The precision variable feeding control system's electrical layout



The precision variable feeding control system for meat rabbit housing integrates human-machine interface (HMI) parameter settings, quantitative feeding, feed adjustment, and spring auger delivery into four key modules. It employs a PLC controller to manage the feeding process, beginning with the setup of a weighing device and stepper motor. The system operates based on HMI-input parameters, performing quantitative weighing and feed adjustments by controlling the stepper motor to adjust the feed volume in the quantitative cup until the desired weight is achieved, as verified by a weight sensor. Feed is then distributed to each box via a spring auger, ceasing when full. The system's electrical layout is depicted in Figure 2, and it is managed via Easybuilder Pro software, which supports functionalities such as startup, login, interface navigation, manual operations, data logging, trend analysis, and alarms, meeting the specific feeding needs of meat rabbit housing.

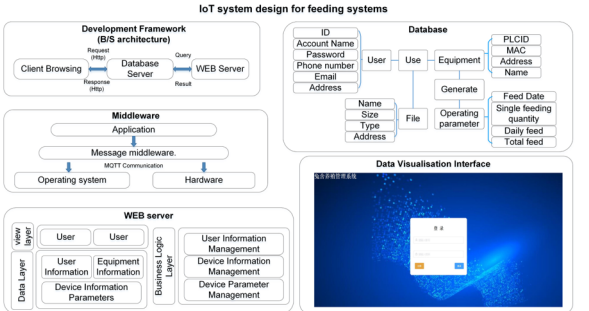


Figure 3: General architecture of IoT system for feeding system

The SCADA system design complies with IoT standards and lower-level specifications, incorporating cloud-based deployment. It leverages 4G technology for remote monitoring and control, ensuring data transmission, storage, and structure management. The system's software architecture follows a Browser/Server (B/S) model, with Docker for cloud deployment of the EMQX broker, and MQTT protocol for SCADA and system communication. The backend uses SpringBoot with a Model-View-Controller (MVC) pattern, featuring designed API interfaces. For the frontend, Vue.js and Element-UI are employed for user interface development, enhancing interaction and visualization, as shown in Figure 3.

The IoT system's comprehensive testing evaluates the electrical control and supervisory systems' performance, focusing on remote control and cloud monitoring functionalities. The testing process encompasses:1.Displaying weight sensor data on the PLC controller's Human-Machine Interface (HMI).2.Using the 485 wiring Modbus protocol to send this data to a 4G wireless module.3.Facilitating data exchange between the 4G module and the cloud IoT server through MQTT.4.Utilizing Modbus and MQTT debugging tools for communication integrity checks.5.Ensuring reliable data transmission between the PLC controller and the 4G-DTU module via the MQTT protocol.

Upon finalizing the designs for the meat rabbit feeding system's mechanical structure, control system, human-machine interface, and IoT system, a test platform was set up for IoT evaluations and feeding efficiency tests. The assessment involved a setup with a high-precision electronic scale (0.1g accuracy), a variable feeding system, specific rabbit pellet feed (572), and a 7-mesh sieve. The setup included 15 rabbit cages over 35 meters, with 30 quantitative cups per cage group. Key steps in the testing process were: sieving feed, setting the weighing auger at 150r/min for feeding, adjusting the quantitative cup's height, and timing the feeding duration from start to feed detection. After feeding, the feed was cleared and weighed for each box, allowing for the calculation of the feeding error rate, duration, and uniformity coefficient. This process quantitatively evaluated the feed quantity adjustment device's efficacy across different feeding volumes.

RESULTS AND DISCUSSION

In the IoT system evaluation, the PLC controller slave's response, "02030400220033292C", was decoded and scrutinized. The examination enabled the capture of the PLC lower-level machine's response on the MQTTX client, aligning with outcomes from the Modbus communication trial. These findings confirm that the communication between the supervisory and lower-level machinery is functioning correctly, the 4G-DTU transmission module exhibits dependable stability, and the comprehensive IoT system testing aligns with the established design criteria.

During the feeding performance test, it was observed that under different feeding volumes, the maximum error for the 30 quantitative cups was less than 3.98%, with the overall distribution around 1%. Moreover, the coefficient of variation (CV) for feeding across the 30 cups was under 2%, also generally around 1%. The average feeding error rate for the 30 cups was 2.06%, and the average coefficient of variation for feeding was 1.68%. For a cage frame with six cage positions, the feeding speed was 0.19 minutes per cage position.

Table 1: Feeder mechanical system feeding performance test results

Evaluation indicators	Feed weight (g)				Mean
	400	500	600	700	
Mean Error	1.95%	2.16%	2.12%	2.02%	2.06%
Coefficient of variation	1.57%	2%	1.68%	1.45%	1.68%

CONCLUSIONS

This study introduced an IoT-based system for industrial meat rabbit farming, designed to boost efficiency and reduce costs through a three-layer architecture for real-time monitoring and remote control. Initial tests confirmed its feasibility for industrial use. Future improvements are necessary in its architecture, user interface, and specific functionalities like the multi-feed box mechanism, PLC system expansion, and web interface optimization for full practical application.

ACKNOWLEDGEMENTS

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ENVIRONMENTAL IMPACT OF RABBIT PRODUCTION: AN ITALIAN SCENARIO

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ABSTRACT

The study aimed at quantifying the environmental impact associated with rabbit production in Italy. Data related to commercial rabbit production were obtained through interviews with farmers and industry experts. Additionally, primary data about the fattening phase were retrieved from several experimental trials published by the research groups involved. Attributional Life Cycle Assessment (LCA) models were constructed based on gate-to-gate and cradle to gate approaches, considering the reproduction and fattening phases and the whole cycle. System boundaries were set to include the impacts related to animal management, manure storage, feed production and transport, enteric fermentation, farm materials (electricity, fossil fuels), and the costs for replaced rabbit does. The LCA model included global warming (GWP, kg CO_2-eq) and eutrophication (EP, g PO_4-eq) potentials as impact categories, considering 1 kg of rabbit weight as the functional unit. Considering the whole rearing cycle, the production of 1 kg of growing rabbit was associated with the emission of 2.57 ± 0.18 kg CO_2-eq and 18.6 ± 5.01 g PO_4-eq. Feed production was the major contributor to both GWP (68-70%) and EP (75-80%). The second contributor to GWP (14-20%) and EP (16-18%) was manure management and storage. This study provides an initial assessment of the environmental impact of rabbit production in Italy, combining field and data from different experimental trials. Future LCA should consider additional impact categories and scenario analyses to test mitigation strategies and reduce highlighted hotspots.

Key words: Life cycle assessment, Global warming, Eutrophication, Emissions, Cradle-to-gate

INTRODUCTION

Rabbit production represents a valuable contribution to human nutrition, given the excellent nutritional profile of rabbit meat and its worldwide accessibility, thus contributing to biodiversity of available meat production (Petracci et al., 2018). In addition, the good feed conversion ratio and the capability of rabbits to exploit various agricultural by-products make this species an interesting candidate for the sustainable development of livestock productions, especially in developing areas. Currently, the assessment of the environmental impact associated with rabbit production is in its early stages. First results relied on primary data obtained from literature (Salou et al., 2015; Cesari et al., 2018), whereas no quantifications are available using field data.

In addition, preliminary results revealed that rabbit production exhibits a slightly higher impact than poultry and is comparable to pork production (Cesari et al., 2018). However, additional studies are necessary to validate these findings. Therefore, the present study aimed at quantifying the environmental impact associated with rabbit production in Italy, one of the major rabbit producers in Europe (Trocino et al., 2019). The assessment used both data obtained from interviews with farmers and technicians, and primary data retrieved from experimental trials related to the fattening phase. Moreover, hotspot analyses were conducted to identify the major contributors to the environmental footprint associated with rabbit farming.

MATERIALS AND METHODS

Data collection

Data from commercial rabbit production were obtained through interviews with farmers (Xiccato et al., 2005) and rabbit industry experts. Additionally, primary data related to the fattening phase were also retrieved from previous experimental published studies (Trocino et al., 2011; Xiccato et al., 2011; Trocino et al., 2013; Birolo et al., 2016; Gasco et al., 2016; Mattioli et al., 2019; Birolo et al., 2020a, 2020b, 2021, 2022). Collected data encompassed information about farm size as for reproducing does, management of feed and manure, feed formulation and composition, and animal performance (Table 1).

Table 1: Descriptive statistics of the breeding and fattening phases of rabbit farms: the Italian scenario in conventional farms.

	Breeding phase		Fattening phase	
	Mean	SD	Mean	SD
Farm data				
Does per farm, *n*	1315	348	-	-
Feed intake, *g/d/head*	487[1]	35	146	12
Dietary crude protein, *%*	16.0	0.8	15.6	8.2
Dietary digestible energy, *MJ/kg*	10.3	0.2	10.1	0.6
Animal performance				
Duration of the production cycle, *d*	-	-	43.0	3.0
Initial body weight, *kg/head*	2.35	0.14	811	142
Final body weight, *kg/head*	4.49	0.25	2823	136
Mortality, *%*	20.5	4.4	3.7	5.0
Feed conversion ratio	3.7	0.4	3.2	0.2
Doe performance				
Kindling-to-kindling period, d	56.8	5.1		
Kits born alive, *n/doe/year*	60.6	7.4	-	-
Weaned kits, *n/doe/year*	51.9	8.4	-	-
Doe replacement rate, *%*	101	12	-	-

[1]Average daily feed intake of the maternal cage (doe + litter) considering the kindling-to-kindling period.

Life cycle assessment

Life cycle assessment models were constructed by following the scheme described in ISO standards 14040-14044 (ISO, 2006). Attributional, gate-to-gate and cradle to gate approaches were adopted to evaluate the environmental impact of rabbit production, including breeding and fattening phases and the whole cycle. System boundaries were set to include the impact related to animal management, manure storage, feed production and transport, farm materials (electricity, fossil fuels), and the purchase of young does for replacement. The LCA model included global warming (GWP, kg CO2-eq) and eutrophication (EP, g PO_4-eq) potentials as impact categories, considering 1 kg of rabbit live weight as the functional unit (1 kg of weaned rabbits for the breeding phase and 1 kg of slaughtered rabbit for the fattening one).

RESULTS AND DISCUSSION

The production of 1 kg live weight (LW) of weaned rabbits during the breeding phase was associated with the emission of 2.67 ± 0.80 kg CO_2-eq and 17.8 ± 5.7 g PO_4-eq. Then, the production of 1 kg LW of growing rabbit from weaning to slaughter was associated with the emission of 2.20 ± 0.19 kg CO_2-eq and 18.6 ± 7.3 g PO_4-eq. Considering the whole production cycle, the production of 1 kg of slaughtered rabbit resulted in a GWP of 2.33 ± 0.15 kg CO_2-eq and an EP of 18.3 ± 5.0 g PO_4-eq (Table 2). The GWP calculated considering a cradle-to-gate approach was similar to previous preliminary assessments (Salou et al., 2015; Cesari et al., 2018) and slightly higher (from +5% to +21%) than that of producing 1 kg LW of broiler chicken (da Silva et al., 2014). On the other hand, EP was notably higher and more variable compared with the above mentioned reports (from +100% to +162%).

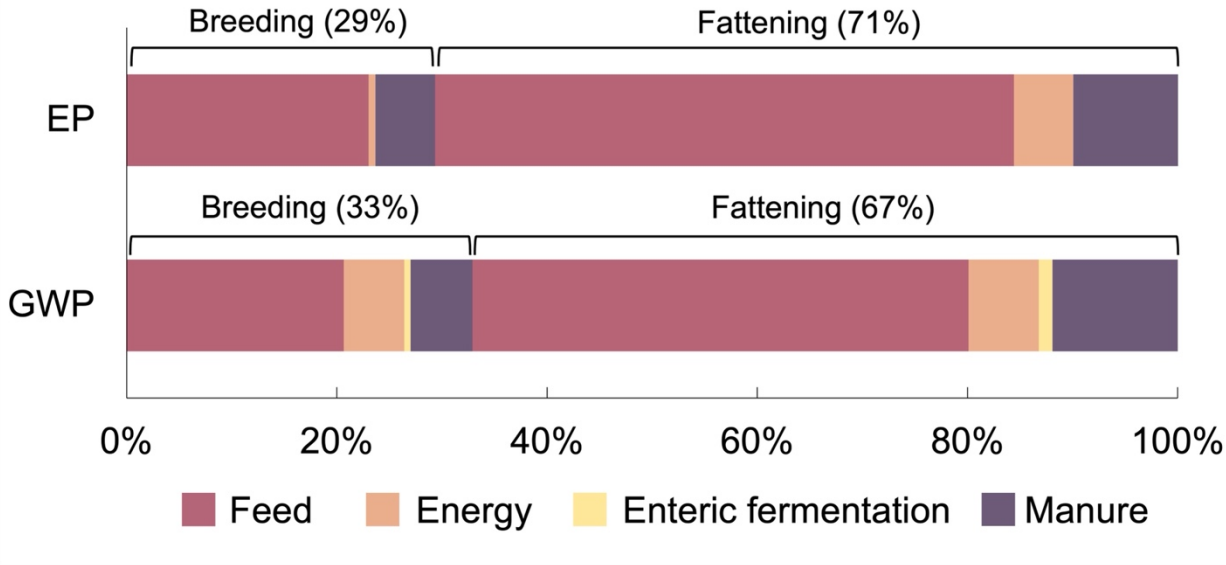
Table 2: Global warming potential (GWP) and eutrophication potential (EP) related to the breeding and fattening phases and whole cycle (cradle-to-gate) of rabbit production in the Italian scenario of conventional farms.

	Mean	SD	Min	Max	CV (%)
Breeding phase[1]					
GWP, kg CO_2-eq	3.51	1.12	1.78	4.41	31.9
EP, g PO_4-eq	17.8	5.7	12.0	30.1	32.0
Fattening phase[2]					
GWP, kg CO_2-eq	2.20	0.19	1.80	2.64	8.6
EP, g PO_4-eq	18.6	7.3	11.8	40.0	39.3
Cradle-to-gate[2]					
GWP, kg CO_2-eq	2.33	0.15	2.01	2.65	6.4
EP, g PO_4-eq	18.3	5.0	13.1	32.5	27.3

[1]Impact expressed per 1 kg live weight of weaned rabbit. [2]Impact expressed per 1 kg live weight of growing rabbit. SD: standard deviation. CV: coefficient of variation.

Hotspot analyses revealed that the breeding phase contributed to 33% of GWP and 29% of EP, whereas the fattening phase contributed to 67% and 71%, respectively. Considering the whole cycle, the major contributor to the environmental impact was feed utilization (69% of GWP and 78% of EP), followed by manure (18% and 16%), energy (11% and 6%), and enteric fermentations (2% and 0%) (Figure 1). Considering the footprint of the feed for the breeding phase, the most impacting ingredients were the soybean meal for GWP (17%) and barley meal for EP (28%). Then, considering the feed for fattening, the most impacting ingredients were alfalfa hay (22% of GWP, 24% of EP), barley (22% of EP), and, when used, sunflower oil (21% of EP).

Figure 1. Hotspot analysis (%) of global warming potential (GWP) and eutrophication potential (EP) per 1 kg of body weight considering the whole production cycle (cradle-to-gate).



CONCLUSIONS

The present study gives a preliminary evaluation of the environmental impacts related to the rabbit production within the Italian scenario of conventional farms, integrating field data with primary data from experimental trials. Feed, especially for the fattening phase, represents the major contributor to both global warming and eutrophication potentials. Future evaluations should include other impact categories and scenario analyses to test the environmental performance of mitigation strategies to reduce the impact of the highlighted hotspots.

ACKNOWLEDGEMENTS

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PRELIMINARY CHARACTERIZATION OF MADAGASCAR'S FARMED RABBITS

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ABSTRACT

A total of 3018 rabbits were surveyed, of which 1060 were measured (35% of all rabbits counted) and 14 breeds were characterized. Among the 1060 rabbits subjected to morphological identification, the Local Breed emerged as the most populous, followed by the Giant Hutia and the White Bouscat Giant. Our findings indicate a wide range of LW (120 to 4860 g), averaging 1320g at around 25wks. While the Flemish Giant exhibited the highest LW, contrasting with the Local Breed's. Other breeds introduced by the FIDA and FORMAPROD projects averaged around 1500g LW. Behavioral observations across farms suviwed, revealed that rabbits primarily engage in static (41.02%), followed by feeding (38.01%) and comfort (11.89%) activities. Motor activities, social interactions, and stereotypies constituted smaller proportions at 5.13%, 3.38%, and 0.67%, respectively. In conclusion, rabbits farmed in Madagascar principally belonge to Local Breeds, are characterized for showing common behavoioural patterns and slaughter LW < 2Kg at < 25wks of age. It appears clear that in term of potential of grown, rabbit farming in Madagascar, has yet to be explored and present huge margins for improvement. In fact rabbit meat is as good food as chickens, there is highly prolific and have excellent taste and a healthy meat profile and can even utilize high-fiber diets better than poultry, reducing the need for high-cereal feed competing with farmers owner for food access, to combat famine.

Key words: Madagascar, Rabbit, Breed, Behaviour.

INTRODUCTION

Rabbit is reared systematically on a vast scale, with global rabbit meat production reaching 1.8 million metric tonnes a year. China is the major rabbit meat producer (735,021 tonnes/year). followed by Italy, Spain, Egypt, and France (FAOSTAT, 2012). The EU is the second largest meat-rabbit producer in the world, after China, where advanced breeding techniques and disease management strategies have evolved (Food and Agriculture Organization of the United Nations, 2019).

While in Madagascar, rabbit farming predominantly follows a family-based model, relying on traditional techniques, and often lacks essential knowledge in rabbit husbandry. Moreover, rabbits were kept with other animals, such as poultry in different housing systems of varying sizes (Randriamandratondrakotonirina, 2019). Rabbit farming has emerged as an innovative solution to address food scarcity in several African countries, including Madagascar, as explored by Dietmar et *al.* (2014).

The objective of this study was to comprehensively characterize (LW, breed and behaviour) rabbit reared in Madagascar, with the aim of advancing knowledge and promoting the development, including the dissemination of appropriate housing, nutrition, health care and best breeding techniques, for rabbit farming in Madagascar.

MATERIALS AND METHODS

Selected farms and survey methods
Direct observations were carried out from June to November 2023, by visiting 272 rural rabbit farms in the Amoron'i Mania region, in the District Fandriana, Ambositra, Ambatofinandrahan and Manandriana. A survey form was conducted at first as a formal discussion with the owner to fill the form. To confirm the data collected by filling the pre-established questionnaire, direct observations were performed.

Characterization of rabbits reared in Madagascar
The questionnaire form contained parameters both qualitative (to characterize farming system owner was asked about rabbits, structures and management procedures; results presented in Sambiazy et *al*., 2024 in WRC2024) and quantitative (characterize reared rabbits).

To characterize reared rabbits, identify rabbit breed from the body conformation, coat and eye color and the shape of the ears (Arnold et *al*., 2005) and the local breed was a cross between medium-sized giant breeds and is generally bicolored (Randriamandratondrakotonirina, 2019). Behavioral observations (Martin and Bateson, 1986) were performed.

A total of 3 018 rabbits were counted in the rural rabbit farms interviewed, of which 1 060 were measured (35% of all rabbits counted) and each rabbit measured was taken between 2 and 152 weeks of age and the live weight (LW) of the rabbit expressed in grams (g).

Moreover rabbit behavior was recorded by direct observation (N=445) by two trained operators. Direct observations were performed for 6 hours a day in two sessions: 9-12h and 14-17h, using the focal animal scan sampling method (Martin and Bateson, 1986). Before each observation, the animals had 30 min to adapt to the presence of observers; during this time, the number of indoor animals was recorded with the necessary information and the individuals marked with a color on his back to facilitate observations. Observed behaviours, were: comfort (C), feeding (F), motor (M), stereotyped (STE), static (STA), and social (SOC) activities.

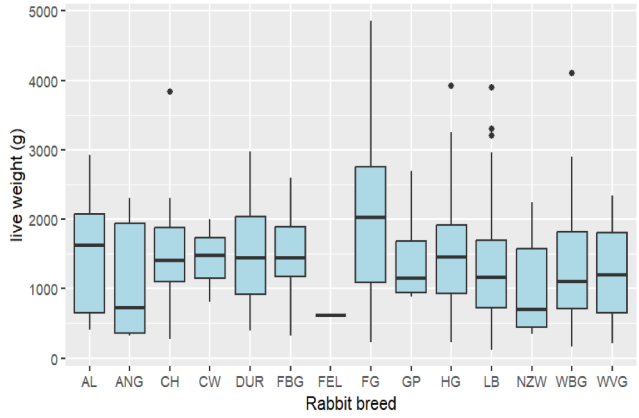
Statistical Analysis
Data analyzes were performed using Stat RStudio software (R 4.2.3) and descriptive statistical analysis. All data were analyzed by the Shapiro-Wilk normality test if the data were normally distributed. Analysis of variance by ANOVA was used to estimate least squares means and analyze the effect of LW of Rabbit breeds and also effects of housing systems on rabbit behaviour and their interactions. Analysis of variance (ANOVA) was used to assess the effects of housing system on rabbit behavior. Multiple comparisons of means were made by calculating by Tukey-Kramer and Bonferroni (interaction between behavior) multiple comparisons ($p<0.05$).

RESULTS AND DISCUSSION

Characterization of rabbit reared in Amoron'i Mania farms
A total of 3018 rabbits were surveyed, of which 1060 were measured (35% of all rabbits counted), and 14 breeds were characterized. The local breed (LB) was the most numerous (413 individuals), followed by the White Bouscat Giant (WBG) (252 Individuals), Hutia Giant (HG) (Hollandais of Madagascar) (139 individuals) and White Vienne Giant (WVG) (87 individuals), while the other breeds were rarely (less) in the Amoron'i Mania region (less than 47 individuals). In 2019, Randriamandratondrakotonirina was counted 9 rabbit breeds in Amoron'i Mania where the White Bouscat Giant was numerous, followed by Chinchilla and local breeds, while the Angora and White Vienne Giant "breeds" were rare in the Amoronimania Region. This difference in the value of several rabbit breeds was due to the presence of new breeds of male breeding rabbits (commercial breeds) such as the Flemish Giant, Giant Papillon, New Zealand White and California White, which were distributed in 2022 (2022-2026) by the FIDA (Fonds International de Développement Agricole) project and

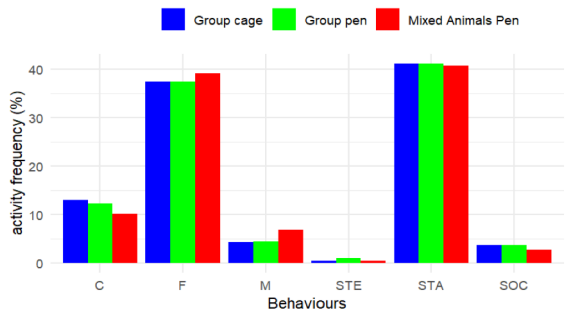
FORMAPROD (Vocational training and agricultural productivity improvement programme) working in the agricultural sector in Madagascar.

Figure 1: LW of rabbit breeds in Amoron'i Mania farms



The LWs of all breeds were recorded (figure 1). FG showed a higher LW, varying from 220.0 to 4860.0g with an average of 2087.97g at the age of 27 wks. While LB rabbits which were the most numerous and raised by the farmers had a lower average LW (between 120 and 3900g with an average LW of 1230g at the age of 23 wks) than the FG breed. However, AL, CH, DUR and FBG rabbits presented the same LW values around 1500g. ANG and WVG showed a relatively low LW (1101.7 and 1759.0g for ANG and WVG, respectivelly). Regarding LW, FG showed higher value (2087.97 g at 27wks of age) than White Bouscat Giant (1528.7g) found by Randriamandratondrakotonirina (2019), Algerian rabbit (1610.49g at 13wks of age) found by Meftik et *al*., 2010 and than of rabbit's local breed (1860g) in Morocco (JAZOUI et *al*., 2006). This difference in LW of rabbits varies depending on the breed (DJAGO et *al*., 2007) and breeding system (Mugnai et *al*., 2014), accordingly in the first part of this study, rabbit housing system was characterised as traditional farming systems with rabbits reared in pen with mixed animals which wich compromise rabbit's performance (Sambiazy et *al*., in WRC2024). LWs of the rabbits presented a significant difference between the breeds recorded ($p < 0.001$).

Figure 2: frequency of rabbit behavioral activities by housing system (%).



Rabbits in Amoron'i Mania spent most of the time on STA (static activities) (41.02%), followed by F (feeding) (38.01%) and C (comfort) (11.89%). Motor (M), social (SOC) and stereotypies (STE) accounted for only a small rate of their activity, 5.13%, 3.38% and 0.67% respectively and also stereotypies (STE) (figure 2). In agreement with many authors (Laura Ozella et *al*., 2022, Trocino et *al.,* 2013 and Dal Bosco et *al*., 2002) static (STA) activities were the most common behaviours observed in rabbits.

CONCLUSIONS

We identified in Amoron'i Mania farms 14 rabbit breeds, with the LB the most abundant, followed by WBG, HG, and WVG. Weights of all breeds showed, with FG showing the highest LW of 2087.97g at 27 weeks, on the contrary LB rabbits, were most commonly raised, had a lower LW.

In terms of behaviour, rabbits in Amoron'i Mania primarily engaged in static activities (STA), followed by feeding (F) and comfort (C), with motor activities (M), social activities (SOC), and stereotypies (STE) being less prevalent.

ACKNOWLEDGEMENTS

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MEAT RABBIT VALUE CHAINS IN NIGERIA

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ABSTRACT

This presentation focuses on meat rabbit value chains (MRVC) in Nigeria, and in Africa, with priority attention on critical stakeholders, agencies and institutions. Overarching goals include chain functionality, empowerment and optimization in relation to inclusiveness and wealth creation. The discourse notes the uniqueness and excellent features of the domestic rabbit as a livestock, as well as the diversity of products and services in the MRVC. Benefits attributable to the MRVC include income, employment, entrepreneurship, gender inclusion and value creation. Critical gaps including absence of frameworks and mechanisms for chain mapping, analysis and feedback to stakeholders in the MRVC, with dire consequences for poverty levels. The presentation assesses all segments and nodes of the MRVC including inputs (feeds, genetics, healthcare, housing), producers, processors, distributors, marketers, consumers and support agencies, with attention to their linkages and functions. Methodologies included semi-structured questionnaires, FGD and KII for stakeholders and professionals in the MRVC. Discussions covered architectural maps of MRVC, challenges, risks, risk mitigation, SWOT analysis, cooperatives, vertical integration models of MRVC, STI in chain functionality and empowerment, traceability, and resilience building through artificial intelligence to mitigate risks and optimize operations in the MRVC. Parallel pathways within the MRVC represented by traditional vs modern operations are dissected. Overall operational mechanisms and frameworks of the MRVC with attention to resilience, empowerment, equity, fairness and inclusiveness are discussed in harmony with Objects of the Constitution of the WRSA, and consistent with UN SDG goals 1, 2, 5 and 8.

Key words: Meat rabbit value chains, Chain optimization, Resilience-building, Wealth creation.

INTRODUCTION

In Africa, Nigeria ranks as the second-largest producer of rabbits after Egypt (FAOSTAT, 2022). In Nigeria, rabbits are recognized as a crucial genetic resource, primarily raised within traditional backyard systems to serve as a source of animal protein for household consumption (Mailafia et al., 2010). Moreover, rabbits hold significant potential for sustainable wealth creation, gender empowerment, enterprise development, and poverty alleviation (Oseni and Lukefahr, 2014). The meat rabbit value chain in Nigeria plays a pivotal role in the country's livestock industry, offering livelihood opportunities for many individuals (Ayeni et al., 2023). It encompasses various stakeholders, including rabbit farmers, input suppliers, processors, and traders, who collaborate to ensure the efficient production, distribution, and consumption of meat rabbit products (Baviera-Puig et al., 2017). Typically, the value chain commences with rabbit farmers, who undertake the breeding and rearing of rabbits for meat production. Subsequently, these farmers process the rabbits themselves or sell to middlemen who facilitate their transportation to processing facilities. At these processing facilities, the rabbits undergo slaughter and processing to produce various meat products, including carcasses, offals, and by-products such as hides and skins. Ultimately, these meat products are distributed to retailers who then make them available to consumers. Currently, the MRVC in Nigeria is not well understood. A comprehensive analysis could help to identify strengths, weaknesses, opportunities and threats (barriers) associated with this value chain to guide policy interventions for chain functionality and the empowerment of rabbit farmers. Further, findings from such analysis could help to harness opportunities and address critical challenges identified in the MRVC, in order to promote efficient and profit-boosting operations in this value chain. Therefore, the objective of the present study is to analyze the meat rabbit value chain in Nigeria.

MATERIALS AND METHODS

Data collection and analysis

A structured questionnaire was employed to interview 156 actors within the meat rabbit value chain (MRVC) in Nigeria. The questionnaire encompassed inquiries concerning demographic and socio-economic characteristics, input supply to the MRVC, meat rabbit production enterprise and entrepreneurship, abattoir operations, meat rabbit processing and value addition, distribution and marketing of meat rabbit products, as well as the involvement of government and non-governmental organizations with the MRVC. Results are as pie charts.

RESULTS AND DISCUSSION

Descriptors of the MRVC in Nigeria

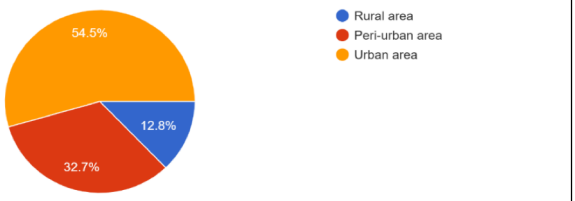

Figure 1: Location of rabbit farm enterprise

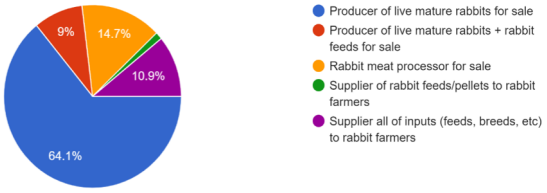

Figure 2: Main rabbit enterprise activity

Figure 1 presents the geographical distribution of rabbit farm enterprises. Majority of stakeholders are situated in urban areas (54.5%) or peri-urban areas (32.7%), with a smaller proportion in rural areas (12.8%). This indicated that urban and peri-urban areas are the primary locations for rabbit farming enterprises in Nigeria. This suggests a potential opportunity for targeted interventions and support programs to enhance rabbit production in rural areas, which may contribute to rural development and poverty alleviation. The distribution of primary rabbit enterprise activities is presented in Figure 2. The most predominant activity among stakeholders is the production of live mature rabbits for sale (64.1%). Other key stakeholders include rabbit meat processors (14.7%), input suppliers (10.9%), producers of live rabbits and feeds (9.0%), and feed producers.

Figure 3 presents the most predominant rabbit breeds kept by MRVC stakeholders. Mixed breeds, also known as heterogeneous stocks are the most predominant breeds (64.7%) followed by New Zealand White (26.9%), Chinchilla and Californian rabbits. According to Lukefahr (1998), non-directional interbreeding of local and exotic rabbit breeds is routinely done in Africa resulting in preponderance of heterogeneous or mixed rabbit populations. Furthermore, these mixed-breed rabbits are highly adapted tot the prevailing climatic and management conditions (Oseni and Lukefahr, 2014).

Input supply to meat rabbit value chain in Nigeria

The ranking of inputs based on recurrent costs of production as reported by stakeholders is presented in Figure 5. Exactly half of the stakeholders (50%) ranked feeds as the largest contributor to recurrent costs, followed by breeding stock and veterinary drugs. Other notable rankings include an equal ranking for these three inputs (26.1%) and a ranking of breeding stocks > feeds > veterinary drugs (18.6%). The high proportion of stakeholders identifying feeds, rabbit stocks, and veterinary drugs as key inputs underscores the critical importance of these inputs in rabbit farming operations in Nigeria. Therefore, ensuring access to quality inputs, as feeds and veterinary drugs, is essential for maintaining the health and productivity of rabbit stocks, ultimately impacting the overall profitability and sustainability of the MRVC.

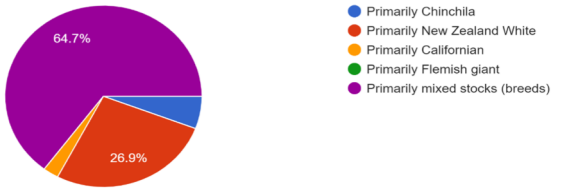

Figure 3: Predominant rabbit breeds kept

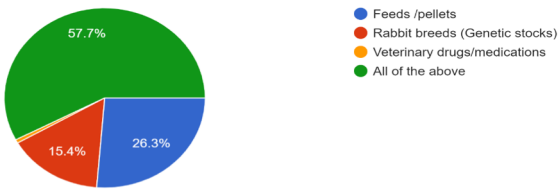

Figure 4: Major inputs for rabbit farm enterprise

Meat rabbit production enterprise and entrepreneurship

Figure 6 illustrates the categories of by-products generated from the MRVC in Nigeria. Majority of respondents (84.6%) reported dealing with by-products such as urine, manure, and pelt, while the remaining respondents (15.4%) do not engage with these by-products. Specifically, 32.1% of stakeholders handle all these by-products, 30.1% deal with urine and manure, and 17.1% manage manure only. Figure 7 depicts the utilization of rabbit skin, a by-product from the MRVC. The analysis indicates that the majority of stakeholders (41%) either flayed or roasted the skin, while 34.6% were not aware of its value. However, 24.4% processed the skin for the leather industry. The high percentage of stakeholders dealing with by-products from the MRVC, such as urine, manure, and pelt, highlights the potential for additional income streams and value addition within the value chain. Proper management and utilization of these by-products can contribute to waste reduction, environmental sustainability, and enhanced profitability for rabbit farmers.

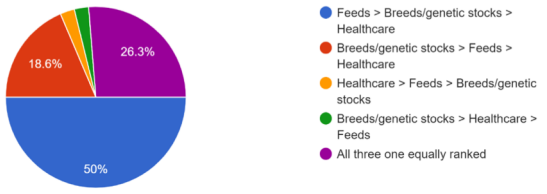

Figure 5: Ranking of inputs in terms of recurrent costs of production

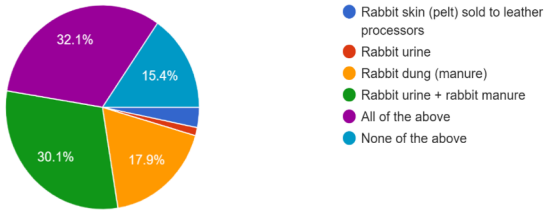

Figure 6: By-products from rabbit enterprise

Distribution and marketing of meat rabbit products

Figure 8 presents avenues for marketing live animals from rabbit enterprise. Majority of stakeholders (47.7%) sell mature and live animals to middlemen who process and sell to consumers while 26.6% of stakeholders process live animals within their farm premises. However, just 3.9% of stakeholders take live animals to abbattoir for processing. Further, 25% of stakeholders practice a combination of these methods.

Main avenue for sale of meat rabbit products by MRVC stakeholders is presented in Figure 9. Overwhelming majority sell directly to consumers (54.5%). Other key avenues include pre-order via WhatsApp group, selling to local market and kiosk while 37.8% of stakeholders reported practicing any of the avenues presented. Figure 10 presents the challenges of marketing meat rabbit stocks along the MRVC. Low awareness of the health benefits and low consumption rate were the most prominent challenges identified by stakeholders. Others include irregular supply channels (9.6%) and rabbits being seen as pet.

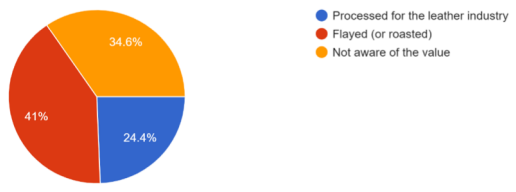

Figure 7: Utilization of the skin of rabbits

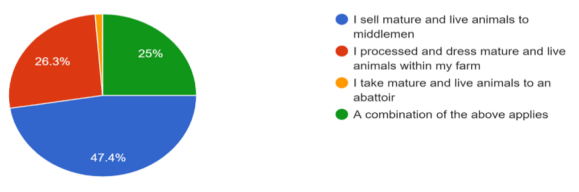

Figure 8: Avenues for marketing live animals

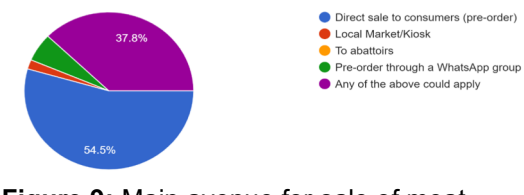

Figure 9: Main avenue for sale of meat rabbit products

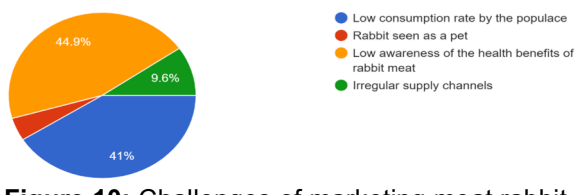

Figure 10: Challenges of marketing meat rabbit stocks

Abattoir operations, meat rabbit processing and value addition

Figure 11shows the proportion of stakeholders that regularly take their finishing stocks to abbatoir for slaughter, dressing and packaging. Overwhelming majority of stakeholders do not take their stocks to any abattoir for processing (85.3%) while others take thier stocks to abattoir, occassionally when price is favourable or not sure of the advantage. Figure 12 presents the distribution of stakeholders based on branding of meat rabbit products. Majority of stakeholders (57.1%) do not brand their meat rabbit products. However, a further 32.1% of stakeholders who do not brand their meat rabbit products expressed their readiness to brand.

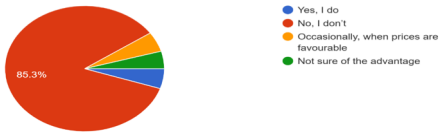

Figure 11: Taking finishing stocks to abbatoir for slaughter, dressing and packaging

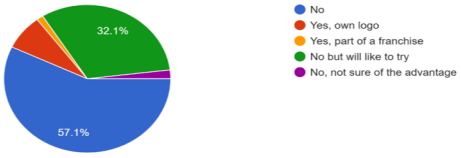

Figure 12: Branding of meat rabbit products

Involvement of government and non-governmental organizations with the MRVC

Figures 13 and 14 present the existence of government support, non-government organization support and platform to access loans and credit facilities for MRVC stakeholders, respectively. Across all three categories, majority of stakeholders (>90%) reported lack of existence of support or intervention programme by the government and NGOs to support the development of rabbit production enterprise. Furthermore, absence of platforms or opportunities to access loans and credit facilities to support rabbit production enterprise was reported.

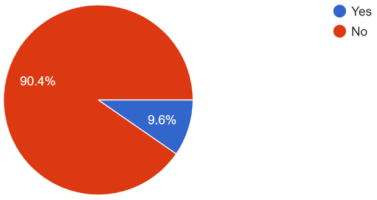

Figure 13: Existence of support or intervention programme by the government to support the development of rabbit production

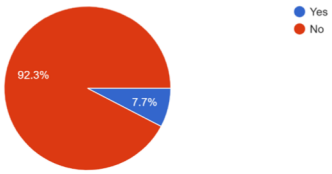

Figure 14: Existence of support systems by NGOs for rabbit enterprise development

CONCLUSIONS

Rabbit farming enterprises in Nigeria are primarily situated in urban and peri-urban areas, indicating potential for targeted interventions in rural areas. Furthermore, feeds constitute the largest recurrent cost for stakeholders, followed by breeding stock and veterinary drugs. In addition, there is significant engagement in the MRVC with by-products such as urine, manure, and pelt, suggesting potential for additional income streams and value addition within the value chain.

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PRELIMINARY CHARACTERIZATION OF RABBIT FARMING SYSTEM IN MADAGASCAR

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ABSTRACT

A total of 272 farmers participated in our survey, with 61% being women and 39% men. The predominant housing system utilized by these farmers was mixed animals pen (50.74%), followed by group pen (25.37%), and single and group cages (13.60% and 10.29%, respectively). Mortality rates varied among housing systems and phase physiological. However, mortality rate before weaning was higher (76.10%). Age at first mating of Amoronimania rabbits ranged from 4 to 10 (months), with a mean age at first mating of 6. Mean LW varied between housing systems, single cage had an advantage in live weight (1389.74 g at age 25.835 weeks). The weight and age at slaughter of Amoron'i Mania rabbits varied according to rearing system, ranging from 4 months to 30 months. Rabbits raised in a single cage gained a weight advantage.

Key words: Madagascar, Rabbit, Housing Systems.

INTRODUCTION

With the ongoing expansion of the global population, the demand for meat poses a significant challenge, reflecting economic disparities between developed and emerging nations.
Rabbit farming has emerged as an innovative solution to address food scarcity in several African countries, including Madagascar, as explored by Dietmar et *al*. (2014). Notably, rabbit meat stands out for its superior nutritional value compared to commonly consumed bovine and pork meat in Madagascar, offering higher protein levels with essential amino acids and lower cholesterol content than meat from other species (Dalle Zotte and Szendrő, 2011).

Despite, in Madagascar, rabbit farming predominantly follows a family-based model, relying on traditional techniques, and often lacks essential knowledge in rabbit husbandry (Randriamandratondrakotonirina, 2019). Furthermore, the limited consumption of rabbit meat among the Malagasy population is attributed to lack of habits (Ministere De L'elevage, 2011). Thus the nutritional benefits, rabbit farming in Madagascar holds the potential to play a crucial role in generating income and addressing the shortage of animal protein in underdeveloped nations, aligning with the Sustainable Development Goals (SDGs) outlined in the 2030 Agenda for Sustainable Development. Additionally, rabbit meat exhibits a relatively lower environmental impact compared to many other meat types (Saga Gunnarsson and Carl-Gustaf Thulin, 2023).

Hence, the primary objective of this study was to comprehensively characterize rabbit rearing systems and traits in Madagascar, with the aim of advancing knowledge and fostering the development of rural rabbit farming in the country.

MATERIALS AND METHODS

Selected farms and survey methods

Direct observations were carried out from June to November 2023, by visiting 272 rural rabbit farms in the Amoron'i Mania region, in the District Fandriana, Ambositra, Ambatofinandrahan and Manandriana. In order to evaluate the rabbit production in the rural areas of Amoron'i Mania region, a survey form was conducted at first as a formal discussion with the owner to fill

the form and to confirm the data collected by filling the pre-established questionnaire, direct observations were performed. The questionnaire form contained parameters both qualitative (to characterize farming system owner was asked about rabbits, structures and management procedures) and quantitative (measurements to characterize reared rabbits; results presented in Sambiazy et *al.*, 2024 in WRC2024).

Characterization of rabbit's farming systems in Madagascar

In order to describe and identify farming systems, was asked: conduction (sex, age, work, if rabbit rearing was the primary activity and level of instruction of owner), used structure (dedicated building, pen, single or group cage), owned rabbit's characteristics (total number of rabbits (does, buck, pups)), breed, sex, age, physiological phase, type of diet (purchased concentrate or self produced and/or family descartes feed), reproductive practices (artificial insemination or natural mating), age at first mating and interbirth interval, number of births and number of deaths at birth, weaning and fattening per doe and age and weight at slaughter.

Statistical Analysis

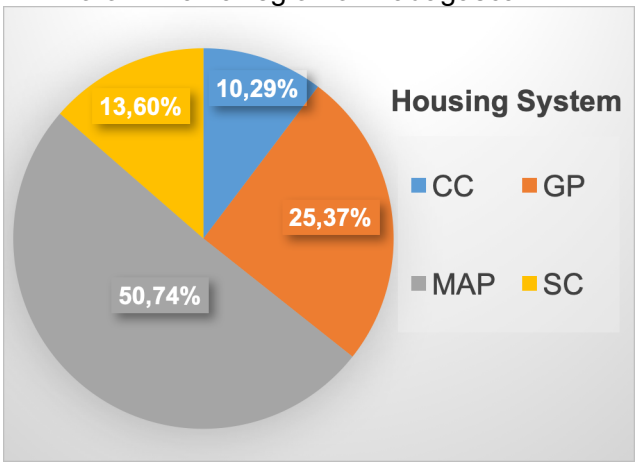
Data analyzes were performed using Stat RStudio software (R 4.2.3) and descriptive statistical analysis. All normally distributed data were analyzed by the Shapiro-Wilk normality test. Kruskal Wallis analysis of variance (data not normally distributed) and ANOVA were used to estimate least-squares means and analyze the effect of housing system on number of deaths per physiological phase, age at first mating, number of pups born per female, interbirth interval per female and age and weight at weaning, mean age and live weight at slaughter. Tukey's multiple comparisons and post-hoc test were tested with $p<0.05$.

RESULTS AND DISCUSSION

Farming systems in Madagascar

The survey revealed that most rabbit farm owners are located in the Amoron'i Mania region (interviewed 272 rural rabbit farms) of Madagascar, greater percentage of farmers were women (61%) and for them, rabbit's farming represents a secondary activity. On the contrary, in Western Algeria, the majority of rabbit farms were generally men owned (80% of farms surveyed), and rabbit farming was considered a secondary activity of the men (Mogharbi et *al.*, 2021). The majority (50.74%) of farmers used to mixed animals pen (MAP) of rabbit and group pen (GP, 25.37%) housing, only 13.60% reared rabbits in single cage (SC) and 10.29% in colony cage (CC) (figure 1). According to Randriamandratondrakotonirina (2019), most farmers (78%) combined rabbits with other animals (mainly poultry) and only 22% isolated (cage or pen) their rabbits.

Figure 1: Distribution of rabbit housing systems in Amoron'i Mania region of Madagascar

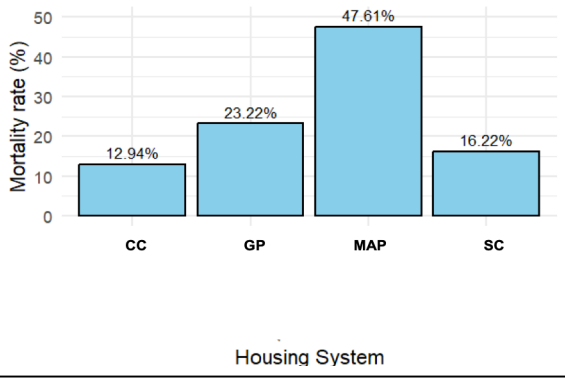


In figure 2 is presented the rate of deaths by housing system. Obviuosly, rabbits raised in MAP had a higheest mortality 47.61% ($p<0.05$), and then GP (23.22%) housing in CC and SC were lower, even if should not be underestimated (not negligible) as they represent

16.22% in S and 12.94% in CC. In figure 3 are showed the weaning, peri-weaning and fattening mortality rates. The highest (p <0.05) mortality was found at peri-weaning physiological phase (76.10%), followed by fattening and at birth mortality rates(12.30 and 11.60%, for fattening and at bith phisiological phases, respectivelly).

Housing system showed only a tendential (P=0.0549) difference in the LW. Obviously, SC reared rabbits had an advantage in mean live weight of 1389.74g at age 25wks. MAP (1323.27g at age of 25 wks) and GP (1345.18g at 26wks) were almost the same LW. CC recorded a LW of 1164.42g at around 19 wks).

Figure 2. Effect of housing system on mortaliy



The measured LWs in rabbits were much lower than that of litterature (Trocino et *al.*, 2004 and 2014) even though the rabbits at Amoron'i Mania were older; this difference is probably due to scarce techical knolowedge of rabbit good practice of breeding. The LW and age at slaughter of Amoron'i Mania rabbits varied according to rearing system (figure 4). Rabbits raised in SC registered the highest (p< 0.001) LW at slaughter (1920.83g at the age of 6 months), followed by CC with 1823.93g at the age of 6 months. While the GP and MAP rabbits showed the lower LWs (1783.91g and 1744.80g at 8 months, in GP and MAP, respectively).

Figure 3: Average mortality rates by physiological phase of rabbits.

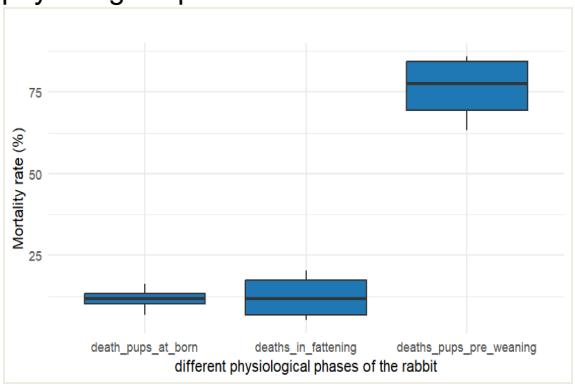
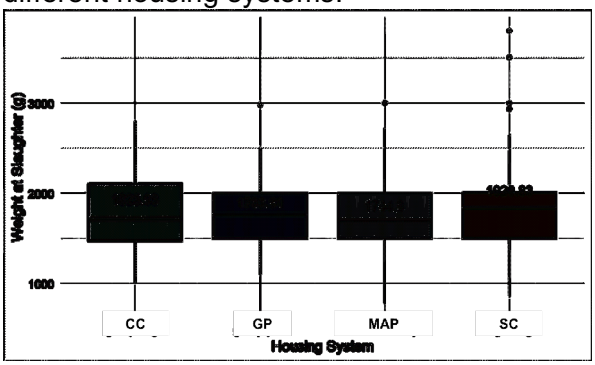


Figure 4: Variation in LW at slaughter in the different housing systems.



CONCLUSIONS

Rabbit farming in Amoronimania region is still traditional and constitutes a secondary activity, it promotes gender equity and in fact is mostly represented by female owners. Rabbit breeding represent the best source of income for people in rural areas, even if their housing system is still traditional, and rabbits are raised especially with other animals directly on the floor which results in high mortality rate, expecially in the periweaning phase. The age at first mating is higher than that of rabbits raised in the EU as the age (>6 months) and also the age at weaning (>2months) in all suvied farms. Rabbit meat is the higher protein level of food source for people in rural areas even if rabbit's weight at slaughter is low, with better slaughter weight in S. Under a thecnichal point of view appear evident the extreme potential of development of this sector and the long-term success of rabbit farming initiatives for future research to combat famine and development in best rearing practices adapted to local resources.

ACKNOWLEDGEMENTS

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EFFECTS OF GROUP SIZE ON PERFORMANCE AND ENERGY BALANCE OF RABBITS KEPT IN A PART-TIME GROUP HOUSING SYSTEM WITH DIFFERENT LITTER SIZE

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ABSTRACT

This study aimed at evaluating the effect of the group size (3 vs. 4 does) in a part-time group housing system and the litter size (9 vs. 10 vs. 11 kits per litter) on the lactation performance and kit mortality, besides body material and energy balance of multiparous rabbit does from kindling to weaning within one reproduction cycle. A total of 72 multiparous pregnant does were used. Nine does, individually housed, were slaughtered immediately after kindling to determine the initial empty body (EB) composition. The remaining 63 does were housed in the D3 or D4 pens with 3 or 4 does/pen, respectively, and kept collectively from their arrival at the experimental farm (11 d before kindling) until 2 d before kindling. Then, the does with their kits were individually kept until 18 d after kindling and then joined again from 18 d to 33 d (weaning). After kindling, the litter size was standardized at 9 kits (L09), 10 kits (L10), or 11 kits (L11), according to a bi-factorial arrangement based on doe group size and litters size. As for the effect of group size in pens, performance of rabbit does at kindling and during lactation and litter growth were not affected, whereas body lipid balance tended to be more favourable in groups with 3 does than with 4 does (P=0.10). As for the effect of litter size, pen feed intake differed during lactation (P<0.05), and kits from L09 and L10 litters showed similar weaning weights (on average 923 g) and higher than what measured in kits of L11 litters (845 g; P<0.001). As for body balance of reproducing does, on the whole, L09 and L10 does showed a better body condition in comparison with L11 does.

Key words: group housing, litter size, body balance, weaning rabbits, lactation.

INTRODUCTION

Part-time housing systems have been proposed with the aim to improve animal welfare of reproducing does as they increase during some time the total available space for movement and allow social contacts with conspecific of the same age, thus mitigating some welfare concerns in of the usual individual housing system such as movement restriction and inability to express positive social interactions (EFSA, 2020). However, group housing systems cannot be recommended yet in the field as they are associated to aggressive behaviors among does and towards kits which produce stress, painful and serious lesions to animals and, in the worst cases, death (Szendrő et al., 2019; EFSA, 2020; Pérez-Fuentes et al., 2022). Increasing doe group size may play a role in enhancing aggression among does (Zomeño et al., 2017), which could result in differences in lactation performance and, as a consequence, litter performance, due to disturbance to normal feeding pattern of reproducing does with their litters. On the other hand, lactation performance and body condition of does and litter growth are also dependent on litter size, which usually is standardized at 8 to 10 kits depending on the parity order of does and the production level of the genotype used (Poigner et al., 2000; Xiccato et al., 2004). High litter size determines a lower milk intake per kit and a worse doe body condition whereas the standardization of litter to low size implies the suppression of exceeding kits, which has strong ethical implications.

Thus, this study aimed at evaluating the effect of the group size (3 or 4 does) and the litter size (9, 10 or 11 kits per litter) on the lactation performance and kit mortality, besides body material and energy balance of multiparous rabbit does in a part-time group housing system.

MATERIALS AND METHODS

Animals and experimental design

A total of 72 multiparous pregnant does (Hycole crossbred) were moved from a commercial farm to the experimental farm of the University of Padova. Nine does were housed individually from their arrival and were sacrificed immediately after kindling to determine the initial empty body (EB) composition, according to the comparative slaughter technique (Xiccato et al., 1995; Xiccato and Trocino, 2020). The remaining 63 does were randomly assigned to D3 and D4 treatments with 3 or 4 does/pen (9 pens per treatment; total of 18 pens), and kept in group from their arrival at the experimental farm (11 d before kindling) until 2 d before kindling. Each pen was open-top, obtained by joining 3 or 4 individual modules of 0.5 m^2, equipped with plastic floor and platform. The modules of each pen were separated and the does with their kits were individually kept from 2 d before until 18 d after kindling; then the modules were joined again from 18 d to 33 d (weaning) according to the D3 and D4 groups. After kindling, litter size was standardized at 9 kits (L09), 10 kits (L10) or 11 kits (L11), according to a bi-factorial arrangement with 6 experimental groups obtained by the combination of doe group size and litters size: D3-L09, D3-L10, D3-L11, D4-L09, D4-L10, D4-L11 (3 replicates per experimental group). During lactation, nests were always open (free lactation).

Recordings and chemical analyses

The following measures were recorded at different times during the trial: weight of does and litters and cage feed intake, health status and body condition score for does. At weaning, the 63 does on trial were slaughtered and their empty bodies (EB) ground, freeze-dried and analysed to calculate material and energy balance, according the comparative slaughter technique, after measuring chemical composition (moisture, crude protein, ether extract and ash) by AOAC (2000) methods and gross energy contents by adiabatic bomb calorimeter.

Statistical Analysis

The data were analysed by a two–way ANOVA using the PROC GLM of SAS (2013) with the doe group size, the litter size, and their interactions as main effects. Differences among means with a $P < 0.05$ were accepted as representing statistically significant differences. Least-square means were compared using the Bonferroni test.

RESULTS AND DISCUSSION

Effect of group size

Performance of rabbit does at kindling were not affected by the doe group size in the part-time housing system (on average 13.0 born alive per does; average weight 58.3 g) (Table 1). Throughout the trial, the weight of rabbit does as well as their feed intake were similar in D3 and D4 pens as it was for the growth rate (25.8 g/d on average) and the weaning weight of kits at weaning (on average 897 g). As for body condition, at weaning, the EB of D3 does had a lower water content ($P<0.05$) and a numerically higher lipid ($P=0.10$) and energy ($P>0.10$) content in comparison with D4 does (data not reported in table). The EB balance (Fig. 1) showed a trend for a more favourable material balance in D3 vs. D4 rabbit does because of a higher fat retention (+7,1% vs −6,8%; $P=0.09$). On the other hand, protein (11,3% vs. 9,3%) and energy retention (+9.9% vs +5.6%; $P>0.10$) were not affected by the group size of does in the pens.

Previous study (Van Damme et al., 2023) using part-time housing systems from 22 d to 35 d after kindling found no significant ($P>0.05$) differences in reproductive performance of does and mortality and growth of kits housed in pens with 3 or 4 does. Under our conditions, we can hypothesize that differences in body balance between D3 and D4 does could depend on the numerically higher feed intake in the former compared to the latter.

Effect of litter size

The litter size significantly affected doe and litter feed intake (DFI) and kit weight gain (DWG). From 18 to 33 days of age, DFI was higher in L10 pens compared with L09 pens (806 vs. 746 g/d; P<0.05) with intermediate values in L11 pens (Table 1). At litter standardization, L09 kits were heavier than L11 kits with L10 kits showing intermediate values (64 g, 62 g, and 59 g in L09, L10, and L11 kits, respectively; P<0.01) (Table 2). At 18 days of age, the average weight of kits decreased with increasing litter size (+49 g from L09 to L10 kits; +26 g from L10 to L11 kits; P<0.001) as a consequence of a decreasing daily weight gain (17.5 g/d, 16.1 g/d, and 14.7 g/d in L09, L10, and L11 kits, respectively; P<0.001). Finally, at weaning, kits from L09 and L10 litters showed similar weights (on average 923 g), whereas kits of L11 litters exhibited a significantly lower weight (on average 845 g; P<0.001). In fact, during the whole lactation (0 to 33 days of age), L11 kits showed a lower daily weight gain (on average 24.2 g/d; P<0.05) compared to L09 and L10 kits (26.5 g/d on average). During the trial, kit mortality was low and averaged 3.1% without significant differences among the experimental groups. As for reproducing does, EB weight and composition at weaning (data not reported in tables) as well as material and energy balance (Fig. 1b) were little affected by litter size. On the whole, L09 and L10 does showed a trend for higher protein body retention in comparison with L11 does (P=0.10), which suggest these latter can have some problem to fully cover protein requirements for lactation.

Indeed, rabbit milk production is known to increase with the number of suckling rabbits with negative consequences on the body energy balance which impact depend on genetics of does, parity order and litter size (Fortun-Lamothe, 2006; Agea et al., 2020; Trocino and Xiccato, 2020).

Table 1: Live weight (LW)[1] of rabbit does and daily feed intake (DFI)[2] of pens with does and litter

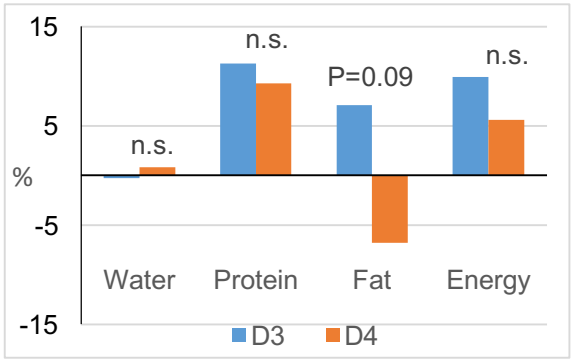
	Doe Size (D)		Litter Size (L)			Probability			MSE
	D3	D4	L09	L10	L11	D	L	G×L	
Rabbit does, n	27	36	21	21	21				
Pens, n	9	9	6	6	6				
LW at -12 d parturition, g	4938	4927	4926	4925	4947	0.903	--	--	339
LW at parturition, g	4385	4348	4360	4337	4403	0.669	--	--	343
LW at 18 d of age	5173	5097	5160	5068	5176	0.387	0.559	0.629	342
LW at 33 d of age	4883	4771	4837	4839	4804	0.240	0.944	0.787	369
DFI -12 d to 0 d, g/d	232	227	230	221	238	0.121	--	--	7
DFI 1 to 17 d, g/d	526	517	522	516	526	0.326	0.623	0.591	18
DFI 18 to 33 d, g/d	784	773	746^{a}	806^{b}	782^{ab}	0.530	0.042	0.538	36

[1]Individual data. [2]Pen data. MSE = mean square error. Means with different letters on the same row differ significantly.

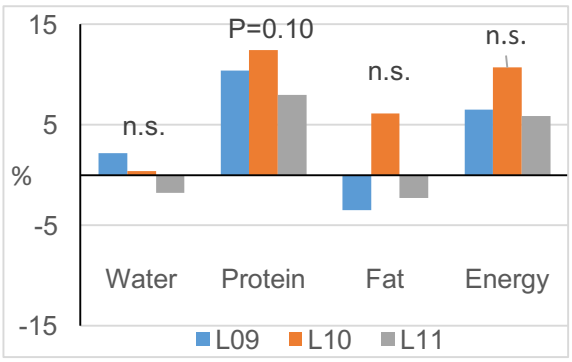
Table 2: Live weight (LW) and daily weigh gain (DWG) of suckling rabbits (pen data)

	Group Size (G)		Litter Size (L)			Probability			MSE
	G3	G4	L09	L10	L11	G	L	G×L	
Pens, n	9	9	6	6	6				
LW at 0 d, g	63	61	64^{b}	62^{ab}	59^{a}	0.139	0.009	0.374	5
LW at 18 d, g	356	347	380^{c}	351^{b}	325^{a}	0.162	<0.001	0.397	24
LW at 33 d, g	905	888	929^{b}	917^{b}	845^{a}	0.083	<0.001	0.113	119
DWG, 0-18 d, g/d	16.3	16.9	17.5^{c}	16.1^{b}	14.7^{a}	0.262	<0.001	0.542	1.3
DWG, 18-33 d, g/d	37.6	37.2	37.7	38.9	35.6	0.732	0.119	0.483	2.5
DWG, 0-33 d, g/d	26.0	25.6	26.7^{b}	26.4^{b}	24.2^{a}	0.559	0.019	0.558	1.4
Kit mortality, %	4.2	2.0	2.8	2.9	3.6	0.159	0.907	0.690	6.2

MSE = mean square error. Means with different letters on the same row differ significantly.



a)



b)

Figure 1. Material and energy balance of empty body of part-time group-housed lactating does from kindling to 33 d of trial, according to rabbit group size (a) and litter size (b). D3 and D4: 3 and 4 reproducing does per group. L09, L10, and L11: litters standardized at 9, 10, and 11 kits.

CONCLUSIONS

Increasing group size from 3 to 4 does in a part-time group housing system had no negative effects on lactation performance and kit survival, but reduced body fat retention which can be associated to changes in feed intake. On the other hand, the increase of litter size in multiparous does from 9 to 10 suckling kits did not affect kit growth and mortality and doe body balance. However, a further increase of litter size to 11 kits lowered the weight at weaning which require further evaluation of the residual effects at the end of the growth period.

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CONSUMPTION OF RABBIT MEAT IN URBAN CHINA: A STATISTICAL ANALYSIS

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ABSTRACT

Rabbit meat, characterized by its low-fat content and high nutritional value, is an excellent source of protein and micronutrients. It is particularly suited to the dietary needs of the increasingly health-conscious urban residents in China. However, the consumption of rabbit meat in urban China is uneven and relatively low compared to other types of meat. This study aims to investigate the consumption characteristics, problems, and potential solutions related to rabbit meat among urban residents in Nanjing and Chengdu, two cities with distinct dietary habits. The study is based on a questionnaire survey conducted among 222 households, 104 valid observations were collected in Nanjing and 118 observations were collected in Chengdu. The findings reveal that rabbit meat has the lowest protein unit price among all meats. This makes rabbit meat a cost-effective choice for consumers seeking to increase their protein intake while minimizing fat consumption. However, the study revealed that rabbit meat consumption was low in terms of quantity, frequency, and proportion, and exhibited strong regional differences. Particularly in Nanjing, rabbit meat consumption was significantly lower and less frequent than other types of meat. In fact, none of the households surveyed in Nanjing consumed rabbit meat more than once per month. Out of the 222 households surveyed, only 35.14% had consumed rabbit meat in the past month, while the remaining households had not. Specifically, the consumption rate of rabbit meat in Chengdu was 79.66%, whereas it was only 10.58% in Nanjing. The study also found that most of the rabbit meat consumption occurred outside of the home and in the form of processed food. This indicates a lack of demand for unprocessed and home-cooked rabbit meat. The main reasons for not consuming rabbit meat were found to be the lack of habit, dislike of the taste, and difficulty in purchasing. Even among consumers, the awareness of the characteristics of rabbit meat was low. Among the 79 households that had consumed rabbit meat in the past month, only 25% knew that rabbit meat was easy to digest, 31% knew that rabbit meat had a low fat content, and 21% knew that rabbit meat had a low cholesterol content. This reveals a gap between the nutritional and health advantages of rabbit meat and consumer knowledge and demand. In light of these findings, the study recommends promoting the consumption of rabbit meat and rabbit products through consumer education, ready-to-cook rabbit meat products, well-known rabbit meat brands. By addressing the identified challenges and leveraging the nutritional benefits of rabbit meat, it is possible to increase its consumption among urban residents in China, contributing to healthier dietary habits.

Key words: Rabbit meat consumption; Urban dweller

INTRODUCTION

As income and living standards improved in China, urban residents have a higher demand for animal products(Fu et al. 2012; Cheng et al. 2022). In China, pork intake increased from 37·1 g/d in 1992 to 64·3 g/d in 2012(He et al. 2016). Additionally, the aging population (Liu et al. 2021)and the prevalence of cardiovascular diseases (Hinton et al. 2018) pose challenges to the dietary health of urban residents in China. Recent studies have shown that consuming high amounts of red meat is strongly associated with cardiovascular and cerebrovascular diseases (Wang et al. 2022; Shi et al. 2023). While meat is the primary source of animal protein for human consumption, it is important to note that different types of meat have varying nutritional and health effects. Compared to red meat, rabbit meat offers several advantages. It is a lean meat rich in proteins of high biological values, with highly unsaturated lipids and low cholesterol

content (Dalle Zotte and Szendrő 2011).Besides, it displays a low content of sodium and a high content of phosphorus, and maybe a good source of B vitamins (Institute for Agricultural and Fisheries Research 2006). This makes it an ideal choice for urban residents seeking healthy and nutritious food. However, the consumption of rabbit meat in urban China is relatively low and uneven, and there are many challenges in the market development. Therefore, it is of great practical significance to explore the factors that influence rabbit meat consumption among urban residents in China.

MATERIALS AND METHODS

Data

This study was based on a survey of urban residents in Nanjing and Chengdu, two cities with different dietary habits and economic development levels. Nanjing is the capital of Jiangsu Province, which is located on the southeastern coast of China. The region is economically developed, but rabbit meat is not commonly consumed. Chengdu, the capital of the southwestern inland province of Sichuan, has a long history of rabbit consumption, accounting for 60% portion of the country's annual rabbit consumption according to statistics from the Department of Agriculture and Rural Affairs of Sichuan. The survey aimed to collect a total of 240 observations, with 120 from Nanjing and 120 from Chengdu. After removing outliers, 104 valid observations were collected in Nanjing and 118 observations were collected in Chengdu. The questionnaire consisted of four parts: the basic information of the respondents, the consumption of livestock products, the consumption of rabbit meat, and the consumption awareness and willingness of rabbit meat.

Basic Characteristics of the Respondents

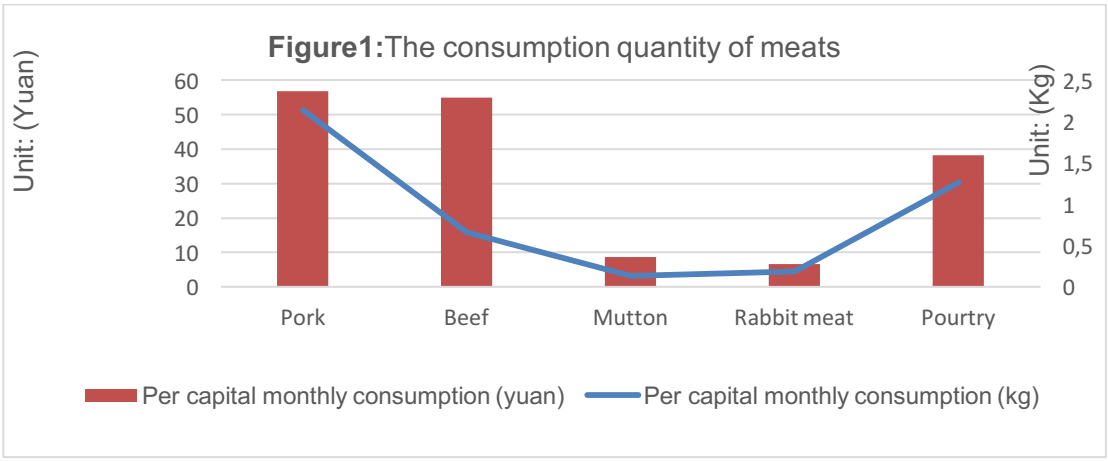
The majority of respondents worked in the private sector, with the most common occupations being company employees and private entrepreneurs or individual businesses, followed by workers or agricultural laborers, freelancers. On average, the respondents had a household size of 3.4. The study analyzed the monthly income and expenditure of the respondents, revealing that the most common income range was between 5000-6999 yuan (about 700-980 USD), while the most common expenditure range was between 3000-4499 yuan (about 420-630 USD). In terms of food expenses, the majority of respondents spent between 1000-1499 yuan (about 140-210 USD).

RESULTS AND DISCUSSION

Consumption Characteristics of Rabbit Meat

The analysis focused on four aspects of rabbit meat consumption: consumption quantity, frequency, mode and value for money of rabbit meat. The results indicate that the consumption rate of rabbit meat is low and varies significantly by region. Out of the 222 households surveyed, only 35.14% had consumed rabbit meat in the past month, while the remaining households had not. Specifically, the consumption rate of rabbit meat in Chengdu was 79.66%, whereas it was only 10.58% in Nanjing.

The consumption of rabbit meat is significantly lower than that of other types of meat, such as pork, beef, mutton, and poultry. According to the data, the average per capita consumption of rabbit meat is only 68g per month, compared to 2100g of pork, 420g of beef,150g of mutton, and 1300g of poultry. Additionally, the frequency of rabbit meat consumption varies greatly among different regions. Of the households that consumed rabbit meat in the past month, 50.68% consumed it once or less, 27.4% consumed it twice, and 21.92% consumed it three times or more. The frequency of rabbit meat consumption in Chengdu was higher than that in Nanjing, with 32% of households in Chengdu consuming rabbit meat twice or more per month, while none of the households in Nanjing did so.

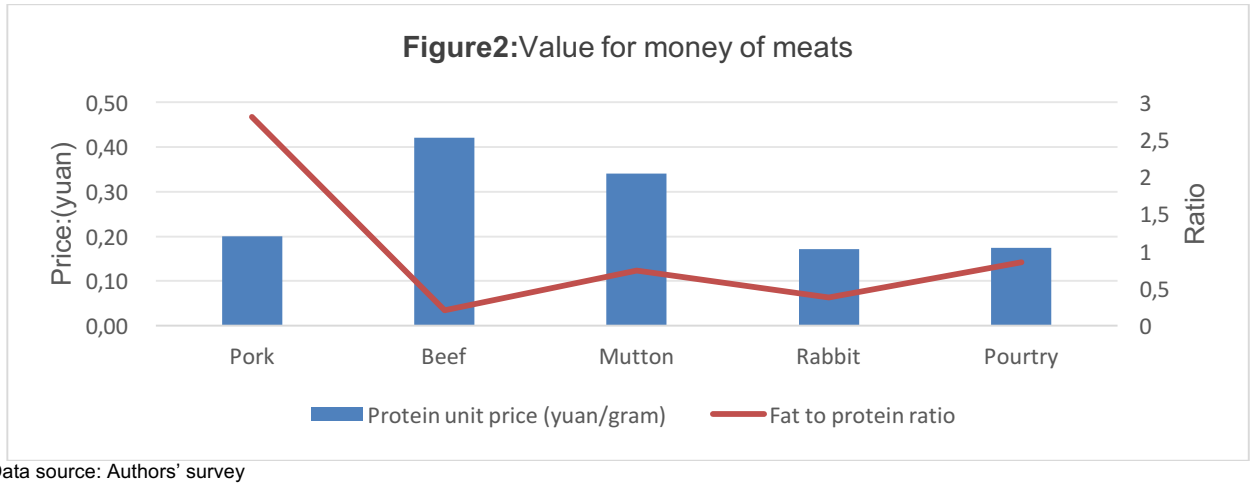


Data source: Authors' survey

The majority of rabbit meat consumption occurs outside of the home and in processed food, with insufficient consumption of unprocessed rabbit meat and home-cooked rabbit meat. Of the households that consumed rabbit meat in the past month, 43.84% consumed it outside of the home, while 56.16% consumed it at home. The primary venues for consuming rabbit meat outside of the home include cooked food shops, rabbit meat specialty restaurants, and hot pot restaurants. Meanwhile, supermarkets, farmers' markets, and online platforms are the main channels for purchasing rabbit meat for home consumption.

Advantages of Rabbit Meat.

Meat is the main source of protein intake for humans, and different types of meat have different protein contents. Pork and poultry, which have relatively low protein ratios, are generally cheaper, while beef and mutton, which have higher protein ratios, are more expensive. Rabbit meat, on the other hand, has a high protein content and a relatively low price. From the perspective of nutritional intake, consumers buy meat mainly for protein intake. Taking the price of protein intake as the starting point, rabbit meat has the lowest unit protein price. From a health perspective, some fat will inevitably be ingested while intaking protein, but excessive fat intake can easily lead to cardiovascular and cerebrovascular diseases, so the lower the fat-to-protein ratio, the better. After calculating the fat-to-protein ratio of several types of meat, the study found that beef has the lowest fat-to-protein ratio, followed by rabbit meat. Considering the cost-effectiveness of protein intake and the fat-to-protein ratio, rabbit meat is a high-quality meat that balances both cost-effectiveness and health.



Data source: Authors' survey

Consumption Problems of Rabbit Meat.

The consumption problems of rabbit meat were analyzed from three aspects: the reasons for not consuming rabbit meat, the awareness of rabbit meat characteristics, and the demand for rabbit meat characteristics. The results showed that:

The main reasons for not consuming rabbit meat were the lack of consumption habit, the dislike of the taste, and the difficulty of purchasing. Among the 143 households that had not consumed rabbit meat in the past month, 37.2% said they did not have the habit of consuming rabbit meat, 22.67% said they did not like the taste of rabbit meat, 17.4% said they could not buy rabbit meat. The awareness of rabbit meat characteristics was low, even among the consumers of rabbit meat. Among the 79 households that had consumed rabbit meat in the past month, only 25% knew that rabbit meat was easy to digest., 31 % knew that rabbit meat had low fat content, 21% knew that rabbit meat had low cholesterol content.

CONCLUSIONS

The consumption of rabbit meat is low and uneven in urban China, and shows strong regional differences. Rabbit meat consumption is low in quantity, frequency, and proportion, and is mainly based on outside consumption and processed food consumption.

The main reasons for not consuming rabbit meat are the lack of consumption habit, the dislike of the taste, and the difficulty of purchasing. The consumers have low awareness of the nutritional and health benefits of rabbit meat, and the demand for rabbit meat does not match its characteristics. The consumers do not pay much attention to the health and nutrition of rabbit meat, and the rabbit meat market lacks well-known brands and ready-to-cook products. To promote the consumption of rabbit meat and rabbit products, it is necessary to strengthen consumer education, develop rabbit meat ready-to-cook products, build well-known rabbit meat brands. These measures can help to increase the consumer awareness, preference, and accessibility of rabbit meat and rabbit products, and tap the potential of the rabbit meat market in urban China.

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ECONOMIC EVALUATION OF THE USE OF *Stylosanthes guianensis* CV CIAT 184 HAY IN GROWING RABBITS FEED

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ABSTRACT

The objective of this work was to study the economic viability of the use of *Stylosanthes guianensis* (*Stylo*) hay, as a source of fiber, in the diet of growing rabbits. To do this, four foods containing 0, 10, 20 and 30% of *Stylo* (for *Sg*0, *Sg*10, *Sg*20 and *Sg*30 respectively) were manufactured and distributed randomly and a*d libitum* to four batches of 12 rabbits (623 ± 29 g) on a period of 8 weeks, from 35 to 91 days of age. Measurements of feed intake, growth and consumption index, carried out throughout the feeding period, made it possible to calculate economic parameters. As a result, the price of feed increased by 18 F CFA with the incorporation of up to 30% of hay. Similarly, total feed costs were 16% higher (P<0.001) for feeds containing 20 and 30% *Stylo* (*Sg*20 and *Sg*30 respectively) compared to the feed without *Stylo* (*Sg*0). However, the effectiveness of feeds with 20 and 30 of *Stylo*, in terms of rapid growth of rabbits and carcass yield, made it possible to achieve higher income per kg of meat produced compared to the control feed. However, the incremental cost and relative benefit revealed a saving of 29.75 F CFA (4.21% benefit) with the *Sg*20 food against an additional expense of 3.52 F CFA (0.5% loss) with food *Sg*30 when compared to the control food *Sg*0. Thus, feed with 20% *Stylosanthes guianensis* hay, which has enabled rabbit production at lower costs, turns out to be the economically viable feed to promote in farms.

Key words: *Stylosanthes guianensis* hay, rabbit, feed price, production cost, economic profitability

INTRODUCTION

The rabbit is an ideal animal to meet the increasingly important protein needs in developing countries. It is renowned for its high prolificacy, its ability to valorize several raw materials into meat of high biological value, and has a relatively low incidence of epidemic diseases compared to other farm animals. Despite these advantages, the development of rabbit farming is hampered by its strong dependence on traditional imported inputs for food production (Kra *et al.*, 2022; Sangaré *et al.*, 2022).

Increasingly, forage resources are exploited for their excellent nutritional value in order to increase the performance of rabbits, but also for their favorable effects on animal health (Safwat et al., 2015; Kimsé et al., 2017). *Stylosanthes guianensis* (Fabaceae) has, for this purpose, been used as a source of fiber for rabbit production. Its hay, incorporated into the pelleted feed at levels of 30%, ensured rapid growth of rabbits, preserved their health, and improved carcass yields, as well as the nutritional quality of the meat (Kouadio et al., 2021; Kouadio et al., 2023). However, the economic aspect of the valorization of this plant material has not yet been the subject of study.

Therefore, the objective of this work was to evaluate the economic viability of using *Stylosanthes guianensis* hay in rabbit feed.

MATERIAL AND METHODS

This economic study was carried out on the basis of the zootechnical performance results obtained in the trial conducted by Kouadio *et al.* (2021). Rabbits were fed pelleted diets containing increasing levels of *Stylosanthes guianensis* hay, as the primary fiber source, for 8 weeks, from 35 to 91 days of age. All price data was expressed in CFA francs (F CFA).

Location and animals

The biological test was carried out in a building at the experimental farm of the Higher School of Agronomy of National Polytechnic Institute Félix HOUPHOUËT-BOIGNY of Yamoussoukro - Ivory Coast, subject to a natural atmosphere (T°: 27 °C, H: 80%).

In total, forty-eight rabbits of local hybrid breeds, male and female, with an initial age of 35 days and average weights of 613, 614 g, 613 g and 615 g, were divided into four batches of 12 per diet, and randomly assigned to one of the experimental groups (*Sg*0, *Sg*10, *Sg*20 and *Sg*30). The rabbits were housed in individual metal digestibility cages (70 x 60 x 55 cm), arranged in a compact battery, until 91 days of age. At the end of the feeding phase, the animals had an average final weight of 2 048 g; 1 923 g; 2 253 g and 2 318 g respectively.

Experimental diets and feeding management

The diets were formulated to meet the needs of the growing rabbit, with similar crude protein contents of 17.5% and lignocelluloses (ADF) of 19%. The foods contained, as the main source of fiber, *Stylosanthes guianensis* (*Stylo*) hay, incorporated at increasing levels of 0, 10, 20 and 30%, i.e. *Sg*0, *Sg*10, *Sg*20 and *Sg*30 respectively. Each batch of rabbits received one of the four pelleted feeds *ad libitum* until the end of the experiment, at the age of 91 days. Water was always available.

Composition and costs of diets

The composition of the feed and the prices of the ingredients are presented in Table 1. The price per kg of each pelleted feed was calculated based on the level of incorporation and the price of the ingredients that compose it. Thus, the prices per kg of *Stylosanthes guianensis* and *Pennisetum purpureum* hay were estimated based on the expenses incurred for the establishment and maintenance of the fields, and the related expenses for hay production. As for the other raw materials, their price on the local market at the time of the experiment (year 2020) was considered. To these various expenses was added the cost of granulation, taking into account the costs of labor, energy used and the depreciation of infrastructure and granulation equipment.

Data collect

Feed ingestion was calculated from daily weighings of the quantities of food distributed and refusals. Growth monitoring was carried out using weekly weighing of the rabbits. The feed conversion was calculated by making the ratio between feed intake and the weight of the rabbits.

Economic analysis

The total feed cost (TFC, F CFA) was calculated by multiplying the total quantity of feed consumed (IA) over the trial period by the price of the feed. The cost price of the weight gain was obtained by multiplying the weight gain by the cost per kg of rabbit. here estimated at 3 250 F CFA (Kra *et al.*, 2022). Net income per kg of weight gain was determined by subtracting the cost price of weight gain, the total cost of power.

Economic efficiency was calculated as the ratio of net income to total feed cost. As for the differential cost, it was obtained by the difference between the food cost per kg of weight gain of the control food and that based on *Stylosanthes guianensis* hay. Finally, the relative benefit

was determined as the ratio between the incremental cost of the feed based on *Stylosanthes guianensis* hay and that of the control feed.

Table 1. Cost per ingredients and composition of diets

Ingrédients (g/kg DM)	Cost/kg	*Sg*0	*Sg*10	*Sg*20	*Sg*30
Stylo (stems and leaves)	87.03	0	100.0	200.0	290.0
Pennisetum purpureum (leaves)	25.43	290.0	190.0	90.0	0
Wheat bran	120.00	240.0	244.0	242.5	237.0
Soybean meal	370.00	105.0	106.0	108.5	114.0
Cottonseed meal	250.00	125.0	120.0	117.0	116.0
Corn grains	160.00	194.0	194.0	196.0	197.0
Cane molasses	60.00	20.0	20.0	20.0	20.0
Salt (NaCl)	200.00	10.0	5.0	5.0	5.0
Shellfish	500.00	10.0	10.0	10.0	10.0
Coccidiostats and Histomonostatics[1]	500.00	2.0	2.0	2.0	2.0
Prémix[2] 0.4%	1 100.00	4.0	4.0	4.0	4.0

[1]Coccidiostats and Histomonostatics: Diclazuril® (Carrier: calcium carbonate) ; [2]Mineral and vitamin composition: A: 2 500 000 IU/kg; D3: 250 000 IU/kg; E: 12 500 mg/kg; K3: 375 mg/kg, B1: 500 mg/kg; B2: 500 mg/kg; Calcium D-pantothenate: ; B5: 1 000 mg/kg; B6: 200 mg/kg; B12: 1.14 mg/kg; PP: 2 500 mg/kg; Choline Chloride: 45 000 mg/kg, Biotin: 5 mg/kg, Folic Acid: 200 mg/kg,: Calcium : 187 g/kg, NaCl (Salt): 400 g/kg, Sodium (Na): 154 g/kg, Magnesium (Mg): 21 198 mg/kg, Copper (Cu): 2 000 mg/kg, Iron (Fe): 12 500 mg/kg, Zinc (Zn) : 12 500 mg/kg, Manganese (Mn): 5 000 mg/kg, Iodine (I): 75 mg/kg, Selenium: 25 mg/kg, Cobalt (Co): 25 mg/kg; citric acid: ; propyl gallate: (Carrier: calcium carbonate, magnesium oxide)

Statistical Analysis

The analyzes were carried out with R software version 4.0.0 (R Development Core Team, 2022). The data obtained were subjected to analysis of variance with diet as the main source of variation. Comparisons of means were made using the Tukey test (5%)

RESULTS AND DISCUSSION

The price per kg of feed (Table 2) increased by 18 F CFA from feed without *Stylosanthes guianensis* hay (*Sg*0) to feed with 30% hay (*Sg*30). This increase in price with the incorporation of *Stylo* can be explained by the higher price of *Stylosanthes guianensis* hay compared to that of *Pennisetum purpureum* hay (approximately 3 times). However, food prices in this study were lower than those of foods commonly used in Ivory Coast, whether locally produced foods (250 CFA francs) or imported foods, varying from 400 to 500 F CFA (Kra *et al.*, 2022). The low price per kg of feed obtained in this study confirms previous observations according to which the use of local resources, to replace conventional raw materials, makes it possible to reduce the costs of feed and production of rabbit meat (Safwat *et al.*, 2015). In addition, the greater consumption of foods with high levels of *Stylo* (*Sg*20 and *Sg*30) led to total feeding costs higher than that of the control food without *Stylo*. But, the efficiency of the feed with 20 and 30%, having favored the development of the edible fraction of the rabbits, made it possible to generate greater net income (+686 F CFA, $P<0.001$) compared to the control feed.

The economic efficiency, although similar for the groups fed with the *Sg*0, *Sg*20 and *Sg*30 feeds, indicates that the profit generated by the sale of rabbits was 3.8 times higher than the dietary burden for the *Sg*20 feed, compared to 3.6 times for feed with 30% *Stylo*. In addition, the analysis of the incremental cost revealed that the production of rabbits with the *Sg*30 feed generated greater expenses on feed (+3.52 F CFA) compared to the use of the *Sg*0 control feed while Savings of 29.75 F CFA were made with the *Sg*20 food. Thus, a relative benefit of 4.21% was achieved for 1 kg of meat produced with *Sg*20 while a loss of 0.5% was observed with the *Sg*30 feed. These results can be explained by the fact that the feed with 20% *Stylosanthes guianensis* hay was consumed less than that with 30% hay to produce similar performances (FC=4.05; $P<0.001$) in the two batches of rabbits (Kouadio *et al.*, 2021). The

*Sg*20 feed with 20% *Stylo*, which recorded low production costs compared to the feed containing 30% *Stylo* (*Sg*30), is positioned as the economically viable feed for a rabbit farm (Gidenne *et al.*, 2017).

Table 2. Production index values of rabbits fed diets containing increasing levels of *Stylosanthes guianensis* hay

Parameters	Experimental feeds				SEM[1]	*P* value
	Sg0	***Sg10***	***Sg20***	***Sg30***		
Live weight at day 35, g	628	627	628	628		
Live weight at day 91, g	2 048[b]	1 923[c]	2 254[a]	2 319[a]	20.13	<0.001
Total weight gain, kg	1.44[b]	1.31[c]	1.64[a]	1.70[a]	0.02	<0.001
Total feed consumption, kg/rabbit	6.35[b]	6.24[b]	6.62[a]	6.78[a]	0.07	<0.001
Feed conversion	4.51[b]	4.84[a]	4.03[c]	4.07[c]	0.07	<0.001
Price per kg live weight, F CFA	3 250	3 250	3 250	3 250		
Price per kg of feed, F CFA	156.48	161.24	167.71	174.54		
Cost of weight gain, F CFA	4 663[b]	4 257[c]	5 334[a]	5 539[a]	71.95	<0.001
Total cost of feed, F CFA	993[c]	1 005[c]	1 110[b]	1 183[a]	11.07	<0.001
Net income per kg of meat produced, F CFA/kg	3 670[b]	3 252[c]	4 224[a]	4 356[a]	67.69	<0.001
Economic efficiency, %.	370.08[a]	323.25[b]	380.84[a]	368.43[a]	6.31	<0.001
Cost per kg of weight gain, F CFA/kg	706.40[b]	781.13[a]	676.65[b]	709.92[b]	10.78	<0.001
Incremental cost, F CFA/kg	-	+74.73	-29.75	+3.52		
Relative profit per weight gain	-	+10.58	-4.21	+0.50		

[1] SEM. : Standard error of mean
[a, b, c] : Averages with different letters on the same line differ significantly (*P*<0.05)

CONCLUSION

The use of *Stylosanthes guianensis* (*Stylo*) hay as a raw material source of fiber has made it possible to produce pelleted and rabbit feed at low costs. Compared to the feed with 30% hay, the feed containing 20% *Stylo* made it possible to achieve benefits in terms of feed. It appears to be the most economically viable food based on *Stylo*.

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RABBIT RESEARCH TRENDS IN AFRICA: LESSONS AND IMPLICATIONS FOR FUTURE RESEARCH DIRECTIONS

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ABSTRACT

The objective of the study was to evaluate rabbit research trend in Africa with a view to drawing lessons and implications for future research directions. A total of four hundred and eleven (411) research articles from 1984 to 2023 were retrieved from World Rabbit Congress Proceedings and World Rabbit Science Journal. Data which included country of research, year, rabbit breed, and core area of rabbit research used were extracted from each article. Data were analyzed using the FREQ procedure of SAS and the output were presented as bar chart and pie chart. The most predominant countries were Egypt, Nigeria and Algeria that accounted for 38, 22 and 20% of all studies evaluated, respectively. For the decadal trend, there was a progressive increase in proportion of rabbit studies evaluated from the first decade (1981-1990) to the last complete decade (2011-2020) considered. The highest proportion (44.1%) of rabbit studies were conducted between 2011-2020 while the least was conducted between 1981-1990. Furthermore, more than 50% of all rabbit studies analyzed (56%) were conducted between 2011 and 2023. Based on the type of rabbit breeds used in the studies, standard rabbit breeds accounted for 38% of all rabbit studies evaluated and were the most predominant breed type. In addition, crossbreds, local breeds and synthetic breeds accounted for 27%, 24% and 11%, respectively, of all rabbit studies evaluated. Overall, New Zealand White which is a standard breed was the most predominant rabbit breed used (36.5%) in all studies. Based on the core area of rabbit research, more than half of all studies evaluated were either in the area of rabbit nutrition (30.0%) or rabbit physiology & reproduction (27.9%). Furthermore, studies on applied rabbit breeding and genetics accounted for about 21% of all rabbit studies while research on rabbit products and value addition accounted for about 11%. Conversely, core area of rabbit research with lower proportion of studies included rabbit health & pathology, production economics and rabbit housing and welfare with 5.5%, 3.8% and 1.4%, respectively, of all studies evaluated. The increasing interest and investment in rabbit research in Africa suggest a recognition of the importance of rabbits in the agricultural landscape, likely driven by their potential for food security and income generation. Furthermore, future studies should prioritize the characterization and utilization of indigenous rabbit breeds for the enhancement of genetic diversity and promotion of local breeds conservation.

Key words: Rabbit, R&D, trend analysis, Africa, breeds.

INTRODUCTION

Across Africa, rabbits are widely raised under backyard, low-input systems especially in sub-Sahara Africa. The most predominant countries in terms of stock numbers are Egypt, Nigeria, Algeria, Sierra Leone and Rwanda with about 6.2, 4.9, 1.7, 1.5, and 1.3 million rabbits, respectively (FAO, 2021). Rabbits play multiple integral roles in the economy and livelihoods of inhabitants of these countries. They have high genetic diversity which makes them reservoir of important genetic materials. They have been identified as key animal genetic resource for bridging animal protein deficiency prevalent in many African countries (Mailafia et al., 2010). Further, rabbits can be utilized for sustainable wealth creation, gender empowerment, enterprise development and poverty alleviation in developing countries (Abu et al., 2008). However, research emanating from Africa on rabbit is low compared with other livestock such as poultry, pigs, cattle and goats (Oseni and Lukefahr, 2014). Therefore, there is a need for a comprehensive evaluation of the current trend in rabbit research in Africa. Such evaluation could lead to identification and prioritization of research needs and gaps, as well as the formulation and implementation of effective and evidence-based policies and interventions. It could also identify capacity building and strengthening needs and guide decisions on future

research directions and policy recommendations. Therefore, the objective of this study was to evaluate rabbit research trend in Africa with a view to drawing lessons and implications for future research directions.

MATERIALS AND METHODS

Literature search

We conducted a comprehensive literature search on rabbit research presented at World Rabbit congresses (http://world-rabbit-science.com/) from 1984 to 2021 and published in World Rabbit Science Journal (https://polipapers.upv.es/index.php/wrs/issue/archive) from 1993 to 2023. A total of four hundred and eleven (411) articles on rabbit research and development in Africa were retrieved. Data extracted from each article were location of study (country), year of research, rabbit breed used, and type of R&D (nutrition, physiology, products and value addition, breeding, etc). Microsoft Excel was used for data management.

Statistical Analysis

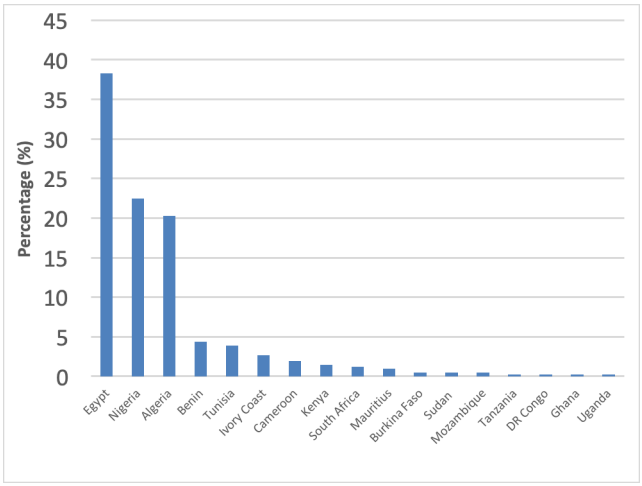
Data were analyzed using the Frequency procedure (percentages, ranges, bar-chart and pie-chart and cross-tabulation) of SAS® (2009) and was presented as bar and pie charts.

RESULTS AND DISCUSSION

Distribution of rabbit studies based on the country of research

Figure 1 shows the distribution of rabbit studies based on the country of research. Three clearly distinguishable clusters can be identified from the Figure. The first cluster represented the most predominant countries which are Egypt, Nigeria and Algeria that accounted for 38, 22 and 20% of all studies evaluated, respectively. Cumulatively, these countries accounted for about 80% of studies evaluated. The second cluster represented other notable countries such as Benin (4.4%), Tunisia (3.9%), Ivory coast (2.7%), Cameroon (1.9%), Kenya (1.5%) and South Africa (1.2%). Cumulatively, these countries accounted for about 16% of all studies analyzed. Furthermore, the third cluster of countries which included Mauritius, Burkina Faso, Sudan, Mozambique, Tanzania, DR Congo, Ghana and Uganda that cumulatively accounted for about 4% of all studies evaluated. The trend for the distribution of rabbit research in Africa seems to follow the FAO (2021) trend for the distribution of African countries in terms of rabbit stock numbers. FAOSTAT data showed that Egypt, Nigeria and Algeria are the top three African countries, in that order, in terms of stock numbers. These countries, also in that order, were found to have the highest number of rabbit studies in Africa from the present study. Other top rabbit producing countries from FAO (2021) data with notable rabbit research outputs include Kenya, Mauritius, Cameroon and Mozambique. Therefore, the dominance of Egypt, Nigeria and Algeria in rabbit research and development outputs could be due to higher stock number and therefore, the relative increased availability of rabbits as experimental animals.

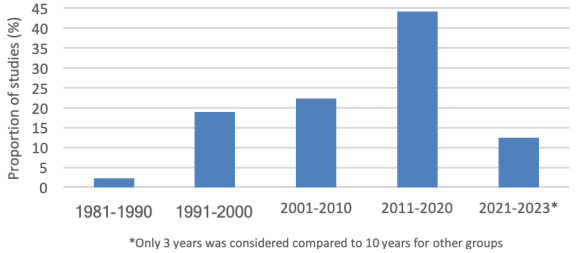
Figure 1: Country distribution of rabbit studies in Africa



Distribution of studies based on decadal and half-decadal trend

Figure 2 shows the decadal distribution of rabbit studies from 1981 to 2023. The highest proportion (44.1%) of rabbit studies was conducted between 2011-2020 while the least was conducted between 1981-

Figure 2: Decadal distribution of rabbit studies in Africa from 1981-2023

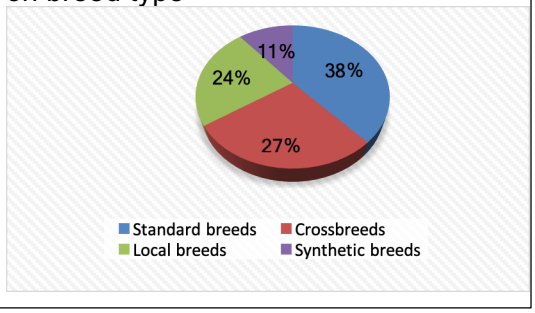


1990. Furthermore, more than 50% of all rabbit studies analyzed (56%) were conducted between 2011 and 2023. The progressive increase in the number of rabbit studies from 1981 to 2023 lends credence to the fact that interest in rabbit a veritable and alternative meat source in Africa is on the rise (Krupová et al., 2020; Siddiqui et al., 2023). The progressive increase in proportion of studies could be an indication of increased investment in rabbit research and development in Africa. This could be due to increasing interest in rabbit as a meat animal (Dairo et al., 2012) and its role in ensuring food and nutritional quality (Johnson et al., 2024). Furthermore, Mutsami and Karl (2020) reported that rabbit meat is fast becoming a widely accepted meat in Africa and there is a gradual shift from subsistence to commercial units.

Distribution of studies based on breed type

Figure 3 presents the distribution of studies evaluated based on the type of rabbit breeds used in the study. Standard and globally recognized rabbit breeds which included the New Zealand White, Californian White, Chinchilla, Dutch, Flemish Giant, Pannon White, Rex, Blue Vienna, Flander and Simmonoire) accounted for 38% of all rabbit studies evaluated and were the most predominant breed type. Crossbreds which were obtained through specific or non-specific crossing between two or more rabbit breeds accounted for 27% of all studies evaluated. Furthermore, local and synthetic breeds accounted for 24% and 11% of studies, respectively.

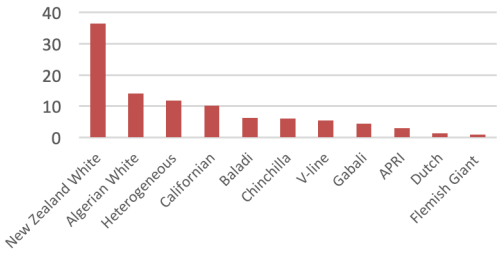
Figure 3: Categories of studies based on breed type



Distribution of studies based on rabbit breed

The overall or pooled ranking of rabbit breeds used for research and development intervention in all studies analyzed is presented in Figure 4. Overall, New Zealand White which is a standard breed was the most predominant rabbit breed used (36.5%) in all studies evaluated followed by Algerian White which is a popular local rabbit breed in Algeria. Other notable breeds include heterogeneous rabbits (11.81%) which are non-specific products of crossing, Californian White (6.3%), Baladi (5.4%) which is a popular local Egyptian rabbit breed and Chinchilla (5.2%).

Figure 4: Overall ranking of rabbit breeds used

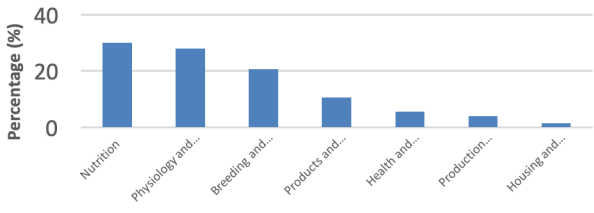


The New Zealand White rabbits were the most predominant standard rabbit breed, demonstrating its popularity as a standard and favourite rabbit breed in Africa. New Zealand White are genetically diverse (Li et al., 2020) and highly adapted to the tropics (Odubote and Akinokun, 1994). Over several decades, heterogeneous rabbits have been subjected to random, non-directional and non-specific mating between local and exotic rabbit breeds (Oseni and Lukefahr, 2014). Lukefahr (1998) reported that heterogeneous rabbits are commonly found in developing countries. These heterogeneous stocks are highly adapted to the hot and humid environmental conditions prevalent in the tropics. Further, they have good survival rates, high fertility and prolificacy under sub-optimal, backyard management systems (Oseni and Lukefahr, 2014).

Distribution of studies based on research area

Figure 5 presents the distribution of studies analyzed based on the core area of rabbit research. More than half of all studies evaluated were either in the area of rabbit nutrition (30.0%) or rabbit

Figure 5: Categories of studies based on core research area



physiology & reproduction (27.9%). Furthermore, studies on applied rabbit breeding and genetics accounted for about 21% of all rabbit studies while research on rabbit products and value addition accounted for about 11%. Conversely, core area of rabbit research with lower proportion of studies included rabbit health & pathology, production economics and rabbit housing and welfare with 5.5%, 3.8% and 1.4%, respectively, of all studies evaluated.

Lessons and implications for future research

The increasing interest and investment in rabbit research in Africa suggest a recognition of the importance of rabbits in the agricultural landscape. This likely driven by their potential for enhancing food security, income generation and supporting livelihoods. In addition, the prominence of standard breeds in research highlights the need for further exploration of local breeds to diversify genetic resources and enhance adaptation to local environments. Further, there is an urgent need to broaden research scope to include neglected areas like health, pathology, production economics, and welfare. Based on this, it is suggested that future studies should prioritize the characterization and utilization of local rabbit breeds. This can enhance genetic diversity, promote local breed conservation, and support smallholder farmers who rely on these breeds. There is also the need to invest in capacity building and strengthening programmes for smallholder rabbit farmers and researchers. This will facilitate the dissemination of research findings, promote adoption of best practices, and ultimately contribute to the development of a vibrant rabbit industry in Africa.

CONCLUSIONS

The study indicates a growing trend in rabbit research in Africa over the past few decades, with significant contributions from countries like Egypt, Nigeria, and Algeria. The predominant focus areas include rabbit nutrition, physiology, and reproduction. Standard rabbit breeds, particularly New Zealand White, were the most frequently used. In addition, there is also a notable proportion of research dedicated to nutrition and physiology, with less emphasis on areas like rabbit health, production economics, and housing/welfare.

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CHARACTERISATION OF SMALLHOLDER RABBIT PRODUCTION IN ANDALUSIA (SPAIN)

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ABSTRACT

Although rabbit farming in Spain is mostly carried out under intensive systems, small-scale rabbit farming still survives in rural and peri-urban areas. In this context, this study characterised small-scale rabbit farming in Andalusia (southern Spain) by surveying 27 rabbit keepers in 2022 throughout a structured questionnaire. On average the smallholder rabbitries were founded 13.2 years ago, 55.6% were located in the province of Seville and 92.6% were owned by men. The smallholder rabbitries in Andalusia had a very small breeding stock (1.9 bucks and 3.9 does; doe-to-buck ratio: 2.1) with a mostly stable (51.9%) or growing (33.3%) trend of the breeding stock in the last five years. The most common genetic types reared were Spanish common agouti-coated rabbits (33.3%), wild rabbits (33.3%) and crossbred rabbits (48.1%). All rabbitries fed the animals with commercial mixed balanced feed and 51.9% of them, in addition, supplemented it mainly with forage. In 70.4% of rabbitries, does were mated only between autumn and winter, to avoid summer heat stress. The first mating of the does took place at an average age of 6.3 months, does were re-mated 13.1 days postpartum and abdominal palpation was performed 11 days after mating. The reproductive lifespan of breeder rabbits lasted on average 3.5 years (2.5 years more frequently). The average litter size at birth was 8.4 kits per litter. Two-third of the breeders vaccinate rabbits against myxomatosis and 37% also vaccinate against viral haemorrhagic disease. Only 51.9% of the breeders marketed the rabbits they produced. In conclusion, in Andalusia smallholding rabbit farming still survives as an amateur activity for self-consumption and to obtain supplemental income.

Key words: Backyard farming, Pet rabbit, Rabbit meat, Livestock systems, Husbandry.

INTRODUCTION

In Spain, rabbit farming experienced, starting in the 1970s, an intense and rapid process of transformation from family and rural breeding systems towards an intensive production model (Roca, 2009). This led to the disappearance of many micro-farms towards the 1990s and the progressive loss of rabbit keeping for self-consumption and under alternative rearing systems. However, in certain areas of the country, small-scale rabbit farming still survives, aimed at various purposes, such as self-consumption of its meat, the sale of pets, the breeding of purebred rabbits and others (González-Redondo, 2014). Small-scale rabbit farming in developing countries is mainly aimed at food security and protein supplementation in the household diet (Oseni and Lukefahr, 2014). However, in developed countries with intensive rabbit farming, small-scale rabbit farming is more oriented to supplementing income and to amateur rabbit breeding. Andalusia is a Spanish region with little implementation of intensive meat rabbit farming, but small-scale rabbit farming still survives in rural and peri-urban areas (González-Redondo, 2010). However, this type of rabbit farming is quite unknown in this region because it has never been the subject of research.

In this context, this work aims to characterise small-scale rabbit farming in Andalusia (Spain), in relation to structure and size of the rabbitries, animal husbandry and marketing of the products.

MATERIALS AND METHODS

Sample selection and field work

The field work was carried out between February and May 2022, by surveying 27 rabbit keepers anonymously, voluntarily and with informed consent. They were located in the 8 provinces of Andalusia (southern Spain) by searching for contacts through the Internet and personal contacts.

Survey design

The survey, arranged as a structured questionnaire, was designed after selecting the questions through a bibliographic search on the subject. The survey questions covered the main aspects related to the age and size of the farm, the genetic types of rabbits kept, the facilities, reproductive, nutritional and health management, as well as technical management and marketing.

Statistical Analysis

The mean, standard error, mode, minimum and maximum were calculated for quantitative variables. For categorical variables, the number and percentage of rabbitries showing the attribute were calculated. The statistical analyses were performed using SPSS v.15.0 (SPSS Inc., 2006).

RESULTS AND DISCUSSION

Ownership, geographical distribution and age of the smallholder rabbitries

Of the 27 Andalusian backyard rabbitries surveyed, 92.6% (n=25) were kept by men and the rest (n=2; 7.4%) by women. The proportion of male owners of backyard rabbitries was higher than the proportion of male owners of rabbit farms (of all productive orientations) registered in Andalusia in 2021, where 61.8% were owned by men, 15.5% by women and 22.7% by legal entities (IECA, 2021). These rabbitries were distributed in 26 municipalities in the eight provinces of Andalusia, with the highest concentration in the province of Seville (n=15; 55.6%). On average the rabbitries were founded 13.2±1.22 years ago, ranging between 3 and 23 years.

Rabbitry size, animal base and productive orientation

The smallholder rabbitries in Andalusia have a very small breeding stock (1.9 bucks, 3.9 does; Table 1), in line with the fact that none of them are officially registered as farm. These are considered by the Royal Decree 1547/2004, which establishes regulations for the management of rabbit farms, as a self-consumption farm, whose maximum census of breeding does must be less than or equal to five and that does not market its production (MAPA, 2004). The number of does per buck (2.1) is very low because when the rabbitry is very small, there is always an excess of males compared to females to have a reserve of males for mating, and because all rabbitries used natural mating, for which more bucks are needed to mate a certain number of females, compared to farms that use artificial insemination (Lebas et al., 1997). The trend in the last 5 years, regarding the size of the breeding stock of smallholder rabbitries in Andalusia, has been to increase in 33.3% (n=9), to remain constant in 51.9% (n=14) and to decrease in 14.8% (n=4) of them.

Table 1: Number of breeding rabbits and sex ratio in smallholder rabbitries in Andalusia (n=27)

	Bucks	Does	Sex ratio (does/buck)
Mean ± standard error	1.9±0.15	3.9±0.49	2.1±0.14
Minimum	1	2	1
Maximum	5	15	4
Mode	2	4	2

The rabbitries reared a diversity of breeds and phenotypes (Table 2). Spanish common agouti-coated domestic rabbits and wild rabbits predominate. Several rabbitries kept rabbits of foreign breeds (Fauve de Bourgogne, Californian, New Zealand White and Butterfly) and only one rabbitry kept the native Spanish Giant breed. In some of the rabbitries, rabbits without a defined

breed were kept, with black or grey coat colours. In almost half of the rabbitries, crosses were carried out between breeds, with commercial and fancy and pet rabbits as well as with wild rabbits. All farms raised rabbits for meat (n=27; 100%), 14 of them (51.9%) raised pet rabbits and 7 of them (25.9%) sold rabbits to train hunting dogs.

Table 2: Breed and genetic types of rabbits kept in the smallholder rabbitries of Andalusia (n=27)

Breed	N (%)	Breed	N (%)
Spanish common agouti-coated	9 (33.3)	Grey coat	2 (7.4)
Wild rabbits	9 (33.3)	Black coat	1 (3.7)
Fauve de Bourgogne	2 (7.4)	New Zealand White	1 (3.7)
Californian	2 (7.4)	Spanish Giant	1 (3.7)
Butterfly	2 (7.4)	Crossbreed	13 (48.1)

Feeding

All backyard rabbitries in Andalusia (n=27) fed the animals with commercial mixed balanced feed, in pelleted form and supplied *ad libitum*. Of them, 48.1% (n=13) used it as the only feed and the rest (51.9%; n=14) supplemented it with forage (fresh, hay or horticultural remains) or additionally with bread (Table 3). Only one rabbitry also supplemented the commercial feed with cereal grains.

Table 3: Supplementary feeds to the mixed feed used in backyard rabbitries in Andalusia (n=27)

	N (%)
Forage (fresh, hay, horticultural remains)	9 (33.3)
Forage (fresh, hay, horticultural remains) and bread	5 (18.5)
None	13 (48.1)

Reproduction

Most of the rabbitries housed the breeders in individual cages (92.6%; n=25). Only 29.6% (n=8) of small-scale rabbit keepers in Andalusia mated breeding rabbits throughout the year, while the remaining 70.4% (n=19) mated them in the period comprised between autumn and winter, avoiding the summer due to the heat stress (González-Redondo, 2014). Table 4 shows the main variables related to the management of mating and abdominal palpation in smallholder rabbitries. In one of them (n=3.7%) the breeding rabbits were free housed in groups and mating occurred spontaneously without control. Abdominal palpation was performed by 40.7% (n=11) of the breeders. The first mating of the does took place at an average age of 6.3 months, during the reproductive life the mating was carried out at 13.1 days postpartum and abdominal palpation was performed on average 11 days after mating. The reproductive lifespan of breeder rabbits ended at an average age of 3.5±0.18 years, varying between 2 and 5.5 years and with a most common age of 2.5 years.

Table 4: Variables of mating and pregnancy diagnosis in Andalusian smallholder rabbitries (n=27)

	Age at first mating (months) (n=27)	Interval birthing-mating (days) (n=26)	Abdominal palpation (days from mating) (n=11)
Mean ± standard error	6.3±0,20	13.1±2.79	11.0±0.67
Minimum	5	1	7
Maximum	8.5	60	14
Mode	5.5	7	10

Nest, birth, lactation and weaning

The materials used for the does to make the nest were predominantly cereal straw (n=18; 66.7%), followed by wood shavings (n=6; 22.2%) and hay (n=3; 11.1%). 29.6% (n=8) of the breeders placed the nest box between 2 and 4 days before the expected birth date (more frequently 3 days before), while the remaining 70.4% (n=19) took the decision of when to place

the nest box by observing when the doe began to prepare to give birth. The average duration of lactation in backyard rabbitries in Andalusia was 31.6±1.4 days, varying between 21 and 45 days and with a more frequent value of 30 days. Lactation was always carried out with free access for the doe to the nest box. The average number of kits in the 21 rabbitries (77.8%) that reported their value was 8.4±0.44 kits born per litter, varying between 4 and 11 and with a most frequent value of 8.5 kits.

Health prophylaxis

A third of the breeders did not vaccinate, while the rest (n=16; 66.7%) all vaccinate against myxomatosis and 37% also vaccinate against viral haemorrhagic disease.

Technical management

Technical management data were recorded only by 55.6% (n=15) of small scale rabbit breeders. All of them recorded reproductive data and 8 of them (29.6%) also recorded productive data. One breeder selected does with greater receptivity to mating and greater prolificacy. Another of the breeders, in addition to selecting the most productive does, selected heavier rabbits.

Marketing

Sixteen breeders (59.3%) reported on the final destination of the rabbits they produced: 7.4% (n=2) of the total did not sell them but gave them as gifts and exchanged them with neighbours and friends, while 14 breeders (51.9%) marketed the rabbits. A quarter of the rabbit keepers (25.9%, (n=7) advertised their animals on advertising websites and in 51.9% of the cases the clients came to the rabbitry to purchase the rabbits. Half of backyard rabbit breeders (55.6%, n=15) reported that the meat-oriented rabbits they sold were bought alive by customers or slaughtered and eviscerated by the breeder on demand.

CONCLUSIONS

In conclusion, in Andalusia small scale rabbit farming still survives as an amateur activity carried out mainly for self-consumption of the meat and to obtain supplemental income for the breeder. The rabbitries are of very low size, rear several breeds (many of them rustic) by using natural mating and fed the rabbits *ad libitum* with commercial mixed balanced feed. Although not officially registered as farm, more than half of the small scale rabbitries in the region sell alive or slaughtered rabbits. Further research could investigate future prospects of this type of farming.

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CAREER ASPIRATIONS AND ENTRY PATHS INTO RABBIT FARMING

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ABSTRACT

This study aimed to understand why rabbit farmers enter the profession and the career aspirations of current and potential farmers. In 2023, we interviewed 21 current or potential rabbit farmers in France (each for ca. 3 hours, with or without farm visits) whose farms represented a variety of systems. We also observed 12 potential farmers during a training session for project leaders of organic rabbit-farming systems. We performed thematic analysis of full transcripts of interviews using Nvivo software. Data analysis identified three main paths for establishing a rabbit farm: i) taking over rabbit farming within the family, ii) creating a new farm or iii) a career change. Detailed understanding of the mechanisms and paths of entry into the rabbit-farming profession, as well as the justifications given by farmers, enabled us to identify 14 career aspirations, which were integrated into a serious game (Insta'Lap) designed to help design rabbit-farming systems.

Key words: aspirations, professional path, rabbit, farmers

INTRODUCTION

The 2020 agricultural census in France showed that more than 66% of farmers are over 50, and ca. 25% are over 60 (Agreste, 2022). Since 2010, the number of rabbit farms in France has decreased by 46%. This decline could increase over the next few years, as some farmers retire and find it difficult to find new owners for their farms. At the same time, the rabbit-farming sector is facing two major challenges: i) decreasing consumption of rabbit meat and ii) public demand for more animal-welfare-friendly farming. These challenges are profoundly challenging the sector's dominant production methods – cages – and are undermining the entire sector.

In recent years, the French rabbit-farming sector has been committed to changing its practices, such as by decreasing the use of antibiotics and developing tools for progress (e.g. Charter of Good Farming Practices, training of animal-welfare specialists; Travel et al., 2023). However, in this critical context, redesign of rabbit-farming systems seems necessary (Hill and MacRae, 1995). New systems have emerged in recent years and are currently being tested or developed (Gohier et al., 2023; André et al., 2023). These approaches, supported by the conventional sector, coexist alongside an alternative form of outdoor rabbit farming, which has been developing and federating a network of ca. 50 farmers since the 2010s, with or without organic certification (Roinsard et al., 2016, Gidenne et al, 2022). These systems are presented as being potentially more attractive to young farmers, as they would help to improve the image of the profession, particularly in terms of animal welfare. But what is the reality?

To contribute to the redesign (and attractiveness) of rabbit-farming systems, we felt it essential to examine the reasons, hitherto little explored, that motivate individuals to enter the rabbit-farming profession. While the factors that influence farmers to adopt innovative animal-welfare practices are now better known (Chiron et al., 2022), little is known about the professional aspirations of farmers and how they enter the rabbit-farming profession. The present study is in line with this perspective. The study thus aimed to understand why individuals enter the rabbit-farming profession, as well as the justifications, reasons for satisfaction and expectations (for potential rabbit farmers) they have of the profession.

MATERIALS AND METHODS

The farmers interviewed and systems

We performed qualitative sociological interviews of 21 current or potential rabbit farmers in France in 2023. The farmers were both men (n=14) and women (n=7). The systems of the farmers interviewed were deliberately varied and did not represent the true distribution of system types in the sector: barns (n=6), barns with outdoor access (n=1) and/or outdoors (n=12), the last of which were either organic (n=7) or not (n=5). Finally, two individuals interviewed were planning to establish a rabbit farm. The farms were located throughout France.

We used the biographical interview method (Bertaux, 2016), which involves collecting life stories based on a central question posed to respondents (i.e. “How and why did you become a rabbit farmer?”). Most of the interviews, which lasted a mean of 3 hours, were performed face-to-face (n=14) and were accompanied, when possible, by observations of the farm during a visit (n=10). They were supplemented by observations of 12 potential farmers who were involved in a 3-day training session for project leaders of organic rabbit-farming systems.

Data analysis

The interviews were audio-recorded and transcribed in full. We then performed thematic analysis (Paillé and Mucchielli, 2012) of the transcripts using Nvivo software (Lumivero, Denver, Colorado, USA) following the principles of grounded theory (Glaser and Strauss, 1967).

RESULTS AND DISCUSSION

The data analysis enabled us to identify three entry paths into rabbit farming and 14 professional motivations.

Establishing a rabbit farm

The three main paths for establishing a rabbit farm were the following:

i) Taking over a family rabbit farm

Five farmers followed this path, which reflected the phenomenon of social reproduction: rabbit farming was the continuation of a specific primary socialization (Bourdieu, 1972) that led these individuals to spend their childhood behind the scenes of rabbit farming, mainly with their mothers. The fact that they "took over" and that this takeover appeared "natural", particularly when their parents retired, partly obscured the need to reflect on technical decisions, as these farmers "inherited" both technical (e.g. barns, material production configurations) and cognitive (e.g. knowledge of rabbit-breeding management) systems that predated them.

ii) Creating a new farm

Seven farmers followed this path, and their main characteristic was the desire to establish a farm after training, whether they came from a farming background or not. They had all showed a strong interest in animal farming (i.e. cattle, goats or sheep), but never specifically rabbit farming. Rabbit farming was largely unknown to them, especially in the absence of specific training (the “Specialization Certificate” was discontinued in France in 2001). For some of them, establishing a rabbit farm was a "disappointing" move because it only partially fulfilled their expectations: they had the satisfaction of becoming farmers, but not of the animals they had initially envisioned. In the absence of specific training, the decision to establish a rabbit farm (whether a barn or outdoor system) was always driven by a third party (e.g. agricultural adviser, cooperative technician, another farmer), but also to overcome certain constraints inherent in establishing a farm (e.g. high cost of land).

iii) Rabbit farming as a career change

Eight farmers had had a professional career outside the agricultural sector before establishing a rabbit farm. For them, establishing an animal farm was a "professional break" (Denave, 2006) and a "biographical bifurcation" (Bidart, 2006). Farming thus appeared as a possible path to a new career (e.g. following assessment of skills, due close ties with the farming world in social circles). They expressed an interest in raising small animals, and the size of rabbits seemed to be an argument to legitimize their career change, making it appear feasible to those outside the agricultural sector. Most decided to develop small rabbit farms, mostly outdoors (7 of 8), often with organic certification, and combined with other professional agricultural or rural activities (e.g. a countryside holiday cottage). Finally, they had higher professional qualification (level III, II or I) than farmers of the other two paths did. This path can be compared to that of certain executives who seek an interesting topic or activity in their work that corresponds to their personal aspirations (Jourdain, 2014). In these cases, rabbit farming made it possible to reconcile several aspirations in relation to a previous career path that served as a point of comparison. As for the previous path, individuals often entered the profession via a third party.

Professional aspirations of rabbit farmers

Investigating the reasons of rabbit farming has enabled us to identify the fundamental motivations behind this choice. The professional aspirations given for becoming rabbit farmers varied greatly depending on the type of system (e.g. barn vs. outdoors) and entry path (Table 1). This was not surprising given the obvious differences in the tasks required by indoor vs. outdoor rabbit farming. We then thought it would be relevant to consider these motivations as potential aspirations for futures farmers and that could be an essential dimension to integrate into their support. After a work session with rabbit farming specialists and futures rabbit farmers, we rework the first motivational list to ensure it reflects the diversity of breeders and farming systems.

Table 1: List of professional and personal aspirations for rabbit farmers

Professional aspiration	Professional aspiration
Work indoors	Be able to develop several skills
Work outdoors	Have work that requires technical skills
Balance professional and personal lives	Establish at a lower cost
Have the freedom to organize the work	Establish with little land
Have a planned job	Respect animal welfare
Be self-sufficient (in inputs, decision making, technology)	Produce a product that meets consumer expectations
Control the selling price	Have a profitable business

We used the diversity of these aspirations to build a serious game (Insta'Lap) designed to help design rabbit farms. Aspirations are a central mechanism of the game because players must choose their aspirations before they can make technical choices. The aim of the game is to enable players to reflect on the ability of the rabbit-farming systems they establish to meet their aspirations.

CONCLUSIONS

This study enabled us to highlight three types of entry paths into the rabbit-farming profession that were characterized by distinct relationships with the animals, types of systems and professional aspirations. Detailed understanding of the mechanisms and paths of entry into the rabbit-farming profession, as well as the justifications given by farmers, could be used in strategies to support change within the sector and to meet the challenge of generational renewal of farm ownership.

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Autodesarrollo comunitario desde una economía feminista a través de la cunicultura.

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ABSTRACT

Despite family dynamics and responsibilities within the family, Cuban rural women are limited in their ability to work in the livestock sector. With the project "Support for the socioeconomic development of the CCS Camilo Cienfuegos, Mayabeque", financed by Norwegian Popular Aid, executed by ACPA Mayabeque, they are offered the opportunity to join agricultural activities from their own homes, to obtain economic benefits and improve the diet of their families by bringing to the table a protein of high nutritional value. Through a participatory rural diagnosis and various surveys, it was determined that women were not involved in livestock activities in the Valle del Perú community, as well as a marked economic dependence on their husbands, and that many women were willing to try to enter the livestock world. Therefore, the objective of this work was to provide productive and reproductive information obtained in rabbit farms managed by empowered women, using non-conventional foods. Through the financing of the project, resources are provided to 40 women in the community who are starting out in rabbit breeding, with the use of local resources to make unconventional mixtures based on rabbit feeding; At the end of the project, among other results, it is expected to have sensitized more than 95% of the cooperative members to gender issues and incorporated 40 women into the private production process with the circular economy. At least 12 tonnes of live rabbits will be produced every year.

KEYWORDS: Rural women, rabbit farming, empowerment, cooperative, community development, circular economy.

INTRODUCTION

With the support of collaborative projects, resources have been acquired to enhance agricultural work on the island and increase production, restore livestock facilities, reestablish grazing areas, and awareness of gender issues has been achieved, promoting equity with the empowerment of women.

Rabbit farming is an activity that can contribute to the production of food in an economical way and without the use of products or other foods that compete with human nutrition. It is a livestock farm that is easy to manage but requires careful attention. In this sense, the objective of this work was to provide productive and reproductive information achieved in rabbit farms managed by empowered women, using non-conventional foods.

This work takes place in the Camilo Cienfuegos Credit and Services Cooperative (CCS), located in the San José de las Lajas municipality, Mayabeque Province, Cuba; This cooperative benefits from a local development project, financed by Norwegian People's Aid (APN).

MATERIALS AND METHODS

Observations and measurements were made in three small production farms, benefited by the project and managed by women, in the Valle del Perú community, San José de las Lajas municipality, Mayabeque province, Cuba. The breeding system used is the traditional one, the feeding of the rabbits is guaranteed with local resources, mainly harvest residues, collected in neighboring farms. the formulation of non-conventional mixtures used was elaborated by the own producers, guaranteeing a nutritional balance with an adequate proportion between fiber, protein and energy. The raw materials used were: Soya flour; Harina de Tithonia; Palmiche flour, Trigo salvado, Maní cascara flour, Maize flour, Blood flour, Boniato forage, Megathyrsus maximus, Bejuco de glycinea, Oro azul (Phyla nodiflora) and Canavalia flour.

In this opportunity, alternative foods were used in the four selected farms, to determine the influence of the offered food on reproductive and productive behavior: Fertility; Mortalidad en los gazapos; Mortality in onions; Finished Production; average peso; onion edad; conejos cebados por reproducora; Kg obtained by the reproducer.

RESULTS AND DISCUSSION

The 40 female producers count with finished ships and employ local resources for animal feed, through non-conventional mixes, with excellent and varied reproductive and productive results.

Through the project, forage seeds were facilitated to all producers to increase their forage areas, who adopted the varieties that they estimated to be the most attractive and appropriate, taking into account the knowledge acquired in the training process promoted by the project; las mujeres que no possessen áreas para tal fin, se apoyan en otros cooperativistas, collective área de la cooperativa y además utilizán residuos de cosecha e industria (blood flour, slaughterhouse residue as a source of protein).

Productive data was taken from 10 percent of the producers who developed this activity in the framework of the project, in this opportunity four producers, selected at random, who use different local sources of food to make non-conventional mixes, as part of their economy circular, en pos de la sustainability de su actividad ganadera; These raw materials can be observed in figure 1, where you can see that there are some coincidences in the use of raw materials, but there are also particularities, such as the husk of Canavalia seed flour, cassava flour, Sangre flour, harina de palmiche, bejuco de boniato, para citar algunas de las más relevantes.

Figura 1: Alimentos utilizados por las productoras para mezclas no convencionales

Productora 1	**Productora 2**	**Productora 3**	**Productora 4**
food composition	food composition	food composition	food composition
Maize meal	Maize meal	Carnavalia semilla flour	Soya flour
Blood meal	Blood meal	Tithonia meal	Tithonia meal
Sesame flour	Sesame flour	Sesame flour	Sesame flour
Salvado de trigo	Salvado de trigo	Yucca flour	Salvado de trigo
Soya flour	Soya flour	Salvado	Palmiche meal
Tithonia.meal	Tithonia meal	Palmiche meal	Maiz meal
Forraje :	Forraje :	Forraje :	Forraje :
Glycine	Glycine	Bejuco de boniato	Bejuco de boniato
Guinea	Guinea	Glycine	Glycine
Oro azul	Oro azul	Guinea	Guinea
Caña	Caña	Oro azul	Oro azul

All producers have the necessary equipment for the production of the rabbit, and all of them receive the necessary training and the constant accompaniment of a specialist in this activity.

The production system used is traditional, to protect the reproductive rabbits; each producer has 10 adult rabbits and a stallion, the productive results are varied and could be attributed to the different food sources employed for non-conventional mixtures used in the feeding of rabbits.

As you can see in figure 2, the Kg produced by breeders in a year are relevant, in the case of breeder 3, it achieves 74 Kg/ breeder, with 80% fertility, achieving the highest number of births per year, 51 parthenic In the same way, production 4 achieves 82% fertility, but only achieves 21 Kg of production per breeder, this can reduce the average sales weight of 1 Kg, as part of its strategy to decrease the occupation time of the cages and decrease the food cost

40 produceras de conejos are doing an excellent job, after the first epidemic of hemorrhagic septicemia of the rabbit in September 2022 that caused the death of more than 80% of the rabbit mass in the scenario of the project, today there is a tendency to recover in a successful way, with the effort of the producers and the accompaniment of the project team, which recently supported the procurement of vaccines to establish a vaccination cycle for the canine mass to protect the animals against possible outbreaks of the previously mentioned epidemic. El 100% of the producers count with the basic mass of reproducers provided by the project 10 females and one stallion, the 65% that was severely affected by the epidemic are on the way to stabilize their productive process, from the same way they are increasing the productions, reporting until the end of December 2023 values superior to 6 400 Kg of rabbit en pie, obteniendo ingresos en el orden de los 745 000 cup por este concepto, además del familiar consumption of proteins with high biological value, como es el case de la carne of conejo

Figura 2: Producciones obtenidas año 2022

Indicadores	**Productora 1**	**Productora 2**	**Productora 3**	**Productora 4**
Breeders	10	10	10	10
Sementales (cabezas)	1	2	1	2
Fertility (%)	70	70	80	82

Cubriciones (un)	60	50	64	50
Parthos	42	35	51	41
Nacimientos por parto(Cabezas)	7,2	9,4	7,8	7,6
Total births	303	328	399	310
Mortality in los gazapos	30	17	25	49
Mortality in los gazapos (%)	10	5	6	5
Destetados (cabezas)	273	311	374	293
Mortality in Ceba (Cabezas)	21	18	23	19
Mortality in Ceba (%)	8	6	6	7
Producción Terminada (cabezas)	252	293	351	274
Producción Terminada (kg)	529	674	737	631
Peso Promedio (kg)	2,1	2,3	2,1	1
Edad de la ceba (días)	94	91	92	33
Conejos cebados / reproductora	25	29	35	27
kg / reproductora	53	67	74	21

The work of gender is priority in our project, this year two meetings were held with the support of the subject matter in the province and the faculty of Social Sciences and Humanities at the Agrarian University of Havana UNAH.

The cooperative today has a total of 82 women associated as producers, of which 60 are involved in the productive process. In addition, 40 women producing conejos are incorporated(Editado)
Recuperar original

CONCLUSIONS

Los resultados evidenciaron que se pueden obtener buenos reproductivos y producivos con la especie cunicola, con el empleo de resources locales en la alimentación de los conejos, en mezclas non convenciales, principale con harina de soya, harina de Tithonia, harina de Palmiche, harina of peanut shell, corn flour, blood flour, boniato forage, Megathyrsus maximus, glycinea bejuco, Oro azul (Phyla nodiflora) and canavalia flour; también evidence que la mujer puede encontrar en la cría de conejos una viable alternative para desarrollar su propia economia y su inserción plena en la actividad ganadera.
It is recommended to carry out more in-depth studies, where you can establish levels of inclusion and influence in reproductive and productive indicators.

SOCIO-ECONOMIC IMPACTS OF MODIFICATIONS TO RABBIT FARMING SYSTEMS TO IMPROVE ANIMAL WELFARE

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ABSTRACT

Consumers are increasingly animal-welfare-conscious and critical of indoor caged-housing systems such as those used in rabbit farming. To help systems transition, we assessed socio-economic consequences of modifying the dominant French commercial production system (670 females, single-batch system with 42-day reproduction rhythm, wire-mesh dual-purpose housing providing 3420 cm²/♀ and 489 cm²/fattener with no enrichment and placed indoors) designed to improve rabbit welfare. We studied the impacts of 22 changes aimed at making rabbits express behaviour or providing them with a more comfortable living environment. The results showed that the changes had impacts, at a null, low, moderate, high or very high level on investment (n=4, 10, 2, 1 and 5, respectively), working time (n=8, 9 1, 3, 1 and 5), production cost (n=0, 16, 4, 1, 1) or income (n=1, 7, 2, 0 and 12). The changes aimed at providing more space for animals had the most important consequences. The selling price of live rabbit should therefore increase to maintain the farmer's income (n=1, 17, 2, 1, 1) but consumers' willingness to pay is uncertain. Significantly improving the welfare of rabbits on farms requires combining several changes. Thus, better meet societal expectations about rabbit welfare seems possible only through significant public financial support or the development of innovative systems based on other economic models.

Key words: rabbit, welfare, modelling, economy, investment

INTRODUCTION

Consumers are increasingly animal-welfare-conscious and critical of indoor caged-housing systems such as those used in rabbit farming. In the European Union (EU), more than 90% of all rabbits raised for meat production live in wire cages indoors (Szendrö et al., 2019) which restrict the expression of behaviour. Because of the European Citizens' Initiative on 'End the Cage Age', the EU has committed to ban caged animal farming. This transition represents a major economic challenge, as rabbit-production units currently have low profitability, and changing them requires new investment. Modelling can help assess socio-economic consequences of modifying existing animal-production systems (Cadéro et al., 2020). Thus, we developed a model of a rabbit farm that represents fixed and variable costs, performance and the farmer's work to estimate the farm's economic results and potential income.

The objectives of this study were to assess socioeconomic consequences of basic changes made to rabbit housing to improve animal welfare. The changes modelled were chosen along with stakeholders of the French rabbit-production sector (e.g. breeders, farmers, nutritionists, geneticists, veterinarians) and two welfare-oriented non-governmental organisations as part of a participatory research project (Living Lab Lapin project).

MATERIALS AND METHODS

The reference system and initial data

The reference system corresponded to the dominant of French production systems in terms of i) management (artificial insemination 10-11 days after kindling, weaning at 35 days), ii) housing (wire-mesh dual-purpose housing with no enrichment of 3420 cm²/♀ and 489 cm²/fattener) inside a building and all-in/all-out management), iii) management (automated feeding and manure management) and iv) sizing in 2021 (670 ♀) . The performance values

used corresponded to the 25% top performing French rabbit farms (Table 1). The data for investment in buildings (207 €/m²; 1020 m² for two breeding rooms), housing (77 € for one dual-purpose housing) and livestock equipment (i.e. ventilation, silo, manure scraping, slurry pit; 121 €/m²) were adapted from the Pays de la Loire region (CRA Pays de la Loire, 2017). The data for variable costs came from France's network on technical and economical reference for rabbit farming (Lalaurette, 2020). The cost of feed was provided by expert opinion (258 €/t). Investments described above are still being amortised (48 €/♀/year of amortization).

Individual changes to the reference system

The modelling consisted of assessing the cost of 22 individual changes (no. 1-22) to the reference system with the aim of improving animal welfare. The changes aimed to allow rabbits to express behaviours that were not, or rarely, expressed in the reference system, or to make them more comfortable. We assessed the following changes (targeted improvement indicated in brackets) i) within the cages : addition of wood or gnawing blocks (gnawing behaviour); a platform (social isolation + vertical jumping); nest covers, PVC tunnel or burrow (hiding); ii) to the housing system : more space (4300 or 5500 cm² for ♀ + 550 or 800 cm² for fatteners) or lower density (25, 30 or 40 kg/m²; ease of movement); longer housing for females (1.8 m long; jumping); group size during fattening (>12 or >20; social interactions); roofless (standing up); plastic mesh floor (20%, 40% or 80% of the floor); iii) to the building : adding artificial light simulating light transitions or natural light (window area equivalent to 3 or 5% of the floor area; being more comfortable).

Table 1: Performance of the reference system modelled

Characteristic	Value
Number of females (♀)	670
Kindling rate (%)	84.6
Total number of. total rabbits	11.1
Stillbirth rate (%)	4.8
Number of weaned rabbits	9.0
Number of rabbits sold/kindling	8.4
Live weight at sale (kg)	2.53
Total rabbit weight sold (kg /♀/year)	158
Seizure + downgrading rate (%)	2.6
Feed-conversion ratio	3.14
Selling price (€/kg alive)	1.91

Modelling principles

We assessed consequences of an individual change in each of the following parameters: i) investment in the building (e.g. decreasing density requires more space), housing (e.g. roofless housing: 82 €/place), livestock equipment (e.g. plastic mesh), or materials (e.g. gnawing block holder, light dimmer, PVC tunnel; which have little or no amortization, unlike livestock equipment); ii) performance (e.g. increasing group size increases the rate of sanitary seizures by 100%, feed conversion ratio by 6%, and fattening mortality by 40%); iii) variable costs (e.g. water and energy use is proportional to building and/or housing area); iv) working time (that required to clean and monitor the animals is proportional to building area). We assumed that i) energy consumption was divided equally between the reproduction and fattening units; ii) energy costs were divided among ventilation (31%), natural gas (30%) and lighting (21%) (among others) according to Menini et al. (2016) and iii) total water consumption was divided among watering (53%), cleaning (19%) and cooling (28%) according to Zened et al. (2013). Results per performance were classified by the degree that each worsened compared to that of the reference system: null (Z; 0%), low (<5%; L), moderate ([5-10%]; M), high (]10-20%]; H) or very high (>20%; VH).

RESULTS AND DISCUSSION

Of the 22 changes studied, only 8 influenced investment in buildings (n=2 and 5 at a M or VH level, respectively), housing (n = 2, 1 and 4 at a L, H and VH level, respectively), livestock equipment (n = 2 and 5 at a M or VH level, respectively) or materials (n = 7 ranging from 1 690 €-12 409 €; Table 2). The changes aimed at providing more space for animals by increasing the area (no. 8-11) or reducing density (no. 15-17) cost the most. Most changes studied had little or no effect on working hours. However, 4 changes (no. 9-11 and 17) had a high or very high effect on working time, due to more time necessary for monitoring animals and cleaning

the housing and building. Warin et al. (2023) found that changing rabbit housing could not only modify working hours, but also influence work ergonomics, sometimes requiring wider movements if the housing was larger or handling of heavier equipment, such as platforms or nest covers. All of the changes studied increased production costs including labour (n = 15, 4, 1 and 1 at a L, M, H or VH level, respectively), except for the platform. All of the changes studied decreased the potential income, except for implementing a lighting transition, which increased it by 323 €/year due to switching from light bulbs to LEDs, which use less electricity. This decrease in income was usually very high (n=12) and could even result in negative income (no. 10-11). For 17 changes, the selling price required to maintain the farmer income increased at a low level (no. 1-8; 12-14; 16-22), but sometimes the increase was moderate (no. 9 and 15), high (no. 10) or very high (no. 11: +40%). Heise and Theuvsen (2017) found that consumers' willingness to pay for animal products is determined by a variety of socio-demographic, personal and product-specific characteristics. No such studies have been performed for rabbit meat. However, as consumption of rabbit meat is already sharply declining, we assume that a price increase of >10% would decrease it significantly.

CONCLUSIONS

Some individual changes made to the dominant French rabbit-breeding system, such as a lighting transition, nest covers or tunnels, can allow rabbits to express some behaviours with few consequences on farm income. However, to significantly improve the welfare of rabbits on a farm, several changes should be combined to allow for expression of a diverse behavioural repertoire. Based on our results, an considering the declining European rabbit meat consumption, we believe that without a paradigm shift in rabbit-farming systems or strong public financial support, the rabbit sector will have difficulty financing the transition to farms that consider societal expectations about animal welfare. Designing innovative systems seems a more promising approach.

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Table 2: Consequences of changes in the reference system (in bold) to improve animal welfare on investment (€), working time (h/week), production costs (€) and income (€/year).

		Investment							
No.	Change	Building (€)	Housing (€)	Livestock equipment (€)	Materials (€)	Working time (h/week)	Production costs[1] (€/kg)	Income (€/year)	Selling price to maintain income[2] (€/kg)
-	**Reference system**[3]	**211 140**	**87 780**	**123 420**	**-**	**31.0**	**1.88**	**10**	**1.91**
1	Gnawing wood	211 140	87 780	123 420	-	32.0^{L}	1.93^{L}	17 459^{VH}	1.96^{L}
2	Gnawing block	211 140	87 780	123 420	12 409	31.7^{L}	1.98^{M}	12 361^{VH}	2.00^{L}
3	Platform	211 140	87 780	123 420	-	31.2^{L}	1.92	18 588^{H}	1.95^{L}
4	Nest covers	211 140	87 780	123 420	5 901	31.2^{L}	1.89^{L}	21 34^{L}	1.92^{L}
5	Tunnel	211 140	87 780	123 420	5 891	31.3^{L}	1.89^{L}	21 342^{L}	1.92^{L}
6	Burrow	211 140	87 780	123 420	11 400	31.5^{L}	1.94^{L}	16 915^{VH}	1.96^{L}
7	Roofless	211 140	143 640^{VH}	123 420	-	31.0	1.94^{L}	16 497^{VH}	1.96^{L}
8	4300 cm² fem. + 550 cm² rab.	225 630^{M}	88 160^{L}	131 890^{M}	-	31.7^{L}	1.94^{L}	16 493^{VH}	1.96^{L}
9	5500 cm² fem. + 800 cm² rab.	279 450^{VH}	105 260^{H}	163 350^{VH}	-	34.2^{H}	2.05^{M}	6 649^{VH}	2.06^{M}
10	5500 cm² fem.+1000 cm² rab.	320 850^{VH}	115 520^{VH}	187 550^{VH}	-	36.1^{H}	2.13^{H}	-243^{VH}	2.12^{H}
11	Housing 1.8 m long for fem.	755 550^{VH}	186 390^{VH}	441 650^{VH}	-	46.6^{VH}	2.75^{VH}	-59 902^{VH}	2.68^{VH}
12	Plastic mesh 20% of the floor	211 140	87 780	123 420	-	31.2^{L}	1.89^{L}	21 728^{L}	1.92^{L}
13	Plastic mesh 40% of the floor	211 140	87 780	123 420	-	31.3^{L}	1.89^{L}	21 246^{L}	1.92^{L}
14	Plastic mesh 80% of the floor	211 140	87 780	123 420	-	31.4^{L}	1.90^{L}	20 763^{M}	1.92^{L}
15	Density 40 kg/m²	223 560^{M}	91 580^{L}	130 680^{M}	-	31.6^{L}	1.91^{L}	20 048^{M}	1.93^{L}
16	Density 30 kg/m²	260 820^{VH}	103 360^{H}	152 460^{VH}	-	33.4^{M}	1.98^{M}	13 582^{VH}	1.99^{L}
17	Density 25 kg/m²	291 870^{VH}	120 080^{VH}	170 610^{VH}	-	34.7^{H}	2.05^{M}	7 482^{VH}	2.05^{M}
18	Artificial light with transition	211 140	87 780	123 420	1 690	31.0	1.88^{L}	22 534	1.91
19	Natural light 3%	211 140	87 780	123 420	-	31.0	1.89^{L}	21 942^{L}	1.91
20	Natural light 5%	211 140	87 780	123 420	-	31.0	1.89^{L}	21 327^{L}	1.92^{L}
21	Group size >12	211 140	87 780	123 420	5 250	31.0	1.94^{L}	15 563^{VH}	1.97^{L}
22	Group size >20	211 140	87 780	123 420	8 780	31.0	1.94^{L}	16 047^{VH}	1.97^{L}

[1] Including labour cost; [2] Selling price of live rabbit to the farmer; [3] See Table 1 for characteristics of the reference system
$^{L, M, H, VH}$ Low (<5%), medium ([5-10%], high ([10-20%] or very high (>20%) decrease compared to the reference system, respectively

WILL CHINA'S MEAT RABBIT FARMS HAVE ECONOMIES OF SCALE DURING THE 13TH FIVE-YEAR PLAN PERIOD?

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ABSTRACT

Based on the investigation data of meat rabbit farms from 2016 to 2020, this paper analyzes the cost-benefit of meat rabbit farms of different scales. Generally speaking, during the 13th Five-Year Plan period, rabbit breeding in China showed economies of scale to some extent. The breeding cost of meat rabbits generally shows a decreasing trend with the increase of scale, but the output value of rabbits also decreases with the increase of scale. From the perspective of net profit, the net profit of small-scale farms was the lowest, while the net profit of medium-scale farms was the highest, and the total profit level of large-scale farmers was lower than that of medium-scale farms.

Key words: meat rabbit breeding, cost-benefit, economies of scale.

INTRODUCTION

China has a very long history of rabbit breeding, but the large-scale breeding developed for the pursuit of economic value is not long. After the founding of the People's Republic of China, many provinces in China gradually regarded rabbit breeding as an industry. In terms of production scale, meat rabbit breeding in China has long been dominated by decentralized small-scale breeding. During the "13h Five-Year Plan" period, China's meat rabbit breeding has experienced great industrial adjustment, which is manifested in the decline of "quantity" and the improvement of "quality". With the increasingly fierce market competition, some small-scale households gradually withdraw from the meat rabbit breeding market in the continuous market competition, and some large and medium-sized rabbit farms continue to emerge.

Nowadays, large-scale breeding of meat rabbits has become the growth trend under the conditions of market economy. Evaluating whether rabbit industry in China has economic scale during the 13th Five-Year Plan period is of great practical significance for reducing costs, increasing farmers' income, and realizing the sustainable development of meat rabbit breeding.

DATA AND METHODS

Data

The data of this paper comes from the investigation of meat rabbit farms conducted by National Rabbit Industry Technology System Team of China in China's main meat rabbit breeding areas from 2016 to 2020, covering 17 provinces and cities such as Sichuan, Shandong, Jilin, Jiangsu, Zhejiang, Fujian and Henan. It is an authoritative and reliable data in the field of rabbit industry, with a sample size of 1103.

Methods

In order to test the economies of scale of China's meat rabbit industry during the "13th Five-Year Plan" period, this paper analyzes the cost, output value and net profit of meat rabbit breeding by statistical accounting analysis.

In the cost accounting section, the production cost and land cost constitute the total cost. Among them, the production cost consists of material and service costs (direct production costs and indirect production costs) and labor costs. Specifically, the direct production costs include the cost of young animals (young rabbits and breeding rabbits), feed costs, medical, epidemic

prevention costs, fuel power costs and water charges, etc. The depreciation of fixed assets (including the depreciation of rabbit huts, rabbit cages and machinery), taxes, management fees and insurance premiums constitute indirect production costs. Household employment and employment expenses constitute labor costs. In the labor costs, when rabbit farming is free-range, there is little labor input through employee management, and it is generally family labor.

The following methods are mainly used to process the data in the process of cost accounting: (1) When the fixed assets are depreciated, rabbit farmers generally do not distinguish between rabbit cages and rabbit huts in the cost accounting, so this paper combines the cost of rabbit cages and rabbit huts, and converts them with a service life of 20 years and a depreciation rate of 8%; The mechanical equipment fee is converted with a service life of 10 years and a depreciation rate of 12.5%; Other fixed assets are converted at a depreciation rate of 20%. (2) The land cost is the rent generated by rabbit farmers renting other people's land, excluding the occupation of their own homestead and contracted land. (3) According to the National Development and Reform Commission's Collection of Cost-Benefit Data of Major Agricultural Products in China, the labor cost is derived from the labor day wage and the number of days of employment, in which the labor day wage refers to the theoretical remuneration of each labor force engaged in animal husbandry production on a standard labor day, and is calculated according to the local standard labor day wage.

The output value of meat rabbits includes two parts, namely, the output value of main products and the output value of by-products. The output value of the main products of meat rabbits includes the income from selling live rabbits and rabbit meat; By-product output includes the income from the sale of eliminated rabbits, material products (such as rabbit manure, internal organs, etc.) and service (such as breeding, etc.).

The net profit is calculated as the output value minus the cost. It should be noted that meat rabbit farmers usually ignore the cost of household labor when estimating their costs, their net profit is lower than farmers' expectations.

As there is no authoritative classification standard for rabbit breeding scale at present, this paper uses the National Development and Reform Commission's Collection of Cost-benefit Data of Major Agricultural Products in China for reference to classify the number of broilers and laying hens, that is, the annual slaughter of meat rabbits as the measurement basis. In this paper, referring to the processing method of "Research Report on the Development of Rabbit Industry System in China", the number of meat rabbits slaughtered in the whole year less than or equal to 5000 is classified as small scale, that between 5000 and 20000 is classified as medium scale, and that above 20000 is classified as large scale, as shown in Table 1.

Table 1: Classification standard of meat rabbit scale

	Small-scale	Medium scale	Large-scale
Meat rabbits are slaughtered all year round, no.	≤ 5000	(5000 ,20000]	＞20000

RESULTS AND DISCUSSION

Total cost

As can be seen from Figure 1, from 2016 to 2020, the overall cost of meat rabbit breeding showed a decreasing trend with the increase of scale, which was in line with the theory of scale economy. Within a certain range, with the expansion of breeding scale, the average cost decreased. In 2019, the total cost of large-scale meat rabbit farms was significantly higher than the other two scales, which was mainly due to the increase in rabbit feeding prices after January 2019, and the large-scale meat rabbit farms were highly dependent on compound feeding, so they could not find alternative feed in the first time.

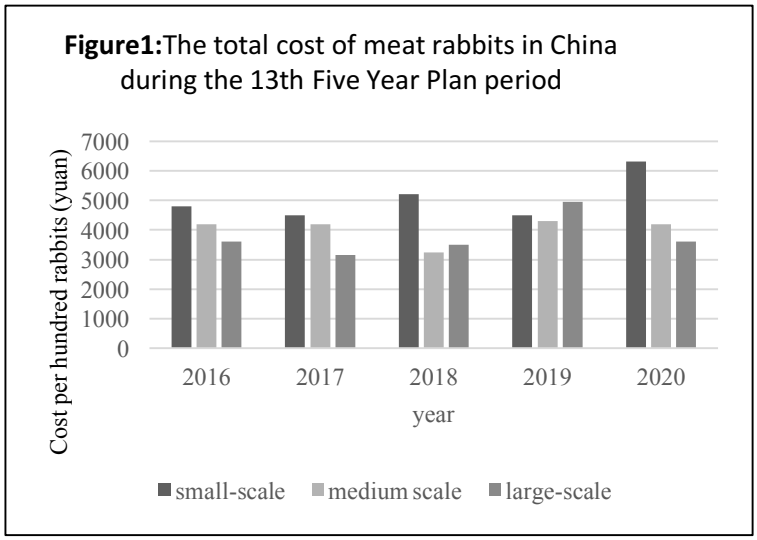

Figure1: The total cost of meat rabbits in China during the 13th Five Year Plan period

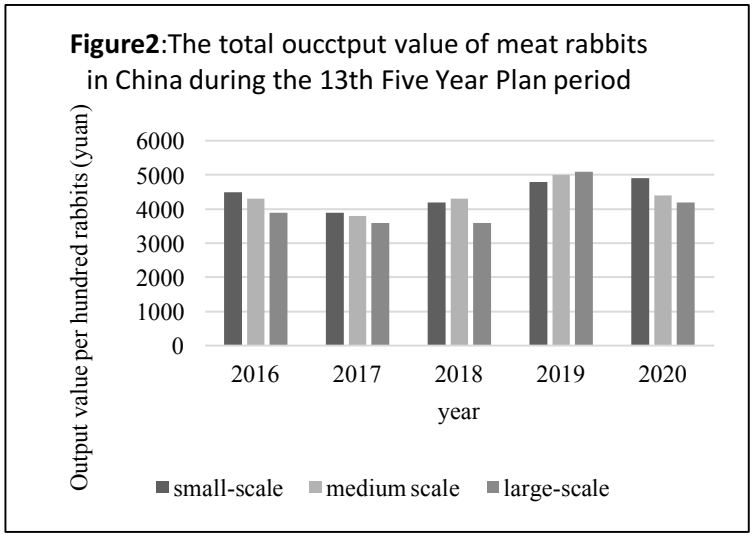

Figure2:The total oucctput value of meat rabbits in China during the 13th Five Year Plan period

Total output value

The Figure2 shows accounting result of the total output value. Overall, the output value per hundred rabbits decreases with the expansion of the scale. This may be due to the sales pressure faced by meat rabbit farmers. As the product sales increase, it will be sold at a reduced price, and the output value per 100 rabbits will also decrease. In 2019, the total output value of large-scale rabbit farms was the largest, followed by medium-scale and small-scale, which was consistent with the change of total cost in that year. In order to make up for the losses caused by higher costs, large-scale farms could only raise the selling price, so the total output value was similar to the other two breeding scales.

From 2016 to 2020, the total output value of meat rabbits showed a changing trend of first decreasing, then increasing and then decreasing. This is mainly because the average selling price of main products showed an overall upward trend in the first four years of the 13th Five-Year Plan, but it dropped significantly in 2020, probably due to the outbreak of COVID-19 epidemic in early 2020.

Net profit

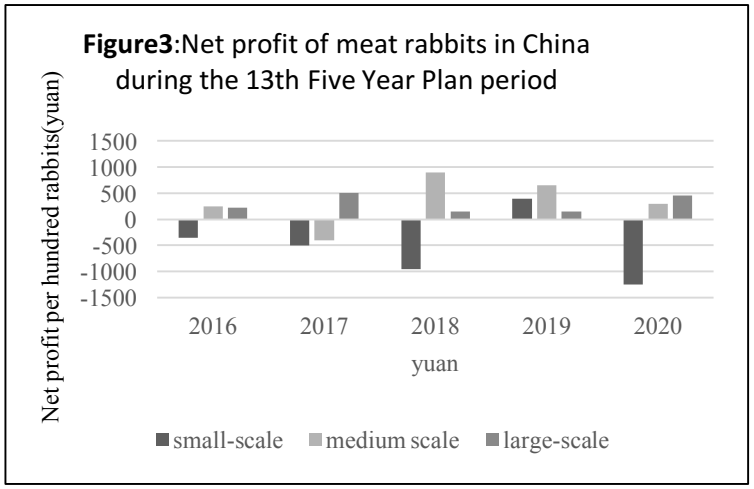

Figure3:Net profit of meat rabbits in China during the 13th Five Year Plan period

From the perspective of net profit, from 2016 to 2020, small-scale farmers were basically in a state of economic loss; Medium-scale farmers were in a state of loss in 2017, while they were in a state of profit in other years; Large-scale farmers were in a profitable state. This shows that only when the scale reaches a certain level, the meat rabbit farm will achieve profitability. From this point of view, small-scale farms may be eliminated, and some medium-scale farms may continue to expand their breeding scale, but moderate-scale operation can obtain the maximum net profit.

CONCLUSIONS

This paper makes a comparative analysis of the cost, output value and net profit of China meat rabbit industry in different scales from 2016 to 2020, and draws the following conclusions: First, moderate scale can improve the net profit of meat rabbit breeding. Through the comparative analysis of the cost-benefit of meat rabbit breeding in different scales, it can be seen that the production cost of medium and large-scale farmers is lower than that of small-scale farmers, but the net profit is greater, which indicates that there is economies of scale in large-scale meat rabbit breeding. Secondly, at present, the development of meat rabbit products still stays on the main products, and the utilization and development of by-products are far from enough. Finally, at present, the material cost and labor cost composed of feed cost account for a large proportion, which indicates that reducing feed cost and labor cost in the future will be an important source to reduce the rising cost of meat rabbit breeding.

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MISCELLANEOUS

World Rabbit Science Association

13th World Rabbit Congress – October 2-4 2024 – Tarragona, Spain – Miscellaneous Session

CHALLENGES AND OPPORTUNITIES FOR THE APPLICATIONS OF PRECISION LIVESTOCK FAMING IN THE RABBIT PRODUCTION SECTOR

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ABSTRACT

Precision Livestock Farming (PLF) is an established field in many livestock sectors. However, when it comes to rabbit production it is still emerging. However, we believe that the rapid advancements in sensor technologies, data analytics, and automation we are witnessing can bring significant and transformative opportunities to the rabbit farming industry. Within this context, this paper explores the potential use of PLF for the rabbit sector. We start by briefly reviewing the current state of the art of PLF applications in other livestock sectors, such as dairy and pig farming, focusing on remote sensing solutions. Then we outline how different technologies can potentially be adapted for rabbit production. Recent rabbit research studies that implement PLF-like technology are then reviewed. We finalise by discussing the challenges of implementing PLF in rabbit farming, including the need for tailored solutions that consider rabbits' specific behavioural and physiological characteristics. When considering the future impact of PLF, early disease detection probably offers the highest potential for rabbit production. Being able to automatically detect early signs of digestive disorders around weaning, particularly in large group-housed growing rabbits, where disease spread is a concern would represent a significant step forward. Additionally, PLF tools can enhance rabbit breeding and genetic programs by providing detailed and accurate individual phenotypic data. Data can be then used to better define animal management practices that promote positive experiences and affective states, reducing negative social interactions. Besides, precision feeding models could contribute to enhancing feed efficiency both for growing and reproductive rabbits, reducing the negative environmental impact of feeding. To this aim, camera monitoring, sound analysis, electronic feeders, accelerometers and other biometric and physiological monitoring technologies can be utilized. The integration of PLF technologies promises to support farmers in meeting the increasingly stringent welfare regulations across the European Union, ultimately enhancing the sustainability and profitability of rabbit production systems. Further research is needed to address the challenges that remain in developing and validating reliable algorithms so that sensors can be used more effectively in diverse rabbit farm conditions.

Key words: rabbit farming, precision livestock farming, remote sensing, animal monitoring,

PLF FOR RABBIT PRODUCTION – AN EMERGING FIELD

Precision Livestock Farming (PLF) represents a modern information-driven approach towards farm animal production. While technology is important part of PLF, technology should never be the core focus, this should instead be the value that PLF creates for the farmer and animal. While research in the field of PLF has been developing for over 30 years now, with many scientific publications in traditional animal production fields, there is still much work needed to confidently take this technology towards producers in many cases. In the smaller production sectors, such as rabbit production, PLF has barely made an impact to date. Nevertheless, with the rapid advancements we see in sensor technologies, data analytics, and automation along with the experience from the other production sectors, PLF in the rabbit production world has the potential for much faster development. This review seeks to give an overview of PLF and to speculate on the potential for PLF technology in the rabbit sector.

PLF centres around the collection and exploitation of data to support animal husbandry practices. In all animal production sectors, data is becoming more effectively used in the daily management of animals. Record keeping of daily farming activities has long been used by farmers to keep account of how the farming system is running. With the transition from the paper to the digital era, farmers now see more opportunities to combine and integrate data all over the farm. With many implementable affordable software solutions being available decision-making of many farmers can be significantly improved. However, these tools have a key limitation. Despite being digital, they often rely on manual inputs from the farmer (or farm workers) to keep account of what is happening. Problems such as forgetting to input data and mis-inputs regularly occur, farmers are, after all, human like us all. Furthermore, when the farmer is not around no data is being collected. The eyes, ears, and perhaps nose of the farmer are currently not represented at the times that they are not there. This is where more advanced PLF tools, which exploit sensor technologies, can make a significant impact.

Until now, PLF research and development has focused on well-established, traditional farming sectors. Rabbit farming presents unique and interesting challenges that make it an ideal candidate for a new wave of PLF developments. For example, when presented with housing-related stressors, the health and welfare of the rabbits can become quickly compromised (Trocino and Xiccato, 2006). Stress responses can be difficult to detect by workers through direct observations (Verga et al., 2007). Given the scale of modern rabbit farms, a more advanced technological approach to monitoring and managing rabbit health and welfare is therefore becoming interesting. Integrated environmental control, and automated health and welfare monitoring, are just a small list of things that have the potential to address key challenges facing the sector. Furthermore, the integration of actuators that can tailor nutrient delivery towards the specific needs of each rabbit, can reduce the environmental impact of feeding while enhancing gut health. Developing PLF technology in the short term can facilitate

pathways towards reducing the negative impacts of the production system. It will not only assist farmers in meeting increasingly stringent welfare regulations across the European Union (EU), but also the general sustainability and profitability of their operations.

In this paper, we review the technology needs of the rabbit production sector. Focusing on remote sensing solutions, we follow this with an overview of the specific advances of PLF in dairy and pig production sectors. We then explore the recent uptake of PLF technology in rabbit research and hypothesize future applications of this technology.

PRODUCTION CHALLENGES FACING THE RABBIT SECTOR FOCUSING ON HEALTH, WELFARE AND PRODUCTIVITY

The rabbit sector in Europe is rapidly changing. These changes have greatly shaped the producing segment, impacting on its sectoral structure. As in many other livestock sectors, but in this case, with a slight time delay, the rabbit farming sector has been undergoing a strong professionalization and renovation process in the last decade. This has resulted in a concentration of farms, particularly those large in size; and the disappearance of smaller, less competitive ones. This situation, however, also presents a perfect opportunity for implementing transformative innovations and improvements in the rabbit farming system.

According to European Commission (2017), new rabbit farms generally have over 1.000 does (in some cases over 2.000 does); whereas farms housing around 500-600 does were the most common in the leading producing countries (Spain, France and Italy) in Europe, the decade before. Large-sized rabbit farms require managing and supervising an increasing number of animals, with a limited workforce, in search for greater efficiency in resource utilization, profitability, as well as improved technical knowledge. Managing a large number of rabbits can be complicated and requires close monitoring. Rabbits can exhibit a range of behavioural, physiological and emotional responses in a relatively short period. This is because they are animals that are constantly vigilant, fast-moving, and mentally occupied with the potential threat of predators (Monclús and Rödel, 2008).

Nevertheless, this scaling up of the sector contrasts with the declining global production metrics, slaughter rates, meat production, census numbers, as well as exports in the EU rabbit sector. Rabbit meat consumption levels are continuously decreasing. This might be linked to changing consumer trends, a loss of traditional habit of eating rabbit meat in young people, the increased perception of rabbits as pets, and consumer price competition at the retail level compared to other meats (European Commission, 2017). However, the rabbit farming system has proven to be resilient. Globally, rabbit systems are diverse and rather atomized, with small-scale farms coexisting with the above-mentioned conventional large-scale ones. For small-scale production, different systems are available worldwide such as open naturally-ventilated

sheds in countries with a warm, tropical climate like Brazil (Machado and Simões et al., 2024). Other niche small-scale examples are based on organic, pastured rabbit systems (Gidenne et al., 2024) or agroforestry models (Savietto et al., 2023) as in France. In the EU, 34% of rabbit meat consumption comes from small-scale backyard farms (European Commission, 2017) – a non-negligible share. In a sector as complex and diverse as rabbit farming, the application of knowledge, technological innovation, and the implementation of digital tools can help improve farm technical management and animal care. This can contribute to both intensive and extensive production models, covering the entire range of intermediate production scenarios in rabbit farming worldwide.

Additionally, similarly to all livestock sectors, rabbit producers must also comply with strict regulations and consumer demands: i.e. the reduction in the use of veterinary antimicrobial substances, the fulfilment of environmental regulations on emission reduction, and improvements in animal welfare. Enhancing rabbit welfare on-farm is particularly challenging, especially given the current transition towards cage-free housing systems as foreseen by the European Citizens' Initiative 'End the Cage Age' (European Commission, 2021). Although alternatives to cage housing exist for other animal species (i.e. floor systems, multi-tier aviaries and free-range for laying hens, and free-farrowing, multi-suckling and fully outdoor systems for sows), there are no well-defined housing alternatives for rabbits, particularly for rabbit does. In 2020, the EFSA AHAW Panel (2020) identified several health and welfare concerns across different rabbit housing systems used in Europe. According to this report (based on a 2-step expert knowledge elicitation process) the welfare of reproducing does and growing rabbits was likely to be lower in conventional cages compared to enriched cages, elevated pens, organic or outdoor systems. Thus, the sector is strongly in need of creative solutions and advanced multidisciplinary tools that can help farmers comply with evolving regulations and assist them in the cage-free transition.

Despite these regulatory advances, practically all rabbits in the EU are housed in cages (112 million) (CIWF, 2020). In this context, key behaviour-related welfare issues that need attention in rabbit farming systems include restriction of movement, resting problems, inability to express maternal, positive social and gnawing behaviour, and the occurrence of abnormal behaviour and fear (EFSA AHAW Panel, 2020). With high levels of animal confinement, rabbits cannot express their natural behaviour freely, which could lead to abnormal behaviours and stereotyped conducts. Furthermore, confined individual housing does not allow social contact and other intrinsically motivated behaviours that could contribute to their positive welfare.

However, shifting towards non-cage systems that allow animals more freedom of movement and stimulate positive behavioural expressions involves trade-offs. Consequently, there are still numerous factors that can be impacted (positively or negatively) by the transition to cage-free systems in rabbit production. These factors include not only increased investment in housing and production costs, but also require a complete evaluation of rabbit's behavioural needs under this new environment. Furthermore, cage-free systems place specific demands

on the management which requires well-trained technical staff to successfully manage the farm and the animals in non-caged large flocks or small groups of animals.

Additionally, rabbit systems with greater freedom of movement exhibit lower feed efficiency, which may impair animal productivity (Machado et al., 2019; Matics et al., 2019), compared with systems where the physical activity of the animals is lower. Additionally, group housing complicates precision nutrition and providing individual tailored feeding for rabbit does which may affect their performance, welfare, health, and lifespan (Martínez-Paredes et al., 2022). Using the population-feeding approach, individual variations according to physiological state, body condition, genetics or animal health may be overlooked (Pomar and Remus, 2019). In fact, rabbits are the only zootechnical species where all reproductive animals (lactating and non-lactating) use a common feed, lacking ad hoc nutrition according to the female's physiological status. Moreover, although group rearing is certainly the best choice to satisfy rabbit social behaviour in growing animals; in breeding does, continuous group housing can lead to aggressive interactions, injuries, stress and, sometimes death (Trocino and Xiccato, 2006). This occurs when competing for resources and to establish a social hierarchy. Pérez-Fuentes et al. (2020) and Szendrő and Dalle Zotte (2011) reported aggressive interactions leading to increased mortality rates, in both does and their kits, when rabbit does were permanently housed in groups. In part-time systems, the most convenient group size and timing for grouping are still under debate.

As regards health aspects, cage-free systems can be linked to a greater health risk and transmission of diseases which can negatively impact animal welfare and farm productivity. Rabbits are highly sensitive animals to disease. In growing rabbits, the risk of intestinal health disorders (i.e. epizootic rabbit enteropathy, ERE) is multiplied when increasing the number of animals housed together (Birolo et al., 2019). Digestive disorders during the growing phase remain one of the main causes of mortality in rabbit farms (Licois et al., 2005). This digestive pathology frequently starts within the first 2 weeks after weaning, affecting rabbits from 3 to 7 weeks of age. The ERE can lead to morbidities of 90% and mortalities of 80% (Bäuerl et al., 2014). The first clinical signs of illness include reduced activity and feed consumption due to a slowdown in the intestinal transit rate and abdominal pain. These early signs of ERE, however, are difficult to detect individually in group housing, and flocks are frequently diagnosed and treated only when final-stage diarrhoea becomes evident. Controlling these digestive disorders remains a challenge, often necessitating antibiotic treatment. This could increase the need to use antibiotics in a context demanding a reduction of their use. Finally, type of housing can also affect air quality, impairing the respiratory health of both animals and workers. Although concentrations of suspended inhalable and respirable dust, bioaerosols (Adell et al., 2012) and gases (Calvet et al., 2011) are low in rabbit farms compared with other animals, their production and/or aerosolization is strongly related to increased animal activity, access to litter or bedded floors (Cambra-López et al., 2010).

In commercial farms, despite the modernization of rabbit rearing practices, housing conditions and other improved farm and animal management practices, mortality rates remain high and

disease outbreaks affecting numerical productivity persist (Huneau-Salaün et al., 2015). Although disease resistance, longevity and resilience have been included in some breeding programmes, particularly in breeding does (García et al., 2021) there are still important health issues causing animal pain, discomfort, mortality and increased production costs to control the disease (EFSA AHAW Panel, 2020).

Some examples of health issues and disease-causing serious consequences for the health and welfare of rabbits are described herein. Besides ERE in growing rabbits, in rabbit does, the main causes of death or illness are related to respiratory conditions (mainly pneumonia), followed by digestive problems (such as enteritis-diarrhoea and mucoid enteropathy) (Rosell and de la Fuente, 2009; Rosell and de la Fuente, 2016). Amongst respiratory disorders, rhinitis is highly prevalent. Consequently, health monitoring for signs of rhinosinusitis - such as sneezing, since rabbits do not cough, nasal secretion or difficulty in breathing - is an important task to diagnose clinical rhinitis (Rosell et al., 2023). Early diagnosis can avoid its progression which can lead to severe pneumonia and death. Other key health-related welfare issues that need attention in rabbit farming systems include pododermatitis, locomotory disorders, skin problems, mastitis, reproductive and neonatal disorders, cold stress, and most notably heat stress - as rabbits are fur animals (EFSA AHAW Panel, 2020). All these problems can greatly impact animal's reproductive life cycle which is on average around 6 kindlings per doe (Rosell and de la Fuente, 2009).

WHAT IN PLF HAS BEEN DONE IN OTHER SECTORS TO SUPPORT ANIMAL HEALTH, WELFARE AND PRODUCTIVITY

PLF leverages advanced Information and Communication Technology (ICT) systems to realise real-time monitoring and control applications. As already mentioned above, these systems can support farmers by automating the monitoring and assessment of animal health and welfare, and in the efficient management of large herds of animals. In the following paragraphs we consider the main developments in sensing in PLF, mainly in dairy and pig farming, drawing out their key benefits and limitations. We focus on remote sensing as this has proven to be an interesting tool for large-scale animal monitoring.

Camera-based monitoring

Camera technology has significant flexibility for PLF applications. For example, in dairy cow applications, cameras have been used to measure body conditions score (e.g. Liu et al. 2020), and cow behaviour concerning cow health and welfare (Chen et al., 2021). The pig production field sees regular improvements in computer vision-based weight monitoring since the original application by Schofield et al. (1999). However, in the current research environment, there is an intense focus on using computer vision to track animal movements in their pen environment,

intending to detect abnormal behaviours and welfare challenges (Parmiggiani et al., 2023). Recent studies have been able to develop technology to track these behaviours at an individual level, which can have a significant advantage for the animal breeding sector. While such work is progressing, it is largely challenged by the difficult conditions experienced in livestock buildings (Liu et al., 2023). Currently, limited tracking of animals can be achievable once certain operating conditions for camera deployment are followed (e.g. overhead view, limited blockages and distortion). Such technology will lead to rapid improvement on long-term health and welfare monitoring on the farm. Therefore, camera-based technologies are showing significant potential as a PLF tool and can find interesting applications in the rabbit production sector.

Sound-monitoring

Sound analysis is increasingly being explored, particularly for animal health and welfare applications. The non-invasive nature of sound monitoring, combined with its ability to listen around corners during the day and night, makes it an interesting tool. While various applications of sound analysis exist, in pig farming sound analysis has been able to monitor respiratory health by using coughing sounds as a primary indicator (Exadaktylos et al., 2008). Additionally, pig vocalisations, including screams, grunts, and coughs, have also been correlated with stress and environmental comfort, providing insights into the pigs' physical and emotional states and ultimately their welfare (Briefer et al, 2022; Nan et al., 2022).

However, sound analysis in typical PLF applications faces important challenges, primarily when it comes to accurately interpreting sounds in noisy farm-building environments. Background noise coming from machinery and overlapping vocalisation from other animals can interfere with the clarity of sound recordings and sometimes makes it difficult to extract meaningful data (Carpentier 2018; Nan et al., 2022). Nevertheless, the potential of sound analysis to enhance animal health monitoring and welfare assessment in commercial farming is improving greatly, with commercial solutions already existing. As technology evolves, it is likely to become an even greater part of animal production, with potential transferrable learnings towards the domain of rabbit production.

WHAT ARE THE OPPORTUNITIES FOR PLF DEVELOPMENT IN RABBIT PRODUCTION

Sensing technology can offer enormous potential to help address current challenges facing rabbit farming that can contribute to maintaining animal productivity, farm profitability, and sustainability. The potential is immense, and technology could serve as an aid in various fields within rabbit production. However, very few technologies are being developed and/or validated for this species. Table 1 summarizes the state of the art of some PLF applications in rabbit science.

TABLE 1. COMPILATION OF PLF APPLICATIONS IN RABBIT FARMING. ADAPTED FROM CAMBRA-LÓPEZ ET AL. (2024).

Monitored trait	Sensor used	Reference
Counting rabbit populations	Thermal camera, camera traps	Psiroukis et al. (2021), Latham et al. (2021)
Body weight, body surface and length	Camera	Giersberg et al. (2015); Negretti et al. (2007)
Body temperature	Thermal camera and implantable temperature loggers	Agea et al. (2021); Chen et al. (2023); Jaén-Téllez et al. (2021)
Feed intake	Camera and RFID[1] systems	Duan et al., (2022); Sánchez et al. (2024)
Physical activity and specific behaviours	Accelerometers and camera	Adedeje et al. (2023); Ipek et al. (2023); Piles et al. (2024); Sánchez et al. (2022); Studd et al. (2019)

[1]Radio frequency identification

In general, although scientific advances in research are sometimes sufficient, implementation is still limited across most species and is particularly pronounced in the rabbit sector. Implementation requires combining scientific advances with practical technological innovation and transfer. To this end, collaboration amongst research, the rabbit sector, and industry is fundamental. In the following paragraphs, we delve deeper into the technology applications that have been so far considered in rabbit science.

Monitoring the presence of rabbits

Monitoring rabbit populations is one important application of sensing technology for ecological purposes. Psiroukis et al. (2021) used Unmanned Aerial Vehicles (UAVs) equipped with thermal cameras to monitor and count free-range rabbit populations on the Lemnos Island in Greece. This research demonstrated how effective UAV-based thermal imaging accurately estimate rabbit populations, particularly over varied terrains and during nocturnal hours. The study also highlighted the advantages of non-invasive technology to provide reliable data for wildlife management. Complementing this, Latham et al. (2021) studied the use of camera traps for monitoring wild rabbit populations in Otago, New Zealand. They showed that these traps are also effective for assessing the success of pest control operations. Certainly, these technologies can be used to improve decision-making in both agricultural and conservation contexts, although limited transferability to commercial rabbit farming.

Physical activity in natural conditions

Recent research currently shows how monitoring techniques can obtain insights into rabbit behaviour, which is important given that this could be used as part of welfare assessment in intensive production systems. Adedeji et al. (2023) developed a deep learning-based approach to classify rabbit behaviours. They utilised state-of-the-art computer vision models to classify various rabbit behaviours, such as digging, eating, and social interactions, with an accuracy of up to 86%. While this work indicates how computer vision could provide information on rabbit behaviour, the use of public datasets that did not represent rabbit farming conditions restricts agricultural use. Another study on the free-range behaviour of shoehorn hares (Studd et al., 2019) employed a combination of accelerator and acoustic analysis to classify behaviour in free-ranging conditions. The study demonstrated how wearable sensors could effectively monitor these activities in their natural environment, and specifically showed that snowshoe hares decreased their activity in response to moonlight conditions. In summary, these studies bring into perspective that behavioural monitoring could be used to assist in understanding behavioural dynamics in free-range situations. These studies have particularly demonstrated the feasibility and effectiveness of remote monitoring (camera and microphone) technologies in capturing and analysing lagomorph behaviours. This is interesting given the opportunities and developments that image and sound technologies have seen in other farming sectors.

Feeding and social behaviour in farming conditions

In intensive rabbit farming situations, the conditions are far different to those experienced in the natural environment. Ipek et al. (2023) studied rabbits in a realistic farm housing environment using computer vision. They considered agonistic interactions among group-housed rabbits, which is a key challenge the animals face when realising a social hierarchy in group housing scenarios. In their analysis, they were able to identify and quantify aggressive behaviours indicative of stress and poor welfare conditions. Another study carried out by Piles et al. (2024) specifically focused on capturing novel phenotypes of feeding and social behaviour that linked with rabbit growth and feed efficiency. Their findings suggest that feeding behaviour and social interactions are connected with growth patterns and optimize feed use. They demonstrate how monitoring of certain production and behavioural traits can enhance both efficiency and welfare in rabbit production systems.

With respect to understanding and managing feed efficiency, Sánchez et al. (2024) introduced the eFeederRab, an electronic feeder designed to measure feed intake in group-housed rabbits. This technology allowed precise monitoring of individual feed consumption and could be used to develop more sustainable nutrition strategies for rabbit production. The ability to monitor and adjust feed intake on an individual basis has long been a big ambition of PLF and

is at the core of enabling more sustainable and cost-effective farming practices throughout all sectors.

Another interesting investigation was carried out by Duan et al. (2022), who quantified an estimate Remaining Feed Weight (RFW), which is the amount of feed left in rabbit feeders, intending to monitor the health of rabbits. The model combined a Deep Learning approach (Mask RCNN) for feed segmentation with technical improvements to image processing (more precise boundary detection) as well as weight estimation. The system achieved high accuracy, with a correlation coefficient of 0.97 and a mean absolute error (MAE) of 10.51 grams. In practical farm applications, the study found that higher RFW, indicating reduced feed intake, was strongly correlated with increased rabbit mortality, thus potentially serving as an indicator of rabbit health.

Taken together these studies highlight potential PLF approaches towards integrated health and production monitoring of rabbits on farms.

Monitoring physiological responses of rabbits with sensors

Recent studies on body temperature measurements in rabbits show the strengths of both infrared thermography (IRT) and telemetry sensor systems. Agea et al. (2021) and Jaén-Téllez et al. (2021) both evaluate the use of IRT as a non-invasive tool for assessing stress-related temperature changes in rabbits. Agea et al. (2021) used IRT to identify rabbits with lower stress responses. Interestingly, their study found that rabbits with lower variability in litter size exhibited smaller increases in body temperature following a stressful event. Jaén-Téllez et al. (2021) also utilized IRT to monitor rabbit stress but instead looked at the impacts of handling and environmental stress production. They showed that, while stress led to increased feed intake and growth rates, the rabbits had less efficient feed conversion as a result. This indicates the importance of stress on productivity, as well as the capability of IRT to capture this information.

Complementing these findings, Chen et al. (2023) developed a telemetry sensor system for continuous, real-time monitoring of body temperature (subcutaneously implanted) in rabbits, to provide a detailed and long-term perspective on rabbit health. The authors showed the telemetry system to be reliable and effective in monitoring temperature deviations beyond expected values. This system can be particularly useful in detecting health issues early, without the need for frequent handling, which can be a source of stress in and of itself. While practical challenges remain, such as how to ensure an appropriate distance between the animal and the antenna, the authors were able to demonstrate that the customized telemetry sensor system could offer useful insights.

Taken together, technologies that monitor the physiological responses of rabbits can give farmers new ways to monitor rabbit welfare accurately. Telematic approaches can also potentially facilitate longer-term monitoring and have potential applications in rabbit farming.

Monitoring the dimensions of rabbits and space utilisation

As already highlighted in previous sections, camera technology has a significant potential to enhance the understanding of animal productivity, with a key example being automated weight analysis. In rabbit production studies by Negretti et al. (2007) and Giersberg et al. (2015), computer vision methods and planimetric measurement methods were used to accurately assess physical traits and space requirements of rabbits in commercial farming systems. Negretti et al. (2007) showed that morphological traits and body weights could be accurately measured using image analysis, being more reliable compared to manual measurements. They also defined a new parameter called “body side surface (BSS)” to estimate live and carcass weights, showing high correlation coefficients (R^2 = 0.87) with live weight. This technology can more efficient and accurate breeding management practices by reducing the need for manual measurements.

Another interesting study was done by Giersberg et al. (2015), who looked at the space occupied by rabbits in different body positions, using the "KobaPlan" method as a basis for designing housing systems that meet welfare standards. They used a camera setup to collect images of rabbits in a controlled environment and then analysed these to determine the floor space occupied by rabbits in different postures (sitting and recumbent positions). They found that the floor space covered by rabbits increased linearly with body weight and that recumbent rabbits occupied significantly more space than sitting ones.

Taken together, these studies illustrate how camera-based technologies can provide tools for different design and monitoring applications, and thereby offer a comprehensive approach to improving both the welfare and productivity of rabbits. Leveraging these technologies can help rabbit farming achieves higher welfare in breeding and management than currently possible.

CONCLUSIONS AND OPPORTUNITIES FOR PLF DEVELOPMENT IN RABBIT PRODUCTION

The type and volume of information that we can handle through PLF technologies is expanding rapidly (i.e. individual identification of animals, their position, behavior, live weight, body condition, body temperature, respiration rate, feed consumption, feed conversion and even emotions, with some attempts being made to measure pain and frustration). Data can be

obtained using different sensing technologies as described in the previous sections of this review. Therefore, outside of research, this information can help professionals in the rabbit sector, especially in key fields such as veterinary medicine, breeding and genetics, and nutrition.

Regarding veterinary medicine, animal monitoring technology can help identify weak and unhealthy animals in real-time, and on a 24/7 basis. In fact, one of the main opportunities offered by PLF in rabbit production is early disease detection. Early detection of signs of disease can help caretakers to take quick actions to reduce the impact of disease on the health of the animals. This application can be highly relevant in rabbit does (e.g. for example, to get an early warning of respiratory problems), but more notably in growing rabbits. Generally, all animals, including rabbits, when ill, reduce their daily feed and water intake and also change their activity patterns. Therefore, measuring feeding behavior and water consumption, as well as the individual activity levels of growing young rabbits, could be used to develop an early warning system for potentially sick animals. This could potentially be done using electronic feeders/drinkers with radio frequency identification (RFID) tags, developing *ad hoc* vision-based monitoring models, tracking rabbit activity using accelerometers, and measuring other biometric and physiological variables (e.g. heart rate). This application could have enormous potential around weaning, when the likelihood of digestive disorders is the highest. Early disease detection enables targeted veterinary treatments for rabbits showing initial signs of illness, improving treatment effectiveness and supporting the rational, minimized use of antibiotics. This application is especially relevant for group-housed animals, where the risk of disease spread is sometimes higher than in other situations.

In terms of breeding and genetics, digital phenotyping allows for the continuous collection of hundreds of traits from many animals simultaneously, that can be recorded non-invasively and individually, even under field conditions. Individual phenotypic data can be used in rabbit breeding programs, for instance, to enhance the adaptation of females to permanent and/or part-time group housing. Precision livestock farming tools could contribute to evaluate management and breeding practices that promote positive experiences (e.g. grooming in rabbit females or play and exploratory behaviour in growing rabbits) and minimize negative ones (e.g. fear, boredom, pain, and frustration). This could be done by measuring objectively specific rabbit behaviours, physiological responses, and body or facial expressions. Cameras, accelerometers, and sound analysis could potentially serve for this aim once the algorithms to do so are developed. These algorithms need to be calibrated to automatedly detect movement patterns and the frequency and type of social interactions to verify that animals exhibit desirable natural behaviors and to identify problems such as aggression. They also must be validated to ensure transferability across different farms and building types.

In the area of nutrition, the use of electronic feeders based on RFID systems in rabbits would allow for continuous and automatic tracking of individual consumption (type and amount of feed), as well as the study of feeding behavior (frequency, duration, and feeding rate). This could certainly enhance feed efficiency and adjustment to individual nutritional needs (both for growing and reproductive animals), towards the practical implementation of a tailored feeding system in rabbits. This application could be highly beneficial in group housing for does, as the formation of stable groups involves animals of different types (mothers and kits) and physiological states (lactating and non-lactating).

Finally, the potential of PLF tools to control indoor environmental conditions in rabbit farms (temperature, relative humidity, air quality, among others) should also be acknowledged as a valuable opportunity. This can be achieved through better integration between the ventilation and manure management systems. Such an approach could ensure optimal environmental comfort for the animals and, therefore, reduce the stress associated with unfavorable environmental conditions. Improved environmental control can lead to better productivity and health outcomes, benefiting both the animals and the farm's efficiency.

Although not exhaustive, these examples also highlight specific challenges where further research is undoubtedly needed in rabbit science. Research challenges reside in transforming sensor data into reliable and robust algorithms that can answer particular questions and predict the response. This requires selecting the appropriate variable(s) to measure, choosing the suitable sensing technology, and establishing an accurate reference standard when developing PLF algorithms – reliable and adapted for rabbits. Then newly developed algorithms should be then validated in different farm conditions before they can be extrapolated and implemented in commercial situations. Nevertheless, our examples also highlight some areas where PLF might be helpful in rabbit production, assisting farmers in animal care by facilitating a greater understanding of animal individual needs on farms. These PLF concepts can help bridge the gap between societal demands and those of the production sector, contributing to achieving high levels of production, efficiency, resilience, and welfare in animal husbandry for the production of high-quality rabbit meat.

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PHYSICOCHEMICAL CHARACTERISTICS AND REPELLENT EFFECT OF RABBIT DROPPINGS FED WITH A GRANULATED FOOD BASED ON STYLOSANTHES GUIANENSIS

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ABSTRACT

Transhumant and nomadic breeding systems are still used in Africans countries. The coexistence between breeders and farmers causes crop damage and conflict. The aim of this work is to use a bio-repellent made from rabbit droppings to protect crops and to reduce conflicts between farmers. This study can contribute to reduce rabbit breeding pollution. Twenty growing rabbits were used. Total droppings of those rabbits were collected. 300 g fresh droppings (SCF) were used to make 1.5 L repellent solution. Qualitative phytochemical compounds in the rabbit droppings solutions such as Alkaloids, Coumarins, Polyphenols, Terpenoids, Tannins, Flavonoids, Saponins and Steroids was analyzed. Physical and chemical parameters of the solutions including pH, Eh and conductivity was carried too. Repellent solution was tested on young corn plants (*Zea mays*). Effect of dropping repellent on maize and growth were tested during one month. Control plot was treated with water only. The results of phytochemical compounds in the rabbit droppings solutions showed the presence of Polyphenols, Tannins, Flavonoids, reducing sugars, aromatic amino acids, steroids and terpenes. Solution temperature, pH, Eh and conductibility were 30 ˚C, 6.3, 2,4 µmhos/cm and 42.8 respectively. Control plot treated with water only was 100% consumed by sheep. However, in plots treated with rabbit droppings solutions, from 11 to 20% leaves was consumed. Rabbit droppings could be used to protect cultures against ruminant attacks.

Keywords: Repellent, droppings valorisation, crops protected, phytochemical

INTRODUCTION

There are three livestock breeding systems in sub-Saharan Africa. These are transhumant, nomadic and sedentary livestock systems. These breeding systems are generally traditional. Animals feed near fields. They cause a lot of damage to crops. This creates often fatal conflicts between breeders and farmers. The main question is how to protect plantations against ruminants.

Raising rabbits emits significant quantities of droppings. This quantity per animal was estimated at 95g/d (Lebas *and* Laplace, 1974). These droppings are used in agriculture. They are often used to fertilize soil.

Poultry droppings and rabbit droppings are found to be the best organic fertilizer for Daphnia production. The highest daily production (15 ind.l-1 .D^{-1}) and the best growth rate (0.24) were obtained in the jars fertilized with poultry droppings and rabbit droppings (Akodogbo *et al*., 2022). Droppings are the undigested part of animal's ration. The rabbit's ration contains a high content of polysaccharides (Gidenne *et al.,* 2015, Kimse *et al.,* 2012). Some of these polysaccharides could be found in droppings. Rabbit droppings fed with a balanced fiber ration could therefore be used against certain plants attacks, particularly those of ruminants.

MATERIALS AND METHODS

Animals and experimental design

Twenty young rabbit (*Oryctolagus cuniculus*) fecal matter were used. Animals were fed with pellet (AGL-STYLO) made by Université Nangui ABROGOUA (Abidjan, Côte d'Ivoire). In this pellet *Alfa alfa* was substituted by *Stylosanthes guianensis* (Table 1). Those rabbit dropping were collected using plastic grille hander cages. Dropping was analyzed to determine them physical and chemical parameters. Sample 300 g (S1) from all dropping collected in morning was made. S1 was diluted and macerated in 3 L water (S2). The dilution S2 was filtrated on a 500 micrometer sieve or using fine fabrics (R). Solution R was stored 3 days at 4˚ C. R was used to spray corn plot (Plot 2) at morning weekly. Second corn plot (Plot 1) was sprayed with water only weekly too. Spraying were made from 8-10 leaf stage to visible panicle stage of corn. Plot size was 15m x 8m. Each plot was composed of 600 plants of corn. Interval between corn plants was 25 cm in the same row and 80 cm between rows. Ten sheep were introduced daily in plot 1 first and plot 2 after. The number of leaves consumed by sheep was counted and percentage were calculated.

Table 1. Ingredients and chemical composition of diet

Ingredient	%	Nutrient	%
S. guianensis	30,0	Dry matter	88,96
Bran	23,7	Ash	6,61
Soybean	11,4	Crude protein	17,44
Cottonseed	10,6	Fats	2,62
Mays	20,7	Raw Cellulose	15,83
Molasses	2,0	NDF	32,58
Salt	0,5	ADF	19,35
Shellfish	0,5	ADL	4,60
Diclazuril + Carbonate	0,2	Total sugars	4,50

CL25 premix	0,4	Digestible Proteins	12,84

Chemical and physical analyses

All analyses were made by Université Nangui ABROGOUA (Côte d'Ivoire).

Phytochemicals analyses

Rabbit droppings solution (S2) was qualitative phytochemical analyzed. These tests were based on precipitation and complexation reactions with the formation of insoluble and colored complexes. The coloring was due to the formation of conjugation or unsaturation in a molecule. Qualitative phytochemical compounds such as alkaloids, coumarins, polyphenols, terpenoids, tannins, flavonoids, saponins and steroids was analyzed.

The alkaloids test was performed on precipitation reactions with alkaloid developer with Dragendorff's reagent (2 drops). 6 mL of each dropping extract was evaporated to dryness. It was supplemented with 6 mL of 70° alcohol. The presence of alkaloids is indicated by the appearance of a precipitate or orange coloring. Coumarins was tested using 10% ammonia. The reaction was positive when the tested tube is transparent. The presence of polyphenols was tested by adding 2 drops of 2% alcoholic ferric chloride solution to a tube containing 2 ml of dropping extract. The appearance of less dark blue-blackish or green color shows the presence of phenolic compounds. The Terpenoids were demonstrated by the addition to extract of 2 ml of Chloroform, 1 ml of acetic anhydrous and 1 ml of concentrated sulfuric acid. The appearance of a purple color indicates presence of terpenoid. Tannin test was carried out with 15 ml of Stiasny reagent added to 5 ml of each dropping extract. The mixture was maintained in a water bath at 80°C for 30 min. It was then cooled under a stream of water. The observation of large flaky precipitates indicated the presence of catechic tannins. Flavonoid test was carried out with NaOH. The appearance of intensive yellow or orange color indicated the flavonoid. Saponin was tested with water addition to extract in tube. Mousse presence after tube agitation indicated saponin in this extract. Steroid was tested with H2SO4. Red band in tube indicated steroid.

Physical and others chemical parameters analyses

pH, temperature, conductivity and redox potential of the dropping solutions (R) were recorded. Multi parameters WTW 3320 cond was used. 0.5 L of R solution was put in a beaker before use. Electrodes were introduced into the beaker then values of parameters were recorded.

Statistical Analyses

Effect of R solution on mays was tested using R Software. Percentage of plants eat by sheep in Plot 1 was compared with plot 2 by G test. Difference was significant if P-values was less than or equal to 0.05.

RESULTS AND DISCUSSION

Chemical and physical parameters rabbit dropping solution

Rabbit farming emits large quantities of droppings. These droppings are regularly used to fertilize the soil. In this study, the objective is to promote droppings to facilitate the association between farmers and breeders in African systems. Adapting breeding practices to preserve biodiversity in agro-ecosystems constitutes one of the principles for ensuring the sustainability of rabbit farming (Lamothe *et al*., 2013). The pH of droppings solution obtained shows that it is not dangerous for the leaves and for animals (Table 2). The pH was close to neutral compared to acid rain (pH=5). However, Eh indicated lower oxygen in this solution than water (Kimsé *et al.*, 2009).

Table 2. Physical and chemical parameters of rabbit dropping solution

	Treatments		*Probability*	
	Water	R solution	*CV*	*P-values*
Repetition	*10*	*10*		
pH	7.43	6.28	*8.4*	*0.01*
T (°C)	30.4	30.3	*0.2*	*0.78*
Cond (µs/cm)	204	2.44	*97.6*	*0.01*
Eh (mV)	301	42.78	*75.5*	*0.01*

R solution contained rabbit dropping; Cond: conductibility; Eh: redox potential

Repulsive effect of dropping solution on mays plants

Results showed that plants in corn plots treated with solutions based on rabbit droppings were less consumed (20% of leaves). On the other hand, the control plot treated with water was 100% consumed. Solutions based on rabbit droppings therefore have a repellent effect on ruminants. Rabbit droppings solution would work in two ways. This is the phytochemical composition and odors left on the leaves.

Indeed, the presence of phytochemical compounds such as Tannins, Flavonoids, Polyphenols, Aromatic Amino Acids, Steroids and Terpenes contained in plant materials which enter into the composition of AGL-STYLO granulated food were translated in droppings. These phytochemicals present in rabbit droppings have a repellent effect on ruminants due to their strong pungent odor and bitter taste Hume *et al*. (1996).

Moreover, the strong and distinct odor of the solutions made from rabbit droppings could disrupt animals' sense of smell. This dissuaded sheep from approaching corn crops. These odors would probably be linked to the high ammonia content of droppings. Nitrogen level in hard droppings exceeds 150 mg/g (Adande *et al*, 2017). It varies depending on the composition of the ration. Olfactory repellents have a long-lasting action (Le Bel *et al,* 2015). This explains the drop of leaves number consumed between week 2 and week 8 (from 20% to 6%).

CONCLUSION

Hard feces of rabbit feed with pelleted which contained *S. guianensis* can be used to protect plants against ruminants attack. When the droppings solution is applied for 8 weeks the consumption is almost zero. Droppings solution can be used as a repellent to protect crops. However, further studies are needed to evaluate the effect of rabbit droppings solution on plant growth.

Table 3. Effect of rabbit dropping solution on mays plant protection of sheep attacks

	Treatment		*Probability*	
	Plot 1	Plot 2	*CV*	*P-values*
Plant number W1	*587*	*576*		
Treatment Duration				
W2	100	20	*66.7*	*0.01*
W4	100	16	*72.4*	*0.01*
W6	100	13	*77.0*	*0.01*
W8	100	6	*88.7*	*0.01*

Plot 1: Mays were treated with water only; Plot 2: Mays were treated with rabbit dropping solution; W: Mays age (week)

Table 4. Qualitative phytochemical composition of droppings

	Polyph	Flav	Tan	Sapo	AA Arom	Stero + Tep	Alcal	Coum	Suc Red
Fresh droppings	+	+	+	-	+	+	-	-	+

Polyph : ; Flav : Flavonoid ; Tan : Tanin ; AA Arom: Aromatic acid: Stero+Tep: Alca: Alcaloids; Suc red: Reductive sugar

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ELECTRONIC FEEDER TO RECORD INDIVIDUAL FEED INTAKE OF RABBITS RAISED IN COLLECTIVE PEN

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ABSTRACT

To meet society's expectations in terms of well-being, while maintaining the possibility of selecting rabbits on the basis of their feed efficiency, we have developed an electronic feeder for animals raised in collective pen. This tool equipped with two feeding stations was tested on a small group of 16 rabbits from 32d. to 70d. of age. It permits to measure feed intake to the nearest 0.5g. The animals quickly adapted to the feeder in just one day. An alert system permits to inform if an animal hasn't shown up to the feeder for too long which enabled us to track the animals more closely. Thanks to an automatic door system preventing several animals from feeding at the same time, 87.5% of feeder visits enabled reliable measurement of feed intake. Animals had a normal growth during the test period (37.11g/d), they made an average of 102 visits per day to the feeder and consumed an average of 149.44 g of feed per day. In the longer term, the objective is to develop a 4 stations system easily disassembled for indoor and outdoor installation.

Key words: electronic feeder, collective pen, feed efficiency, rabbits.

INTRODUCTION

The European Citizens' Initiative "end of the cage age" aims to ban the use of cage systems for all animals (EFSA AHAW Panel). Raising rabbits in collective pens instead of individual cages is a way of meeting this society's expectations in terms of animal welfare. Indeed, this type of accommodation enables animals to enjoy greater freedom of movement, more frequent and varied social contacts, as well as to express specific behaviors. However, such a rearing system does not allow us to measure individual feed consumption, which is a prerequisite for estimating feed efficiency (FE). Yet, because of the relatively high importance of feed-related costs in animal production systems (Gilbert et al., 2007; Calenge et al., 2014), selecting animals for a better FE is one of the best levers of action to improve farm profitability. In addition, improving FE reduces the environmental impact of livestock farming (Basarab et al., 2013; Saintilan et al., 2013). Improving such trait is thus a key element of the agroecological transition (Ducos et al., 2021). To overcome this problem, we developed a tool to measure the individual consumption of rabbits raised in collective pen and tested it on a small group of 16 animals.

MATERIALS AND METHODS

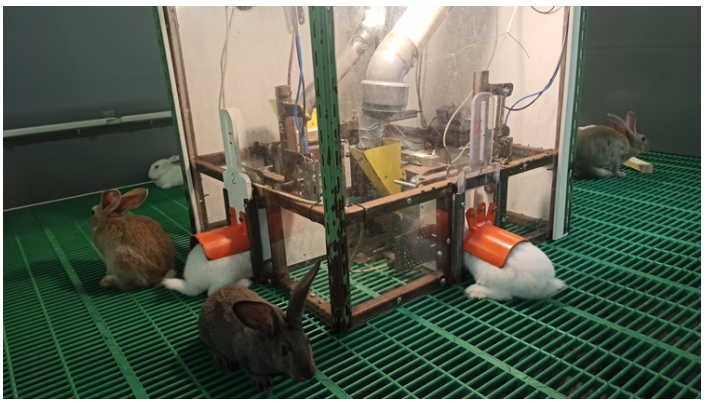
Animals management and identification

The test batch was made up of 16 pure (6) and crossbred (10) rabbits (5 of INRA1777 X the commercial line HYPHARM PS119 and 5 of INRA1777 X the commercial line HYPHARM PS59). At 3 days of age, animals were equipped with a RFID ear tag designed by INRAE and manufactured using 3D printer technology. This RFID buckle is fitted in the same way as the standard buckle. Kittens are weaned on the Friday of the fourth week after farrowing (31 ± 2d of age) and moved to the fattening room. The animals were reared in collective pen (floor pen type) in an environment-controlled building from November to December 2023. The pen surface was 5.5 m^2 (2 m wide by 2.75 m long), with plastic slatted floors enriched with pieces of wood. The average temperature in the building over the fattening period was 18.9 C°, with a humidity level of 59.26%. The lighting duration was 10 hours, from 7 a.m. to 5 p.m.

Equipment

An electronic feeder (EF) was placed in the center of the pen (Figure 1). This EF is a prototype adaptation of a duck pellet dispenser. However, adjustments were necessary to adapt the EF to the specificity of rabbits: gnawing animal of smaller size. The EF consisted of 2 stations equipped with: a scale, an automatic door to close the feeder, a photocell to detect the animal entering, a RFID antenna to identify the animal and a hopper with a flow hatch. The device is protected from being damaged by animals. As the rabbit approaches the EF, it is detected by the photocell, identified by the RFID antenna and the door opens. When the animal has finished eating and the photocell beam is re-established, the door closes. The recording of the visit to the EF starts when the animal cuts the photocell beam cell and end when the photocell beam is reestablished.

Figure 1. Electronic Feeder in park © J. Ruesche



The quantity of feed consumed, with an accuracy of 0.5g, is calculated as the difference between the weight of the feed is calculated as the difference between the weight of food at the beginning and end of the visit. On each test day, the scales were checked for zeroing and tare. The visit data are automatically computed at the end of each visit (one line per visit in the database: animal ID, date time enter and exit, visit duration and amount of feed consumed).The door in front of the feeder permits to obtain accurate measurement of the feed intake for two reasons: it prevents the animal to press on the scale and to disturb the measurement when arriving to the feeder; it also prevents that more than one animal are feeding at the same time by closing the door if the system detects another RFID number during a visit. This stop the feeding of the first identified animal that is nonetheless recorded. All the data are recorded in the EF SQlite database, and copy to a local server MySql database. A supervisor Web application (Webdistri CATI SICPA Automated Information Processing Center, Information System and Calculation for Animal Phenotyping) permits real time visualization of the data by several graphs' analysis (weight curve, amount curve, by animal or by group, …). In addition, the system can provide an alert if an animal hasn't shown up to the EF for too long (defined by the user). Furthermore, we also had 24/7 video surveillance. During the fattening period, animals were fed with a commercial pellet diet. During the first week of the fattening period, that corresponds to an adaptative period, animals were 85% feed restricted using a collective feeder. Then from 38d. ± 2d. to 70d. of age they were fed *ad libitum* with the EF. Animals were weighted at weaning, one time a week during the fattening period and at 70d. of age. At each weighing session, the animal's health status was also recorded (healthy or main symptom: digestive, respiratory disorders, skin lesions…).

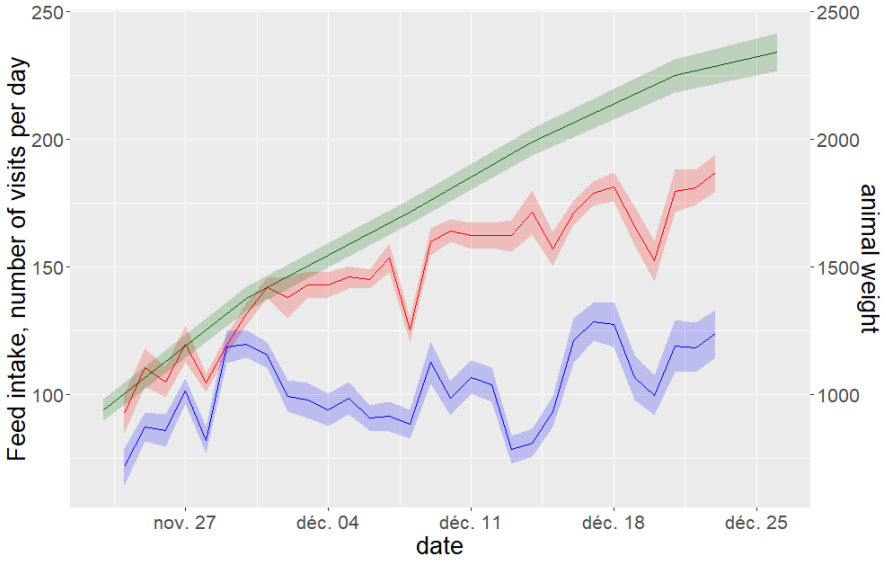
From the data provided by the EF and the weight of the animals, variables describing the feeding behavior, growth performances and FE of the animal were calculated. The feeding behavior was assessed for each animal using the amount of feed intake and the time spent to the feeder at different timescales (visit, day, week, period), the number of visits with feed intake per day, the preferred station. The average daily gain of each animal was computed as the difference between weight at 70d. and at weaning divided by the number of days elapsed. The FE was assessed by calculating the feed conversion ratio (FCR) as the ratio between total feed intake and total weight gain over the period. Other variables were also computed at the group level: the distribution of the visits across the day and the percentage of time the hooper is occupied per day. All variable calculation and descriptive statistics were performed using SAS software (version 9.1, 2003).

RESULTS AND DISCUSSION

Except the death of one animal at 42 days of age due to digestive problems, no major problems in this trial were encountered. We observed a rapid adaptation of the animals to the EF after just one day. No competitive or dominant behavior between animals has been observed. However, we did observe some crowding at certain times. Thanks to the automatic door system, which opens only on identification, we didn't observe visit with unallocated food intake which was one of the main issues reported by Sanchez et al. (25%). A small part of the visits was at 0 g (12.5%). This can be explained by rabbits that enter the device but are disturbed

by a fellow rabbit, and then leave without eating. The 15 alive animals were all in good health at the end of the test period.

Figure 2: Daily feed intake (red), number of visits per day (blue) and animal weight (green) over the growing period



line=average value, shaded area=1 standard deviation

The average weaning and at 70d. of age weights were 855 ± 91.5g and 2,340±289g, respectively. The average daily gain was 37.1±6.2g/d. The average FCR was 3.61±0.46. These data are in line with the performance of the INRAE maternal line. At the last check in June 2023, its average daily gain was 33.48 g/d and its average FCR was 3.11±0.22 (Helies personal communication). The average number of visits per day per animal with a feed intake equal to or greater than 0.5 gr was 102 (±15) over the period, which is in line with the total of 1515 visits (41.5% without feed intake) for a group of 7 rabbits reported by Sanchez et al. (2021) leading to ~127 visits with feed intake /animal/day. The average duration of a visit was 0.8±1.08 min, with an average consumption per visit of 1.45±1.47 g leading to an average total daily consumption per animal of 149±26g. Average consumption per animal increased steadily from 92g/d at D1 to 178g/d at D32 (Figure 2). Time spent on feed remained stable, average feeding time per day was 102 min per animal. The average occupancy time at the 2 stations was 21±3h, indicating a high level of animal activity. The rabbits went to eat at both stations and the feeder attendance rate was balanced: 48.79% for station 1, 51.21% for station 2. The access to the EF was not uniform over the day. We observed 2 periods from 4pm to 00am (49.98%) and from 5am to 7am (12.96%) when rabbits eat more. There was also a rest period from 9 a.m. to 12 p.m. (6.33%). These periods correspond to those described by Gidenne et al (2005). All in all, during busy periods, the occupancy rate is 100%. So, we can't add more animals per feeding station.

CONCLUSIONS

The new measuring equipment we have developed is giving satisfactory results. Feeding behavior and performance are fully consistent with literature results. In the longer term, the objective is to develop a system on a larger scale, with the collaboration of the ARIAS company. The final food distributor must include 4 feeders instead of 2, while limiting the size so that the EFs can be used easily (indoor and outdoor parks) according to experimental needs. Software adaptations are also to be expected to reduce manufacturing costs. Once operational, these EFs will open new field of research thanks to the availability of high throughput phenotyping data. These are useful information to study the animal's resilience for instance by studying the dynamic of the different phenotype over the time. These data will also be interesting to study the social behaviors of animals during food intake, to estimate the grass consumption in free-range systems or to compare palatability in food tests.

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NUTRITION & FEEDING

ALTERNATIVE METHODS IN RABBIT NUTRITION TRIALS

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ABSTRACT

Animal studies are essential to nutrition research, particularly in investigating the effects of dietary changes on animal growth, reproduction, health, and metabolism. These studies provide quantitative data on feedstuffs' nutritive value and animal's response to diets, indispensable for building accurate nutrient databases and defining animal nutrient requirements, respectively. However, advancements in (bio)technologies have encouraged the development of non-animal alternatives in rabbit nutrition research. Moreover, Europe's commitment to replacing animals for scientific purposes emphasizes the need to regulate and harmonize animal experimentation according to the principles of the 3Rs (Replacement, Reduction and Refinement). While animal methods remain necessary in some cases, attention must be paid to their reliability and validity, alongside the adoption of alternative methods. Alternative approaches in rabbit nutrition include prediction equations to estimate the nutritional value of feedstuffs based on their chemical composition, *in vitro* models to simulate the digestibility and fermentability of diets and feedstuffs, and the use of near-infrared spectroscopy (NIRS) to calculate feed composition and nutritive value. Other non-animal-based approaches using mathematical modelling and cell/tissue/organ culture models are also rapidly evolving to test animal's response to dietary changes. Reduction can be achieved through extensive literature searches, careful experimental design, statistical methods, and sharing data to avoid unnecessary duplication of experiments. Refinement includes appropriate housing, care, and enrichment to minimize the suffering of animals used in research. Additionally, integrating precision livestock farming technology into research practices, and omics tools through non-invasive procedures can contribute to refining rabbit trials, as well. The objective of this work was to critically review these approaches following Replacement, Reduction and Refinement principles for rabbit nutrition. We first examine already existing possibilities and practical methods and later discuss their adequacy. Recommendations for designing rabbit nutrition trials and further research needs, opportunities and challenges for the use of alternative methods that pursue any of the 3Rs will also be reviewed in the light of rabbit nutrition trials.

Key words: rabbit nutrition, animal research, replacement, reduction, refinement.

INTRODUCTION

Nutrition research requires *in vivo* animal studies to verify hypotheses related to the effects of dietary changes on animal growth, reproduction, nutrient use, feed conversion efficiency, metabolic processes, and overall animal and gut health. Furthermore, they are necessary to generate evidence-based quantitative information on the nutritive value of feedstuffs indispensable to create a well-characterized and accurate nutrient database for feed ingredients, together with properly defined animal nutrient requirements. These studies need to be specifically conducted in different types of animals of the same species to account for sex, physiological, genetics and health-based differences, and cannot be easily extrapolatable from other physiologically similar animal species. In rabbit nutrition research, this knowledge is key to enhancing animal performance and health, feed efficiency, farm profitability and overall system sustainability.

While animal research has contributed to significant scientific breakthroughs, in the last decades there have been huge (bio)technological advancements in the methodologies and approaches applicable to rabbit nutrition research, including non-animal alternatives. Non-animal-based methods are rapidly evolving and proven to be effective, thus leading to a growing debate over the ethical implications and scientific value of animal studies.

Regarding animals in science, Europe is committed to the goal of the complete replacement of animals used for scientific and educational purposes and is taking concrete action towards that goal (Holley et al., 2016). In addition to the replacement of the animal model, alternative approaches also include methods of refining and reducing the use of animals for scientific purposes (the "3Rs") as described by Russell and Burch (1959). Therefore, there is still a need to regulate and harmonize animal experimentation, fully integrating the 3Rs principles.

In this framework, animals are required in many circumstances to achieve the desired specific goals. However, special attention should be given to the reliability, validity and transferability of the animal methods used, as well as when alternative methods are employed. Using non-validated methods for a particular purpose can result in unreliable and misleading results. This can not only result in distorted data but also a waste of resources and animal lives. Moreover, such practices may highly hinder scientific advancements.

Current regulations on the protection of animals in science are also focused on the principles of the 3Rs. All research involving animal experimentation should conform to the specifications outlined in European Directive 2010/63/EU on the protection of animals used for scientific purposes (European Commission, 2010) which also requires replacing, reducing, and refining the use of experimental animals.

Replacement advocates for finding approaches that do not involve the use of live animals when possible. In animal nutrition trials, these alternatives may include prediction equations for estimating the nutritive value of feedstuffs based on their chemical composition, *in vitro* models to simulate the digestibility and fermentability of diets and feedstuffs, near-infrared spectroscopy (NIRS) to calculate feed composition and nutritive value, mathematical modelling and other cell/tissue/organ culture approaches. The Reduction principle pursues minimizing the number of animals used in experiments and thus includes any approach that will result in fewer animals being used to achieve the same objective. This can be achieved through extensive literature reviews, careful experimental design, statistical methods, and sharing data to avoid unnecessary duplication of experiments. Refinement includes procedures focused on minimizing the suffering of animals used in research and enhancing their welfare. This comprises providing appropriate housing, care and enrichment to enhance animal positive emotions and reduce the frequency of negative ones such as pain, fear, anxiety and boredom, amongst others. The use of technology to monitor animals automatically, omic tools and non-invasive sampling procedures can be key in this regard. By adhering to these principles, researchers can conduct experiments in a way that is both scientifically valid and ethically responsible, ensuring that animal use is minimized and any potential harm is mitigated to the greatest extent possible. However, in rabbit research, there is still a lack of guidance for these principles that would be deemed acceptable.

The objective of this work was to critically review approaches that can be potentially useful in rabbit nutrition trials following the principles of 3Rs: Replacement, Reduction and Refinement. Therefore, we first examine already existing possibilities and practical methods and later discuss their adequacy. Recommendations for designing rabbit nutrition trials and further research needs, opportunities and challenges for the use of alternative methods that pursue any of the 3Rs will also be reviewed in the light of rabbit nutrition trials.

REPLACEMENT

The successful replacement of animals for the estimation of the nutritive value, e.g. gross energy digestibility (GEd), digestible energy (DE), crude protein digestibility (CPd), digestible crude protein (DCP), true ileal digestibility amino acid (TIDAA) of ingredients and diets, requires deep knowledge on animal's digestive physiology. In fact, *in vivo* results provide the reference data needed to develop and validate robust and precise alternative methods. For this reason, it is essential to have a representative *in vivo* database, including the effects of the main factors that influence nutritive value and the potential interactions with different feeds (Villamide et al. 1990, 1991). In rabbits, unlike other non-ruminants (swine, poultry), there is a single nutritive value available for each feedstuff, which is recommended to be used for all types of animals, ignoring differences related with age (young *vs.* adult animals; Xiccato et al., 1992; de Blas et al., 1995), physiological state (lactating *vs.* no lactating does; Maertens and De Groote, 1982; Read et al., 2017), or feed intake (restricted *vs. ad libitum;* Lebas and Cousin, 1979; Ledin, 1984). Not all the studies, however, observed these differences suggesting potential interactions with type of diet (fibre and energy level), animal management and/or housing/environment. This implies that more *in vivo* data involving rabbits are still needed to improve the nutritional evaluation of feeds and optimize the efficiency of the overall feeding process.

Non-animal-based methods to predict nutritive value

Prediction equations of the nutritive value based on chemical composition

The use of equations to predict the energy or protein value of a single ingredient or compound feeds based on its chemical composition and *in vitro* digestibility was probably one of the first activities that replaced the use of live animals in rabbit nutrition. These values could be obtained rapidly, simply and cost-effective, and could be readily utilized within the feed manufacturing industry. Several authors have developed different equations to estimate the nutritive value of ingredients and compound feeds for energy (Table 1) and protein (Table 2).

The single predictor most used to estimate the GEd or DE was dietary acid detergent fibre content (ADF) (Table 1); a simple and repeatable laboratory chemical analysis (Xiccato et al., 1996). However, while ADF is useful to differentiate the energy value among diets with wide differences in ADF, its sensitivity decreases when comparing diets with ADF values close to each other, and/or high levels of pulps, straw or fat (Maertens et al., 1988; de Blas et al., 1992), even when combined with other chemical constituents like ash and fat (Villamide et al., 2009). This was partially solved by providing different equations for different types of diets. To obtain a better fit in energy value predictions, the value of the three-step *in vitro* dry matter (DM) digestibility can be included, highly increasing the precision (Villamide et al., 2009). The prediction equations proposed by these authors are the only validated ones with independent data for rabbits.

As regards the prediction of CPd or DCP of diets (Table 2), they are mainly correlated with the dietary CP and acid detergent lignin content (ADL) (Villamide et al., 2009). Generally, prediction equations obtained for protein values were more precise than those obtained for energy. In this case, the three-step *in vitro* DM digestibility did not improve the prediction, while the three-step *in vitro* CP digestibility was not available.

The information to predict the nutritive value of single ingredients is limited to alfalfa (Maertens et al., 1988; Pérez, 1994; García et al., 1995) and wheat bran (Blas et al., 2000), while some studies have focused on providing prediction equations for families of ingredients (e.g. high-fibre feedstuffs) (Wiseman et al., 1992; Villamide and Fraga, 1998; Villamide et al., 2016). More recently, the apparent and true ileal nitrogen digestibility of single ingredients were successfully predicted by using the three-step *in vitro* nitrogen digestibility method, as well as

the apparent and true ileal amino acid digestibility, although the precision was lower in the latter (Villamide et al., 2016; Table 2).

Table 1. Equations to estimate the gross energy digestibility (GEd) and digestible energy (DE) value of diets and ingredients in rabbits. Each line (shaded or not) represents a regression equation and the independent variable(s) used. The range of values expressed in g/kg dry matter is included in parentheses

Compound feeds	n	GEd (%)	RSD	R^2	VE (%)	DE (MJ/kg DM)	RSD	R^2	VE (%)
Maertens et al. (1988)	31					ADF (148-387)[3]	0.05	0.76	-
						ADF (148-387) Fat (23.3-62.2)	0.04	0.83	-
de Blas et al. (1992)	58	ADF (98-327)	0.040	0.73	-				
		ADF (98-327) ADL (10-149) CP (81-255)	0.031	0.85	-				
Fernández-Carmona et al. (1996)[1]	22	ADF (87-525)	0.031	0.95	-	ADF (87-525)	0.82	0.90	-
Villamide et al. (2009)	111	ADF (135-284)	0.033	0.49	5.04	ADF (135-284)	0.62	0.43	5.49
		ADF (135-284) ADL (26.6-195)	0.032	0.54	4.99	ADF (135-284) Ash (63.6-115) Fat (9.9-71.2)	0.57	0.54	5.38
		DMdinv (0.538-0.810)	0.025	0.70	4.25	Dmdinv (0.538-0.810)	0.58	0.52	5.42
		DMdinv (0.538-0.810) Fat (9.9-71.2) ADL (26.6-195)	0.025	0.81	3.90	DMdinv (0.538-0.810) Fat (9.9-71.2) ADL (26.6-195)	0.57	0.74	4.42
Trocino et al. (2013)	76					ADF (102-281)	0.13	-	-
Various types of ingredients									
Wiseman et al. (1992)	31	CF (13.0-510)	0.086	0.84	-	CF (13.0-510)	2.28	0.76	-
Alfalfa									
Maertens et al. (1988)	10					CP (-) CF (-)	-	0.76	-
Pérez (1994)	12					CF (208-308)	0.35	0.83	-
García et al. (1995)	5					NDF (387-550)	-	0.73	-
Wheat bran									
Blas et al. (2000)	8	ADF (98-142) CP-ADF (12.3-33)	0.030	0.75	-	ADF (98-142) CP-ADF (12.3-33)	0.57	0.72	-

RSD: residual standard deviation; R^2: regression coefficient of determination; VE: validation error.
NDF: Neutral detergent fibre, ADF: Acid detergent fibre, ADL: Acid detergent lignin, CF: Crude fibre, CP: Crude protein, DMdinv: Dry matter *in vitro* digestibility.
[1] Differentiation according to the type of diet in feeds varying widely in fibre content.

It is well accepted that these types of equations might be improved by characterizing more precisely the diet/ingredient and including the physical characteristics (particle size, water holding capacity, density, etc.), fat properties (free fatty acids, unsaturation degree, oxidation level), or the real impact of lignin on the digestibility of other cell wall constituents (Shurson et al., 2021). However, the inclusion in the prediction equations of the physical characteristics does not usually improve the precision of the estimation (Martens et al., 2019).

In vitro estimation of digestibility and fermentability

An enzymatic *in vitro* digestibility method to evaluate the nutritive value of feedstuffs in rabbits was developed by Ramos et al. (1992) adapting the one developed by Boisen (1991) for pigs. This method showed a good repeatability and reproducibility in rabbits (Carabaño et al., 2008)

although results improved when predicting digestibility of DM rather than CP. This multienzyme method shows better accuracy, repeatability, and reliability than those based on the use of digestive inocula (Pascual et al., 2000). Although the *in vitro* value does not perfectly match the *in vivo* one of diets or ingredients, both are closely correlated. Therefore, prediction equations were developed to estimate *in vivo* DM digestibility using the *in vitro* DM digestibility values, which can considerably improve the prediction based only on the chemical composition previously described.

Table 2. Equations to estimate the crude protein digestibility (CPd) and digestible crude protein (DCP) value of diets and ingredients in rabbits. Each line (shaded or not) represents a regression equation and the independent variable(s) used. The range of values expressed in g/kg dry matter is included in parentheses

Compound feeds	n	CPd	RSD	R^2	VE (%)	DCP (g/kg DM)[3]	RSD	R^2	VE (%)
Villamide et al. (2009)	111	ADL (26.6-195)	0.032	0.49	4.49	CP (134-232)	7.94	0.67	4.65
		ADL (26.6-195) CP (134-232)	0.031	0.53	4.28	ADL (26.6-195) CP (134-232)	5.53	0.84	4.35
Various types of ingredients									
Villamide and Fraga (1998)	93					CP (59-627)	7.36	0.95	-
Villamide et al. (2016)[1]	11	Ndiv (0.764-0.946)	0.053	0.80	-				-
Wheat bran									
Blas et al. (2000)	8	ADF (98-142) CP-ADF (12.3-33)	0.009	0.54	-	ADF (98-142) CP-ADF (12.3-33)	0.009	0.63	-

RSD: residual standard deviation; R^2: regression coefficient of determination; VE: validation error.
ADF: Acid detergent fibre, ADL: Acid detergent lignin, CP: Crude protein, Ndinv: Nitrogen *in vitro* digestibility.
[1]Nitrogen true ileal digestibility

Recent studies suggest that this methodology might be used to estimate the faecal digestibility of neutral detergent fibre (NDF), as it effectively ranks diets according to their NDF digestibility at the ileal level (Abad-Guamán et al., 2024). Despite the advancements in *in vitro* methods, studies conducted in pigs reported that this method does not discriminate accurately the nutritive value according to specific feed characteristics (presence of antinutritional factors, added enzymes, solubility, protein-rich ingredients, insoluble fibres, fat or minerals) or according to animal age (Noblet and Jaguelin-Peyraud, 2007; Swiech, 2017; Noblet et al., 2022).

Improving *in vitro* digestion methods is considered a priority to deepen our understanding of animal digestion physiology. Novel methods have emerged that, while not as fast, straightforward, or economically viable for immediate integration into practical feed formulation, hold great interest in providing new physiological insights. For instance, the INFOGEST static *in vitro* digestion model is an example of this trend (Minekus et al., 2014; Brodkorb et al., 2019). It is static because it uses constant ratios of food to digestive fluids and a constant pH for each digestive segment. In this method, the digestibility is not measured by the disappearance or solubility of nutritional constituents, but the residue is analysed. There are more complex *in vitro* digestion models like the dynamic ones, where the lab conditions are fitted more closely to the physiological climate, and thus the rate of passage, environmental gut conditions (pH, enzymes concentration, absorption, etc.) in the different stages (Meunier et al., 2008; Gerrits et al., 2021). In this context, the evaluation of digestion kinetics (either using static or dynamic systems) of both protein and starch seems to be of interest in practical feeding, confirming that protein solubilization does not imply a complete hydrolysis (Chen et al., 2019), and can help elucidate the potential implications of fast digested protein after weaning (Jaworski et al., 2019).

The *in vitro* gas production technique was adapted from ruminants (Menke et al., 1979) and it has been used in rabbits and other non-ruminants to estimate the GEd and DE of diets as well as to determine ingredient fermentability. It rendered positive results in the GEd prediction (Calabró et al., 1999), but it did not improve that obtained with the chemical composition-only (Stanco et al., 2003). The proposed technique used the caecal digesta as inoculum and required the slaughter of rabbits, although the number was much lower compared with those required for *in vivo* digestibility tests. More recent studies used a similar methodology to evaluate ingredient fermentability. One of the first objectives achieved was the replacement of the use of caecal digesta as inoculum. The gas production kinetics of the caecal inoculum was correlated with the values obtained with either hard or soft faeces inocula using different substrates (Bovera et al., 2006; Abad-Guamán et al., 2018, Figure 1). This indicated that the slaughter of rabbits is not mandatory, although they are required to obtain faecal samples.

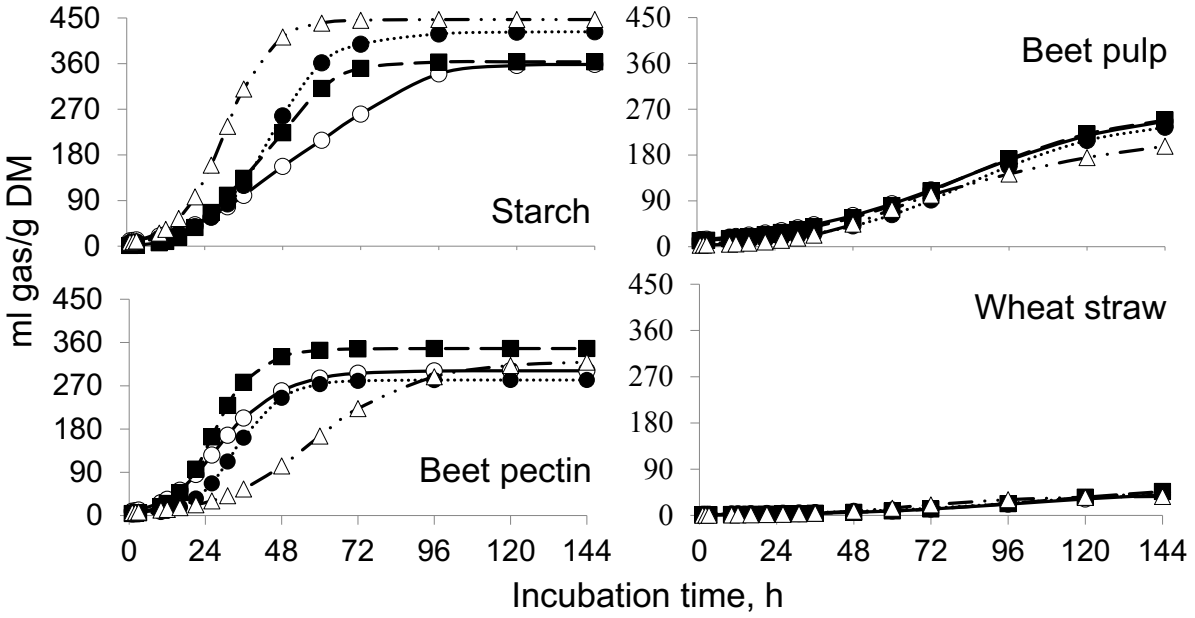

Figure 1. *In vitro* gas production (ml gas per g dry matter, DM) kinetics of four substrates with different inocula [Ileal digesta: △; caecal digesta: ■; soft faeces:○; hard faeces: ●]. (Abad-Guamán et al., 2018).

Probably the most interesting application of this method is the *in vitro* study of dietary fibre fermentability, mainly from a qualitative rather than a quantitative point of view. This is because the use of Viscozyme (a commercial cellulolytic enzyme mixture) in the *in vitro* digestibility method does not allow the study of the potential interactions between the diet and the intestinal microbiota (Ocasio-Vega et al., 2018a). However, there is still a need to further refine the methodology to better mimic rabbit physiology (pH, inclusion of endogenous substances, type of substrate fermented, etc.), as well as the standardization of the method. One of the key points is the type of sample used to evaluate intestinal fermentability. Ingredients require a pre-digestion to simulate the conditions of the digestive tract (e.g. acidity in the stomach and neutral pH in the small intestine solubilize part of the insoluble fibre; Abad-Guamán et al., 2024) and to remove the nutritional constituents that would be digested before the caecum, and thus will not be fermented in the caecum (mainly sugars, starch, protein; Rodríguez-Romero et al., 2011; Ocasio-Vega et al., 2018b). However, this pre-digestion also removes the soluble fibre, unless it is precipitated with ethanol, and the residue for fermentation would be the insoluble fibre fraction (Abad et al., 2013). This insoluble residue rendered around 30-40% of the gas produced (for 48 h) compared to the complete samples like straw or beet pulp (Al-Soufi et al., 2023) or pulps (Ocasio-Vega et al., 2024) and would not resemble the fermentability of the whole sample. In any case, this separation of the fibrous fractions according to their

fermentability before or after the caecum would be an approximation considering that a fraction of the dietary fibre is fermented before the caecum (Gidenne, 1992; Carabaño et al., 2001; Abad-Guamán et al., 2015). All these problems become less significant when fermenting low-protein low-starch high-fibrous ingredients.

Use of near-infrared spectroscopy (NIRS) for nutrient composition and nutritive value

NIRS technology is based on the interaction between electromagnetic radiation in the near-infrared region and molecules in a sample. When light in the near-infrared range falls on a sample, some wavelengths are absorbed by the covalent bonds of the molecules present in the sample. The higher the concentration of the molecules with this bond, the higher the absorption of light at this wavelength. Under that premise, the amount of light absorbed at different wavelengths provides information about the chemical composition of a sample (Siesler, 2007). The main advantages of NIRS technology are that routine analyses are non-destructive, cost-effective, fast and safe. Furthermore, once the equipment is calibrated it allows for the determination of multiple constituents in a single scan of the sample. As a counterpart, it requires the acquisition of expensive equipment and is a secondary method that requires the development of calibrations for each constituent by specialized technicians. In any case, the use of this technology is widespread in all agri-food fields, and practically all feed factories have a NIRS equipment to determine the chemical composition of their raw materials and feeds.

Although the predictive capabilities of NIRS primarily pertain to the chemical components within samples, the spectra can be correlated with more intricate parameters, such as nutritive value. This correlation is established based on the relationships that may exist between these parameters and the chemical or chemical-physical properties inherent in the samples (Bastianelli *et al.*, 2015). In this regard, we can indicate that rabbits were one of the pioneers in trying to determine the nutritive value of a feed from their NIRS spectrum (Xiccato et al., 2003a). In this trial, NIRS was used to predict the chemical constituents, digestibility and energy value of 164 experimental compound feeds for rabbits using harmonized methods in six European institutes (European Group for Rabbit Nutrition, EGRAN). Figure 2 shows the digestibility of gross energy (GE) ($R_v^2 = 0.81; SEP = 1.9\%$) and the DE content of the feed ($R_v^2 = 0.77; SEP = 0.39\ MJ/kg\ DM$) can be predicted for samples external to the calibration with good precision (R_v^2, coefficient of determination in validation; SEP, standard error of prediction). This high prediction of DE may be due to the high precision of NIRS in predicting DE's main components: starch ($R_v^2 = 0.90$) and ether extract ($R_v^2 = 0.93$), although the prediction of the NDF content was lower ($R_v^2 = 0.50$).

On the contrary, although the prediction of feed CP content is quite good ($R_v^2 = 0.86$), the ability to determine protein digestibility by NIRS is quite poor ($R_v^2 = 0.44; SEP = 2.6\%$). The digestibility of protein depends greatly on its availability and association with the fibre in the feed, especially in feeds rich in fibrous by-products, being more difficult to predict.

More recently, an alternative has been proposed in pigs based on NIRS analysis of faeces. This method does not require animal housing in digestibility cages but requires faecal sampling (Bastianelli et al., 2015). The method has a similar precision for determining the digestibility of the GE ($R_v^2 = 0.67; SEP = 1.2\%$), but it seems to improve the prediction of the digestibility of the CP ($R_v^2 = 0.62; SEP = 1.5\%$) in comparison to the values obtained for rabbit feeds.

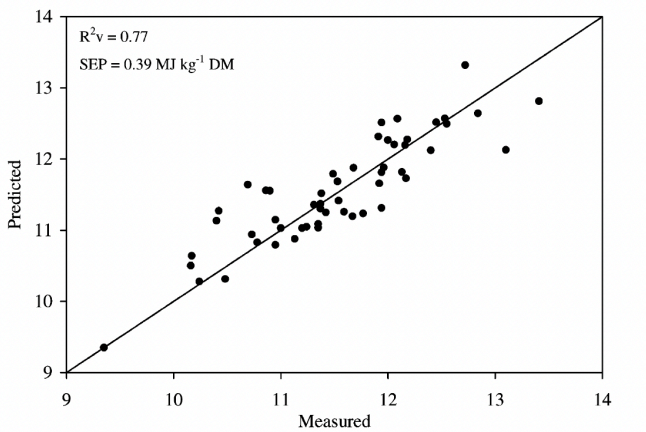

Figure 2. Relationship between near-infrared spectroscopy (NIRS) predicted and *in vivo* values for digestible energy (MJ/kg dry matter, DM) in compound rabbit feeds. R_v^2, coefficient of determination in validation; SEP, standard error of prediction (Xiccato et al., 2003a).

Other replacement methods

In silico simulations

In silico experiments (mathematical modelling) can be further used to explore changes in rabbit performance resulting from the interactions between genetic, nutrition and other environmental factors, through computational simulations. Growth in the fattening phase of rabbits is influenced by genetics and numerous environmental factors, including feed characteristics, housing conditions and the farm's health status. A reliable mathematical model can effectively predict rabbit growth during the fattening phase, eliminating the need for live animals in such predictions.

For over 50 years, numerous models have been tailored to rabbit growth (Baron et al., 1970), with the Gompertz growth curve emerging as the most widely accepted (Gompertz, 1825). This sigmoid function fits very well to the rabbit's growth curve and includes three coefficients related to biological aspects of the animal (maximum or adult weight, maximum growth rate and age at maximum growth). Several studies have studied how these coefficients change depending on the genetic type (Blasco and Gómez, 1993; Blasco et al., 2003; Juárez et al., 2020) or feeding level (Yang et al., 2020), or to verify correct animal growth (Sampaio et al., 2005). Nevertheless, there is a lack of studies exploring the impact of feed characteristics and housing conditions on these parameters. It may be prudent to conduct a meta-analysis of extensive rabbit growth data considering genetics, feeding, and housing conditions. Such an analysis could contribute to the development of valuable models for predicting animal responses to these factors.

Another function frequently modelled in other species and in rabbit females is milk yield. This trait is very important in defining rabbit females' effort and litter survival and development before weaning. In addition, the milk yield of rabbit females can be affected by many factors such as genetic type, litter size, diet, environmental conditions, age or reproductive management (Maertens et al. 2006). Typically, measuring milk production in rabbits involves separating the mother from her litter throughout the day and weighing her before and after nursing. This method takes advantage of the kits' habit of nursing only once a day. However, to minimize handling of females and their litters, and considering that rabbits consume milk only during the first three weeks, a practical alternative is to rely on the high correlation observed between the litter weight at 21 days and milk production during that period (milk yield (kg) = 0.75 + 1.75 litter weight 21 d (kg), correlation coefficient (r)=0.87 (de Blas et al., 1995); milk yield (g) = 362 + 1.69 × litter weight gain (g), r=0.91 (Fortun-Lamothe and Sabater, 2003)). Additionally, if we want to avoid the use of animals, we can estimate milk production from

lactation curve models. The first models were fitted to quadratic curves (Lebas, 1968; McNitt and Lukefahr, 1990; Sabater et al., 1993), due to their perfect fit to said model. However, the low biological interpretation of quadratic model parameters limits its usefulness. Casado et al. (2006) proposed a modified beta model [Milk yield (g/day)=k x (day/30)^{a} x (1-(day/30))^{b}] that shows a good fit, having three parameters related to the curve height (k) and milk production at the beginning and end of lactation (a and b, respectively). In fact, parameters of the beta-modified milk curve significantly change in function of feed, genetic type, reproductive rhythm and parity order, being also highly correlated with rabbit female performance and body condition.

Modelling approaches recognize animals not solely as passive production units but as active living systems. These systems intricately integrate physiological functions, including growth, reproduction, and nutrition, orchestrating the complex interaction that influences nutrient partitioning and overall performance. In other species, teleonomic models have been developed to allocate the proportions of energy of the different physiological functions (growth, maintenance, gestation and lactation) as animals grow and undergo reproductive processes, according to their genotype and nutritional environment (Martin and Sauvant, 2010). The conceptual foundation of these models relies on the integration of a regulatory sub-model, generating teleonomic impulses that govern an operational sub-model scaled with genetic parameters. Once trained, these models allow us to assess the effects of some decisions (such as genetic selection for a certain trait, changing feed energy content or using another reproductive rhythm) on the reproductive performance and lifespan of the female. Although there are no models in rabbit females yet, some first attempts have already been made (Figure 3).

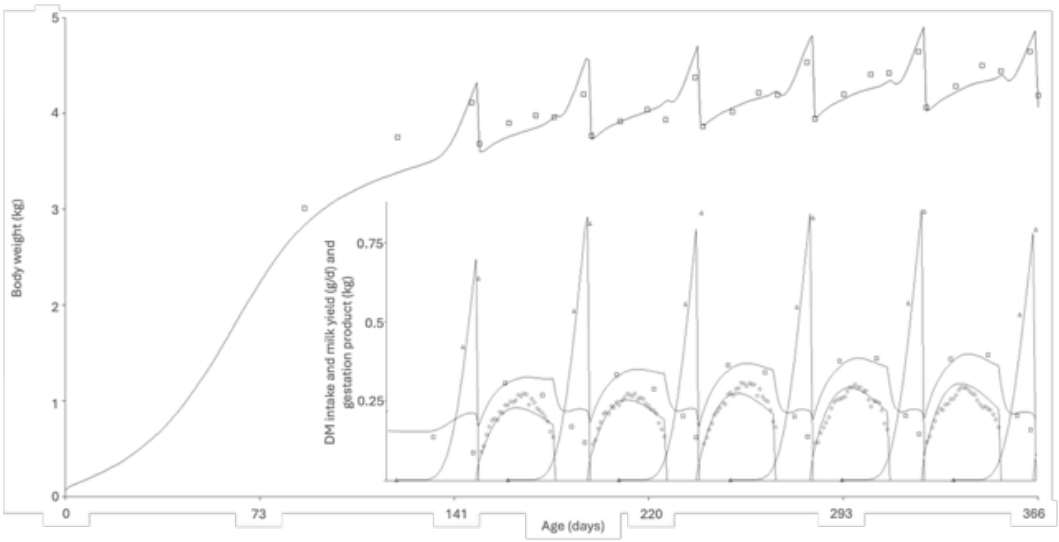

Figure 3. Model simulations of body weight (BW, kg), dry matter intake (DMI, g/d), milk yield (MY, g/d) and gestation product (GP, kg) from 3 months of age to the 6th kindling plotted with own date (Quevedo *et al.*, 2005; 2006) in reproductive rabbit does using a teleonomic model (Pascual, personal communication).

Mathematical models are being developed, with greater or lesser success, to: i) evaluate carcass conformation from *in vivo* data of growing rabbits (Deltoro et al., 1985; Hernández et al., 1996); ii) evaluate the environmental impact of rabbit farms based on the productive, reproductive and health indices obtained in the literature (Méda et al., 2014); iii) consumption of grass in open systems based on genetic, environmental and economic indices (Joly et al., 2018).

Cell/tissue/organ culture models

In vitro and *ex vivo* culture models allow simplified and controlled conditions to explore specific interactions that involves the gastrointestinal tract (Ghiselli et al., 2021). These models and techniques could be useful to understand how nutrients, feed additives and microorganism affects the gut function, gut health and host-microbiome interactions. These techniques revolve around the creation of an animal model designed to explore gut interactions by culturing cells, tissues or organs for a specified assay duration while preserving their *in vivo* characteristics.

In vitro cell-based models utilize specialized cells that grow to form a monolayer on permeable membranes, effectively mimicking the physiological structure and functionality of the *in vivo* intestinal barrier (Yeste et al., 2018). The best-known model is that of Caco-2 cells, immortal cells from human colorectal carcinoma, which when cultured create monolayers with apical microvilli that have the main morphological and functional characteristics of absorptive intestinal cells (Sambuy et al., 2012). However, there are cell lines for production animals such as pigs and cattle (e.g. IPEC-J2, Wu et al., 2020; or IBEC, Föllmann et al., 2000). In poultry, immortalized lines are not available, and primary cells from the intestinal epithelium and even epithelial cell lines from the intestinal tissue of adult chickens are frequently used (Immerseel et al., 2004). Intestinal stem cells can also be used to create 3D spherical organoids "mini guts" (Sato et al., 2009). Their use in rabbits until now has been very limited, but they have a great potential to evaluate the effect of those new additives aimed at improving the digestive health of growing rabbits (organic acids, medium chain fatty acids, phytogenic, probiotics, prebiotics...). Rabbit caecum epithelial cells has been already cultured as organoids (Mussard et al, 2020), allowing accessible apical side cells when cultures as monolayers. Caco-2 cells *in vitro* models have been also used to explore the interaction of microbiota-intestinal barrier in maturation at the suckling to weaning transition (Beaumont et al., 2020).

Ex vivo models entail cultivating living functional tissues or organs in an artificial environment outside the organism. Ussing chamber (Ussing and Zerahn, 1951) or inTESTine™ system (Roeselers et al., 2013) involves removing a segment of intestinal mucosa from an animal, which is then placed between two halves of a chamber filled with a buffer. This setup simulates the flow of mucosal and basolateral passages. These chambers are the most used systems in production animals to study how certain additives affect transepithelial transport, intestinal permeability, intestinal physiology, etc. (Ghiselli et al., 2021). An alternative is the in everted intestinal rings, where the animal's intestine is cut into ring slices and transferred into oxygenated culture media, useful to study the metabolism in different regions of the intestine (Le Ferrec et al., 2001). These systems have seen limited application in rabbits to date, primarily involving fragments of rabbit cecal tissues utilized in Ussing Chambers for paracellular permeability studies (Beaumont et al., 2020).

Other animal models (Caenorhabditis elegans)

Caenorhabditis elegans is a small nematode measuring approximately 1 mm (with only 959 cells) that has emerged as a magnificent animal model in the last decade (Figure 4). This choice is mainly because *C. elegans* is easily and rapidly reproducible in the laboratory, it is a non-pathogenic nematode, its entire genome has been sequenced, transgenic know-out mutants are available and, despite the enormous evolutionary distance, it shares common metabolic pathways and homologous genes identified with mammals (Chakravarty, 2022). It is also a bacterivore, capable of growing and reproducing in a wide variety of microorganisms. All these characteristics make *C. elegans* an adequate model of animal nutrition to carry out studies of metabolism, microbiota composition, oxidative stress and survival. To date, *C. elegans* has not been used for nutrition studies in rabbits, but they have already shown its usefulness for choosing candidates, through screening tests, before using animals in other species. *C. elegans* has been used to evaluate the effects of some nutraceuticals (ginseng, grape seed extracts) on reactive oxygen species (ROS) formation, oxidative stress gene expression and lifespan, before its evaluation on *in vivo* broiler chickens (Sandner et al., 2020, 2023). With the aim of finding a probiotic to fight against the porcine enterotoxigenic

Escherichia coli (ETEC), Zhou et al. (2014) infected *C. elegans* with ETEC isolated from porcine and tested 10 combinations of possible probiotics, controlling *C. elegans* immune response (through the expression of the clec-60 and clec-85 genes) and survival. It would serve as an animal model with significant potential for digestive health studies in rabbits, enabling the preliminary screening of potential candidates in *C. elegans*. This initial step allows for the careful selection of candidates deemed promising for further evaluation in *in vivo* tests.

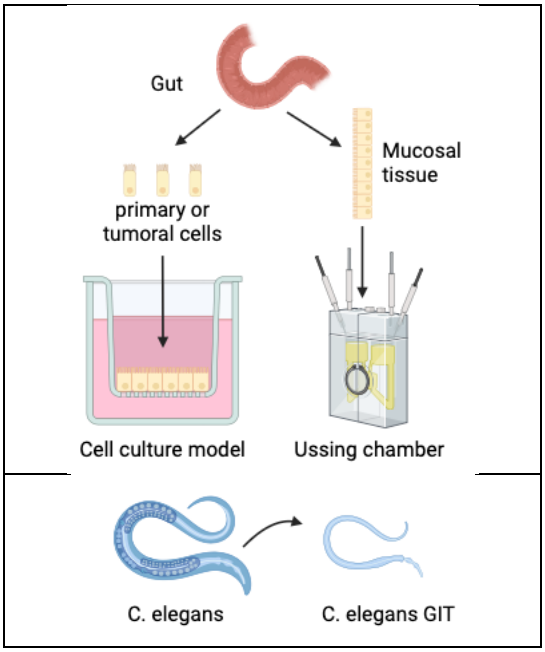

Figure 4. Scheme of the main cell/tissue culture models and of *Caenorhabditis elegans* and its gastrointestinal tract (Source: Drawn using Biorender).

REDUCTION

The first step in adhering the reduction principle is literature search. Identifying already existing knowledge is fundamental to minimize the number of animals used in experiments. Furthermore, by carefully planning and designing experiments, researchers can ensure that they gather the necessary information without the need for additional animal trials. This involves considering factors such as sample size, control groups, and experimental conditions. In nutrition research, prior characterization of the product to be evaluated, precise feed formulation and analytical verification of nutrient levels are considerably relevant, as well. Amongst these factors, sample size plays a critical role in the reliability and validity of research findings. When sample size is not appropriately addressed it can lead to failed experiments. Finally, collaborative work between researchers is increasingly promoted every day, both at an inter- and intradisciplinary level. This collaboration allows us to progress faster, avoid duplicated efforts and open up to new approaches not yet explored, also contributing to reducing the number of trials and animals involved.

Understanding previous work and existing methodologies before designing an experiment

The European Reference Laboratory for alternatives to animal testing (EURL ECVAM) provides fundamental tools, databases and inventories that can significantly contribute to finding available, validated and accepted alternative methods to animal testing. The EURL ECVAM Search Guide (Roi and Gune, 2013) provides guidelines for good search practices on alternatives to animal experimentation. Additionally, EURL ECVAM Dataset on Alternative Methods to Animal Experimentation (European Commission JRC, 2019) includes a comprehensive database (available in Excel) with detailed protocols on methods that can be

used in practice. Moreover, there is also an inventory of the 3Rs knowledge sources (Holley et al., 2017). This inventory includes more than 800 knowledge sources (including hubs, organisations, events, expert groups, etc.) and identifies the ways in which these share information (available in Excel, as well). Finally, the EURL also includes TSAR online tool (TSAR - Tracking System for Alternative methods towards Regulatory acceptance, https://tsar.jrc.ec.europa.eu) where progress towards acceptance of non-animal methods, for testing chemicals or vaccines, for instance can be monitored.

Also following the reduction concept, the European Food Safety Authority (EFSA) accepts extensive literature searches to provide evidence of safety and efficacy of feed additives (EFSA FEEDAP Panel, 2021). In its guidelines, it includes specifications on how to conduct systematic reviews including temporal scope, databases, keywords, etc. (Glanville et al., 2014). Literature searches should be done in a structured manner, including all relevant sources (conferences, books, journals and others). This can not only help identify research gaps in knowledge and areas where further research is needed; but more importantly, it can be valuable to discard others where sufficient and valid research has already been conducted.

Meta-data analysis is a term that refers to any secondary analysis of the findings of other primary research studies (Edwards, 2014), bringing a synthetic perspective to the large-scale evaluation of empirical literature. In this way, meta-analysis allows the summarization of the literature data through statistical procedures that produce empirical models. Sometimes, nutritional effects can lead to contradictory results depending on the experimental conditions, making it difficult to reach conclusions. Meta-analyses can allow us to draw main effects and obtain valuable conclusions from available studies, creating evidence from this variability and avoiding the need for additional studies. Its main enemy is the sources of heterogeneity, so the aforementioned harmonization efforts contribute to the execution of more robust meta-analyses. Some recommendations for animal nutrition meta-analysis can be found in Sauvant et al. (2008). In rabbit nutrition, few meta-data-analyses are available in the literature (e.g. Trocino et al., 2013).

Finally, open research data initiative is based on the data obtained in each experiment being available in open-access repositories, enabling anyone to access them. For its proper use, researchers and research institutions recommend that databases comply with the FAIR principles of findability, accessibility, interoperability, and reusability (Wilkinson et al., 2016). Allowing open access to our data can be useful to assist in the development of mechanistic systems, such as those *in silico* animals set out above, that allow us to model complex biological systems, or evaluate existing ones, without the need to use animals. In this framework, Open Science can also contribute to reducing the use of animals (Janssens et al., 2023). Diederich et al (2022) described the main reasons why Open Science can be useful to the 3Rs principle, among which we could project: i) to learn from prior knowledge and outcomes can avoid the repetition of studies; ii) to understand which approaches have been successful and which have not, and discard failed or biased designs; iii) to accelerate the access to information and non-animal alternative methods or methods with less suffering; and iv) to allow access to data, as well as validated harmonized protocols and methods, that contribute to walking a common path.

Failed experiments and sample size calculation

The decision about how many replicates per treatment are needed requires the researcher to dedicate time to calculate the proper number. Experiments carried out with an insufficient number of replicates (rabbits) are useless, and those carried out with an excess of replicates (rabbits) breach the ethical codes of research. Scientists are obliged before beginning experiment i) to define accurately what will be the hypothesis/objectives to evaluate, ii) to identify the experimental resources available (number of pens, cages, animals, etc.), iii) to elaborate an experimental design and setting the number of treatments according to the

objectives and the resources available. Today the main problem is that there are still too many useless experiments, that are carried out with no hope of success, just from the beginning because the 'iii' point was not done properly, because of the insufficient number of experimental units used. Usually, the only fixed factor for the scientist is the number of pens/cages available in the facilities, and it conditions the number of treatments or even if the experiment is worth being done or not. There are some recommendations regarding the minimal number of replicates that should be used (García et al., 2001; Fernández-Carmona et al., 2005; Gómez-Conde et al., 2011). However, each researcher should be able to calculate the minimal number of replicates per treatment required to detect a fixed difference between two means. To identify the number of replicates per treatment required it is necessary to know: a) the size of the difference that we want to detect as significant (*D*), b) the level of significance, usually $\alpha = 0.05$ (that fixes the maximal type I error), it allows to calculate the t-student value depending on the degree of freedom, c) the power of the analysis, usually β ranges from 0.70 to 0.80, depending on the objective (to minimize the type II error), that also indicates the t-student value depending on the degree of freedom, and d) the standard deviation (*s*) of the trait studied that is expected in the experiment (*s*). The expression would be:

$$\text{Sample size (n/treatment)} = s^2 \times (t_\alpha + t_\beta)^2 \times 2 / D$$

When the number of replicates per treatment is higher than 10, we could 'simplify' and consider $t_\alpha \sim 2$ and $t_\beta \sim 0.86$, obtaining a simpler expression:

$$\text{Sample size (n/treatment)} = s^2 \times (2.86)^2 \times 2 / D$$

Collaborative networks and use of harmonized methods

In the case of rabbit science, collaboration is extremely valuable. Rabbit farming must face the same challenges as the rest of the zootechnical species, with a small number of researchers and resources, so the effective organization of the scientific effort is essential (Pascual, 2009). The development and agreement of harmonized procedures contribute to all research groups working under similar conditions. The use of these harmonized methods favours the direct comparison of the results obtained and the reach of effective conclusions with a fewer number of studies. In the field of rabbit science, there are harmonized methods in the field of meat research (Blasco and Ouhayoun, 2010), as well as in the field of rabbit nutrition, where the EGRAN have dedicated significant effort (Table 3) to harmonize and improve the main methods used for feed evaluation (Gidenne, 1999). The development and use of these harmonized methods should always be promoted.

REFINEMENT

Opportunities for refinement involve modifying protocols to minimize pain and other negative emotions, distress, or discomfort experienced by animals. This implies numerous aspects related to husbandry and conditions of care. Therefore, refinement measures are complex to assess, as husbandry, animal care and management conditions are variable and depend on the objective of the research. In rabbit nutrition trials, as a first refinement step, special attention should be given to housing. Moreover, technology has a high potential to make animal research less invasive as well as more objective, informative, and efficient. Sensors enable continuous and automatic monitoring of animals on a massive number of animals, without the need to disturb them (Pérez-Enciso et al., 2021). It can also be useful to avoid confined housing of animals to obtain individual data. Nevertheless, most of these applications are still in the early phases of ongoing research and development and require further validation. Finally, refinement in scientific research also includes identifying other animal species with lower neurological development. For example, using rodents instead of rabbits, with quite similar digestive physiology, can be considered such a refinement approach.

Table 3. European Group for Rabbit Nutrition harmonized methods, in chronological order.

Methods	Title	Reference
Chemical composition	European ring-test on the chemical analyses of feed and faeces: influence on the calculation of nutrient digestibility in rabbits	Xiccato et al. (1996)
	Attempts to harmonize chemical analyses of feeds and faeces for rabbit feed evaluation	EGRAN (2010)
Faecal digestibility	European reference method for *in vivo* determination on diet digestibility in rabbits	Pérez et al. (1995b)
	European ring-test on *in vivo* determination of digestibility in rabbits: reproducibility of a reference method in comparison with domestic laboratory procedures	Pérez et al. (1995a)
	A critical approach of the calculation procedures to be used in digestibility determination of feed ingredients for rabbits	Villamide et al. (2001)
Ileal digestibility	Effect of ileal cannulation on rabbit digestion and caecotrophy: an interlaboratory study	Gidenne et al. (1994)
	Measurement of ileal digestibility in rabbits: an interlaboratory study to compare two markers and two frequencies of digesta collections	Blas et al. (2000)
	Interlaboratory study on ileal digestibility in rabbits: the effect of digesta collection time and a simplification of the procedure	Blas et al. (2003)
Nutritive value prediction	Nutritive evaluation and ingredient prediction of compound feeds for rabbits by near-infrared reflectance spectroscopy (NIRS)	Xiccato et al. (1999)
	European harmonisation of Near Infrared Reflectance Spectroscopy (NIRS) analysis on rabbit feeds: first steps	Xiccato et al. (2000)
	Comparison among methods of nutritional evaluation of fibrous ingredients	Villamide et al. (2000)
	Prediction of chemical composition, nutritive value and ingredient composition of European compound feeds for rabbits by near infrared reflectance spectroscopy (NIRS)	Xiccato et al. (2003a)
	In vitro analysis, an accurate tool to estimate dry matter digestibility in rabbits. Intra- and inter- laboratory variability	Carabaño et al. (2008)
	Prediction of the nutritional value of European compound feeds for rabbits by chemical components and *in vitro* analysis	Villamide et al. (2009)
Others	Collaborative studies on caecotrophy in adult rabbits: Effect of feed intake and methodology	Carabaño et al. (2000)
	Nutritive value of raw materials for rabbits: EGRAN tables 2002	Maertens et al. (2002)
	Recommendations and guidelines for applied nutrition experiments in rabbits	Fernández-Carmona et al. (2005)

Next, we describe some applications and advancements that could be applicable (at present and in the future) for refining rabbit nutrition trials. These also include omic tools to optimize the use of experimental animals and obtain as much information as possible from them using non-invasive methods, whenever possible.

Enriching of individual and group housing

Housing characteristics, specifically in terms of floor space and vertical space, type of flooring and enrichment materials (platforms, hiding places, and gnawing objects) need to be considered. Barren cages isolate animals from their surroundings. According to Reihardt and Reinhardt (2006), these types of enclosures, which are very common in all types of rabbit experimentation settings, are probably a significant stressor for animals, because space is biologically extremely frightening as it exposes them to the eyes of potential predators. Therefore, providing enrichment material might help animals perform natural behaviour and ameliorate the effect of stressors in the environment (Rommers et al., 2014). Some recommendations for rabbit enrichment materials are hiding pipes (Van Damme et al., 2023) to provide animals with escape areas, gnawing sticks or blocks for chewing (Huang et al., 2021; Rommers et al., 2014) and elevated platforms (Mikó et al., 2012).

Additionally, social housing is a fundamental aspect of refinement and yet controversial and contradictory. Wild rabbits usually live in small groups comprising one to four males and one to nine females (Trocino and Xiccato, 2006). Hawkins et al. (2008) reported group housing can

represent a stimulating form of enrichment when establishing refining guidelines for those working with rabbits in animal research. As a counterpart, group housing in rabbits is associated with aggressions, bullying, and fighting to establish a social hierarchy, particularly when these hierarchies have not been previously defined. In rabbit females permanently housed in groups, aggressive interactions and mortality of reproducing does and kits have been reported (Pérez-Fuentes et al., 2020; Szendrő and Dalle Zotte, 2020). Despite these problems, some authors indicate that the overall benefits of group housing could exceed those for single caging (Morton et al., 1993). This could also be achieved with semi-group or part-time housing (Van Damme et al., 2022). Moreover, individual housing can alter feeding behaviour (more intake) and digestive processes (usually a lowering of nutrient digestibility). However, for specific experimental reasons or health conditions, rabbits may still need to be housed individually (i.e. digestibility trials). But limiting the confined periods, applying specific group management practices and incorporating functional areas and the right enrichment materials (i.e. refuges and hiding places for subordinate animals to evade potential harm or aggression) are considerably helpful when addressing refinement without causing unwanted social problems, injuries and aggressions.

Morton (1993) describes there are five major factors to be considered in rabbit group housing: compatibility of individual animals, size of pens, stocking densities, husbandry practices and environmental enrichment. When possible, animals should be of the same sex, of similar size and, preferably, related and grouped when young, i.e. around the time of weaning. Moreover, rabbits housed in social groups benefit not only from the company of others but also from more functional space for exercise, hopping or other behaviours. The structural complexity of housing gives animals things to do (other than eating or drinking) providing them the opportunity for positive emotional experiences with intrinsically motivated behaviours that could contribute to their positive welfare (Boissy et al., 2007).

Finally, training animals and personnel can serve as complementary refinement tools in rabbit nutrition research. Non-aversive handling and training techniques for experimental animals are required to facilitate experimental and routine husbandry procedures, improving human-animal interactions and both animal welfare and scientific quality (Hohlbaum et al., 2024).

Automatic animal monitoring using precision livestock farming (PLF) tools

Refinement methods should be compatible with the experimental needs and should not interfere with research data. Sensors (particularly remote sensors) can help monitor animals in a non-invasive way. Wearable sensors can furthermore help collect data from individual animals while still housed in groups. PLF can be defined as the management of livestock production using the principles and technology of process engineering (Wathes et al., 2008). PLF technologies can provide real-time, continuous feedback from animals' physiological changes (e.g. body weight and body condition) and behavioural changes (e.g. feeding and drinking) and can thus serve as fundamental tools in minimizing the manipulation of animals in nutrition experiments. Therefore, there is a need to develop research and acquire more knowledge on PLF applications for individual rabbit identification, tracking and automated management that can help monitor individual or small groups of animals without disturbing them.

Accelerometers, temperature sensors and radio-frequency identification (RFID) devices are commonly used as wearable sensors attached to animals to measure physiological variables. Some examples of sensors and applications include: (1) thermometers and infrared cameras to measure body temperature, (2) implants, sensors based on photoplethysmography or electrocardiography to measure heart rate, (3) digital humidity sensors to measure sweating, (4) boluses to measure intestinal health, (5) accelerometers, pedometers, and GPS to measure activity, location, and movement speed, and (6) RFID to identify and measure

location and behaviour such as electronic feeders to measure feeding behaviour (Cambra-López, 2023).

However, remote vision and sound technologies are becoming highly relevant and are preferred because they are non-invasive. Remote sensors are associated with cameras or microphones. In recent years, there has been a significant transformation in computer vision and image analysis, primarily driven by the emergence of specialized machine learning tools such as deep learning. Digital image analysis, which represents the process of extracting meaningful information from images, can be used as input for imaging processing techniques with direct application for livestock phenotyping (Silva et al., 2021). Cameras for individual animal identification can be used by varying the appearance (conformation and coat colour) of the entire animal, visual marking, or working in combination with RFID tags.

Body weight estimation and biometric measurements

Live weight can be accurately measured using 3D imaging and conformation features can be measured from images (Pérez-Enciso et al., 2021). In rabbits, computerized image analysis has been used to predict biometric measurements and weight in adult rabbits (Negretti et al., 2007). These authors reported low standard errors of this technique and reported it was a viable, quick and practical mean to measure and select for weight and morphological traits as head length, ear length, body length and body side surface of rabbits.

Additionally, visible spectrum cameras have been suggested both to predict group feed consumption based on images of remaining feed in the feeder (Duan et al., 2022) and to predict the area they occupy in their space/housing using planimetric data (Giersberg et al., 2015). These approaches, however, although obtained with training image datasets and validated with different ones, have not been extrapolated to practical settings/images (Duan et al., 2022). The models defined in Giersberg et al. (2015) were not validated against real allometric measurements, but serve as a valuable reference for ethological spatial needs and housing design.

Body condition and composition estimation

Traditionally, the reference method used to determine body condition, carcass composition and nutrient retention has been the comparative slaughter (Xiccato et al., 2003b and 2004), but it is a highly invasive method and does not allow following the nutrient retention or the evolution of body and carcass composition over time on the same animal. An alternative to comparative slaughters is the *in vivo* monitoring techniques of body condition and composition (Pascual et al., 2005). Although they are not exactly refining methods, their use reduces the number of slaughtered animals. Several methods validated in other species have been tested in rabbits, such as infrared radiation (Masoero et al., 1992), deuterium oxide dilution (Fekete, 1992), X-ray tomography (Romvari et al., 1996), nuclear magnetic resonance (Köver et al.,1998), and total body electrical conductivity (Fortun-Lamothe et al., 2002). However, the most extended methods in the last decades were ultrasound measurement of perirenal fat thickness (PFT) and the bioelectrical impedance analysis (BIA) to evaluate body condition and composition in rabbits, respectively.

Perirenal adipose tissue is the most important lipid storage in rabbits and its size varies the most depending on the physiological state (Pascual et al., 2004). For that reason, PFT measurement with ultrasound equipment has frequently been used to assess the evolution of body condition in rabbit females (e.g., Savietto et al., 2016). PFT is a non-invasive and brief method (1-2 min), which requires shaving the area to be scanned (8^{th}-9^{th} thoracic vertebra area), without the need for anaesthesia and using portable equipment (Pascual et al., 2000). PFT is reliable in young (R^2=0.90; CV=5.1%; Pascual et al. 2000) and reproductive rabbit does at different reproductive stages (R^2=0.67-76; CV=7.6-9.8%; Pascual et al., 2004).

The BIA is a non-invasive approach that can accurately assess *in vivo* changes in the body composition of rabbit does (Nicodemus et al., 2009; Pereda, 2010) and growing rabbits (Saiz del Barrio et al., 2017 and 2022). It has been used in rabbit does and has been correlated with their reproductive success (Rebollar et al., 2011; Taghouti et al., 2021). The regression equations developed and validated so far can successfully predict growing rabbits and doe's body content in moisture, protein, fat, ash and energy. Measurements are conducted with a four-terminal analyser that generates an alternating current of 425 μA of intensity at a frequency of 50 kHz. It is provided with 2 black electrodes to conduct the electrical current through the doe's body, and 2 red electrodes to register the resistance and reactance resulting from the passing current. When it is used with rabbit does and growing rabbits, a needle has to be inserted in the extremity of each electrode and introduced through the animal's skin at four reference points along the loin. Impedance is defined by the equation: Impedance = $(\text{Resistance}^2 + \text{Reactance}^2)^{1/2}$. One of the main advantages of BIA is that it allows us to estimate the evolution of body composition over time and determine nutrient retention without the need to slaughter different aged animals. On the contrary, it requires manipulation of the animals, which need to remain still during the procedure and the subcutaneous placement of the electrodes.

Electronic feeders with individual animal identification

Individual identification of animals and automated feeding systems have already been tested and successfully applied in animal species (Norton et al., 2019). In rabbits, an electronic feeder prototype, specifically designed for fattening rabbits has been developed and is currently being used for genetic selection purposes (Sánchez et al., 2024). This feeder enables the automatic and individualized recording of the feed intake and efficiency in rabbits housed in groups using RFID. However, this prototype is still susceptible to improvement in terms of reducing errors. In rabbit performance experiments, feed efficiency is registered in individual pens, where animals are kept in isolation during the whole fattening period following Fernández-Carmona et al. (2005) guidelines. By using electronic feeders, individual feed intake data could be obtained without the need to confine animals, replicating the real rearing conditions more accurately. A similar prototype is being developed for rabbit does (Cambra-López et al., 2023).

Although these tools are still in the developmental stage, in parallel with the use of data from electronic feeders in the rabbit species, the use of wearable sensors such as triaxial accelerometers is also being explored (Piles et al., 2023). The acceleration information from each of the spatial axes has been used to quantify the level of activity in fattening rabbits and predict the type of behaviour. This work opens the door to quantifying the energy expenditure associated with the physical activity of the animals, which can explain individual variation in feed efficiency, obtained with electronic feeders as previously explained.

In summary, the real implementation of technological PLF tools is still complex due to robustness, integration and reliability issues. The availability of robust hardware is still challenging. Although there is a wide range of sensors available, not all are designed for use in small animals like rabbits or optimized to work in harsh farm environments. Once we have robust sensors, the next challenge lies in collecting data and proper labelling. The challenge lies in transforming sensor data into reliable and robust algorithms that can answer the question at hand and predict the response accurately. The key is to carefully choose the variable(s) to measure, as animals can express the same phenomenon (e.g., heat stress) in various ways; and to have a correct reference criterion to verify the entire process. This process is complex, lengthy, and requires manual labelling of numerous data (e.g., animal behaviours) to train artificial intelligence-based algorithms. Connectivity and the integration of data from various sensors is another limitation in many cases, as is data storage capacity. Lastly, probably one of the greatest barriers to implementation is the reliability of some automated systems. Reliability increases as the algorithms they use are trained with broad and sufficiently representative databases of the diversity and complexity found on commercial farms. Consequently, we still need further research to validate already existing PLF

applications and sensors, but also to develop new prototypes in close collaboration among researchers, the sector, and the industry.

Other refinement methods: non-invasive sampling for physiological and genetic traits

Genetic tools have become indispensable in animal science including nutrition. The preferred methods used for DNA collection are blood or tissue sampling. However, non-invasive genetic analysis is a powerful tool that avoids animal over-handling (Ben Larbi et al., 2012). Hair, saliva, skin, buccal swabs and intestinal epithelial cells recovered from faeces are the preferred biomaterial for genetic studies (Zemanova et al., 2020). In rabbits, Fontanesi et al. (2007) reported the use of hair and buccal swab sampling for DNA gene expression analysis. More recently, Ben Larbi et al. (2012) evaluated blood, hair, ear biopsies and faeces as DNA sources. However, they concluded that the use of such non-invasive samples as a source of genetic material is a recent and very promising technique, especially for the study of endangered species, but these techniques are still too unreliable and costly to altogether replace invasive techniques when the latter is possible.

Omics techniques, including RNAseq, represent a powerful toolset for precisely examining gene expression changes in response to dietary interventions. With RNAseq, investigation on specific genes that are turned on or off when subjects are exposed to different dietary compositions can be performed. This level of precision allows for a comprehensive understanding of how dietary factors influence gene regulation, providing valuable insights into the mechanisms underlying nutritional responses and potential health outcomes, with minimal animal intervention (i.e. acquiring meaningful and massive data from a minimal number of individuals). Some attempts have been conducted to evaluate the effect of nutritional interventions on liver metabolism (Yasoob et al., 2022; Wang et al., 2008), feed restriction on expression of skeletal muscle differentiation-related genes (Li et al., 2023).

Finally, Pietro et al. (2019) reviewed specific biomarkers of digestion and absorption capacity, intestinal barrier function and gut microbiota, in urine and faeces, that could be interesting in rabbit nutrition research but none have been specifically validated in rabbits. When evaluating a rabbit's stress response related to feeding management and/or housing conditions, determining corticosterone levels in the rabbit's hard faeces and hair (Trocino et al., 2014; Trocino et al., 2022), rather than in blood, has been addressed in both growing rabbits and reproducing does. Trocino et al. (2014) proposed that measuring corticosterone levels in faeces can be useful for assessing acute stress, while corticosterone levels in hair can indicate chronic stress in rabbits. Further research is thus encouraged in this field.

CONCLUSIONS AND RECOMMENDATIONS

The 3Rs principles encourage researchers to prioritize replacing animal use with alternative methods whenever feasible, minimize the number of animals in experiments, and enhance measures to refine procedures and mitigate the suffering and stress experienced by animals. Achieving complete replacement of rabbits in nutrition research remains a distant goal. However, in the last decades, there has been a notable effort to provide reliable methods, particularly to predict the nutritive value of ingredients and diets which can contribute to the replacement of a significant number of animal trials.

Validated replacement methods in rabbit nutrition research such as prediction equations based on chemical composition, *in vitro* digestibility and fermentability methods, and the use of NIRS technology are aimed at this goal. The equations and methods reviewed herein can be readily utilized and are therefore recommended to estimate the energy and protein value of ingredients and compound feeds. Their limitations, however, in terms of accuracy, repeatability and reliability have been discussed, together with their ability to account for changes in diet

components and feed characteristics (i.e. physical). To sum up, recommendations and limitations of these alternative/replacement methods are presented in Table 4.

Table 4. Recommendations of methods implementing the 3Rs principle of Replacement.

Recommended replacement method	Instead of	Limitations
Prediction equations based on chemical composition to estimate energy and protein value of ingredients and compound feeds	Animal digestibility trials	Estimation of protein values is more precise than energy values. Energy values show high variability depending on differences in ADF value, levels of pulp, straw or fat. Method does not account for intrinsic physical characteristics of feed, fatty acid profiles and real impact of lignin.
In vitro digestibility and fermentability techniques	Animal digestibility trials and slaughter	Alternative methods do not account for animal-related factors (i.e. age) or changes in feed characteristics such as antinutritional factors, exogenous enzymes, solubility, protein-rich ingredients, insoluble fibres, fat and minerals.
NIRS for nutritive value	Animal digestibility trials	Higher accuracies for energy than for protein value. Expensive equipment is needed. The efficacy depends on calibrations for each constituent and specialized staff.
In silico simulations	Animal performance and reproductive trials	Lactation curves can successfully predict milk yield in rabbit does, but developed growth models still do not account for changes in feed characteristics or housing/environment conditions. No validated teleonomic models exist in rabbits.
Culture models	Animal trials and slaughter	Only caecum epithelial cells and Caco-2 cells have been successfully cultured in rabbits. *Ex-vivo* models are still very scarce.
Other animal models	Animal trials and slaughter	Yet not validated in rabbits.

Although scientists are encouraged to replace the use of animals with alternative methods in rabbit research, however, these methods must be analysed in the light of the current knowledge and unique characteristics of rabbits. There is still a notable lack of comprehensive understanding of interactions between feed composition and animal-related (age and physiological state), feeding management (*ad libitum* or restricted) and housing/environmental-related factors that directly affect nutritive value. To improve current procedures and develop robust and precise alternatives, an exhaustive and representative *in vivo* database is still fundamental. Therefore, more *in vivo* rabbit trials are still necessary to elucidate such interactions and improve the nutritional evaluation of ingredients and diets, allowing a precise estimation for each animal category (*vs.* the single value currently available). More research in this direction is thus needed to achieve successful replacement, together with emphasizing the ongoing efforts to validate and harmonize these methods. Reduction and refining recommendations have been established and substantially rely on extensive literature reviews and sound statistical methods, promoting Open Science principles, and collaboration networks for achieving the Reduction principle; and enriching the housing environment, the use of non-invasive PLF applications, non-lethal and alternatives to blood samples, and omic tools for contributing to Refinement.

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EFFECTS OF DIGESTIBLE ENERGY SOURCES AND LEVELS IN DIETS ON REPRODUCTIVE PERFORMANCE OF NEW ZEALAND WHITE CROSSBRED RABBITS

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ABSTRACT

Molasses was a by-product of the sugar processing industry which could be supplemented for rabbits. In the Mekong Delta of Vietnam, rabbit producers fed locally available feedstuffs including natural grasses, wild vegetables, and agro-industrial by-products. This study aimed to evaluate the effects of maize and molasses supplementation, at different levels, in diets on the reproductive performance of does through two litters. The experiment included 30 New Zealand White crossbred doe rabbits, aged between 6-6.5 months and with an average weight of 2680 ± 58.69 g/per. The rabbits were arranged in a factorial design with two factors and five replications, with one doe rabbit per experimental unit. The first factor was the energy source supply in the diet (maize and molasses), and the second factor was the levels of digestible energy (DE) in the diet (10.5; 11.0; and 11.5 MJ/kgDM). The results showed that DM,OM, CP, EE, DE intake were improved at the level of DE11.5 and CP intake had an interaction between energy sources and DE levels in both two litters. Postpartum weight gain increased following increasing DE levels in the first litter, however, it was improved in the molasses group in the second litter. Milk yield was increased following increasing DE levels in the second litter. There was an interaction between energy sources and DE levels in terms of litter size at birth and litter size at weaning in the second litter. In conclusion, disgestible energy at level of 11.5 MJ/kgDM improved in terms of nutrient intake and milk yield.

Keywords: crossbred rabbits, digestible energy, energy sources, molasses, maize.

INTRODUCTION

Rabbits are herbivores that not only consume agro-industrial by-products but also convert 20% of their protein intake into meat, compared to 16-18% in pigs and 8-12% in cattle (Thu and Dong, 2011). Therefore, it is possible to meet the requirements for high-quality protein in human food. Molasses is a by-product of the sugar processing industry that contains sucrose, glucose, and fructose (Trach, 2003). Additionally, corn kernels are an excellent source of energy in the diet and are rich in carotene, which can be supplemented for cattle.

The disgestible energy requiremennts for different stages of rabbits were 2550 DE/kg diet for weanling rabbits (Maertens, 1992), 10.7 MJ/kg diet for breeding does, and a DE content of fattening rabbits of 9.7-11.5 MJ without any adverse effects (de Blas and Mateos, 2020). The use of maize and molasses in rabbit diets as energy sources not only helps utilize agricultural by-products but also reduces production costs. However, studies on these feeds in terms of livestock production in Vietnam have been still limited. The objectives of this study were to determine the optimal level of digestible energy in the diet for the reproductive performance of crossbred rabbits and to identify an appropriate energy source for reproductive rabbits.

MATERIALS AND METHODS

The study was performed at Experimental Farm, Can Tho University, Vietnam. All procedures were carried out in compliance with the ethical standards stated in the Helsinki Declaration of 1975, revised in 2000, in addition to following the national laws.

Animals and Experimental Design

The experiment consisted of 30 New Zealand White crossbred doe rabbits (New Zealand White x local) that were produced at the farm and were 6-6.5 months old with an average weight of 2680 ± 58.69 g/per rabbit. These rabbits had been fully vaccinated against parasitic diseases, rabbit hemorrhagic disease, and respiratory diseases.

Table 1: Ingredients and chemical composition of experimental diets

Ingredients (%)	Molasses			Maize		
	10.5	11.0	11.5	10.5	11.0	11.5
Soya waste	29	30	23	27	24	24
Soybean extraction meal	30	33	34	29	30	30
Maize	0	0	0	7	17	25
Molasses	4	9	18	0	0	0
Pennisetum purpureum	37	28	25	37	29	21
Total, %	100	100	100	100	100	100
CP (%DM)	22.0	22.7	21.9	21.7	21.5	21.3
DE, MJ/kgDM	10.5	11.0	11.5	10.5	11.0	11.5

CP: crude protein, DM: dry matter
DE 10.5, DE 11.0, DE 11.5: Digestible energy levels in the diet.

The experiment was arranged in a factorial design comprising two factors and five replications. Factor 1 was the energy source supplied in the diet, namely maize and molasses, while factor 2 was the digestible energy (DE) levels in the diet, which were 10.5, 11.0, and 11.5 MJ/kgDM. Each unit of the experiment consisted of a 6.0-6.5-month-old New Zealand White crossbred doe. The rabbits were housed in individual cages and provided with free access to clean drinking water nipples. The composition and ingredients of the experimental diets were presented in Tables 1 and 2.

Table 2: The chemical composition of raw materials used in the experiment

Ingredients	DM	OM	CP	EE	NDF	ADF	CF	Ash	DE, MJ/kgDM
Soya waste	12.7	96.0	18.0	9.23	32.4	27.8	15.5	4.00	10.9
Soybean extraction meal	92.3	93.8	42.3	3.90	23.7	13.5	6.60	6.20	12.6
Maize	90.5	98.2	8.20	3.30	15.8	3.90	1.70	1.80	13.6
Molasses	69.1	87.2	3.50	-	-	-	-	12.8	15.3
Pennisetum purpureum	17.9	89.2	10.8	2.66	64.1	45.9	31.5	10.8	7.91

DM: dry matter, OM: organic matter, CP: crude protein, EE: Ether extract, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, CF: Crude fiber, DE: digestible energy (Perez *et al.,* 1998)

Data collection and management

The rabbits were fed three times a day at 7:00, 12:00, and 17:00h. The experimental diets contained between 21.3 g to 22.7 %DM and DE from 10.5 – 11.5 MJ/kgDM. The feed offered and refused were weighed daily in the morning to calculate feed intake. All feeds were analyzed for chemical composition and calculated DE for the treatments. The samples were collected every two weeks and dried at 55^0C for 48 hours until the weight remained unchanged. They were then finely ground for chemical composition analysis.

Rabbits were weighed individually every week. The monitoring indicators included: feed daily intake, pregnancy, and post-partum weight weekly, litter size at both birth and weaning, litter weight at both birth and weaning, milk yield of doe, weight of opening-kits, weight of 21-day kits, interval of partum two litters and economic efficiency.

Statistical Analysis

All data were analyzed with the General Linear Model of Minitab 13.21 program (Minitab, 2016). Data were analyzed using the model yijk = μ + Ti + Aj + Pk + eijk; where yijk: the

dependent variable, μ: the overall mean, Ti = the effect of treatment (i = 1 to 4), Aj: the effect of animal (j = 1 to 4), Pk= the effect of period (j = 1 to 4), and eijk = the random error. The significance of pairwise comparisons was determined by Tukey posttest. Significance was declared at $P<0.05$.

RESULTS AND DISCUSSION

Effect of digestible energy on nutrient intake and reproductive performance in the first litter

DM, OM, EE, and DE intake between the two groups of maize and molasses tended with higher in the maize and lower in the molasses group ($P<0.05$, Table 3). DM, OM, EE, and DE intake increased ($P<0.05$, Table 3) with the gradual increase of DE levels in the diet. CP and EE intake had the interaction between energy sources and DE levels ($P<0.05$, Table 3). Postpartum weight gain was gradually increased ($P<0.05$, Table 3) followed by increasing levels of DE in the diet.

Table 3: Nutrient intake and reproductive performance of doe rabbits in the first litter

Items	Energy sources		DE levels, MJ/kgDM			SE/P-value		
	Maize	Molasses	10.5	11.0	11.5	E.S	DEL	E.S*DEL
Nutrient intake on average pregnancy and lactation period, gDM/day								
DM	132	127	117^{c}	131^{b}	141^{a}	1.471/0.032	1.802/0.001	2.549/0.880
OM	124	117	109^{c}	122^{b}	131^{a}	1.312/0.002	1.607/0.001	2.272/0.901
CP	28.8	28.4	26.2^{c}	29.0^{b}	30.7^{a}	0.158/0.066	0.193/0.001	0.273/0.015
EE	6.30	5.66	5.68^{c}	6.03^{b}	6.23^{a}	0.039/0.001	0.048/0.001	0.068/0.001
DE, MJ/day	1.461	1.416	1.242^{c}	1.453^{b}	1.621^{a}	0.012/0.011	0.014/0.001	0.020/0.297
Reproductive performance								
PPWG (g)	427	467	350^{b}	375^{b}	615^{a}	49.39/0.572	60.48/0.009	85.54/0.980
LSAB (kits/litter)	5.00	5.20	4.80	5.30	5.20	0.370/0.705	0.453/0.714	0.640/0.650
LSAW (kits/litter)	4.67	4.80	4.20	4.90	5.10	0.353/0.792	0.432/0.320	0.611/0.883
Birth/weaning (%)	91.9	94.0	87.4	93.1	98.3	3.798/0.709	4.652/0.269	6.579/0.976

DM: dry matter, OM: organic matter, CP: crude protein, EE: Ether extract, E.S: energy sources, DEL: digestible energy levels, DE: digestible energy.
PPWG: Postpartum weight gain, LSAB: Litter size at birth, LSAW: Litter size at weaning
^{abc}Means within a row with different letters differ significantly ($P<0.05$), according to the Tukey test.

Effect of digestible energy on nutrient intake and reproductive performance in the second litter

DM, OM, EE, CP, and DE intake increased ($P<0.05$, Table 4) followed by the gradual rise of DE levels in the diet. CP intake had an interaction between energy sources and and DE levels ($P<0.05$, Table 4).

The postpartum weight change was different ($P<0.05$) in both maize and molasses groups, there was a decrease in the weight of does in the maize group and an increased in the molasses group. Milk yield was a gradual rise in the milk yield ($P<0.05$, Table 4) and followed by the DE levels. Litter size at birth and at weaning had an interaction between energy sources and and DE levels ($P<0.05$, Table 4).

Comparison between some reproductive performances of does in both two litters

Litter size at birth, litter size at weaning, milk yield, and the rate of live birth/weaning were higher in the second litter compared to the first litter.

Table 4: Nutrient intake and reproductive performance of doe rabbits in the second litter

Items	Energy sources		DE levels, MJ/kgDM			SE/P-value		
	Maize	Molasses	10.5	11.0	11.5	E.S	DEL	E.S*DEL
Nutrient intake on average pregnancy and lactation period, gDM/day								
DM	131	131	119[c]	129[b]	145[a]	1.809/0.968	2.215/0.001	3.133/0.164
OM	123	121	110[c]	120[b]	136[a]	1.614/0.343	1.976/0.001	2.795/0.317
CP	28.8	28.9	26.4[c]	28.9[b]	31.4[a]	0.196/0.873	0.241/0.001	0.340/0.002
EE	6.28	5.77	5.73[c]	5.97[b]	6.37[a]	0.048/0.001	0.059/0.001	0.083/0.182
DE, MJ/day	1.449	1.448	1.255[c]	1.428[b]	1.663[a]	0.014/0.939	0.018/0.001	0.025/0.056
Reproductive performance								
PPWC (g)	-44.6	86.5	22.5	-68.0	108	41.43/0.035	50.74/0.068	71.76/0.286
LSAB (kits/litter)	5.6	6.3	5.5	6.2	6.1	0.245/0.066	0.300/0.224	0.424/0.051
LSAW (kits/litter)	5.4	6.0	5.3	5.8	6.0	0.216/0.061	0.265/0.178	0.374/0.023
Milk yield (g/doe)	69.8	71.2	65.3[b]	68.0[b]	78.3[a]	2.327/0.667	2.850/0.009	4.031/0.019
IP (days)	53.3	57.7	66.9	51.0	48.6	4.475/0.487	5.481/0.055	7.752/0.420

DM: dry matter, OM: organic matter, CP: crude protein, EE: Ether extract, E.S: energy sources, DEL: digestible energy levels, DE: digestible energy.
PPWC: Postpartum weight change, LSAB: Litter size at birth, LSAW: Litter size at weaning, IP: interval parturium
[abc]Means within a row with different letters differ significantly (P<0.05), according to the Tukey test.

Table 7: Comparison of some reproductive performances of does in both 2 litters

Item	Litter 1	Litter 2	SE	P
Postpartum weight gain (g)	213	183	47.0	0.001
Litter size at birth (kits/litter)	5.10	5.93	0.315	0.013
Litter size at weaning (kits/litter)	4.73	5.70	0.297	0.003
Milk yield of the doe rabbit (g)	66.6	70.5	2.47	0.121
Live birth/weaning rate (%)	93.0	97.0	2.93	0.225

CONCLUSIONS

The results indicated at the level of DE11.5, DM,OM, CP, EE, DE intake were improved in both two litters and CP intake had an interaction between energy sources and DE levels. Postpartum weight gain increased following increasing DE levels in the first litter, however, it was improved in the molasses group in the second litter. Milk yield was increased following increasing DE levels in the second litter. There was an interaction between energy sources and DE levels in terms of litter size at birth and litter size at weaning in the second litter.

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EFFECT OF ARGININE, ISOLEUCINE AND VALINE FEED SUPPLEMENTATION ON RABBIT DOES PERFORMANCE AND THEIR LITTERS

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ABSTRACT

Litter size and weaning weight significantly impact the technical and economic aspects of rabbit production. Protein nutrition and amino acids play crucial roles in various physiological processes affecting reproductive and growth performance. However, research on amino acid requirements for rabbit production is limited. The study aimed to assess arginine, valine, and isoleucine supplementation in rabbit does' feed on rabbit kits' growth performance from birth to 70 days of age. One hundred thirty-six Optima Hyplus female rabbits were randomly assigned to two groups: control lactation feed (L) and an experimental diet (L+AA), supplemented with 15% of L-Arginine, 15% of L-Valine and 10% of L-Isoleucine (=AA). Kits were monitored until 70 days. Amino acid supplementation did not affect doe weight or kit numbers at birth and weaning. However, individual kit weight at weaning was higher with amino acid supplementation (963 vs 982g; $p<0.05$), with a better ADG (average daily gain) between 4 and 35d (27.8g vs 28.4g; $p<0.05$). Similarly, improved growth during the early fattening period was observed with amino acid supplementation during lactation (weight at 56d: 1765g vs 1828g; $p<0.05$). Supplementing secondary amino acids like arginine, valine, and isoleucine during lactation could enhance rabbit performance, especially under high-production or challenging environmental conditions. Further research is needed to determine optimal amino acid levels for the best technical and economic outcomes.

Key words: amino acid, lactation (parturition -7d until 28d), reproduction performances, litter weight, rabbit

INTRODUCTION

Litter size at birth and weaning weight are critical factors rabbit production, significantly impacting the technical and economic performance of farms. Protein nutrition and amino acids are involved in many physiological processes that greatly influence the reproductive and growth performance of animals. However, there is a notable scarcity of studies regarding the amino acid requirements of rabbit production. While Blas and Wiseman (2010) have compiled the various existing nutritional recommendations for Lysine, Threonine and Sulfur amino acids; there remains gaps for other essential amino acids. Arginine, for instance, is a precursor to nitric oxide and plays an important role in fetal development but also in many metabolic functions. Delgado and al. (2019) showed an improvement in litter weight during lactation with 0.4% arginine supplementation in the rabbit feed. Similarly, in pig production, many studies show an increase in fetal growth and piglet birth weight with arginine supplementation (Costa and al. 2019). Although Valine has not been studied in rabbit nutrition, its positive effect on milk production and litter growth has been observed in sows (Strathe and al, 2016). Finally, no data on isoleucine were found in rabbits. However, belonging to the branched-chain amino-acid family, these amino acid is also implicated in various physiological, including reproduction and stimulation of the mammary gland (Holen et al., 2022).

The aim of this study was to evaluate the effect of a supplementation of arginine, valine and isoleucine in the feed of rabbit does on the growth performances of rabbit kits from birth to 70 days of age.

MATERIALS AND METHODS

Animals, diet, and experimental design

136 Optima Hyplus female rabbits, confirmed as pregnant through palpation, were randomly assigned to two groups (68 per diet) 7 days before the parturition, with a body weight (BW) of 4.8 ± 0.3 kg. Following birth, does were inseminated 11 days after parturition corresponding to a theoretical kindling-to-kindling interval of 42 days, and weaning occurred 35 days of lactation. Adoptions were done until 4 days after parturition among rabbit does belonging to the same diet group to ensure 8 kits under primiparous, 9 kits under parturition 2, and 10 kits under multiparous. The trial concerned 3 successive cycles (lactation+gestation = 42d).
Two experimental feeds were used during the experiment (Table 1). The L (lactation) diet was formulated to meet the nutrient requirements of reproductive females, with a relatively high level in digestible energy (DE; 10.4 MJ DE/kg), crude protein (CP; 172 g/kg), crude fiber (142g/kg) and fat (32 g/kg). The AA (L+AA) diet was formulated from the same base supplemented with 15% of L-Arginine, 15% of L-Valine and 10% of L-Isoleucine. These two diets were distributed from 7 days before parturition until 28 days after parturition. The PW (pre-weaning) diet was formulated to meet the nutrient needs of fattening rabbits with lower digestible energy (9.30 MJ /kg) and protein (150 g/kg) than the other two diets, but higher crude fibre content (CF; 180 g/kg). It was distributed from 28 days after parturition until weaning at 35 days for all the rabbit does and their litter.
At weaning, 7 homogeneous kits per litter were chosen to be placed in fattening cages and monitored for performance until 70 days of age.

Growth, intake, and health status measurements

At parturition, the number of total born and alive was counted per doe. Rabbit does were individually weighted at 4 days, 11 days (AI), 25 days and 35 days after parturition. The litters were weighted at 4 days, 11 days (AI), 25 days and 35 days after parturition, along with the number of kits to calculate the mean weight.
In fattening period, the weight were measured per cage at 35, 56 and 70 days, along with the number of rabbits to calculate the mean weight. The feed intake was measured by feed line in the building with a mean data per cage.

Statistical Analysis

All statistical analyses were performed using the R software. The selected data included only positive lactation. Growth performances were evaluated using a linear mixed model, with the experimental group and the reproductive cycle as fixed effects. Interactions between group and cycle were noted in the results. The number of kits was compared using the Man-Whitney test.

RESULTS AND DISCUSSION

In the present study, we observed a statistical effect of the cycle on all criteria, but no significant interaction between the cycle and group.

The number of kits at birth and weaning was not affected by amino acid supplementation (Table 2). This latter finding is consistent with the results of Delgado et al. (2019), which did not demonstrate any effect of 0.4% arginine intake on the number of rabbits.

Table 1: Chemical composition of experimental diets.

(g/kg on fresh)	Diet L	Diet AA	Diet PW
Digestible energy (MJ/kg)	10.4	10.4	9.3
Crude Fiber	146	146	180
Fat	32	32	27
Crude protein	172	172	150
Total Lysine	8.5	8.5	7.2
Total Methionine+cystein	6.8	6.8	5.4
Total Threonine	6.8	6.8	6.1
Total Arginine	11.3	13	9.5
Total Valine	8.2	9.4	7.3
Total Isoleucine	6.3	6.9	5.5

Table 2: Effect of feed on number of kit

	Group			P-value
	L	**L+AA**	**Sem***	Group
Number of kits per litter				
Total born per doe	12.17	12.31	0.24	0.856
Born dead	0.49	0.81	0.11	0.673
Born alive	11.68	11.50	0.26	0.251
At 4d (after adoption)	9.63	9.68	0.05	0.489
At 35d (weaning)	9.18	9.18	0.08	0.271

* Sem = standard error mean

There was no significant effect of amino acid feed supplementation on litter weight, but the mean individual weight at weaning (35 days) was higher with amino acids ($p < 0.05$). This increase in weaning weight can be explained by a significant increase of ADG (average daily gain) between 11 and 25 days ($p<0.05$) and a tendency for increased ADG between 25 and 35 days ($p=0.063$), resulting in a significantly higher ADG for the overall period 4 to 35 days ($p<0.05$) (Table 3). Once again, this finding is in accordance with the results obtained by with Delgado et al. (2019) with arginine supplementation. Moreover, our results with AA supplementation can also be corroborated by the effects observed in sows on the stimulation of milk production and piglet weight with valine and isoleucine supplementation (Che et al, 2022; Wang et al., 2021).

Table 3: Effect of the feeding strategy on growth performances of young rabbits.

	Group			P-value		
	L	**L+AA**	**sem**	Cycle	Group	Cycle x Group
number of Rabbit does	190	197				
mean kit individual weight (g)						
4d	100.6	100.6	1.01	0.001	0.966	0.899
11d	211.2	212.1	2.03	0.001	0.786	0.970
25d	481.6	491.0	4.82	0.001	0.120	0.363
35d	963.4 b	982.6 a	8.47	0.001	0.049	0.094
kit's ADG (g/d)						
4-11d	15.8	15.9	0.21	0.001	0.721	0.947
11-25d	19.3 b	19.9 a	0.23	0.001	0.030	0.136
25-35d	48.2	49.2	0.45	0.001	0.065	0.047
4-35d	27.8 b	28.4 a	0.26	0.001	0.036	0.077

Lastly, regarding rabbits' weight during the fattening period, growth at the beginning (35-56d) was improved by the amino acid feed supplementation in lactation ($p < 0.05$ on ADG 35-56d) (Table 4), but there was no effect at the end, at 70d. These findings suggest that amino acid supplementation during lactation leads to higher quality rabbits, more robust at weaning with improved growth at the beginning of fattening period.

Table 4: Effect of feed lactation on fattening performance

		Group			P-value		
		L	**L+AA**	**sem**	Cycle	Group	Cycle x Group
cage number		60	60				
mean weight (g/rabbit)	35d	990 b	1020 a	12.23	0.001	0.001	0.314
	56d	1765 b	1828 a	20.96	0.001	0.003	0.269
	70d	2344	2376	21.51	0.001	0.098	0.023
ADG (g/d)	35-56d	36.91 b	38.49 a	0.65	0.001	0.045	0.521
	56-70d	40.66	38.79	0.93	0.001	0.090	0.106
	35-70d	38.70	38.73	0.42	0.001	0.955	0.015

CONCLUSIONS

In conclusion, supplementing with secondary amino acids, like arginine, valine, and isoleucine during the lactation phase of rabbits has the potential to enhance lactation and rabbit performance, particularly in high-production context or challenging environmental conditions. However, further studies are needed to better refine each level of amino acids to archive the best technical and economic compromise.

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EFFECTS OF PROBIOTICS ON PLASMA BIOCHEMICAL, IMMUNE AND ANTIOXIDANT PROPERTIES AND FECAL MICROORGANISMS OF PREGNANT REX RABBITS

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ABSTRACT

The purpose of this experiment was to explore the effects of probiotics on blood biochemistry, immune and antioxidant properties and fecal microorganisms of pregnant rex rabbits. 120 female rabbits with 3-4 parities were randomly divided into 4 groups, each consisting of 30 rabbits. Rabbits in the control group were fed a basal diet, while groups A, B, and C received 15.0 g/kg, 30.0 g/kg, and 45.0 g/kg of probiotics added to their basic diet, respectively. The results indicated that compared with the control group, the levels of IgA, IgM, and IgG in the plasma of group B were significantly increased by 8.32%, 23.11%, and 24.22%, respectively ($P<0.05$). Moreover, the activities of GSH-Px and CAT in the plasma of group B were significantly elevated by 41.16% and 23.72%, respectively ($P<0.05$), while the concentration of MDA in the plasma of group B was significantly reduced by 24.35% ($P<0.001$). Additionally, the relative abundance of Rikenellaceae_RC9_gut_group in the fecal flora of rex rabbits in group B was significantly higher than that in the other groups ($P<0.05$). In conclusion, the application of a 30.0 g/kg dose of probiotic preparations in the diet can effectively enhance the plasma immune and antioxidant properties and regulate the structure of intestinal flora in pregnant female rex rabbits.

Key words: probiotics; pregnant rex rabbit; blood biochemistry; immunity and antioxidation; fecal microbes

INTRODUCTION

The intestinal environment of rabbits is fragile and susceptible to environmental influences, leading to inflammatory intestinal diseases (Pickard et al., 2017). Adding antibiotics to rabbit feed can achieve the effect of preventing and treating inflammation, which can also lead to imbalance of gut microbiota and residual antibiotics in agricultural products (Mohammadzadeh et al., 2022; Bacanli et al., 2019; Yang et al., 2020). Composite probiotics are feed additives composed of several live microbial, and have become the most promising alternative to antibiotics due to their residue free and non-toxic properties (Shen et al., 2018; Deriu et al., 2013). The aim of this research was to evaluate the effects of a composite probiotic on the immune function, antioxidant capacity and structure of intestinal flora in pregnant female rex rabbits.

MATERIALS AND METHODS

Animals and experimental design

At 24 days of gestation, a cohort of 120 healthy female rabbits with comparable parity and weight were randomly assigned to 4 groups (30 rex rabbits/group), and fed with basic diet (CON), basic diet plus probiotics at a dose rate of 15.0 g/kg (Group A), 30.0 g/kg (Group B), 45.0 g/kg (Group C), respectively. The compound microecological preparation (Fushite Biotechnology Co. LTD, Yangli, China) used in this study was composed of *Bacillus subtilis*, *Bacillus licheniformis* and *Saccharomyces cerevisiae*. It was guaranteed to contain at least 2.5

* 10^7 CFU/g of *Bacillus subtilis*, 2.5 * 10^7 CFU/g of *Bacillus licheniformis* and 1.0 * 10^8 CFU/g of *Saccharomyces cerevisiae*. Throughout the 31-day experimental period, free access to feed and water was provided to all female rabbits, with a pre-feeding phase of 7 days followed by a formal feeding phase of 24 days. The basic diet was designed according to NRC (1977), the average daily feed intake (ADFI) of female rabbits was calculated.

Chemical Analyses

The levels of biochemical, immune, and antioxidant indicators were measured using standard commercial kits following the manufacturer's instructions (Quanzhou Ruixin Biotechnology Co., Ltd., Quanzhou, China). Simultaneously, fresh fecal samples were collected from specifically selected female rabbits and then placed into 2ml frozen tubes for further analysis of gut microbiota (conducted by Beijing Biomarker Technologies Co., Ltd., in Beijing, China).

Statistical Analysis

The experimental data were compiled for statistical analysis using Excel. Statistical significance was tested using one-way ANOVA in IBM SPSS Statistics 27 software, with (P<0.05) considered significant and (P<0.001) highly significant. The results are presented as the mean ± standard error (SE), and multiple comparisons were made using Duncan's correction.

RESULTS AND DISCUSSION

Effect on plasma biochemical indexes of rex rabbits

The results presented in Table 1 indicate that there were no significant differences observed in the biochemical indicators and ADFI between the experimental and control groups of rex rabbits (P>0.05). This suggests that supplementation with probiotics can maintain the normal functioning of the body without increasing the body load of pregnant Rex rabbits.

Table 1: Effect on plasma biochemical indexes of rex rabbit

Item	CON	Group A	Group B	Group C	*P*-value
Parity	4.5±0.5	4.25±0.25	3.75±0.48	4.00±0.58	0.705
ADFI/(g)	255.04±2.71	259.65±5.96	252.06±6.35	246.93±8.88	0.085
LDH/(nmol•mL^{-1})	11.84±1.69	9.57±1.65	8.99±2.12	10.84±2.18	0.208
TP/(mg•mL^{-1})	26.12±1.72	25.86±1.14	24.8±3.85	25.37±1.58	0.861
ALB/(g•L^{-1})	20.75±4.44	18.11±1.38	21.13±2.81	21.78±3.5	0.429
TG/(mmol•L^{-1})	0.76±0.16	0.83±0.15	0.89±0.08	0.77±0.12	0.448
BUN/(mmol•L^{-1})	4.17±0.72	4.87±1.76	5.84±0.79	5.51±1.05	0.238
AST/(nmol•mL^{-1})	7.33±1.01	8.05±1.38	7.86±0.99	7.59±0.95	0.806
ALT/(nmol•mL^{-1})	5.96±2.4	5.1±0.6	7.85±1.16	6.78±2.26	0.208
TCHO/(mmol•L^{-1})	3.24±0.68	2.9±0.16	3.86±0.25	3.35±0.56	0.08

Effect on the immune and antioxidant indexes of rex rabbits

According to Table 2, compared with the control group, the IgG in the plasma in group A and group B increased by 13.79 % and 24.22 %, respectively (P<0.05); the IgA in group A, group B and group C increased by 8.05 %, 8.32 % and 7.11 %, respectively (P<0.05); the IgM in the plasma in group A and group B increased by 17.31 % and 23.11 %, respectively (P<0.05). Compared with the control group, the activity of GSH-Px in plasma of rex rabbits in groups A, B and C was increased by 22.90%, 41.16% and 22.05%, respectively (P<0.001); the activity of CAT in plasma of group B was increased by 23.72% (P<0.05); the content of MDA in groups A and B decreased by 13.27% and 24.35%, respectively (P<0.001). There were no significant differences in the activity of SOD in plasma among all groups (P>0.05). The results show that the immune and antioxidant properties of female rabbits can be improved by adding a certain dose of probiotic preparations in the basic diet, and the appropriate dosage is 30.0 g/kg.

Table 2: Effect on the immune and antioxidant indexes of rex rabbits

Item	CON	Group A	Group B	Group C	*P*-value
IgG/(mg•mL^{-1})	14.37±0.4^{a}	16.35±0.64^{bc}	17.85±0.77^{c}	15.02±1.89^{ab}	0.003
IgA/(μg•mL^{-1})	208.28±9.84^{a}	225.06±6.54^{b}	225.61±5.03^{b}	223.1±7.41^{b}	0.019
IgM/(μg•mL^{-1})	581.19±21.96^{a}	681.81±27.53^{b}	715.49±6.86^{b}	602.34±33.33^{a}	<0.001
GSH-Px/(ng•mL^{-1})	83.27±3.98^{a}	102.34±6.47^{b}	117.54±3.84^{c}	101.63±13.34^{b}	<0.001
SOD/(ng•mL^{-1})	540.04±58.19	570.62±30.34	572.69±13.03	522.27±33.29	0.216
CAT/(ng•mL^{-1})	46.83±2.65^{a}	51.39±2.99^{a}	57.94±3.13^{b}	46.57±5.72^{a}	0.004
MDA/(nmol•mL^{-1})	6.07±0.69^{c}	5.27±0.35^{b}	4.6±0.21^{a}	6.04±0.17^{c}	<0.001

^{a,b} Different superscripts within a row indicate significant differences (P<0.05).

Effect on the intestinal microbiota of rex rabbits

As can be seen from Table 3, compared with the control group, the relative abundance of *Rikenellaceae_RC9_gut_group* in the fecal flora of female rabbits in group B was significantly increased (P<0.05), the relative abundance of *Rikenellaceae_RC9_gut_group* in the feces of female rex rabbits in groups C showed an increasing trend (P>0.05). The results showed that the relative abundance of *Rikenellaceae_RC9_gut_group* in the fecal flora of rex rabbits increased with the addition of probiotic preparations, indicating that the probiotic preparations can improve the decomposition and utilization of crude fiber in the diet by the intestine, so as to produce enough energy to meet the needs of the body and reduce dependence on external energy sources (Chen. 2022).

Table 3: Effects on fecal microbiota of rex rabbits

Item (%)	CON	Group A	Group B	Group C
Rikenellaceae_RC9_gut_group	1.95±0.81^{a}	2.03±0.45^{a}	6.12±2.03^{b}	2.59±0.98^{ab}
NK4A214_group	5.31±0.53	5.92±0.49	5.76±1.65	6.4±0.83
Monoglobus	2.08±0.54	2.76±0.34	2.97±0.24	3.2±0.38

^{a,b} Different superscripts within a row indicate significant differences (P < 0.05).

CONCLUSION

Under the experimental conditions, probiotic preparations at a dosage of 30.0 g/kg can enhance the immune and antioxidant properties of pregnant rabbits and regulate the flora structure.

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EFFECT OF ORGANIC ACID SUPPLEMENTATION IN FEED OR WATER ON GROWTH PERFORMANCE, NUTRIENT DIGESTIBILITY, AND GASTROINTESTINAL TRACT CHARACTERISTICS IN FATTENING RABBITS

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ABSTRACT

The aim of this study was to evaluate the effect of organic acid (OA) type and route of administration (water *vs.* feed) on fattening rabbit's performance, gastrointestinal tract (GIT) characteristics, pepsin activity, and ileal protein digestibility and flow. We used seven experimental treatments, in which three OA (formic, acetic, and citric) administered via drinking water or feed were tested. In addition, a negative control was included (without OA). A total of 1624 weaned rabbits were used divided into four batches. In each batch, animals were distributed into the seven treatments in individual (64 replicates per treatment) and collective cages (28 replicates per treatment with 6 animals/replicate). The OA administered in the drinking water were dosed to achieve pH=4. The concentration of OA in feed was calculated to equal the amount of OA ingested *via* water. Mortality was determined daily in all animals. At 43 and 57 days of age, in each batch, all animals were individually weighed and feed consumption was recorded. On these days, 6 animals per treatment, day and batch, were slaughtered, and pH was measured along the GIT (*fundus, antrum*, duodenum, jejunum, ileum, and caecum). Moreover, gastric content was collected to measure pepsin activity. At the end of the study, an ileal digestibility trial was performed. The administration of OA through drinking water improved animal performance during the postweaning period (29 to 43 days of age) and globally, compared to feed. Tested OA administered in water (W-FOR, W-ACET, and W-CIT) could be a suitable in rabbit farming, compared with CON. However, they showed limited capacity to modulate GIT pH. The administration of OA through feed improved protein digestibility and reduced ileal CP flow compared to water. Further research is needed to assess the effect of OA on mortality in rabbits.

Key words: weaned rabbits, additives, acetic acid, formic acid, citric acid, gut health.

INTRODUCTION

Organic acids (OA) exhibit antimicrobial properties, reducing the pH of the matrix into which they are incorporated, and limiting the growth of microorganisms. In addition, undissociated OA molecules show another mechanism of antimicrobial action, crossing the bacterial cell wall, releasing H+ and reducing the cytoplasmic pH, with deleterious effects on bacteria (Diebold and Eidelsburguer, 2006). Additionally, the use of OAs in animals improves gastric acidification, enzymatic activity and consequently the digestibility of nutrients (Falcão-e-cunha *et al*., 2007). Therefore, the use of these compounds could be useful for the control of digestive disorders in rabbits. In a previous study, Ramón-Moragues *et al*. (2023) tested six OA at three different pHs in the drinking water of post-weaned rabbits and determined that the OAs with the greatest potential for use in rabbits were formic, acetic, and citric acid at pH 4. These OAs were selected based on their potential to modulate gastrointestinal tract (GIT) pH and promote gastric pepsin activity. The aim of the present work was to determine the most suitable type of OA (formic, acetic, and citric) and the route of administration (water *vs.* feed) in fattening rabbits, and to evaluate their effect on productive performance, mortality, GIT characteristics, pepsin activity and ileal protein digestibility and flow during fattening.

MATERIALS AND METHODS

Animals and experimental design

The trial was carried out at the experimental farm of the Universitat Politècnica de València (UPV). The experimental procedure was authorised with code 2022 VSC PEA 0111.

Three OAs (formic, acetic, and citric) administered via feed or drinking water were tested. Seven experimental treatments were used: negative control (CON; without addition of OA), F-FOR (formic acid in feed), F-ACET (acetic acid in feed), F-CIT (citric acid in feed), W-FOR (formic acid in water), W-ACET (acetic acid in water) and W-CIT (citric acid in water). For water treatments, the OAs were dosed to achieve a pH equal to 4 in the drinking water. For feed treatments, the OA concentration was calculated to equal the amount of OA ingested via the water. For this, a water:feed intake ratio of 2.73 was used (Ramón-Moragues et al., 2023). For F-FOR, F-ACET and F-CIT treatments, a commercial meal feed containing, on dry matter (DM) basis, 18.4% crude fibre, 15.8% crude protein (CP), 4.8% starch, 3.1% ether extract, and 9.0% ash (NANTA, Spain) was added respectively with 0.06% sodium formate (AlbioFerm, 97% purity), 0.23% acetic acid (BioChemica, 100% purity) and 0.12% citric acid (Sigma Aldrich, 99% purity), and then pelleted. For the treatments via water, the same OAs were added, except for W-FOR where formic acid (Sigma Aldrich, purity >95%) was used, and no OAs were added to meal before pelleting.

A total of 1624 rabbits (LP genetic line, UPV) weaned at 29 days of age were used. The trial was divided into four batches of 29 days of duration each (until day 57 of age). In each batch, animals were distributed into the seven treatments in individual cages (44 × 52 × 32 cm) – 64 replicates per treatment – for performance measurements, and collective cages (50 x 80 x 32 cm) – 28 replicates per treatment, 6 animals/replicate – for mortality observations. Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated for periods of 29 to 43 days of age, 43 to 57 days of age, and globally. Mortality was recorded daily. Also, at 43 and 57 days of age, in each batch, all animals in a cage of each treatment (6 animals per treatment, day and batch) were slaughtered to measure pH along the GIT (fundus, antrum, duodenum, jejunum, ileum and cecum), and to collect gastric samples to determine pepsin activity according to Ramón-Moragues et al. (2023). Finally, at the end of batch 3, an ileal digestibility trial was performed using ytterbium as indigestible marker to calculate apparent and true ileal CP digestibility and flow. Endogenous protein losses were estimated according to Marín-García et al. (2024).

Statistical analysis

All data were statistically analysed using SAS System Software® SAS 9.3, to analysed the effect of type of OAs and route of administration on the variables studied. Significance was set at P-value≤0.05. Orthogonal contrast to compare route of administration of OAs were used.

RESULTS AND DISCUSSION

Effect on animal performance and mortality

Table 1 shows the effect of OA and route of administration on animal performance variables. Animals were weaned with an average body weight (BW) of 620 g and reached an average BW of 1538 g on day 57. Compared with CON, the addition of OA did not affect BW on day 43. On day 57, BW of the animals that received W-FOR was higher (P=0.05) than CON. Regarding the rest of the performance variables, the addition of W-FOR, W-ACET, and W-CIT could be suitable in rabbit farming as they improved (P<0.05) global ADG and ADFI (W-FOR), global FCR (W-ACET), and global ADG and FCR (W-CIT), compared with CON. The rest of OA treatments did not show significant differences in performance compared with CON. Considering the administration route (water *vs.* feed), animals receiving OA through drinking water showed higher BW on day 43 (+35 g; P=0.010) and on day 57 (+50 g; P=0.006) compared to those receiving OA through feed. Additionally, animals receiving OA through drinking water exhibited higher ADG, higher ADFI, and improved FCR in the 29 to 43-day period (+2.3 g/day, +3.5 g DM/day and -0.09 g feed/g, respectively; P<0.05) and globally (+1.7 g/day, +2.9 g DM/day and -0.07 g feed/g, respectively; p<0.05), compared to those receiving feed additives through feed. These results are similar to those obtained by Zhu *et al.* (2014)

where the addition of a mixture of formic acid, acetic acid and ammonium formiate in drinking water (pH=4.3) significantly improved growth performance.

Table 1: Body weight (BW; g), average daily gain (ADG; g/day), average daily feed intake (ADFI; g dry matter/day), and feed conversion ratio (FCR; g feed/g) according to the organic acid and route of administration.

	CON	F-FOR	F-ACET	F-CIT	W-FOR	W-ACET	W-CIT	SEM	P-value	Water-Feed
BW										
29 d	624,7^{abc}	603,6^{a}	638,1^{c}	618,4^{abc}	631,3^{bc}	610,8^{ab}	615,7^{abc}	9,3	0,093	-0.8±7.0
43 d	1056^{abc}	1022^{a}	1055^{abc}	1026^{ab}	1081^{c}	1056^{abc}	1070^{bc}	17	0.116	35±13*
57 d	1526^{ab}	1511^{a}	1517^{ab}	1516^{ab}	1586^{c}	1535^{abc}	1571^{bc}	23	0.099	50±18*
ADG										
29-43 d	30,4^{abc}	29,6^{abc}	29,5^{ab}	28,7^{a}	31,6^{bc}	31,5^{bc}	31,7^{c}	0,8	0.032	2.3±0.7*
43-57 d	33,2^{ab}	34,3^{ab}	32,6^{a}	34,6^{ab}	35,5^{b}	33,8^{ab}	35,0^{ab}	1,0	0.311	1.0±0.8
Global	31,8^{ab}	31,9^{abc}	31,0^{a}	31,6^{ab}	33,6^{d}	32,7^{bcd}	33,3^{cd}	0,5	0.005	1.7±0.4*
ADFI										
29-43 d	70,5^{abc}	68,0^{a}	69,6^{abc}	68,6^{ab}	73,0^{c}	72,0^{c}	71,8^{bc}	1,31	0.039	3.5±1.2*
43-57 d	104,9^{a}	106,7^{ab}	103,5^{a}	108,2^{ab}	110,4^{b}	105,7^{ab}	108,8^{ab}	2,1	0,196	2.2±1.6
Global	87,7^{ab}	87,4^{ab}	86,6^{a}	88,4^{abc}	91,7^{c}	88,9^{abc}	90,3^{bc}	1,34	0.071	2.9±1.0*
FCR										
29-43 d	2,64^{abc}	2,59^{ab}	2,73^{c}	2,70^{bc}	2,63^{abc}	2,57^{a}	2,57^{a}	0,04	0.031	-0.09±0.03*
43-57 d	3,62	3,48	3,61	3,52	3,48	3,50	3,49	0,07	0.518	-0.05±0.05
Global	3,13^{bc}	3,04^{a}	3,17^{c}	3,11^{abc}	3,06^{ab}	3,03^{a}	3,03^{a}	0,03	0.006	-0.07±0.03*

n: 64 animals per treatment. Periods: 29 to 43 days of age; 43 to 57 days of age, and Global from 29 to 57 days of age. Treatments: CON (control), F-FOR (formic acid in feed), F-ACET (acetic acid in feed), F-CIT (citric acid in feed), W-FOR (formic acid in water), W-ACET (acetic acid in water), W-CIT (citric acid in water). ^{a,b,c,d}Means within a row with different superscripts differ significantly (P<0.05). SEM: Standard error of the mean. Water *vs.* Feed orthogonal contrast for comparing means: *= P<0.05.

Effect on pH along the GIT

The OA tested showed a limited capacity to reduce GIT pH. Compared with CON, the addition of OA did not affect pH along the GIT. Average pH values were: 2.5 in *fundus*, 1.6 in *antrum*, 7.0 in duodenum, 7.6 in jejunum, 7.7 in ileum and 5.9 in caecum (data not shown). Considering the administration route (water *vs.* feed), the pH in the *fundus* of the animals that received OA *via* drinking water was significantly higher compared with feed (+0.36±0.13; P=0.005). There were no significant differences in pH regarding the administration route in the rest of GIT.

Effect on pepsin activity and ileal protein digestibility and flow

Compared with CON, the addition of F-FOR and F-CIT improved (P<0.05) pepsin activity in the stomach on day 43 (Table 2). The rest of OA treatments did not affect pepsin activity on day 43. On day 57, there were no significant differences in pepsin activity amongst treatments. Although the addition of OA did not statistically affect ileal CP flow, apparent and true ileal CP digestibility, were significantly higher in F-ACET (63.5 vs. 56.7%; P=0.045) and F-CIT (63.8 vs. 56.7%; P=0.037) compared with CON. Considering the administration route (water *vs.* feed), animals receiving OA through feed showed a lower ileal CP flow compared to water (1.46 g/day; P= 0.010) and higher ileal apparent and true ileal CP digestibility (-6.27, P= 0.001, and -5.92, P=0.0004, respectively). Therefore, the addition of OA during the first weeks after weaning seems to improve the functionality of the still immature digestive tract by increasing the activity of the enzyme pepsin. In addition, when OA is administered in feed, protein digestibility is improved.

Table 2: Pepsin enzyme activity at 43 and 57 days of age, and apparent and true ileal crude protein (CP) digestibility at 57 days of age according to organic acid and route of administration.

		CON	F-FOR	F-ACET	F-CIT	W-FOR	W-ACET	W-CIT	SEM	P-value	Water-Feed
Pepsin activity[1]	**43 d**	81,9^{a}	145,3^{b}	117,7^{ab}	154,5^{b}	111,9^{ab}	141,1^{ab}	132,7^{ab}	22,8	0,297	-10,6±18,1
	57 d	137,2	138,9	95,5	127,7	111,8	83,8	94,9	23,3	0,444	-23,8±18,3
Ileal CP flow (g/day)		9,68^{ab}	8,48^{ab}	8,25^{a}	8,15^{a}	9,62^{ab}	9,32^{ab}	10,32^{b}	0,74	0,187	1,46±0,55*
Apparent ileal CP digestibility (%)		56,7^{ab}	61,9^{bcd}	63,5^{cd}	63,8^{d}	57,2^{abc}	57,8^{abcd}	55,3^{a}	2,28	0,036	-6,27±1,85*
True ileal CP digestibility (%)		89,3^{ab}	93,9^{bc}	95,9^{c}	95,1^{c}	89,1^{ab}	90,4^{abc}	87,5^{a}	2,11	0,015	-5,92±1,59*

Pepsin n: 24; Digestibility n: 14. Treatments: CON (control), F-FOR (formic acid in feed), F-ACET (acetic acid in feed), F-CIT (citric acid in feed), W-FOR (formic acid in water), W-ACET (acetic acid in water), W-CIT (citric acid in water). ^{a,b,c,d}Means within a row with different superscripts differ significantly (P<0.05). SEM: Standard error of mean. Water *vs.* Feed orthogonal contrast for comparing means: *= P<0.05. [1]: Pepsin enzyme activity (U/g stomach content in DM).

Animal mortality ranged from 8.2% to 16.8% (data not shown). No significant differences were observed between the animals that received OA compared with CON, except for W-FOR group, which exhibited higher mortality (9.5% *vs.* 16.8%; P=0.020) than the CON group. Chamorro *et al.* (2007) indicated that a decrease in ileal protein flow two weeks after weaning resulted in reduced overall mortality attributed to digestive disorders in fattening rabbits. More research is required to assess if this factor influenced mortality in the OA water treatments in our study.

CONCLUSIONS

The administration of OA via drinking water improved animal performance during the post-weaning period (29 to 43 days of age) and overall, compared to feed. The tested OCs administered in water (W-FOR, W-ACET and W-CIT) could be suitable in rabbit farming, compared to CON. However, they showed limited ability to modulate GIT pH. Administration of OA via feed improved protein digestibility and reduced ileal CP flux compared to water,but did not result in higher production performance. Further research is needed to evaluate the effect of OA on rabbit mortality.

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SUSTAINED RELEASE OF TWO TYPES OF DIETARY MICROENCAPSULATED BUTYRIC ACID IN FATTENING RABBITS

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ABSTRACT

This work aims to evaluate the effect of adding encapsulated butyric acid in slow-release carriers (i.e. montmorillonite and kaolinite) on the productive performance of post-weaning rabbits. Additionally, we determined the release kinetics of butyric acid along the small intestine from three experimental treatments: control group (CON, without butyric acid), CON + montmorillonite impregnated with butyric acid (MONT) and CON + kaolinite impregnated with butyric acid (KAO). A total of 762 rabbits of the genetic line LP from 29 to 63 days of age were used, 522 animals were housed in collective cages to control mortality (29 cages of 6 animals/treatment) and 240 animals were housed in individual cages to control animal performance (80 animals/treatment). At 45 days of age, 16 animals per treatment were slaughtered to determine the release kinetics of butyric acid. Although the addition of microencapsulated butyric acid in fattening rabbit feed did not significantly improve animal performance and survival, which were good in general, both MONT and KAO clay-based supports proved capable of conducting a sustained release of this bioactive molecule. MONT seemed to release a more sustained butyric acid load throughout the small intestine compared with KAO. Further research is necessary to study whether the inclusion of butyric acid through laminar clay carriers can have beneficial effects on digestive health, gastrointestinal tract development, and mucosal functionality.

Key words: fattening rabbits, additives, butyric acid, encapsulation, weaning rabbits.

INTRODUCTION

Butyric acid is a short-chain fatty acid with many benefits on intestinal health (Vital *et al*., 2014), being a major source of energy for enterocytes and colonocytes. This compound is key in maintaining epithelial integrity, improving the morphology and function of the luminal mucosa, the development of the intestinal epithelium, accelerating its maturation, and increasing the intestinal absorptive surface area (Moquet, 2018).
Nevertheless, due to its small number of carbon atoms, butyric acid is very volatile (Brody, 1998). The undissociated molecules of butyric acid are easily absorbed in the stomach (Ichikawa *et al*., 2002) and by the most proximal portion of the intestine epithelium. Consequently, the bioavailability of butyric acid in the subsequent sections of the gastrointestinal tract (GIT) is hindered, making its protection necessary (Silva *et al*., 2020). With the aim of protecting and achieving a more balanced and sustained effect throughout the GIT, this molecule is commonly used in animal feeding as coated or free butyrate salt (i.e. sodium butyrate) or as mono-, di- and triglyceride butyrins (Moquet 2018; Sadurní *et al*., 2023). However, its use in rabbit rearing and its effect on productive performance, as well as the release kinetics of protected butyric acid in the rabbit's GIT, has barely been studied. Moreover, natural two-laminar clays, which are cost-effective and have been proven to be effective in

immobilizing other types of compounds (Bernados *et al*. 2019), could be a potentially suitable carrier for butyric acid protection.
Thus, the aim of this work was to evaluate the effect of adding encapsulated butyric acid in two laminar clay carriers on the productive performance and health of post-weaning rabbits, as well as in the release kinetics of butyric acid along the small intestine.

MATERIAL AND METHODS

Animals and experimental design

The trial was carried out at the experimental farm of the Universitat Politècnica de València (UPV). The experimental procedure was authorised with code 2022 VSC PEA 0111.
We encapsulated butyric acid using two laminar clays (montmorillonite and kaolinite), added to a conventional feed and compared with a negative control (without butyric acid). Laminar clays used were authorised as feed additives. In total, there were three experimental treatments: control group (CON), CON + montmorillonite impregnated with butyric acid (MONT) and CON + kaolinite impregnated with butyric acid (KAO). Control feed consisted of a no-medicated commercial pelleted feed (analytical composition in dry matter: 9.7% ash, 40.8% neutral detergent fibre, 16% crude protein, 13.1% starch, 3,6% ether extract).
Both clay supports were prepared in the Nutrition Lab of the Institute of Animal Science and Technology (UPV). Butyric acid was encapsulated via impregnation method 1:1. Before impregnation, the laminar structure of kaolinite was opened by soaking it in water and then oven dried and consequently ground to a powder. After impregnation, the butyric acid concentration in each support was measured (analysed butyric acid concentrations of 2.37 g butyric acid/kg feed in MONT treatment and 2.85 g butyric acid/kg feed in KAO).
A total of 762 rabbits of the genetic line LP of the UPV, weaned at 29 days of age were used in the trial. The duration of the trial was 35 days (until day 63 of age). After weaning, 522 animals were housed in collective cages (50 x 80 x 32 cm) to control mortality (29 cages/treatment; 6 animals/cage), and each cage was assigned to one treatment. In addition, 240 animals per treatment were housed in individual cages (44 × 52 × 32 cm) to control animal performance (80 animals/treatment). At 29, 43, and 63 days of age, the animals were individually weighted, and their feed consumption was recorded to determine the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). Mortality was recorded daily and calculated per treatment using the total number of animals. Additionally, at 45 days of age, 16 animals per treatment (from two collective cages) were slaughtered to determine the release kinetics of butyric acid in the small intestine (duodenum, jejunum, and ileum). Measurements of butyric acid concentration were performed by collecting 1 g of digestive contents from each section into Eppendorf (5 mL) containing 2 mL of preservative (2% H_3PO_4 + 0.5% ClH_3) and then frozen at -20°C until further analysis. The analysis of the samples for butyric acid concentration per section was carried out according to Jouany (1982).

Statistical analysis

All data were statistically analysed using a general linear model (SAS System Software® SAS 9.3), to assess the effect of butyric acid supplementation on the variables studied. Significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Effect of microencapsulated butyric acid on animal performance and mortality

Table 1 shows the effect of microencapsulated butyric acid on growth performance and mortality compared with CON. No significant differences were observed amongst treatments for body weight (BW), ADG and FCR throughout the trial. Mortality ranged between 3.94 to 5.51%, not being significantly different ($p<0.05$) among treatments. However, the addition of microencapsulated butyric acid affected ADFI. Globally, animals from MONT and KAO treatments had a slightly lower ADFI (av. -2.8 g dry matter/day; $p<0.05$) compared with the CON group. Animals from MONT also showed a slightly lower ($P<0.05$) ADFI between 43 and

63 days of age compared with the CON group. These differences in ADFI could be related to an increased feed hardness of the MONT and KAO pellets due to a higher particle cohesion caused by the clay matrix.

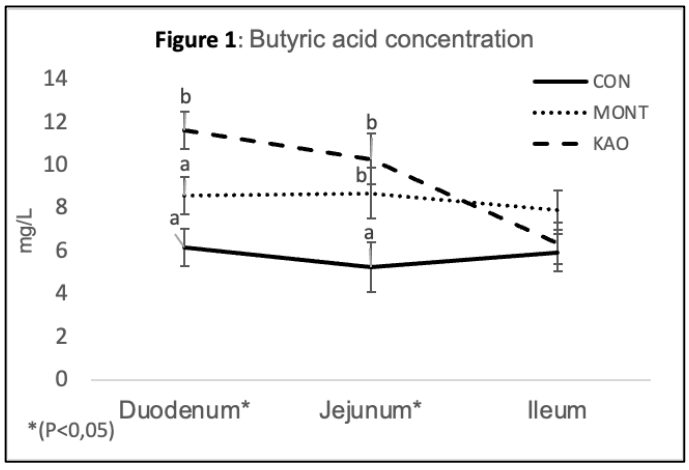
Table 1: Body weight (BW; g), average daily gain (ADG; g/day), average daily feed intake (ADFI; g dry matter; g/day), feed conversion ratio (FCR) and mortality (%) per microencapsulated butyric acid supplementation.

		CON	MONT	KAO	SEM	P-value
BW	**29 d**	610.5	602.0	602.2	7.9	0.669
	43 d	1113	1091	1087	12.1	0.233
	63 d	1912	1865	1885	17.2	0.177
ADG	**29-43 d**	35.7	35.1	34.3	0.5	0.159
	43-63 d	37.8	36.9	37.8	0.5	0.430
	Global	36.7	36.0	36.0	0.4	0.253
ADFI	**29-43 d**	72.7	71.0	70.9	1.0	0.310
	43-63 d	120.9^{b}	116.2^{a}	117.8^{ab}	1.2	0.021
	Global	96.8^{b}	93.6^{a}	94.4^{a}	0.9	0.025
FCR	**29-43 d**	2.05	2.04	2.08	0.02	0.508
	43-63 d	3.21	3.16	3.14	0.03	0.267
	Global	2.63	2.60	2.61	0.02	0.555
	Mortality	5.12	3.94	5.51	-	0.691

n: 80 animals per treatment. Periods: 29 to 43 days of age; 43 to 63 days of age and the entirety of the trial, 'from 29 to 63 days of age. Treatments: CON (control, without butyric acid), MONT (montmorillonite impregnated with butyric acid), KAO (kaolinite impregnated with butyric acid). ^{a,b} Means within a row with different superscripts differ significantly (P<0.05). SEM: standard error of the mean.

Release kinetics of microencapsulated butyric acid along the small intestine.

Figure 1 shows the concentration of butyric acid in each section of the rabbit's small intestine per treatment. Both clays showed a sustained release of butyric acid. Butyric acid concentration was higher in the duodenum in KAO (p=0.002), and in the jejunum in MONT and KAO (p<0.0001) compared to CON. No significant differences were observed in the distal section of the small intestine (ileum), probably due to the absorption of butyric acid by the intestinal epithelium. Differences between encapsulation supports were only observed in the duodenum, where KAO released a significantly higher amount of butyric acid compared to MONT, whereas MONT seemed to release a more sustained butyric acid load throughout the small intestine compared to KAO.



The inclusion of butyric acid as a feed additive in different livestock species has shown positive results (da Silva *et al*., 2022; Zhang *et al*. 2023). However, there is limited information available on its application in rabbit farming. Our results show a reduced potential of butyric acid to produce changes in growth performance when administered via slow-release porous carriers in a farm with good digestive health conditions (mortality <5%). Nevertheless, Sadurní *et al*. (2023) did not observe differences in animal performance when evaluating the inclusion of

0.1% sodium butyrate in feed. It is possible that in this case, the concentration of butyrate was too low to observe changes in performance parameters. On the other hand, Zhang *et al*. (2023), observed a significantly positive effect on growth performance when sodium butyrate was included in the feed of early-weaned rabbits at a concentration similar to this study (0.3%). These authors observed an increase in ADFI and ADG throughout the trial but reported no significant improvements in FCR. Likewise, da Silva *et al*. (2022) also observed improvements in performance (BW, ADG, and FCR) when encapsulated sodium butyrate was included in the feed of weaned piglets. Considering the positive effects attributable to butyric acid in the GIT (improving digestive health, epithelial tissue integrity and anti-inflammatory properties), together with the verification in our data of its sustained release when using laminar clays; its inclusion in the diet of fattening rabbits may also be beneficial for other traits not studied in this study. Therefore, further research should examine its effects on growing rabbits' digestive health traits and GIT function.

CONCLUSIONS

In conclusion, although the addition of microencapsulated butyric acid in growing rabbit feed did not significantly improve growth performance and mortality, both montmorillonite (MONT) and kaolinite (KAO) supports proved capable of conducting a sustained release of this bioactive molecule. The montmorillonite seemed to release a more sustained butyric acid load throughout the small intestine compared with kaolinite. Further research is necessary to study whether the inclusion of butyric acid through laminar clays can have beneficial effects on digestive health, gastrointestinal tract development, and improve mucosal functionality.

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PERFORMANCE AND MEAT QUALITY OF GROWING RABBITS FED DIETS INCLUDING *CHLORELLA VULGARIS*

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ABSTRACT

The present study aimed at evaluating the growth performance, diet digestibility, and meat quality of growing rabbits fed diets with different levels of chlorella (*Chlorella vulgaris)* and fat. A total of 576 mixed-sex crossbred rabbits (841 ± 140 g live weight; 33 days of age) were randomly distributed in 72 pens with 8 animals and assigned to six experimental groups according to a bi-factorial arrangement consisting of three dietary inclusion levels of chlorella (0%, 1%, and 2%) and two levels of crude fat (CF: 3.0% and 5.0%). The six diets were formulated to be isonitrogenous (15.5% crude protein) with two levels of DE (9.48 and 9.98 MJ/kg) according to CF level. Individual live weights were recorded weekly; pen feed intake and rabbit health status were monitored daily. From 47 to 51 days of age, a digestibility trial was performed. At 71 days of age, the rabbits were slaughtered and their meat burgers analysed for proximate composition and fatty acid profile. Growth performance of rabbits was not affected by the dietary treatments, whereas feed conversion improved by 5% as CF increased (P<0.001). Diets with 2% chlorella decreased the digestibility of CF. Diets with 5% CF improved the digestibility of dry matter, hemicelluloses, CF and gross energy. As for meat quality, the inclusion of 2% chlorella increased the proportion of n-3 polyunsaturated fatty acids and the n-3/n-6 ratio in meat burgers. Overall, the inclusion of chlorella up to 2% in the diets for growing rabbits did not affect rabbit performance, whereas slightly improved the quality of the meat products in terms of fatty acids profile.

Key words: microalgae, digestibility, fatty acids, soybean oil.

INTRODUCTION

Microalgae, such as chlorella (*Chlorella vulgaris)*, are rich in crude proteins (50–70%) and offer essential nutrients like n-3 fatty acids, carotenoids, amino acids, and vitamin B_{12} (Bature et al., 2022). Positive effects of microalgae inclusion in livestock diets include enhanced productivity, immune response, and meat quality (Bature et al., 2022). Although the role of chlorella in rabbit feeding is scarcely explored, the existing research testing low inclusion rates (0.2% to 1%) indicates positive effects on growth, reduced oxidative stress, and improved immune status (Peiretti and Meineri, 2008; Abdelnour et al., 2019), whereas the impact of chlorella on meat quality has been scarcely investigated. Therefore, the present study aimed at evaluating the growth performance, diet digestibility, and meat quality of growing rabbits fed diets with different inclusion levels of chlorella (0%, 1%, and 2%) and two levels of crude fat (3.0% and 5.0%).

MATERIALS AND METHODS

Animals and experimental design

At 33 days of age, a total of 576 mixed-sex Grimaud rabbits (841 ± 140 g live weight) were housed in 72 pens with 8 rabbits per pen under controlled conditions in the experimental farm of the University of Padova and distributed into six dietary treatments (96 rabbit per group), homogeneous in average live weight and variability, according to a bi-factorial arrangement

consisting of three dietary inclusion levels of chlorella (0%, 1%, and 2%) and two levels of crude fat (3% and 5%) obtained with the inclusion of 1% and 3% soybean oil. Diets were formulated to be isonitrogenous (15.5% crude protein; CP) with two levels of digestible energy (9.48 and 9.98 MJ/kg) according to crude fat level (Table 1).

Table 1. Ingredients (%) and proximate composition (% as fed) of the experimental diets.

	Diets					
Ingredients	**C0-F3**	**C1-F3**	**C2-F3**	**C0-F5**	**C1-F5**	**C2-F5**
Dehydrated chlorella (49% CP)	0	1.0	2.0	0	1.0	2.0
Soybean oil	1.0	1.0	1.0	3.0	3.0	3.0
Dehydrated alfalfa meal (16% CP)	19.0	19.0	19.0	16.0	16.0	16.0
Dehydrated alfalfa meal (14% CP)	11.0	12.0	13.0	11.0	12.0	13.0
Wheat bran (20% of starch)	24.8	24.8	24.8	24.8	24.8	24.8
Barley meal (10% CP)	10.0	10.0	10.0	8.0	8.0	8.0
Dried beet pulp (8% CP)	14.0	14.0	14.0	14.0	14.0	14.0
Sunflower meal (30% CP)	17.0	15.0	13.0	20.0	18.0	16.0
Other ingredients[1]	3.2	3.2	3.2	3.2	3.2	3.2
Proximate composition						
Dry matter	90.2	89.6	89.9	89.9	90.6	90.7
Crude protein	15.5	15.4	15.2	15.6	15.7	15.3
Crude fat	2.9	2.9	3.1	5.0	5.2	4.9
aNDF	38.9	38.5	39.0	39.1	38.6	38.4
ADF	22.0	21.2	21.0	22.0	21.5	21.5
ADL	5.2	4.7	4.8	5.1	4.7	4.8
Starch	8.9	7.4	9.3	8.2	8.0	9.3

[1]Cane molasses, 1.5%; limestone, 0.65%; salt, 0.4%; L-lysine-HCl, 0.15%; DL-methionine, 0,10%; Vitamin–mineral premix, 0.4%. C0, C1, C2: diets with 0, 1, and 2% of *C. vulgaris*; F3 and F5: diets with 3% and 5% of crude fat.

Recordings and chemical analyses

Diets were fed *ad libitum* from 33 to 71 days of age. Individual live weights of rabbits were recorded weekly; pen feed intake and rabbit health were monitored daily. The coefficients of total tract apparent digestibility for nutrients and energy were evaluated using 72 rabbits (12 per diet) distinct from those involved in the growth trial, from 47 to 51 days of age, kept in individual digestibility cages, according to Pérez et al. (1995). The growth performance of these rabbits was not included in the analysis. At 71 d, rabbits were slaughtered. A total of 72 meat burgers (121 ± 4 g; 12 burgers per treatment) were prepared using hind leg muscles (about 80 g) and *longissimus lumborum* meat (about 40 g). The proximate composition of diets, faeces, and meat burgers was determined according to AOAC (2000) methods and gross energy content by adiabatic bomb calorimeter. The fatty acid profile of rabbit meat burgers was analysed by gas chromatography.

Statistical Analysis

Data were analysed by a two–way ANOVA using the PROC GLM of SAS (2013) with chlorella inclusion, crude fat level, and their interactions as main effects. Differences among means with a $P<0.05$ were accepted as representing statistically significant differences. Least-square means were compared using the Bonferroni test.

RESULTS AND DISCUSSION

Rabbit growth performance

Chlorella inclusion did not affect growth performance (Table 2) consistently with previous studies in growing rabbit fed diets with chlorella from 0.05% to 0.15% (Hassanein et al., 2014;

Abdelnour et al., 2019). On the other hand, the increase of crude fat improved feed conversion ratio by 5% P<0.001), in agreement with previous studies (Ayyat et al., 2021) which depended on the contemporary increase of daily weight gain (P=0.10) and the decrease of daily feed intake (P=0.16), consistently with the chemostatic regulation of appetite.

Table 2: Performance from weaning until slaughtering (33 to 71 days of age) of growing rabbits fed diets with different inclusion levels of chlorella and fat.

	Chlorella (C)			Crude fat (F)		Probability			RSD
	0%	1%	2%	3%	5%	C	F	C×F	
Rabbits, n	174	168	168	253	257				
Pens, n	24	24	24	36	36				
Initial live weight[1], g	839	834	842	840	836	0.83	0.73	0.95	137
Final live weight[1], g	2723	2672	2702	2676	2722	0.42	0.16	0.54	278
Weight gain[1], g/d	49.4	48.1	48.8	48.1	49.4	0.40	0.10	0.48	5.52
Feed intake[2], g/d	159	160	158	161	157	0.89	0.16	0.22	10.8
Feed conversion ratio[2]	3.22	3.33	3.25	3.34	3.18	0.08	<0.001	0.37	0.17

[1]Individual data. [2]Pen data. RSD: residual standard deviation.

Diet digestibility

CF digestibility was lower in rabbits fed the diet with the highest inclusion rate of chlorella, whereas ADF digestibility significantly decreased with both 1% and 2% of chlorella compared to rabbits fed the control diet (Table 3). Likely, both results depended on the higher inclusion level of alfalfa meal (14% CP) in diets containing chlorella. On the other hand, the inclusion of chlorella did not affect the digestibility of dry matter, gross energy, and protein as observed with low or moderate (0.075%-10%) inclusion levels of chlorella (Hassanein et al., 2014) and spirulina (Peiretti and Meineri, 2008). Differently, protein digestibility decreased on rabbits fed high levels of spirulina (>15%) (Peiretti and Meineri, 2008).

Conversely, digestibility of dry matter (+2%; P<0.01), CF (+6%; P<0.001), hemicelluloses (+10%; P < 0.01), and gross energy (+2%; P<0.001) increased with the fat inclusion level. The dietary inclusion of fat (2-6%) has been shown to enhance the digestibility not only of the fat itself, but also of the other nutrients (Santomá et al., 1987; Xiccato, 2020), as we observed in our study.

Table 3: Coefficients of total tract digestibility and nutritive value of experimental diets with different inclusion levels of chlorella and fat.

	Chlorella (C)			Crude fat (F)		Probability			RSD
	0%	1%	2%	3%	5%	C	F	C×F	
Rabbits, n	24	24	24	36	36				
Dry matter, %	57.6	57.9	58.0	57.4	58.3	0.43	<0.01	0.70	0.72
Crude protein, %	75.1	75.5	74.3	74.7	75.3	0.11	0.16	0.43	1.06
Crude fat, %	85.6[b]	84.6[ab]	83.8[a]	82.4	86.9	<0.01	<0.001	<0.001	0.79
aNDF, %	30.5	30.0	30.8	30.0	30.8	0.63	0.24	0.63	1.61
ADF, %	23.2[b]	20.7[a]	21.1[a]	22.3	21.1	<0.05	0.11	0.10	1.79
Hemicelluloses, %	39.9	41.6	42.6	39.5	43.2	0.11	<0.01	<0.05	2.45
Starch, %	98.1	97.8	98.4	98.0	98.2	0.07	0.34	0.09	0.49
Gross energy, %	57.9	57.9	58.2	57.3	58.7	0.61	<0.001	< 0.05	0.76

RSD: residual standard deviation.

Meat quality

The inclusion of 2% chlorella increased the proportion of n-3 polyunsaturated fatty acids (PUFA) (+6%; P<0.05) and the n-3/n-6 ratio (+7%; P<0.01) compared with diets without chlorella. A similar increase of n-3 PUFA due to dietary inclusion of chlorella have been observed in chicken meat with α-linolenic acid (18:3 n-3) accounting for most of n-3 PUFA (Spínola et al., 2023). On the other hand, the increase of crude fat decreased the proportions of saturated and monounsaturated fatty acids (−20% and −5%, respectively; P<0.001), while

increasing (P<0.001) total PUFA (+22%), PUFA n-3 (+19%), and PUFA n-6 (+18%) (Table 4), consistently with the fatty acid profile of the added fat (i.e. soybean oil).

Table 4: Fatty acid profile of meat burgers in rabbits fed diets with different inclusion levels of chlorella and fat.

	Chlorella (C)			Crude fat (F)		Probability			RSD
	0%	1%	2%	3%	5%	C	F	C×F	
Burgers, n	24	24	24	36	36				
SFA	32.5	33.0	33.0	37.5	30.0	0.36	<0.001	0.19	1.13
MUFA	29.2	29.0	28.7	29.8	28.2	0.33	<0.001	0.13	0.75
PUFA	38.4	37.9	38.2	34.5	41.8	0.80	<0.001	0.09	1.52
PUFA n-3	4.6[a]	4.7[ab]	4.9[b]	4.3	5.1	<0.05	<0.001	0.16	0.25
PUFA n-6	33.8	33.3	33.3	30.2	36.7	0.64	<0.001	0.07	1.36
n-3/n-6	0.14[a]	0.14[a]	0.15[b]	0.14	0.14	<0.01	0.15	0.04	0.01

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. RSD: residual standard deviation.

CONCLUSIONS

The inclusion of chlorella up to 2% in the diets for growing rabbits did not affect growth performance, whereas slightly improved the quality of meat products in term of fatty acid profile. The increase of crude fat up to 5% improved nutrient digestibility and feed conversion, therefore increasing the efficiency and profitability of rabbit production.

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IMPACT OF DIETARY ENRICHMENT WITH OMEGA-3 POLYUNSATURATED FATTY ACIDS FROM EXTRUDED LINSEED AND ALGAE ON THE GROWTH PERFORMANCE OF RABBITS

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ABSTRACT

Currently, the use of n-3 polyunsaurated fatty acids (n-3 PUFA) in rabbits' nutrition represents an interesting strategy not only to enhance the productive and reproductive performances, but also the nutritional value of the meat, potentially creating a functional food for humans. This study investigated the effects of n-3 PUFA dietary integration with extruded linseed alone and in combination with algae *Padina pavonica* extract on the growth performance and metabolic status of fattening rabbits. Sixty New Zealand White growing rabbits, from weaning (35 days of age) until slaughter (85 days of age), were divided into three different groups (n=20) and fed different pelleted diets: commercial diet (CNT group), commercial diet integrated with 5% extruded linseed (L5% group), and commercial diet integrated with 3.5% extruded linseed in combination with 0.2% algae *Padina pavonica* extract (LPP group). In order to evaluate the growth performances of the rabbits, live weight (LW), average daily gain (ADG), and feed conversion ratio (FCR) were measured weekly, while feed intake was monitored daily. Moreover, to evaluate the metabolic status of the animals, plasma concentrations of insulin as well as glucose and non-esterified fatty acids (NEFA) were evaluated at 35, 60, and 85 days of age. In terms of productive performance, the fattening rabbits in the L5% group exhibited the highest marginal mean for LW, associated to higher average ADG and lower average FCR (for all: $P<0.05$), suggesting that the use of linseed alone improved feed efficiency compared to the LPP group. Furthermore, the integration with both extruded linseed and algae *Padina pavonica* extract did not significantly alter plasma concentrations of insulin, glucose, or NEFA, indicating no negative impact on the animals' energy metabolism.

Key words: Live weight, insulin, fattening rabbit, metabolites, NEFA.

INTRODUCTION

In rabbit farming, the productive performance of fattening rabbits is associated with different parameters related to the health status, growth rate, and metabolic efficiency of the animals. Optimizing the growth performances in fattening rabbits represents a key factor in increasing the rabbit farmers' profitability, especially considering the high feeding costs. Moreover, a well-balanced diet is crucial for these animals to meet their energy requirements and prevent nutrient deficiencies. Currently, the use of n-3 polyunsaurated fatty acids (n-3 PUFA) in animal nutrition appears an interesting strategy to enhance the rabbits' productive and reproductive performance. In addition, reaserch suggests that supplementing rabbits' feed with n-3 PUFA offers a promising strategy to enhance the nutritional value of rabbit meat, potentially creating a functional food with benefits for human health (Dalle Zotte and Szendrő, 2011). Both plant and animal products rich in n-3 PUFA have been explored in the rabbits' nutrition, although most studies have focused on linseed-derived products which gave the most interesting results (Dal Bosco et al., 2004). Among potential sources of plant-derived n-3 PUFA, marine algae have gained significant interest due to their established beneficial effects and their environmental sustainability (Al-Soufi et al., 2022). Notably, our study is the first to investigate the use of the brown algae *Padina pavonica* in rabbit diets to evaluate its effect on fattening rabbits. In this context, the objectives of this study were to investigate the impact of the integration with extruded linseed alone and in combination with algae *Padina pavonica* extract on the growth performance and energy metabolism of fattening rabbits.

MATERIALS AND METHODS

Animals, diets, and experimental design

Sixty male New Zealand White rabbits were individually housed in conventional cages (L×W×H: 75×38×25 cm) under controlled environmental conditions, in a commercial farm located in Central Italy. The experimental protocol was approved by the Ethical Committee of the Department of Veterinary Medicine of the University of Milano (OPBA_18_2021). At weaning (35 days of age), the rabbits were randomly divided in three experimental groups (n = 20 animals/group), each receiving a different pelleted diet. The experimental diets were formulated in accordance with current nutritional recommendations for fattening rabbits, ensuring they contained similar proximate chemical composition. The three diets were formulated as follows: a control diet consisting of commercial feed (CNT), the same commercial feed with adjusted ingredient proportions and the substitution of 5% extruded linseed (L5%), and commercial feed with the adjustment of ingredient proportions and the substitution of 3.5% extruded linseed combined with 0.2% algae *Padina pavonica* extract (LPP). The proximate chemical composition of the diets, as detailed in Table 1, was determined following the AOAC methods (2016). The animals were slaughtered at 85 days of age. Throughout the study (35 to 85 days of age), the rabbits received a daily ration gradually increasing from 100 g/day to 160 g/day. Fresh water was always available. The rabbits' live weight (LW), the agerage daily gain (ADG), and feed conversion ratio (FCR) were recorded weekly, whereas the individual feed intake (FI) was daily monitored.

Table 1: Chemical composition of the experimental diets.

g per 100 g of dry matter	Experimental diets[1]		
	CNT	L5%	LPP
Dry matter	89.34	89.49	89.94
Crude protein	15.23	15.28	15.23
Total fat	3.56	3.75	3.57
Starch	12.07	12.07	11.87

[1]CNT: Commercial control diet; L5%: CNT diet integrated with 5% of extruded linseed; LPP: CNT diet integrated with 3.5% extruded linseed and 0.2% *Padina pavonica* extract.

Plasma hormones and metabolites

Blood samples were taken at 35, 60, and 85 days of age from the ear marginal vein. The samples were immediately centrifuged at 3500 rpm for 15 minutes, then plasma and serum were stored until the assay. From each sample, insulin, glucose, and non-esterified fatty acids (NEFA) were analysed as previously described by Menchetti et al., 2018.

Statistical Analysis

The productive performance were analyzed using the Linear Mixed model including days as repeated factor, with a first-order autoregressive covariance structure. The procedures of Generalized Estimating Equations were used to analyze insulin and metabolites concentrations using Normal distribution and Identity link function or gamma distribution and log link. The models evaluated the main effects of time, group, and the interaction between group and time. Correlations were evaluated using Spearman's coefficient (ρ). Statistical analyses were performed with SPSS Statistics version 25 (IBM, SPSS Inc., Chicago, IL, USA). We defined $P<0.05$ as significant.

RESULTS AND DISCUSSION

Productive parameters

Studies conducted so far have found little influence of n-3 PUFA on the productive performance of fattening rabbits, independently from the source, wether linseed or marine algae (Matics et al., 2017). In our study, the L5% group showed the highest marginal mean for LW, compared to both CNT and LPP groups ($P<0.05$, data not shown). Notably, no significant differences in LW were observed between the CNT and LPP groups. Furthermore, ADG and FCR were significantly affected by the different diet ($P<0.01$). Indeed, rabbits fed the L5% diet exhibited higher marginal means for ADG (41.0±1 g per day) and lower for FCR (4.1±0.2) compared to those fed the LPP diet. These findings suggest that incorporating 5% extruded linseed into the diet might improve growth performance and feed efficiency compared to a combination of linseed and algae. Thorughout the entire experimental trial, no differences in FI were found among groups (data not shown). This is an interesting outcome, particularly given that several previous studies have raised concerns about feed palatability when integrating various marine algae into rabbits' diets at 2% (Okab et al., 2013). It's important to note that our study represents the first experimental trial to employ the alga *Padina pavonica*, distinct from the seaweeds documented in existing literature and incorporated in the diets at 0.2%.

Hormones and metabolites

Figure 1: Insulin concentrations at 35, 60 and 85 days of age of the three groups

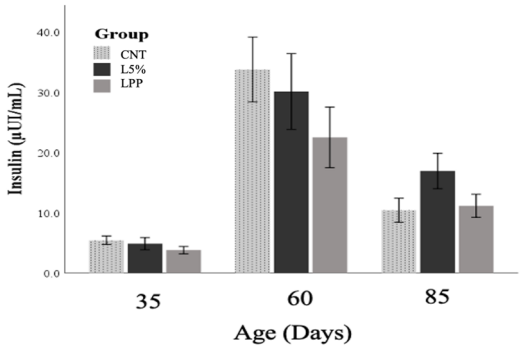
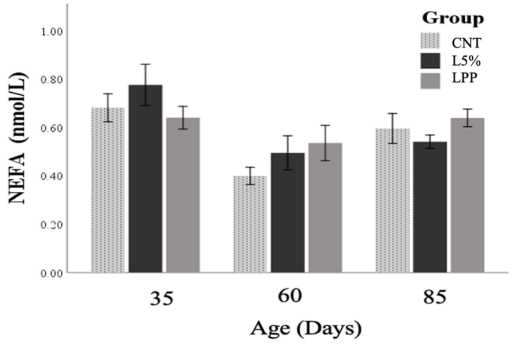


Figure 2: NEFA concentrations at 35, 60 and 85 days of age of the three groups



The LPP group had the lowest marginal means for insulin concentrations (12.4±1.0, 13.5±1.4, and 9.8±0.9 µIU/mL for CTN, L5%, and LPP, respectively; Figure 1; $P=0.048$), although pairwise comparisons did not reveal statistically significant differences between groups. Scientific literature suggest that marine algae contain different bioactive compounds, including

high levels of soluble dietary fibers, which may reduce glycaemia and insulin levels by slowing down both nutrients' intestinal absorption and hormonal response through their capacity to increase the viscosity in the digestive tract (Vaugelade et al., 2000). Circulating NEFA are important metabolic energy sources which change in response to the energy status of the animals. In our study, NEFA concentrations were influenced by time and, regardless of group, the lowest values were found at 60 days (P=0.001; Figure 2). NEFA concentrations were also influenced by group x time interaction (P=0.01), but multiple comparisons did not highlight differences among groups. The absence of significant differences in NEFA concentrations among groups suggest that diets integrated with linseed and algae do not impair energy metabolism, as evidenced by the same level of lipolysis compared to the CNT group. Notably, a negative correlation was observed between NEFA and insulin concentrations (ρ=-0.401, P<0.001). This finding aligns with one of the established roles of insulin as an anti-lipolytic hormone, suppressing the release of NEFA from the adipose tissue into circulation.
Finally, glucose concentrations were only affected by age, with the highest values observed at 35 days (110.7±6.1 mg/dL; P<0.001), whereas neither group (P=0.985) nor its interaction with time (P=0.597) had significant effects on glucose levels. The observed peak in glucose levels at weaning is consistent with known age-related changes in insulin sensitivity and glucose metabolism of young animals. The lack of significant differences in glucose levels among groups suggests that neither 5% extruded linseed nor 0.2% alga *Padina pavonica* had a negative impact on glucose metabolism.

CONCLUSIONS

Overall, the results suggest that both extruded linseed and algae *Padina pavonica* extract can be safely incorporated into rabbit diets at 5% and 0.2% respectively without compromising the productive performance and the energy metabolism of the animals.

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ARGININE RECOMMENDATIONS IN HIGH GROWTH RATE RABBITS: GROWTH PERFORMANCE, DIGESTIVE UTILISATION AND BODY RETENTION.

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ABSTRACT

For this experiment, five isonutritive experimental feeds, differing only in their arginine content (6.5, 9.6, 11.1, 13.2 and 14.9 g/kg DM), were formulated, manufactured, and distributed ad libitum to a total of 345 young rabbits 28-day old rabbits divided into four batches. The animals belonged to two different genetic lines: the maternal line LP and the paternal line RFLP. The animals were housed in individual cages and received feed and water *ad libitum* throughout the experiment. Mortality and morbidity were recorded daily, while weight and feed intake were recorded at 49 and 63 days of age. During the first and second batches, 64 animals between 49 and 53 days of age were used for a digestibility trial. At the end of the digestibility trial, 103 rabbits were sacrificed by intracardiac puncture. The empty carcasses of the animals were frozen, chopped and analysed. There were no differences between P3, P4 and P5 in weight gain, feed intake, digestibility, protein intake and retention. The recommended arginine level of 10 g arginine/kg DM optimises protein retention and average daily gain in fattening rabbits.

Key words: requirements, digestibility, genetic line, arginine, rabbit

INTRODUCTION

Evidence suggests that amino acid requirements may differ among animals with different growth rates, influenced by the growth of different tissues in rabbits (Carabaño et al., 2009) and other animals (Armero et al., 1999). The lack of one essential amino acid could have the same result as a general shortage of protein, impairing their growth. Consequently, current guidelines on the dietary composition of essential amino acids might be constraining the full expression of genetic potential in rabbits with high growth rates. On the other hand, an excess of protein can cause lower feed intake or the appearance of digestive disorders associated with an excess flow of ileal nitrogen (De Blas and Mateos, 2020).

Since the guidelines for arginine content in fattening rabbit feed rely on studies conducted over 45 years ago (McWard et al., 1967; Colin, 1975; Adamson and Fisher, 1976), it seems reasonable to verify their relevance for current genetic lines. This study investigates the effect of different arginine levels in feed for growing rabbits of two different genetic lines.

MATERIALS AND METHODS

The experimental procedure was approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València (UPV; code: 015/VSC/PEA/00061) and carried out following the recommendations of the European Group on Rabbit Nutrition (Fernández-Carmona et al., 2005) and the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes.

To a basal mixture, with an initial level of 5 g/kg of arginine, synthetic L-arginine was added in different proportions to achieve 5, 7.5, 10, 12.5, and 15 g/kg of theoretical arginine content for

diets P2, P3, P4, and P5, respectively. The arginine content of the diets were 6.5, 9.6, 11.1, 13.2, and 14.9 g/kg for P1, P2, P3, P4, and P5, respectively (Table 1).

A total of 345 rabbits weaned at 28 days, belonging to 69 different litters, were used and distributed in four batches. The animals belonged to the robust maternal LP line, founded by reproductive longevity (Savietto et al., 2015) and to a new paternal line named RFLP. Further information about the RFLP genetic line can be found in Peixoto-Gonçalves et al. (2023). Animals were housed in individual cages and fed *ad libitum*. Weight and feed intake controls were carried out at 49 and 63 days. In addition, a digestibility trial was performed from days 49 to 53. After the digestibility trial, 103 animals were sacrificed, frozen, cut, and ground and four Petri dishes per animal were lyophilised.

Table 1. Ingredients and chemical composition of the experimental diets (g/kg DM).

Ingredients %		Chemical Composition	P1	P2	P3	P4	P5
Barley	19.5	Dry matter	884	886	885	886	887
Gluten meal	9.0	Ashes	76.9	77.4	78.4	73.5	74.7
Wheat bran	17.4	Crude Protein	184	188	189	193	199
Alfalfa hay	35.0	Crude Fat	31.7	30.9	28.0	28.8	28.4
Beet pulp	11.7	Neutral Detergent Fibre	384	387	388	387	383
Arbocel®	3.0	Acid Detergent Fibre	210	210	209	211	209
Soybean oil	1.0	Acid Detergent Lignin	31.1	32.0	31.3	31.4	30.4
L-lysine	0.541	Starch	179	174	184	177	176
DL-methionine	0.216	Lysine	8.4	8.5	8.5	8.5	8.4
L-threonine	0.177	Methionine	4.5	4.4	4.2	4.4	4.6
L-tryptophan	0.100	Cystine	2.5	2.7	2.4	2.5	2.4
L-histidine	0.107	Methionine+Cystine	7.0	7.2	6.6	6.8	7.0
L-arginine	0.036	Threonine	7.2	7.3	7.7	7.7	7.4
Calcium carbonate	0.073	Isoleucine	5.5	5.4	5.4	5.6	5.3
Dicalcium phosphate	1.326	Valine	7.6	7.5	7.3	7.8	7.5
Sodium chloride	0.404	Histidine	3.5	3.7	4.0	3.6	3.5
Vitamin-trace element premix	0.500	Arginine	6.5	9.6	11.1	13.2	14.9

Chemical analysis

Diets and individual faeces were analysed following the procedures of AOAC (2002). Fibre fractions (neutral detergent fibre, acid detergent fibre and acid detergent lignin) were sequentially determined following Van Soest et al. (1991). Starch was analysed by a two-step enzymatic hydrolysis (Batey, 1982), adjusting the resulting glucose with the hexokinase/glucose-6-phosphate dehydrogenase system. The amino acid content was determined after acid hydrolysis with 6N HCL at 110 °C for 23 h, according to Bosch et al. (2006). The fresh meat samples were analysed for dry matter. The lyophilised meat samples were analysed for dry matter, ashes, and crude protein.

Statistical analysis

Data from the empty bodies were analysed using a PROC MEANS analysis in SAS (Statistical Analysis Systems Institute, 2009). Growth, protein balance and digestibility data were analysed with the SAS GML procedure, incorporating a model that considered diet (5 levels), genetic line (2 levels), and their interaction.

RESULTS AND DISCUSSION

Table 2 shows no differences in the digestibility coefficients for the different diets and between the genetic lines. As expected, RFLP animals were significantly heavier at weaning and had higher weight gain during the growing period than LP animals, consistent with prior research

(García-Quirós et al., 2014). Regarding the arginine content, increasing arginine in the diet from 6.5 to 9.6 g/kg DM (P1 vs P2) led to a significant rise in intake, average daily gain (ADG), and weight at 63 days of age (+24 g DM/day, +5 g/day, and +228 g, respectively; P<0.05).

Table 2. Effect of the diets and the genetic lines on growth, digestive utilisation and body retention.

	Diets					Genetic Line			**P-value**	
	P1	**P2**	**P3**	**P4**	**P5**	**LP**	**RLP**	SEM	Diet	Genetic Line
Digestibility coefficients										
No.	14	10	16	10	9	34	25			
DM (%)	56.3 (0.38)	55.9 (0.45)	56.7 (0.35)	56.9 (0.45)	56.9 (0.58)	56.8 (0.25)	56.3 (0.29)		0.421	0.148
OM (%)	56.7 (0.40)	56.5 (0.47)	57.4 (0.37)	57.8 (0.47)	58.0 (0.50)	57.6 (0.26)	56.9 (0.30)		0.101	0.111
CP (%)	72.0 (0.66)	71.7 (0.79)	71.2 (0.61)	73.1 (0.78)	72.2 (0.83)	72.0 (*0.43*)	72.1 (0.51)		0.483	0.894
No.	24	16	23	20	20	61	42			
BW at 28 days (kg)	0.613	0.689	0.642	0.643	0.608	0.530	0.749	0.018	0.3569	<0.0001
Feed intake (g DM/d)	112.5^{a}	136.1^{c}	127.1^{b}	130.7^{b}	119.8^{ab}	104.4	146.0	2.5	0.0004	<0.0001
ADG (g/d)	46.18^{a}	51.20^{c}	49.92^{b}	50.90^{b}	47.60^{ab}	40.90	57.42	0.74	0.0073	<0.0001
BW at 63 days (kg)	2.252^{a}	2.480^{b}	2.398^{b}	2.455^{b}	2.288^{a}	1.977	2.772	0.036	0.0166	<0.0001
Carcass composition										
DM (%)	29.53	30.12	30.37	30.50	29.69	28.64	31.45	0.25	0.3019	<0.0001
CP (%)	63.50	60.92	61.49	61.65	63.34	67.33	57.03	0.51	0.0926	<0.0001
Protein intake (g/d)	15.08^{a}	18.51^{b}	17.41^{b}	18.56^{b}	17.36^{b}	14.52	20.25	0.35	<0.0001	<0.0001
Protein retention (g/d)	9.48^{a}	10.25^{b}	9.88^{ab}	10.40^{b}	9.80^{ab}	8.82	11.11	0.16	0.0613	<0.0001
Protein retention efficacy	0.640^{b}	0.573^{a}	0.584^{a}	0.567^{a}	0.568^{a}	0.617	0.556	0.10	0.0031	<0.0001

DM: dry matter; OM: organic matter; CP: crude protein; BW: body weight; Means with different letters on the same row differ significantly

Concerning the genetic line, the RFLP line exhibited higher daily protein intake and retention (+39% and +26%, respectively; P<0.001) but had lower protein retention efficiency (-0.06; P<0.001), resulting in a 10.3 percentage point decrease in body protein content (P<0.001). Prior research (Marín-García et al., 2020) had already noted that high-growth rate animals tend to have lower protein retention, possibly indicating a potential protein shortage in current commercial rabbit diets. Concerning the impact of arginine levels on protein retention, diet P2 maximises digestible protein intake, showing no further improvement with higher arginine levels. Protein retention in rabbits' empty bodies peaks with P2. These findings support current arginine recommendations for rabbits (De Blas and Mateos, 2020), indicating a 10 g/kg DM for optimal growth and protein retention. However, it's notable that increased arginine levels reduce protein retention efficiency. This may be linked to interaction with other amino acids, as described by D'Mello and Lewis (1970), dependent on specific transporters in the ileal mucosa, facilitating selective amino acid absorption and exchange.

CONCLUSIONS

Regardless of the genetic line, a content of 10 g arginine/kg dry matter is adequate to optimise weight gain and protein retention during the growing period.

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EFFECT OF A TWICE A WEEK FASTING ON PERFORMANCE, HEALTH AND FEEDING BEHAVIOR OF FATTENING RABBITS

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ABSTRACT

In rabbit nutrition, many studies described the benefits of applying feed restriction in fattening, in order to manage the sanitary digestive risk of animals (SDR). The aim of this study was to determine if a twice a week fasting improved performances and sanitary status of fattening rabbits. 224 rabbits of 34 days old were divided in 2 groups and fed ad libitum with a single fattening feed: a group was not fasted (AL group) whereas the other group had a twice a week fasting of 16 hours (F group). Feed consumption, growth performances and sanitary status (digestive mortality and morbidity) were measured. Results showed that a twice a week fasting reduced the average daily feed intake (ADFI) by 8.8% (149.4 versus 163.9 g/d; $p<0.001$), and the final live weight by 4.3% (2625 g versus 2742 g ; $p<0.001$), without significant improvement of the Feed Conversion Ration (FCR). The decrease of ADFI caused by fasting did not improve the SDR of rabbits, which tended to be higher for F group (26.8% versus 16.1% for AL group; $p=0.051$). This finding is probably correlated with increased feed intakes of rabbits for the days following the realimentation : +9% at 41-42 days, +18% at 48-49 days ($p<0.01$), +10% at 55-56 days, +19% at 62-63 days ($p<0.05$) and +31% at 69 days ($p<0.001$). In conclusion, applying a twice a week fasting as it was done in this study does not seem to be appropriated for improving the sanitary status nor the FCR of growing rabbits. It may be wiser to use fasting for a temporary sanitary action more than a fully-fledged feed restriction method.

Key words: rabbit, feeding program, fasting, performances, digestive sanitary risk

INTRODUCTION

In rabbit nutrition, many studies described the benefits of applying feed restriction on the decrease of digestive troubles in fattening, with a quantitative (distribution of a part of feed in comparison with an ad libitum theorical ingestion) (Tudela & Lebas, 2006; Martignon et al., 2009) or hourly (reduction of the access duration to the feeder) (Foubert et al., 2007 ; Rebours et al., 2021) feed restriction. However, due to constraints of equipment, it is not always easy to apply feed restriction in the farms. That is the reason why some breeders use a 24 hours (h) default fasting every week, in order to create a vacuity in the digestive tract of rabbits, which would contribute to reduce digestive disorders.

There is no scientific references about effects of this feeding method on both performances and sanitary status of fattening rabbits. Lebas & Laplace (1982) studied the influence of a twice a week 24h fasting (with realimentation ad libitum or fed restricted at 71% of ad libitum) with rabbits of 5 weeks old. This feeding method decreased the Average Daily Feed Intake (ADFI) from 111.9 g/day to 83.4g/day (i.e. 74.5% of ad libitum), and the Live Weight (LW) but without information on the sanitary status of rabbits. Masoero et al. (1992) studied the effect of a 24h fasting, but it was applied only the last fattening day (95 days old). Brecchia et al. (2006) compared rabbits fed ad libitum with or without fasting (24h or 48h). But these animals were productive does. Thus, no scientific work indicates the effect of a weekly fasting on performances, sanitary status and feeding behavior of fattening rabbits. It is important to consider that applying a fasting could impair the animal welfare, which can be defined as « an absence of a prolonged hunger » (EFSA, 2020). In order to preserve the animal welfare, in this study we applied a twice a week fasting of 16h, instead of 1 fasting of 24h.

MATERIALS AND METHODS

Animals and experimental design

The trial took place in the experimental station of Saint Symphorien (France). At weaning at 34 days old, 224 Hyplus Optima rabbits were divided in 2 groups of 16 cages of 7 rabbits each. Rabbits of these 2 groups had the same average weaning weight, and were fed with one single fattening feed (12.0% moisture, 3.4% crude fat, 16.2% crude fibre, 15.0% crude protein, crude ash 7.8%), ad libitum until 40 days old. From 40 days, 2 different feeding methods were applied : distribution continuous ad libitum (AL group) or discontinuous with 2 fastings (F group) per week (Tuesday and Friday) from 5.00 pm to 8.00 am (i.e. 16h of fasting). Thus, the F group had 9 fasting periods: at 40, 43, 47, 50, 54, 57, 61, 64 and 68 days old. Rabbit weights and feed intakes were measured at 34, 50 and 71 days old. These data allowed to calculate ADFI, Average Daily Gain (ADG) and Feed Conversion Ratio (FCR). Mortality and morbidity were recorded everyday.

Feed intake was measured for 1 fasting per week for the 2 groups (9 cages per group) for the 2 days following the realimentation of the F group, excepting at the end of the trial where there was only 1 measure. Finally data were recorded at 41-42, 48-49, 55-56, 62-63 and 69 days old.

Statistical Analysis

Statistical analysis were done with R software (version 3.6.0). Zootechnical parameters were analysed with a linear model with the weaning weight as covariate. Mortalities and morbidities were analysed by categorial analysis (chi-deux).

RESULTS AND DISCUSSION

Performances

For the first fattening period (34-50d old), ADFI was 10.7% lower for F group (124.3 versus 139.2 g/d) ($p<0.001$) (Table 1). Thus, ADG was reduced by 9.3% (49.5 g/d versus 54.6 g/d) ($p<0.001$). FCR were not significantly different: 2.51 for F group and 2.55 for AL group. For the second fattening period (50-71d old), ADFI is also lower (-7.7%) for F group (168.7 g/d versus 182.8 g/d) ($p<0.01$), without significantly impacting ADG and FCR. For the total fattening period (34-71d old), ADFI and final LW were reduced for F group (respectively 149.4 g/d versus 163.9 g/d, $p<0.001$; and 2625 g versus 2742 g, $p<0.001$), without improving the FCR. Even though fastings decreased the access duration to the feed of 17% (in average 20h versus 24h per day for the whole trial), the reduction of feed intake was only 8.8%. With two 24h fastings per week, (i.e. in average 17h per day of access to the feed), Lebas & Laplace (1982) measured for rabbits of 5 and 8 weeks old a drop of ADFI (-21.1%) and LW (-17.0%), but a rise of the FCR (+45.6%). Differences observed with results of this trial can be explained by a more severe feed restriction.

Table 1 : Performances of rabbits according to the experimental group.

	34-50d			50-71d			34-71d		
	AL	F	p-value	AL	F	p-value	AL	F	p-value
Live Weight (g)	1843	1772	< 0.001	2742	2625	< 0.001	-	-	-
Weight Gain (g/d)	54.6	49.5	< 0.001	42.8	40.6	NS	47.9	44.5	< 0.001
Feed Intake (g/d)	139.2	124.3	< 0.001	182.8	168.7	< 0.01	163.9	149.4	< 0.001
Feed Conversion Ratio (g/g)	2.55	2.51	NS	4.30	4.18	NS	3.42	3.36	NS

AL group : rabbits with a continuous ad libitum (AL) feed distribution. F group : rabbits with a discontinuous feed distribution with 2 fastings (F) per week (Tuesday and Friday) from 5.00 pm to 8.00 am (i.e. 16h of fasting).

Sanitary status

The sanitary digestive risk (SDR), which takes into account dead and sick rabbits only for digestive reasons, tended to be significantly higher for F group (26.8% versus 16.1%)

(p=0.051). This deterioration of the sanitary status of rabbits, which feed intake was reduced by fastings, causes concern because the aim of this strategy was to lower digestive troubles.

When we detail SDR in mortality and morbidity, we note that digestive mortality is significantly higher for F group (14.3% versus 5.4%) (p<0.005). But digestive morbidity is similar between the 2 groups (10.7% for AL group and 12.5% for F group).

The effect of fastings on the evolution of the digestive morbidity is shown on the Figure 1. It seems that after most of fasting, morbidity of F group increased. Thus, there would be a correlation between morbidity and fastings.

This sanitary result, added with counter-performances, leaded to an economical margin on feed cost lower for F group (2.79€/rabbit versus 3.20€/rabbit).

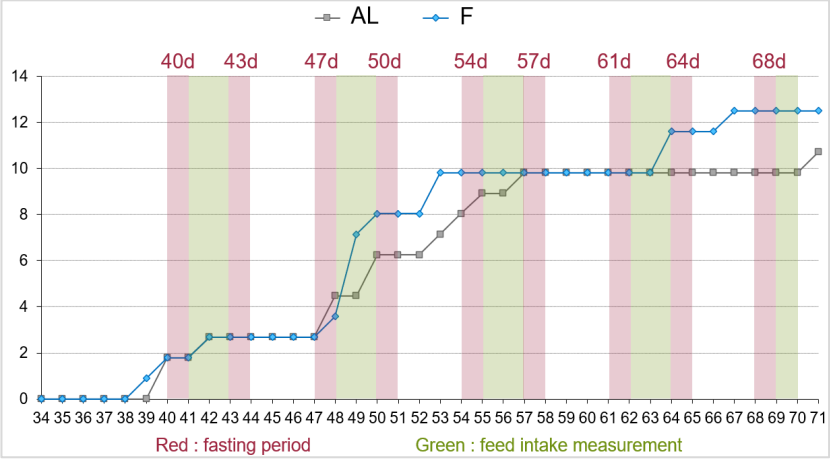

Figure 1 : Evolution of the cumulated digestive morbidity(%) of Ad Libitum (AL) and Fastings (F) groups according to the age of rabbits, the fasting period of F group, and the feed intake measurement periods.

Follow-up of feed intakes at realimentation

Calculation of average speeds of feed consumption (related with the access duration to the feed) showed a quicker feed ingestion (+8.8%) for F group (7.4 versus 6.8 g/h ; p<0.001). This result is similar to Rebours et al. (2021), where more the duration of access to the feeder is short and more rabbits have a fast feed intake. Moreover, related with animal' LW, average speeds of feed consumption are also higher for F group: 4.3 g/h/kg vs 3.7 g/h/kg for AL group (p<0.001).

The measurement of feed intakes for the 2 days following fastings indicated an over-consumption of F group in comparison with AL group: +9% at 41-42d, +18% at 48-49d (p<0.01), +10% at 55-56d, +19% at 62-63d (p<0.05) and +31% at 69d (p<0.001) (Table 2). Thus, ADFI related to each fattening period did not highlight these large variations of feed consumption following fastings. This behavior indicates that fastings leaded to an over-consumption at realimentation, which would increase morbidity.

The difference of feed consumption between F and AL groups seemed to increase with time. But we note that on period 55-56d, the difference is lower, and could be linked with a bad sanitary status of rabbits. This relative increase with time could be explained by a progressive rise of the stomach volume, which influences the quantity of feed intake. However, litterature is contradictory: some authors prove that feed restriction does not impact stomach weight (Gidenne & Feugier, 2008; Lebas et al., 1974 ; Ledin, 1984 ; Tumova et al., 2007). On the other hand, some publications show an increase of the stomach weight related to LW (Birolo, s.d.) and gastric contents (Lebas & Laplace, 1982) ; and a decrease of stomach weight related to the weight of digestive organs at 43 and 51 days old (Bergaoui et al., 2008).

Other fasting modalities could be studied to find a convenient and effective feeding method for improving sanitary status and performances of rabbits. However, the over-consumption post-fasting that was observed during this trial could suggest that whatever would be the fasting modalities, rabbits would keep the same feeding behavior leading to digestive troubles. Thus, an everyday feed restriction, which is more regular than intermittent weekly fastings, seems to be a better technic for improving sanitary and FCR by limiting the feed intake.

Table 2 : Daily Feed Intake (g) measured for the 2 days following realimentation of Ad Libitum (AL) and Fastings (F) groups, and difference between 2 groups (%)

	Ad libitum (AL) (g)	Fastings (F) (g)	Difference (%)	p-value
Days 41-42	143	156	+9	NS
Days 42-43	148	159	+7	NS
Average Days 41-43	145	158	+9	NS
Days 48-49	154	183	+19	<0.01
Days 49-50	160	190	+19	<0.01
Average Days 48-50	157	186	+18	<0.01
Days 55-56	181	206	+14	0.09
Days 56-57	174	182	+5	NS
Average Days 55-57	177	194	+10	NS
Days 62-63	189	230	+22	<0.01
Days 63-64	181	210	+16	<0.05
Average Days 62-64	185	220	+19	<0.05
Days 69-70	182	239	+31	<0.001

CONCLUSIONS

This study shows that applying a twice a week discontinuous fasting reduces the ADFI related to the fattening period. However, it leaded to an over-consumption of rabbits during at least the 2 days following realimentation, and induced more digestive troubles. Moreover, applying a fasting according to the studied strategy did not allow to improve the FCR, contrary to other restriction methods. That is why, in case of impossibility to reduce the feed intake of rabbits everyday (with quantitative or hourly feed restriction), it is recommended to avoid applying fastings as it was done in this study for technical, economical, and ethical (animal welfare) considerations.

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PRESENCE OF VINASSE IN BEET PULP: IMPACT ON THE HEALTH AND GROWTH OF FATTENING RABBITS

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ABSTRACT

The growth performances and the health status of rabbits fed diets containg 20% of different beet pulps, containing or not vinasses, were analysed in two trials. The trial 1 compare 3 diets (pulp N without vinasse, pulp Bv containing vinasse, pulp N added with 2% of vinasse B "N+vb") fed to 3 groups of 40 weaned rabbits. The trial 2 compare 5 diets (one pulp S without vinasse, two pulps Bv and Tv containing vinasse, the pulp S added with 2% of vinasse B "S+vb", or T "S+vt") fed ad libitum to 5 groups of 120 weaned rabbits. In trial 1, the total number of deads was higher when vinasse was present in the diets, either added or inside the pulp (P=0.039), and the contrast (N vs [N+vb & Bv]) that compared the presence of vinasse or not gave a P level of 0.02 (0 vs 4.5 deads). The health risk index reached meanly 12.5% for the two groups having vinasse compared to the N group (P= 0.015). In trial 2, no significant differences was detected among the 5 diets. However, the contrast (S vs (Bv & Tv & S+vb & S+vt) was almost significant (P=0.055), suggesting that the presence of vinasse increased the Health risk index (10 vs 15,5%). We assumed that a higher risk of digestive trouble in the trial 1 could be linked to the high potassic level of the vinasse of the factory B (7.1%), compared to lower potassic levels of vinasse in the trial 2 (4%). Growth performances did not differ according the diets, either in trial 1 or 2. In trial 2, the feed intake and the feed conversion of the group S was 8% lower compared to the two groups with the same pulp but with added vinasse (S+vb & S+vt; P=0.035). This suggest that adding vinasse to a pulp may increase the intake but without improving the feed conversion. We recommend the use the beet pulp containing vinasse at a moderate level (around 10%) in rabbit diets, and particularly if the potassium level is high (over 4%).

Key words: rabbit feeding, beet pulp, vinasse, growth, health

INTRODUCTION

The beet pulp is a major ingredient of the rabbit feeds at least in Europe and is subjected to large exchanges either in animal feeding or and for energy. Factories producing beet pulps could belong to two main classes. First, factories that are adjacent to a distillery and thus produce beet pulp containing vinasse. In such process, for each liter of ethanol produced, 10 to 15 liters of vinasse must be removed from the distillation units. After concentration, agricultural spreading as fertilizer is the main valorization, but other uses are also possible (Decloux and Bories, 2001). Some of the recycled vinasse is then discharged from the sugar factory in wet beet pulp (92% moisture), which is then pressed and dehydrated. In this case, the factory produces beet-pulps containing already vinasses. Vinasses can be used positively in animal feed (Fisher *et al.,* 1999), but they can lead to poor performances and health status in rabbits (Morisse *et al.*, 1983), in relation to high potassium contents. In return, with a particular vinasse having a very low potassic content (<1%) no effect on rabbit health status was found (Maertens et al., 1994). Second, factories not associated to a distillery and thus producing beet pulp not containing vinasse. Thus, we analyse the performances and the health status of growing rabbits fed with different type of beet pulps, containing or not vinasses, in two trials, at ADM and at Inrae facilities.

MATERIALS AND METHODS

Animals and experimental design

Trial 1 ADM facilities (March 2016).

Three diets (without antibiotics or coccidiostatics) with 20% of beet pulp were formulated and pelleted (table 1): the feed N contained a beet pulp from the factory N, not associated with a distillery and thus not containing vinasse; the feeds B(v) contained a beet pulp from the factory B, associated with a distillery and thus containing vinasse; the feeds N+vb contained a beet pulp from the factory N, and 2% of vinasse from the factory B was added, to simulate the beet pulp of factory B. Beet pulps of factories B and N contained respectively 6.6% and 8.9 of crude ash, and the in-vitro degradability of organic matter were 82.4 and 79.1% (Aufrère 1982). Vinasse of the factory B contained 7.1% of potassium.

Table 1: Ingredient and composition of experimental diets used in trial 1.

Ingredients, g/kg Feeds:	N	N+vb	B(v)
Beet pulp	200.4	180.4	200.4
Vinasse added (factory B)	0	20	0
Cereals and soja	150	150	150
Alfalfa, wheat straw and bran	417	417	417
Rapeseed and sunflower meals	112	112	112
Beet molasse and grapeseed meal	100	100	100
Minerals, premix	20.6	20.6	20.6
Composition,%			
Crude protein	14.3	14.6	14.3
ADFom	23.6	22.6	21.8
ADL	7.9	7.6	7.0
Crude ash	7.5	7.4	7.2

The three diets were given to three groups of 40 weaned rabbits from weaning to slaughter (35-70d). A feeding program was defined as follow: 88g/d in week 1, then 102, 116, 130, and 144 g/d respectively for weeks 2 to 5. Rabbits were housed individually (0.153m²), the feed intake was controlled weekly, the live weight and morbidity were controlled at 49 and 70d of age, while mortality was checked daily.

Trial 2 INRAE facilities (October 2017)

Five diets (without antibiotics or coccidiostatics) with 20% of beet pulp were formulated and pelleted (table 2): the diet S contained a beet pulp from the factory S, not associated with a distillery and thus not containing vinasse; the diet B(v) contained a beet pulp from the factory B, associated with a distillery and thus containing vinasse; the diet T(v) contained a beet pulp from the factory T, associated with a distillery and thus containing vinasse; the diet "S+vb" contains a beet pulp from the factory S, and 2% of vinasse from the factory B was added to simulate the beet pulp of factory B; the diet "S+vt" contained a beet pulp from the factory S, and 2% of vinasse from the factory T was added to simulate the beet pulp of factory T. Beet pulps of factories S, B and T contained respectively 6.2, 6.6% and 11.1 of crude ash, and their ADF content were 21.1, 21.1, and 20.6%. Vinasse of the factories T and B contained 4.0 and 4.1% of potassium.

The 5 diets were fed ad libitum to five groups of 120 rabbits, from weaning to slaughter (34 to 70d old) to analyse more precisely the health status of rabbits. Rabbits were housed collectively (6 rabbits per cage of 0.35m², 20 cages per group). The feed intake was controlled at 49d and 70d of age on 10 cages per group. The live weight and morbidity were controlled at 49 and 70d of age, while mortality was checked daily.

Statistical Analyses

No outlier was found for growth performances. All data were analysed using SAS software. For each trial, a single factor variance analysis was used to estimate the diet effect on performance traits (live weight and growth), while mortality and morbidity were analysed with Catmod procedure of SAS.

RESULTS AND DISCUSSION

Presence of vinasse and health status of the growing rabbit

The diets of the trial 1 were formulated to reach a relatively high level of ADF and ADL to reduce the risk of digestive pathologies. In return, in the trial 2 the level of ADF was limited to 19%, to reach a "moderate" digestive security. Accordingly, for the whole period (35-70d), the health risk index was meanly of 8% in trial 1, and 14% in trial 2 (table 3 and 4). For the two trials, the mortality and morbidity was always due to diarrhoea.

In trial 1 (table 3), when vinasse is added to pulp N (group N+vb), the number of morbids increased (n=3 on 40) two weeks after weaning (P=0.043). In addition, the total number of deads was also higher when vinasse was present in the diets, either added or inside the pulp (P=0.039), and the contrast (N vs [N+vb & Bv]) that compared the presence of vinasse or not gave a P level of 0.02 (0 vs 4.5 deads). Finally, and although the number of replicates was moderate, the health risk index reached meanly 12.5% for the two groups having vinasse compared to the N group (P= 0.015).

Table 2: Ingredient and composition of experimental diets and beet pulps used in trial 2.

Ingredients, g/kg Feeds:	S	Bv	Tv	S+vb	S+vt
Beet pulp	200.0	200.0	200.0	184.9	180.4
Vinasse added				15.1	19.6
Cereals	167	167	167	167	167
Alfalfa, wheat straw and bran	376	376	376	376	376
Soja and sunflower meals	190	190	190	190	190
Grapeseed pulp	50	50	50	50	50
Minerals, premix	17	17	17	17	17
Composition,%					
Crude protein	17.6	17.1	17.0	17.5	17.7
ADFom	19.1	19.9	19.8	18.7	19.1
Crude ash	7.7	8.3	8.5	8.0	8.2

Table 3: Health status of growing rabbits according to the groups in the trial 1, from weaning to slaughter (35-70d)

	Groups:	N	N+vb	Bv	*P level*
deads/alives (mortality rate, %)	at 49 d	0/40 (0.0)	1/39 (2.5)	0/40 (0.0)	*NS*
	at 70 d	0/40[a] (0.0)	6/34[b] (15.0)	3/37[ab] (7.5)	*0.039*
morbids/healthy (morbidity rate, %)	at 49 d	0/40[a] (0.0)	3/36[b] (7.7)	0/40[a] (0.0)	*0.043*
	at 70 d	0/40 (0.0)	0/34 (0.0)	1/36 (2.7)	*NS*
deads+morbids/total (health risk index, %)	at 49 d	0/40[a] (0.0)	4/36[b] (10.0)	0/40[a] (0.0)	*0.016*
	at 70 d	0/40[a] (0.0)	6/34[b] (15.0)	4/36[b] (10.0)	*0.047*

Table 4: Health status of growing rabbits according to the groups in the trial 2, from weaning to slaughter (35-70d).

Groups:	S	Bv	Tv	S+vb	S+vt	*Sign. Stat*
deads/alives	11/109	12/108	19/101	18/102	13/107	
mortality rate, %	9.2	10.0	15.8	15.0	10.8%	*>0.20*
morbids/healthy	1/119	5/115	1/119	2/118	3/117	
morbidity rate, %	0.8	4.2	0.8	1.7	2.5	*>0.20*
deads+morbids/total	12/108	17/103	20/100	20/100	16/104	
Health risk index, %	10.0	14.2	16.6	16.6	13.3	*>0.20*

In trial 2 (table 4), with a higher number of replicates, the number of morbids and dead rabbits was numerically lower for the group S (without vinasse), but no significant differences was detected among the 5 diets. However, the contrast (S vs Tv) tended to be significant (P=0.12). The contrast (S vs (Bv & Tv & S+vb & S+vt) was almost significant (P=0.055), suggesting that the presence of vinasse increased the Health risk index (10 vs 15,5%).

Table 5: Live weight, growth and feed conversion according to the groups in the trial 1.

Groups:	N	N+vb	Bv	*VCr, %*	*P level*
Live weight at 35 d, g	1039	1039	1039	*5.6*	*NS*
Live weight at 70 d, g	2552	2531	2549	*3.5*	*NS*
Weight gain, g/d	43.2	42.5	43.0	*5.7*	*NS*
Feed intake, g/d	116.5	115.8	116.8	*1.9*	*NS*
Feed conversion	2.71	2.74	2.72	*8.4*	*NS*

When comparing the two trial, we assumed that a higher risk of digestive trouble in the trial 1 could be linked to the high potassic level of the vinasse of the factory B (7.1%), compared to lower potassic levels of vinasse in the trial 2 (4%).

Presence of vinasse and growth performances of the rabbit

Growth performances did not differ according the diets, either in trial 1 (table 5) or 2 (table 6). In trial 2, the feed intake and feed conversion differed according to the diets, but without a main effect of the presence of vinasse. However, the feed intake and the feed conversion of the group S was 8% lower compared to the two groups with the same pulp but with added vinasse (S+vb & S+vt; P=0.035). This suggest that adding vinasse to a pulp may increase the intake but without improving the feed conversion.

Table 6: Live weight, growth and feed conversion according to the groups in the trial 2.

Aliments	S	B(v)	T(v)	S+vb	S+vt	*VCr, %*	*P level*
Live weight at 34 d, g	858	857	857	857	857	*8.9*	*NS*
Live weight at 70 d, g	2387	2416	2400	2404	2394	*6.3*	*NS*
Weight gain, g/d	42.5	43.6	42.8	42.9	42.8	*3.9*	*NS*
Feed intake, g/d	145.0 ^{b}	146.7 ^{b}	156.9 ^{ab}	159.1 ^{a}	155.8 ^{ab}	5.4	<0.001
Feed conversion	3.45 ^{ab}	3.37 ^{b}	3.67 ^{a}	3.71 ^{a}	3.64 ^{a}	5.1	<0.001

CONCLUSIONS

The compilation of two trials strongly suggested that the presence of vinasse, either inside the beet pulp or added to the pulp, increased the risk of digestive trouble for the growing rabbit. The post-weaning growth of the rabbit remained similar whatever the type of beet pulp. Therefore, adding vinasse gave no improvements in growth or feed conversion. We thus recommend to use the beet pulp containing vinasse at a moderate level (around 10%) in rabbit diets, and particularly if the potassium level is high (over 4%).

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NUTRITIONAL ASSESSMENT OF LOCAL AGRICULTURAL PRODUCTS AND BYPRODUCTS AND THEIR COMBINATIONS IN NON-CONVENTIONAL EXPERIMENTAL'S DIETS FOR RABBITS

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ABSTRACT

Food constitutes the most expensive component in the meat production systems of the rabbit species and with greater weight in tropical countries, where conventional raw materials are not available for the majority of producers. The research work was carried out with the objective of nutritionally analyzing products and local agricultural byproducts and three experimental's diets, based on the chemical characterization and estimation of digestive utilization by rabbits (Oryctolagus cuniculus). Were used for the study, the foliage gold button (*Tithonia diverifolio (Hemsl.*) A. Gray), fruits breadfruit tree (*Artocarpus altilismapen*), fruits of royal palm (*Roystonea regia o.F.Cook*); shell of the fruit of the cocoa plant (*Theobroma cacao L*.), sugar cane stem (*Saccharum officinarum L*.) Which were processed until obtaining meal, Coconut meal (*Cocos nucifera L*.) was purchased directly from the Baracoa oil extraction factory, from these ingredients three experimental's diets and a control diet was formulated with conventional raw materials, the chemical composition of the ingredients and the processed diets was determined. To determine the digestibility coefficients of the experimental's diets and the control diet were used eight adult rabbits of the Chinchilla breed with an average live weight of 2234 g ± 102.21 g. a latin square design was used (2 rabbits x 4 diets x 4 periods). The treatments were a control diet and the three experimental's diets, the controlled variables were the digestibility coefficients of DM, CP, CF and OM. The products and byproducts used showed an adequate balance of nutrients, otherwise, the formulated experimental's diets revealed nutrient content that meets the nutritional requirements of rabbits in the growth and fattening phase, also promoted percentages of digestibility coefficients in normal ranges for dry matter, crude protein, crude fiber and organic matter.

Keywords: Agricultural product and byproducts, rabbits, nutrition and non-conventional diets

INTRODUCTION

In recent years, livestock systems for meat production in Cuba have experienced a marked decrease, motivated by various factors, among which feeding stands out, with monogastric species being the most affected. One of the effective ways to mitigate this decrease is the promotion of rabbit farming, by reducing its production costs, with the use of cheaper foods, available in large quantities, that are characterized from a nutritional point of view and not competitive with human nutrition (Apocalypse et al., 2008).

On this issue Acosta *et al.* (2016) point out that in Cuba there is a large amount of waste derived from agricultural activity that is currently a subject of study in order to use them as alternative sources in feeding rabbits (*Oryctolagus cuniculus*). The objective of this work was to nutritionally analyze products, agricultural byproducts local and their combination in

experimental's diets, based on chemical characterization and estimation of digestive utilization by rabbits.

MATERIALS AND METHODS

To prepare the experimental's diets, gold button (*Tithonia diverifolio (Hemsl.) A. Gray*), Breadfruit tree (*Artocarpus altilismapen*), Royal palm fruits (*Roystonea regia o.F.Cook*), Shell of the fruit of the cacao plant (*Theobroma cacao* L.) and sugar cane stem (*Saccharum officinarum L.*), were dehydrated separately to approximately 90 % dry material. Coconut meat meal (*Cocos nucifera L.*) was purchased directly from the Baracoa oil extraction factory. The grinding of these agricultural products and byproducts was carried out in a hammer mill with a 2.5 mm sieve. The formulation of the experimental's diets was carried out based on the nutritional requirements of growing rabbits reported by the literature (Table 1). For the chemical analysis, 2 kg of each raw material and the formulated experimental diets were used. The chemical determinations were carried out in the chemistry laboratory of the National State Inspection Office in Santiago de Cuba.

Table 1. Composition of the experimental's diets made with agricultural products and byproducts and the control diet.

	Experimental's diets			Control diet	
Raw material	A	B	C	Raw material	%
Gold button meal (%)	35	35	35	Corn meal	24,72
Breadfruit meal (%)	27	26	27	Alfalfa meal	55,33
Coconut meat meal (%)	30	30	30	Soya meal	12,87
Plan nut meal (%)	4	-	-	Dicalcium phosphate	1,06
Shell of cacao fruit meal (%)	-	5	-	Sodium chloride	0,30
Sugar cane stem meal (%)	-	-	4	DL-Methionine	0,12
Dicalcium phosphate (%)	1	1	1	L-Lysine	0,00
Calcium carbonate (%)	1	1	1	Min-Vit1 Premix	2,00
Common salt (%)	1	1	1	Zeolite	1,50
Mineral premix (%)	1	1	1	Coconut oil	2,10

To determine the digestibility coefficients of the experimental's diets and the control diet were used eight adult rabbits of the Chinchilla breed with an average live weight of 2234 g ± 102.21 g, a Latin square design was used (2 rabbits x 4 diets x 4 periods). The treatments were a control diet and the three experimental's diets described above, the controlled variables were the digestibility coefficients of DM, CP, CF and OM.

The experiment was carried out in 48 days, divided into four successive periods of 12 days (six days of adaptation of the animals, three days of collection of excreta and food waste and three days of rest for sanitization of the facility and accessories). The animals were placed individually in metabolic cages at a rate of two rabbits per treatment in each experimental period. The temperature ranged between 23 and 28 °C, and the hours of light/dark were 12/12. The diets were offered ad libitum in the form of flour throughout the experiment.

The chemical determination data were analyzed based on the determination of the mean, standard deviation and coefficient of variation. While to evaluate the effect of the experimental diets on the nutrient digestibility coefficients, an analysis of variance was carried out based on the experimental design used and the differences between the means were determined with the Duncan (1955) test. In all cases, STATGRAPHICS plus 5.1 statistical software was used.

RESULTS AND DISCUSSION

The chemical composition of the fruits of royal palm, sugar cane stem and shell of the fruit of the cocoa plant (table 2). It shows a discrete protein content and a high content of fibrous material, this last component is very valuable, since it regulates the speed of digestion through the rabbit's gastrointestinal tract and promotes adequate digestive functioning (García et al., 1999). Gold button meal foliage flour and coconut meal have crude protein contents higher than those required for growing rabbits according to de Blas and Mateos (2010); On the other

hand, it is observed that most of these ingredients have a high calcium and phosphorus content. The diets prepared with these products and byproducts showed CP and FC levels within the recommended range for growing rabbits according to the nutritional value tables reported by these authors, who reported values between 14.2-16.0% for the first and 15.0-16.0% for the second.

Table 2. Nutrient composition of local agricultural products and byproducts and diets prepared with their incorporation.

Ingredients	DM (%)	CP (%)	CF (%)	NDF (%)	NDF (%)	Ca (%)	P (%)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Gold button	87.16±0.005	23.36±0.005	27.02±1.06	27.65±1.37	43.74±1.37	2.30±0.02	0.30±0.03
FBT	84.64±0.005	8.36±0.005	4.49±0.45	17.58±0.53	27.80±0.71	0.98±0.16	0.14±0.01
Coconut	84.34±0.005	19.46±0.005	9.78±0.65	41.52±0.64	70.48±0.44	0.23±0.02	0.54±0.03
SCS	80.84±0.005	2.12±0.004	31.23±2.01	35.41±0.81	68.77±0.17	1.01±0.06	0.33±0.04
RPF	86.98±0.44	5.84±0.005	34.32±1.50	55.65±0.73	55.54±0.82	0.38±0.10	0.68±0.09
SCFF	86.18±0.004	8.44±0.005	33.11±2.33	52.54±0.80	58.20±0.48	0.78±0.01	0.12±0.01
ED A	86.56±0.005	16.68±0.55	15.57±0.48	29.40±0.50	44.07±0.06	1.05±0.07	0.46±0.06
ED B	85.44±0.005	16.61±0.45	15.54±0.36	29.13±0.74	46.87±0.21	1.01±0.07	0.45±0.02
ED C	85.96±0.005	16.48±0.27	15.57±0.28	28.41±0.13	48.48±0.20	1.07±0.06	0.45±0.03
Control diet	88.87±0.004	17.78±0.005	14.08±0.03	27.18±0.12	60.82±0.20	1.13±0.05	0.50±0.03

- FBT: Fruits Breadfruit tree; SCS: Sugar cane stem; RPF: Royal palm fruits; SCF: Shell of cacao fruit.
- ED A: Experimental diet A; ED B: Experimental diet B; ED C: Experimental diet C

Table 3 shows the apparent digestibility coefficients of diet made with local agricultural products and byproducts. In general, the digestibility values of the nutrients in these diets showed, values in the range of those reported for diets made with non-conventional ingredients. Only the digestibility coefficients of DM and CP for experimental diet C and for organic matter for experimental diets B and C showed differences with respect to the control diet. Are several factors that influence the digestibility of the nutrients in the diets, in this case, the most probable cause could be the content of indigestible material reflected by the content of neutral detergent fiber present in experimental diet C, which comes from the sugar cane stem meal used in its formulation.

Table 3. Digestibility coefficients of diets made with local agricultural products and byproducts and the control diet.

	Experimental's diets					
Indicators (%)	A	B	C	Control diet	EE	Valor P
DMCD	63.12^{ab}	64.27^{ab}	60.29^{b}	71.83^{a}	2.53	0.015
CPCD	72.86^{ab}	74.06^{ab}	70.15^{b}	75.22^{a}	1.85	0.039
CFCD	46.70	46.79	48.38	49.71	2.07	0.696
OMCD	70.15^{b}	73.68^{ab}	69.84^{b}	78.54^{a}	1.92	0.007

^{ab} *Different Super index in the same row indicates significant differences at p<0,05, Duncan p<0,05*
DMCD*: Dry matter digestibility coefficient,* CPCD*: Crude protein digestibility coefficient,* CFCD *Crude fiber digestibility coefficient and* OMCD *Organic matter digestibility coefficient*

CONCLUSIONS

Agricultural products and byproducts could be considered promising for use in rabbit diets, since they present a balanced content of nutrients grouped mainly in ingredients that provide crude protein (coconut meal, gold button foliage meal) and fibrous material (fruits of royal palm, shell of the fruit of the cocoa plant and the stem of sugar cane). The digestibility coefficients of the nutrients evaluated did not present high variability with respect to the control diet, except for diet C, which differed from the control diet for the three nutritional components.

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EFFECT OF A FOOD BASED ON *STYLOSANTHES GUIANENSIS* ON THE DIGESTIVE HEALTH, GROWTH AND REPRODUCTIVE PERFORMANCE OF RABBITS IN IVORY COAST

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ABSTRACT

The objective of this work is to evaluate the zootechnical and health performances of Oryctolagus cuniculus rabbits fed with a pellet formulated with a fibrous core based on *Stylosanthes guianensis*. The rabbits used are hybrids resulting from informal crossbreeding. The study involved 300 breeding animals aged 90 days, 240 females and 60 males. Of the 240 females, 60 were used for the growth study from 90 to 174 days and 180 for the reproduction study from 90 to 239 days. For each test, two groups were formed. One group C served as control and the other experimental Sg. The Sg group received diet based on *Stylosanthes guianensis*. This diet contained a level of 46% *Stylosanthes guianensis*. Group C was fed a commercial feed containing alfalfa used in Ivory Coast. Growth, reproduction and health parameters were measured. The results showed that the Sg group and the C group had similar animal growth and reproduction performance. The mean values of average weight, average daily gain and feed conversion ratio were 3500 g, 20 g/d and 5.8, respectively. The observed reproductive parameters were also similar. Thus, the receptivity, parturition and fertility rates observed were 84%, 72.5% and 66% respectively. The numerical productivity was respectively 5.7 total born, 4.8 born alive and 4.7 born weaned. On the other hand, mortality and health risk were respectively 4 and 6% higher in group C compared to group Sg ($P<0.05$). The stillbirth rate of the experimental group was also better.

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Keywords: Rabbit, Diet, Growth, Reproduction, Mortality.

INTRODUCTION

Le développement de l'élevage de lapins en Côte d'Ivoire se heurte à de nombreux obstacles. La qualité et le coût élevé des aliments font partie de ces obstacles majeurs (PSDEPA, 2014 ; Kimsé et al., 2017). La luzerne, principale source de fibres utilisée dans l'alimentation des lapins en Côte d'Ivoire, est non seulement importée mais constitue l'une des principales causes du coût élevé de l'alimentation industrielle.

Ainsi, l'objectif de ce travail est de trouver une alternative en utilisant une source locale de fibres, à savoir *Stylosanthes guianensis*, dans l'alimentation des lapins en Côte d'Ivoire. *Stylosanthes guianensis* possède de bonnes caractéristiques, dont 84,8 % de matière sèche (MS), 10,4 % de protéines brutes (CP), 35,6 % de fibres brutes (CF) et 1980 kcal/kg d'énergie digestible (DE).

MATERIALS AND METHODS

Animals and experimental design

Oryctologus cuniculus hybrid rabbits from a cross between Californians and New Zealanders. A population of 300 breeders comprising 240 females and 60 males aged 90 days with an average weight of 1890 ± 14 g were used. The assessment of growth and health status was carried out on 60 females. Measurements were taken until 174 days of age. Breeding parameters required an additional 180 nulliparous females. They were also divided into two

batches (Diet C and Diet Sg) of 90 females for 30 males. Measurements were taken between 90 and 239 days.

Batches C were fed with a FACI® brand industrial feed containing alfalfa produced in Ivory Coast. The Sg groups were fed pelleted diet containing only *S. Guianensis* as the fibrous core *S. Guianensis* was incorporated at 46% into the diet (Table 1).

Ingestion was monitored daily. Health monitoring was carried out by daily monitoring of morbidity and mortality of animal's morning and evening. The health risk index (IRS) was evaluated by adding the number of cases and the number of deaths. To measure reproductive parameters, females were placed in contact with the male from the age of 120 days. Receptivity, fecundity and parturition rate (MTb) were calculated.

Table 1: Theoretical chemical composition* of experimental diets

Chemical composition (%)	Diet C	Diet Sg
Dry matter	90.33	88.9
Fat content	2.40	2.6
Crude protein	15.4	17.2
Neutral detergent fiber	21.10	32.6
Acid detergent fiber	14.03	19.3
Acid detergent lignin	2.50	4.6
Extractable non-fiber carbohydrates	23.73	24.6
Digestible energy (Kilocalories per kilogram)	1811	2401

Statistics Analysis

The mean weight, weight gain and feed efficiency of rabbits in the two groups were compared using Student's t test. The chi-square test was used to compare proportions linked to reproductive parameters such as receptivity, fertility, parturition rate and numerical productivity. The same test was applied to compare mortality parameters such as morbidity, mortality and health risk index. The recorded parameters of the two batches were compared using the statistical software R version 3.1.0 at the threshold of 5%.

RESULTS AND DISCUSSION

Effect of *Stylosanthes guianensis* on growth and ingestion

This study showed that the use of *S. guianensis* as a source of fiber in the diet of the experimental batch did not affect the animals consumption **(table 2)**. The fiber content of this diet would meet the needs of rabbits. Indeed, intake drops when the fiber content of the ration does not cover the animals' needs (Gidenne *et al*. 2012). This would lead us to say that *S. guianensis* is a source of quality fiber allowing a good level of ingestion. Consumption is comparable to that usually observed in Côte d'Ivoire in various studies (Kimse *et al*., 2014). However, it is 20 g lower due to the high temperature and relative humidity which reduces feed intake. These climatic conditions also reduce the growth of animals from tropical countries compared to their counterparts from temperate countries of the same age. This slow growth is justified by the very advanced age of these does. Indeed, at 3 months, there is a strong increase in IC and a strong deposition of fat (Gidenne *et al*., 2019). Adult rabbits generally have a low health risk index.

Effect of *Stylosanthes guianensis* on health

Rabbits fed the *S. guianensis ration* showed better health. The better health performance obtained with this ration could be linked to a better balance of nutrients and better digestibility **(table 3)**. The diet would have no impact on fertility, much less on receptivity. These parameters are rather linked to the strain used (Lebas *et al*., 2010). However, for parturition,

the most suitable diet could improve this factor. This would partly explain the strong tendency of rabbits fed a ration containing *S. guianensis* to have a better birth rate.

Effect of *Stylosanthes guianensis* on the numerical productivity of does

In does, the ration seems to impact productivity parameters **(table 4)** in primiparous animals. From birth to 20 days of age, rabbits feed exclusively on mother's milk. Their survival therefore depends on the quality and quantity of this milk. *S. guianensis* could therefore have a galactogenic effect. Indeed, certain fodder improves the quantity of milk in rabbits (Kouakou *et al.*, 2012). The ration containing *S. guianensis* allows the rabbits' needs to be covered with milk from the 3rd week of age. These characteristics of the ration with *S. guianensis* would explain the low stillbirth and mortality of young rabbits at the nest. The recorded stillbirth is twice as low as that observed in Morocco by Jaouzi *et al*. (2004).

Table 2: Effect of the ration based on *Stylosanthes guianensis* on the growth and feed efficiency of future breeding does.

	Diets		*ESM*	*P value*
	C	Sg		
Number of animals	*30*	*30*		
Average weight				
90d	1880 ± 4	1900 ± 14	*7.5*	*0.59*
118d	2516 ± 8	2526 ± 2	*17.5*	*0.29*
146d	3065 ± 1	3080 ± 5	*5*	*0.21*
174d	3523 ± 4	3513 ± 4	*0.57*	*0.97*
daily weight gain (g/d)				
90 - 118d	22 ± 1	23 ± 1	*2.88*	*0.76*
119 - 146d	20 ± 0	21± 1	*0.04*	*0.20*
147 - 174d	16 ± 1	17± 0	*0.005*	*0.40*
90 - 174d	20 ± 1	20 ± 0	*0.32*	*0.47*
Food consumption (g/d)				
90 to 118 d	96	98	*0.14*	*0.07*
119 to 146 d	116	115	*4.00*	*0.59*
147 to 174 d	129	132	*3.24*	*0.64*
90-174 d				
conversion index				
90 - 118d	4.1 ± 0.4	4.2 ± 0.5	*0.02*	*0.81*
119 - 146d	5.5 ± 0.2	5.8 ± 0.2	*0.04*	*0.59*
147 - 174d	7.6 ± 0.3	7.4 ± 0.2	*0.08*	*0.59*
90 - 174d	5.7 ± 0.5	5.9 ± 0.2	*0.04*	*0.59*

Table 3: Effect of the ration based on *Stylosanthes guianensis* on the health of breeders

	Diets		*P value*
	C	Sg	
Number of animals	***30***	***30***	
90 to 174 d			
Morbidity	3(10%)	2(6.7%)	*0.34*
Mortality	3(10%)	2(6.7%)	*0.01*
Health Risk Index	6(20%)	4(13.3%)	*0.01*

Table 4: Number of total rabbits born, born alive per delivery and number of rabbits weaned per weaning

	Diets		*ESM*	*P value*
	C	Sg		
Number of animals	*90*	*90*		
90-239d				
Total rabbits born	5.9	5.5	*0.1*	*0.57*
Rabbit born alive	5.1	4.5	*0.32*	*0.21*
Weaned rabbit/litter	4.9	4.5	*0.1*	*0.12*

CONCLUSIONS

The objective of this study was to evaluate the effect of a feed based on *Stylosanthes guianensis* on mortality, growth, and reproduction parameters in rabbits. The results obtained indicate that using *S. guianensis* as an alternative to alfalfa in the diet of growing and breeding rabbits is beneficial. The main advantages of this feed are its local availability and favorable nutritional characteristics, such as high crude protein and digestible energy content. Additionally, it has positive effects on the growth and reproduction parameters of rabbit's while being a safe option. Therefore, *S. guianensis* can be used effectively and safely to replace alfalfa in the diet of growing and breeding rabbits in tropical countries.

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DEHYDRATED BANANA PEEL IN DIETS FOR NEW ZEALAND WHITE RABBITS ON MEAT QUALITY AND OXIDATIVE STABILITY

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ABSTRACT

Rabbit meat presents high biological protein value, low fat, tenderness and flavor. Besides that, rabbits have some peculiar characteristics such as the efficient use of fiber, thus providing residues and by-products is possible, which reduces the application of products that may be used in human nutrition, in addition to improve meat properties. In this sense, the aim of this study was to evaluate the quality and oxidative stability of meat from New Zealand White rabbits fed increasing levels of dehydrated banana peel (DBP). Forty animals (mixed sex) were used, distributed in a completely randomized design, from 35 to 85 days of age, with ten replications per treatment. Four pelleted experimental diets were provided, with increasing levels of DBP inclusion (0.00; 3.33; 6.67 and 10.00%). At 85 days of age, all animals were slaughtered to determine meat quality parameters (pH 45 min, pH 24h, color, cooler carcass shrinkage, unfreeze loss, cooking loss and shear force) and oxidative stability (TBARS, DPPH and ABTS). There was no difference among treatments ($P>0.05$) for meat quality parameters. For all oxidative stability parameters there was no interaction between treatments and periods ($P>0.05$). Even so, there was a linear increase in oxidation depending on the days of storage, as well as a reduction depending on the levels of DBP added to the diets ($P<0.001$). In conclusion, the addition of up to 10% dehydrated banana peel in diet for rabbits can keep meat quality attributes and improve the antioxidant capacity of chilled meat for up to 72h.

Key words: Fruit waste, Rabbit farming, Shelf life.

INTRODUCTION

Rabbit farming stands out for being a viable and sustainable alternative of animal protein, as animals achieve high fertility and productivity rates in small areas. Thus, it is possible to have a significant capacity for balance with the environment, considering reducing the environmental impact, through the use of agro-industrial waste in rabbit food (Ferreira et al., 2012).

In this context, research on the efficient use of agro-industrial by-products is relevant, especially in developing countries where rabbit farming is not consolidated. This way, it is possible to minimize food insecurity, reduce costs and achieve environmental sustainability in production systems (Falcone et al., 2023). Banana peel have a high concentration of polyphenols, which present nutraceutical, antimicrobial, and antioxidant properties at moderate levels (Qamar and Shaikh, 2018). The advantages and benefits in the feeding of rabbits and poultry with dehydrated banana peel (DBP) has been studied by several researchers in recent years (Falcone et al., 2020; Sugiharto et al., 2020; Shumye et al., 2022). Thus, DBP could add extra nutritional components, with bioactive properties, able to mitigate oxidative stress in meat and improve shelf life.

The aim of this study was to evaluate the quality and oxidative stability of meat from New Zealand White rabbits fed increasing levels of DBP.

MATERIALS AND METHODS

Animals, experimental design and treatments

The experiment was carried out at the State University of Maringá, Brazil, in the Rabbit Farming Sector, between October and November 2021 (temperature: 26,1±3,8ºC; relative humidity: 72,7 ±5,4%). All experimental procedures were approved by the University's Committee for Ethical Conduct in the Use of Animals in Experimentation (CEUA/UEM) (Protocol no. 7526270720).

Forty 35-days-old New Zealand White rabbit (mixed sex) with initial weight of 775 ±22g were used. The experimental design was completely randomized, with four treatments and ten replicates, with one animal per experimental unit. Treatments were a basal diet (0.00% DBP) and three test diets (3.33, 6.67 and 10.00 % DBP). Basal diet was formulated with alfalfa hay, tifton 85 hay, wheat bran, corn, soybean meal, amino acids, minerals, vitamins and additives, according to the requirements of adult rabbits (De Blas and Mateos, 2010). All diets were isonutritive. Animals received water and food *ad libitum* from 35 to 85 days.

Rabbits were housed individually in experimental cages (0.6 x 0.8 x 0.4 m), equipped with automatic drinker (nipple) and semi-automatic metal feeder. The ripe banana peels were collected immediately after disposal at the Popular Restaurant of Maringá, Brazil. The fractions were individually disintegrated in an electric organic waste crusher, and subsequently dehydrated in the sun until they reach a minimum dry matter of 90%. After obtaining the dehydrated material, the DBP was crushed in a knife-type mill (sieve of 2.5 mm holes), which resulted in an average geometric diameter of 1048 µm.

Meat quality and antioxidant analyses

At 85 days of age, all animals were slaughtered, after a period of 12 hours of fasting, through electrical stunning and subsequent bleeding. Then, the skin was removed and evisceration was performed.

The pH of loin muscle (l*ongissimus lumborum*) was measured using a portable digital pH meter HI 99163 (Hanna Instruments) in hot carcasses, 45 min after slaughter (pH 45 min) and in chilled carcasses, kept in the cold room (1 - 2ºC) for 24 h (pH 24 h), wrapped in transparent plastic bags. For qualitative evaluation of the carcass, 24 hours after slaughter, the cold carcasses were weighed, to calculate the cooler carcass shrinkage. Samples (1.5 cm thick) of the loin muscle were taken for subsequent determination of water loss due to thawing and cooking.

The color of *longissimus lumborum* muscle was measured 24 h after slaughter, using Minolta luminosity measurements (L*, a* and b*), the portable colorimeter CR-400 Konica Minolta's, (settings: Illuminant D65; 0º angle of vision and 4 auto-average). The components L* (brightness), a* (red-green component) and b* (yellow-blue component) were expressed in the CIELAB color system.

The cooked *longissimus lumborum* samples were used to measure the shear force. From each sample, six sub-samples were taken in a cylindrical shape (diameter 1.27cm), longitudinally, in the direction of the muscle fibers, according to recommendations by Ramos and Gomide (2007). The analyzes were carried out on a Stable Micro System TA-XT2i texturometer, coupled to the Warner-Bratzler Shear Force probe and the Texture Expert Exponent – Stable Micro Systems software.

In periods 0, 24, 48 and 72 hours after obtaining the chilled carcass, meat samples from the right leg (thigh + drumstick) were collected for lipid extraction, which were immediately stored in a freezer (-18ºC) until analysis. Oxidative stability was assessed by measuring malonaldehyde, through the analysis of Thiobarbituric Acid Reactive Species (TBARs), according to the methodology used by Vital et al. (2016). For antioxidant capacity analysis, a sample of ± 5 g of meat was crushed and 10 mL of methanol was added to the sample, which was homogenized and filtered. The analysis was performed using the stable free radical

scavenging method 2,2-diphenyl-1-picrylhydrazyl (DPPH), according to Li et al. (2009) and the total antioxidant capacity, using the 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical capture method or ABTS, described by Erel (2004).

Statistical Analysis

Analysis of variance was performed using GLM procedure of SAS statistical software. For levels of DBP inclusion in diets, the degrees of freedom were unfolded into orthogonal polynomials, to obtain the regression equations. The evaluation of oxidative stability was carried out in a 4 *vs* 4 factorial scheme, with four DBP levels (0.00; 3.33; 6.67 and 10.00%) and four cooling times at 4ºC (0, 24, 48 and 72h). For all analyses, a significance level (P) of 0.05 was adopted.

RESULTS AND DISCUSSION

There was no difference among treatments (P>0.05) for meat quality parameters (Table 1). It is likely that at the levels evaluated, DBP was not able to result in effects on the meat quality, considering that DBP has a high bioactive compounds content. Banana peel has an appreciable concentration of tannins, tocopherol, carotenoids, flavonoids and other bioactive compounds with potent antioxidant activity, even after dehydration and pelletizing diet (Falcone et al., 2023). This work is part of a larger study where productive parameters have been measured, but growing performance was not influenced by the same levels of DBP.

Table 1. Meat quality of rabbits fed diets with increasing levels of dehydrated banana peel (DBP)

Parameters	DBP levels (%)				SEM[1]	P-value
	0.00	3.33	6.67	10.00		
pH 45 min	6.87	6.99	6.99	6.96	0.02	0.113
pH 24 h	5.63	5.50	5.54	5.66	0.02	0.621
Minolta L*	57.30	55.68	57.20	56.91	0.23	0.501
Minolta a*	12.46	12.01	12.23	12.89	0.19	0.792
Minolta b*	5.26	4.73	5.55	5.45	0.11	0.534
Cooler carcass shrinkage (%)	1.88	1.76	1.85	1.77	0.19	0.117
Unfreeze loss (%)	1.50	1.54	1.60	1.53	0.16	0.811
Cooking loss (%)	43.70	45.29	44.47	41.63	0.84	0.216
Shear force (N)	17.36	18.85	18.22	17.88	0.59	0.506

1 - Standard error of mean.

There was no interaction (P>0.05) between DBP levels and period of meat cooling (shelf life) for any parameter of oxidative stability (Table 2). DBP levels increased (P<0.001) the capture of DPPH and ABTS radicals, and stopped the generation of TBARS in meat, indicating an improvement in antioxidant defense. The evaluation days reduced (P<0.001) the capture power of DPPH and ABTS radicals and increased the generation of TBARS, a progressive oxidation due to the meat storage time.

In general, the phenolic compounds present in banana peel have a marked antioxidant characteristic (Sundaram et al., 2011). Antioxidants are substances capable of stabilizing free radicals when donating a hydrogen, preventing oxidative stress (Moure et al., 2001). Another mechanism related to phenolic groups is accept an electron to form relatively stable complexes, inhibiting chain oxidation reactions of cellular components (Scalbert et al., 2005).

Bioactive compounds, even in small concentrations, when exposed to an oxidizable substrate can delay or inhibit the oxidation of these substrates. Among the oxidizable substrates in cellular tissues are lipids, proteins, nucleic acids and other molecules (Yang, 2012). Even after three days under storage conditions, meat from rabbits that consumed levels of up to 10% of

DBP maintained high oxidative stability, indicating DBP can be a nutritional tool to improve meat stability.

Table 2. Effect of increasing levels of dehydrated banana peel (DBP) in diets for New Zealand White rabbits on the generation of thiobarbituric acid reactive substances (TBARS - mg MDA Eq/kg), percentage capture (%) of 2,2- radical diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in drumstick, at different storage periods (4ºC).

Variables	DBP levels (%)				SEM[1]	Period (h)				SEM	P-value		
	0.00	3.33	6.67	10.00		0	24	48	72		DBP *vs* Period	DBP	Period
TBARS	13.89	1.20	0.87	0.81	0.39	2.61	3.63	4.63	5.91	0.32	0.548	<0.001[2]	<0.001[3]
DPPH	31.88	52.87	56.11	62.10	0.75	58.73	52.14	48.84	43.24	1.04	0.751	<0.001[4]	<0.001[5]
ABTS	52.91	57.11	62.44	66.34	0.52	65.46	61.50	58.17	53.67	1.07	0.645	<0.001[6]	<0.001[7]

1- Standard error of mean.
2- Linear effect: Y = 14.087 - 3.957X (R^2=0.62)
3- Linear effect: Y = 1.465 + 1.091X (R^2=0.99)
4- Linear effect: Y = 27.264 + 9.3908X (R^2=0.85)
5- Linear effect: Y = 63.183 – 4.977X (R^2=0.98)
6- Linear effect: Y = 48.290 + 4.564X (R^2=0.99)
7- Linear effect: Y = 69.382 – 3.873X (R^2=0.98)

CONCLUSIONS

In conclusion, the addition of up to 10% dehydrated banana peel in diet for rabbits can keep meat quality attributes and improve the antioxidant capacity of chilled meat for up to 72h.

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EFFECTS OF THE REPLACEMENT OF *IPOMOEA AQUATICA* FOR COMMERCIAL PELLET FEEDS IN DIETS ON GROWTH PERFORMANCE AND CARCASS CHARACTERISTICS OF GROWING CROSSBRED RABBITS

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ABSTRACT

This study aimed to evaluate the effects of replacing water spinach (*Ipomoea aquatica*) (WS) for commercial pellet feed in the diets on growth performance, nutrient digestibility, carcass characteristics, and meat quality of growing crossbred rabbits. The experiment was a completely randomized design with 6 treatments, 5 replications, and 2 rabbits at 35 days old (365±11.9 g/per) with balanced sexes per trial unit. The six treatments consisted of different levels of replacing commercial pellet feed with water spinach, namely 0%, 20%, 40%, 60%, 80%, and 100% dry matter (%DM) in the diets, corresponding to WS0, WS20, WS40, WS60, WS80, and WS100, respectively. The experiment was conducted for 12 weeks. The experimental nutrient digestible period was conducted during the 6th week of the experiment and carried out for 6 days continuously. After completing the feeding trial, all rabbits were slaughtered to evaluate the carcass characteristics and meat quality. The results indicated that increasing levels of WS in the diet decreased ($p<0.05$) dry matter intake and daily weight gain with the lower values observed in the WS100 treatment. The dry matter digestibility, organic matter digestibility, and acid detergent fiber digestibility also decreased ($p<0.05$), along with lower nitrogen retention ($p<0.05$), as the levels of WS in the diets increased. The values of the WS0 treatment were significantly different ($p<0.05$) compared to the WS100 treatment but not significantly different from the WS60 treatment. The final live weight and carcass weight were reduced ($p<0.05$) by increasing the levels of WS in the diet. However, the composition of meat quality did not change among the treatments. In conclusion, WS60 (60% *Ipomoea aquatica* and 40% commercial pellet feed) could be used for growing rabbits while maintaining growth performance, nutrient digestibility, and carcass weight.

Keywords: Carcass characteristics, crossbred rabbits, *Ipomoea aquatica,* growth performance, commercial feed.

INTRODUCTION

In recent years, rabbit husbandry has been developing rapidly for several reasons, one of which is that rabbits could take advantage of many local by-products or available vegetables and green plants. However, In the Mekong Delta, rabbit farmers currently mainly rely on experience on a small and spontaneous scale. The diets for rabbits are mainly based on locally available food sources, without regard to nutritional needs. The dietary nutrient content and nutrient intake of rabbits are lower than domestic recommendations, while research on the nutritional needs of crossbred rabbits is still very limited (Chau & Thu, 2015).

Besides, water spinach (*Ipomoea aquatica)* has the most potential for livestock growth performance, including chickens, pigs, cattle, rabbits, and goats, especially in tropical countries. It is not only an alternative feed source but also one kind of supplemented feed for goat raising (Channy *et al.,* 2023). Commercial pellet feed (CPF) as a source provides more energy immediately and it helps reduce the time raising and decreasing labor costs. However, currently, studies on the replacement levels between forage and CPF are still very limited in

terms of rabbit production in the Mekong Delta of Vietnam. This study aims to determine the optimal replacement level of water spinach in the diet for growth performances and carcass characteristics of crossbred rabbits.

MATERIALS AND METHODS

The study was performed at Experimental Farm, Can Tho University, Vietnam. All procedures were carried out in compliance with the ethical standards stated in the Helsinki Declaration of 1975, revised in 2000, in addition to following the national laws.

Animals and Experimental Design

The experiment consisted of 60 New Zealand White weaned crossbred rabbits (New Zealand White x local rabbits) at 35 days old (365±11.9 g/per) produced at the experimental farm. Rabbits were vaccinated against parasitic diseases, polio, and respiratory diseases. The experiment was a completely randomized design with 6 treatments and 5 replications with 1 male and 1 female rabbits per trial unit. Six treatments were 0, 20, 40, 60, 80, and 100% replacing water spinach for commercial pellet feed (%DM) in the diets corresponding to WS0, WS20, WS40, WS60, WS80, and WS100, respectively. The experiment was carried out for 12 weeks. The rabbits were housed in cages and had free access to clean drinking water. The experimental rabbits were fed commercial pellet feed first following treatments. Rabbits were fed *ad libitum* water spinach after they consumed commercial pellet feed. The ingredients and chemical composition of experimental diets were shown in Table 1.

Table 1: Chemical composition and metabolizable energy of feeds used in the experiment, %DM

Feed	DM	OM	CP	EE	NDF	ADF	CF	Ash	ME, MJ/kgDM
Ipomoea aquatica	9.72	89.3	20.1	4.65	36.0	20.4	16.3	10.7	10.0
Commercial pellet feed	90.8	92.9	14.9	4.53	45.2	28.3	16.4	7.10	9.98

Note: DM: dry matter, OM: organic matter, CP: crude protein, NDF: neutral detergent fiber, Ash: total mineral, ME values of feeds were calculated according to Maertens et al. (2002)

Chemical Analyses and Measurements

Feed offered and refusals were recorded daily in the morning during the experiment. The samples were collected every 2 weeks. Samples were dried at 55^0C for 48 hours until the weight was unchanged, then finely ground for chemical composition analysis. The experimental nutrient digestible period was conducted during the 6th week of the experiment and carried out for 6 days continuously. The feeds offered, refusals, feces, and urine were collected for nutrient digestibility and nitrogen retention measurement. Samples of feed offered refusals and feces were dried and finely ground. Then mix the samples of 3 days according to each experimental unit to analyze the nutrient components such as DM, OM, CP, EE, NDF, ADF, CF, and Ash. After finishing the feeding trial, all the rabbits were slaughtered for evaluating the carcass characteristics and meat quality. The rabbits were fed twice daily at 7:00h and 17:00h. All rabbits were weighed once a week throughout the experiment and before feeding in the morning.

Chemical composition of feeds and feces including dry matter (DM), organic matter (OM), crude protein (CP), crude fat (EE), crude fiber (CF), and ash were analyzed following the methods described by AOAC (1990). NDF analysis was done according to the Van Soest et al. (1991) and ADF was analyzed according to Robertson and Van Soest (1981). The metabolizable energy (ME) values of feeds were calculated according to Maertens et al. (2002). The slaughtering procedure was implemented according to the standards of QCVN 01-75: 2011/BNNPTNT (2001). Carcass (after removing blood, head, 4 feet, hair, skin, and internal organs), lean meat, and thigh meat were weighed for an evaluation. One hundred grams of the loin and thigh meat were sampled and put into a thermos containing ice and immediately brought to the laboratory for analyzing DM, OM, CP, EE, and ash (AOAC, 1990) within a day. The nutrient digestibility was determined following the method described by McDonald et al. (2010).

Statistical Analysis

All data were analyzed with the General Linear Model of Minitab 13.21 program (Minitab, 2016). The significance of pairwise comparisons was determined by Tukey posttest. Significance was declared at P<0.05.

RESULTS AND DISCUSSION

Effects of the replacement levels of water spinach in the diet on the growth period

Table 2 showed the nutrient daily intake and growth performance of experimental rabbits in the growth period. The DM, OM, EE, NDF, ADF, CF, and ME intake were reduced (P<0.05) by increasing levels of water spinach in the diets. In the growth performance, the daily weight gain decreased gradually (P<0.05) followed by the increase in the water spinach ratio. In contrast, FCR decreased from WS100 (4.27) to WS0 (2.79). There was a close linear relationship (R^2= 0.83) between water spinach intake and daily weight gain of experimental rabbits. (Y= -0.199X +26.3).

Table 2: Effects of the replacement levels of the water spinach on nutrient intake and growth performance in the crossbred rabbits

Items	Treatments						SE	p
	WS0	WS20	WS40	WS60	WS80	WS100		
Dry matter, g/per/day								
Ipomoea aquatica	0.00^{e}	20.0^{d}	26.7^{cd}	39.1^{bc}	43.0^{ab}	54.6^{a}	3.37	0.001
Commercial pellet feed	67.2^{a}	58.5^{a}	44.9^{b}	29.8^{c}	15.4^{d}	0.00^{e}	2.97	0.001
Nutrients intake daily, g/per/day								
Dry matter	67.2^{ab}	78.5^{a}	71.7^{ab}	68.9^{ab}	58.4^{ab}	54.6^{b}	4.98	0.028
Organic matter	62.5^{ab}	72.2^{a}	65.6^{ab}	62.6^{ab}	52.7^{ab}	48.8^{b}	4.53	0.015
Crude protein	10.0	12.7	12.1	12.3	10.9	11.0	0.89	0.290
Ether extract	3.05^{ab}	3.58^{a}	3.28^{ab}	3.17^{ab}	2.70^{ab}	2.54^{b}	0.23	0.041
Neutral detergent fiber	30.4^{ab}	33.6^{a}	29.9^{ab}	27.5^{abc}	22.4^{bc}	19.7^{c}	2.01	0.001
Acid detergent fiber	19.0^{a}	20.6^{a}	18.2^{ab}	16.4^{ab}	13.1^{bc}	11.1^{c}	1.20	0.001
Crude fiber	11.0^{ab}	12.8^{a}	11.7^{ab}	11.3^{ab}	9.54^{ab}	8.91^{b}	0.81	0.025
Ash	4.77	6.29	6.05	6.30	5.70	5.85	0.46	0.217
Metabolizable energy, MJ	0.67^{ab}	0.78^{a}	0.72^{ab}	0.69^{ab}	0.58^{ab}	0.55^{b}	0.05	0.028
Growth performance								
Initial weight, g	361	365	367	373	362	365	5.46	0.635
Final weight, g	2423^{a}	2277^{a}	2256^{a}	2027^{a}	1940^{a}	1440^{b}	113	0.001
Daily weight gain, g/day	24.6^{a}	22.8^{a}	22.5^{a}	19.7^{a}	18.8^{a}	12.8^{b}	1.35	0.001
Feed conversion ratio	2.79	3.62	3.27	3.59	3.14	4.27	0.39	0.171

$^{a, b, c, d, e}$ Mean values with different superscripts within the same row are different at P<0.05. WS0, WS20, WS40, WS60, WS80, and WS100 were the treatments replacing water spinach for commercial pellet feed (%DM) in the diets at 0, 20, 40, 60, 80, and 100%, respectively.

Effects of the replacement levels of water spinach in the diet on the nutrient digestibility

Table 3 showed the nutrient digestibility, and nitrogen retention of rabbits during the nutrient digestibility period. The dry matter digestibility, organic matter digestibility, ether extract digestibility, and N retention decreased (P<0.05) following the increasing water spinach levels.

The results of slaughter weights, carcass traits, and chemical composition of the rabbit meat

The live weight at slaughter period and carcass weight decreased (P<0.05) by increasing water spinach in the diets. This result was higher than the result of Dong & Thu (2021) with the highest live weight and carcass weight corresponding to 2160 g and 1057 g.

CONCLUSIONS

The results indicated that increasing water spinach levels in the growing rabbit diets reduced performance. Using 60% water spinach and 40% commercial pellet feed in the growing rabbit

diet did not affect the growth performance, nutrient digestibility, and carcass characteristics of crossbred rabbits.

Table 3: Nutrient digestibility, and Nitrogen retention of the nutrient digestibility period

Items	Treatments						SE	p
	WS0	WS20	WS40	WS60	WS80	WS100		
Nutrient digestibility, %								
Dry matter	75.5[a]	75.8[a]	74.9[a]	71.9[ab]	68.6[b]	68.5[b]	1.86	0.020
Organic matter	75.6[a]	75.9a	75.1[a]	72.1[ab]	68.9[b]	69.0[b]	1.81	0.022
Crude protein	77.1	78.9	77.4	79.2	78.1	77.4	1.63	0.919
Ether extract	86.7[a]	87.2[a]	84.2[ab]	78.6[abc]	75.7[bc]	69.4[c]	2.21	0.001
Neutral detergent fiber	64.9	65.3	66.1	60.6	58.4	56.2	3.25	0.198
Acid detergent fiber	57.3[a]	56.7[a]	55.9[ab]	50.3[abc]	45.4[bc]	43.3[c]	2.45	0.001
Nitrogen balance								
N urine, g	0.17[ab]	0.13[b]	0.21[ab]	0.33[ab]	0.24[ab]	0.36[a]	0.05	0.017
N retention, g	1.54[ab]	1.68[a]	1.54[ab]	1.34[ab]	1.26[ab]	1.06[b]	0.13	0.023

[a, b, c, d] Mean values with different superscripts within the same row are different at P<0.05. WS0, WS20, WS40, WS60, WS80, and WS100 were the treatments replacing water spinach for commercial pellet feed (%DM) in the diets at 0, 20, 40, 60, 80, and 100%, respectively. N: nitrogen.

Table 4: The slaughter weights, carcass traits, and chemical composition of the rabbit meat

Items	Treatments						SE	p
	WS0	WS20	WS40	WS60	WS80	WS100		
LWS, g	2530[a]	2519[a]	2302[b]	2068[c]	1929[c]	1552[d]	35.3	0.001
CS, g	1500[a]	1375[b]	1260[c]	992[d]	808[e]	643[f]	11.9	0.001
Chemical composition of the rabbit meat								
Dry matter	27.3	27.1	27.0	27.1	27.3	27.0	0.27	0.914
Organic matter	98.1	98.1	98.0	98.2	98.2	98.0	0.14	0.778
Crude protein	20.5	20.6	21.7	21.3	21.9	22.2	0.51	0.122
Ether extract	4.50	4.46	4.70	4.56	4.70	4.50	0.12	0.610
Ash	1.91	1.94	2.00	1.78	1.78	1.99	0.14	0.778

[a, b, c, d, e, f] Mean values with different superscripts within the same row are different at P<0.05. WS0, WS20, WS40, WS60, WS80, and WS100 were the treatments replacing water spinach for commercial pellet feed (%DM) in the diets at 0, 20, 40, 60, 80, and 100%, respectively. LWS= Live weight at slaughter; CW= Carcass weight

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EFFECTS OF DIETARY METABOLIZABLE ENERGY LEVELS ON CROSSBRED RABBIT SEMEN CHARACTERISTICS IN MEKONG DELTA, VIETNAM

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ABSTRACT

The study aimed to evaluate the effects of metabolizable energy levels in the diet on rabbit semen characteristics in Mekong Delta conditions (Temperature Humidity Index (THI) 28.2±0.50). Thirty-five crossbred bucks (New Zealand White x local), with an average weight of 2.38 ± 0.16 kg, were used in a completely randomized design consisted of five treatments and seven replications, with one buck as an experimental unit. The five experimental treatments were diets containing 9.5, 10.0, 10.5, 11.0, and 11.5 MJ/kg DM metabolizable energy corresponding to ME9.5, ME10, ME10.5, ME11, and ME11.5, respectively. The experiment was carried out for 18 weeks in which the sperm of bucks was collected by an artificial vagina and analyzed weekly from the sixth week until the eighteenth week. The results showed that there was no difference (P>0.05) in terms of the final live weight, daily weight gain, and testosterone concentration. However, the testicular measurement results (testis length, testis weight, and testis volume) were significantly different (P<0.05) among treatments. The semen ejaculate volume and pH ranged from 0.49-0.57 mL and 6.99-7.18, respectively. Results of semen characteristics showed higher values with the ME11.5 diet in terms of viscosity (1.17 cp), and total motile sperm/ejaculate (80.6x106 sperm), compared to other treatments (P<0.05). Experimental results showed that the diet containing 11.5 MJ/kg DM ME is suitable for rabbits in the Mekong Delta.

Keywords: Buck rabbits, crossbred rabbits, metabolizable energy, molasses, sperm.

INTRODUCTION

In the Mekong Delta of Vietnam, rabbit husbandry mainly takes advantage of available green grass, vegetables, and agro-industry by-products such as molasses, beer waste, and soya waste... Therefore, nutrient intake may not be sufficient to meet requirements, especially during growth and reproductive period. In recent years, the application of artificial insemination in rabbit livestock has been developing. However, it requires bucks with higher semen characteristics and greater reproductive performance. Additionally, bucks have a lower semen concentration of 200-600x10^6 sperm/mL (Battaglini *et al.,* 1993), and ejaculate volume was around 0.3-0.6 mL/ejaculation (Adams and Singh, 1981). These results decreased the number of inseminations, since it is recommended to use 0.5 mL of diluted fresh semen containing about a minimum 10x10^6 sperm/mL/insemination (Carluccio *et al.,* 2004).

The higher energy level of the diets gave the higher semen volume and was recommended for bucks (Papadomichelakis *et al.,* 2000). DE at 10.5 MJ/kgDM is used for growing crossbred rabbits in the Mekong Delta of Vietnam (Truong *et al.,* 2021). However, studies on dietary energy content in Vietnam have been still limited, especially on buck nutrition. The study aimed to determine the effects of metabolizable energy levels in the buck's diet on the ability of growth, testicular parameters, and sperm characteristics in the Mekong Delta of Vietnam.

MATERIALS AND METHODS

The study was performed at Experimental Farm, Can Tho University, Vietnam. All procedures were carried out in compliance with the ethical standards stated in the Helsinki Declaration of 1975, revised in 2000, in addition to following the national laws.

Animals and Experimental Design

The experiment included 35 crossbred (New Zealand White x local) buck rabbits (2.38 ± 0.16 kg/buck, 6 months of age) and was arranged completely randomly with 5 treatments

(corresponding to 5 levels of metabolizable energy in the diet: 9.5, 10, 10.5, 11.0, and 11.5 MJ/kgDM, respectively) and seven replications (one buck as an experimental unit). They were kept indoors, in individual cages, with the same environmental conditions of temperature, relative humidity, lighting time, and free access to water in nipple drinkers. Rabbits were vaccinated against common diseases before starting the experiment.

All feedstuffs for the experimental rabbits were in fresh form. Before the experiment, the rabbits were fed ad libitum for a week to monitor feed intake and determine the amount of dry matter required by the rabbits. The animals were fed three times a day: at 7:00 a.m. with half of the heated soya waste, soybean extraction meal, and molasses mixture; at 12:00 p.m. with the other half of this mixture; and at 5 p.m. with para grass, fed ad libitum after consuming all of the mixture. The feed intakes were recorded, calculated, and adjusted every day to ensure the dietary nutrient composition of the experimental design. The experimental diets (Table 1) were calculated based on the DM of feedstuffs, balancing the nutrient requirements, and to obtain different ME level in each treatment.

Table 1: Feed formula and chemical composition of experimental diets

Feed, %DM	ME, MJ/kgDM				
	9.5	10	10.5	11	11.5
Heated soya waste	35	34	36	35	34
Soybean extraction meal	13	15	16	18	20
Brachiaria mutica	49	42	34	26	19
Molasses	3	9	14	21	27
Total	100	100	100	100	100
OM	89.0	89.1	89.4	89.5	89.7
CP	18.0	18.0	18.0	18.0	18.0
EE	4.20	3.90	3.80	3.50	3.20
NDF	41.8	38.1	34.6	30.3	26.7
ADF	29.7	27.3	25.3	22.6	20.3
CF	20.1	18.1	16.2	13.9	11.9
Ash	11.0	10.9	10.6	10.5	10.3
ME, MJ/kgDM	9.50	10.0	10.5	11.0	11.5

Chemical Analyses and Measurements

The chemical composition of feeds and feces including dry matter (DM), organic matter (OM), crude protein (CP), crude fat (EE), crude fiber (CF), and ash were analyzed following the methods described by AOAC (2000). NDF analysis was done according to Van Soest et al. (1991) and ADF was analyzed according to Robertson and Van Soest (1981). The metabolizable energy (ME) values of feeds were calculated according to:

ME (MJ/kgDM) = DE (0,995 - 0,048 DCP/DE) (Maertens *et al.,* 2002) in which:

DE (MJ/kgDM) = 14,9 - 0,22 ADF + 0,35 EE (De Blas *et al.,* 1992)

DCP (%/DM) = -1,15 + 0,82 CP – 0,06 ADF (Fernandez-Carmona *et al.,* 2004)

Table 2: The chemical composition of the ingredients used in the diet (%DM)

Feed	DM	OM	CP	EE	NDF	ADF	CF	Ash	ME, MJ/kgDM
Heated soya waste	15.2	91.0	18.7	6.35	32.2	27.8	15.5	9.00	10.4
Soybean extraction meal	89.0	93.8	42.3	5.68	25.8	18.6	6.60	6.20	11.2
Brachiaria mutica	20.2	86.4	11.9	3.54	55.5	35.2	28.1	13.6	8.04
Molasses	69.1	87.2	3.51	-	-	-	-	12.8	15.0

DM: dry matter, OM: organic matter, CP: crude protein, EE: ether extract, CF: crude fiber, NDF: neutral detergent fiber, ADF: acid detergent fiber, ME: metabolizable energy

The temperature and relative humidity were recorded each 2 hours, from 6:00 am until 6:00 pm. THI was calculated following Marai *et al.,* 2002: THI = t - {(0.31 – 0.31RH)(t – 14.4)} while t is the temperature (ºC) and RH is relative humidity (%).

Semen samples were collected weekly starting from the sixth week until the eighteenth week. Semen samples were collected from individual bucks using an artificial vagina (Ewuola *et al.,* 2014). Semen would be evaluated for color, ejaculation volume, and pH (after 30 minutes of semen collection). Fresh semen samples would be mixed into the medium at a ratio of 1:10 in solution (cold stored at 12-17ºC) and analyzed for sperm characteristics.

Semen was evaluated as described by Hafez and Hafez (2000). The ejaculate volume was determined by reading the volume directly from the calibrated collecting tube and the gel-free

ejaculate volume recorded. Ejaculate pH was determined immediately following collection using pH paper (SpezialIndikatorpapier pH 5.5-9.0, Macherey-Nagel, Germany). Viscosity was determined according to the method of Bootwalla and Froman (1988) with sperm diluted with Ostwald viscometers (funnel diameter 0.8).

The sperm membrane integrity rate was calculated following HOS-Test method by taking 10 µL of semen mixed with 90 µL of HOS solution (100 ml of HOS solution, 70 mOsmol: content sodium citrate 0.343 g and D-fructose 0.630 g). The sperm membrane integrity rate was the ratio of hypoosmotic swelling sperm per observation sperm after incubation at 37^0C for 10 minutes. Sperm motility (%) is the number of active sperms out of the total number of sperm present in each microsphere. The percentage of live/dead spermatozoa was determined using eosin nigrosin stained smears. Semen smears were prepared using one drop of eosin stain on a clean glass slide which was then dried at room temperature. The slide was examined at x400 magnification, using an Olympus CX21 microscope, (Olympus Corporation, Tokyo, Japan). At least 100 cells were counted and the percentage was calculated.

Testicular measurements were recorded at the end of the experiment period from each buck and averaged over the measurements of the two testicles. Testis length (TL) and width (TW) were measured using a flexible measuring tape calibrated in centimeters and millimeters. Testis weight (TM) and testis volume (TV) were estimated using the mathematical model of Bailey et al. (1996), where TM - 0.5533*(TL)*$(TW)^2$ and TV - 0.5236* (TL)*$(TW)^2$. Testosterone concentration was determined by taking the blood samples in the early morning, before feeding by Chemiluminescent Immuno Assay: CLIA method (AutoLumo A1000 machine).

Statistical Analysis

Data were analyzed by ANOVA using the General Linear Model of the Minitab 13.21 program (Minitab, 2016). The significance of pairwise comparisons was determined by Tukey posttest. Significance was declared at P<0.05.

RESULTS AND DISCUSSION

Effects of ME on the growth performance of buck rabbits

The growth performance and feed intake results were similar among different ME levels (Table 3, P>0.05).

Table 3: The growth performance of buck rabbits during the experiment period

Items	ME, MJ/kgDM					SEM	P
	9.5	10	10.5	11	11.5		
Initial live weight (g)	2311	2417	2386	2406	2383	63.5	0.793
Final live weight (g)	2721	2826	2849	2808	2913	102	0.760
Weight gain (g)	532	523	619	503	582	102	0.928
Daily weight gain (g/day)	4.22	4.15	4.91	3.99	4.62	0.81	0.928
Feed intake (gDM/day)	63.4	68.5	67.7	69.0	76.7	3.12	0.086

Effects on testis measurements and testosterone concentration

Testis measurements: length, weight, width, and volume had a linear tendency according to the ratio of ME in the diet (Table 4, P<0.05). There was a higher correlation between testosterone concentrations and testis weight ($y = 240.67x^2 - 4468x + 25293$; $R^2 = 0.7056$). The results showed that the testis volume of bucks had a greater correlation to testis length ($R^2 = 0.9723$). The higher of DWG, the lower of testosterone concentration ($y = -369.34x + 6488.8$; $R^2 = 0.5306$), this result agrees with Marco-Jiménez and Vicente (2018), so that the overweight of bucks would affect testosterone concentrations.

Table 4: Testicular measurements results of bucks during the experiment period

Items	ME, MJ/kgDM					SEM	P-value
	9.5	10	10.5	11	11.5		
Testis length (cm)	6.61^b	7.22^{ab}	6.88^{ab}	7.20^{ab}	7.34^a	0.15	0.011
Testis width (cm)	1.49	1.61	1.60	1.63	1.62	0.05	0.212
Testis weight (g)	8.14^b	10.4^{ab}	9.79^{ab}	10.6^{ab}	10.7^a	0.62	0.039
Testis volume (cc)	7.71^b	9.86^{ab}	9.27^{ab}	9.99^{ab}	10.1^a	0.58	0.039
Testosterol (ng/dL)	4871	4801	4616	5141	4930	132	0.158

Effects on semen characteristics

Bucks fed the ME11.5 diet showed higher semen viscosity, and total motile sperm/ejaculate compared to other treatments (P<0.05).

Table 5: Semen characteristics of bucks fed with the different experimental diets

Item	ME, MJ/kgDM					SEM	*P*
	9.5	10	10.5	11	11.5		
Volume (mL)	0.49	0.57	0.50	0.56	0.57	0.03	0.185
pH	7.12	7.18	7.17	7.08	6.99	0.06	0.209
Viscosity (cP)	1.14^{b}	1.14^{b}	1.13^{bc}	1.12^{c}	1.17^{a}	0.003	0.001
Sperm concentration (x10^6/mL)	207^{ab}	211^{ab}	230^{ab}	191^{b}	239^{a}	10.3	0.013
Motility (%)	58.1^{a}	56.7^{ab}	50.0^{b}	55.3^{ab}	59.0^{a}	1.84	0.014
Total motile sperm (x10^6/ejaculate)	57.6^{b}	67.1^{ab}	58.2^{b}	61.5^{b}	80.6^{a}	4.59	0.007
Live sperm (%)	62.3	57.6	58.0	61.0	64.3	1.83	0.072
Membrane integrity (%)	53.1^{ab}	48.2^{b}	51.0^{ab}	53.6^{ab}	57.3^{a}	1.88	0.026

$^{abc:}$ Means with different letters on the same row differ significantly (*P*<0.05)

CONCLUSIONS

A diet containing 11.5 MJ/kgDM ME is suitable for buck rabbits in the Mekong Delta of Vietnam.

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EFFECT OF CHLOROGENIC ACID ON ILEAL MORPHOLOGY AND PERMEABILITY OF MEAT RABBITS

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ABSTRACT

This study was conducted to evaluate the effects of dietary chlorogenic acid (CGA) supplementation on ileal morphology and intestinal permeability in meat rabbits. Eighty healthy rabbits were randomly allotted to two groups, and fed with a basal diet supplemented with 0 or 1.6 g CGA/kg in a 35-day trial, respectively. The results indicated that CGA decreased (P<0.05) the crypt depth and increased (P<0.05) the ratio of villus height to crypt depth of ileum. Rabbits in CGA group had lower (P<0.05) serum diamine oxidase, lipopolysaccharide, and D-lactate levels, and higher (P<0.05) mRNA expression of ileal claudin-1 and occludin than those in CON group. Our findings suggested that CGA supplementation could promote the ileal development through improving the morphology and decreasing mucosal permeability in meat rabbits.

Key words: Rabbit, Chlorogenic acid, Intestinal morphology, Intestinal permeability

INTRODUCTION

Small intestine is the main site for the absorption of nutrients and serves as the primary barrier against commensal and pathogenic bacteria (Turner, 2009). Nevertheless, the morphological structure change and intestinal barrier dysfunction are the important causes of intestinal diseases. A study found that epizootic rabbit enteropathy is one of the leading causes of rabbit mortality, particularly in meat rabbits (Carabaño *et al*., 2008). Ileum mucosal epithelium serves as a crucial intestinal barrier, separating the internal milieu from potentially hostile external environments in the gut (Turner, 2009). Therefore, it is imperative to explore strategies for preventing ileal mucosal damage in rabbits in a safe and effective manner.

Chlorogenic acid (CGA) is one of the most abundant polyphenols, which has been confirmed possessing positive effects on maintaining intestinal health of piglets (Chen *et al.*, 2018). However, until now, little information is available concerning the effects of CGA on ileal morphology and epithelial barrier function of rabbits.

This study aimed to investigate the effects of CGA supplementation on ileal morphology and mucosal permeability in meat rabbits, which was helpful to clarify the benefit effects of CGA on rabbits.

MATERIALS AND METHODS

Animals and experimental design

A total of 80 healthy meat rabbits (1.05±0.01 kg) were randomly assigned into two treatment groups, with forty replicates of one rabbit each, in a 35-d study. The rabbits received either a basal diet (CON group) or a basal diet added with 1.6 g CGA/kg. The basal diet (4-mm-diameter pellets) was formulated according to the recommended requirements of rabbits in Mateos *et al*. (2010). The ingredient composition and nutrient levels are shown in Table1. The animals were kept in individual cages and had free access to fresh water and feed. On day 35 of the trial, 8 rabbits per group, with the body weight closest to the average, were selected and blood samples were collected from the ear edge vein for harvesting serum samples. Then, the 16 rabbits were slaughtered to collect the distal ileal segment for histomorphological observation and the determination of intestinal barrier-related genes expression.

Table 1: Ingredient composition and nutrient levels of basal diet (as-fed basis)

Ingredients	Content (%)	Nutrient concentrations	Content (%)
Corn	13.3	DM	84.5
Bean pulp	13.0	CP	16.4
Wheat bran	19.0	EE	3.01
Corn germ meal	19.0	CF	15.4
Alfalfa	12.0	NDF	37.9
Soya bean stem meal	19.0	ADF	20.6
Soya oil	0.70	Ash	5.36
Premix[1]	4.00	Ca	1.13
Total	100	P	0.49

[1]Premix provided per kg diet: vitamin A, 8,000 IU; vitamin D3, 1,000 IU; vitamin E, 50 mg; vitamin K3, 2.3 mg; thiamine, 1.75 mg; riboflavin, 6.9 mg; niacin, 28.45 mg; pantothenic acid, 6.7 mg; biotin, 2.75 mg; folic acid, 0.6 mg; vitamin B12, 2.2 mg; choline, 420 mg; lysine, 1.5 g; methionine, 1.5 g; copper, 50 mg; iron, 100 mg; manganese, 30 mg; magnesium, 150 mg; iodine, 0.1 mg.

Histomorphological and chemical analyses

The ileal morphology including villus height (VH) and crypt depth (CD) was examined as previously described by Chen *et al.* (2021). The diamine oxidase (DAO) activity and the D-lactic acid and lipopolysaccharide (LPS) levels in the serum were determined by commercial kits following the manufacturer's instructions. The RNA extraction and quantitative real-time PCR of ileum sample was performed according to the method described by Chen *et al.* (2021).

Statistical Analysis

Data were analyzed by *t*-test using the statistical program SAS 9.4. The results were expressed as means with standard error. Statistical significance was displayed by $P < 0.05$.

RESULTS AND DISCUSSION

The integration of intestinal villus-crypt morphology is vital to the digestion and absorption of nutrients. Intestinal morphological changes, including villous shedding, crypt hyperplasia, and villus atrophy, could result in an invasion of pathogenic bacteria, impairing digestion and absorption of nutrients, which consequently resulted in retarded growth of animals (Turner, 2009). As shown in Figure 1, the CGA rabbits showed significantly reduced CD and increased VH/CD ratio relative to the control rabbits, demonstrating that dietary supplemented with 1.6 g CGA/kg improved the ileal villus-crypt structures.

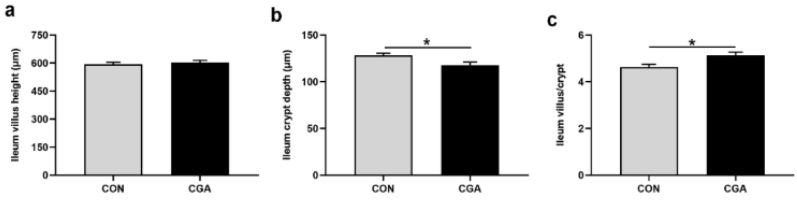

Figure 1: Effects of CGA on intestinal morphology in the ileum of rabbits

On the other hand, dietary CGA supplementation was found to reduce the serum levels of DAO, LPS and D-lactate in the present study (Figure 2). The integrity of intestinal barrier is essential for the proper functioning of epithelial cells and to inhibiting the invasion of pathogenic bacteria causing inflammatory responses (Martel *et al.*, 2022). Numerous studies have indicated that weaning stress can destroy the small intestinal barrier function rapidly after weaning in rabbits (Oglesbee and Lord, 2020). The intestinal barrier injury is often accompanied by increased epithelial permeability. Serum DAO, LPS and D-lactate concentrations are commonly used as quantitative and sensitive circulating markers for evaluating the intestinal permeability (Yang *et al.*, 2003). Once the intestinal barrier integrity is destructed, excessive DAO, LPS and D-lactate will be released into the blood stream via the damaged mucosa. Therefore, our results suggested that 1.6 g CGA/kg decreased the ileal permeability of rabbits.

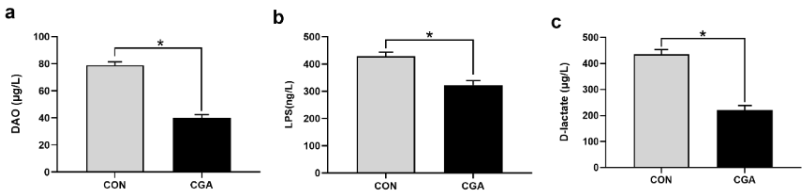

Figure 2: Effects of CGA on serum DAO, LPS and D-lactate of rabbits

The tight junctions between epithelial cells play a crucial role in maintaining and regulating the integrity of the intestinal epithelial barrier. The assembly of tight junctions relies on a variety of distinct proteins, such as the claudins, occludins, and zonula occludens (ZOs) families, which establish a selective permeable barrier by anchoring to the actin-based cytoskeleton (Shen, 2012).

Hence, the levels of tight junction proteins are closely linked to the function of intestinal barrier. In our study, CGA significantly up-regulated the mRNA expression of claudin-1 and occludin in ileum of rabbits compared with the control group (Figure 3), which could potentially elucidate why CGA had the ability to decrease ileal permeability in rabbits.

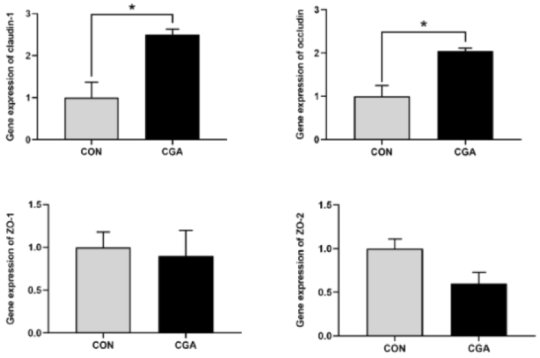

Figure 3: Effects of CGA on mRNA levels of intestinal barrier-related genes in the ileum of rabbits

CONCLUSIONS

In conclusion, dietary supplemented with 1.6 g CGA/kg could promote the ileal development through improving the morphology and decreasing mucosal permeability in rabbits.

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EFFECT OF SAMPLING HOUR, FEED MANAGEMENT AND SOLUBLE FIBRE LEVEL ON DUODENAL, JEJUNAL AND ILEAL DIGESTIBILITY IN POST-WEANED RABBITS

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ABSTRACT

The aim of this work was to evaluate two alternatives to minimize the potential influence of soft faeces on the determination of ileal digestibility in young rabbits fed two different soluble fibre levels (LSF: 8.7 vs. HSF: 12.7% DM): i) feed withdrawal from 8 to 13 h or not, and ii) sampling ileal digesta at 9 h or at 20 h. A total of 240 25-d weaned rabbits were randomly assigned to each of the 8 experimental treatments (30 rabbits/treatment). At 38 d of age all rabbits were slaughtered by head concussion and intestinal digesta samples collected at duodenum, jejunum and ileum. They were pooled by segment within each treatment combination. Feed withdrawal during the morning had no effect on feed intake from 29-35 d or 35-38 d (101 g/d on average. P ≥ 0.53) and did not modify the presence of soft faeces in the stomach at 38 d of age (P = 0.19). At 20 h there were no soft faeces in the stomach (0.0%), but at 8.30 h appeared them as expected (60%. P < 0.001). The duodenal digestibility showed a higher standard error than jejunal and ileal ones for DM (4.85 vs. 0.71 and 0.87), CP (19.4 vs. 1.37 and 1.15) and starch (4.29 vs. 0.87 and 0.47). Intestinal digestibility increased in the more distal parts of the small intestine for DM (9.7, 36.3 and 47.3% for duodenal, jejunal and ileal. P < 0.001), CP (-45.3, 51.8 and 66.6%. P < 0.001) and starch (70.0, 86.4 and 93.9%. P < 0.001). Neither feed withdrawal nor diet influenced intestinal DM and CP digestibility (P ≥ 0.47). When ileal traits were analysed alone, sampling time during the morning increased the variability and mean value of the ileal Yb concentration f(3.1 vs. 1.7), but it did not influence the variability of ileal DM digestibility or other ileal traits. Sampling ileal digesta at 9 h compared with the standard sampling at 20 h also increased CP concentration and reduced the starch one, and increased DM and starch digestibility (P ≤ 0.007), with no effect on CP digestibility. Feed withdrawal did not reduce the variability of any ileal trait or influenced ileal digestibility. In contrast, the variability of the ileal starch concentration was lower in the HSF than in the LSF group (0.074 vs. 0.24). The HSF group had a higher ileal starch digestibility than LSF group 96.1 vs. 92.5%. P < 0.001). Rabbits fed HSF diet showed a lower difference between ileal and jejunal starch digestibility than the LSF group ($P_{diet\ x\ segment}$ = 0.070). In conclusion, collecting an ileal sample in the evening is recommended to determine ileal digestibility for this type of animal.

Key words: methodology, small intestine, digestibility, rabbit

INTRODUCTION

The determination of ileal digestibility of dietary constituents provides useful information either to evaluate nutritive value or gut function. It is a complex methodology that implies the use of indigestible markers, and the ileal canulation when adult rabbits are used, while in young rabbits this is difficult to apply due to the recovering period required (Gidenne et al., 1988). For this reason, in young rabbits the ileal digesta is obtained from slaughtered rabbits. However, the coefficient of variation of the ileal DM digestibility is higher in young (18.5%, ranging from 9.0 to 40.2%) than in adult-cannulated rabbits (11.3%, ranging from 6.0 to 18.3%) (cannulated does: Gidenne, 1992; Merino and Carabaño, 2003; Blas et al., 2003; Abad-Guamán et al., 2015; growing rabbits: Gutiérrez et al., 2003; Gómez-Conde et al., 2007; Chamorro et al., 2007; Martínez-Vallespín et al., 2013; Delgado et al., 2019a, b; and unpublished results). In young rabbits ileal digesta is collected in the evening (from 19 h onwards) to avoid the presence of soft faeces at ileal level (Merino and Carabaño, 2003; Blas et al., 2003) and allow to evaluate the digestibility of the feed ingested rather than the mix of feed and soft faeces. It

simplifies the procedure, as soft faeces collection and analysis is not required, and the balance between the concentration of the indigestible marker in the diet and in the ileal digesta (assumed to be free of soft faeces) provides a direct ileal DM digestibility value. However, the pattern of feed and soft faeces intake differed among young and adult rabbits (Bellier et al., 1995; Carabaño and Merino, 1996; Orengo and Gidenne, 2007; Gómez-Conde et al., 2011) and might account for part of the higher variability observed. No methodological study was performed in young rabbits to evaluate ileal digestibility methodology, and the reduction of the variability might help to reduce the number of rabbits required in these type of trials. The aim of this work was to evaluate two alternatives to minimize the potential influence of soft faeces on the determination of ileal digestibility in young rabbits fed two different soluble fibre levels: i) remove the feed during the morning or not, to help to synchronize soft faeces intake, and ii) collect the ileal digesta early in the morning when soft faeces intake period should be beginning in most rabbits. Besides, the duodenal and jejunal digestibility were evaluated.

MATERIALS AND METHODS

Animals and experimental design

Eight treatments in a factorial arrangement were used: 2 feed management (ad libitum vs. fasting from 8 to 13 h) × 2 sampling hour of ileal contents (20 h vs. 8.30 h) x 2 diets differing in soluble fibre (8.7 vs. 12.7% DM) and starch (20.7 vs. 16.5% DM), and with similar NDF and crude protein (31.4 and 19.7% DM, respectively). Diets included 5 g/kg of alfalfa fibre labelled with ytterbium (Yb_2O_3) (García et al, 1999). Two hundred forty 25-d weaned rabbits (345±46 g liveweight) from primiparous does were blocked by litter, randomly assigned to each of the 8 experimental treatments (30 rabbits/treatment) and housed individually. Growth traits were recorded from 25 to 38 d of age. At 38 d of age all rabbits were slaughtered by head concussion and intestinal digesta samples at duodenum (15 cm ahead of the duodenal flexure), 20 cm in middle jejunum and in the last 20 cm of ileum. Due to the small quantities of sample, digesta from the small intestine was pooled within each treatment combination. The number of rabbits/pool and pools/treatment were: duodenal digesta: 2-8 and 3-6, jejunal digesta: 1-3 and 9-16, and ileal digesta: 1-4 and 8-13. For starch there was only 0-2 pools/sampling hour, feed management and diet. All the experimental procedures used were in compliance with the Spanish guidelines for care and use of animals in research (BOE-A-2013-1337) and authorized by the Dirección General de Agricultura y Ganadería from the Community of Madrid (PROEX 328/15).

Chemical Analyses

Procedures of the AOAC (2006) were used to determine DM (method 934.01), crude protein (method 968.06), starch (amyloglucosidase--amylase method; method 996.11), and total dietary fibre (985.29; TDF). Dietary NDF was determined using a thermo-stable amylase without any sodium sulphite added and corrected for ash and protein. The dietary SF was calculated, as TDF–NDF (both corrected for ash and protein). Ytterbium content of diets and intestinal digesta were assessed by atomic absorption spectrometry (García et al., 1999).

Statistical Analysis

The pool made from digesta of different rabbits was the experimental unit. The results were analyzed by using a mixed model that included as fixed effects feed management, sampling hour, intestinal segment and soluble fibre level, and their interactions. The potential heterogeneity of variances caused by any of the factors studied was considered and evaluated by using the AIC and BIC criteria.

RESULTS AND DISCUSSION

Feed withdrawal during the morning only reduced feed intake in HSF group from 25 to 29 d of age (47.8 vs. 38.2 g/d), but it had no effect in the periods 29-35 d or 35-38 d (101 g/d on average. $P \geq 0.53$). It indicated that restricted rabbits had a higher intake from 13.00 to 8.00 h. In contrast, from 35 to 38 d of age feed withdrawal reduced growth rate (60.8 vs. 56.6 g/d. $P = 0.018$) and feed efficiency (0.630 vs. 0.570 g/g. $P = 0.030$). Feed withdrawal did not modify the presence of soft faeces in the stomach at 38 d of age ($P = 0.19$). At 20 h there were no soft

faeces in the stomach (0.0%), but at 8.30 h appeared them as expected, and were more usual in the HSF than in LSF group (69.4 vs. 51.2% of rabbits. $P_{sampling\ hour\ x\ diet}$ = 0.050).

When the intestinal segments were analysed all together, the duodenal digestibility showed a higher standard error than jejunal and ileal ones for DM (4.85 vs. 0.71 and 0.87), CP (19.4 vs. 1.37 and 1.15) and starch (4.29 vs. 0.87 and 0.47). Intestinal digestibility of samples collected at 20 h was lower than those collected at 9 h for DM (42.3 vs. 52.3%. P < 0.001) and starch (87.9 vs. 93.5%. P < 0.001. Only jejunal and ileal samples for starch). Jejunal CP digestibility was higher when samples were collected at 20 than at 9 h (56.8 vs. 49.9%. P < 0.001), with no hour effect at ileal level (66.8 vs. 66.4%. P = 0.45). Intestinal digestibility increased in the more distal parts of the small intestine for DM (9.7, 36.3 and 47.3% for duodenal, jejunal and ileal ones. P < 0.001), CP (-45.3, 51.8 and 66.6%. P < 0.001) and starch (70.0, 86.4 and 93.9%. P < 0.001). Neither feed withdrawal, diet nor their interactions with sampling hour or segment influenced intestinal DM and CP digestibility (P ≥ 0.44).

When ileal traits were analysed alone, sampling time during the morning increased the variability and mean value of the ileal Yb concentration probably associated to the variable presence of soft faeces (Table 1). Gidenne et al. (1985) also observed in growing rabbits a higher chromium concentration in the ileum at 9 than at 21 h. In this way, Blas et al. (2003) found a higher Yb concentration in soft faeces than in the diet, and also a higher ileal Yb concentration in the morning than in the evening, although the latter was not the case of Merino and Carabaño (2003), both using cannulated rabbit does. In the current study, Yb in soft faeces was not determined. Anyway, the Yb variability did not influence the variability of ileal DM digestibility or other ileal traits. Sampling ileal digesta at 9 h, instead of the standard at 20 h, increased the concentration of CP and improved the digestibility of dry matter and starch, while decreasing the concentration of starch, with no impact on CP digestibility (Table 1). The higher CP and lower starch ileal concentration at 9 h might be compatible with the presence of soft faeces residues (lower starch and higher Yb and protein contents than the diet). A higher ileal CP concentration in the morning was also reported by Gidenne et al. (1985), Merino and Carabaño (2003), and by Blas et al. (2003). In contrast, Gidenne et al. (1988) did not found significant variation in the ileal protein content of cannulated does along the day. Feed withdrawal did not reduce the variability of any ileal trait or influenced ileal digestibility.

Table 1: Effect of sampling time on ileal digestion traits

	20 h	9 h	SEM	P-value
Ileal concentration				
Yb, ppm	101 ± 1.7	123 ± 3.1	--	< 0.001
Crude protein, %	10.2	12.6	0.47	< 0.001
Starch, %	2.39	1.70	0.17	0.007
Ileal digestibility, %				
Dry matter	42.3	52.3	1.00	< 0.001
Crude protein	66.8	66.4	1.62	0.84
Starch	92.9	95.7	0.54	< 0.001

Different standard error values are included when heterogeneous variances exist. N = 47 (20 h) and 37 (9 h).

On the opposite, the diet influenced the variability of starch digestion traits. The variability of the ileal starch concentration was lower in the HSF than in the LSF group (Table 2). The HSF group had a higher ileal starch digestibility than LSF group (P < 0.001). Rabbits fed HSF diet showed a lower difference between ileal and jejunal starch digestibility than the LSF group ($P_{diet\ x\ segment}$ = 0.070). It might indicate a better starch digestibility in the proximal small intestine in the HSF group. It might be associated with the lower starch content of HSF diet, or a higher endogenous digestive capacity and/or microbial activity in the small intestine produced by the increase of soluble fibre (Gómez-Conde et al., 2007; Delgado et al., 2019), or a slower rate of passage in the small intestine.

Table 2: Effect of dietary soluble fibre on ileal starch digestibility

	LSF	HSF	SEM	P-value
Ileal starch concentration, %	2.86 ± 0.24	1.23 ± 0.074	--	< 0.001
Ileal digestibility, %				
Dry matter	47.6	47.0	1.00	0.66
Starch	92.5 ± 0.71	96.1 ± 0.29	--	< 0.001

Different standard error values are included when heterogeneous variances exist. N= 44 (LSF) and 39 (HSF).

CONCLUSIONS

In post-weaned rabbits, neither feed withdrawal from 8 to 13 h nor sampling intestinal digesta at 9 h (to avoid the period of soft faeces intake) helped to reduce variability in the intestinal digestibility traits. Collecting ileal digesta at 9 h can increase the likelihood of soft faeces being present in the sample. As a result, it is advisable to collect ileal samples from these types of animals during the evening.

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EFFECT OF SOURCE OF SOLUBLE FIBRE (APPLE, BEET AND CITRUS), TYPE OF SAMPLE AND TYPE OF GRINDING ON CAECAL *IN VITRO* GAS PRODUCTION IN RABBITS

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ABSTRACT

This work intends to evaluate whether the reduction of particle size of highly fermentable fibrous sources (apple, beet, and citrus pulps) improves the caecal *in vitro* fermentability of the complete sample or their insoluble fraction. The soluble fibre of these ingredients was removed, and the insoluble residue was also incubated. Furthermore, each sample (complete and insoluble residue) was grounded at 1.0 mm or grounded at 1.0 mm and powdered. Both the complete and the insoluble residue of each ingredient, powdered or not, were predigested and incubated with caecal inoculum (4 different inoculums, 3 rabbits/inoculum) for 144 h. The total dietary fibre of the samples was (g/kg DM): 646 and 836 for complete and insoluble beet pulp, 646 and 630 for complete and insoluble apple pulp, and 542 for complete citrus pulp. The type of grinding did not modify the *in vitro* gas production at 12 h (24.4 vs. 22.6 ml/g DM, for 1mm and powdered, respectively. P = 0.19), 24 h (61.2 vs. 59.8 ml/g DM. P = 0.75) or 48 h (108 vs. 113 ml/g DM. P = 0.41). No effect of type of grinding was also observed for the total gas production at 144 h (139 vs. 135 ml/g DM. P = 0.62), and there were no interactions among type of grinding and the other factors. These results suggest that the reduction of particle size did not enhance the fermentability of these ingredients, especially that of the fibrous fraction, which in general is characterized by a relatively high fibre digestibility. The removal of the soluble fibre fraction decreased the *in vitro* gas production at 12, 24 and 48 h (P < 0.001), as expected. Apple pulp (either the complete sample or the insoluble one) showed the highest gas production at 12, 24 and 48 h (P < 0.001). An interaction fibre source × type of sample was observed at 48 h (P = 0.002), due to the much more fermentability of the insoluble fraction of apple pulp than those of beet or citrus pulp. In conclusion the reduction of particle size of high fermentable fibre sources did not increase their *in vitro* caecal fermentability in rabbits. The removement of soluble fibre reduced the fermentability of apple and beet pulps, while apple pulp showed the best fermentability.

Key words: gas production, fermentable fibre, rabbit, type of grinding.

INTRODUCTION

The benefits observed by the increase of dietary soluble fibre on gut health in farms affected by epizootic rabbit enteropathy might be accounted for by its influence on mucosa functionality (higher villus/height ratio, goblet cell numbers and mucin production) and/or by their higher ileal and caecal total dietary fibre fermentability (Gómez-Conde et al., 2007; Abad-Guamán et al., 2015; Delgado et al., 2019). The increase of dietary fibre is expensive and may be interesting to limit its inclusion but maintaining its effectiveness. One option would be to reduce the particle size of sources of fermentable fibre to increase their fermentability. The evaluation of fermentability of fibre-rich ingredients may be done by using *in vivo* and *in vitro* methodologies. The latter is faster and cheaper, limits the use of rabbits and allows the sorting of fibrous ingredients according to their fermentability (Abad-Guamán et al., 2018; Ocasio-Vega et al., 2018ab).

The aim of this work was to evaluate whether the reduction of particle size of highly fermentable fibrous sources (apple, beet and citrus pulps) improves the caecal *in vitro* fermentability of the complete sample or their insoluble fraction.

MATERIALS AND METHODS

Animals and Experimental Design

Three fibrous, low-starch, and low-protein ingredients, having a wide range of fermentation rate and extent were used as substrates for the *in vitro* incubations: sugar beet pulp, apple pulp, and citrus pulp. The soluble fibre of these ingredients was removed (in our lab for beet, and citrus pulp, and in the industry was obtained a depectinized apple pulp) and the residue was also incubated. Furthermore, each sample (complete and insoluble residue) was grounded at 1.0 mm using an ultra-centrifugal mill (ZM 200) and the other half was additionally powdered with a ball mill (Retsch MM400). Both the complete and the insoluble residue of each ingredient, powdered or not were predigested and incubated with caecal inoculum according to Ocasio-Vega et al. (2018ab). Twelve adult rabbits fed a commercial standard diet (g/kg DM: 180 crude protein (CP); 403 neutro detergent fibre (NDF); 93.4 soluble fibre (SF)) were slaughtered at 9:00 h by head concussion. The caecal contents were collected, placed in thermal flasks, and transported immediately to the laboratory. The caecal contents each 3 rabbits were pooled (3 rabbits/pool), obtaining 4 different inoculums. All the experimental procedures used were in compliance with the Spanish guidelines for care and use of animals in research (BOE-A-2013-1337) and authorized by the Dirección General de Agricultura y Ganadería from the Community of Madrid (PROEX 328/15).

Chemical Analyses

Procedures of the AOAC (2006) were used to determine DM (method 934.01), ash (967.05), crude protein (method 968.06), total dietary fibre (985.29; TDF), and acid detergent fibre (973.187). Dietary neutral detergent fibre was determined using a thermo-stable amylase without any sodium sulphite added and corrected for ash and crude protein (NDFom-cp. Mertens et al., 2002). The dietary SF was calculated, as TDF – NDFom-cp (Abad et al., 2015). Dietary acid detergent lignin (ADLom) were analysed according to Van Soest et al. (1991). Insoluble citrus pulp was not analyzed.

Table 1: Chemical composition of fibrous ingredients

Substrates	Ash	CP	TDF	SF	NDFom-cp	ADFom	ADLom
Sugar beet pulp	73.4	102	646	271	375	255	18.9
Insoluble sugar beet pulp	34.0	107	836	75.5	761	513	24.7
Apple pulp	16.8	50.8	646	208	437	308	93.5
Insoluble apple pulp	79.1	92.7	630	68.7	561	438	154
Citrus pulp	131	51.2	542	370	172	123	23.6

CP: Crude protein. TDF: Total dietary fibre free. SF: Soluble fibre NDFom-cp: Neutral detergent fibre, both free of ash and protein. ADFom: Acid detergent fibre free ash. ADLom: Acid detergent lignin free of ash.

Statistical Analysis

Gas production kinetics were modelized according to the logistic model described by Schofield et al. (1994) and the values obtained at 12, 24 and 48 h for each inoculum. Data was analysed as a factorial arrangement by using a mixed model that included as fixed factors the source of fibre, type of sample, and the type of grinding. The inoculum was considered a random effect.

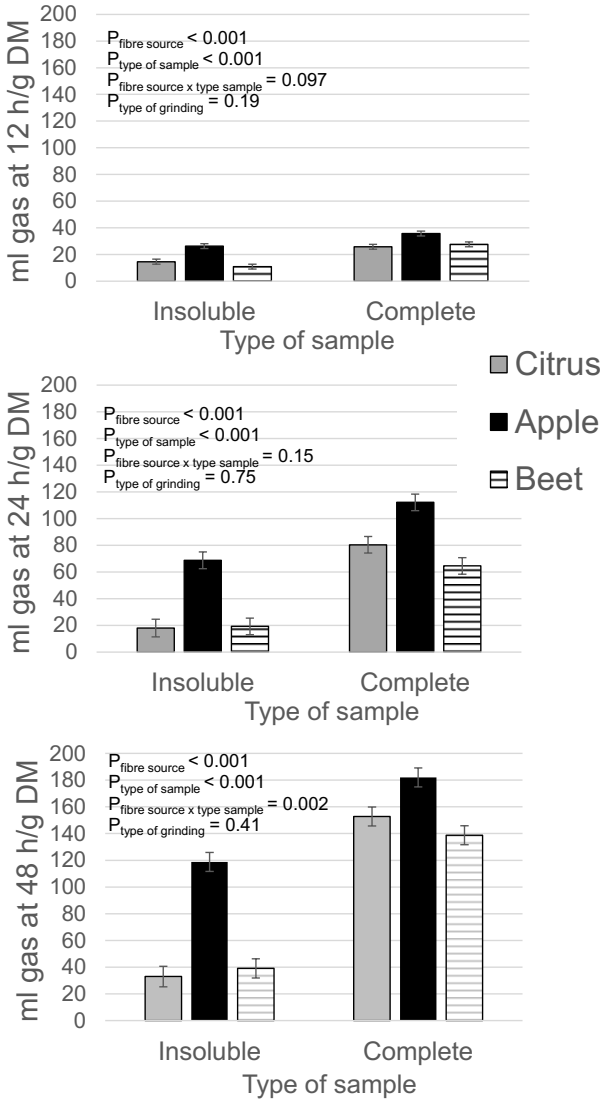
RESULTS AND DISCUSSION

Chemical composition of fibrous sources is shown in Table 1. The type of grinding did not modify the *in vitro* gas production at 12 h (24.4 vs. 22.6 ml/g DM, for 1 mm and powdered, respectively. P = 0.19), 24 h (61.2 vs. 59.8 ml/g DM. P = 0.75) or 48 h (108 vs. 113 ml/g DM. P = 0.41). No effect of type of grinding was also observed for the total gas production at 144 h (139 vs. 135 ml/g DM. P = 0.62), and there were no interactions among type of grinding and the other factors. These results suggest that the reduction of particle size did not enhance the

fermentability of these ingredients, especially that of the fibrous fraction, which is characterized by a relatively high fibre digestibility (Gidenne et al., 2020).

There are no studies evaluating the type of grinding of these ingredients on their fermentability. Previous results indicated no effect of grinding of insoluble fibre on insoluble fibre digestibility (Gidenne et al., 1991; Romero et al., 2011), both obtained grinding alfalfa, or even a negative effect of the reduction of particle size (Lambertini et al., 2000). In contrast, the reduction of particle size or an increase of short particles were associated with an increase of insoluble fibre digestibility in some trials (García et al., 1999; Gomes et al., 2000; Nicodemus et al., 2006; Romero et al., 2024). These discrepancies might be due to the different range of particles sizes and the different ingredients used. Besides, in this study, the powdered of the sample might have negatively affected some potentially fermentable constituents (fibre, sugar, protein).

Figure 1: Effect of the source of soluble fibre and fibrous fraction on in vitro caecal gas production at 12, 24 and 48 h.



The removal of the soluble fibre fraction decreased the *in vitro* gas production at 12, 24 and 48 h (Figure 1. P < 0.001), as expected. Apple pulp (either the complete sample or the insoluble one) showed the highest gas production at 12, 24 and 48 h (Figure 1. P < 0.001). However, it contained less soluble fibre and more lignin than beet and citrus pulp, so this effect might be associated with a higher sugar content (not analysed) or an unexpected higher insoluble fibre degradability.

An interaction fibre source × type of sample was observed at 48 h (P = 0.002), due to the much more fermentability of the insoluble fraction of apple pulp than those of beet or citrus pulp. This result agrees with the closer faecal TDF digestibility between diets based on the complete and the insoluble sample of apple pulp (33.8 vs. 36.0%) than similar diets based on beet pulp (32.1 vs. 38.1%) (Abad-Guamán, 2015; Abad-Guamán et al., 2015). However, it does not fit with the higher lignin and lower soluble fibre content of the insoluble fraction of apple compared to beet pulp (analysis of insoluble citrus pulp was not available).

CONCLUSIONS

The reduction of particle size, from 1 mm to powder, of high fermentable fibre sources like citrus, apple and beet pulps did not increase their *in vitro* caecal fermentability in rabbits. The removement of soluble fibre reduced the fermentability of apple and beet pulps, while apple pulp showed the best fermentability.

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EVALUATION OF MILK PRODUCTION USING TWO METHODS AND RELATIONSHIP WITH REPRODUCTIVE PERFORMANCE

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ABSTRACT

A lot of parameters have an impact on milk production of rabbit does: genetics, the lactation stage, the number of suckling kits, the parity order, as well as environmental conditions as temperature and feed for example. Milk production is well described for multiparous females, but few information are available for nulliparous, primiparous and secondiparous. The aim of this article is 1/ to compare 2 methods evaluating the milk production, by weighing the does or the litters, and 2/ to study the relationship between milk production and reproductive performances. For this, females were weighted 10 days after farrowing before and after suckling during the first 3 lactations, whereas their litters were weighted before and after suckling for the first 2 lactations; the difference of weight before and after suckling being the milk production. Number of total born and born alive were recorded as well as the weight of the litter (with only the alive young rabbits). Milk production at D10 was quite close in bands 1 and 2, but in band 3, it was higher compared to the first 2 bands; 130g of milk more for total daily production, and 10g/d more per young rabbit. Weight at the 1st artificial insemination, total newborn, born alive or litter weight did not impact the total milk production of the rabbit does. The parameters studied in this trial seemed to have no clear effect on milk production regarding nulliparous, primiparous and secondiparous does.

Key words: Milk production, Rabbit doe, reproductive performance

INTRODUCTION

The milk production of the rabbit does is quite variable from one individual to another, and is very dependent on environmental factors as demonstrated in the articles of Savietto et al.(2013) and Maertens et al. (2006). But no study linking this milk production and the reproductive performance of the rabbit does has been published until now. So it seems interesting to study if the number of total rabbits born, born alive, or if the litter weight would have an effect on rabbit does milk production.

The evaluation of milk production can be done by 2 ways as already described in previous studies (Lebas and Zerrouki, 2011, Maertens et al., 2006).

The objective of this article is twofold. The first is the comparison of 2 methods to evaluate the milk production of does, by measuring the weight difference of rabbits does or of litters before and after suckling. The second objective is to study the relationships between milk production and the reproductive performance of young rabbit does.

MATERIALS AND METHODS

Animals and experimental design

The trial was conducted at the ADM-NEOVIA research station located in Saint Nolff (France, 56) between February and August, 2023. This protocol received the approval of the ethics committee and the French Ministry of Research.

136 rabbits does (Optima x PS59) were followed from their 17 weeks of age to the end of their 3rd cycle of reproduction. Animals were placed in cages respecting European regulation on animal experimentation. The number of kits left under the mother was 9, 9 and 10 for farrowing 1, farrowing 2 and farrowing 3, respectively. The reproduction cycle was 42 days long, young rabbits were weaned at 35d of age.

The trial was carried out on a melting herd: no young female was integrated into the herd as the trial progressed. Females empty after inseminations were excluded from the trial.

Thus, females in band 1 (B1) were all nulliparous, in band 2 (B2), they were all primiparous, and in band 3 (B3), they were all secondiparous.

Until 10 days before weaning of the 1st band, females received a commercial feed (2450 kcal/kg, 16% crude protein, 13% starch, 15.8% crude fiber). Between 10d before weaning and weaning, animals receive a preweaning feed containing 2350 kcal/kg, 15% crude protein, 11% starch, and 17% crude fiber. After, female received a feed containing 2530 kcal/kg, 17.5% crude protein, 14.8% starch, and 14.3% crude fiber until 10d before weaning, when whey received again the preweaning feed.
PMSG was not used, and there was no increase in energy intake via a distribution of a complementary feed before farrowing.
In order to evaluate milk production, females were weighted 10 days after farrowing before and after suckling during the 3 lactations, whereas their litters were weighted before and after suckling for the first ant the second lactations; the difference of weight before and after suckling being the milk production. During the weighing used to evaluate milk production, the water was cut off so as not to bias the measurements.
Number of total born and born alive were recorded as well as the weight of the litter (with only the alive young rabbits).

RESULTS AND DISCUSSION

Comparison of 2 methods to evaluate milk production

Table 1: Total milk production and milk production per young rabbit, by weighing the does or the young rabbits, 10 days after farrowing, per band (B1 = nulliparous, B2 = primiparous, B3 = secondiparous).

Band	Total milk production (g) ± SD			Milk production per young rabbit (g) ± SD	
	n	via weighing of does	via weighing of young rabbits	via weighing of does	via weighing of young rabbits
B1	93	243.2 ± 60.2	204.7 ±60.2	27.4 ± 6.4	23.1± 6.6
B2	71	202.0 ± 60.2	239.8 ± 53.5	22.5 ± 6.6	26.7± 5.9
B3	73	352.9 ± 50.2		35.7 ± 5.0	

Milk production per doe or per rabbit is presented in the table 1. In band 1, for nulliparous, quantity of milk produced by the rabbit does (total or per rabbits) was higher when it was evaluated by weighing the doe compared to the evaluation by the young rabbit weighing, with a difference of 38.5g. In band 2, for primiparous, contrary to the band 1, quantity of milk produced by the rabbit does was lower when it was evaluated by weighing the doe compared to the evaluation by the young rabbit weighing, with a difference of 37.8g.
In bands 1 and 2, quantity of milk produced was quite close, but in band 3, it was higher compared to the first 2 bands; 130g of milk more for total production, and 10g more per young rabbits.
The stagnation of milk production in B2 could be explained by the fatigue of the does during cycle 2. Indeed, it is quite common to observe drops in prolificacy and fertility during the 2nd cycle of rabbit does.

Effect of reproduction performance on milk production

The Fig.1, Fig. 2 and Fig. 3 present respectively the relationship between the total newborn, the born alive and the litter weight of alive rabbits and the total production of milk, evaluated by weighing the rabbit does, per band. These results demonstrate that there is no correlation between these parameters and the total quantity of milk produced by the female on D10 after farrowing whether in band 1, 2 or 3, contrary to the result presented in the study of Chibah-Ait et al., (2015) and in the book of Gidenne (2015). Given the lack of effects seen in Figures 1, 2 and 3, it is probably worth noting that milk production could be more related to the lactating litter than to the born litter, (the size of the lactating litter was equalized).

Figure 1: Effect of total newborns on total milk production, per band

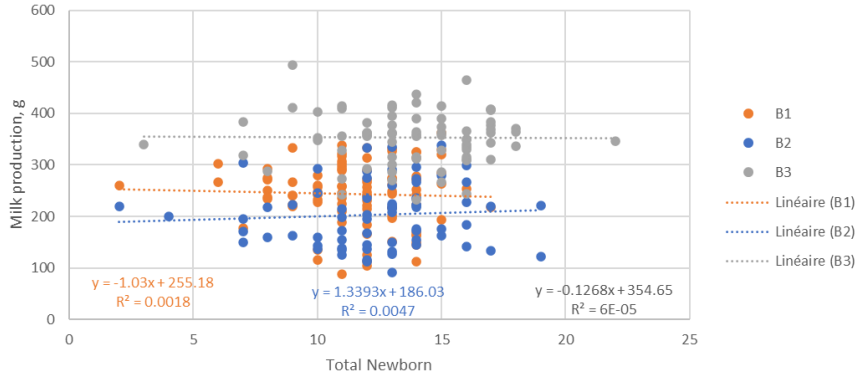


Figure 2: Effect of born alive on total milk production, per band

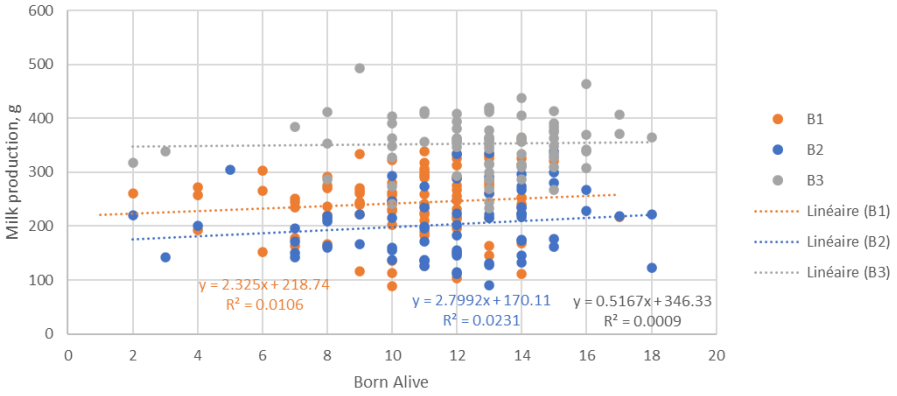


Figure 3: Effect of litter weight on total milk production, per band

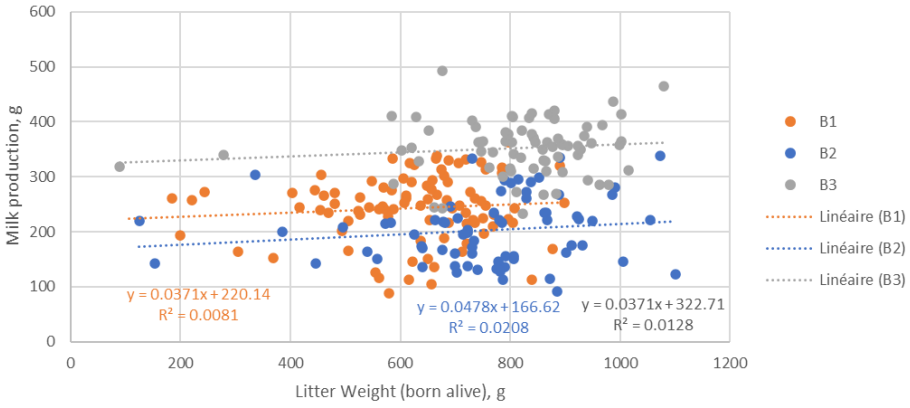
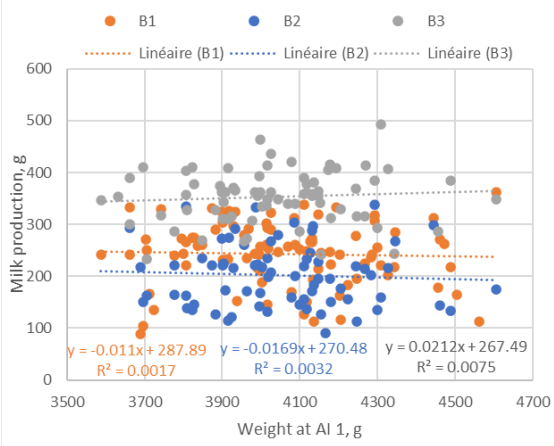
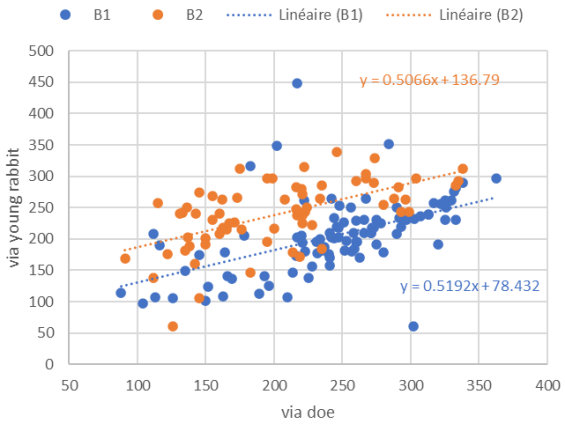


Figure 4: Effect of weight at AI1 on total milk production, per band



There was no effect of the body weight of rabbit does at artificial insemination 1 on the total milk production of the does, weather the band (Fig. 4).

Figure 5: Correlation between the evaluation of milk production via the rabbit doe or via the young rabbits



The correlation between the 2 methods (via weighing the rabbit doe of the young rabbits) was not very good; in band 1, r=0.52 and in band 2, r=0.58 (Fig. 5).

CONCLUSIONS

Milk production at D10 was quite close in lactations 1 and 2, but in lactation 3, it was higher compared to the first 2 lactations; 130g of milk more for total production, and 10g more per young rabbits. Several criteria were studied in order to evaluate if they had an impact on the total milk production of the rabbit does: weight at the first artificial insemination, total newborn, born alive or litter weight. None of these criteria seems to impact the total milk production of the rabbit doe.

The correlation between the 2 methods to evaluate the milk production was not very good. This is difficult to explain because all precautions were taken to avoid possible bias: the water was cut off before weighing, breastfeeding was checked individually, etc.

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USE OF PHYSICALLY SEPARATED SUNFLOWER MEAL WITH 20% CRUDE PROTEIN CONTENT AS AN ALTERNATIVE SOURCE OF PROTEIN IN THE DIET OF GROWING RABBIT

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ABSTRACT

While the EU is self-sufficient in the most important cereals, the level of self-sufficiency in protein rich raw matterialls are particularly low. As a result, it is extremely important to use alternative protein-rich by-products of human food production industry as an alternative protein source for the nutrition of our animalls. With the help of increased usage of these protein rich by-products we can reduce the high import protein dependence of the EU. In our research, we investigated the determination of the optimal usage of the separated sunflower meal with a crude protein content of 20% in the feed of growing rabbits. We examined a control feed without including 20%CP sunflower (D) and five feeds including increasing proportions of 20 % CP sunflower meal 6% (D06), 8% (D08), 10% (D10), 12% (D12) and 14% (D14) both in feed for growing and finishing rabbits. During the experiment, carried out under large scale farming conditions, the performance of fattening animals was monitored until slaughter (79-84 days). We found a significant difference between the different groups in the mortality under the fattening period. The most favourable and statistically proved mortality rate was obtained in the case of the D10 group (5.39%) compared to the control group (10.3%), as a statistically not verifiable worst mortality rate (12.8%) was find at group D14. Although there was a significant difference between the groups (D12 vs. D14) in the evolution of the AI index (total slaughter weight/number of the inseminated does), none of the experimental groups differed significantly from the control group. The conclusion that can be drawn from the results is that also in an increased (up to 14%) use of the 20% CP sunflower meal in the diet of growing rabbits did not result in statistically significant differences in any examined key performance index compared to the control diet

Key words: extracted sunflower meal, fattening rabbit, AI index, feeding

INTRODUCTION

The feeding industry of the European Union basically requires protein imports. These protein-rich feed raw materials are mainly imported from Brazil, Argentina and the USA (Link1). While the EU's self-sufficiency in soybean meal is only 3%, i.e. the import share is 97%, the level of self-sufficiency in rapeseed is also only 69%. The EU has a 100% or close to self-sufficiency level only with regard to the protein supply from low-concentrated bulk feed.

Sunflower oil is a basic human food. During its production, the extracted meal with a crude protein content of 34% have a limited use due to its fiber content, but it is suitable for animal feeding. At the same time, it cannot completely replace soybean meal due to its higher protein content. The extracted sunflower meal exceeds the needs of rabbits due to its high sulfur amino acid content, but its low lysine content can only cover the needs of rabbits in 70% (LEBAS, 2004), so it is justified to give it together with other ingredients with a higher lysine content, such as soybean meal or legumes (CARABAÑO and FRAGA., 1992). Several studies have investigated the role of sunflower meal in the feeding of rabbits and a review prepared by LEBAS and RENAUF (2009) recommended an average mixing ratio of 12%, however, other authors found a much higher mixing ratio of 20% or more to be safe (MARTINA, 1976; KPODEKON et al., 2019; SIDDARAMANNA et al., 2009).

However, from the previously mentioned 34% extracted sunflower meal it is possible to get two fraction after physical separation method. After the separation we get a material with 44% crude protein and low fiber content, similar to soybean meal, and the rest fraction is with a high fiber content matterial, but still containing 20% crude protein. This fiber rich product is no longer sufficiently digestible for monogastric animal and poultry. However, it can be suitable for

ruminants and rabbits feeding, because these two species are close to each other from digestibility point of view as both can use fiber fractions as a source of energy. Since sunflower meal can be considered a safe raw material from a digestive physiology point of view due to its high lignin level (GIDENNE et al. 2010) the 20 % crude protein separatum can be good for the safe feeding of ruminants and rabbit.
The aim of our study was to determine the effect of using different proportions (0, 6, 8, 10, 12 and 14%) of separated sunflower meal with a crude protein content of 20%.

MATERIAL AND METHODS

The experiment was carried out on Olivia Kft's HUSU farm (Pannon white x PannonKa) x Pannon Large crossbred growing rabbits. We formed 6 groups based on their diets. A total of 60,067 rabbits were examined in 6 repetitions in the control group, while 31,090, 29,683, 28,951, 30,292 and 28,958 growing rabbits were examined in 3-3 repetitions (3 different production group in each repetition) respectively in groups D06, D08, D10, D12 and D14. In the present study, we examined the development of KPI's (key performance indexes) from 37 days to the slaughter age (12 weeks). In the experiment, the temperature of the stables was kept at 18-25 °C and 16 hours of lighting was used. The rabbits were housed in a special Landkaninchen welfare technology. The base level was 103x53 cm and from this level via a special passage (13.5 cm x 22 cm) rabbits can climb down to the basin level. The basin level has a floor area (48 cm x 62 cm). 25 cm above the base level is the 2nd level made of plastic (41.5 cm x 53 cm), the 3rd level has a metal grid floor (53 cm x 32 cm), which is also 25 cm above the 2nd level. Thus, the total usable area for the animals, including raised and lowered surfaces, is 1.2322 m^2. The experimental animals were already born in these boxes, and at the weaning (37 days) the mother animals were moved to another production room, while the selected animals remained in their original cages (8-10 animals per cage).
The experimental feeds contained 0, 6, 8, 10, 12, 14% of 20 % CP sunflower meal with a crude protein content of 20% (D, D06, D08, D10, D12, D14; Table 1). The animals received the supplemented diet from the age of 28 days. The main chemical parameters of experimental feeds were similar (digestible energy, DE: 10-10.1 MJ/kg; crude protein, CP: 16.7-17.3%; crude fiber, CF: 17.4-18.0%) the content values were calculated based on the aggregate content (CP, CF based on laboratory examination, DE was calculated with the method DErabbit (Mj/kg RM= 0,0221 x total protein + 0,0398 x total fat + 0,0176 x total fibre + total NFE (Link2)) of the raw materials. From the kindling until the age of 28 days, the does and their kits received a lactation feed including 6% of 20 % sunflower meal except for the control group. After 28 days of age, we changed to the weaning (29-69 days) and fattening (70-slaughtering) experimental feed. The animals could consume feed and water *ad libitum*.
In the experiment carried out under large scale farming conditions, we recorded the feed consumption and mortality at group level and the final fattening weights were determined at the slaughterhouse. The FCR (feed conversion ratio), the average weights and the AI index (artificial insemination index, kg sold rabbit/insemination) was determined by calculation.
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Table 1: Composition of the diets used for the experiment

	Weaning diet						Fattening diet					
Rowmatterialls (%)	D	D06	D08	D10	D12	D14	D	D06	D08	D10	D12	D14
Sugarbeet pulp	11.8	15.8	17.2	17.2	17.2	17.2	10	15	17	16.3	16.3	15
Alfalfa	32	21	17.4	16.4	15.4	13.9	33	20.2	15.5	15.5	14.5	14.5
Extracted sunflower 34 % CP	11.7	12.3	12.5	11.5	10.5	10	13	14.1	14.8	13.5	12.5	10.5
Wheat midlings	15	15	15	15	15	16	16	16.3	16	16	16	18
Wheat bran	27	27	27	27	27	26	26	26	26	26	26	25.3
Extracted sunflower 20 % CP	-	6	8	10	12	14	-	6	8	10	12	14
Premix	2.5	2.9	2.4	2.9	2.9	2.9	2	2.4	2.7	2.7	2.7	2.7

Statistical analysis

The production results were evaluated using one-factor analysis of variance using the SAS statistical program package. Tukey's test was used to evaluate differences between groups.

RESULTS AND DISCUSSION

The results of the experiment are summarized in Table 2.

Table 2: Effects of feed including different proportions of 20% CP sunflower meal on the performance of fattening rabbits.

	Groups						RMSE	P
	D	D06	D08	D10	D12	D14		
AI index kg/AI	14.4ab	14.8ab	14.1ab	14.7ab	15.3b	13.7[a]	0.57	0.0482
FCR kg/kg	4.31	4.29	4.29	4.08	4.14	4.44	0.15	0.1033
Mortality under fattening, %	10.3bc	9.78bc	8.29ab	5.39a	7.22ab	12.8c	1.63	0.0008
Final weight, kg	2.76	2.72	2.68	2.81	2.73	2.78	0.06	0.1786

Means marked with different letters differ at the 0.05 significance level per line

AI index is affected not only by the effect of feeding on growth and mortality during fattening but also depends on many maternal factors, especially the fertility and prolificacy of the does, also influenced by their feeding. Table 2 and Figure 1 clearly shows that only the D12 and D14 groups significantly differed from each other in the case of the AI index. In the case of the other groups, we did not find any significant differences in terms of the AI index, although the 0.5 kg difference between the D12 group, which proved to be the best, and the second best, D06 group can still be considered as a high difference from professional point of view, even though this difference could not be statistically verified (Figure 1).

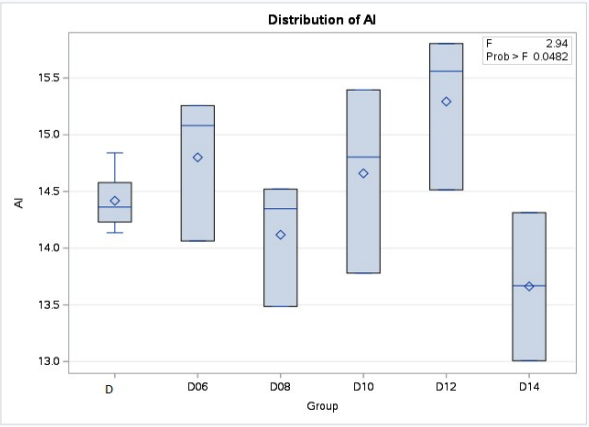

Figure 1: AI index

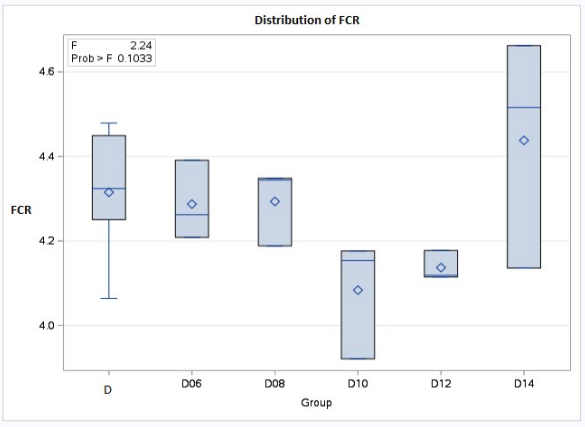

Figure 2: FCR

Regarding the feed conversion ratio, we could not find any statistically proved difference between the feeding groups, however, as can be seen from Figure 2, the FCR of D10 (4.08 kg/kg) and D12 (4.14 kg/kg) can be said to be better from a professional point of view, than the values of the other groups between 4.29 kg/kg and 4.44 kg/kg.

We did not find any significant differences between the groups in terms of final weights either. In this investigated trait, the average final slaughtering weight of the D08 group of 2.68 kg was the lowest compared to the other groups. Another interesting result is that in the D10 group, a large deviation can be observed compared to the average within the group (Figure 3), which indicates the high heterogeneity of the group.

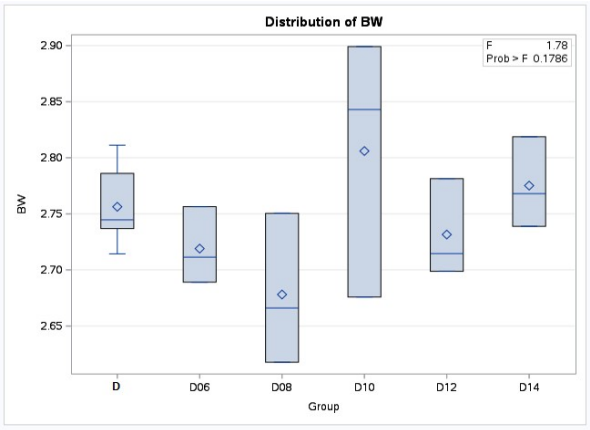

Figure 3.: Final slaughtering weight

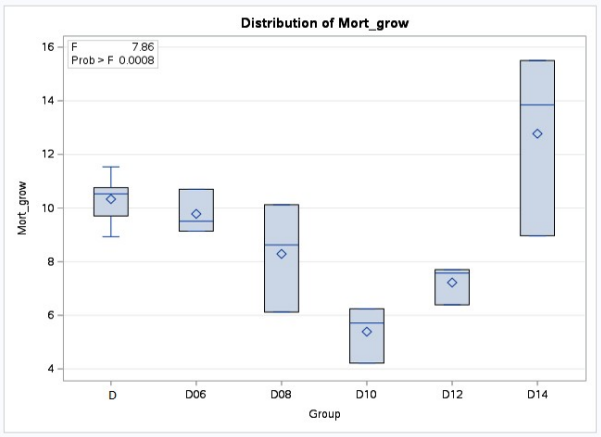

Figure 4.: Mortality under the fattening

Figure 4 shows the evolution of fattening mortality. Based on the results, we obtained a statistically proved difference between the groups. D10 (5.39%) differed significantly compared to group D (control, 10.3%), while mortality of D06 (9.78%), D08 (8.29%), D12 (7.22%) and D14 (12,8%) was not statistically different compared to the control group (D), although the differences can be said relevant from professional point of view. Presumably, the low death rate observed in the D10 group could have had an effect on the development of the large deviation in the weights measured at slaughter. To confirm or reject the connection, further tests are required.

CONCLUSION

After evaluating the results of the experiment, it is clear that the use of 20% CP sunflower meal in the feed of fattening rabbits can have statistically proved favourable effect only on the mortality of D10 group compared to the control (D) group. Using higher rates of 20% CP sunflower meal in the diets do not have any statistically proved negative effect on any examined KPI's compared to control group. As the further increased use of locally produced 20% CP sunflower meal can decrease the dependent of Europe from the protein imports, the continuation of the research with even higher mixing rates may still bring interesting results.

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PRODUCTION AND MEAT QUALITY TRAITS OF GROWING RABBITS FED GRADED LEVELS OF COMPOSITE MEAL FROM TWO VARIETIES OF SWEET POTATO

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ABSTRACT

The sweet potato root could be a suitable replacement for the pricey maize and offer growing rabbits an inexpensive source of energy. The quality of sweet potato root as energy source can be improved by mixing with the sweet potato vines in the right composition. Hence, the study investigated how varied levels of Composite sweet potato meal affected rabbit production and its meat quality attributes. Seventy-five 35-day old crossbreed (Chinchilla × New Zealand White) weaned doe rabbits weighing 570.76 ± 42.09g were allotted to five dietary treatments (15 rabbits/treatment; 5 rabbits/replicate). The diets were labeled T1-control 0%, 25%, and 50% of orange flesh sweet potato composite meal as T2 and T3, respectively, and 25% and 50% of white flesh sweet potato composite meal as T4 and T5, respectively. The diets comprised 10.6-12.6% crude fibre, 16.4-17.6% crude protein and metabolizable energy of 2610-2788 kcal. The experiment was carried out in a completely randomized design and lasted for 63 days. At the end of the feeding trial, meat quality characteristics was determined by sampling nine rabbits from each treatment weighed, starved overnight, stunned and slaughtered. The inclusion of Composite sweet potato meal had a quadratic ($P<0.05$) effect on final live weight, daily weight gain and Feedcost per rabbit. Over the course of all treatments, there were no significant differences in the meat pH. The test ingredient had no effect on the levels of meat lightness (L*) or redness (a*). However, the yellowness (b*) and chroma (c*) significantly varied. The findings suggest the level of inclusion of the two variety of composite sweet potato meal did not have an impact on the overall quality of the rabbit meat.

Key words: Meat attributes, Performance, Growing rabbit, sweet potato composite.

INTRODUCTION

Animal production in developing countries faces several difficulties, including high costs and a lack of energy and protein feed ingredients (Balehegn *et al*., 2020). For instance, in Nigeria Fulani herdsmen and farmers conflicts has drastically affect maize production which is the major source of energy in livestock feed. Changing climatic conditions including inconsistent rainfall all year round has also impacted on maize production in economic quantity across Nigeria. Also, the rapidly expanding population's demand for feed, and fuel, prices of the main energy sources in animal diets, such as maize are continuing to rise sharply (FAO, 2022). In light of this, it is crucial to look for alternative feed sources that are reasonably priced, widely accessible, and capable of simultaneously boosting animal output and meat quality. The sweet potato root, leaf and vines may be a better source of digestible energy, fibre, crude protein, and neutral detergent fibre when compared to other root and tubers in livestock feed (Olaleru, and Abu, 2022). It also has greater concentrations of bioactive phytochemicals, which may benefit livestock meat production and quality due to their nutritional and bioactive features (Olaleru and Abu, 2019).Therefore, the objective of the study was to investigate how feeding growing rabbits diets with increasing amounts levels of composite sweet potato meals in place would affect growth performance, carcass characteristics, and meat quality.

MATERIALS AND METHODS

Study area: The feeding trial was conducted at the University of Ibadan's Rabbitry Unit, Teaching and Research Farm, in Ibadan, Oyo State, Nigeria. The University of Ibadan is situated in the tropical rain forest zone of Nigeria with latitude 7°47.05"N and longitude 3°96.74"E of the Greenwich Meridian, with a mean altitude ranging from 185 to 230m above

sea level. Temperature range and the average relative humidity of the location were between 20-37°C and 60-80%, respectively (SMUI, 2018).

Animals and experimental design

Five diets were formulated in a completely radomised design (CRD). The values of CP, CF, and ME were determined (on a DM basis) (Table 1). The composite meal contains a mixture of 65% root and 35% leaf and vines of the sweet potato. The sweet potato root and leaf were shade dry for 3 to 5 days before being milled and included in the feed afterwards the feed were pelletizes. Seventy-five 35-day old crossbreed (Chinchilla × New Zealand White) growing rabbits weighing 570.76 ± 42.09 g were allotted to five dietary treatments (15 rabbits/treatment; 5 rabbits/replicate). The rabbits were housed in wire-meshed cages, accommodated in a well-ventilated pen, offered water and experimental diet ad libitum. For the duration of the 63-day experiment, the cages were sanitised and cleaned every day. During nine weeks of the experiment, rabbits were fed one of the 5 diets ad libitum, with a weekly weight and daily feed consumption recorded. The daily weight gain (DWG), daily feed intake (DFI) and the feed conversion ratio (FCR) were calculated, respectively, for the experimental period (63 days). At the end of the 9th week, three rabbits per replicate (Nine per treatment) whose weights were representative of the average weight of the rabbit contained in each treatment were tagged. The animals were restricted feed overnight but had access to drinking water. Animals were weighed the next day and taken to the slaughter slab and stunned, properly bled, scalded, de-haired and eviscerated before dissecting into separate parts for carcass.

Meat proximate analyses

The rabbit meat samples were analysed in triplicate. The dry matter (DM) of the samples was determined by weight upon drying the sample at 105^{o}C as described in the AOAC (2002) methods. The Kjeldahl method using a Buchi analyser (Centec Automatika, spol.s.r.o., Prague, Czech Republic) was performed to determine the crude protein (CP) content. A Soxhlet method was used to determine the ether extract by a Soxtec apparatus (Thermo Stientific, Warrington, UK). The ash was determined by weighing the sample after incineration at 550 ◦C.

Statistical analysis

The design of the experiment was a completely randomized design. Data were subjected to One-way ANOVA followed by Duncan's Multiple Range Tests were used to compare differences among individual means. All statistics were performed using SPSS 20.0 (SPSS, Chicago, IL, USA).

Table 1: Gross composition (on a DM basis) of experimental diets fed to growing rabbits

Ingredient		Levels of orange flesh CSPM		Levels of white flesh CSPM	
	T1(0%)	T2(25%)	T3(50%)	T4(25%)	T5(50%)
Maize	50.00	37.50	25.00	37.50	25.00
*CSPM	-	12.50	25.00	12.50	25.00
Soya bean meal	16.00	16.00	16.00	16.00	16.00
PKC	19.00	19.00	19.00	19.00	19.00
Fish meal	1.00	1.00	1.00	1.00	1.00
Wheat offal	8. 50	8. 50	8. 50	8. 50	8. 50
CRM	2.00	2.00	2.00	2.00	2.00
Limestone	2.00	2.00	2.00	2.00	2.00
Bone Meal	1.00	1.00	1.00	1.00	1.00
Vitamin-mineral premix	0.25	0.25	0.25	0.25	0.25
Table Salt	0.25	0.25	0.25	0.25	0.25
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Calculated Nutrients					
Crude Protein	16.90	17.29	17.01	16.72	16.60
Crude Fibre (%)	9.45	10.40	11.09	10.51	11.01
ME(Kcal/Kg)	2788.00	2760.00	2690.00	2780.00	2680.00

CSPM: Composite sweetpotato meal, CRM: Cassava root meal; ME-metabolizable energy. PKC- Palm kernel cake. (Olaleru and Abu, 2018).

RESULTS AND DISCUSSION

Effect of growth performance and carcass attributes of growing rabbits.
Results of the study (Table 2) showed growth performance and carcass attributes of growing rabbits. The final live weight of rabbits on diets 1 (1633.40g), 2 (1604.30g) and 3 (1653.30g), 4 (1602.10g) and 5 (1545.70g) were not significantly higher (P<0.05) compared to each other. The similarities in the final live weight recorded by the rabbits fed composite sweet potato diets could be a direct consequence of excellent utilization, so the rabbits had eaten to satisfy their energy requirement. The values from this study did not follow the trends observed by Tewe (2002), who found out in the experiment to replace maize with oven-dried and sun-dried sweet potato meal (SPM), that there was a reduction in body weight gain and nutrient utilization of birds in the SPM-substituted compared to the maize-based control diets. It must be pointed out that all the initial rabbits were still alive after the 9 weeks of experiment, this is an indication that the rabbits were healthy during the period of the experiment and didn't not experience digestive disorder that could be attributed to the diet. The feed intake, growth, weight gain and feed conversion ratio in this experiment ranged within the values mentioned in the literature, measured for the growing rabbits.

The fasted weight was not significantly affected by the inclusion of the composite sweet potato meal the average weight reported in the study was higher than the local market weight (1400.0 ± 0.05 g),which as reported by Kadi *et al.* (2008). For the values of the dressing percentages only T1 (56.98%) and T5 (58.05%) were within the range of 56.53 − 66.35 % obtained by Chikaodi *et al.* (2017), the others values were lower however the dressing percentage obtained in the study were all not significantly different from each other.

Table 2: Effect of substituting increasing levels of two varieties of composite sweetpotato meal for maize on growth performance and carcass attributes of growing rabbits

	T1	T2	T3	T4	T5	SEM	P-value
Initial live weight, g	557.70	569.20	537.60	528.70	568.10	8.18	0.3864
Final live weight, g	1633.40	1604.30	1653.30	1602.10	1545.70	18.20	0.3217
Daily wt gain (g)	17.08	16.43	17.71	17.04	15.52	0.36	0.1441
Daily feed intake (g)	72.75	73.07	72.45	72.77	72.71	0.09	0.0949
Feed Conversion Ratio	6.19	6.09	6.00	7.39	7.41	0.32	0.2309
Feedcost/ rabbit / wk **(₦)**	56.02[a]	53.71[b]	50.71[d]	53.99[b]	52.42[c]	0.88	0.00
Survialabilty (%)	100	100	100	100	100	-	-
Fasted weight(g)	1556.00	1521.70	1513.00	1467.70	1441.30	20.33	0.4989
Bled wt (g)	1494.00 [a]	1460.30 [b]	1446.30 [b]	1413.30 [bc]	1364.70 [c]	21.98	0.0214
Skinned wt (g)	1216.00	1241.67	1234.50	1185.67	1205.67	10.06	0.0856
Carcass wt (g)	846.67	799.33	784.83	737.00	781.00	17.63	0.3092
Dressing percent (%)	56.98	54.45	54.11	52.27	58.05	1.04	0.4392

T1(0 %), T2(25% OFCSP), T3 (50% OFCS), T4 (25% WFCS) and T5 (50% WFCS) OFCS – Orange flesh composite sweetpotato; WFCS – White flesh composite sweetpotato
Mean values in the same row having different superscripts are significantly (P < 0.05) different.
1US Dollar=₦360 Nigeria Naria

Effect of growth performance and carcass attributes of growing rabbits.
Results of the study (Table 3) showed meat quality attributes of growing rabbits. There was no significant difference in pH across all treatments. The levels of meat lightness (L*) and redness (a*) were not altered by the test substance. However, there was a noticeable change in yellowness (b*) and chroma (c*). The ability of myoglobin to express red and bind to water is directly regulated by pH, which influences how pre-slaughter management affects meat color (Daszkiewicz and Andrzej 2020). In the current study, the pH levels were remarkably similar between treatments, resulting in comparable lightness (L*) and values. The presence of the test component had minimal effect on the meat's immediate composition, while moisture, fat, and ash content varied dramatically among the dietary treatments. The increased metabolic activity in the rabbit body could explain the considerable change in proximate composition.

Table 3. Meat quality of rabbit fed graded levels of sweetpotato composite meal from two varieties

	T1	T2	T3	T4	T5	SEM	P-value
pH							
Fore Limb	6.53	6.35	6.27	6.31	6.39	0.32	0.5000
Hind Limb	6.58	6.43	6.49	6.34	6.43	0.22	0.1513
Colour							
L*	62.06	62.82	63.85	62.84	63.80	0.33	0.4107
a*	5. 38	6.52	6.94	5.50	5.96	0.29	0.4161
b*	15.36^{c}	15.60^{b}	15.91^{a}	15.63^{b}	15.87^{a}	0.10	0.0011
c*	16.14^{b}	16.84^{a}	16.92^{a}	16.54^{ab}	16.58^{ab}	0.13	0.0304
Proximate composition							
Moisture	74.26	72.16	71.64	72.02	73.99	0.01	0.0796
Protein	21.36	21.13	21.89	21.33	21.31	0.45	0.2309
Fat	2.75^{ab}	2.89^{a}	2.80^{ab}	2.86^{a}	2.57^{b}	0.15	0.0045
Ash	1.29^{a}	1.24^{b}	1.23^{b}	1.23^{b}	1.30^{a}	0.02	0.0112

T1(0 %), T2(25% OFCSP), T3 (50% OFCS), T4 (25% WFCS) and T5 (50% WFCS) OFCS – Orange flesh composite Sweet potato; WFCS – White flesh composite Sweet potato
Mean values in the same row having different superscripts are significantly (P < 0.05) different.

CONCLUSIONS

The composite sweet potato meal from the two varieties can replace maize in rabbit's diet enhancing weight gain and slaughter performance. To find the ideal blend of sweet potatoes for a composite that would be better suited for adding to developing rabbit diets without compromising their health or growth, as well as the meat quality more investigation may be needed. The overall meat quality was not affected by the level of inclusion of the two varieties of composite sweetpotato meal.

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BIODIVERSITY AND NUTRITIONAL QUALITY OF PLANT RESOURCES FOR TRADITIONAL RABBIT FEEDING IN KABYLIA, ALGERIA

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ABSTRACT

This study aimed to inventory plant resources used for rabbit feeding on farms in Kabylia, Algeria. During interviews with farmers, 54 different plant species were sampled in the field. Of these, 30 plants are of interest and could be used in industrial feeds, and their nutritional value was estimated on the basis of their chemical composition using prediction equations available in the scientific literature. Particular attention should be paid to the green leaves of Malva sylvestris and the aerial part of Plantago lanceolata, the leaves of Robinia pseudoacacia and the green leaves of Morus alba, which can be considered as alternative sources of energy, protein and fibre. Secondary attention should be paid to species that are likely to be sources of fibre and protein as the early stages of Hedysarum flexuosum. Finally, other species such as the fresh leaves of Anthyllis vulneraria, the aerial part of Cynara cardunculus L., the bracts and stems of Cynara scolymus L. and the fresh leaves of Taraxacum officinale and Erodium moschatum can be considered as potential sources of energy and fibre. Tree leaves include those of Celtis australis, Pyrus communis and dried branches of Quercus ilex. However, the characterisation of these raw materials requires more in-depth studies using in vivo tests.

Key words: Rabbit, Family farming, Plant resources, Nutritional value.

INTRODUCTION

There have always been traditional rabbit farms in Algeria. Traditional rabbit farms are family farms with a small number of animals in comparison to the rational farms. This type of farming, considered a secondary source of income and a high quality protein, is natural for village families. In this type of farming, rabbits are fed on cooking waste and especially wild plants and leaves from trees and bushes.

In rational rabbit farming, the rabbits are fed with complete compound feed in the form of pellets made from raw materials that are imported into Algeria at high prices. Furthermore, the commercial feeds available on the market are usually unbalanced. This is the main obstacle to the development of rabbit farming. As a result, the farmers irrationally use those low-cost feedstuffs as a supplement to the pellets because they are not aware of its nutritional value (Mouhous *et al.*, 2017). However, knowledge of the nutritional value of the feed is a prerequisite for the improvement of production performance. In view of this situation, studies are being carried out to replace these imported materials with local products (Dorbane et al., 2019; Kadi et al., 2011, 2017, 2018). The aim of the present study is the inventory of the plant resources used in the traditional breeding of rabbits in Kabylie and the estimation of the nutritional value of the plant resources which seem best suited in the industrial diets.

MATERIALS AND METHODS

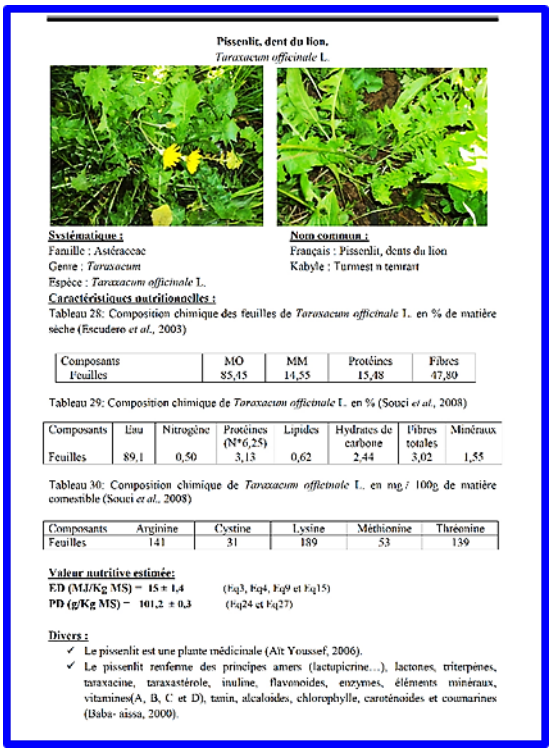
Description of the study area

The region of Kabylia is located in the north of Algeria. It is located in the Tell Atlas mountain range, which runs parallel to the coast of the Mediterranean Sea. The landscape of the province is characterised by rugged terrain. Mountains, valleys and plateaus dominate the landscape.

In economic terms, Tizi-Ouzou is mainly an agricultural region, with agriculture being an important sector of the local economy. The region is well known for the production of olives, figs, citrus fruits and a variety of cereals. Livestock farming is also widespread in the area, including sheep, goats, poultry and rabbits.

Methodology

The inventory of the plants used for the feeding of rabbits in the traditional breeding in the region of Tizi-Ouzou was carried out by means of a series of interviews with the farmers. A sampling of the 54 plants that were praised by the farmers and considered to be the most useful was carried out in order to identify them. In addition, a photographic database and a herbarium of the pre-selected plants have been set up. The taxonomy of the listed species has been determined with the help of the lecturers of Botany and Phytosociology of our Faculty. After identification, each source was subject to a bibliographical search in the various databases available on the Internet in order to determine its nutritional value. When this was not available, prediction equations available in the scientific literature and compiled by Dorbane (2011) were used to estimate it (30 cases listed). These equations were selected depending on the available chemical composition parameters and their R2 and RSD. Finally, data sheets were drawn up for the plants that were identified. These sheets summarise the information collected on the species, in particular one or more photographs of the plant, its systematic identification (family, genus and species) and Kabyle vernacular name(s), its chemical composition, its nutritional value for rabbits and a "Other " section containing specific and useful information (Figure 1).



Pissenlit, dent du lion.
Taraxacum officinale L.

Systématique :
Famille : Astéraceae
Genre : *Taraxacum*
Espèce : *Taraxacum officinale* L.

Nom commun :
Français : Pissenlit, dents du lion
Kabyle : Turmest n temirant

Caractéristiques nutritionnelles :

Tableau 28: Composition chimique des feuilles de *Taraxacum officinale* L. en % de matière sèche (Escudero *et al.*, 2003)

Composants	MO	MM	Protéines	Fibres
Feuilles	85,45	14,55	15,48	47,89

Tableau 29: Composition chimique de *Taraxacum officinale* L. en % (Souci *et al.*, 2008)

Composants	Eau	Nitrogène	Protéines (N*6,25)	Lipides	Hydrates de carbone	Fibres totales	Minéraux
Feuilles	89,1	0,50	3,13	0,62	2,44	3,02	1,55

Tableau 30: Composition chimique de *Taraxacum officinale* L. en mg / 100g de matière comestible (Souci *et al.*, 2008)

Composants	Arginine	Cystine	Lysine	Méthionine	Thréonine
Feuilles	141	31	189	53	139

Valeur nutritive estimée:
ED (MJ/Kg MS) = 15 ± 1,4 (Eq3, Eq4, Eq9 et Eq15)
PD (g/Kg MS) = 101,2 ± 0,3 (Eq24 et Eq27)

Divers :
- ✓ Le pissenlit est une plante médicinale (Ait Youssef, 2006).
- ✓ Le pissenlit renferme des principes amers (lactupicrine…), lactones, triterpènes, taraxacine, taraxastérole, inuline, flavonoides, enzymes, éléments minéraux, vitamines(A, B, C et D), tanin, alcaloides, chlorophylle, caroténoides et coumarines (Baba- aissa, 2000).

Figure 1: Example of a data sheet compiled for a selected herbaceous plant

RESULTS AND DISCUSSION

Fifty-four species, including sixteen shrub and tree species (Table 1) and thirty-eight herbaceous (Table 2), belonging to 14 plant families, were recorded.

Table 1: List of trees and shrubs used for feeding rabbits in traditional farming systems in the Kabylia region of Algeria

Latin name	Kabyle name
Ceratonia siliqua	Axarrub
Robinia pseudoacacia.	Cilmoum
Acacia cyanophylla	Mimusa
Quercus ilex	Aveludh lehlu
Quercus suber	Akarruc
Morus alba	Tut amellal
Morus nigra	Tut aberkan
Ficus carica	Thaneqlets
Olea europaea	Azemmur
Fraxinus exelcior	Asslene
Pyrus communis	Thifiresthe
Eryobotria japonica	Zaârore
Mallus pumila Mill.	Tefahe
Celtis australis	Iviqesse
Ulmus campestris	Ulmu
Vitis vinifera	Thara

Table 2: List of herbaceous plants used for feeding rabbits in traditional farming systems in the Kabylia region of Algeria

Latin name	**Kabyle name**	**Latin name**	**Kabyle name**
Calendula arvensis	Zeghdar	*Lotus ornithopodioides*	Adjilvane
Centaurea sp	Lehlafa	*Scorpiurus vermiculatus*	Thahdhurthe
Cichorium intybus	Thifaf	*Lotus edulis*	Ajilvana
Coleostephus myconis	Wazedduz	*Vicia sativa*	Ahvac, Tuga
Cynara scolymus	Thifeɣwa	*Hedysarum flexiosum*	Thasulla
Picris sp	Lahlafa	*Lupinus* sp	Iviw bucen
Taraxacum officinale	Tuɣmest temɣart	*Erodium moschatum*	Thimcet tegmerth
Scolymus hispanicus	Thaɣediwt	*Asphodelus microcarpus*	Averwaqe
Sonchus oleraceus	Thifafe	*Malva sylvestris*	Megire
Sonchus sp	Thifafe	*Papaver rhoeas*	Thiwahririne
Picris sp	Thizedla	*Plantago lanceolata*	Elguz n imeksawen
Cynara cardunculus	Thaga	*Ampelodesma mauritanica*	Adles
Echium plantagineum	Lahlafa	*Avena sterilis*	Azekoune
Borago officinalis	Iles tfounassthe	*Cynodon dactylon*	Affar
Anagallis arvensis	Acnafe	*Phragmite australis*	Aranim
Convolvulus arvensis	Anaraffe	*Polygonum aviculare*	Izrem bbufrux
Anthyllis vulneraria sp	Ifelfel tɣetene	*Rumex* sp	Tassemoumt bezger
Trifolium campestre	Ikffise	*Portulaca oleracea*	Amarmour
Medicago minima	Ikffise	*Potentilla reptans*	Thiwcicine

Following the recommendations of Lebas (2004), three groups of plants were formed according to their levels of Digestible energy (DE ≥ 11 MJ/kg DM), Digestible protein (DP ≥ 120 g/kg) and fibre (CF ≥ 15%, NDF ≥ 31%, ADF ≥ 17%, ADL ≥ 5%):

Potential sources of digestible energy: *Anthyllis vulneraria*, *Calendula arvensis, Cichorium intybus, Cynara cardunculus, Cynara scolymus, Cynodon dactylon, Erodium moschatum, Lupinus* ssp, *Malva sylvestris, Plantago lanceolata, Taraxacum officinale, Vicia sativa* for herbaceous plants and *Acacia Cyanophylla, Celtis australis, Ceratonia siliqua, Ficus carica, Fraxinus angustifolia, Malus pumila, Morus alba, Morus nigra, Pyrus communis, Quercus ilex* and *Robinia pseudacacia* for tree leaves

Potential sources of digestible protein (ranging from 145,2 to 260 g/kg DM): *Calendula arvensis, Hedysarum flexuosum, Lupinus* ssp, *Malva sylvestris, Plantago lanceolata, Vicia sativa for* Herbasious plants *and Morus alba and Robinia pseudocacia* for tree leaves.

Potential sources of fibre: *Ampelodesma mauritanica, Anthyllis vulneraria, Cynara cardunculus, Cynara scolymus, Cynodon dactylon, Erodium moschatum, Hedysarum flexuosum, Malva sylvestris, Phragmite australis, Plantago lanceolata, Taraxacum officinale* for herbaceous plants and *Acacia Cyanophylla, Celtis australis, Ficus carica, Morus alba, Olea europaea, Pyrus communis, Quercus ilex, Quercus suber, Robinia pseudocacia, Ulmus campestris* and *Vitis vinifera* for tree leaves.

CONCLUSIONS

The leaflets that have been produced will serve as a useful source of information for farmers who are interested in improving the diet of their rabbits.

This work has also been the basis for the compilation of a list of interesting plants which will be the subject of further investigation with a view to their possible use in rational breeding.

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EFFECT OF MORINGA AND PERENNIAL SOY ON THE PRODUCTIVE PERFORMANCE OF RABBITS

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ABSTRACT

Proper nutrition significantly influences the growth, fertility, and health of rabbits. Certain foods are rich in protein, particularly fresh vegetables, while others serve as energy sources, such as grasses and tubers. Therefore, a balanced diet that includes adequate protein, energy, and minerals is crucial for optimal rabbit performance. Despite many alternatives for feeding the animals, rural rabbit breeders limit the feeding of this species only to grasses that are sources of energy and thus cannot meet the animals' protein needs, which may be one of the factors contribute to suboptimal performance of rabbits under these conditions. The objective of this study was to evaluate the effect of Moringa and Perennial Soy on the productive performance of rabbits, aiming to introduce new feeding alternatives. Twenty-seven female New Zealand White rabbits, aged between 35 and 46 days, were assigned to three treatment groups. Treatment 1 (T1) consisted of 100% pelleted feed, while treatments 2 (T2) and 3 (T3) comprised 90% pelleted feed supplemented with 10% Moringa and 10% perennial soybeans, respectively. The experiment followed a Randomized Complete Block Design (RCBD), with three blocks used to control for initial weight variation. Food consumption, weight gain, and feed conversion efficiency were evaluated as performance indicators. Statistical analysis using the Tukey test at a significance level of 5% revealed no significant differences ($P > 0.05$) in these parameters among the treatments. The findings suggest that replacing 10% of commercial feed with either Moringa or perennial soybeans did not adversely affect the productive performance of rabbits. Therefore, incorporating these forages at up to 10% inclusion levels is recommended as a viable feeding strategy for rabbits.

Keywords: Rabbit nutrition; Moringa; Perennial soybeans; Productive performance; Alternative feeding strategies.

INTRODUCTION

Animal production is the main source of protein for the human population, and has great economic and strategic value for countries (OLIVEIRA 2013). In rabbit farming, food is one of the limiting factors in the growth, fertility and health of animals, therefore, their diet must be complete, containing sufficient protein, energy and minerals for their physiological activities (SCHIERE 2004).

The high content of crude protein in moringa leaves (24%), the presence of adequate levels of essential amino acids and the low level of anti-nutritional factors such as saponins (80g/kg), phytates (21g/kg) and tannins (12g/kg) kg), combined with its regrowth capacity and adaptability to different climatic conditions, make this plant a promising option for animal feed and/or protein supplementation for animals farmed in semi-arid regions (BAKKE et al 2018) .

However, perennial soybeans have been known for many years for their importance as fodder in bird feed, probably due to the high content of vitamins and minerals present in this food. The green part of this plant, still in the growth phase, has been considered the basis of animal nutrition as it presents appreciable amounts of many nutritional principles such as carotenes, vitamins C, E, K, thiamine, riboflavin, pyridoxine, vitamin B5, niacin, biotin and choline. (GUARACHI, ROJAS, JOAQUIN 2010).

In view of the above, this work aims to evaluate the effect of including two tropical forages, Moringa oleifera and Neonotonia wightii (perennial soybean) on the productive performance of rabbits in order to observe the extent to which these forages provide good performance in

rabbits for that they are recommended to communities that are unable to purchase conventional food (feed) but that want their production to perform well.

MATERIALS AND METHODS

Animals and experimental design

Twenty-seven female New Zealand White rabbits, aged between 35 and 46 days, were used in a randomized complete block design, blocking for initial weight effect (B1 - 542±33.65g; B2 - 644.67±22.05g; B3 - 754±28.62g) with three treatments (T1 - 100% pelleted feed, T2 - 90% pelleted feed + 10% moringa hay, T3 - 90% pelleted feed + 10% perennial soybean hay), and three animals per experimental unit.

To assess productive performance, variables including average feed consumption (AFC), average forage consumption (AFC), average weight gain (AWG), and feed conversion ratio (FCR) were evaluated.

Chemical Analyses

The nutritional composition of the ingredients was determined based on a literature review. And the calculation method was used to determine the nutritional composition of the diets in the different treatments, as illustrated in Table 1.

Table 1. Nutritional Composition of Ingredients and Diets.

Ingredient	DM (%)	CP (%)	GE (kcal/kg)	CF (%)	Ca (%)	P (%)
Pelleted feed (PF)[1]	-	15	2500	14	0,8	0,5
Moringa (MO)[2]	94	27,2	3391	19,4	2,98%	3,5
Perennial Soybean (PS)[3]	88,9	16,46	4500	28,93	2,54%	0,26
Nutritional Composition of Diets in Different Treatments						
T1 (100% PF)	-	15	2500	14	0,8	0,5
T2 (90% PF+10%MO)	-	16,22	2589,1	14,54	1,02	0,8
T3 (90% PF+10%SP)	-	15,15	2700	15,49	0,97	0,46

Source: 1. (HIGEST, 2021); 2. (VIEIRA, et al., 2018); 3. (PADUA, et al., 2006) & (GUARACHI, et al., 2010).

DM - dry matter; CP - crude protein; GE - gross energy; CF - crude fiber; Ca - calcium; P - phosphorus

Statistical Analysis

The data were analyzed using the statistical software package MiniTab 18. Analysis of variance was conducted at a 5% significance level, and mean comparisons were performed using the Tukey test at a 5% significance level.

RESULTS AND DISCUSSION

Feed Consumption

Regarding the average daily feed intake, there were no significant differences ($p > 0.05$) among the treatments, with means of 107, 105, and 103 grams/day/animal for treatments T1, T2, and T3, respectively, as illustrated in Table 2.

Similar results were reported by Sun et al. (2016) when studying the effect of replacing alfalfa meal with Moringa meal in rabbit diets at different levels (0%, 10%, 20%, and 30%), where no significant differences ($p > 0.05$) in average daily intake were observed among the diets. Fierro (2019) also found no significant differences ($p > 0.05$) in total dry matter intake among treatments during the first 2 weeks of evaluating rabbit performance during the fattening phase (starting from 42 days of age) with the inclusion of 0%, 15%, and 30% perennial soybean in their diet.

However, when analyzing the average consumption of forages (Moringa hay and perennial soybean hay) in different treatments (T2 and T3), significant differences were noted ($p < 0.05$), with Moringa hay being consumed more (T2 – 9.76g) compared to perennial soybean hay (T3 – 4.76g).

This difference is likely due to the higher energy content in perennial soybean hay (4500 kcal/kg) compared to Moringa hay (3391 kcal/kg). According to PESSÔA (2003) and Simionato (2012), feed intake is limited by high energy levels, and rabbits consume feed more accurately when energy levels are between 2500 to 3000 kcal/kg, without affecting animal performance. Alternatively, it could be attributed to the considerable concentration of saponins in perennial soybean, which are anti-nutritional substances in plant-based foods responsible for the bitter taste of these plants. Nepomuceno et al. (2009) suggested that saponins can reduce food intake.

Table 2. Statistical comparisons of treatment means for different evaluated parameters.

Tratamento	T1 (100% PF)	T2 (90% PF+10%MO)	T3 (90% PF+10%SP)	SD	p-Value
Average Consumption (g/day/animal)	107,00	104,93	102,64	2,18	p> 0,05
Average Weight Gain (g/day/animal)	32,80	32,51	31,09	0,92	p> 0,05
Feed Conversion Ratio	3,28	3,23	3,31	0,04	p> 0,05
Average Consumption of Hay (g/day/animal)	-	9,76[a]	4,76[b]	1,46	p<0,05

Means with different letters on the same row differ significantly (Tukey's test at 5% probability)
PF – pelleted feed; **MO** – moringa hay; **SP** – perennial soybean hay

Weight Gain

Regarding the average daily weight gain (Table 4), no differences were observed among the treatments ($p > 0.05$). However, the observed means were higher compared to the growth rate of rabbits in tropical regions (20 to 30 g/day) as reported by PESSÔA (2003). Similar results were obtained by Sun et al. (2016), where no significant differences in daily weight gain were found when substituting 10% of alfalfa meal with Moringa meal compared to 0% substitution in their study, with means of T0 – (31.5) and T1 – (33.2) in g/day.

Fierro (2019) also did not find significant differences in daily weight gain when comparing different inclusion levels (0%, 15%, and 30%) of perennial soybean in the diet of rabbits during the fattening phase starting from 42 days of age. At 56 days of age, the average daily weight gain (g/day/animal) was T0 – (25.8); T1 – (33.4); and T2 – (48.8).

These results suggest that the treatments in your study have led to favorable weight gain rates compared to typical tropical rabbit growth rates and are consistent with previous findings on the effects of dietary substitutions with Moringa or perennial soybean in rabbit diets.

Feed Conversion Ratio

The feed conversion ratios did not differ significantly among the treatments ($p > 0.05$), with values of 3.28, 3.23, and 3.31 for treatments 1, 2, and 3, respectively, as illustrated in Table 2. However, there were differences in feed conversion ratio among blocks ($p < 0.05$), where the block with the highest initial average weight (Block 3) had a higher feed conversion ratio of 3.58, which was poorer compared to Blocks 2 and 1 with ratios of 3.20 and 3.04, respectively, as shown in Table 3.

The feed conversion ratios obtained in this study are favorable compared to those reported by Falemara et al. (2020) when evaluating the supplementation effect of Gmelina, Neem, and Leucaena combined with Moringa. Falemara et al. (2020) obtained the following feed conversion ratios: Control Diet (5.12), Gmelina + Moringa (7.06), Neem + Moringa (8.66), and Leucaena + Moringa (4.65). Similar values (around 2.7) were reported by PESSÔA (2003) when substituting alfalfa hay with perennial soybean at different levels (25%, 50%, 75%, and 100%).

Fierro (2019) did not observe significant differences in feed conversion ratios in rabbits fed diets with different levels of perennial soybean inclusion (0%, 15%, and 30%), with the following average ratios observed: 4.58, 2.87, and 2.34, respectively. In treatments with perennial soybean inclusion, the ratios were better compared to those observed in the present study.

The block with the highest initial average weight (Block 3 – 754g) exhibited the poorest feed conversion ratio. This result may be attributed to the fact that these animals were closer to adult weight compared to lighter animals, where heavier animals tend to consume more feed but have diminishing meat deposition due to reaching growth stability.

Table 3. Feed Conversion Ratio between Blocks.

Block*	B1	B2	B3
Feed Conversion Ratio	3,04[a]	3,20[a]	3,58[b]
Standard Deviation	0,12	0,05	0,04

Means with different letters on the same row differ significantly (Tukey's test at 5% probability)
*__B1__ – initial average weight 542.00g; __B2__ – initial average weight 644.67g; __B3__ – initial average weight 754.00g.

CONCLUSIONS

Based on the data obtained in this study and the analyses conducted, it can be concluded that both Moringa and perennial soybean can be used as alternative feeds in rabbit production without affecting feed intake, weight gain, and feed conversion ratio at an inclusion level of 10% as a replacement for commercial feed in their diet.

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CHEMICAL COMPOSITION STABILITY OF RABBIT FEED IN THE ALGERIAN MARKET

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ABSTRACT

The aim of this study was to assess the stability of the chemical composition, and thus the nutritional content, of the rabbit diets marketed in Algeria. An exhaustive nationwide survey of the appropriate service departments, breeders' associations and resource persons permitted to identify only six feed manufacturers that included rabbit diets in their product types. Diets were sampled in two different periods, 10 consecutive months apart, whenever this was possible (diets were available on the market). 15 samples of pelleted feeds were collected and analysed for their chemical composition. All the types of diets contained the same level of energy and appeared to be well above the recommended levels. Six diets showed a significant excess of crude fibre (from 0.65 to 8.95%), while six other diets had a significant deficit in relation to the recommendations (from 0.54 to 12.90%). The levels of protein in the majority of the diets were well in excess. The results of this study show differences, sometimes significant, in the composition of identically labelled diets taken at 10-month intervals: from 0.7 to 14.6 points for crude fibre, from 0.2 to 5.7 points for crude protein and from 0.2 to 1.2 points for fat. The composition of commercial rabbit diets in Algeria was found to vary in two ways. Firstly, the chemical composition was not in accordance with the recommended values. Secondly, their composition and nutritional value showed heterogeneity over time.

Key words: Rabbit, Feed, chemical composition stability, Algeria.

INTRODUCTION

In rabbit production, feed is the most important production factor and represents the largest item of expenditure. These costs are tending to increase as raw material prices rise. In Algeria, the increase in demand for rabbit meat (Kadi et al., 2021) and the development of the young rabbit industry (Mouhous et al., 2019) have led to an increase in production and the diversification of feed on the market. The feed is also one of the factors explaining the poor performance recorded, the often insufficient and discouraging level of profitability (Mouhous et al., 2021) and the persistently high prices of this meat on the local market (Kadi et al., 2008, Benabdelaziz et al., 2020). The raw materials that constitute the feeds currently available on the Algerian market are largely of import origin and are prohibitively expensive (Kadi, 2012). The high cost of those imported raw materials indirectly contributes to the nutritional imbalances in commercial feed. In fact, the more expensive the raw material, the more manufacturers need to regularly update feed formulations to adapt to these changing market conditions, and it is essential that formulations are adjusted accordingly to maintain cost effectiveness without compromising animal nutrition.

The Aim of this study is to assess nutrition quality of these diets in relation to recommended nutritional levels and temporal stability of chemical composition.

MATERIALS AND METHODS

Samples collection

In order to identify the feeds to be sampled, an extensive nationwide survey was carried out with the Agricultural Service Directions, Chambers of Agriculture, feed companies, breeders' associations and some resource breeders. Six feed manufacturers, four in the centre of the country, one in the east and one in the west, were found to have rabbit feed in their product

type. Three brands offer only mixed feeds for both reproduction and fattening. The other three brands offer both types of differentiated feed, i.e. 'maternity' or 'reproduction' feed for breeding rabbit does and 'fattening' feed for fattening rabbits. One brand offers, in addition to these last two types, a 'finishing' diet for fattening rabbits. The feed samples were collected at two different times, 10 months apart, where this was possible (feed available in the market): 09 samples in February and 09 samples in December including 05 of the same commercial brand and feed type as the samples collected in February. In total, 15 samples (one kg/feed) were collected from feed distributors and sellers in several wilayas (districts). The types of feed collected were as follows: five maternity feeds, six fattening feeds and four mixed feeds. Each sample taken was accompanied by the label on the bag of feed. The feed samples were then stored in a refrigerator at 4°C until analysis.

Chemical Analyses

The chemical analyses were performed according to ISO methods and recommendations of EGRAN group. Digestible Energy (DE) was estimated by the equation of Villamide et al. (1989) and Digestible Protein (DP) by the one of Villamide and Fraga (1998).

Statistical Analysis

The data for the different parameters analysed were expressed as the mean ± standard deviation and were compared using the Student's t-test with the R 383 3.3.2 software. The statistical difference was considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Chemical composition and nutritional values of rabbit diets and situation regarding recommendations

Comparing the main chemical and nutritional parameters of the different diets (Table 3) shows that their composition does not differ, except for protein and digestible protein.

Table 1: Comparison of the main parameters of chemical composition and nutritive value between rabbit diets available in the Algerian market

Feed Type /Parameter	CA (%)	CF (%)	CP (%)	F (%)	DE (MJ/Kg DM)*	DP (g/Kg DM) **
Maternity	6.72 ± 1.53 a	18.97 ± 6.93 a	19.80 ± 1.83 a	3.16 ± 0.95 a	13.81 ± 0.20 a	138.70 ± 16.50 a
Fattening	6.47 ± 0.24 a	15.63 ± 1.54 a	16.40 ± 2.16 b	2.50 ± 10.17 a	13.87 ± 0.06 a	109.20 ± 18.96 b
Mixed	6.30 ± 1.56 a	15.40 ± 8.91 a	17.50 ± 2.72 ab	2.80 ± 0.89 a	13.88 ± 0.25 a	119.10 ± 23.81 a
P-value	0.7398	0.3463	0.0173	0.1996	0.6712	0.0216

CA: Crude ash. CF: Crude fibre. CP: Crude Protein. F: fat. DE: Digestible Energy. DP: Digestible Protein. DM: Dry Matter. * Estimated according to Villamide et al. (1989). **: Estimated on the basis of Villamide and Fraga (1998). Means in the same column with different letters are significantly different at $p<0.05$

These results contradict the dietary recommendations found in the literature, which recommend specific intakes for "maternity" and "fattening", as well as a single, less balanced "mixed" diet. Some of the diets in our sample are far from meeting the nutritional requirements of the rabbit, while others are in excess of the requirements for certain nutrients. This situation will result in economic losses for farmers. This situation leads to economic losses for the rabbit farmer, as well as being paid different rates for different types of feed, despite the fact that the nutritional content is virtually the same and sometimes deficient and the zootechnical performance of the rabbits is compromised.

According to Figure 1, energy concentration, at the same level for all samples, is well in excess of the recommended levels. It is well known that fattening rabbits that are fed ad libitum will

adjust their food intake according to the energy concentration of the diet. A diet containing too much ED will cause the rabbit to reduce its intake, which may result in other nutrients not getting in. Excess energy will also affect carcass fatness. When compared with the recommendations for six diets, the analysis also revealed a significant deficiency in crude fibre (Figure 1). It's know that when fibre intake is too low, growth rates are reduced and this is often associated with digestive problems and mortality due to diarrhoea. It should be noted that fibre deficiency in rabbit diets marketed in Algeria is chronic. Therefore, Mouhous et al. (2017) recommended the supplementation of pelleted diets with high-fibre diets.

Unexpectedly, protein levels (Figure 1) in most diets were well above the requirements recommended by De Blas and Mateos (2020) for all types of diets. The rate of incorporation of soybean meal may partly explain this excess. The second explanation is related to the protein content of wheat bran, which is underestimated, according to Harouz-Cherifi et al. (2018). In fact, in the formulation of feed, the values used are often those indicated in foreign tables, otherwise it is the EGRAN table of Maertens et al. (2002). According to these tables, the protein content of wheat bran is around 15% of dry matter, whereas Boudouma (2009) reports that the protein content of wheat bran produced in Algeria would be close to 19% DM. Of the fifteen diets collected, only two (F2 and M3) were slightly deficient in crude protein.

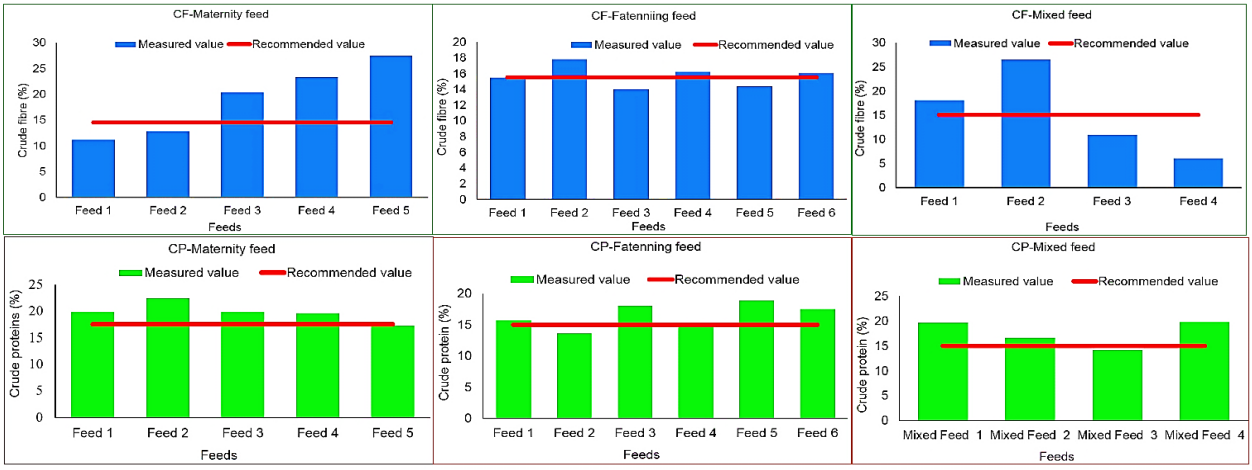

Figure 1: Situation of the crude fibre (CF) and crude protein (CP) contents of the rabbit feeds available on the Algerian market in comparison with the values recommended by De Blas and Mateos (2020).

Chemical composition stability of rabbit feeds

There are differences, sometimes significant, in the composition of identically labelled diets taken at 10-month intervals: from 0.7 to 14.6 points for crude fibre, from 0.2 to 5.7 points for crude protein and from 0.2 to 1.2 points for fat (Figure 2). The situation is even more unfavourable in the case of maternity feed. The composition raw materials is not constant and variations in their composition are likely to have a significant impact on formulating and therefore feed quality. Fluctuations in raw material prices lead some manufacturers to change their feed formulations in order to maintain acceptable production costs, sometimes at the expense of the nutritional quality of the feed, without any change in the content of the information on the label.

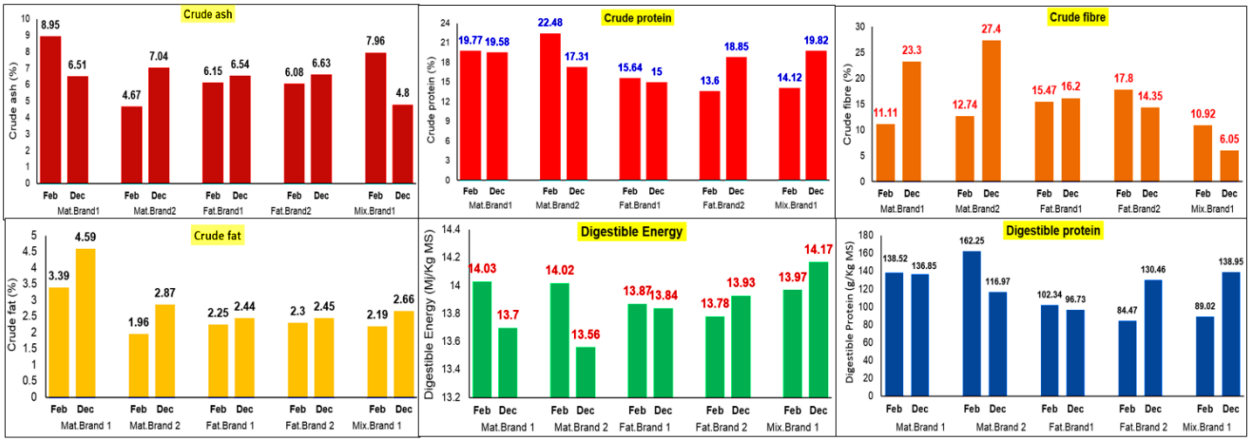

Figure 2: Chemical composition and nutritional value of the same types of rabbit feed from the same manufacturers (brand) at ten-month intervals (February vs. December)
Feb: February. Dec: December. Mat. Maternity. Fat: Fattening. Mix: Mixed

CONCLUSIONS

The chemical analysis of rabbit diets marketed in Algeria revealed a double variability. On the one hand, their chemical composition did not comply with the recommendations, with unacceptable differences in particular in the protein and fibre contents. On the other hand, the composition of the same diets, produced at different times in the same factory, could vary considerably. This situation hurts stockbreeders, who pay the same or even more for the same feed purchased a few months apart, but whose nutrient content differs significantly and often does not meet recommended standards. Depending on the composition of available raw materials, local feed manufacturers have to update their feed formulations.

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Dietary *Moringa oleifera* leaf meal did not affect feed intake but improves weight gain and feed conversion efficiency in rabbits : A systematic review and meta-analysis.

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ABSTRACT

The aim of the study is to use meta-analytical procedure to resolve uncertainty, identify knowledge gaps, and create new insights using published data on the effects of feeding rabbits diets containing *Moringa oliefera* leave meal (MOLM) on feed intake (FI), average daily gain (ADG), and feed conversion ratio (FCR). The search engine, google and electronic databases were employed to search literatures. The data generated were analysed using OpenMEE software. Results revealed that rabbits fed MOLM-based diet (MOLMBD) inclusion were not significantly affected FI (SMD= -0.07; 95% CI: -0.19,-0.05; *P*= 0.05; I^2 = 56.1%), but showed improved ADG (SMD = 0.86; 95 % CI: 0.64, 0.09; P < 0.001) and FCR (SMD= 0.89; 95% CI= -1.11, -0.68; P<0.001). In conclusion, MOLM could be used in the diets of rabbits to utilizing its quality nutrients and phytochemical to promote growth performance.
Key words: Feed intake, feed ingredients, rabbit production, weight gain.

INTRODUCTION

Rabbits are a valuable source of meat that meets the animal protein requirements of medium-sized families and can be raised under small-scale rural farming systems (Abdulmali, 1994). The meat is high in essential nutrients and low in cholesterol, making it a healthy option for consumers (Slim *et al*., 2021). However, the cost of soybean meal in commercial rabbit diets limits their potential contribution to food and nutrition security. Researchers have turned to feeding rabbits with MOLM due to its nutritional and medicinal properties (Sebola *et al*., 2019). Studies have shown that anti-nutritional factors and fibre in MOLM can interfere with mineral and protein utilization, but fibre has been found to improve growth rates in rabbits (Chiou *et al*., 1998). Vast researches were conducted on the rabbit fed MOLM on growth performance and the results remained inaccurate and this study aims to predict the response to MOLM using meta-analysis. We hypothesize that meta-analysis will provide a clear conclusion on the effects of MOLM on rabbit growth performance.

MATERIALS AND METHODS

Search strategy and inclusion criteria

Pre-defined scientific databases, such as Google Scholar, Scopus, and PubMed were used, along with the Google search engine, to search the literatures. The database searches used the terms *Moringa oliefera leaves "OR ("rabbits" AND "growth performance" OR "blood")* but were not restricted to many keywords. A maximum of 782 publications was found and all duplicate publications were identified and removed. Once the duplicate publications had been removed, the databases were recorded in Microsoft Excel 2003. The title and the abstract of each publication were used to exclude irrelevant publications and were read, and the growth performance parameters were recorded for possible classification of eligibility. The following inclusion criteria were applied: 1) publications that compared different MOLM inclusion with the control diet, 2) publications with detailed descriptions of a chemical analysis of the diet. 3)

any publications that reported four of the following parameters: Rabbits, FI, ADG, FCR and MOLM and that were published in English. However, studies where enzymes were incorporated with MOLM were excluded. A total of 30 peer-reviewed articles were considered eligible for this meta-analysis, using the selection process shown in Figure 1.

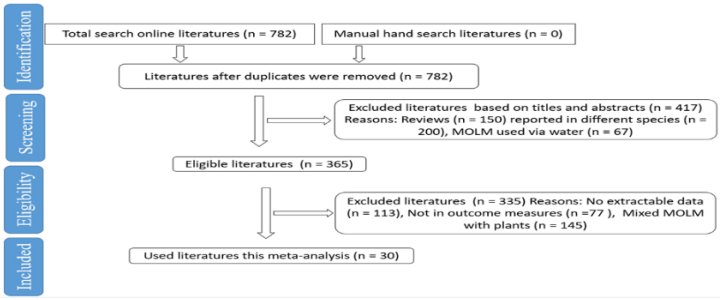


Figure 1: Literature search and selection process following the PRISMA procedure.

Data synthesis

Data were extracted (Table 2) on study identification, study country, continent, and growth performance. Data were also collected on MOLM inclusion levels, breed of rabbit, sex and study periods. Where authors whose studies were used for the analysis did not state the breed and sex of rabbits used in their studies, these rabbits were named not reported and unknown respectively.

Table 2: Characteristics of eligible studies used for the analysis.

		Moderators					
	Studies	Country	Inclusion	Breed	SD	Sex	Outcome
1	Mankga et al. 2022	S. Africa	0, 5, 10, 15	Nzealand	12	Male	1, 2, 3
2	Abiodun and Olubisi 2017	Nigeria	0, 2.5,5, 7.5	Cross	>12	Male	1, 2, 3
3	Aljohani and Abduljawad 2018	S. Arabia	0, 0.5, 1.0	Nzealand	8	unknown	1, 2, 3
4	Ayo-Ajasa et al. 2017	Nigeria	0, 15, 30, 45	Unknown	10	Unsexed	1, 2, 3
5	Bhatt et al. 2023	India	0, 70, 95	Chinchilla	8	Male	1, 2, 3
6	Egu et al. 2021	Nigeria	0, 5, 10.5, 15	Nzealand	8	Male	1, 2, 3
7	El-badawi et al. 2014	Egypt	0, 0.15, 0.30	Nzealand	8	Male	1, 2, 3
8	El-Desoky et al. 2018	Egypt	0, 3, 6	Nzealand	10	Unsexed	1, 2, 3
9	El-kashef et al. 2022	Egypt	2.5, 5, 7.5	Nzealand	18	Unsexed	3
10	Ewuola et al. 2012	Nigeria	0, 5, 10, 15	Cross	10	unknown	1, 2, 3
11	Helal et al. 2017	Egypt	0, 1	Nzealand	10	Male	1, 2, 3
12	Jiwuba and Ogbuewu. 2019	Nigeria	0, 10, 20, 30	Mixed	<8	unknown	1, 2, 3
13	El-Dawy et al. 2020	Egypt	0, 1, 1.5	Nzealand	12	Male	1, 2, 3
14	Ndofor-Foleng et al. 2019	Nigeria	0, 10, 20, 30	Cross	12	unknown	1, 2, 3
15	Ogunlade et al. 2019	Nigeria	0, 5, 10, 15	Mixed	>12	Male	1, 2, 3
16	Omara et al. 2018	Egypt	0, 10, 20, 30	Nzealand	12	Male	1, 2, 3
17	Saka et al. 2019	Nigeria	0, 0.5,1, 2	mixed	10	Male	1, 2, 3
18	Selim et al. 2021	Egypt	0.5, 1.0, 1.5	Nzealand	<8	Unsexed	1, 2, 3
19	Sun et al. 2016	China	0, 10, 20, 30	Nzealand	<8	unknown	1, 2, 3
20	Yakubu et al. 2013	Nigeria	0, 25, 50, 75, 100	Mixed	8	Unsexed	1, 2, 3
21	Zendrato et al. 2019	Indonesia	0, 20, 40, 60	Nzealand	8	unknown	1, 2, 3
22	Gommaa et al. 2017	Egypt	0, 5.2	Nzealand	8	unknown	1, 2, 3
23	Hernández-Fuentes et al. 2020	Egypt	0, 10, 20, 30	Cross	10	unknown	1, 2, 3
24	Bakr et al. 2019	Egypt	0, 3, 6	Nzealand	8	Unsexed	1, 2, 3
25	Bolarin et al. 2017	Nigeria	0, 15, 30	Nzealand	12	Unsexed	1, 2, 3
26	Alatrony et al. 2022	Nigeria		Moshtoher	10	Male	1, 2, 3
27	Rahmy et al.2023	Egypt	0, 1, 2, 3	Nzealand	>12	unknown	1, 2, 3
28	Singer et al. 2018	Egypt	0, 25, 50, 75	Nzealand	8	unknown	1, 2, 3
29	Dougnon et al. 2012	Benin	0, 10, 15	Cross	10	unknown	1,3
30	Elkloub et al. 2018	Egypt	0, 0.25, 0.50, 0.75	Californian	8	Unsexed	1,3

S. Africa= South Africa; S. Arabia= Saudi Arabia; Nzealand = New Zealand;1= Feed intake;2= Average weight gain; 3=Feed conversion ratio.

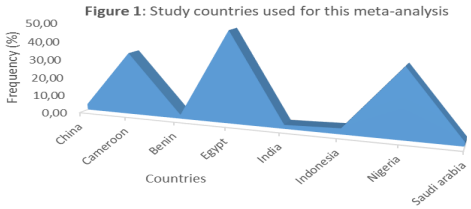
Statistical Analysis

OpenMEE software was used to analyse the data. The MOLM effects on the growth performance of rabbits were assessed using a restricted maximum likelihood (REML) random effects model. The impact of MOLM inclusion on growth performance was assessed using SMD (95%CI). The Q-statistic and I^2-statistic were used to assess heterogeneity (Higgins and Thompson, 2002). Publication bias was determined by the Rosenberg fail-safe (Nfs) number. The significance level was set at P<0.05. Pooled results were deemed robust in the presence of publication bias if Nfs = (5N + 10), where n is the number of studies (Jennions et al., 2013).

RESULTS AND DISCUSSION

Overview of studies included in meta-analysis.

Figure 2 indicates that most of the experiments are conducted in Egypt (48.94%), followed by Nigeria (36.72%) and Cameroon (34.04%) while other countries are less than 3.2% frenquency. This meta-analysis covered 2 continents (Africa and Asia) in which thirty studies were used from 2012 to 2023 (11 years).



Growth performance

Table 3 showed that FI of rabbit was not significantly affected by feeding MOLMBD (SMD= -0.07; 95% CI: -0.19,-0.05; *P*= 0.23; I^2 = 56%) suggesting that MOLM inclusion did not affect the palatability of the diet. Similar pattern was also reported by Sun et al. (2018). Nevertheless, it appears that ANFs in MOLM did not reduce FI in the rabbits. The MOL are rich in high biological value protein and essential amino acids. Methionine and lysine are vital for muscle development (Fang et al., 2021). The best (P>0.05) ADG (SMD = 0.86; 95 % CI: 0.64, 0.09; P < 0.001; I^2 = 87.5% and FCR (SMD= 0.89; 95% CI= -1.11, -0.68; P<0.001) in rabbits fed MOLMBD implies that the rabbits were able to utilise nutrients in the MOLMBD which could be linked with improved morphometric of the intestinal mucosa and gut microbiota. Indeed, Ogbuewu and Mbajiorgu (2022) highlighted intestine as the principal site for immunity, nutrient digestion, and uptake in animals. This results agreed with Jiwuba and Ogbuewu. (2019) who reported improved ADG and FCR when 30% MOLM was included in rabbit diets.

Table 3: Growth performance of rabbit fed *Moringa oleifera* leave meal.

Outcomes	SMD	95% CI		SE	*p*-value	Heterogeneity			
		Lower	Upper			Q	df	*P*-value	I^2(%)
FI	-0.07	-0.19	0.05	0.06	0.23	212	93	<0.001	56
ADG	0.89	0.68	1.11	0.12	<0.001	841	105	<0.001	85
FCR	-0.89	-1.11	-0.68	0.11	<0.001	844	114	<0.001	94

FI= feed intake; ADG = average daily weight; FCR = feed conversion ratio; SMD = standardised mean difference; CI = confidence interval; SE = standard error; P = probability; df = degree of freedom; I^2 = Inconsistency index; Q = Cochran statistic.

Analysis of publication bias

Figure 3 showed a weak tendency for smaller studies to be associated with greater negative effects.

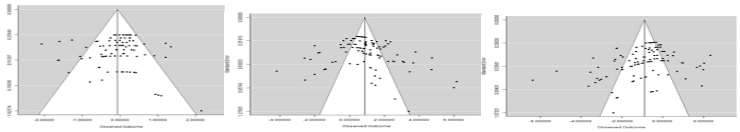


Fig A Fig B Fig C

Figure 3: Funnel graphs on MOLMBD fed to rabbits on Fig A (FI), B (ADG) and C (FCR)

CONCLUSIONS

The meta-analysis showed MOLMBD did not significantly affected FI, but significantly improves AWG and FCR.

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UTILISATION OF BOVINE RUMEN FILTRATE FOR BIODEGRADATION OF SWEET ORANGE PEEL (*CITRUS SINENSIS*) AND EFFECT ON RABBIT GROWTH

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ABSTRACT

A feeding trial was conducted with four to six-week old rabbits (n=30) to determine the maize replacement value of sweet orange peel (SOP) biodegraded with rumen filtrate (RF). Rumen filtrate was obtained from the rumen content and liquor of four randomly selected slaughtered cattle, to which water was added in ratio 1:1, and filtrate manually squeezed out. Freshly collected SOP was divided to five batches of 5 kg each. Rumen filtrate was added to and mixed with one each of 5 kg SOP in ratio 1:5 (SOP1), 2:5 (SOP2), 3:5 (SOP3), 4:5 (SOP4) and 5:5 (SOP5) and, biodegraded for 24 hrs, sundried and milled. Each of SOP replaced 50% maize in the control diet (T1) of rabbits to obtain diets T2, T3, T4 T5 and T6, respectively. Mixed breed exotic rabbit of both sexes were randomly allocated to six diets of five rabbits each. Rabbits were fed *ad libitum* and provided adequate drinking water in a 77-day feeding trial. Performance indices measured were live body weight (LBW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), protein intake (PI) and protein efficiency ratio (PER). Experimental diets had significant effect ($p<0.05$) on PER while, they had no significant effect ($p>0.05$) on final LBW, FI, BWG, FCR and PI. 24-hr biodegradation of SOP, which was mixed with RF in a ratio of 4 L RF : 5 kg SOP, at 50% maize replacement level gave a significantly higher PER of 2.10.

Keywords: Rumen filtrate, biodegradation, peel, rabbit

INTRODUCTION

Food is a necessity in human life as it helps in body growth and sustenance of diverse life activities. However, the world is facing food insecurity which is a threat to human survival most especially in the developing countries. In Nigeria, over 70% of the citizens are involved in agriculture and mainly at subsistence level (Babamaaji and Ekwe, 2023). In spite of its contribution to the nation's economy, the sector confronts a myriad of problems which affect productivity, prominent among which are climate change, land degradation which reduces cultivable land mass, inadequate local production of feed ingredients, post-harvest losses and high production cost. These have adverse effect on agricultural productivity, causing increased food importation due to population increase and decreased food sufficiency. Nigeria's population is estimated at 223.8 million with a growth rate of 2.41% (Worldometer, 2024), and dietary protein deficiency is a major problem partly, because the demand for protein of animal origin is greater than supply. The recent average animal protein intake in Nigeria put at 6 – 8 g/day (Have *et al*., 2020) is dismal. The global average daily total protein intake based on a reference intake value of 0.83 g/kg of body weight is 64 g, and in advanced economies more than 60% of protein intake comes from animal sources whereas, in Africa, India and other food deficient countries it ranges from 20 – 25% of total protein (Capper *et al*., 2013). Animal production in Nigeria remains underexploited, thus, a dire need for an appreciable step-up in livestock production to mitigate the critical shortage of animal protein using animals like the domestic rabbit which when compared with other livestock is characterized by early sexual maturity, high prolificacy, relatively short gestation period, short generation interval, high productive potential, rapid growth, in addition to its ability to utilize forages and fibrous plant materials and agricultural wastes. Sweet orange peels are abundant citrus wastes in most part

of Nigeria because of the heavy consumption of the fruit. It is high in metabolisable energy content, and similar to maize in crude protein (Jobo and Oluremi, 2023) and can be an alternative feed ingredient to maize in rabbit nutrition because of its relatively high crude fibre. The study determined the feed value of sweet orange peels biodegraded with rumen filtrate from bovine species in grower rabbit performance

MATERIALS AND METHODS

Experimental site

The feeding trial was carried out in the Rabbitary unit at the T & R Farm, Federal University of Agriculture, Makurdi, Nigeria located in latitude 60^0 - 80^0 N and Longitude 6^0 - 10^0 E. Makurdi has an annual precipitation ranging between 508 mm to 1016 mm, for a six to eight month period, minimum temperature of 22.80 ^oC and maximum temperature of 40.03 ^oC and, relative humidity between 37.3% and 59.2% (Audu *et al*., 2022).

Collection of sweet orange peel (test Ingredient) and biodegradation

Fresh sweet orange peels (SOP) were collected from orange fruit retailers on the Campus and Wadata, in Makurdi metropolis and treated with RF. Fresh rumen content together with liquor was collected in covered plastics buckets from four randomly selected slaughtered cattle in a Government abattoir within the metropolis. The RF was analysed for its microbial content (Cheesbrough, 2005) in the University's Veterinary Microbiology Laboratory. Water was added to rumen content and liquor in the ratio of 1 L : 1 kg of rumen content, thoroughly stirred and squeezed manually to extract the rumen filtrate. The RF was then added to and mixed with SOP in the ratio of 1 L : 5 kg, 2 L : 5 kg, 3 L : 5 kg, 4 L : 5 kg and 5 L : 5 kg, to obtain biodegraded SOP1,, SOP2, SOP3, SOP4 and SOP5,, respectively. Each mixture was poured in a feed sack, kept in a shade and allowed to ferment for 24 hours. Thereafter, biodegraded SOPs were spread thinly on a concrete floor and sundried until about 10% moisture content was attained within 24 – 48 hours. Samples of SOP1,, SOP2, SOP3, SOP4 and SOP5 were milled and analysed for its proximate constituents (A.O.A.C., 2015) at the University's Animal Nutrition Laboratory and the metabolisable energy calculated (Pauzenga. 1985) as shown in Table 1.

Table 1: Proximate constituents of sweet orange peel (%DM**)**

Nutrients	SOP1	SOP2	SOP3	S0P4	SOP5
Dry matter	89.40	88.86	87.78	88.73	85.48
Crude protein (CP)	5.87	6.40	6.98	7.39	9.73
Crude fibre	9.97	10.03	10.48	11.75	12.20
Ether extract (EE)	1.84	2.23	2.10	2.30	2.11
Ash	9.32	10.38	10.21	10.10	10.40
Nitrogen free extract (NFE)	73.00	70.42	70.23	68.45	65.56
ME[1] (kcal/kg)	2958.01	2917.56	2921.74	2890.29	2858.51

[1]ME = Metabolisable energy, SOP = sweet orange peel.

Preparation of diets, experimental animals and management

Six experimental diets were formulated, using biodegraded SOP1, SOP2, SOP3, SOP4, and SOP5, as replacement for 50% maize in the control diet (T1), to obtain diets T2, T3, T4, T5 and T6, respectively (Table 2). The experimental animals were four to six-week old 36 mixed breed exotic rabbits of both sexes, housed in separate cages, and randomly assigned to the six diets at the rate of five rabbit per diet group. Each rabbit in a diet group served as a replicate, all rabbits were fed *ad-libitum* and drinking water supplied without restriction. The feeding trial lasted for 77 days. The experiment was a completely randomized design. Medications given were antibiotics (Tridox) intramuscularly, coccidiostat (Amprolium) and Zantriviral were given orally using water as the administration route. Vitalyte was given as antistress.

Table 2: Gross composition of experimental diets

Ingredients (%)	T1	T2	T3	T4	T5	T6
Maize	41.44	20.72	20.72	20.72	20.72	20.72
Sweet orange peel	0	20.72	20.72	20.72	20.72	20.72
Fixed ingredients[1]	58.56	58.56	58.56	58.56	58.56	58.56
Total	100	100	100	100	100	100

[1]Fixed ingredients (%); soybean 5.00, blood meal 0.50, rice bran 27.30, brewers dried grain 22.45, bone ash 2.81, vitamin/mineral premix 0.25, table salt 0.25.

Performance data collected and statistical analysis

The performance data collected were IBW, FBW, DFI, BWG, FCR (FI / BWG), PI (FI x % crude protein in feed), PER (BWG / PI) shown in Table 3. Data generated were subjected to the analysis of variance (ANOVA) using SPSS (2012), and the means of significantly different ($p<0.05$) indices separated with the least significant difference (LSD).

RESULTS AND DISCUSSION

Microbes in rumen filtrate and proximate nutrients in biodegraded sweet orange peel

The microbial analysis of the rumen content showed that its homogenous mixture contained bacteria *E.coli* and *Klebseilla* sp.. in the range of $2.4 \times 10^6 – 2.8 \times 10^6$ cfu/g and *Aspergillus* sp. *a* fungal *spp*. in the range of $0.5 \times 10^6 – 0.6 \times 10^6$ cfu/g. The ruminal ecosystem has a large diversity of microorganisms; bacteria, fungi and protozoa (Castillo-Gonzalez, 2014), working synergistically for the nutritional transformation of forage, the main feed of ruminant animals to produce metabolic energy for various physiological activities. The presence of these bacterial and fungal isolates in RF shows that there are some ruminal microbes which can survive outside the ruminal ecosystem and can be useful to stimulate biodegradation process *in vitro* to improve the feed value of some crop residues and agricultural wastes such as sweet orange peels.

The crude protein content of biodegraded SOP increased as the quantity of RF added to SOP increased from 1:5 to 5:5. The microbial load in the RF was expected to increase the higher the quantity of RF added to SOP. This can cause higher cell multiplication and more microbial cell mass thereby causing the increase in the crude protein content of SOP. The crude fibre level of the biodegraded SOP increased while, the nitrogen free extracts decreased as the ratio of the mixture of RF to SOP increased from 1:5 to 5:5, implying that crude fibre in the biodegraded SOP may contain higher indigestible fractions. The microbial isolates in the rumen filtrate *Klebseilla* sp., *E.coli* and *Aspergillus* sp. appeared unable to alter the structure of SOP by reducing its crude fibre content. Ash content increased as the RF : SOP ratio increased from 1:5 to 5:5, probably because of the mineral content in the rumen mass as a result of mostly forage consumed by the cattle. The higher ether extract in the rumen treated SOP might be the consequence of the lipolytic activity of *E.coli*, one of the microbial isolates. There was a slight decrease in the metabolisable energy content of biodegraded sweet orange peel as the RF added increased from 1:5 to 5:5. This may have been due to increased fibre and ash, and reduced digestible carbohydrate in the form of nitrogen free extract.

Growth Performance of Rabbit

The diets had no significant ($p>0.05$) effect on the FBW, daily BWG, DFI, FCR and daily PI except PER ($p<0.05$) as shown on Table 3.

Rabbits in diet T5, which contained 50% maize replacement with SOP treated with RF in ratio 4:5 had the significantly ($p<0.05$) higher PER of 2.10. This result showed that as the quantity of RF added to SOP for its biodegradation increased, PER of growing rabbits tended to increase significantly ($p<0.05$). This, most likely stimulated the relatively higher mean final body weight of 1461.40 g of the rabbits in T5 compared to the other dietary treatments. The non-significant effect ($p>0.05$) of the experimental diets on rabbit FBW, BWG, DFI. FCR and daily PI across the dietary treatments is an evidence of the nutritional potential of biodegraded SOP

to serve as a replacement feed resource for maize, an energy feed ingredient in growing rabbit nutrition. Biodegradation of SOP using bovine RF enhanced the nutritive value of SOP for growing rabbit feeding. Thus biodegraded SOP obtained when bovine RF is added to SOP in ratio of 4:5 can be used to replace 50% of maize in the diet of growing rabbits. This observation has elicited increased level of utilization of SOP in the diet of rabbit as against the 30% maize replacement value earlier reported (Oluremi *et al.,* 2018).

Table 3: Effect of experimental diets on the performance of grower rabbits

Indices	T1	T2	T3	T4	T5	T6	SEM	p-values
IBW (g)	418.33	425.83	425.83	425.83	425.83	376.66	14.78	0.93
FBW(g)	1317.00	1338.40	1318.50	1187.00	1461.40	1335.00	32.55	0.32
BWG(g)	11.80	12.70	11.76	9.61	12.99	12.46	0.44	0.26
DFI(g)	58.88	59.62	61.58	52.58	58.82	53.61	1.64	0.60
FCR	5.07	5.45	5.32	5.57	4.56	4.41	0.17	0.19
PI(g)	6.19	7.41	6.45	5.79	6.17	6.07	0.20	0.30
PER	1.89^{ab}	1.58^{b}	1.61^{b}	1.65^{b}	2.10^{a}	2.07^{a}	0.06	0.02

^{ab}Means with different superscripts on the same row are significantly different ($p<0.05$), SEM = standard error of mean, IBW=Initial body weight, FBW=Final body weight, BWG=Body weight gain, DFI=Daily feed intake, FCR=Feed conversion ratio, PI=Protein intake, PER=Protein efficiency ratio.

CONCLUSION

The RF used contained three microbial isolates *Klebseilla* sp., *Escherchia Colli* and *Aspergillus* sp. which possibly improved the feed value of raw SOP during biodegradation. The FBW, BWG, FI, FCR and PI were similar irrespective of the experimental groups, except PER and was highest in T5. The growth response of rabbits fed biodegraded SOP based diet in ratio 4:5 at 50% maize replacement have a comparative advantage over the other treatment groups and can be used to replace 50% of maize in the diet of growing rabbits.

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IN VITRO DIGESTIBILITY AS A PREDICTOR OF APPARENT FIBRE DIGESTIBILITY IN RABBITS

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ABSTRACT

The aim of this work was to determine the potential use of the *in vitro* digestibility methodology to assess the digestibility of neutral detergent fibre (NDF) at ileal and fecal level. Seven experimental diets from two experiments were used, providing values of ileal and fecal digestibility obtained both through *in vivo* and *in vitro* methodologies. The experiment was considered a random factor. *In vivo* ileal NDF digestibility was clearly underestimated by the direct measurement obtained *in vitro* (10.2 vs. 3.25%. P = 0.028). In fact, the *in vitro* ileal NDF digestibility was not different from zero (P = 0.32). In contrast, the mean value of the *in vitro* faecal NDF digestibility showed no difference with the *in vivo* one (22.8 vs. 18.6%. P = 0.35) indicating a good direct estimation. A high correlation was observed between *in vivo* and *in vitro* NDF digestibility both at ileal or at faecal level (P ≤ 0.018). In conclusion, *in vitro* NDF digestibility in rabbits serves as a reliable methodology for understanding differences in digestibility or solubility among diets. However, at the ileal level, it may underestimate digestibility values. Nonetheless, at the fecal level, the estimation remains much more precise irrespective of diet type.

Key words: digestibility, faecal, fibre, ileal, *in vitro, in vivo.*

INTRODUCTION

In recent years, there has been a growing interest in developing *in vitro* methodologies to assess nutrient availability, driven by the limitations inherent in *in vivo* methods, particularly those concerning animal welfare issues. Regarding rabbit nutrition, Ramos et al. (1992) pioneered the development and validation of an *in vitro* method for estimating digestibility in rabbits. This method, comprising three steps, enables the determination of feed digestibility at the gastric, ileal, and fecal levels.

Additionally, the study of fibre and its fractions is relevant because of their connection with intestinal health. Understanding the digestibility or solubility of fibre as it passes through the digestive tract could provide valuable insights into its relationship with intestinal health (Gidenne et al., 2020).

The present study aims to determine the interest of using the *in vitro* method to assess the digestibility of insoluble fibre at the ileal and fecal levels.

MATERIALS AND METHODS

Diets

Seven diets were used to conduct this experiment from two separate experiments (3 from Abad-Guamán et al., 2015, and 4 from Abad-Guamán, 2015 using), each with determined in vivo ileal and fecal NDF digestibility, data obtained in the Department of Agricultural Production at Polytechnic University of Madrid.

The average and variation range in chemical composition of the seven diets are shown in Table 1. Raw materials were consistent across all diets. The primary fibre sources included cereal straw, sugar beet pulp, sunflower hulls, oat hulls, dehydrated alfalfa and purified pectins.

Table 1: Range of variation of the chemical composition of the seven diets used in the study (% DM)

	Crude protein	aNDFom	Soluble fibre	Lignin	Starch	Ether extract
Average	18.1	32.8	8.14	4.19	27.8	5.11
Min	16.5	31.2	3.03	3.34	20.5	3.74
Max	20.3	36.3	14.5	4.84	37.8	5.91

Chemical Analyses

The *in vitro* method developed by Ramos et al. (1992) was modified using Ankom filter bags, as described by Abad et al. (2013). The two-step *in vitro* procedure was employed to estimate ileal digestibility, while the three-step method was used for fecal digestibility assessment. The analysis of aNDFom was done according to Mertens et al. (2002), by using a filter bag system with Ankom technology and a thermo-stable amylase, no added sulphite and expressed free of ash.

Statistical Analysis

Data were analyzed by using a mixed model for the methodology comparison. The method was included as a fixed factor, while the study and the diets were included as random factors. Regression analysis was conducted using a mixed model, where the study was treated as the random variable, with *in vivo* digestibility serving as the dependent variable and *in vitro* digestibility considered as the predictor variable. It was done with SAS on Demand for Academics.

RESULTS AND DISCUSSION

In vivo ileal NDF digestibility was clearly underestimated by the direct measurement obtained *in vitro* (P = 0.028. Table 2). In fact, the *in vitro* ileal NDF digestibility was not different from zero (P = 0.32). What is probably happening is that the low *in vitro* ileal NDF solubilization is due to the lack of any fibrolytic enzymes compared with the *in vivo* one (Marounek et al, 1995), and the limited NDF solubilization was mainly accounted for the action of the stomach acidity.

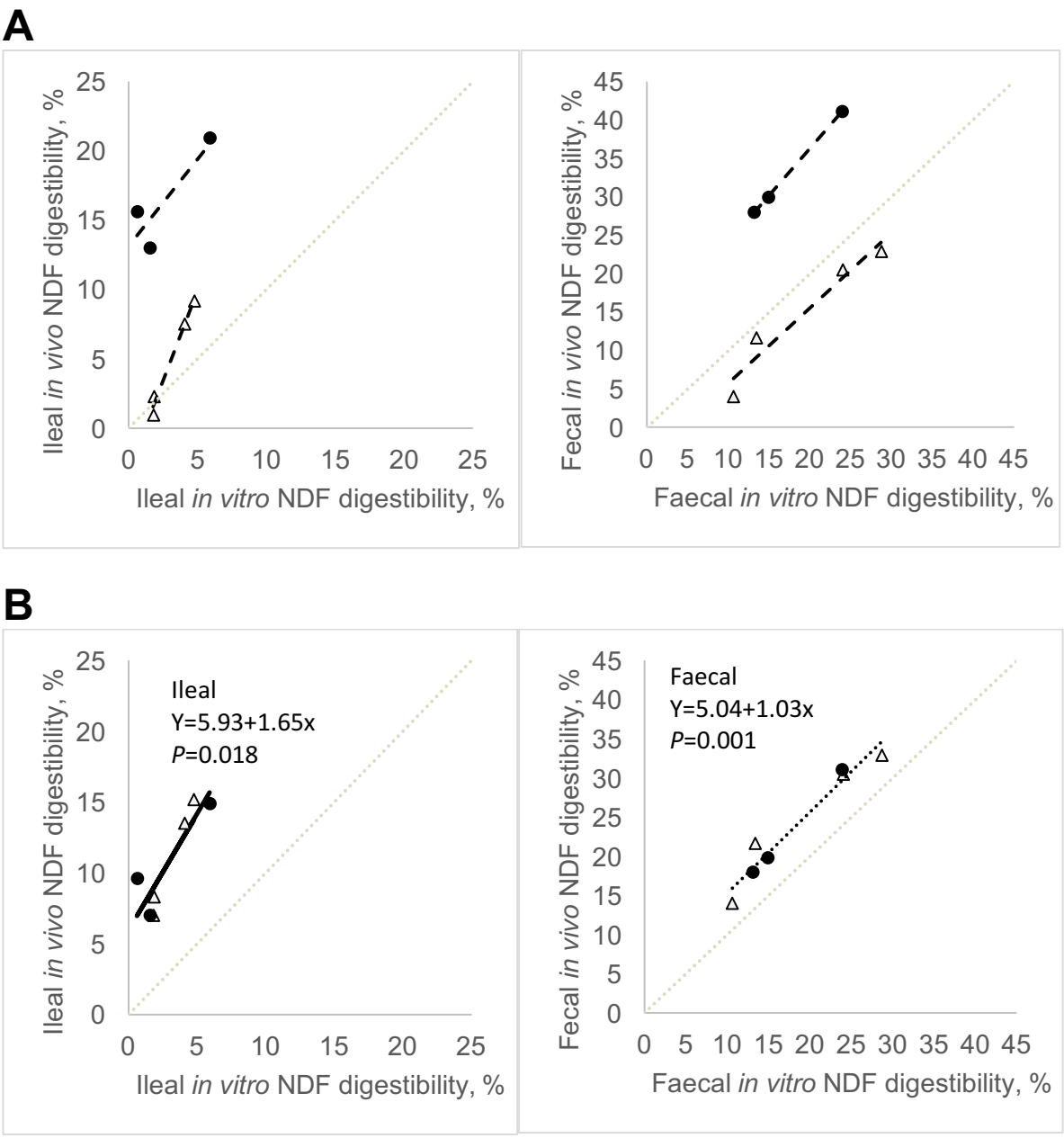
Table 2: Comparison of *in vitro* and *in vivo* ileal and fecal insoluble fibre (NDF) digestibility in rabbits

	Method		SEM[1]	P-value
	In vivo	*In vitro*		
Ileal digestibility	10.2	3.25	3.02	0.028
Fecal digestibility	22.8	18.6	4.57	0.35

[1]Standar error mean, n=7

Several studies indicated a relevant disappearance of total dietary fibre (insoluble and soluble) before reaching the cecum (Gidenne, 1992; Carabaño et al., 2001; Abad-Guamán et al., 2015; Farias-Kovac, 2021). It was hypothesized that dietary fibre would be hydrolyzed partially by microbial fibrolytic enzymes, although this fraction would be majorly fermented in the caecum (Abad-Guamán et al., 2015), which would be in agreement with the negative relationship between total dietary fibre digested before the caecum and caecal pH (Gidenne et al., 2020). In contrast, the mean value of the *in vitro* faecal NDF digestibility showed no difference with the *in vivo* one (P = 0.35) indicating a good direct estimation.

Figure 1: Relationship between *in vitro* and *in vivo* ileal and fecal insoluble fibre (NDF) digestibility. (**A**) Original observations plotted with the mean regression line for each study (• Experiment A; ∆ Experiment B). (**B**) Adjusted observations by the study effect plotted with the mean regression line across studies.



Furthermore, *in vivo* and *in vitro* NDF digestibility were highly correlated either when determined at ileal or fecal level (P ≤ 0.020), considering the random effect of the studies.

In previous studies on rabbits, it has been demonstrated that incorporating *in vitro* digestibility values aids in predicting dry matter digestibility, while the digestibility of crude protein is better predicted by diet composition rather than *in vitro* digestibility values (Villamide et al. 2009). In our study, the ileal and fecal digestibility of insoluble fibre showed a strong correlation with *in vitro* digestibility. The findings of this study reinforce the notion that *in vitro* methodologies play a crucial role in elucidating the differences in nutrient availability and digestibility among various diets. The *in vitro* approach offers a more qualitative assessment, shedding light on the intricate interactions between diet composition and fibre digestibility at different

gastrointestinal levels. Providing valuable insights into the complexities of nutrient utilization and intestinal health.

CONCLUSIONS

In vitro digestibility of NDF in rabbits proved to be a fairly reliable methodology for understanding the differences in the *in vivo* faecal NDF digestibility among diets. However, at the ileal level, it underestimates NDF digestibility, although a significant relationship existed. Further studies would be required with a higher number of diets and a wider variability in dietary NDF to confirm and validate the results of this study.

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EFFECT OF DIETS WITH DIFFERENT FIBER SOURCES ON SLAUGHTER PERFORMANCE, MEAT QUALITY AND INTESTINAL PH OF MEAT RABBITS

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ABSTRACT
The aim of this experiment was to investigate the effects of different fiber source diets on intestinal ph and meat quality of rabbits. A total of 160 healthy 35-day-old Eora rabbits with similar body weights were randomly divided into four groups of eight replicates of five rabbits each, fed full-price rabbit diets with soybean hulls, rice straw, peanut seedling and alfalfa as the only fiber sources, set as T1, T2, T3 and T4, respectively, for 7 days of pre-feeding and 28 days of formal experiment.The carcass weight of T3 was significantly higher than that of T2 and T1, and did not differ significantly from that of T4.In the T4 group the Longissimus dorsi muscle a* and b* were significantly higher ($P<0.05$) than that of T1 group, while Longissimus dorsi muscle L*, cooking loss and shear force did not differ significantly ($P>0.05$) among the groups.The pH of cecum was significantly lower ($P<0.05$) in T1 and T4 groups than that of T2 and T3 groups, while the gastric , duodenum, jejunum, ileum and dorsal longest muscle pH were not significantly different ($P>0.05$). In conclusion, different fiber source diets significantly affected the internal environment and meat color of rabbit cecum. Under the conditions of this experiment, T4 had the best combined performance on all indices and was the most suitable fiber source for rabbit diets.

Keywords: Fiber source, slaughter performance, meat quality, intestinal pH.

INTRODUCTION
Roughage is a relatively large and indispensable component of rabbit diets and the main source of fiber in rabbit diets. By comparing soybean hulls, rice straw, peanut seedlings and alfalfa, dietary fiber sources suitable for meat rabbits were screened to provide theoretical guidance for the application of different fiber feeds on meat rabbits.

MATERIAL AND METHODS
Thirty-five-day-old Hyla meat rabbits with similar body weight and good health condition were selected and randomly divided into four groups, with eight replicates in each group and five rabbits in each replicate. The composition and nutrient content of the full-price ration are shown in Table 1. Before the start of this study, the site and cages were completely sterilized. These meat rabbits were housed individually with free access to feed and water. The experimental data were analyzed by one-way ANOVA using SPSS 26.0 software, Duncan's multiple comparison test, and the results of the data were expressed as "mean±standard error", with $P<0.05$ as significant difference.

Slaughtering performance
Test rabbits were weighed and slaughtered and skinned, limbs and hooves, gastrointestinal tract and contents and urogenital organs were removed and weighed as carcass weight (head, trachea, esophagus, thoracic organs, liver and kidneys and perirenal fat were retained). Carcasses were weighed at the first cervical vertebrae after removing the head, as well as the trachea, esophagus, and retaining the liver (with gallbladder removed), kidneys, and perirenal fat, which were weighed to determine the weight of the semi-clean-bored carcasses. Semi-

clean-bored carcasses are weighed after removing all viscera and perirenal fat. The ratio of carcass weight, semi-clean chambers carcass weight and full clean chambers carcass weight to pre-slaughter live weight was slaughter rate, semi-clean chambers slaughter rate and full clean chambers slaughter rate.

Table 1: Composition and nutritional level of complete diet (air-dry basis)

Items	groups			
	T1	T2	T3	T4
Ingredients				
Corn	10.00	5.00	5.00	11.00
Flour	17.00	9.50	7.00	17.00
Dehulled Soybean Meal	21.00	27.50	23.50	11.00
Soybean Hull Powder	46.00			
Rice Straw Powder		49.00		
Peanut Seeding Powder			58.00	
Alfalfa Meal				55.00
Soybean Oil	2.00	4.00	2.50	1.00
Bentonite		1.00		1.00
Nacl	0.50	0.50	0.50	0.50
Premix[1)]	3.50	3.50	3.50	3.50
Total	100.00	100.00	100.00	100.00
Nutrient Levels[2)]				
Digestible Energy/(MJ/Kg)	9.74	9.13	9.52	9.59
Crude Protein	17.12	16.94	17.01	17.13
Ether Extract	3.11	3.42	2.38	2.85
Crude Fiber	20.17	18.38	20.75	18.61
Neutral Detergent Fiber	30.39	33.32	29.88	28.18
Acid Detergent Fiber	22.19	20.05	23.40	20.74
Calcium	1.25	1.69	1.26	1.23
Phosphorus	0.60	0.71	0.70	0.69

T1, T2, T3 and T4 were fed a complete rabbit diet with soybean hulls, rice straw, peanut seedlings and alfalfa as the sole source of fiber

Meat quality

After meat rabbits were slaughtered, a 205 pH meter was inserted at 45 min and 24 h to determine the pH value, and the mean value was calculated after three trials. The brightness (L*), redness (a*) and yellowness (b*) of the three meat samples were measured using a Wilford WR-18 precision colorimeter and the mean values were calculated. The meat samples were placed in a sample bag, water-bathed at 80°C for 2 h, cooled under tap water for 30 min, cut into 1.5x1.0x0.5 cm, and tested for shear force using a C-LM3B digital muscle tensiometer. The meat samples to be tested were weighed and placed in a sample bag, placed in a water bath at 80°C for 1 hr, cooled under tap water for 30 min, then removed from the meat samples, surface moisture removed and weighed. The formula is calculated as follows.

Cooking loss (%) = [(weight before cooking a weight after cooking)/ weight before cooking1X100.

Intestinal pH

After meat rabbits were slaughtered, the jejunum, ileum, and cecum were removed. A 205 pH meter was inserted in the jejunum, ileum and cecum to determine pH and the mean values were calculated after three trials.

RESULTS

Pre-slaughter live weight was significantly lower in the rice straw group than in the other groups. Carcass weight was significantly higher in the peanut seedling group than in the rice straw and soybean hull groups, and the difference with the alfalfa group was not significant. There was no statistically significant difference in half and full clean carcass rates among the groups.

Table 2 Effect of different fiber sources on the slaughtering performance of meat rabbits

Items	groups				SEM	*P-value*
	T1	T2	T3	T4		
Pre-slaughter weight/g	2165.38a	2051.88b	2210.5a	2206.75a	19.10	0.005
carcass weight/g	1327.5bc	1307.5c	1433.13a	1393.25ab	15.32	0.007
Half eviscerated slaughter ratio/%	52.39	53.86	54.88	53.92	0.42	0.21
Full eviscerated slaughter ratio/%	48.27	49.23	50.01	48.69	0.38	0.421

Same as the table above.

The Longissimus dorsi muscle a* and b* were significantly higher in the alfalfa group than in the soybean hull group (P<0.05), and there were no significant differences in the Longissimus dorsi muscle L*, cooking loss, and shear force between the groups (P>0.05). The results are shown in Table 3.

Table 3 Effect of different fiber sources on meat quality of meat rabbits

Items	Different fiber materials				SEM	*P-* Value
	T1	T2	T3	T4		
Longissimus dorsi muscle						
45min pH	6.88	6.91	6.86	6.94	0.54	0.442
24h pH	5.92	5.85	5.77	5.88	0.75	0.256
Flesh color						
L*	33.25	33.49	32.48	32.73	0.91	0.675
a*	3.50^{b}	3.59^{ab}	4.05^{ab}	5.06^{a}	0.69	0.119
b*	3.30^{b}	3.87^{ab}	3.84^{ab}	4.70^{a}	0.44	0.031
Steaming losses/%	0.17	0.19	0.16	0.17	0.03	0.734
Shearing force/N	21.05	23.29	25.67	25.40	3.75	0.585

Same as the table above.

The pH of the cecum in the soybean skin and alfalfa groups was significantly lower than that in the rice straw and peanut seedling groups (P<0.05), and the pH of the

stomach, duodenum, jejunum, ileum, and longest dorsal muscle did not differ significantly between groups (P>0.05). The results are shown in Table 4.

Table 4 Effect of different fiber sources on intestinal ph in meat rabbits

Items	Different fiber materials				SEM	*P*-Value
	T1	T2	T3	T4		
Gastric	2.01	1.74	1.73	1.96	0.16	0.245
Duodenum	6.46	7.20	6.49	6.72	0.62	0.615
Jejunum	7.54	7.42	7.55	7.62	0.11	0.308
Ileum	7.65	7.54	7.60	7.50	0.11	0.519
Cecum	6.91^{b}	7.29^{a}	7.33^{a}	6.94^{b}	0.13	0.003

Same as the table above.

DISCUSSION

Previous studies have shown that fiber destroys the villous structure of the intestinal wall, promotes apoptosis of villous cells (Priester et al., 2020), and stimulates cell proliferation. Fiber fermentation produces short-chain fatty acids, which maintains the pH in the intestines at a low level, which is conducive to the stimulation of cell division, and short-chain fatty acids can also directly provide energy for the proliferation and development of intestinal epithelial cells. This experimental study found that the alfalfa group was able to reduce the pH value in the cecum of rabbits compared to the soybean hull, rice straw and peanut seedling groups, which may be due to the fact that the diets with different fiber sources entered the digestive tract of rabbits and changed the structure of the intestinal flora, which promoted the proliferation of beneficial bacteria, and facilitated the digestion and absorption of nutrients. Combined with this experiment, it was hypothesized that the fiber content of alfalfa grass meal was the most suitable for rabbits among the four fiber sources, which was conducive to the promotion of intestinal health in rabbits.

CONCLUSION

In summary, different fiber source rations can significantly affect the internal environment of the cecum and meat color of rabbits. Under the conditions of this experiment, the alfalfa group performed the best in all indicators combined and was the most suitable fiber source for rabbit rations.

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CANNABIDIOL IN RABBIT NUTRITION: EFFECTS ON GROWTH PERFORMANCE AND CARCASS TRAITS

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ABSTRACT

Cannabidiol (CBD), a non-psychoactive component of *Cannabis sativa*, has attracted interest due to its potential application as a dietary supplement aimed at improving animal health and welfare. Aim of the present study was to investigate the effect of CBD extract on growth and slaughter traits in New Zealand x California rabbits. The animals were divided into experimental and control groups, with the first group subjected to a daily dose of 10 mg of CBD per animal via a cannabis extract. Observations were conducted up to 92 days of age, analyzing parameters such as live weight, feed intake, body weight gain and feed conversion ratio. At the end of the study, carcass traits were assessed, including chilled carcass weight, organ weight, fat deposits and muscle pH. Chemical analysis of the cannabis extract showed a predominance of total cannabidiol (CBD + CBDA) at 10.35%, with total tetrahydrocannabinol (THC + THCA) levels limited to 0.19%. In vivo results indicated that the live weight of rabbits in the CBD group was significantly affected at the end of the study ($P<0.05$), with a significant increase in body weight ($P<0.01$) during the experimental period (from 65 to 92 days) compared to the control group. The dynamics of feed intake presented a non-linear trend, with an average consumption of approximately 160 g/rabbit/day in both groups. The feed conversion ratio (FRC) was significantly lower ($P<0.01$) in the CBD group at 65-70 days, 86-92 days and in the entire experimental period 65-92 days. Hot carcass weight and carcass yeld were higher in the CBD group compared to the control ($P<0.05$ and $P<0.01$, respectively). The spleen, as a percentage of the warm carcass, was heavier in the CBD group, unlike the liver which was heavier in the control group ($P<0.01$). The reference carcass was heavier in the CBD group ($P<0.05$) while the groin fat was heavier in the control group ($P<0.05$). Regarding gender effect, although in animal species males usually show greater growth potential, in rabbits these disparities are not evident since the animals are slaughtered early, without reaching puberty. Despite this, the results revealed significantly superior ($P<0.05$) in vivo performance (LW, BWG, FI and FCR) and post-mortem outcomes (LW, carcass traits, spleen and liver) in males compared to females, with an opposite trend observed for perirenal fat. In conclusion, the use of 10 mg CBD/animal/day produced positive effects on the growth performance of rabbits. These results were confirmed by a higher carcass yield in the rabbits of the treated group.

Key words: CBD, rabbits, growth performance, carcass traits, animal health and production

INTRODUCTION

In recent years, there has been a notable evolution in feeding techniques, including feed production and formulation, and in rabbit feeding recommendations. This progress is aimed at obtaining a more effective adaptation between the composition of the feed and the specific needs of the animals (Lebas and Laplace, 1994). The use of feed additives in animal nutrition has long aroused interest in scientific research, with the aim to improve their health and well-being (Fallahi et al., 2022) as well as to optimize the growth performance and slaughter characteristics of animals intended for food production. In this context, cannabidiol (CBD), a phytochemical compound, extracted from the *Cannabis sativa* plant, emerges as a substance of potential interest for improving rabbit diets. In the context of rabbit breeding, the main

objective lies in the production of high-quality meat. Despite this, various factors, including stress and pathologies, can have an unfavorable impact on growth performance and carcass characteristics. Several studies have shown that CBD is proposed as a potential dietary supplement capable of modulating the inflammatory response (Fallahi et al., 2022). In such context, the aim of the present study was to investigate the effect of CBD extract on growth and slaughter traits in New Zealand x California rabbits.

MATERIALS AND METHODS

Animals and experimental design

The experimental procedures were approved by the Ethical Animal Care and Use Committee of the University of Napoli Federico II, Italy (prot. N. 2019/0058989) according to the principles stated by the EC Directive 2010/63/UE, regarding the protection of animals used for experimental and other scientific purposes. A total of 42, sixty days old New Zealand x California rabbits (sex ratio 1:1, average weight 1621.3 g ± 46.2) were homogeneously divided into 2 groups (21 animals/group). The animals were raised in individual cages (25 cm length x 45 cm depth x 30 cm height) in a room under controlled environmental conditions; 3 individual cages were considered as a replicate (7 replicates of 3 cages/group). During the adaptation period (60 – 64 days of age) the groups fed the same commercial diet with 15 % CP, 3.5 % of fat and 11.9 MJ/kg of digestible energy. From 65 days of age, both groups continued to be fed the basal diet, but the Cannabidiol (CBD) group (11 females, 10 males) received 0.1 ml of a cannabis extract in coconut-based oil (Giantec, Isernia, Italy), corresponding to 10 mg of CBD/animal/day. The cannabis extract was administered individually by putting it on a wafer of alfalfa dehydrated meal (15 g) and waiting until the wafer has been completely consumed. The control group (10 females, 11 males) received the same amount of alfalfa wafer with 0.1 ml of coconut oil but without cannabis extract. Up to 92 days of age (for 27 d), the live weight and feed intake were individually controlled weekly. Then, the body weight gain and the feed conversion ratio were calculated. CBD extract was characterized using HPLC-DAD (High Performance Liquid Chromatography - Diode Array Detector) according to Ph.Eur. 2.2.29 (European Pharmacopoeia).

Slaughteing traits

At 92 days of age the rabbits were moved to a specialized slaughterhouse and the carcass traits were evaluated on 1 rabbit per replicate (7 per groups) following the World Rabbit Science Association recommendations (Blasco and Ouhayoun, 1996). The carcasses were weighed and then chilled at 4°C for 24 h in a ventilated room. After 24 h chilling, the carcasses were weighed again to obtain the chilled carcass (CC) weight, then head, liver, heart, the lungs + oesophagus + trachea + thymus gland package, and kidneys, were removed to obtain the reference carcass (RC). With a portable instrument (Model HI 9025; Hanna Instruments, Woonsocket, RI, USA), equipped with an electrode (FC 230C; Hanna Instruments), the value of pH was measured in the Biceps femoris (BF) and Longissimus lumborum (LL) muscles, 1 and 24 h after slaughtering.

Statistical Analysis

Data were analyzed by ANOVA, using the GLM procedure of SAS (2002) according to the following model:

$$Yijk = m + Gi + Sj + G*Sij + eijk$$

Where Y is the single observation, m the general mean, G the effect of the dietary treatment, S the effect of the sex, G*S the interaction between dietary treatment and sex and e the error. For in vivo performance, the replicate has been considered the experimental unit.

RESULTS AND DISCUSSION

The chemical analysis of the cannabis extract showed that the Total Cannabidiol (CBD + CBDA) was the highest active compound (10.35%) whereas very low levels (0.19%) of Total

Tetrahydrocannabinol (THC + THCA) were detected. Concerning the in vivo results (Table 1), rabbits Live Weight (LW) was significantly affected by CBD supplementation at the end of the trial (P=0.05), according to a significant (P<0.01) Body Weight Gain (BWG) registered in the CBD group along the experimental period (65 up to 92 days) compared to the Control one. Feed intake (FI) was significantly increased by CBD supplementation from 65 to 70 days (P<0.01), while in the period 79-85 d the control group had a higher (P<0.01) feed intake than the CBD group, so that, considering the entire experimental period, no significant differences can be found for feed intake between the groups. In our trial, daily feed consumption showed a non-linear trend, recording an average consumption of approximately 160 g/rabbit/day in both groups. The ability of the cannabinoid system to control appetite, feed intake, and energy balance has recently received great attention, particularly in the light of the different modes of action underlying these functions.
Feed Conversion Ratio (FRC) resulted significantly (P<0.01) lower in CBD group at 65-70d, 86-92d and all along the experimental period 65-92d. No interaction effects between group and sex were recorded.

Table 1. Effect of cannabis extract and sex on in vivo performance of rabbits

	Control	CBD	Females	Males	RMSE	P-values		
						Group	Sex	Interaction
LW 65d, g	1727.1	1707.1	1625.7^{b}	1808.6^{a}	91.6	0.95	0.03	0.38
LW 75d, g	1905.7	1948.6	1827.1^{b}	2027.1^{a}	93.4	0.57	0.02	0.56
LW 78d, g	2191.4	2245.7	2108.6^{B}	2328.7^{A}	89.4	0.33	<0.01	0.39
LW 85d, g	2364.3	2422.9	2284.3^{b}	2502.9^{a}	92.1	0.09	0.01	0.60
LW 92d, g	2637.1^{b}	2731.4^{a}	2608.6^{b}	2760.0^{a}	101.3	0.05	0.03	0.62
BWG 65-70d, g	35.71^{b}	48.27^{a}	40.29^{b}	43.71^{a}	3.96	0.01	0.04	0.49
BWG 71-78d, g/d	35.71	37.14	35.18	37.68	2.99	0.07	0.07	0.33
BWG 79-85d, g/d	30.95	29.76	29.52	31.19	3.01	0.12	0.16	0.68
BWG 86-92d, g/d	38.98^{B}	44.08^{A}	46.33^{A}	36.74^{B}	3.26	<0.01	<0.01	0.51
BWG 65-92d, g/d	33.70^{B}	37.94^{A}	36.40	35.23	2.75	<0.01	0.12	0.28
FI 65-70d, g/d	126.6^{B}	145.4^{A}	135.1	136.9	10.0	<0.01	0.63	0.38
FI 71-78d, g/d	153.7	153.9	145.3^{B}	162.3^{A}	11.3	0.54	<0.01	0.79
FI 79-85d, g/d	170.7^{A}	157.1^{B}	147.6^{B}	180.2^{A}	13.5	<0.01	<0.01	0.61
FI 86-92d, g/d	186.8	179.2	169.3^{B}	196.8^{A}	12.63	0.11	<0.01	0.74
FI 65-92d, g/d	161.9	161.4	151.8^{B}	171.5^{A}	11.9	0.45	<0.01	0.57
FCR 65-70d, g/g	3.54^{A}	3.02^{B}	3.35	3.13	0.26	<0.01	0.07	0.70
FCR 71-78d, g/g	4.30	4.14	4.13	4.30	0.32	0.15	0.012	0.80
FCR 79-85d, g/g	5.51	5.27	5.01^{B}	5.77^{A}	0.39	0.06	<0.01	0.47
FCR 86-92d, g/g	4.79^{A}	4.06^{B}	3.65^{B}	5.35^{A}	0.30	<0.01	<0.01	0.82
FCR 65-92d, g/g	4.80^{A}	4.25^{B}	4.17^{B}	4.86^{A}	0.32	<0.01	<0.01	0.63

CBD: cannabidiol extract; RMSE: root mean square error; ^{a,b}: P < 0.05, $^{A, B}$: P < 0.01; LW: live weight; BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio.

In Table 2, the rabbit carcass traits are reported. The hot carcass weights and the carcass yield resulted significantly higher (P<0.05 and P<0.01, respectively) in CBD group, compared to the Control one. Spleen, in % of hot carcass, resulted heavier in the CBD group while the liver was heavier in the control group (P<0.01). The reference carcass weight was higher in the CBD group (P<0.05) while the inguinal fat resulted significantly (P<0.01) heavier in the control group. Our results indicated a significant difference (P<0.05) in in vivo performance, specifically in terms of body weight gain (BWG), where males showed higher BWG at 65-70 days (P<0.05) compared to female. However, at 86-92 days, BWG was lower in males (P<0.01), with no significant differences observed across the overall period. Additionally no significant disparities were found between males and females in post-mortem findings, with the exception of carcass yield, reference carcass weight and hot carcass weight higher in males (P<0.01, P<0.05 and P<0.05, respectively) and perineal fat %, higher in females (P<0.05). No interaction effects between group and sex were recorded.

Table 2. Rabbit carcass traits according to dietary treatments, sex and their interaction

	Control	CBD	Females	Males	RMSE	P-values		
						Group	Sex	Interaction
LW, g	2617.1	2668.3	2585.2^{b}	2700.2^{a}	165.4	0.06	0.03	0.79
Skin, % LW	14.23	15.31	13.72	15.81	1.05	0.19	0.06	0.27
Full GUT, % LW	17.65	16.69	17.86^{a}	16.49^{b}	1.39	0.72	0.04	0.36
Empty GUT, % LW	8.80	8.44	8.76	8.49	0.87	0.42	0.51	0.24
Hot Carcass, g	1604.3^{a}	1702.9^{b}	1581.4^{a}	1725.8^{b}	109.1	0.03	0.02	0.75
Carcass Yield, %	61.30^{B}	63.82^{A}	61.17^{B}	63.91^{A}	1.65	<0.01	<0.01	0.16
Spleen, % HC	4.25^{B}	6.70^{A}	5.41	5.54	0.23	<0.01	0.59	0.20
Urinary+genitals, % LW	0.66	0.62	0.64	0.63	0.04	0.74	0.95	0.49
pHLD1h	7.11	6.86	6.97	7.06	0.33	0.11	0.80	0.95
pHBF1h	6.91	6.75	6.71	6.96	0.40	0.56	0.30	0.44
Chilled carcass, g	1577.1	1688.6	1591.4	1674.3	98.6	0.07	0.08	0.91
CC Yield, %	60.26^{B}	63.28^{A}	61.56	62.00	0.47	<0.01	0.63	0.84
pHLD24h	5.87^{A}	5.76^{B}	5.81	5.83	0.045	<0.01	0.94	0.31
pHBF24h	5.93	5.89	5.91	5.92	0.048	0.28	0.95	0.43
Reference carcass, g	1029.6^{b}	1116.5^{a}	1022.1^{b}	1124.0^{a}	84.93	0.03	0.03	0.70
Head, % RC	14.33	13.04	14.02	14.15	1.06	0.24	0.63	0.35
Liver, % RC	8.47^{A}	6.91^{B}	7.55	7.82	0.43	<0.01	0.92	0.20
Kidney, % RC	1.71	1.60	1.66	1.66	0.14	0.22	0.85	0.46
Lungs, % RC	1.43	1.43	1.41	1.45	0.12	0.96	0.66	0.59
Heart, % RC	1.29	1.33	1.26	1.36	0.09	0.31	0.28	0.23
Inguinal fat % RC	0.19^{A}	0.15^{B}	0.18	0.16	0.01	0.01	0.45	0.84
Perirenal fat, % RC	2.39	2.40	2.55^{a}	2.23^{b}	0.16	0.96	0.02	0.76
Scapular fat, % RC	0.49	0.49	0.50	0.47	0.03	0.97	0.80	0.94

CBD: cannabidiol extract; RMSE: root mean square error; ^{a,b}: $P < 0.05$, $^{A, B}$: $P < 0.01$; LW: live weight; GUT: gastro-intestinal tract; HC: hot carcass; CC: chilled carcass; RC: reference carcass.

CONCLUSIONS

In conclusion, this study highlights that the use of a cannabis extract at the dosage of 10 mg of CBD/animal/day positively affects growth performance in rabbits in terms of live weight, body weight gain and feed conversion ratio and the data are confirmed by a better carcass yield in rabbits belonging to the treated group. These results are independent of gender. Concerning our extract, different dosages of this product should be tested to assess the optimal supplementation to achieve best results.

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EFFECTS OF ADDING DIFFERENT PROBIOTICS TO FEED ON THE PRODUCTION PERFORMANCE, INTESTINAL MUCOSAL IMMUNITY, AND CECAL FERMENTATION OF RABBITS

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ABSTRACT

This experiment was conducted to study the effects of dietary add different probiotics on production performance, intestinal mucosal immunity and cecal fermentation of rabbits. A total of 400 commercial Ira meat rabbits were randomly divided into 4 groups, and the control group was fed basic diets, while the experimental group added 10^8 CFU/kg *Lactiplantbacillus plantarum*, *Pediococcus pentosaceus* and *Clostridium butyricum* to the basic diet, respectively. After a 7-day pre-feeding period, it entered a 40-day formal experiment. The results showed that: Adding different probiotics in diet had no significant effect on growth performance of rabbits (P>0.05), but the diarrhea rate of meat rabbits in the experimental group with probiotics decreased (P=0.0595) and the survival rate increased (P=0.0603). The villi height of the experimental group with probiotics was significantly higher than control group, and the crypt depth of *Clostridium butyricum* group was significantly higher than the other groups. Besides, the content of secretory immunoglobulin A (sIgA) in the experimental group with probiotics was significantly higher than control group, while the content of tumour necrosis factor α (TNF-α) and interferon-γ(IFN-γ) was significantly lower than control group. The pH of the experimental group with probiotics was significantly lower than control group, the contents of acetic acid and total volatile fatty acids were significantly higher than control group, and the ammonia nitrogen content of *Clostridium butyricum* group was significantly lower than control group. Threrfor, adding probiotics to the diet could improve the intestinal morphology and structure, change the composition of volatile fatty acids in cecum contents, improve the immune performance of intestinal mucosa, and promote the intestinal health of meat rabbits. Based on the results of this experiment, *Clostridium butyricum* could maintain the intestinal morphology and structure of meat rabbit better.

Keywords: *Lactiplantbacillus plantarum*; *Pediococcus pentosaceus*; *Clostridium butyricum*; rabbit; production performance; immune performance; cecal fermentation.

INTRODUCTION

There are a large number of microorganisms in the animal gastrointestinal tract, which play an important role in nutrient digestion and absorption, maintaining the integrity of the intestinal barrier structure, and resisting pathogens. Dysfunction of gastrointestinal microbiota not only leads to digestive dysfunction, but also causes various extraintestinal diseases (Gentile and Weir, 2018; Tilg et al., 2018; Zuo et al., 2020). Probiotics were initially defined as living microorganisms that, when given in sufficient quantities, bring health benefits to the host, thereby regulating the balance of gut microbiota and exerting preventive and therapeutic effects on diseases. Therefore, using probiotics instead of antibiotics in animal production to maintain animal health is of great significance. This experiment was conducted to study the effects of dietary add different probiotics on production performance, intestinal mucosal immunity and cecal fermentation of meat rabbits, so as to provide theoretical reference for application of probiotics in rabbit production.

MATERIALS AND METHODS

Experimental design

A total of 400 commercial Ira meat rabbits (male and female half) with 28-day-old weaned, similar weight and good health were randomly divided into 4 groups, with 10 replicates in each

group and 10 rabbits in each replicate. The control group was fed basic diets, while the experimental group added 10^8 CFU/kg *Lactiplantbacillus plantarum*, *Pediococcus pentosaceus* and *Clostridium butyricum* to the basic diet, respectively. The composition and nutritional level of the raw materials are shown in Table 1. After a 7-day pre-feeding period, it entered a 40-day formal experiment.

Table 1: Composition and nutrient levels of basal diets (air-dry basis) %

Raw material composition	Percentage	Nutrient levels [2)]	Content
Corn	5.0	Digestible energy (MJ/kg)	10.23
Soybean meal	8.0	Dry matter	89.82
Barley	6.0	Crude protein	16.12
Wheat bran	15.0	Ether extract	2.80
Corn germ meal	16.0	Crude fiber	17.38
Corn husk	17.0	Neutral detergent fiber	38.74
Alfalfa meal	15.0	Acid detergent fiber	23.08
Soybean straw powder	7.0	Acid detergent lignin	6.29
Rice hull powder	8.0	Crude ash	9.03
Calcium hydrogen phosphate	1.5	Calcium	0.95
Sodium chloride	0.5	Total phosphorus	0.45
Premix[1)]	1.0	Lysine	0.60
Total	100.0	Methionine	0.65

[1)] Premix provided the following per kg of diets: VA 8 000 IU, VD_3 1500 IU, VE 45 mg, VK_3 2.0 mg, VB_1 1.0 mg, VB_2 3.0 mg,VB_6 1.5 mg, nicotinic acid 30 mg, pantothenic acid 50 mg, folic acid 0.5 mg, choline chloride 100 mg, Fe 50 mg, Cu 10 mg, Zn 50 mg, Mn 10 mg, I 0.5 mg, Se 0.05 mg, Lysine 1.5 g, Methionine 0.5 g, the rest is maifan stone carrier complement. [2)] Digestible energy was a calculated value, while the others were measured values.

Statistical analyses

Perform analysis of variance on the data using GLM in SAS Inst, Inc, Cary, NC, USA, and perform multiple comparisons using Duncan's test.

RESULTS AND DISCUSSION

Growth performance

During the growth experimental trial, adding different probiotics in diet had no significant effect on growth performance of meat rabbits (P>0.05), but the diarrhea rate of rabbits in the experimental group with probiotics decreased (P=0.0595) and the survival rate increased (P=0.0603; Table 2).

Table 2: Effects of dietary add different probiotics on growth performance of rabbits

Items	Groups				*RMSE*	*P*-value
	Control group	*Lactiplantbacillus plantarum*	*Pediococcus pentosaceus*	*Clostridium butyricum*		
Initial body weight /g	961.7±16.7	939.0±17.2	958.0±11.3	936.0±15.3	48.4137	0.5576
Final body weight /g	2632.2± 26.5	2582.6±19.6	2597.3±16.2	2563.0±34.9	80.1536	0.2809
Average daily gain /(g/d)	41.79± 0.49	41.09±0.64	40.98±0.19	40.68±0.68	1.6958	0.5269
Average daily feed intake /(g/d)	171.17± 2.43	174.94±2.99	177.03±3.14	178.59±3.74	9.8362	0.3762
Feed/Gain	4.10±0.08	4.27±0.11	4.32±0.07	4.40±0.11	0.3001	0.1725
Survival rate /%	89.00±2.77	92.00±3.27	91.00±3.14	92.00±2.49	8.2796	0.0603
Diarrhea rate /%	11.00±3.79	8.00±3.27	9.00±2.77	7.00±3.00	9.2062	0.0595

Notes. Means ± std. error and root mean error (RMSE), n= 10.

Histological structure

As shown in Table 3, the villi height of the experimental group with probiotics was significantly higher than control group, and the crypt depth of *Clostridium butyricum* group was significantly higher than the other groups (P<0.05).

Table 3: Effects of dietary add different probiotics on histological structure of rabbits

Items	Groups				*RMSE*	*P*-value
	Control group	*Lactiplantbacillus plantarum*	*Pediococcus pentosaceus*	*Clostridium butyricum*		
Villus height/μm	686.06±41.41[b]	831.45±28.86[a]	830.28±37.35[a]	825.96±45.42[a]	122.5305	0.0275
Crypt depth/μm	110.29±5.93[b]	114.10±4.98[b]	114.69±7.18[b]	138.68±5.50[a]	18.8256	0.0068
Villus width/μm	106.15±4.73	113.86±7.15	117.10±7.49	120.90±5.05	19.6984	0.3984
Mucosal layer thickness/μm	930.80±28.21	984.00±43.24	1019.20±47.59	1081.23±42.12	129.4580	0.0860
Muscle layer thickness/μm	127.44±10.77	141.20±11.76	157.75±7.84	130.70±8.91	31.4333	0.1495
Villus height/Crypt depth	6.26±0.32	7.45±0.48	7.45±0.49	6.08±0.46	1.4008	0.0516

Means ± std. error and root mean error (RMSE), n= 10. [a, b] Different superscript letters means different (P < 0.05).

Intestinal mucosal immunity

Adding different probiotics in diet had significantly different effect on sIgA, TNF-α and IFN-γ of meat rabbits (P<0.05; Table 4), among which the content of sIgA in the experimental group with probiotics was significantly higher than control group, while the content of TNF-α and IFN-γ was significantly lower than control group.

Table 4: Effects of dietary add different probiotics on intestinal mucosal immunity of rabbits

Items	Groups				*RMSE*	*P*-value
	Control group	*Lactiplantbacillus plantarum*	*Pediococcus pentosaceus*	*Clostridium butyricum*		
sIgA/ (mg/g)	3.96±0.16[b]	4.89±0.18[a]	5.19±0.19[a]	5.09±0.16[a]	0.5508	<0.0001
TNF-α /(ng/g)	5.14±0.26[a]	4.08±0.23[b]	3.81±0.33[b]	3.96±0.22[b]	0.8336	0.0111
IFN-γ/ (ng/g)	7.00±0.37[a]	5.60±0.44[b]	5.41±0.29[b]	4.97±0.30[b]	1.1213	0.0093
IL-6 / (ng/g)	1.24±0.04	1.34±0.06	1.33±0.10	1.24±0.10	0.2429	0.6997

sIgA, secretory immunoglobulin A; TNF-α, tumour necrosis factor α; IFN-γ, interferon-γ; IL-6, interleukin-6; Means ± std. error and root mean error (RMSE), n= 10. [a, b] Different superscript letters means different (P < 0.05).

Cecal fermentation

As shown in Table 5, the pH of the experimental group with probiotics was significantly lower than control group, the contents of acetic acid and total volatile fatty acids were significantly higher than control group, and the ammonia nitrogen content of *Clostridium butyricum* group was significantly lower than control group (P<0.05).

CONCLUSIONS

Adding probiotics to the diet could improve the intestinal morphology and structure, change the composition of volatile fatty acids in cecum contents, improve the immune performance of intestinal mucosa, and promote the intestinal health of meat rabbits. Based on the results of this experiment, *Clostridium butyricum* could maintain the intestinal morphology and structure of meat rabbit better.

Table 5: Effects of dietary add different probiotics on cecal fermentation of rabbits

Items	Groups				*RMSE*	*P*-value
	Control group	*Lactiplantbacillus plantarum*	*Pediococcus pentosaceus*	*Clostridium butyricum*		
pH value	7.04±0.11[a]	6.73±0.07[b]	6.82±0.07[b]	6.61±0.09[b]	0.2703	0.0087
Ammoniacal nitrogen /(μg/g)	74.93±1.74[a]	71.47±1.84[ab]	73.29±1.35[ab]	66.34±1.59[b]	3.3463	0.0325
Acetic acid/ (mg/g)	0.90±0.05[b]	1.25±0.09[a]	1.19±0.07[a]	1.13±0.06[a]	0.2166	0.0050
Propionic acid/ (mg/g)	0.13±0.01	0.14±0.01	0.12±0.02	0.10±0.02	0.0457	0.2427
Butyric acid /(mg/g)	0.69±0.08[b]	1.03±0.08[a]	0.76±0.10[b]	1.11±0.08[a]	0.2706	0.0024
Total volatile fatty acid /(mg/g)	1.71±0.08[c]	2.42±0.10[a]	2.07±0.11[b]	2.35±0.09[ab]	0.3067	＜0.0001
Acetic acid/(Propionic acid+ Butyric acid)	1.21±0.14	1.14±0.14	1.55±0.23	0.98±0.10	0.4998	0.0890

Means ± std. error and root mean error (RMSE), n= 10. [a, b] Different superscript letters means different ($P < 0.05$).

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EFFECTS OF A OF OIL-BASED CANNABIS EXTRACT ON BEHAVIOR AND OXIDATIVE STRESS INDEX IN GROWING RABBITS

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ABSTRACT

A cannabis extract in coconut-based oil was administered for 27 days to growing rabbits in order to explore its potential effects on behavior and oxidative stress. The extract was analyzed showing that the total cannabidiol (CBD Cannabidiol + CBDA Cannabidiolic Acid) was the highest active compound (10.35%) whereas very low levels (0.19%) of total tetrahydrocannabinol (THC + THCA) were detected. A total of 42 rabbits aged 60 days were included in the trial and divided in two groups (control and treated). Behaviors were recorded weekly between 12.00h to 15.00h, watched and coded by an expert observer. A second operator coded 20% of the videos, and the interobserver reliability was found to be very high for all observed behaviors, ranging from 90 to 96%. Results showed a general increase of both locomotion (moving, stretch) and exploring (sniffing, look around) behaviors, and a decrease of lying (resting position), thus suggesting an improvement of attention span with a consequent increase of activity.A significative increase of the biological antioxidant potential (BAP) ($P<0.01$),) led to a reduction of free radicals, finally resulting in a significantly lower oxidative stress index (Osi) r in the treated group. The increase of positive behaviors and the improvement of oxidative stress suggest a beneficial effect of the extract.

Key words: Rabbit, Cannabidiol, Oxidative stress, Behavior.

INTRODUCTION

Cannabidiol (CBD) has shown potential in reducing inflammation and discomfort in rabbits (Ozawa S. et al. 2023). Various conditions, including arthritis and digestive issues, can cause pain and inflammation in these animals. CBD interacts with the rabbit's endocannabinoid system (ECS), which plays a role in regulating immune response and pain perception (Rooney T. et al. 2021). By modulating cannabinoid receptors, CBD may also contribute to the overall well-being of rabbits, improving their quality of life, but the literature on this topic is limited for this species. By modulating the ECS, which plays a role in regulating mood and anxiety (Zuardi A.W. et al .2017), CBD may promote a sense of calm and relaxation in rabbits, helping them cope better with stressful situations. It can potentially reduce anxiety-related behaviors, such as excessive grooming, hiding, or aggressive behavior (Ceballos M.G. et al. 2016). CBD's ability to address anxiety and stress in rabbits without inducing intoxication or severe side effects makes it an appealing tool improve animals welfare.

In order to explore these potential benefits of CBD administration to rabbits, two aspects were considered: the behavioral repertoire and the oxidative status changes in growing rabbits supplemented with a commercial cannabis extract designed for pets for 27 days.

MATERIALS AND METHODS

Animals and breeding conditions

The experimental procedures were approved by the Ethical Animal Care and Use Committee of the University of Napoli Federico II, Italy (prot. N. 2019/0058989) according to the principles

stated by the EC Directive 2010/63/UE, regarding the protection of animals used for experimental and other scientific purposes. A total of 42, sixty days old New Zealand x California rabbits (sex ratio 1:1, average weight 1621.3 g ± 46.2) were homogeneously divided into 2 groups each including 7 replicates of 3 animals. The animals were raised in individual cages (25 cm length x 45 cm depth x 30 cm height) in a room with controlled environmental conditions. During the adaptation period (60 – 64 days of age) the groups fed the same diet with 15 % CP, 3.5 % of fat and 11.9 MJ/kg of digestible energy.

At 65 days of age, both groups continued to be fed the basal diet, but the Treated (CBD) group (11 females, 10 males) received 0.1 ml of a cannabis extract in coconut-based oil (Giantec, Isernia, Italy), corresponding to 10 mg of CBD/animal/day. The cannabis extract was administered individually by putting it on a wafer of alfalfa dehydrated meal (15 g) and waiting until the wafer has been completely consumed. The control group (10 females, 11 males) received the same amount of alfalfa wafer with 0.1 ml of coconut oil but without cannabis extract. Up to 92 days of age (for 27 d), the live weight and feed intake were individually controlled weekly. Then, the body weight gain and the feed conversion ratio were calculated. The cannabis extract was analyzed was further analyzed to evaluate the amount of bioactive compounds (cannabidiol, CBD+CBDA, and tetrahydrocannabidiol, THC + THCA).

Video Recording and Coding

Behaviors were recorded weekly between 12.00h to 15.00h, the periods were selected after preliminary observations, which characterized the time of greatest daytime activity of the animals. The footages were captured using four AP-320S C&Xanadu cameras strategically positioned in the corners of the cages. Throughout the recording periods, measures were implemented to ensure that no individuals had access to the animals, preventing any interference with their usual behavioral patterns. The recordings for each group were watched and coded by an expert observer. A second operator coded 20% of the videos, and the interobserver reliability was found to be very high for all observed behaviors, ranging from 90 to 96%.

Oxidative Stress Analysis

Blood samples were collected at slaughtering after 27 days of extract administration. Serum free oxygen radicals and total antioxidants were determined by spectrophotometer using the d-ROMs and the BAP tests (Diacron International, Grosseto, Italy) (Chiofalo et al., 2020). The OSi parameter was calculated as d-ROMs/BAP*100.

Statistical Analysis

Data were analyzed by ANOVA, using the GLM procedure of SAS (2002) according to the following model:

$$Yijk = m + Gi + Sj + G*Sij + eijk$$

Where Y is the single observation, m the general mean, G the effect of the dietary treatment, S the effect of the sex, G*S the interaction between dietary treatment and sex and e the error. P values between 0.05 and 0.10 has been considered as “tendency”. For in vivo performance, the replicate has been considered the experimental unit.

RESULTS AND DISCUSSION

Effects on behavior

As depicted in Table 1., results showed a general increase of both locomotion (moving, stretch) and exploring (sniffing, look around) behaviors, and a decrease of lying (resting position), thus suggesting an improvement of attention span with a consequent increase of activity. Indeed, the differences were significant only for stretching ($P<0.01$) whereas only a trend was observed for the other behaviors. One potential approach to improve animals’ welfare involves to providing them opportunities to express species-specific behavioral repertoires (Baumans,

2005). Rabbits, in their natural environment, exhibit significant exploratory behavior (Trocino et al., 2006).

Our findings, according with Verga et al. (2004), report an increase in locomotion (moving, stretch) and exploring (sniffing, look around). This could be possibly indicative of an improved state of comfort and well-being in animals. However, further hours of observation will be necessary to validate this hypothesis.

Table 1: Behavioral changes in rabbits treated with the CBD (Cannabidiol) extract for 27 days vs control.

	Group		Sex			P-Value		
	Control	CBD	Females	Males	RMSE	Group	Sex	Interaction
	seconds							
Gnawing	21.3	22.9	25.8	18.4	14.58	0.841	0.371	0.577
Sniffing	97.3	115.9	87.3	125.9	81.73	0.681	0.402	0.090
Moving	40.0	126.5	73.3	93.4	82.35	0.080	0.659	0.986
Lyng	7413.6	5893.3	6435.6	6871.3	1472.7	0.080	0.596	0.822
Sitting	902.0	941.7	923.5	920.2	536.07	0.894	0.991	0.641
Stretch	27.5	44.2	38.0	33.7	9.2	0.007	0.404	0.316
Look Around	12.9	37.5	24.6	25.7	24.7	0.090	0.937	0.961
Self- Grooming	1208.0	1796.1	1747.3	1256.8	675.4	0.138	0.208	0.679

Such result is consistent with those reported in the literature for several animal species. The behavioral changes detected in the treated group should be related to the beneficial activity of CBD on the central nervous system (CNS).

Effects on Oxidative Stress

In table 2, the differences of d-ROMs and BAP, as well as the OSi are reported. As seen, rabbits treated with CBD showed significant decrease of dROMs and increase of BAP, thus suggesting that CBD is able to improve the antioxidant barrier thus resulting in a reduction of total reactive oxygen metabolites in rabbits.

Table 2: Serum free oxygen radicals and total antioxidants in rabbits treated with the CBD extract for 27 days.

		Group		Sex			P value		
		Control	CBD	Females	Males	RMSE	Group	Sex	Interaction
d-ROMs	*UCarr*	104.75	69.46	91.62	82.58	14.26	<0.01	0.05	0.227
BAP	*mcmol/l*	1649.29	2530.46	2178.79	2000.95	372.45	<0.01	0.134	0.988
OSI		6.446B	2.739A	4.66	4.52	0.501	<0.01	0.387	0.197

d-Roms: Derivates of Reactive Oxigen Metabolites; BAP: Biological Antioxidant Potential; OSi: oxidative stress index

A large body of data suggest that cannabinoids have therapeutic properties, alleviating symptoms of several CNS disorders. Indeed, cannabinoids can mitigate inflammation, reduce CNS spasticity, alleviate neuropathic pain, and a body of evidence indicates that cannabinoids provide neuroprotection following injury or inflammation in the CNS (Pereira et al. 2021). In particular, a neuroprotective effect was observed for CBD, which is able to prevent hydroperoxide-induced oxidative damage much better than other antioxidant compounds. As an example, CBD has more protective effect against glutamate neurotoxicity than either ascorbic acid (ASC) or α-tocopherol (TOC), indicating that it is a potent antioxidant compound (Borges, R.S. and da Silva, A.B.F. 2017). The lower significance of behavioral changes may be due to the time and amount of cannabidiol administered, thus, further studies could improve the beneficial effects on behavior.

CONCLUSIONS

The cannabis extract tested in this trial was able to improve the antioxidant barrier, thus resulting in a reduction of free radicals. Also, the extract seemed to induce a general increase of locomotion and exploring behaviors, thus suggesting an improvement of the attention span which could have a beneficial effect on animal wellbeing. Both effects should be ascribed to cannabidiol, which represented the main active compound in the extract. Further studies could confirm the potential use of the extract to ameliorate health and wellbeing in rabbits, probably improving their quality of life.

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EFFECT OF A DIET CONTAINING CAMELINA CAKE (*CAMELINA SATIVA* (L.) CRANTZ) ON GROWTH PERFORMANCE AND PANCREATIC ENZYME ACTIVITIES IN GROWING-FATTENING RABBITS

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ABSTRACT

The study was aimed at evaluating the effect of the dietary inclusion of *Camelina sativa* (L.) Crantz cake (CS) on the growth performance and activities of pancreatic enzymes. Three experimental diets, differing in their main crude protein source, were formulated: the control diet (100 g/kg rapeseed cake), the CS5 diet (50 g/kg rapeseed cake and 50 g/kg camelina cake), and the CS10 diet (100 g/kg camelina cake). A total of 168 hybrid rabbits (line Hyplus PS 19 x Hyplus PS 40; of both sexes, 32 days old) were allocated to one of the three experimental groups (56 animals per group, 4 rabbits per cage) and were fed one of the three experimental diets for 42 days, at which time the animals were 74 days old. In addition, 30 hybrid rabbits (line Hyplus PS 19 x Hyplus PS 40; 32 days old, 830±107 g live weight, both sexes) were used in a digestibility trial (10 rabbits per diet; data in process, not shown here). At the end of the digestibility trial, all rabbits (62 days old) were slaughtered, and samples of small intestinal content were collected for the analysis of pancreatic enzyme activities and the concentration of sialic acid. A lower average daily weight gain between 32 and 74 days of age (by 2.3 g; P=0.003), resulting in a lower final live weight at 74 days of age (by 122 g; P=0.007), was detected in rabbits fed the CS10 diet than in those fed the other diets. There was also a significantly lower average daily feed intake (by 5.5 g; P=0.031) and proteolytic activity in small intestine content (by 29.8 mg azocasein/g dry matter digesta/h; P=0.032) in rabbits fed with the CS10 diet than in rabbits fed the other diets. No mortality or morbidity occurred during the whole fattening period. In conclusion, adding 5% camelina cake to the diet had no detrimental effects on the rabbits' growth performance, health or the hydrolytic activities of pancreatic enzymes. The poorer growth performance in rabbits fed a diet with 10% CS cake necessitates determining the threshold of the dietary level of CS.

Key words: Rabbit, Diet, Rapeseed cake, Camelina cake, Feed efficiency

INTRODUCTION

Camelina sativa (L.) Crantz (CS) is considered a valuable crop for human and animal nutrition due to its relatively high content of oil and fatty acid profile (Singh *et al*., 2023). Regarding rabbit feeding, there is scant information concerning the dietary inclusion of CS seeds in the literature, and to our knowledge, no information exists regarding using CS cake for rabbit feed (Heuzé *et al*., 2017). Considering the current discussion in the EU concerning the transition from a linear economy to a circular economy, with an emphasis on reducing food-feed

competition (Sandström *et al*., 2022), dietary inclusion of CS cake might extend the range of by-products used for rabbits.
Thus, the aim of the study was to gain the original results considering the effect of dietary inclusion of CS cake on the growth parameters and activities of pancreatic enzymes.

MATERIALS AND METHODS

Animals and experimental design

The CS cake was obtained by an improved camelina line selected to have a reduced content of glucosinolates (GLSs) (Alan line, CNR Milano, Italy). Three diets, differing in their main crude protein source (CP), were formulated to have the same basic mixture of ingredients and their proportions as well (Table 1). The control diet contained 100 g/kg of rapeseed cake, the CS5 diet contained 50 g/kg of rapeseed cake and 50 g/kg of camelina cake, and the CS10 diet contained camelina cake (100 g/kg).The diets were formulated to have similar levels of CP, neutral detergent fibre, acid detergent fibre, and acid detergent lignin (without acid-insoluble ash) and to comply with dietary recommendations for growing-fattening rabbits (de Blas and Mateos, 2020). There was a higher level of ether extract (EE) in the CS10 diet, due to its higher level in camelina cake, than in other diets.

Table 1: Ingredient and chemical composition (g/kg on an as-fed basis unless otherwise stated) of camelina cake, and the experimental diets

		Experimental diets[1]		
	Camelina cake	Control	CS5	CS10
Ingredient				
Alfalfa meal		280	280	280
Rapeseed cake		100	50	0
Camelina cake		0	50	100
Sugar beet pulp		70	70	70
Wheat bran		322	322	322
Oats		200	200	200
Vitamin-mineral supplement[2]		10	10	10
Dicalcium phosphate		5	5	5
Limestone		8	8	8
Salt		5	5	5
Analyzed composition				
Dry matter	903	910	915	909
Crude protein	308	154	157	154
Ether extract	203	33	37	46
Ash	46	76	76	74
Starch	21	159	158	153
Neutral detergent fibre	210	346	336	345
Acid detergent fibre	115	197	192	193
Acid detergent lignin	29	46	46	47
Gross energy (MJ/kg)	21	16.9	17.1	17.1

[1]Control: 10% rapeseed cake, CS5: 5% rapeseed cake and 5% camelina cake, CS10: 10% camelina cake; [2]Expressed per kilogram of complete diet: vitamin A (retinol), 12,000 IU; vitamin D3 (cholecalciferol), 2,000 IU; vitamin E (α-tocopherol), 50 mg; vitamin K3 (bisulfite menadione complex), 2 mg; vitamin B1 (thiamine mononitrate), 3 mg; vitamin B2 (riboflavin), 7 mg; vitamin B6 (pyridoxine), 4 mg; niacinamide, 50 mg; Ca-pantothenate, 20 mg; folic acid, 1.7 mg; biotin, 0.2 mg; vitamin B12.

A total of 168 hybrid rabbits (line Hyplus PS 19 x Hyplus PS 40; of both sexes) at the time of weaning (i.e., 32 days old) were selected from prospering litters and were randomly allocated to one of the three experimental groups (56 animals per group). After moving rabbits from the maternal sector to the fattening sector, 4 rabbits per cage were placed in wire-net cages (80 x 60 x 45 cm; 0.12 m^2 floor space for one rabbit). These rabbits were fed one of the three experimental diets until the end of the fattening period (for 42 days). Rabbit live weight (weekly period) and feed intake (daily) were recorded per cage. The average daily weight gain (ADWG), average daily feed intake (ADFI), and feed conversion ratio (FC) were calculated afterwards.

In addition, 30 hybrid rabbits (line Hyplus PS 19 x Hyplus PS 40; 32 days old, 830±107 g live weight, both sexes) were used in a digestibility trial (data in process, not shown here). Animals were individually placed in cages (50 x 40 x 42.5 cm) and randomly assigned to one of the three experimental diets (10 rabbits per diet). At the end of the digestibility trial, all rabbits (62 days old) were slaughtered, and samples of small intestinal content were collected for the analysis of pancreatic enzyme activities and the concentration of sialic acid.

Chemical Analyses

The experimental diets and camelina cake were analyzed by AOAC (1984) methods. Total amylase, protease, and lipase activities were analyzed according to Taubner *et al*. (2023) with soluble starch, azocasein, and tributyrin as substrates. The analysis of the sialic acid was performed according to Salcedo *et al*. (2011).

Statistical Analysis

Data on growth performance, enzymatic activities, and concentration of sialic acid in the intestinal content were examined by the GLM procedure in the Statistical Analysis System (2006), with type of diet as the fixed effect. For growth performance, the cage was used as the experimental unit. The individual rabbit represented the experimental unit for enzymatic activities and the concentration of sialic acid in the small intestinal content. The data in tables are presented as least squares means and root mean square error (RMSE). Differences between least squares means with $P \leq 0.05$ were accepted as statistically significant.

RESULTS AND DISCUSSION

Due to their similar nutritional composition and membership in the Brassicaceae family, camelina cake and rapeseed cake were compared in this study. The diet containing 10% rapeseed cake was regarded as the control diet because rapeseed meal is frequently utilized as a crude protein source in commercial and experimental rabbit diets (Heuzé *et al*., 2020).The results concerning the growth performance of rabbits are presented in Table 2. A lower ADWG between 32 and 74 days of age (by 2.3 g; P=0.003), resulting in a lower final live weight at 74 days of age (by 122 g; P=0.007), was detected in rabbits fed the CS10 diet than in those fed the other diets. These results are most likely related to the fact that rabbits given the CS10 diet had a lower ADFI than the rabbits in the other two groups (by 5.5 g; P=0.031).

Table 2: Growth performance of rabbits (32 to 74 days of age)[1] fed the control diet, the CS5 diet, or the CS10 diet[2]

	Experimental diets[1]				
	Control	CS5	CS10	RMSE	P-value
Live weight 32 d (g)	840	823	804	55	0.231
Live weight 74 d (g)	2617[a]	2613[a]	2493[b]	112	0.007
Average daily weight gain (g)	42.3[a]	42.6[a]	40.2[b]	1.9	0.003
Average daily feed intake (g)	139[a]	136[a]	132[b]	6	0.031
Feed conversion ratio	3.28	3.19	3.29	0.11	0.056
Morbidity	0	0	0	-	-
Mortality	0	0	0	-	-

[1]56 rabbits per group (4 rabbits/cage); [2]Control: 10% rapeseed cake, CS5: 5% rapeseed cake and 5% camelina cake, CS10: 10% camelina cake; Means with different letters on the same row differ significantly.

In general, the feed efficiency of protein sources is associated with their impact on ileal digestibility (Gutiérrez *et al*., 2003). In this respect, there was a lower proteolytic activity in small intestine content (by 29.8 mg azocasein/g dry matter digesta/h; P=0.032) in rabbits fed with the CS10 diet than in rabbits fed with the control or CS5 diet (Table 3).

This finding is probably associated with the antinutritional properties (trypsin inhibitors, condensed tannins, etc.) of CS varieties (Pozzo *et al*., 2023; Singh *et al*., 2023). There is no information in the literature about the dietary impact of camelina cake on the growth

performance of rabbits, making it impossible to compare results. It was observed in poultry, however, that growth performance can be affected by increasing the dietary level of camelina cake above threshold (Singh *et al*., 2023). No mortality or morbidity occurred during the whole fattening period.

Table 3: Hydrolytic activities of pancreatic enzymes and concentration of sialic acid in the small intestinal content of rabbits[1] fed the control diet, the CS5 diet, or the CS10 diet[2]

	Experimental diets[1]				
	Control	CS5	CS10	RMSE	P-value
Amylolytic activity[3]	19.9	19.8	19.5	5.8	0.989
Proteolytic activity[4]	127.2[a]	121.9[a]	94.8[b]	27.8	0.032
Lipolytic activity[5]	8.24	8.15	9.07	2.0	0.528
Sialic acid[6]	1.29	1.35	0.95	0.5	0.181

[1]10 rabbits per group at 62 days of age; [2]Control: 10% rapeseed cake, CS5 5%: rapeseed cake and 5% camelina cake, CS10: 10% camelina cake; [3]mg sugar/g dry matter digesta/h; [4]mg azocasein/g dry matter digesta/h; [5]mmol butyrate/g dry matter digesta/h; [6]µg sialic acid/g dry matter sample. Means with different letters on the same row differ significantly.

CONCLUSIONS

The addition of 5% camelina cake to the diet had no detrimental effects on the health status, growth performance, or hydrolytic activities of pancreatic enzymes. These results encourage us to consider camelina cake as a new by-product to extend the range of by-products used for rabbit diets. Even if the rabbits' growth and, in particular, their health condition were satisfactory, the lower growth performance in the case of adding 10% of CS to the diet necessitates determining the threshold of the dietary level of camelina cake.

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EFFECTS OF *ASTRAGALUS* POLYSACCHARIDE ON GROWTH PERFORMANCE, ANTIOXIDANT STATUS AND ANTI-RHDV ANTIBODY TITERS OF WEANED RABBITS

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ABSTRACT

This study aimed to evaluate the effects of *Astragalus* polysaccharide (APS) supplementation in dietary on the growth performance, antioxidant status and anti-RHDV antibody titers of weaned rabbits. A total of 120 New Zealand White rabbits, 35d weaned, healthy and similarly weighted, were randomly divided into 3 groups (40 rabbits per group). Rabbits in the control group (APS 0) received a basal diet without additives, while the other two groups (APS100, APS 200) received the same feed supplemented with APS at 100 and 200 mg/kg diet, respectively. Results showed that APS improved the growth performance, affected protein metabolism of weaned rabbits. APS 200 group showed a significant increase in ADG ($P < 0.05$) and final weight. The serum total protein (TP), globulin (GLB), and alanine aminotransferase (ALT) activity were significantly higher than those in control group ($P < 0.05$). The GSH-Px activity was significantly higher than the other two groups ($P < 0.05$), and there was also a certain degree of increase in SOD activity ($P>0.05$). The anti-RHDV antibody titers in APS increased by 11.1% ($P > 0.05$). In conclusion, adding APS to the diet is beneficial for promoting the health of weaned rabbits, with the optimal effect observed at a supplementation level of 200 mg/kg.

Key words: *Astragalus* polysaccharide; weaned rabbits; antioxidant status; anti-RHDV antibody titers

INTRODUCTION

Astragalus polysaccharide (APS) is an active polysaccharide extracted from *Astragalus*, which has biological functions, such as immune regulation, antioxidant, anti-inflammatory and antiviral effects (Zhou, 2017). With the in-depth research on APS, it has attracted much attention from researchers as a substitute for antibiotics. Recent studies have shown that APS can promote the development of immune organs, enhance immune cell function, improve the growth performance and product quality of piglets and poultry species (Yuan et al., 2006; Liu Xu, 2016). Now, research has mainly focused on piglets, poultry, etc., with less research on young rabbits. To our knowledge, there are no reports on adding APS to the diets of weaned rabbits. Hence, this experiment aimed to study the effects of different levels of APS on the growth performance, antioxidant power, and anti-RHDV antibody titers in weaned rabbits. We hypothesized that APS supplementation would enhance growth, improve blood biochemistry and antioxidant status, and alleviate the weaning stress of the rabbits.

MATERIALS AND METHODS

Animals and experimental design

The trial was carried out in the experimental farm of the Jiangsu Academy of Agricultural Sciences, Nanjing, China. APS (60% effective) was purchased from Shengtai'er ,Beijing, China.

A total of 120 New Zealand White rabbits, 35d weaned, healthy and similarly weighted, were randomly divided into 3 groups (40 rabbits per group) with 5 replicates of 8 rabbits per each. Rabbits in the control group (APS 0) received a basal diet without additives, while the other

two groups(APS100, APS 200) received the same feed supplemented with APS at 100,200mg/kg diet, respectively. The trial lasted for 30 days after an adjustment period of 5 days.

Diets and rabbit rearing

The basal diet was prepared following the recommendation of NRC (1977) and the Agricultural Industry Standard of the People's Republic of China (NY/T 4049-2021, 2021). The analysis of the ingredients was performed according to the Standardization Administration of the People's Republic of China (GB/T 18868-2002,2022) (Table 1).

Rabbits were housed in galvanized wire battery cages (60 × 45 × 40 cm) in a well-ventilated rabbitry with freshwater and feed provided *ad libitum*. and a standard pelleted ration was provided twice daily at 8am and 16pm. Rabbit cages were regularly cleaned and disinfected. Urine and feces dropped beneath the batteries were removed every morning.

Table 1 Composition and nutrient levels of the diet

Ingredients	rate(%)	Nutrient levels	
Corn	24.0	DE (MJ/kg)[2]	10.28
Wheat bran	18.9	CP(%)	16.25
Soybean meal	13.8	CF(%)	12.57
Salt	0.3	EE(%)	3.48
Chrysanthemum	38	Ca(%)	1.62
Premix[1]	5.0	AP(%)	0.48
		Lys(%)	0.93
Total	100	Met+Cys(%)	0.51

Growth performance

At the start of the 40d, the animals were weighed individually until the end of the experiment (70 d of age), and average daily gain (ADG) was estimated.

Biochemical and antioxidant status

At the end of the trial, blood samples were collected from the lateral ear vein, 8 rabbits with similar body weights in each group. Serum was separated and stored at -20°C until analysis.Total protein (TP), albumin (ALB),globulin (GLB),urea nitrogen (BUN), alkaline phosphatase (ALP),alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA) were evaluated using commercial kits (kits obtained from Jiancheng, Nanjing,China).

Hemagglutination Test

The determination of the anti-RHDV antibody titers was entrusted to the Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Nanjing China.

Statistical analysis

All data were statistically analyzed using SPSS 17.0, using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test to examine the difference among different treatments, at significance level of $P \leq 0.05$. The data were expressed as means ± SEM.

RESULTS AND DISCUSSION

Effect of astragalus polysaccharide on the growth performance

APS can improve the growth performance of weaned rabbits. APS 200 group showed a significant increase in ADG ($P < 0.05$) and final weight, but there was no statistically significant difference ($P > 0.05$). This is consistent with the results of Tang Jiaoyu et al. (2014), where adding 200 mg/kg APS to the diet of weaned rabbits showed the best growth performance. Some studies have also found that adding 400 and 1600 mg/kg APS to the diet of hare rabbits can significantly increase their feed intake and average daily gain. This may be related to the

purity of APS, the breed of experimental rabbits, and different physiological stages, which need further research.

Table 2 Effect of astragalus polysaccharide on the growth performance

Item	Control group	APS100 Group	APS200 Group
Initial weight/g	982.0±25.5	987.6±20.6	987.3±18.4
Final weight/g	1753.5±66.7	1785.6±48.2	1820.3±36.6
Average daily gain/g.d^{-1}	24.3±1.05^{b}	25.1±0.84^{ab}	26.8±0.58^{a}

Note: In the same row, values with no letter or the same letter superscripts mean no significant difference ($P>$ 0.05), while with different small letter superscripts mean significant difference (P<0.05). The same as below.

Effect of astragalus polysaccharide on the biochemical indexes

APS can affect protein metabolism in the body. TP, GLB, and ALT levels in APS200 group were significantly higher than those without additives (P < 0.05), while BUN content in APS100 group was significantly lower (P < 0.05). Blood biochemical parameters are the main indicators reflecting the metabolism of animal bodies, among which TP, BUN, and antioxidant indices can reflect the nutritional metabolism and health status of animal bodies. This is similar to the results of Zhao Tianzhang et al. (2018).

Table 3 Effect of astragalus polysaccharide on the biochemical indexes

Item	Control group	APS100 Group	APS200 Group
SOD activity (U/mL)	319.9±14.6	322.3±22.0	356.5±16.1
GSH-PX activity (U/mL)	383.6±39.5	390.3±38.2	505.1±27.9
MDA (nmol/mL)	4.12±0.27^{a}	3.15±0.42^{b}	4.16±0.15^{a}

Effect of astragalus polysaccharide on the antioxidant status

APS can improve the antioxidant capacity of the body. The results showed that the GSH-Px activity in APS 200 group was significantly higher than the other two groups (P < 0.05), and there was also a certain degree of increase in SOD activity, but it was not statistically significant (P>0.05). Antioxidant indices were measured in this experiment, and adding 200 mg/kg APS to the diet significantly increased the activity of GSH-PX, and there was also a certain degree of increase in SOD activity, indicating that adding an appropriate amount of APS can improve the antioxidant capacity of the body, thereby improving the health status of weaned rabbits. Xia Mingming (2023) found that 200 mg/kg APS significantly improved the total antioxidant capacity of weaned rabbits, but GSH-Px and SOD were not detected

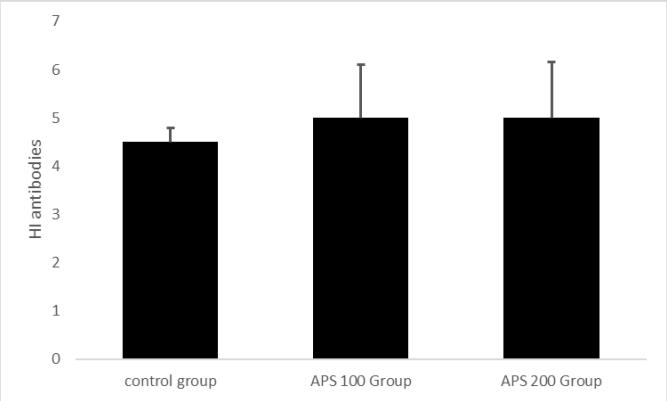
Table 4 Effect of astragalus polysaccharide on the antioxidant status

Item	Control group	APS100 Group	APS200 Group
TP (g/L)	64.5±3.25^{b}	58.7±1.85^{b}	78.3±1.86^{a}
ALB (g/L)	25.9±0.53^{ab}	24.8±1.15^{b}	28.7±1.08^{a}
GLB (g/L)	38.6±3.25^{b}	34.0±1.35^{b}	49.9±1.94^{a}
BUN (mmol/L)	5.54±0.44^{a}	4.17±0.37^{b}	4.72±0.33^{ab}
ALP activity (King unit /100 mL)	8.88±1.09	13.3±2.98	11.8±1.91
ALT activity (U/L)	4.69±0.56^{b}	3.47±0.12^{b}	6.36±0.63^{a}
AST activity (U/L)	1.22±0.20	1.24±0.16	1.77±0.35

Effect of astragalus polysaccharide on the anti-RHDV antibody

The anti-RHDV antibody titers in APS increased by 11.1%, but there was no significant difference (P > 0.05). APS could increase the antibody titer, enhance the protection of vaccine immunity against young rabbits, and reduce the occurrence of epidemics.

Figure 1 Effect of astragalus polysaccharide on the anti-RHDV antibody



CONCLUSION

APS supplementation in rabbits significantly enhanced antioxidant power, improved protein metabolism, immune function and health status of weaned rabbits, thereby encouraging rabbit growth. In this experiment, a dose of 200mg/kg had the best effect.

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EFFECTS OF YEAST PEPTIDE ON ANTIOXIDANT CAPACITY OF MEAT RABBITS

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ABSTRACT

This study endeavors to investigated the effect of yeast peptides on antioxidant capacity in meat rabbits. A total of 200 Hyla meat rabbits (35-day-old, mean weight: 1.17±0.04 kg) were randomly divided into 5 treatment groups (8 replicates in each treatment group and 5 rabbits per group in each replicate) and fed the basal diet added with 0 (Control group), 100, 200, and 300 mg/kg yeast peptides (Test group I, II and III) or 20 mg/kg engramycin and 150 mg/kg gentamycin (Antibiotic group) for 35 d. The results showed that, compared to control group, all test groups (I, II and III) showed significantly higher concentration of GSH-Px in liver, and also in ileum in the case of test groups I and II (P<0.05). Compared with the control group, the concentration of MDA in the liver was significantly reduced in all test groups (P<0.05). These results revealed that diets supplemented with yeast peptides exerted a beneficial effect on antioxidant function of meat rabbit.

Key words: Yeast peptides, Meat rabbit, Antioxidant capacity

INTRODUCTION

Antibiotics have always played an important role in the prevention and treatment of livestock and poultry diseases. But with the indiscriminate abuse of antibiotics, pathogen-acquired resistance and residues in animals has emerged as one of the world's greatest health threats (Ramanan *et al.*, 2013). Subsequently, antibiotics have been widely prohibited in animal husbandry in many countries (Martin *et al.*, 2015). In 2020, China had also banned feed manufacturers from adding antibiotics to feed processing, and the development of feed additives that can replace antibiotics has become a hot research direction of animal nutrition and feed industry.

Yeast peptides, produced by induced fermentation of *Saccharomyces cerevisiae*, are antimicrobial peptides with diverse biological activities, encompassing anti-stress, anti-inflammatory, and growth promotion (Hu *et al*., 2017; Ding *et al*., 2020). These peptides are produced microbially and have found extensive application in livestock and poultry farming. However, there has been no report on the effect of yeast peptides on meat rabbits. This study aims to investigate the effect of yeast peptides on antioxidant capacity in meat rabbits.

MATERIALS AND METHODS

Animals and experimental design

A total of two hundred healthy 35-day-old Hyla line meat rabbits (mean weight: 1.17±0.04 kg, equally distributed by gender) were randomly divided into five treatment groups with 8 replicates in each treatment group and 5 rabbits per group in each replicate. The basal feed used in this study was produced on the basis of "NY/T 4049-2021 Nutrient Requirements of Meat Rabbits" (China). The control group was fed the basal diet, the antibiotic group was fed the basal diet added with 20 mg/kg engramycin and 150 mg/kg gentamycin to the basal diet, and the test groups I, II and III were fed with the basal diet supplemented with 100, 200 and 300 mg/kg yeast peptides, respectively. An adaptation to experimental diet lasted for 7 days

before experimental feeding. The experiment lasted for 28 days. All experimental rabbits were housed in a closed rabbit shed, with one rabbit per cage. The experimental rabbits had access to water and food ad libitum.

On day 28 of the trial period, one rabbit per replicate and group was selected, and the rabbits were euthanized with sodium pentobarbital, the liver and ileum samples were collected in sterile sampling tubes. After that, anti-oxidation index, including T-AOC, SOD, GSH-Px and MDA in liver and ileum, were measured using individual ELISA kits by means of the manufacturer instructions.

Statistical analysis

All experimental data were analyzed using one-way analysis of variance (ANOVA) of SPSS (version 26.0), and then the Duncan multiple-comparison test was performed. Results were expressed as mean and SEM, $P < 0.05$ was considered significant , while a trend was denoted when $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

As show in Table 1, compared with the control group, the concentration of GSH-Px in the liver was significantly increased in all test groups and antibiotic group (P<0.05); the concentration of T-AOC in the liver tended to increase in test group III and antibiotic group (P<0.10). The concentration of MDA in the liver was significantly reduced in all test groups (P<0.05). However, the concentration of SOD in the liver was significantly reduced in all test groups and antibiotic group (P<0.05)。

Table 1. Effect of yeast peptides on the antioxidant capacity in the liver of meat rabbits

Items	Control group	Antibiotic group	Test group Ⅰ	Test group Ⅱ	Test group Ⅲ	*P*-value
T-AOC/(mmol/L)	14.16±0.41	15.45±0.87	14.70±0.78	14.46±0.16	17.00±0.78	0.053
SOD/(U/ml)	165.81±3.54^{a}	151.72±1.85^{b}	150.44±6.21^{b}	152.42±2.92^{b}	144.69±3.93^{b}	0.025
GSH-Px/(U/L)	87.97±4.18^{e}	201.71±7.14^{a}	164.95±6.5^{b}	148.04±5.47^{c}	117.48±3.03^{d}	＜0.001
MDA/(nmol/L)	9.94±0.4^{a}	9.44±0.43^{ab}	7.05±0.22^{c}	8.03±0.98^{bc}	7.96±0.65^{bc}	0.022

In the same row, values with different letter superscripts mean significant difference (P<0.05), while with the same or no letter superscripts mean no significant difference (P>0.05).

As show in Table 2, compared with the control group, the concentration of GSH-Px in the ileum was significantly higher in the antibiotic group, test group I and test group II (P<0.05); the concentration of T-AOC in the ileum tended to increase in all test groups (P<0.10). There were no significant differences in the concentration of SOD and MDA in the ileum among all groups (P>0.05).

Table 2. Effect of yeast peptides on the antioxidant capacity in the ileum of meat rabbits

Items	Control group	Antibiotic group	Test group Ⅰ	Test group Ⅱ	Test group Ⅲ	*P*-value
T-AOC/(mmol/L)	10.81±1.09	10.86±1.83	13.50±0.18	14.21±1.41	14.62±0.80	0.094
SOD/(U/ml)	105.38±10.09	129.00±18.55	113.90±19.29	108.53±6.36	124.13±24.94	0.822
GSH-Px/(U/L)	66.20±6.90^{d}	99.79±3.59^{a}	97.26±4.49^{ab}	82.21±4.45^{bc}	72.50±5.00^{cd}	0.001
MDA/(nmol/L)	6.65±0.90	6.94±0.94	6.74±0.45	7.18±0.83	5.51±0.56	0.591

In the same row, values with different letter superscripts mean significant difference (P<0.05), while with the same or no letter superscripts mean no significant difference (P>0.05).

Reactive oxygen species (ROS) are a series of molecules produced continuously by oxygen consumption and play an important role in immune cell signaling and homeostasis. But excessive production of ROS can lead to oxidative stress, which can lead to chronic diseases and aging, so the homeostasis of ROS in the body is mainly balanced by a complex antioxidant system (Ma, 2014). Antioxidant enzymes are capable of scavenging free radicals and producing non-toxic compounds. GSH-Px is an enzyme that protects from the toxicity of hydroperoxides whereas MDA is one of the final products of peroxidation and is used as an indicator of oxidative stress. It have been reported that antimicrobial peptides can increase the concentration of antioxidant enzymes in zebrafish and broiler chickens, while reducing the concentration of MDA in animals and enhancing antioxidant capacity (Rashidian *et al*., 2021; Sholikin *et al*., 2021). Similar results were obtained in the study, yeast peptides significantly increased the concentration of GSH-Px in liver and ileum and decreased the concentration of MDA in liver. This may be due to the fact that peptides rich in sulfur and aromatic amino acids, such as Cys, Met, Trp, Tyr and Phe, then these peptides often exhibit greater antioxidant capacity (Murase *et al*., 2002; Stdal *et al*., 1999).

CONCLUSIONS

Result from our study indicate that diets supplemented with yeast peptides exerted a beneficial effect on antioxidant function of meat rabbit. The effect of yeast peptides on improving the antioxidant properties of rabbits is comparable to that of antibiotics.

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PATHOLOGY & HYGIENE

STUDY ON NOVEL VACCINES AND ADJUVANTS FOR RABBIT INFECTIOUS DISEASES

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ABSTRACT

Epidemic diseases have seriously damaged the development of the global rabbit industry. Vaccination plays a vital role in improving the health and welfare of livestock and preventing animal-to-human transmission, thereby constituting a major public health strategy. There is continued demand for effective and safe adjuvants capable of enhancing antigen-specific responses to a target pathogen of rabbits. Pathogens such as Rabbit Hemorrhagic Disease Virus type 2 (RHDV2), *Pasteurella multocida* and *Eimeria* sp. coccidium are lethal and highly contagious for rabbits worldwide. Scientists have been working on screening for immunogenic proteins and designing adjuvant systems for safer and more effective vaccines against these highly prevalent pathogens. With the rapid development of genetic engineering technology and biotechnology, recombinant vaccines, genetic engineering vector vaccines, nucleic acid vaccines and other new vaccines have been gradually developed. Novel adjuvants such as new oil adjuvants, herbal medicine adjuvants, cytokine adjuvants, nano-adjuvants, and polysaccharide adjuvants have been applied in the new vaccines to enhance their immunogenicity of new vaccines. Researchers are committed to developing new vaccines with broad spectrum and high efficiency. This review will briefly overview the current research on vaccines and adjuvants against viral, bacterial and parasitic pathogens prevalent in rabbits.

Key words: Vaccine; Adjuvant; Rabbit; Infectious diseases; Prevention

INTRODUCTION

Rabbit infectious diseases have become the technical bottleneck of the development of the global rabbit industry. Several infectious organisms have been identified as the cause of emerging diseases in rabbits. Vaccination is one of the essential strategies for preventing and controlling rabbit infectious diseases. There is continued demand for effective and safe adjuvants capable of enhancing antigen-specific responses to a target pathogen of rabbits. In recent years, significant progress has been made in researching novel vaccines and adjuvants for preventing and controlling domestic rabbit infectious diseases. With the rapid development of genetic engineering technology and biotechnology, recombinant vaccines, genetic engineering vector vaccines, nucleic acid vaccines and other new vaccines have been researched and developed. At the same time, novel adjuvants such as new oil adjuvants, herbal medicine adjuvants, cytokine adjuvants, nano-adjuvants, and polysaccharide adjuvants have been applied in new rabbit vaccines to enhance their immunogenicity. This review will briefly overview the current research on vaccines and adjuvants against viral, bacterial and parasitic pathogens prevalent in rabbits.

VACCINES AND ADJUVANTS AGAINST VIRAL PATHOGEN

At present, one of the major rabbit virus diseases in the world is rabbit hemorrhagic disease (RHD), which is caused by rabbit hemorrhagic disease viruses, e.g. RHDV1/RHDVa and the more recent RHDV type 2 (RHDV2). RHDVs belong to the Lagovirus genus within the Caliciviridae family. According to the proposed taxonomy based on the genomic organisation(Jacques et al., 2017), RHDV2 is provisionally classified as *Lagovirus GI.2*. It is thought to be a newly emerged serotype related to RHDV1 (*Lagovirus GI.1*) both of which are lethal and highly contagious in rabbits. Since RHDV2 was first detected in France in

2010, it has replaced RHDV1 by spreading in many parts of the world, including Europe, Asia, Africa, Oceania, and America (Neimanis et al., 2018, Toh et al., 2022, Hu et al., 2021, Asin et al., 2021, Rouco et al., 2019, Ambagala et al., 2021). To date, vaccination remains the most effective method for the prevention of RHD. RHDV2 is antigenically distinct from RHDV1, so there is no immunological cross-protection between RHDV1 and RHDV2 (O'Toole et al., 2022, Calvete et al., 2018). As a result, research on vaccines and adjuvants against RHDV2 is a hot spot in rabbit infectious disease prevention and control.

Inactivated vaccines

Previously, commercially available vaccines were inactivated forms obtained from the livers of experimentally infected rabbits since RHDV is not known to replicate in any cell lines (Guo et al., 2016, Zheng et al., 2016). The inactivated vaccines can effectively induce antibody production, and maternally derived antibodies can be transmitted to young susceptible rabbit kits through transplacental (Baratelli et al., 2020). However, producing the tissue-inactivated vaccine is expensive and does not meet animal welfare requirements. It also raises potential safety concerns owing to the possible risk of disseminating such a highly contagious virus from vaccine manufacturing facilities. It is, therefore, necessary to develop new vaccines and adjuvants.

To make the inactivated vaccine more effective, RHDV-inactivated vaccines in conjunction with Taishan *Robinia Pseudoacacia* polysaccharides (TRPPS) were administered to rabbits. The study showed that TRPPS-supplemented vaccines could enhance the immune function which was comparable to those of propolis. In addition, the TRPPS-supplemented RHDV-inactivated vaccines could significantly improve the survival rates of the immunised rabbits against RHDV infection (Yang et al., 2017).

Subunit vaccines

Novel rabbit vaccines mainly include genetically engineered subunit vaccines and nucleic acid vaccines. VP60 is the major immunogenic protein that induces the production of virus-neutralizing antibodies in infected rabbits. The research and development of novel vaccines are based on VP60. Subunit vaccines have the advantages of low production costs and good safety.

RHDV VP60 proteins have been expressed in *E. coli* using a prokaryotic expression vector containing a SUMO tag to promote soluble expression. After cleavage of the SUMO tag by proteases, the virus-like particles (VLPs) of RHDV undergo self-assembly, which was shown to demonstrate excellent immunogenicity (Guo et al., 2016).

A recombinant *Lactobacillus casei* (*L. casei*) expressing the major structural capsid protein VP60(VP1)-eGFP fusion protein of RHDV was developed. It was proved to be an efficient and safe oral vaccine that could induce strong mucosal and systemic immune responses (Wang et al., 2019). Changjin Liu constructed a TK-deactivated recombinant swinepox virus (rSWPV) expressing the VP60 protein and showed that it protected rabbits from lethal RHDV infection. No histopathological changes or antigenic staining were found (Liu et al., 2022).

Manman Yin expressed VLPs by the yeast of *Pichia pastori*. The gene of RHDV VP60 was cloned into the pPIC3.5k vector; then, the resulting recombinant plasmid was transformed into *P. pastoris* KM71 strain to construct the recombinant yeasts. This study showed an ideal immune protection effect (Yin and Lou, 2019).

The baculovirus vector expression system has been widely used for the expression of proteins for more than 30 years. Mainly subunit vaccines manufactured at large scale by this technology, denominated CrisBio (Escribano et al., 2020). Inactivated baculovirus-derived recombinant subunit vaccine against RHDV2 could effectively induce humoral immunity and provide complete protection against lethal infection in rabbits with no significant effect on the health status of rabbits (Li et al., 2023b, Bosco-Lauth et al., 2022). VLPs self-assembled from VP60 protein expressed by the baculovirus-silkworm pupae system could confer durable protection against RHD (Zheng et al., 2016). Since there is no cross-protection between

RHDV1 and RHDV2, a multivalent RHDV vaccine is preferred over monovalent vaccines to protect domestic rabbits. A bivalent VLPs vaccine was developed by constructing a recombinant baculovirus (Bac-classic RHDV VP60-RHDV2 VP60) containing the VP60 genes of RHDV1 and RHDV2 (Qi et al., 2020), which could protect rabbits from RHDV1 and RHDV2 infection. Co-infecting the insect pupae with two baculovirus vectors expressing the RHDV GI.1- and RHDV GI.2-derived VP60 proteins, a chimeric VLPs incorporating both proteins was obtained. The resulted bivalent vaccine was proved to be able to protect against a lethal challenge infection with the two RHDV serotypes (Dalton et al., 2021). In China, two subunit vaccines against RHDV2 based on the baculovirus expression system have been officially approved for clinical trials in 2022, four years after the RHDV1 vaccine based on the baculovirus vector got the national certificate.

Subunit vaccines also rely on the activity of adjuvants to enhance immunogenicity and generate sufficiently robust immune responses against the target pathogen. Most of the commercially available subunit vaccines are based on the use of traditional adjuvants (oil and alum hydroxide). Recently, a sulfated lactosyl archaeol (SLA) archaeosome-adjuvanted RHDV subunit vaccine was constructed by incorporating SLA with recombinant RHDV2 VP60. The study demonstrated that SLA enhanced antigen-specific antibody titers and cellular responses in rabbits significantly higher than those immunized with antigen alone (Akache et al., 2023).

DNA vaccines

Administration of DNA vaccines, in association with appropriate adjuvants, to strengthen the immune response presents a novel optimisation strategy to generate more efficient vaccines. Deng and his co-workers (Deng et al., 2019) constructed a eukaryotic vector expressing a fusion gene based on IL-2 of rabbits and VP60 of RHDV. The recombinant DNA vaccine SL7207-pVAX1-IL2-VP60 induced a higher level of antibodies than the vaccine SL7207-pVAX1-VP60 and inactivated vaccines significantly. Furthermore, the fusion gene vaccine provided higher protection (93.33%) after viral challenge than the immunisation with the single gene (SL7207-pVAX1-VP60). Moreover, the study also demonstrated that IL-2 enhanced both humoral and cellular responses, highlighting the utility of rabbit IL-2 as an effective adjuvant.

VACCINES AND ADJUVANTS AGAINST BACTERIAL PATHOGEN

Pasteurellosis is an economically important disease of rabbits worldwide caused by *Pasteurella multocida,* and vaccination is an effective tool to control disease outbreaks. Various efforts have been made to develop different recombinant or synthetic immunogenic antigens using different adjuvant formulations. A novel phage lysate was prepared from isolated lytic phage, significantly increasing humoral immunity and protecting the rabbits against P. multocida challenges (Durairajan et al., 2021).

Bordetella bronchiseptica causes infections in the respiratory tract of rabbits and acts as a precursor for secondary infection with *P. multocida*. Vaccination is one of the most effective methods to control and prevent *B. bronchiseptica* infection in rabbits. The outer membrane porin protein (OMPP) of *Bordetella bronchiseptica* is an important adhesion factor and protective immunogen. Soluble rOMPP was successfully expressed in *Escherichia coli*, and the purified recombinant protein was mixed with the ISA 201 VG adjuvant to prepare a subunit vaccine for *B. bronchiseptica*. Rabbits were immunised with the rOMPP subunit vaccine and then infected with the virulent *B. bronchiseptica* strain QDBb01. Rabbits immunised with the subunit vaccine were completely protected compared to the control group, and the protective effect was better than that of the inactivated whole-cell vaccine. Moreover, analysis of the immunisation duration showed that the rOMPP subunit vaccine provided immune protection for at least four months after the second immunisation (Zhang et al., 2019).

The outer membrane vesicle (OMV) is a bilayer membrane vesicle with a diameter of about 20-300 nm, secreted during Gram-negative bacteria's growth process (Zhuang et al., 2021,

Gan et al., 2021). OMV carries a large number of pathogen-related molecular patterns (PAMPs), such as bioactive proteins, lipopolysaccharide (LPS), nucleic acids and peptidoglycan (PG), which make OMV a potent antigen candidate (Maiti et al., 2021, Li et al., 2022). Combining OMV with a nanoparticle core could maintain OMV's stability better (Wu et al., 2022). It was reported that chitosan nanoparticles adjuvant could enhance the immune efficacy of rabbit *B. bronchiseptica* outer membrane vesicles (Li et al., 2023a).

Xiao prepared a water-in-oil emulsion by combining Rg1 (a ginsenoside) with white oil (mineral oil) and explored the immune effect of this Rg1-oil formulation on inactive rabbit *B. bronchiseptica* vaccine. The result showed that Rg1 (100 μg) plus oil significantly improved the humoral and cellular immune effect of the *B. bronchiseptica* vaccine (Xiao et al., 2021). Cui developed a vegetable oil adjuvant (E515) containing soybean oil, vitamin E and ginseng saponins. E515 adjuvant was proven to be safe, and it could effectively elicit antigen-specific immune responses and provide reasonable protection against *B. bronchiseptica* infection in rabbits (Cui et al., 2022).

Epsilon toxin (Etx) produced by *Clostridium perfringens* type D is responsible for the pathogenesis of enterotoxaemia in animals. An artificial chimeric epitope construct (CEC) was prepared by joining tandem repeats of a peptide containing amino acids (aa) 134-145 of Etx B-cell epitope and universal T-cell epitopes. It was found that CEC yielded high titers of neutralising antibodies (≥ 1.035 IU/ml) in immunised mice and rabbits, which indicated the potential ability of CEC as a vaccine against *C. perfringens* (Singh et al., 2020).

Regarding market demand, the application prospect of monovalent bacterial vaccines is minimal, so more emphasis is placed on developing multivalent vaccines. Since RHD and *P. multocida* infection, the two most harmful diseases of rabbits, are both characterised by high morbidity and mortality, many researchers have turned their attention to the development of multivalent vaccines. A bivalent vaccine containing a minimum of 2^8HAU inactivated RHDV & 10^8 CFU inactivated *P. multocida* was prepared and emulsified with Montanide™ ISA70 oil adjuvant. The study reveals that the bivalent vaccine candidate provided lower stress to rabbits by decreasing the required manipulation by 50% and induced better protection and higher antibody response for both antigens (El-Jakee et al., 2020). A recombinant strain of Pasteurella C51-17 was constructed with a VP60 gene inserted. Although the idea is innovative, the efficiency of its expression needs to be improved, and its immunogenicity needs to be further confirmed (Zhou, 2018).

Another new idea was based on the analysis of the epitopes of VP60 of RHDV (SZ/2016) and OmpH/OmpA of *P. multocida* (SZ/2016). The 1~102 amino acid region of VP60 protein, 121~216 amino acid region of OmpH protein and 139~231 amino acid region of OmpA protein were selected and connected by a flexible linker. The resulting sequence has a total length of 978bp, which encodes 323 amino acids. The result showed that recombinant protein could simultaneously induce rabbits to produce neutralising antibodies against these two pathogens (Lin, 2018). Zhu and his co-worker selected a different epitope of *P. multocida* protective antigen PlpE. The gene fragments encoding 26-45 and 51-95 amino acid sequences of PlpE were ligated to the 5 '-and 3'-terminus of the VP60 gene, and the recombinant protein VP60-PlpE chimeric with PlpE epitopes showed a good immune protective effect against both pathogens (Zhu et al., 2021).

In a study on a novel trivalent vaccine, VP60 protein was combined with *P. multocida* and *C. perfringens* inactivated antigen. Results showed that the trivalent vaccine could confer 210-day protection (RHDV 5/5, P. multocida 5/5, C. perfringens 4/5) after challenge with no adverse reactions (Han et al., 2021).

The effective immune protection for rabbits against α, β, and ε exotoxins of *C. perfringens* is provided by an oral tetravalent bait probiotic vaccine delivering α, ε, β1, and β2 toxoids of *C. perfringens*. Oral administration of the probiotic vaccine can effectively elicit significant levels of antigen-specific mucosal sIgA and sera IgG antibodies with exotoxin-neutralizing activity.

The protection rate was 80% after challenging rabbits with a combination of *C*. *perfringens* (toxinotypes A, C, and D) and an exotoxin mixture (Bai et al., 2020).

The commercially available vaccines in China are mainly multivalent, such as RHD-*P. multocida* inactivated vaccine (LQ strain+C51-17 strain), propolis adjuvanted RHD-*P. multocida* inactivated vaccine (YT strain+JN strain), RHD-*P. multocida* inactivated vaccine (CD85-2 strain+C51-17 strain), RHD-*P. multocida*-*C*. *perfringens* (type A) inactivated vaccine (VP60+SC0512 strain+ LY strain), etc.

VACCINES AND ADJUVANTS AGAINST PARASITIC PATHOGEN

Coccidia are the major parasitic pathogens in rabbits, and they can be responsible for high morbidity and mortality [illegible]. Eimeria intestinalis is one of the most pathogenic rabbit coccidia species, causing severe intestinal damage and an increased risk of secondary infection from opportunistic pathogens, which results in severe economic losses in rabbit farms (Xiao et al., 2023). A vaccine against *Eimeria* with perfect safety and effectiveness seems necessary to face this parasitosis.

Apical membrane antigen 1 (AMA1) and immune mapped protein 1 (IMP1), as surface proteins, are associated with host invasion and might have the potential as candidate vaccine antigens (Jenkins et al., 2015, Santos et al., 2011). A recombinant IMP1 (rEiIMP1) and AMA1 (rEiAMA1) vaccine expressed by *E. coli* BL21, with Quil-A as the adjuvant, showed ideal immunoreactivity and immunoprotective effects (Xiao et al., 2023). Another two recombinant proteins, E. intestinalis 14-3-3 and rEi-GRA10, obtained via prokaryotic expression, were investigated. The immunoadjuvant was also Quil-A. Both the proteins could effectively induce humoral immunity and were protective against *E. intestinalis* infection in rabbits, with r*Ei*-14-3-3 showing a better protective effect (Xiong et al., 2023).

E. stiedae parasites in the liver invade the bile duct epithelial cells and cause severe liver coccidiosis. The prokaryotic expression of *E. stiedae* ubiquitin-conjugating enzyme (*rEs*-UCE), elongation factor G (*rEs*-EFG), and dense granule protein (*rEs*-DG32) recombinant proteins was carried out, and the result showed that immunisation of *rEs*-UCE effectively induced cellular and humoral immune responses in rabbits (Bai, 2022). Another five genes, *Es*-GAPDH, *Es*-HSP40, *Es*-HSP60, *Es*-HSP70 and *Es*-ROP38, were screened from *E. stiedae* transcriptome data. And five recombinant proteins were obtained by prokaryotic expression. Quil-A saponin was used as an adjuvant. The study suggested r*Es*-GAPDH can be used as a candidate antigen for *E. stiedae* subunit vaccine (Zheng, 2022).

E. magna is another common pathogen in rabbits, which results in lethargy, weight loss, diarrhoea, and even death in severe cases after infection. According to Chen Hao's study, the rEmMIC2 and rEmMIC3 proteins expressed in the *E.coli* system could induce cellular immunity, reduce oocyst output, weight loss, and intestinal damage, and improve the host against *E. magna* infection (Chen et al., 2023). Another recombinant *E. magna* GAM56 (rEmGAM56) and ROP17 (rEmROP17) proteins obtained from a prokaryotic expression system elicited cellular and humoral immune responses and are potential vaccine candidates against *E. magna* (Xiao et al., 2022). Besides, the *EmSAG10* and *EmSAG11* showed a higher average weight gain, meat ratio, the oocysts decrease rate and significantly reduced intestinal lesions. The result also showed that r*EmSAG10* could induce humoral and cellular immunity, while rEmSAG11 could only induce humoral immunity (Pu et al., 2022). Therefore, r*EmSAG10* is a candidate antigen for *E. magna* recombinant subunit vaccines.

A precocious line trivalent vaccine, including *E. magna*, *E. intestinalis*, and *E. media,* was formulated as a candidate vaccine. After immunisation, it showed no clinical symptoms, and the daily weight gains were similar to those of unimmunised, unchallenged controls. The oocyst outputs in the vaccinated challenged groups decreased with the increase in the immunisation dose (Fang et al., 2019). In China, a live trivalent rabbit coccidiosis vaccine (*E. media* PMeGX strain + *E. magna* PMaSD strain + *E. intestinalis* PInGX strain) was licenced in 2023.

Studies on recombinant vaccine vectors based on transgenic rabbit *Eimeria* spp. have also been conducted. A transgenic rabbit E*. magna* line expressing the P2 subdomain of VP60 (EmagE-VP60) was constructed, and immunisation with the *EmagE-VP60* induced exogenous antigen-specific intestinal immunity in rabbits (Tao, 2017).

In addition to coccidia, another parasitosis, *Sarcoptes scabiei,* was also studied. Recombinant *S. scabiei* serpin (rSs-serpin), recombinant *S. scabiei* chitinase-like protein-5 [rSs-CLP5], and -12 [rSs-CLP12] (three proteins) were expressed separately in E. coli. The mixture of the three proteins proved to be a promising cocktail vaccine candidate that induced robust immune protection and could significantly decrease mite populations to reduce the direct transmission between rabbits (Shen et al., 2023).

DISCUSSION

Epidemic diseases, such as RHD, *P. multocida* infection and *E. intestinalis* infection, are lethal and highly contagious in rabbits, which has seriously damaged the development of the global rabbit industry. Prophylactic immunisation offers a safe, effective, and economical preventive measure in case of rabbit infectious diseases.

According to reports in recent years, scientists have been struggling with vaccine design strategies that lead to improved, cross-protective vaccines offering the best method of effective control. Strengthening the research and development of multivalent vaccines is necessary to effectively prevent and control the occurrence of multi-serotypes of bacteria/viruses or prevent multiple diseases with one injection in clinical practice. This can better meet the market demand and clinical needs.

Since the widespread prevalence of RHDV2, most of the development of rabbit novel multivalent vaccines is basically designed around RHD, such as the bivalent vaccine of RHDV and *P. multocida* (Lin, 2018, Zhu et al., 2021), the bivalent vaccine of RHDV and *Eimeria* (Tao, 2017).

Alum and mineral oil adjuvants are widely used in rabbit vaccines. As new vaccines are developed, more safe and effective immune adjuvants are needed, and natural vegetable oil adjuvants (Cui et al., 2022) and traditional Chinese medicine adjuvants (Yang et al., 2017, Xiao et al., 2021) have good market application prospects in animal vaccines.

In developing new rabbit vaccines, we also need to pay attention to the efficiency and cost of production. Different expression systems were tried on VP60 expression, including *E. coli*, yeast, baculovirus, and transgenic plants. The prokaryotic expression of *E. coli* has the advantage of simple preparation, low cost and easy production. However, the *E. coli* system cannot make post-translative modifications to proteins, so proteins tend to form inclusion bodies. Especially when recombinant proteins come from eukaryotic host cells, it is challenging to form soluble VLP (Wu, 2021). The baculovirus expression system belongs to the eukaryotic expression system, in which insect cells can recognise and process signal peptides and have certain post-transcriptional modifications (Xia, 2023). This system has the advantages of high efficiency, high positive rate and large expression.

CONCLUSIONS

In recent years, vaccine and adjuvant research has made continuous progress, providing a new way to prevent and control rabbit infectious diseases. Introducing new vaccine technology and adjuvants is expected to improve rabbit vaccines' protective power and immune effect significantly. However, further research and experimental validation are still needed to ensure the safety and efficacy of these new technologies.

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WHY SOME FARMS ARE REGULARLY AFFECTED BY RABBIT HEMORRHAGIC DISEASE? AN EPIDEMIOLOGICAL SURVEY IN FRANCE

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ABSTRACT

About one third of the yearly outbreaks of Rabbit Hemorrhagic Disease (RHD) in France occur in farms previously affected by the disease during the last three years. Limiting recurrence of RHD outbreaks is a key objective to reduce the global burden of the disease for the French rabbit production sector. A prospective epidemiological study was set up aiming to reduce the recurrence of RHD in previously affected farms by improving decontamination measures in case of RHD outbreaks and by improving the vaccination quality of reproductive females. Fourteen farms affected several times by RHD were investigated between December 2021 and October 2023. Efficacy of cleaning and disinfection measures after an RHD outbreak was evaluated on environmental samples analysed by RT-PCR for RHDV genome detection in the 14 farms. Viral genome was detected in 10 farms after cleaning and disinfection, mostly on rendering container (7 farms) and on floor and walls of rabbitries (three farms). The results show that the reinforcement of decontamination measures is needed in a majority of farms. On four farms, vaccinated females showed positive rectal swabs for RHDV2 genome, despite exhibiting no clinical signs. The potential risk associated to the excretion of RHDV2 virus by females for the exposure of kits to the disease could not be evaluated in the study but a reinforcement of sanitary barriers between farrowing and fattening parts of the farm is advised. Assessment of the vaccination quality of females by an ELISA test for anti-RHDV2 antibodies showed that primiparous does were improperly vaccinated on three farms out of 12. Transmission of maternal immunity to kits is another key factor in the prevention of RHD on fattening rabbits. Improvement of the vaccination scheme and practices for females before the first gestation is a potential way to limit the risk of RHD recurrent outbreaks.

Key words: epidemiology, rabbit, viral hemorrhagic disease, decontamination, viral phylogeny

INTRODUCTION

Rabbit Haemorrhagic Disease (RHD) is a highly contagious viral hepatitis that affects domestic and wild European rabbits (*Oryctolagus cuniculus*). This generally fatal disease is caused by a virus (RHDV) belonging to the *Lagovirus* genus of the *Caliciviridae* family. A new genotype of RHDV (RHDV2) emerged in rabbits in 2010 and almost replaced RHDV strains in France. According to the results of the surveillance system set up by the rabbit sector in France in 2018, about one third of the outbreaks reported each year occur on farms previously affected by RHD during the last three years. A similar situation was also reported in Spain, with multiple outbreaks on some farms accounting for a significant proportion of the cases recorded (Rossel et al., 2019). These observations demonstrate the importance of recurrent outbreaks in the epidemiology of RHD. Controlling of reoccurrences is therefore a priority for reducing the impact of RHD on the rabbit sector. Our study aims to reduce the number of recurrent outbreaks of RHD by strengthening outbreak management measures and improving vaccination practices. The study, which will run for three years from September 2021, is a prospective epidemiological survey on farms regularly affected by RHD outbreaks. This article presents the results obtained during the first two years of the study and the initial practical lessons to be learned.

MATERIALS AND METHODS

The objective of the prospective epidemiological study was to monitor the management of outbreaks of RHD on farms that had been affected several times by the disease. Fourteen farms were reported by the vets and rabbit farming groups taking part in the study when a new outbreak of RHD occurred on the farms. We visited the farms twice to gather information on the occurrences of RHD and the measures taken to prevent the disease, based on biosecurity measures and on the regular vaccination of young and productive does (but not of the growing rabbits). The first visit, during the RHD outbreak, was used to carry out a serological evaluation of the vaccination quality on 14 females (seven primiparous does and seven multiparous does) by measuring total antibodies against RHDV2 with a method adapted from the kit ELISA 2 (KIT IZSLER Code 78751) (Lavazza et al., 2018). Rectal swabs taken from the same females were tested for virus excretion. Environmental swabs were also taken to identify surfaces positive for the RHDV2 genome (Huneau-Salaün et al., 2021). The same samples were taken during the second visit after decontamination of the fattening room to assess the quality of cleaning and disinfection (C&D). A positive result on an environmental sample denotes the presence of RHDV2 genome but does not inform about virus viability. Nevertheless, this type of protocol showed its interest for monitoring the effectiveness of C&D (Huneau-Salaün et al., 2021). Rectal swabs and environmental samples were tested for RHDV2 genome detection using specific real-time RT-PCR (Le Gall-Reculé et al., 2017). Total RNAs were previously extracted from 200 µL of each swab eluate using the NucleoMag®VET kit (Macherey-Nagel, KingFisher Flex ^{TM} instrument).

RESULTS AND DISCUSSION

Efficacy of cleaning and disinfection after a RHD outbreak

At the first visit (8 to 15 days after the outbreak), 50 samples out of 138 (36%) were positive for RHDV2 genome, on 12 farms; no positive sample was obtained on one farm and the last one was not sampled before cleaning and disinfection (Table 1).

Table 1: Detection of RHD genome before cleaning and disinfection (C&D) in rabbit farms

Area	Before cleaning and disinfection (13 farms)		After cleaning and disinfection (14 farms)		Comments after C&D
	Detected	%	Detected	%	
Floor	9/20	45	1/23	4	
Walls	3/12	25	2/12	17	Detected on 2 farms
Anteroom	7/25	28	1/29	3	
Aeration system	5/31	16	2/32	6	Detected on 2 farms
Slurry system	6/14	43	2/17	12	Detected on 2 farms
Rendering	19/21	90	8/24	33	Detected on 7 farms
Surrounding	1/15	7	0/16	0	
Total	50/138	36	16/143	11	Detection after C&D on 10 farms

The most frequently contaminated surfaces were the rendering container and the freezer containing dead rabbits (19/21, 90%), the floor of the fattening room (9/20, 45%) and the slurry evacuation system – deep pits or scrapping systems (6/14, 42%). After C&D (3 to 30 days after disinfection, 14 farms), 16 positive samples (out of 143, 11%) were observed. No positive sample was obtained on 4 farms out of 14. Up to 3 positive samples (out of 13) were obtained in one farm. On this farm, only one fattening room of the rabbitry had been disinfected while the other room was still housing vaccinated fattening rabbits. A positive sample was observed in the anteroom shared between the two fattening rooms and on the floor of the fattening room housing rabbits. A complete disinfection and a sanitary break in

the fattening sector was not possible in this farm with two batches of rabbits at three weeks apart. Two-third of the samples taken on the rendering containers and freezers were still positive after C&D: the surfaces had not been treated during C&D operations on seven farms. The results obtained are on line with our previous observations in four farms studied after a RHD outbreak (Huneau-Salaün et al., 2021). Rendering equipment (container and freezer) are rarely decontaminated on farms. The risk is to maintain the presence of potential infectious material inside the rabbitry, as most of the freezers for stocking dead rabbits before the collection by the render operator are located in the main building.

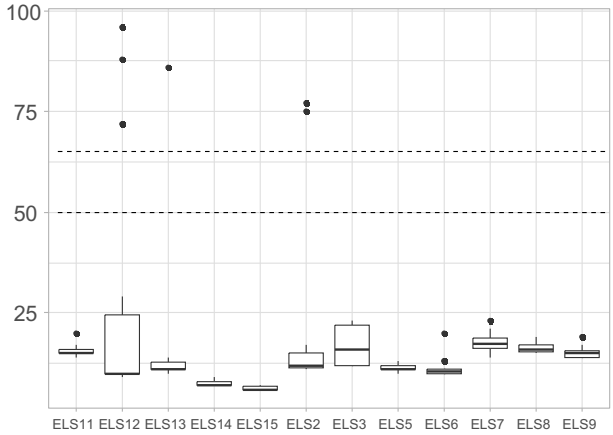
Fecal excretion of RHDV2 in females

Rectal swabs taken on vaccinated females were positive for RHDV2 genome in four farms. The twenty females (out of 124 tested, 16%) that had positive rectal swabs did not show clinical signs of RHD but were exposed to the virus during the outbreak. This result is not suprising as the vaccination prevents from the disease but not from the infection or from a residual excretion of virus (Müller et al., 2021). The positive females were both primiparous females (10) and multiparous females (10). Females were resampled after three weeks on two of those four farms to follow RHDV2 excretion but samples could not be done on the same females as the first visit. One female (out of 14) and two females (out of 14) had a positive rectal swab for RHDV-2 genome on the two resampled farms at three weeks after the end of the RHDV2 outbreak. The females with positive rectal swabs may not excrete infectious RHDV2 virus (Calvete et al., 2021). Nonetheless, presence of females potentially excreting RHDV2 virus without exhibiting clinical symptoms may contribute to maintain the disease on the farm. This risk is potentially high when we take into account that the excretion was still detectable three weeks after the outbreak and after the C&D of the fattening rooms. Reinforcement of sanitary barriers is needed between the farrowing and the fattening parts of the farm to prevent young rabbits after weaning from being exposed to potential infectious feces of the excreting females. Emergency vaccination of growing rabbits is recommended for the two batches produced after an RHD outbreak but some farms cannot afford to vaccinate young rabbits.

Quality of anti-RHDV2 vaccination of females

Serological results were available for 12 farms (Figure 1). High and homogeneous levels of anti-RHDV2 antibodies were observed in 9 farms, showing satisfactory vaccination coverage. In three farms, one to three females did not exhibit anti-RHDV2 antibodies. In all cases, these were females giving birth for the first time and had never had a booster vaccination after the initial immunisation scheme. The absence of antibodies could be explained by a failure of the initial vaccination. This observation is of interest as in three farms with recurrent RHD outbreaks, the disease affected fattening rabbits issued from primiparous females only. Lenormand et al. (2019) observed on a commercial farm that kit rabbits issued from vaccinated females with low titles of anti-RHDV-antibodies did not present anti-RHDV2 antibodies at 35 days of age. Baratelli et al. (2020) experimentally demonstrated that kits inherited a maternally derived antibody response against RHDV2 from the vaccinated females that lasted at least four weeks. Fattening rabbit from females improprely vaccinated, especially primiparous females, may be more sensitive to RHDV2 infections. The quality of anti-RHDV2 vaccination of young females is a key element to protect fattening rabbits from infection after weaning and a high level of maternal antibodies has to be reached before or during the first gestation.

Figure 1: Serological results for ELISA anti-RHDV2 test on vaccinated females (12 farms, 14 females sampled per farm). *Results are expressed as % S/N. The horizontal lines denote the thresholds for the test interpretation with results below 50 % S/N being associated with the presence of anti-RHDV2 antibodies.*



CONCLUSIONS

The study on farms facing recurrent outbreaks of RHD showed the key role of the vaccination quality of young reproductive females before the first parturition. Assuring a high and homogenous protection level during the first gestation is all the more important to ensure the transmission of the maternal protection to the kits that may be exposed to a contaminated environment after an RHD outbreak. Cleaning and disinfection procedures might be insufficient if not applied on all contaminated surfaces and in particular on surfaces in contact with dead rabbits. Similarly, strong sanitary barriers and measures are required in the farrowing room to limit the exposure of weaned rabbits to potential infectious material that could be excreted by females.

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OCCURRENCE AND CHARACTERIZATION OF RABBIT CALICIVIRUS (RCV) STRAINS IN ITALY OVER 20 YEARS

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ABSTRACT

Lagoviruses are a group of riboviruses from the *Caliciviridae* family whose host spectrum is represented mainly by lagomorphs. Focusing on viruses affecting rabbits, both pathogenic and non-pathogenic viruses are phylogenetically distinguished: Rabbit Haemorrhagic Disease Virus (RDHV/GI.1) and Rabbit Haemorrhagic Disease Virus-2 (RDHV-2/GI.2) belong to the first group, while in the second several genotypes of Rabbit Calicivirus (RCV/GI.3-GI.4) are present. The existence of non-pathogenic rabbit caliciviruses (RCV) was proposed in the '90, following the detection of RHDV-seropositive but healthy rabbits. In 1996, examining the intestine of such seropositive rabbits with no history of the disease, the first RCV strain was found and characterised in Italy. Since the first case, several studies have been conducted to characterise RCV strains in Europe and Australia, highlighting how recombination events with other lagoviruses are possible and relatively frequent. They were all isolated from the duodenum or, more rarely, from faeces, indicating a substantial difference in the tropism compared to the pathogenic hepatotropic RHDVs. Following these studies, this work aimed to genetically characterise RCV strains diagnosed in Italy in samples of faeces and intestines of both wild and farmed rabbits collected in various areas of Italy from 2000 to 2022. Out of 257 analysed samples, 67 resulted positive in RT-PCR for the lagovirus genus but negative for RHDV. Eleven selected strains were confirmed to be RCVs by complete vp60 sequencing. Phylogenetic analysis showed that the strains identified in this work, circulating in Italy, are located in both the European (RCV_E1/GI.3) and Australian (RCV_E2/GI.4) RCV clusters. Based on the VP60 gene sequences, these strains form separate clusters from the other RCV genotypes. The level of genetic variation above 15% suggests that these viruses could belong to one or more new genotypes.

Key words: non-pathogenic lagovirus, serology, phylogeny, evolution.

INTRODUCTION

Viruses of the genus lagovirus, Caliciviridae family, infect rabbits and hares. Lagoviruses are both hepatotropic agents (RHDVs and EBHSVs), causing acute, fulminant viral hepatitis, and enterotropic viruses (RCVs and HaCVs) that are almost entirely benign.

Lagoviruses are classified based on the nucleotide capsid protein sequences (VP60) into genogroups (e.g. GI and GII), genotypes (e.g. GI.1, GI.2, GI.3 and GI.4), and variants (e.g. GI.1a, GI.1B, GI.1c) (Le Pendu et al., 2017). Rabbit haemorrhagic disease (RHD) is a highly contagious and acute fatal hepatitis of the European rabbit (*Oryctolagus cuniculus*). RHDV (GI.1) was first reported in 1984 in China and rapidly spread worldwide, becoming endemic in almost all countries where European rabbits are present as domestic or wild animals. In 2010, a new RHDV strain was identified in France (Le Gall-Reculé et al., 2011), causing extended epidemics in vaccinated adult rabbits and young rabbits considered typically resistant to RHDV. Since this virus is phylogenetically and antigenically distinct from RHDV, it has been called RHDV2 (GI.2). The new virus spread all over Europe in a few years, and it is now present and prevalent all over the world, including Australia, North America and Africa. The other lagovirus group, named rabbit calicivirus (RCV/GI.3), was detected in domestic and wild rabbits in Italy in 1996 (Capucci et al., 1996) and then in other European countries (Le Gall-Reculé et al., 2011), as well as in 2007 in Australia (RCV-A1/GI.4) (Strive et al., 2009). These viruses cause a silent intestinal tract infection without inducing clinical

signs and relevant pathological lesions. The nomenclature based on the phylogenetic relationships of the full-length VP60 capsid gene collocates the rabbit non-pathogenic viruses into two distinct genotypes: a) the European Rabbit Calicivirus, RCV-E1 (GI.3); b) the Australian RCV-A1 and European RCV-E2 (GI.4). In particular, the European strains (GI.3) cluster with classical RHDV (GI.1), suggesting that they are more closely related to these pathogenic lagoviruses than the more divergent RCV-A1, which forms a distinct monophyletic branch (Mahar et al., 2016, Strive et al., 2009). While the first Italian RCV (X96868/GI.3) provides complete cross-immunity to RHDV (GI.1), the Australian RCV-A1 (GI.4) does only partially, with up to 50% protection.
Based on serological results, the study aimed to identify and characterise the RCV strains circulating in Italy by molecular approaches.

MATERIALS AND METHODS

Sampling

Rabbit sera were collected from 1999 to 2017 in over 100 farms in different Italian regions. Almost all sera were collected at slaughterhouses from rabbits with an average age of 3 months belonging to RHD-free herds. In addition, the proximal duodenum was sampled during necropsies of dead farmed rabbits collected from 2000-2022 and from feral rabbits living in a city park in Forli. In some farms, faeces from live animals were also collected.

ELISA tests

Several RHD-ELISA tests were used as already described (Cooke et al. 2000), but the results of the competition ELISA (cELISA) and IgG RHDV were the most indicative. In fact, cELISA mainly detects antibodies specific to the virus surface, while IgG ELISA also detects antibodies against epitopes buried in the virus structure.

Extraction, detection, and sequencing of viral RNA

Total RNA was extracted from faeces or duodenum (Cavadini et al., 2020) using the Trizol reagent (Qiagen), according to the manufacturer's instructions. To detect the viral RNA, a first PCR was performed using the universal primers for lagovirus Rab1/Rab2 (Strive et al. 2009). The entire VP60 gene was amplified using several overlapping PCRs, and the products were gel purified and sequenced with an ABI Prism 3500 Series Genetic Analysers in both directions (Applied Biosystems, Foster City, CA, USA). Contig assembling and genome sequence analysis were done using Seqman NGen DNASTAR version 11.2.1 (DNASTAR, Madison, WI, USA). Phylogenetic analysis was performed with MEGA X (Kumar et al., 2018).

RESULTS AND DISCUSSION

Serological analysis

Overall, in about 30% of the tested farms, we found RCV antibodies but with two distinct patterns: 1) farms with negative or sera just above the cut-off value in cELISA and medium to high titres in the IgG ELISA test, and 2) farms with cELISA titres similar to those found in vaccinated rabbits (4-16 times higher than in pattern 1) and similar medium to high titres in the IgG ELISA test.
The serological results were then used to select the farms where sample duodenum and /or faeces were collected for viral identification.

Genome sequencing and phylogenesis of RCV isolates in Italy from 2000 to 2022

Out of 257 analysed samples, most of which were from farms where the serological results suggest a non-pathogenic calicivirus, 69 resulted positive for lagovirus but negative for RHDV by RT-PCR. Eleven samples positive for lagoviruses were confirmed to contain RCV by complete vp60 sequencing. The inability to amplify and sequence the entire vp60 gene from all the RT-PCR positive samples is likely due to too low viral RNA amounts to permit successful complete amplification (Mahar et al., 2019; Cavadini et al., 2020).

Phylogenetic analysis based on vp60 sequences (**Figure 1**) showed that the strains identified in this work and circulating in Italy are located in both the European (RCV-E1/GI.3) and Australian (RCV-E2/GI.4) RCV clusters. In particular:

- Two strains from the same farm in the Brescia province were identified after several years of interval. These strains have a nucleotide identity of 98.99% (RCV_BS2000) and 90,49% (RCV_BS2007), respectively, compared to the first RCV "Italian" strain (RCV X96868-1996), which indeed was first identified in the same farm.
- In a farm in Bergamo province, the RCV-E1 was found twice with a three-year interval (RCV_Bg2012 e RCV_Bg2015), but with only 84.4% of nucleotide identity between the two strains, suggesting that these are different viruses rather than the outcome of the persistence and evolution of the same virus.
- Viruses phylogenetically related to the Australian RCV strains (RCV-E2/GI.4) were detected in three farms located in geographically distant areas (RCV FG-2013 and RCV FG-2016 Foggia province, South Italy; RCV PN-2012/2 Pordenone Province, North-East Italy; RCV BS-2010, Brescia province, North Italy), and over a relatively significant period (2010-2016). Note that the two strains identified in the same farm in Foggia (FG) province with a three-year interval presented a nucleotide identity of 98%, suggesting, in this case, also, the persistence of the virus in the farm. The other two strains (RCV FC-2017 and RCV PN-2012/1), belonging to the RCV-E2/GI.4 genogroup, are closer phylogenetically to the strains identified in France between 2008 and 2015 (LT08121-LT08130).

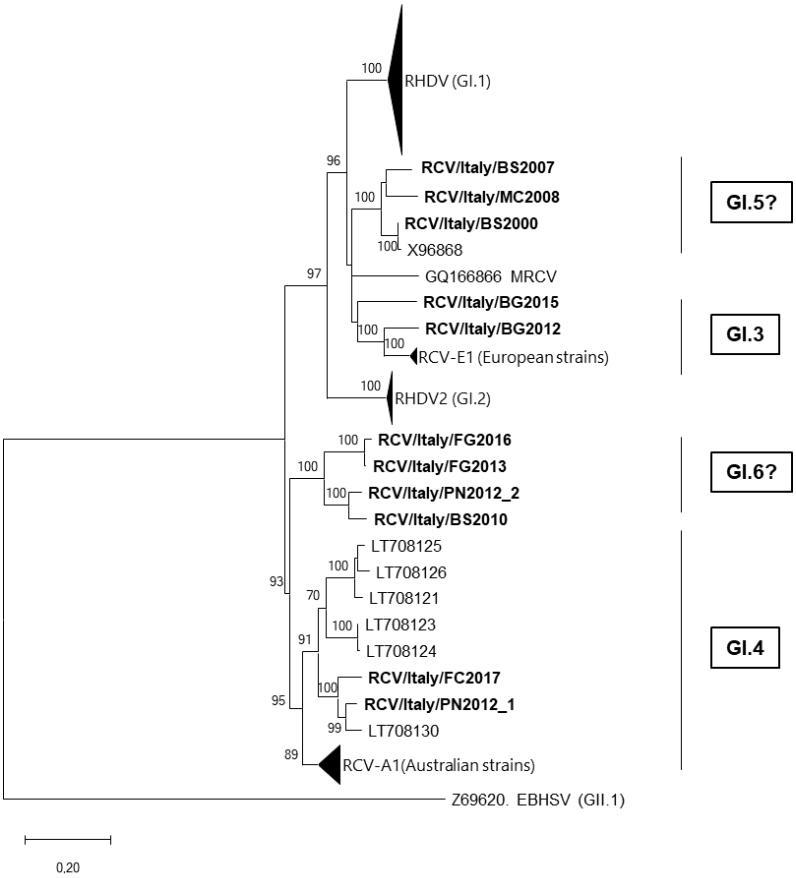

Figure 1: Maximum Likelihood (ML) phylogenetic trees performed for the structural genes vp60 (n= 122 sequences; nucleotides 5240-6869; nucleotide substitutions model GTR+G+I). Support for eachcluster was obtained from 1.000 bootstrap replicates. Bootstrap values >60% are shown.

According to the genotype definition proposed (Lependu et al., 2017) and stating the nucleotide differences of vp60 found in this study, we can suggest that within the cluster of non-pathogenic viruses, two other genotypes might exist: GI.5 and GI.6 (Figure 1).

CONCLUSIONS

In this study, which covers an extensive period (about two decades) and territory (most Italian regions), we investigated the presence of RCVs, first by a serological approach using different ELISA tests and then by virological methods. The genomic characterisation of a selected number of RCV strains permitted the identification of new putative members of the Lagovirus genus within the Caliciviridae family.
This data further expands the knowledge for better defining the evolution of pathogenic lagoviruses, likely starting from non-pathogenic ones. This is especially true in light of recent evidence that RHDV2 originated by recombination between a non-pathogenic virus as a donor for the non-structural part of the genome and the new lagovirus (GI.2) that formed the structural part.

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PASSIVE PROTECTION FROM RHD: THE ROLE OF IMMUNOGLOBULINS IN MILK

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ABSTRACT

At the time of birth, newborns of Mammalia use nursing as a source of nutrition and protection from infectious diseases since their immune system needs time to mature and become autonomous. It is known that the main transmission of maternal antibodies from rabbit mothers to the foetus occurs at the level of the placenta, which is the haemochorial type. Therefore, colostrum and milk contain immunoglobulins, mainly IgA, to passively protect the young rabbit's mucous membrane during the lactation period. However, the origin of specific immunoglobulins in relation to the vaccination protocol still needs to be clarified. In this study, we analysed the quantity and specificity of IgA and IgG present in the milk of breeding rabbits vaccinated against the two lagoviruses (RHDV and RHDV2) causing Rabbit Haemorrhagic Disease (RHD), a lethal hepatitis with a high mortality rate.

Blood and milk samples were collected from 20 pluriparous does vaccinated with two monovalent vaccines for RHDV and RHDV2. Samples were tested with specific competitive ELISAs to detect total immunoglobulins against both viruses. Furthermore, we also performed direct and reverse ELISA using specific MAbs anti-Igs and/or anti-viruses to detect total and specific Igs only against RHDV2. Vaccinated mothers' titres showed protection for RHDV (cELISA titres~1/320) and RHDV2 (cELISA titres~1/160). Interestingly, titres obtained with cELISAs performed on sera were comparable with those obtained from milk. Moreover, the amount of IgA in the sera was 350 ± 100 ug/ml, with ≈ 20% of secretory IgA (sIgA) and 5-10 mg/ml of sIgA in the milk, while the amount of IgG in the milk ranges from 1.5 to 2.6 mg/ml.

Results of this study show that rabbit milk contains specific IgA and IgG at relevant titres, similar to those found in the serum, suggesting that young rabbits born and taking milk from vaccinated mothers benefit from passive protection that, in addition to the systemic protection, is especially important for the mucosal level.

Keywords: Rabbit, RHD, milk, passive protection.

INTRODUCTION

The 1980s represented an important year for the rabbit industry, marked by the emergence of a highly pathogenic virus in China that quickly reached Europe as well. This virus is nowadays known as the Rabbit Haemorrhagic Disease Virus, a Lagovirus (Caliciviridae family) that principally affects European wild rabbits (*Oryctolagus cuniculus*) and causes severe hepatitis and often death within 48 hours (Liu et al., 1984). In addition, in 2010 appeared in France a new virus related to RHDV, called RHDV2 (Le Gall-Reculè et al., 2013), which shows some significant differences, such as extended infectivity even to young rabbits and a broader host range, being pathogenic also for other lagomorph species (Puggioni et al., 2013; Velarde et al., 2017). Several commercial vaccines currently available can induce a humoral immunity that prevents rabbits from developing the disease. Moreover, it is known that maternal antibodies represent a type of passive immunity transmitted from mothers to offspring during gestation. In rabbits, this phenomenon occurs thanks to the haemochorial placenta, which allows the exchange of antibodies (especially IgGs) from the mother's serum to the foetus (DeSesso et al., 2012). It has also been shown that antibodies provided from vaccination follow transplacental transmission and are inherited by kits up to one year from vaccination of breeding does (Baratelli et al., 2020).

Regarding lactation, we know that milk is the primary source of nutrition, but its role in passive immunity has yet to be fully known. Since colostrum and milk originating from the mammary glands are closely connected to the mucosal system, IgA is the most abundant immunoglobulin compared with IgG and IgM (Butler and Kehrli, 2004). While there is evidence for other mammals that lactation is necessary to ensure passive immunity and, consequently, their survival, this aspect still needs to be clarified in rabbits.
For this purpose, this study aimed to clarify the presence of immunoglobulins in milk and to demonstrate that lactation from vaccinated mothers could confer protection to the litter, especially at a mucosal level, in addition to that derived from the transplacental mechanism.

MATERIALS AND METHODS

Sampling and matrix processing

Twenty commercial pluriparous does were selected from an Italian farm. All these rabbits had been vaccinated one month before parturition with two monovalent commercial vaccines containing inactivated viruses, respectively, for RHDV and RHDV2. Titres of protection were tested from sera taken at 5 and 9 weeks post-vaccination with competitive ELISA as reported in the WHOA Manual Chapter 3.7.2, while milk (approximately 1 ml) was collected at 7 days from birth. Milk was centrifuged at 13000 rpm for 1 hour at 4°C to separate the whey proteins from the fat, which were then collected and stored at -20 °C, waiting for analysis. Moreover, 1 ml of milk was treated with Vertrel XF (Fluka Sigma-Aldrich) to further remove fats and any aggregates before processing ~250µl to gel filtration in FPLC NGC Bio Rad®, with column Superose 6 10/300 (GL-Cytiva). Gel filtration was used to separate milk components, immunoglobulins included. Fractions obtained through this passage were also tested in cELISA for specificity against RHDV/RHDV2 and quantitative ELISA for IgA and IgG.

Sandwich ELISA for total IgA and RHDV2 specific IgA

Two sandwich ELISA assays were used to study IgA, i.e., total IgA and RHDV2-specific IgA, by using monoclonal antibodies (MAbs) produced in our laboratory.
1) To quantify total IgA, standards to make a reference calibration curve were included on each plate. Plates were adsorbed with the specific anti-IgA MAb (2E2) followed by a 1-hour incubation with the whey or serum and then three washes (with PBS1X + Tween 0.1%), another hour of incubation with the anti-IgA 3E8 or 2F2 HRP labelled antibodies. In particular, the 3E8 MAb is specific for the secretory fragment present in secretory IgA (sIgA), while 2F2 recognises an epitope common to all rabbit Igs.
2) For the analysis of RHDV2-specific IgA, two types of sandwich ELISA tests were performed: direct and reverse. In the reverse ELISA, plate adsorption and sample addition are similar to the procedure described at point 1) followed by incubation with RHDV2 antigen and subsequent detection with an RHDV2 specific MAb (4H12 HRP-conjugated). In the direct one, the same MAb (4H12) was used for adsorption, followed by incubation with the antigen and then with the samples and further incubation with anti-IgA MAbs (3E8 or 2F2 HRP-conjugated).

ELISA for total IgG and RHDV2 specific IgG

The same study performed for IgA was also carried out to detect total and specific IgG. The procedures used were as described above for IgA. Specifically, for the quantification of total IgG, an IgG-specific MAb (MAb 4H9) was adsorbed to the plate, and a second IgG-specific Mab was used as a tracer (3D6 HRP); for the detection of RHDV2 specific IgG, direct and indirect assays were performed. For the direct method, also reported in the WOAH Terrestrial Manual (Chapter 3.7.2), MAb 4H12 was adsorbed to the plate as the catcher of RHDV2. After incubation of the samples, MAb 4H9 HRP was used as a tracer. In the reverse method, 4H9 was adsorbed to the plate, followed by incubation with samples, then with RHDV2 virus and finally with MAb 4H12 HRP-conjugated as the tracer.

RESULTS AND DISCUSSION

Quantification of IgA and IgG in serum and milk

Sandwich ELISAs were used to quantify IgA and IgG using specific MAbs. IgG has no subclasses in rabbits, so it was possible to use a single pair of MAbs (4H9 and 3D6 HRP-conjugated). On the other hand, IgA has a variable structure; thus, to include as many types as possible, we used MAb 2E2 as a catcher, which binds the IgA heavy chain in all their forms to ensure ELISA specificity. Depending on the MAb used as a tracer, we could quantify both the secretory IgA (MAb 3E8, which binds the secretory fragment of sIgA) and all other IgA (MAb 2F2 that recognises an epitope common to all rabbit Ig). Table 1 shows the results obtained from interpolation with the calibration curves for each pair of MAbs used. The already known majority of IgG in serum has an average of 13 mg/ml compared to milk with only 2.2 mg/ml, even if this amount is higher than the one reported by Butler and Kehrli in 2004. On the contrary, the highest amount of IgA is found in milk where average levels of sIgA and non-secretory IgA are similar, and the quantity is concordant with what has already been shown (Butler and Kehrli, 2004), while their difference is most evident in serum where sIgA levels are the lowest.

Table 1: sIgA, IgA and IgG concentrations in serum and milk matrices.

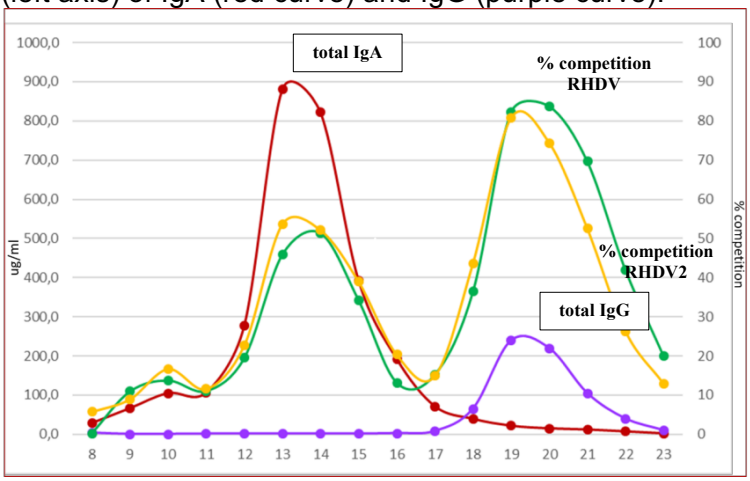
		MAbs couple	Min	Max	Mean	Ds
Serum	sIgA	2E2-3E8	40 µg/ml	60 µg/ml	47,4 µg/ml	11,1 µg/ml
	IgA	2E2-2F2	0,33 mg/ml	1,05 mg/ml	0,67 mg/ml	0,30 mg/ml
	IgG	4H9-3D6	10 mg/ml	18 mg/ml	13 mg/ml	3,5 mg/ml
Milk	sIgA	2E2-3E8	6 mg/ml	9 mg/ml	8,1 mg/ml	1,1 mg/ml
	IgA	2E2-2F2	7 mg/ml	12 mg/ml	9,4 mg/ml	2,2 mg/ml
	IgG	4H9-3D6	1,5 mg/ml	2,6 mg/ml	2,2 mg/ml	0,5 mg/ml

The presence of specific vaccine-induced immunoglobulins

Vaccination-induced immunity was first tested on serum in cELISA (WHOA Manual Chapter 3.7.2) since this method allows measurement of the total humoral response given by all immuno-globulins in the matrix. The vaccine's protection level was good in all animals, with normal variation between the first and the second sampling and among individual animals and titres, averaging 1/320 for RHDV and 1/160 for RHDV2.

The competitive ELISA was also used for the milk sampling to evaluate the specific antibody response against the two viruses (RHDV and RHDV2). However, the cELISA, which has a cut-off value dependent on the matrix, was not developed to investigate the titre of immunoglobulins in milk. Although pre-treated to reduce fat and casein micelles, milk has characteristics that could result in "nonspecific" competition. Nevertheless, the results indicated a direct relationship with sera titres for both RHDV and RHDV2 in milk. However, they were 3-4 times lower than in sera and more similar to those obtained testing the last blood sampling than the first ones. The specificity of the reaction was demonstrated by comparing milk from non-vaccinated farms: serum titres were close to the threshold value, and milk titres were negative. At the same, the specificity of the IgA and IgG in milk was determined with the two types of ELISA described above. The results demonstrated that although IgGs are present in a smaller amount than IgAs, their specificity is higher, and the type of ELISA that best represents this difference is the direct ELISA. Regarding IgA, the reverse ELISA makes it possible to eliminate competition with IgG because the binding to the adsorbed solid phase with the specific anti-IgA MAb is favoured over the binding due to virus affinity.

Figure 1: Percentage of competition (right axis) relative to fractions (x-axis) examined in cELISA toward RHDV and RHDV2, green and yellow curves, respectively. Quantifications (left axis) of IgA (red curve) and IgG (purple curve).



A pool of milk samples was fractionated by gel filtration chromatography with separation, based on molecular weights, of the IgA component from the IgG components to further investigate the results obtained. The fractions were then tested in sandwich ELISA to quantify total IgA and IgG, and in cELISA to assess their specificity toward RHDV and RHDV2 viruses, as shown in Figure 1. Quantification performed on the fractions by ELISA resulted in a value for total IgA of ~900µg, which was higher than that for IgG, which was found to be ~300µg. Despite this, the higher competition rate in cELISA is associated with the IgG peak, in agreement with the results of the direct ELISA on unfractionated milk.

CONCLUSIONS

Interesting data from this study suggest that vaccination-induced antibody levels in the milk matrix may play a role in preventing infection in young rabbits. This shows that does not only protect infants by the systemic route through transplacental transfer but also extends protection at the mucosal level, first via colostrum and then with normal lactation until the weaning period, and that the main contribution to protection is due to IgG although lesser represented in this matrix.

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PRIMARY INSIGHTS INTO POTENTIAL MOLECULAR BIOMARKERS RELATED TO INNATE IMMUNITY IN *LAGOVIRUS EUROPAEUS* INFECTION

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ABSTRACT

RHDV2 virus (Lagovirus europaeus GI.2) poses a significant threat to global rabbit populations. This study investigates gene expression during infection, focusing on autophagy, apoptosis, and RIG-I-Like Receptors (RLR) receptors. We observed decreased autophagy gene expression early in infection and apoptotic changes, while RLSs showed increased activity. These findings contribute to understanding disease progression, offering potential insights for therapeutic strategies and prognostic markers.

Keywords: Rabbit Haemorrhagic Disease, biomarkers, autophagy, apoptosis, RLR, receptors

INTRODUCTION

RHDV2 virus (Lagovirus europaeus GI.2), the causative virus of Rabbit Haemorrhagic Disease (RHD), has posed a significant challenge to the global rabbit industry and wild as well as domestic rabbit populations since its discovery in the 1980s. Scientific research involving experimental use of RHDV holds substantial value. The acquired insights contribute to the veterinary field, aiding in enhancing our understanding of RHD for better and quicker responses to new outbreaks. Additionally, this research is crucial for advancing human medicine, serving as a model for various diseases, particularly those associated with liver damage (Bębnowska and Niedźwiedzka-Rystwej, 2019). Immunological prognostic markers are currently a focus of research, enabling the monitoring of therapeutic effectiveness and patient prognosis (Mao et al., 2023). The study specifically aimed to assess the expression of key autophagy, apoptosis-related genes and receptors that recognise the molecular patterns of the virus in the blood of RHDV2-infected rabbits to track the progression of the infection.

MATERIALS AND METHODS

Animals and experimental design

The animal experiment was conducted at the Pomeranian Medical University in Szczecin with the consent of the Local Ethical Committee for Animal Experiments in Poznań No. 35/2022. The study used 20 Oryctolagus cuniculus/Crl:KBL (NZW)/0052 rabbits purchased from a licensed breeder. Rabbits (6 months old, body weight 4.5 kg ± 10%) were not vaccinated against *L. europaeus* and divided into a control group (n=10) and an experimental group (n=10). The inoculum (1 ml) administered intramuscularly to experimental group (Bębnowska et al. 2024) and the control group received placebo (1 ml) - PBS. Blood was collected from each animal at selected time points: 0, 12, 24, 36 and 48 hours after inoculation (h pi).

Real-time PCR

Procedures for extracting total RNA from blood were consistent with those described by Bębnowska et. all (2024) and followed the manufacturers' protocols. To analyze relative gene expression quantitative real-time PCR was performed using the LightCycler® 480 Instrument II (Roche Diagnostics GmbH, Mannheim, Germany) and the PowerUp™ SYBR™ Green Master Mix Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to

manufacturer's recommendations. In the study specific primers were used. Obtained threshold cycle (Ct) values were normalized by β-actin gene expression, and the relative gene expression was calculated using the Pfaffl method.

Statistical Analysis
To perform statistical analysis Tibco Statistica 13.3 (StatSoft, Palo Alto, CA, USA) was used. The values of the parameters were presented as arithmetic means and standard error (SE). Shapiro-Wilk test was used to test the normal distribution of continuous variables. Student's t-test was used to compare data of 2 groups data with a normal distribution and the Mann-Whitney U test was used for data with a non-normal distribution.

RESULTS AND DISCUSSION

Clinical signs of infection
Observation of infection symptoms showed that in some animals (n = 6) no clinical symptoms were recorded, and death occurred suddenly 12-36 h p.i. The remaining animals (n=4) developed an acute form of the disease (apathy, conjunctival hyperemia, shortness of breath, body temperature > 41°C, anorexia). Symptoms were observed from 24 hours after challenge. Experimental endpoints for these animals were reached at 36-48 h. p.i.

Genes expression results
In our study, we noted a decrease in the expression of key autophagy genes in PBMCs in rabbits infected *with L. europaeus*/GI.2 during the initial stage of infection. At 12 h p.i. there was an inhibition of Beclin-1, Atg5, Atg12, Atg16L and MAP1LC3B expression, while at 24 h p.i. a decrease in Beclin-1 expression was recorded (Table 1).

We also assessed the expression of genes encoding RLR receptors, such as RIG-I, MDA5 and LGP2. The result of the qPCR analysis was in line with the current state of knowledge, showing expression of all three receptors in blood samples (Table 2). RIG-I, achieved a significant increase in activity at 24 ($p \leq 0.01$) and 36 h p.i. ($p \leq 0.03$). Also, a statistically significant increase in expression was observed for LGP2 at 12 ($p \leq 0.01$), 24 ($p \leq 0.01$) and 36 h p.i. ($p \leq 0.03$). However, for the MDA5 gene there was an increase only at 24 hours ($p \leq 0.01$).

In our study, we also noted a decrease in the expression of several important autophagy genes during the initial phase of infection. We speculate that this may correlate with the high mortality rate of the animals. At 12 and 36 h p.i., increased expression of caspase-3 was observed in the experimental group. Infection was also accompanied by changes in the expression of proapoptotic Bax and anti-apoptotic Bcl2. There was increased expression of Bax at 12, 36 and 48 h p.i. and increased expression of Bcl2 at 12 and 24 h p.i., which decreased at 36 and 48 h p.i.. In addition, specific values of the Bax/Bcl2 ratio were higher at 36 and 48 h p.i. and lower at 24 h p.i. These results suggest that apoptosis can occur in infected rabbits from 12 h p.i. and was silenced at 24 h p.i., which is also confirmed by the reduced expression of caspase-3 at this time point, however, a re-increase in proapoptotic markers was noted in surviving animals after this time point, which we qualified as a prognostic factor. In COVID-19 patients, caspase-3 expression levels correlate with disease severity (Yildiz Gulhan et al., 2022). Similarly, an increased ratio of Bcl-2 to Bax expression correlated with disease stage in bovine leukaemia virus-induced leukaemogenesis in cattle (Reyes and Cockerell, 1998). Jounai et al. emphasize that the activation of RIG-I and MDA5 is affected by various factors, including autophagy, a critical aspect during RHDV2 infection. A notable rise in RIG-I expression at 24 and 36 h p.i. suggests that RIG-1 likely plays a pivotal role in the immune reaction to RHDV2 virus infection in rabbits. Elevated expression of this gene may signal the initiation of antiviral pathways in response to the infection.

Table 1. Relative genes expression of autophagy genes in blood samples.

Gene	Parameter	Time after inoculation									
		0h		12h		24h		36h		48h	
	Group	CG	EG	CG	EG	CG	EG	CG	EG	CG	EG
Beclin-1	Mean	1,00	1,16	1,00	0,26	1,00	0,26	1,00	0,54	1,00	1,27
	SE	0,37	0,20	0,30	0,08	0,28	0,07	0,28	0,27	0,23	-
	p-value	NS		0,002***		0,021**		NS		NS	
UVRAG	Mean	1,00	0,83	1,00	1,34	1,00	0,62	1,00	2,74	1,00	0,35
	SE	0,16	0,1	0,27	1,05	0,17	0,24	0,16	1,66	0,34	-
	p-value	NS		NS		NS		0,034*		NS	
Atg5	Mean	1,00	0,87	1,00	0,63	1,00	0,75	1,00	3,09	1,00	5,42
	SE	0,24	0,19	0,27	0,15	0,17	0,14	0,29	0,01	0,29	-
	p-value	NS		0,035*		NS		NS		NS	
Atg12	Mean	1,00	0,98	1,00	0,47	1,00	0,67	1,00	2,94	1,00	5,39
	SE	0,23	0,14	0,09	0,09	0,12	0,21	0,26	1,34	0,20	-
	p-value	NS		<0,001***		NS		NS		<0,001***	
Atg16L	Mean	1,00	0,61	1,00	0,08	1,00	0,85	1,00	6,84	1,00	3,75
	SE	0,09	0,28	0,24	0,03	0,21	0,32	0,25	1,52	0,21	-
	p-value	NS		<0,001***		NS		0,014**		0,004***	
MAPLC3B	Mean	1,00	0,70	1,00	0,33	1,00	0,53	1,00	1,04	1,00	0,43
	SE	0,21	0,19	0,11	0,18	0,12	0,11	0,10	0,48	0,15	-
	p-value	NS		NS		0,012**		NS		NS	
Caspase-3	Mean	1,02	0,70	1,04	4,04	1,02	0,91	0,98	11,98	0,99	2,27
	SE	0,21	0,23	0,34	0,67	0,14	0,17	0,29	2,45	0,25	-
	p-value	NS		<0,001***		NS		<0,001***		NS	
Bax	Mean	1,02	1,11	1,05	4,06	1,01	1,78	1,00	4,41	0,99	11,26
	SE	0,12	0,09	0,14	0,34	0,08	0,70	0,22	0,01	0,23	-
	p-value	NS		<0,001***		0,003***		<0,001***		0,005***	
Bcl2	Mean	1,02	0,86	1,05	3,23	1,01	4,74	0,99	0,46	0,99	0,39
	SE	0,36	0,26	0,13	0,53	0,25	0,99	0,21	0,09	0,28	-
	p-value	NS		0,004***		<0,001***		NS		NS	
Bax/Bcl2 *ratio*	Mean	1,00	1,30	1,01	1,26	1,00	0,37	1,01	9,63	1,00	29,01
	SE	0,38	0,31	0,15	0,40	0,30	0,31	0,19	3,76	0,37	-
	p-value	NS		NS		0,005***		0,014**		<0,001***	

CG-control group; EG-experimental group; NS- non significant; ***p<0,01; **p<0,03; p<0,05

Table 2. Results of RLR expression in blood samples

Gene	Parameter	Time after inoculation									
		0h		12h		24h		36h		48h	
	Group	CG	EG	CG	EG	CG	EG	CG	EG	CG	EG
RIG-1	Mean	1.01	1.06	1.02	1.21	1.01	15.54	0.99	33.30	1.00	42.42
	SE	0.06	0.14	0.13	0.50	0.14	4.40	0.20	3.61	0.14	-
	p-value	0.911589		0.203277		0.000100***		0.012417**		NS	
MDA5	Mean	1.01	1.22	1.02	2.85	0.99	3.08	1.00	14.86	1.00	7.91
	SE	0.15	0.32	0.09	0.99	0.21	0.65	0.11	5.64	0.19	-
	p-value	0.528553		0.719967		0.002083***		0.134133		NS	
LGP2	Mean	1.00	0.85	1,00	5,17	1,01	7,29	1.00	5.57	1.00	9.48
	SE	0,15	0,09	0,13	2,10	0.14	2.04	0.16	1.83	0.14	-
	p-value	0,053194597		0,002827***		0.000100***		0.014248**		NS	

CG-control group; EG-experimental group; SE- standard error; NS- non significant; * $p \leq 0.05$; ** $p \leq 0.03$; *** $p \leq 0.01$

Although there appears to be a trend towards increased MDA5 expression in the experimental group compared to the control group, differences in the expression of this gene are only noted at 24 hours. Additionally, there is a significant increase in LGP2 gene expression observed in the experimental group at both 24 and 36 hours after inoculation. This increase may indicate LGP2's involvement in the immune response to RHDV infection and its supportive role in the function of the RIG-I receptor. Moreover, the mosaic of RLR activity may serve as a potential biomarker of the infection phase. These results align with the earlier work of Pichlmair et al. (2006) and Schlee et al. (2009), which illustrated the activation of RIG-I and MDA5 by RNA viruses.

CONCLUSIONS

The obtained results are the first in literature on the primary investigation of apoptosis-, autophagy- and RLR-signalling related expression in GI.2 expression. Further studies are needed to evaluate the potential of RLRs as biomarkers of the infection and is crucial for understanding the pathogenesis of the disease caused by this virus.

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ROLE OF FLIES IN THE TRANSMISSION OF RHDV2: A STUDY OF CONTAMINATION IN *LUCILIA SERICATA* (DIPTERA: CALLIPHORIDAE)

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ABSTRACT

Rabbit Haemorrhagic Disease Virus (RHDV) belongs to the genus *Lagovirus*, family Caliciviridae. Two distinct serotypes are known: the "classic" RHDV/RHDVa and the RHDV2, which first appeared in 2010 in France and has since spread globally. RHDV2 can also infect and cause disease in a wide range of lagomorph species, including various hares (*Lepus spp.*). Transmission of RHDV occurs directly via contact of a susceptible rabbit with an infected animal or carcass or indirectly through contaminated surfaces, food, burrows, cages, and vectors such as insects, scavenging birds, and mammals. Limited studies have investigated the ability of immature stages of brush and scavenger flies in the mechanical transmission of lagoviruses. Therefore, our study aimed to investigate the role of various stages of life of blowfly *Lucilia sericata* in the possible transmission of the RHDV2 virus. Laboratory-reared larvae of stage I flies were exposed to rabbit livers infected with RHDV2 and incubated under controlled temperature, relative humidity, and photoperiod conditions. During the study, third-instar larvae, post-feeding larvae, pupae, and emerged adults were analysed by qPCR for the presence of viral DNA. The virus was also detected following washing with buffer solution and displacement from the contaminated food substrate of the different tested specimens up to 14 days after the start of the experiment. There was no increase in viral load, consolidating the role of *L. sericata* as a mechanical vector of RHDV2. The persistence of RHDV2 in the immature stage of L. sericata, in combination with the long distances travelled by post-feeding larvae of this abundant and widespread fly, could have implications for the epidemiology of RHDV2 and the implementation of control measures during disease outbreaks.

Keywords: Rabbit Haemorrhagic Disease Virus, *Lucilia sericata*, Larvae, Pupae, Mechanical vector.

INTRODUCTION

Rabbit haemorrhagic disease virus (RHDV) emerged in China in 1984 and spread throughout Europe over the rest of the decade (Henning J et al. 2005). Although much is known about the characteristics of the virus and the disease, relatively less is known about the mechanisms of virus transmission. Among the other several ways of indirect transmission, favoured by the high environmental resistance of the virions, many studies have shown that adult flies belonging to the family Calliphoridae and Muscidae can transmit the disease under laboratory conditions (Sassan Asgari et al. 1998; Lopes et al. 2023). Under laboratory conditions, fleas (*Spilopsyllus cuniculi* and *Xenopsylla cunicularis*) and mosquitoes (*Culex annulinostris*) (Sassan Asgari et al. 1998) are also capable of transmitting RHDV to susceptible rabbits. Muscidae could theoretically transmit RHDV by visiting dead rabbits and then transmitting the virus to the conjunctiva of healthy rabbits, but for blowflies (Calliphoridae), which feed on dead animals but rarely visit live rabbits, a less direct method of transmission must be considered (Sassan Asgari et al. 1998).

Synanthropic blowflies of *Calliphora* Robineau-Desvoidy and *Lucilia* Linnaeus (Diptera: Calliphoridae) can act as vectors of the causative agents of bacterial (Washington Pruna et al. 2019) or viral infections (Turčinavičienė et al. 2021). The larvae of these blowflies are necrophagous or polyphagous; adult blowflies feed on sugars (e.g., vegetable nectar, ripe fruit juices, sweets, aphid honeydew) and proteins (e.g., animal carcasses). Larvae and

adults of blowflies come into contact with pathogenic microorganisms, including viruses found in animal carriers' excretions, secretions, and tissues, and may become contaminated (Fischer et al. 2001). The sheep blow fly, *Lucilia sericata* (Meigen, 1826), is a widespread species of carrion fly with worldwide distribution. It is likely one of the most commonly found Calliphoridae worldwide, including Europe. *L. sericata* is considered a synanthropic species associated with human habitation and urban areas. Still, it does not have a habitat preference, being able to efficiently colonise also rural areas and farms (Turčinavičienė et al. 2021).
There are few studies in Europe on fly species that could be vectors of RHDV (Fischer et al. 2001), while studies have yet to examine the imaginal stages of flies. The main objectives of this study were to search for evidence of mechanical transmission of RHDV2 by *L. sericata* and to determine the potential of immature stages in facilitating the spreading of RHDV2.

MATERIALS AND METHODS

Rearing conditions, sampling and insects' exposures to RHDV2

An *L. sericata* stable laboratory colony was established at the Laboratory of Entomology in the Virology Unit of IZSLER. The rearing was conducted in climatic chambers under controlled conditions (25°C±1°C Temperature, 70%± 5% Relative Humidity, and 16:8 light:dark photoperiod. Adults were reared with a glucose-saturated solution *ad libitum*.
The larvae of I instar were housed in plastic containers. The containers' dimensions were 12.5 × 11 × 11 cm, yielding a 3.3 larvae/cm2 density. The containers were fitted with a lid containing a mesh-covered surface to allow air circulation but prevent larval escape. The larvae were reared on 300gr of liver *ad libitum* until the pupal stage.
The experiment design included two set-ups: i) specimens of III instar larvae, pupae and adults reared on contaminated substrate were unwashed, and ii) larvae, pupae, and adults were washed before virus detection by real-time RT-PCR. All experimental set-ups were performed with three replicates (n=5) for each rearing cycle. *L. sericata* and substrate were sampled at each stage change: III instar larvae, post-feeding larvae, pupae and adults in aseptic conditions. For trial two, the specimens were washed with 3ml of PBS 1X buffer solution (15 seconds at 35Hertz on a laboratory shaking) with two rinsing steps.
The insect larvae for positive controls were reared on pig livers, whereas the insect larvae for experimental exposure were reared on samples of RHDV2-infected rabbit livers. The concentration of RHDV2 in the liver and wash liquids was determined by real-time RT-PCR.

RNA extraction and real-time RT-PCR absolute quantification

For the RNA extraction, 10 µl of the liver/larvae/pupae and adult insects' homogenate (10% w/v in standard PBS1X) were resuspended in 1 ml of the TRizol reagent following the manufacturer's instructions. The RNA pellet was resuspended in 50µl of H_20 RNAse-free and 2µl amplified by real-time RT-PCR with the following primers and probe specific for the vp60 gene: RHDV2-F 5'- CCTGACGACGAATTTGTGAA -3', RHDV2-R 5'- AATCGACGTGGACGCRTTCTC -3' and FAM-CCC TGG AAC CAC AAC CGA CGG TAT GGA CCC-TAMRA.
For the standard curve, an RHDV2 fragment of 324 bp was in vitro transcribed, and the RNA concentration was determined using a NanoDrop 1000 (Thermo Scientific, USA). Tenfold dilutions series, ranging from 10^8 to 10^4 genomic copies, of *in vitro* transcribed RNA were prepared in RNAse-free water. Each dilution was tested by RT-qPCR in triplicate. The calibration curves were generated by the CFX ManagerTM Software (Bio-Rad, USA).
The RT-qPCR was performed using a CFX-96 real-time system (Bio-Rad) in a 96-well optical plate format. Amplification was carried out in 20 µl volume reactions using the One Step PrimeScript III RT-PCR Kit Kit (Takara Bio Inc) according to the manufacturer's recommendations. Optimal assay performance was obtained using using 0.2µM final concentrations of each primer and probe, respectively. Thermal cycling conditions included one cycle at 52°C for 5 min and 95°C for 10 sec for reverse transcription and 40 cycles of cDNA amplification (95°C for 5 s, 60°C for 30 s).

RESULTS AND DISCUSSION

L. sericata larvae were exposed to livers with a viral load of 10^8 to 10^9 genomic copies of RHDV2 RNA/mg of liver. During the whole two sets of study, no apparent symptoms (lethargy, behavioural changes) and no mortality were observed among the *L.sericata* larvae following exposure to RHDV2.

Using quantitative real-time RT-PCR, RHDV2 RNA was detected in 100% of the *L. sericata* larvae III instar, larvae post-feeding and pupae sampled at different days after exposure. No viral RNA was detected in the control positive and adult flies (Table 1).

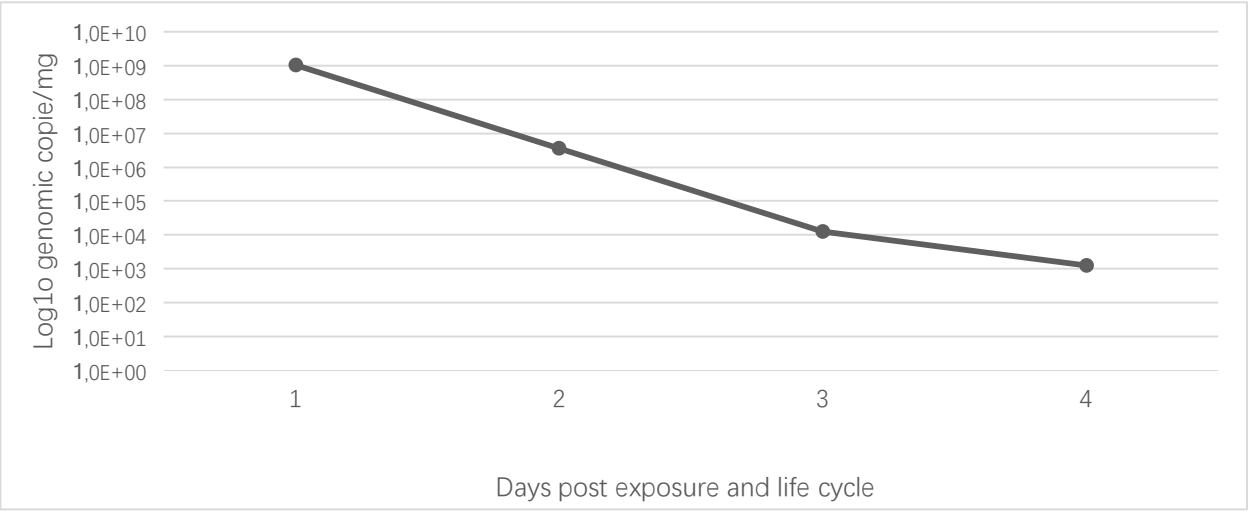
Table 1: Number of RHDV2 genome copies/mg of insects contaminated and Wash liquids (copies/µl) on different sampling days in two set-ups (Exp_1 and EXP_2).

Exp_	Days	Stage on life cycle	Pos. Control	Log10 genome copies /mg in insects	Log10 genome copies /µl in wash liquids
1	3	Larvae III instar	0	$2.4*10^9$	/
	6	Pupae	0	$1.43*10^6$	/
	14	Adults	0	0	/
	2	Larvae III instar	0	$1.07*10^9$	$1.78*10^7$
2	4	Larvae post-feeding	0	$3.6*10^6$	$3.59*10^6$
	7	Pupae	0	$1.25*10^4$	$5.6*10^4$
	9	Pupae	0	$1,23*10^3$	$5.8*10^4$
	14	Adults	0	0	0

*Means obtained from the values of the 3 replications for each sampling

In both experimental sets, the highest presence of RHDV2 RNA was found in larvae III instar and a progressive decrease in later developmental stages was observed. In the three preimaginal stages, the number of genome copies/mg varied from 10^9 to 10^3 (Figure 1). Similarly, it is noted that in the washing fluids, the number of genome copy/µl varied from 10^7 to 10^4 (Table 1)

Figure 1: Presence of RHDV2 RNA in the *L. sericata* and wash liquids during virus exposure



Although epidemiological observations had already suggested the role of adult flies as vectors of RHDV2 (Lopes et al. 2023), in this study we formally demonstrated under experimental conditions the mechanical vector capacity of RHDV2 by larvae and pupae of flies.

In fact, after feeding on the contaminated liver, RHDV2 viral RNA was detected for the entire pre-imaginal period of *L.sericata*, up to 12 days after exposure. At 9 days, pupae were positive with a lower amount of viral genome than larvae III instar and larvae in post-feeding, thus suggesting that no replication occurred. However, virus contamination and mechanical transmission role of larvae post-feeding may play an important role in virus dispersal into the habitat. Indeed, the larvae, when they start to search a pupation site, activate a dispersal behaviour known as post-feeding larval dispersal. In this movement, larvae may move several meters from the food source. In particular, *Lucilia sericata* is among the necrophagous species with a wide dispersal ability being able to move 3-8m away from the food source (Gomes et al. 2006).

CONCLUSIONS

Since this study suggests that fly larvae and pupae play a noteworthy role as mechanical vectors of RHDV2 under experimental conditions, other necrophagous arthropods such as burying beetles (Coleoptera: Silphidae) should be taken into consideration as players in RHDV2 epidemiology.

Infection and transmission experiments should be conducted with them in the field and laboratory conditions, in order to verify the existence of further and alternative vectors of RHDV2.

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MUCOID ENTEROPATHY IN FARMED RABBIT DOES

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ABSTRACT

In this study we determined mortality in rabbit females due to mucoid enteropathy /ME (similar to Epizootic Rabbit Enteropathy/ ERE), in Portugal and Spain from 1996 until 2023. We obtained our information by carrying out 10,593 visits to 1372 rabbitries. We performed 830 farm-visits due to ME in rabbits of different ages. The diagnoses were based on the clinical signs and gross post-mortem findings observed by a veterinarian attending farmed rabbits. In this 28-yr study we necropsied 4363 females on 504 farms gathered in 2101 cohorts. On a sub-set of 92 farms we found 227 does with disorders of the gastrointestinal tract compatible with ME. The mean Monthly Mortality Risk (MMR%) and 95% Binomial confidence interval (CI) in does was 0.19 (0.18–0.21%). We highlight several risk factors. First parity females were more predisposed to ME.

Keywords: Animal Welfare, Disease prevention, Rabbit female, Mucoid enteropathy

INTRODUCTION

Mucoid enteropathy/ ME of rabbits (Flatt *et al.,* 1974) is a disease characterized by caecal impaction, in the distal segment of the ileum or proximal colon, gastric dilation or mucous secretions in the colon and other parts of the intestine. This condition was described by Hurt (1949) in kits, young weaned and breeding rabbits; he called it mucoid *enteritis*. Marcato and Sja'ban (1967) pointed out that *enteropathy* was the most appropriate term because in the initial phase there was no inflammation in the intestine, although the loops were dilated. Breeders on small farms and commercial producers remember that during the 1950s-1980s, there were rabbits with a hard intestine, mucus in their droppings, and grinding teeth. From 1996 onwards there were outbreaks of a similar process: epizootic rabbit enteropathy/ERE (Licois, *et al.,* 2005).

We visited a farm affected by ME on April 16th, 1996 (50 dead females over 450 does). From 1997, there were several days of visits, for ME in kits, weaned or adult rabbits. We diagnosed ME through clinical examination and necropsy. Furthermore, since 1997, we have collaborated with analytical labs and research centers (Badiola *et al.,* 2000, Pérez de Rozas *et al*., 2005). ME was frequent in young rabbits. Thus, during 2002, in the middle of the pandemic, we performed ME necropsies on 125 weaned rabbits, 43 kits, 2 young does and 4 females (Rosell, 2003). Lund (1951) also found few affected does in a 3-yr study. ME causes severe morbidity, mortality and economic damage. Currently, there are gaps in the knowledge of this disease. ME continues to be the subject of interest, as in the review by van der Sluis *et al*. (2024). Our aims were (1) describe on-farm occurrence of ME, (2) calculate Monthly Mortality Risk in farmed rabbit females, and (3) estimate risk factors: season, age and physiological status.

MATERIALS AND METHODS

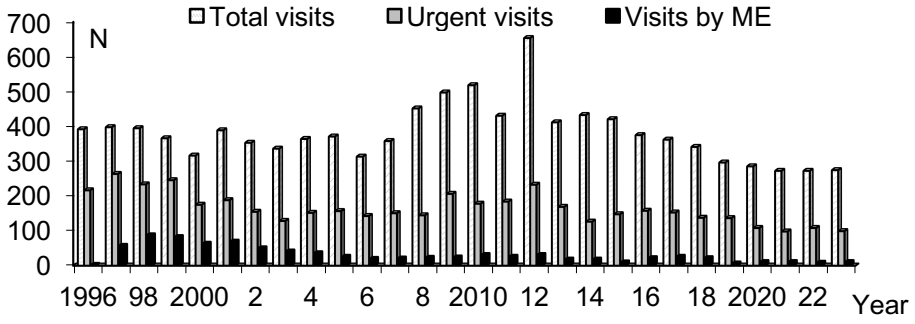
We made this retrospective study from January 1996 to December 2023 in 1372 farms visited in Portugal and Spain. The rabbitries were closed cycle, with domestic does, males (23 artificial insemination/AI centers), with males and females, or only with weaned rabbits (13 farms). In 1996, AI was used on 6% of farms visited, but >95% in 2023. In this study we

refer to visits inside the farms, with access to all the rabbits housed. The diagnostic protocol was based on clinical signs and post-mortem findings described in Rosell *et al.* (2009), besides the classification of visits, without repetitions for the same reason, to the same farm, in one month. We estimated incidence risk of ME by the mean Monthly Mortality Risk as explained in Rosell and de la Fuente (2016).

RESULTS AND DISCUSSION

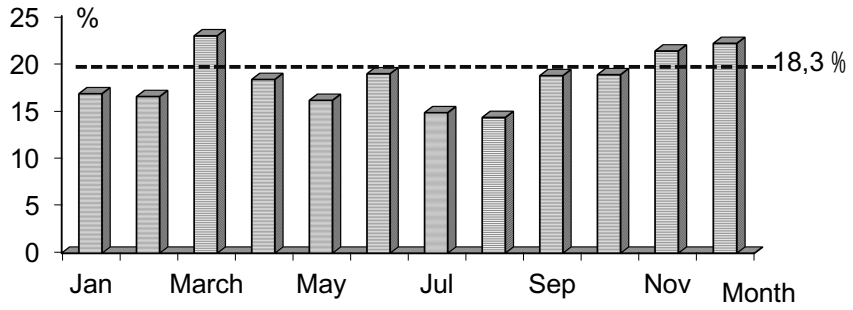
The present observational study includes the findings made by the first author in 10,593 visits. In this 28-yr study we made 6043 check-up visits without problems; 4450 (42%) were emergencies. We performed 4363 necropsies of females (504 farms, 2101 cohorts). On a sub-set of 92 farms we found 227 does with lesions compatible with ME. Concerning differential diagnosis, we visited one farm with does affected by gas gangrene (*Clostridium septicum,* confirmed), and two rabbitries with Tyzzer's disease (*C. piliforme*, confirmed). In addition, we analyzed several specimens to rule out RHD, due to post-mortem serosanguinous secretions on the snout, perhaps due to other clostridiosis, or respiratory disorder, as we show in Rosell *et al*. (2023). In the 28 years of the study, we found 22 does with cecal impaction or other intestinal obstruction, not classified as ME. In Figure 1 we show the visits made during the 28-yr of this study.

Figure. 1. Yearly distribution of 10,593 total visits, 4450 urgent visits, and 830 visits due to mucoid enteropathy; one visit monthly per farm, for the same reason.



The frequency of visits for ME was high during the years 1997-2005. Afterwards, we may accept that the ME was better controlled. However, it was cause for emergency visits until the end of this study. In Figure 2 we include a description with the proportion of ME visits (kits, weaned or adults) with monthly risk emergencies during 28 years.

Figure 2. Monthly distribution of 830 visits due to mucoid enteropathy, in relation to emergencies in each month. (1996-2023). Dotted line was the monthly mean.



There was no seasonal effect or slightly, as we had previously observed (Rosell *et al.,* 2009). Concerning ME in does, in this 28-yr study we examined 227 affected does (92 farms, 175 cohorts); 204 were dead and 23 were moribund-euthanized does. Carcasses were not frozen. The median size of the 92 farms was 725 does (minimum to maximum 126-4200 does). The mean Monthly Mortality Risk (MMR%) and 95% Binomial confidence interval (CI) in does was 0.19 (0.18–0.21%). From 2006 to 2014, the MMR due to ME was 0.11% (Rosell and de la Fuente, 2016). Besides, in the present study we have analyzed the ages. The parity number was available in a sub-set of 116 /227 females. In figure 3 we show the distribution of dead does with 1 ≥ parities. We compared it with the age distribution described in preliminary work with 50,230 lactating (live) females, randomly examined on 256 farms (Rosell *et al.,* 2023).

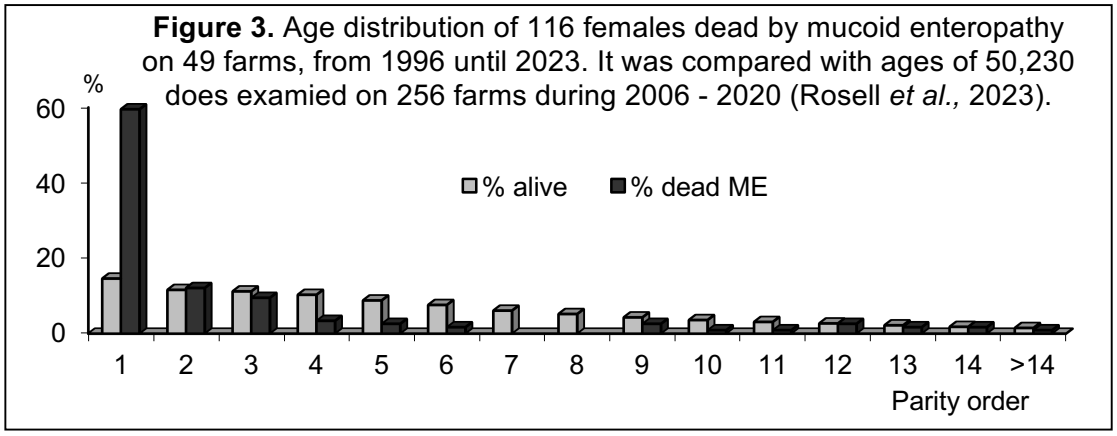


Figure 3. Age distribution of 116 females dead by mucoid enteropathy on 49 farms, from 1996 until 2023. It was compared with ages of 50,230 does examied on 256 farms during 2006 - 2020 (Rosell *et al.,* 2023).

We found 69/116 (59.5%) deaths due to ME at first lactation. This mortality risk was high, in relation to the 50,230 does mentioned, with 14.5% of primiparous. Coutelet (2015) found 13.5% of does served for the first time, by batch (769 farms). Figure 4 shows the rabbit females dead due to ME during lactation, pregnancy or both.

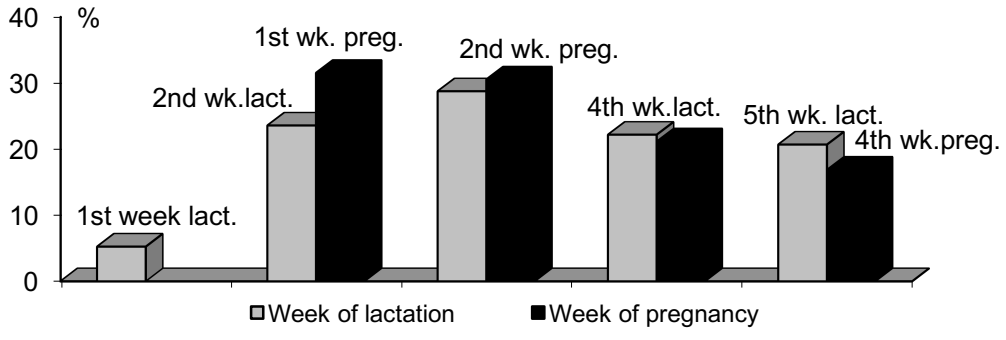


Figure 4. Proportional distribution of 136 lactating does dead by mucoid enteropathy (grey columns) and 95 dead during pregnancy (black columns). Data from 68 farms, grouped by weeks, from 1996 until 2023.

Most of the 95 pregnant does (89.5%) in figure 4 were served 11 d post-partum. There were only 2 nulliparous. For these 2 reasons, we have distributed the deaths in the 2nd week of lactation coinciding with the deaths in the 1st week of gestation. From 8th day after kindling there was increase of deaths, with a peak in the 3rd week of lactation. We agree with Hurt (1949) that females die from ME with acute or over-acute evolution, in the absence of treatment. Nevertheless this author described that mortality was especially around

parturition; in our study, we found dead females throughout lactation. In fact, mucoid enteropathy affects all stages of farmed rabbits.

In our opinion, the following factors might influence the occurrence of ME in does: a) age at first service (4.5-5.5 mo old), and other aspects of reproductive management, such as early weaning, or extensive rhythm (service ≥ 32 d post-partum), b) genetic predisposition (does from heavy lines, with high ingestion capacity), c) feeding strategy: restricted feed or *ad libitum,* d) feed composition: high protein and energy diet to enable doe production, which is antagonist with requirements of kits before weaning (Gidenne *et al.,* 2020), and with the gut health of 1st parity does, according to our opinion. We prefer a high content of dietary fiber (and digestive fiber) coarsely ground, low starch and moderate protein and fat content, for pre-weaning diet; in some cases, also for 1st lactation diet. Lastly, e) medical measures might palliate ME in females, with *prevention*: (1) antacid in water (sodium bicarbonate), (2) probiotics and, *treatment* with antimicrobials at kindling or pre-weaning period (for mother and weanlings), among others. It is likely that the problem of does dying from ME, in our practice has been underestimated due to the numerical effect of deaths in rabbits.

CONCLUSIONS

From this 28-yr analysis on 1372 commercial rabbitries, we found farms affected of mucoid enteropathy/ ME throughout the year. We performed 830 farm-visits due to ME in rabbits of all ages. There was no or weak seasonal effect on the occurrence of ME. We found 227 females with lesions compatible with ME, out of a total of 4363 necropsied. The mean Monthly Mortality Risk, MMR% (95% CI) in does was 0.19 (0.18–0.21%). Primiparous were most predisposed to ME, mainly during 2nd and 3rd week of lactation. In our opinion, it is necessary to reduce the gap of our knowledge of this disease in does, during rearing of junior does, first pregnancy and lactation. We would have to explain it to the producers and they should put it into practice.

ACKNOWLEDGEMENTS

The authors appreciate the cooperation of rabbit producers and the collaboration of several technicians and veterinarian peers during 1996–2023. Our recognition goes also to Dr Hollis N. Erb, former Prof. of Population Medicine and Diagnostic Sciences at Cornell University College of Veterinary Medicine. This paper is dedicated to them.

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ASSESSMENT OF RABBIT CORONAVIRUS (RbCoV) AND LAPINE BOCAPARVOVIRUS (LBoV) IN SPAIN: REKINDLING THE INTEREST OF VIRAL ENTERITIS AGENTS.

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ABSTRACT

Digestive problems represent a major challenge in the rabbit meat industry increasing morbidity and mortality rates in rabbitries. The complex infectious etiology concerns numerous bacteria, viruses, and parasites. Although Lapine Rotavirus is well recognized as a viral cause, there is evidence suggesting the involvement of other virus such as Rabbit Coronavirus (RbCoV), Lapine Bocaparvovirus (LBoV), and Rabbit Kobuvirus. This research aimed to assess the importance of these viruses in digestive illnesses by integrating novel molecular detection strategies and histopathologicaly (HP) studies. The statistical association between this virus and the rest of digestive infectious agents, signs of disease and HP findings was researched. RbCoV was associated ($p<0.05$) with illness status whereas LBoV detection agreed with compatible histological alterations and demonstrated relationship ($p<0.05$) with that of other primary bacterial agents such as *Escherichia. coli*, *Bacteroides fragilis* and *Clostridium spiroforme*. The results support the inclusion of RbCoV and LBoV in the diagnostic profile of enteric syndrome in the cases routinely submitted to the Exopol laboratory.

Key words: coronavirus, bocaparvovirus, enteritis syndrome, qPCR, histopathology.

INTRODUCTION

Digestive issues are one of the most significant causes of monetary losses in the rabbit meat industry. These enteric problems mainly affect young animals, including suckling and growing rabbits, and significantly increase morbidity and mortality rates in rabbitries. The infectious etiology is complex, including different bacteria, viruses, and parasites: enteropathogenic *Escherichia coli* (EPEC), *Clostridium spiroforme*, *Clostridium perfringens*, *Salmonella*, *Enterococcus hirae*, *Bacillus fragilis*, Eimeria spp., and Rotavirus(Solans et al., 2019).

The multifactorial enteropathy is known as "enteric syndrome" and notably affects rabbit intestinal tissues, causing severe diarrhea and malabsorption. The diagnosis of this illness is frequently challenging due to the variability of clinical signs and pathological lesions, the range of pathogenic agents involved simultaneously or consecutively, and the participation of conditioning factors, which often hinder quick and accurate diagnosis.

To date, only Lapine Rotavirus (Rotavirus group A) is widely known as viral primary cause of enteric disease. Nevertheless, it is commonly accepted that other enteric viruses can act as potential pathogens either on their own or by facilitating the action of other microbes. Rabbit Coronavirus (RbCoV; HKU-14) was first described in the 1980s; however, its role was never completely revealed(Lapierre et al., 1980). This is a Betacoronavirus belonging to subgroup A. Phylogenetic studies(Lau et al., 2012) of several HKU-14 strains have been reported. Additionally, rabbit parvovirus was first described in 1977(Matsunaga et al., 1977). Initially, it was considered to have very low pathogenicity potential. More recent studies(Lanave et al., 2015) described a novel lapine bocaparvovirus (LBoV) in Italian rabbits, although its role is still uncertain. Nevertheless, their widespread presence among rabbitries leads to consideration that they could potentially have any effect in combination with other viruses or bacteria. Another virus under study is Picornavirus. This virus was found in feces from

rabbits on Hungarian farms(Pankovics et al., 2016). Subsequently, the virus was classified within the genus Kobuvirus: rabbit Kobuvirus (RKoB). This virus infection often leads to enteric illness in many other mammalian species; nonetheless, its role in lagomorphs needs to be further defined. Other virus such as different calicivirus(Martín-Alonso et al., 2005), adenovirus, reovirus or enterovirus, have been previously described(Cerioli and Lavazza, 2006) and considered of minor impact. Therefore, they were not the subject of study in this research.

The aim of this study was to assess the presence of RbCoV, LBoV and RKoB, which have been generally neglected thus far, and evaluate their significance in digestive illness by combining novel molecular detection methods with histopathological (HP) studies.

MATERIAL AND METHODS

Molecular biology tests: qPCR and Sanger Sequencing

Three new real time PCR assays were developed for detection of RbCoV, LBoV and RKoB based on respective sequences reported previously. The assays utilized a hydrolysis probe labelled with FAM fluorophore, targeting RdRp gene in RCoV and Kobuvirus, and VP1 in LBoV. Simultaneously, the detection of a housekeeping gene within the host cells, using the HEX fluorophore, served as an endogenous control.

The presence of these 3 viruses was determined in a limited collection of samples originated from diverse animals suffering from enteric illness (n=60) and fattening animals without apparently signs of disease (n=30). Statistical relationships between the presence of each virus and health status were studied through Chi square test.

The samples encompassed rectal swabs, intestinal tissues or feces. Four samples which had resulted RCoV positive were selected for RdRp gene sequencing in order to confirm the specificity of the qPCR test. The product of amplification of 440 base pairs was generated with primers and protocol adaptated from previous publication(Lau et al., 2012). Additionally, the genetic variability of RCoV strains was studied through S1 gene from 5 RCoV positive samples following an adapted protocol and primers described for Bovine Coronavirus(Martínez et al., 2012). Similarity of the sequences were studied through multiple sequence alignment (MAFFT; version 7) and matrix generated with Sequence Demarcation Tool software (version 1.2).

Histopathological studies

Furthermore, 6 outbreaks with clear signs of enteric disease were studied under histopathology and qPCR techniques (RCoV, LBoV and Rotavirus A). Each outbreak included jejunum or ileum of 5 growing animals, with a total of 30 animals studied. Basically, four types of tissue alterations were categorized: a) atrophy and fusion of intestinal villi; b) vacuoles in enterocytes, c) citomegaly and d) depletion of Peyer's patches. The enteric HP diagnosis focused two different processes particularly linked to viral infection: NRACE non-regenerative atrophic catarrhal enteritis (a+c), mostly related with parvovirus infection and RACE regenerative atrophic catarrhal enteritis (a) which is usually caused by coronavirus and rotavirus.

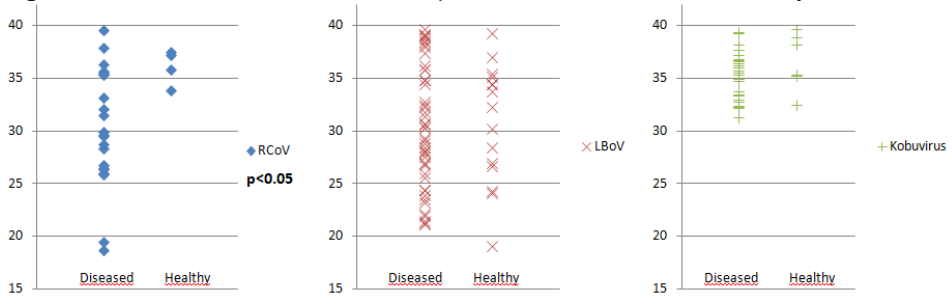
Spanish epidemiology survey

The epidemiological situation of RCoV and LBoV in Spanish rabittries was investigated through the analysis of 1359 enteric samples submitted to the laboratory, with limited clinical information provided. The samples belonged to adult (n=107), growing (n=885) and suckling (n=233) animals; 133 samples had unknow ages. The collection originated from 1201 outbreaks occurring between 2020 and 2024, which were reported from 440 different farms located in 39 diverse Spanish provinces. The complete profile of infectious agents described before was tested in each sample by qPCR using EXOone kits series (Exopol). The normality of Cq values obtained for RCoV and LBoV was tested using Saphiro-Wilk test. The statistical associations between the presence of both viruses, and also between any of them with other agents such as enterotoxigenic *Bacteroides fragilis*, eae *E.coli*, and *C. Spiroforme*, were assessed by the Chi-square test.

RESULTS AND DISCUSSION

RCoV, LBoV and Kobuvirus were respectively detected in 28%, 63% and 30% of the samples from limited collection (n=90). The Cq values ranged from 18 to 38 for RCoV and LBoV, whereas they varied from 32 to 38 for Kobuvirus. Statistical association between presence of the virus and disease status was determined only for RCoV ($p<0.05$).

Figure 1. RCoV, LBoV and Kobuvirus Cq values from diseased and healthy animals from validation collection.



The cutoff Cq value was initially defined in 38, however, decreasing values (38, 35, 32 and 30) were tested to search for statistical significance. RCoV unvariably obtained very significative results ($p<0.01$), while the statistics for LBoV and Kobuvirus did not achieve significance. These results would strengthen the pathogenic role of RCoV in enteric disease, warranting further investigation of this virus. Furthermore, LBoV was widely detected, with remarkably low Cq values in some cases. Although its p-value was >0.05 its assessment through histopathology was considered pertinent. On the contrary, the non significant and minor presence of Kobuvirus coupled with its very low viral titters discouraged its consideration within the enteric syndrome.

The RdRp gene sequences obtained confirmed the specific detection of the RCoV qPCR test. Additionally, five sequences of the S1 gene were obtained, showing nucleotide identity values ranging from 70% to 85%. These values indicate a wide genetic diversity and suggest a long evolution of the virus since its introduction long ago.

The histopathological findings revealed characteristic lesions of enteric disease in the 6 outbreaks studied. The qPCR detection of the virus and histopathological descriptions are summarized in Table 1. Interestingly, animals from 5 out of 6 outbreaks showed compatible lesions with NRACE, which is consistent with the widespread detection of LBoV, suggesting its potential infectious cause. Moreover, in all cases except one either RCoV or Rotavirus A was found and would have been characterized as RACE.

Table 1. HP description and presence of the studied virus through qPCR.

Case	qPCR detection	HP description
1	RCoV, LBoV, Rotavirus A	NRACE
2	RCoV, LBoV	NRACE, RACE
3	RCoV, LBoV, Rotavirus A	NRACE, RACE
4	RCoV, LBoV	NRACE, RACE
5	LBoV, Rotavirus A	NRACE, RACE
6	LBoV, Rotavirus A	RACE

The epidemiological study of Spanish rabbitries demonstrated a wide presence of RCoV and LBoV, with detection rates of 23% and 51%, respectively. Notably, these findings corroborate previous observations made through electron microscopy(Cerioli and Lavazza, 2006) and serological techniques(Deeb et al., 1993), underscoring the presence and spread of these viruses within Spanish rabbit population. Growing animals showed remarkably higher detection rates for both RCoV and LBoV compared to adults or suckling rabbits, as illustrated in Figure 2. The Cq values for both viruses ranged from 17 to 38, with a median of 29. None of them resulted in $p > 0.05$ in the Shapiro-Wilk test, indicating a non-normal distribution of their values.

The prevalences of the rest of the tested agents from the enteric syndrome are showed in figure 3.

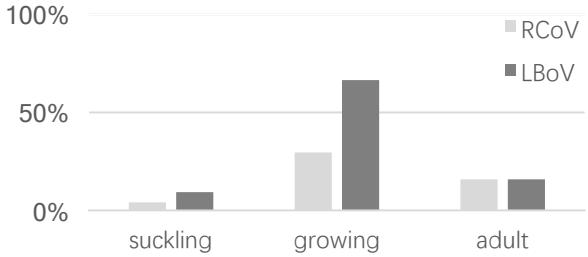


Figure 2. Rate (%) of detection of studied virus within different age groups.

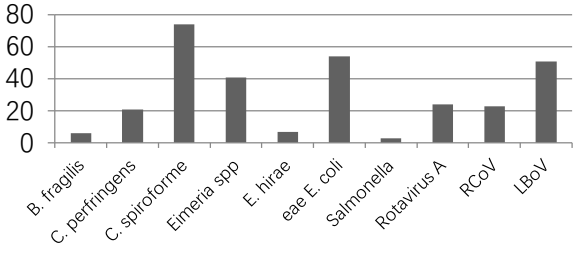


Figure 3. Rate (%) of positive cases of each agent studied within enteric syndrome, (n=1359).

Significative association ($p<0.05$) was found between the presence of RCoV and LBoV. In addition, RCoV also presented association with eae *E. coli*. Curiously, LBoV revealed statistic relationship with *B. fragilis*, eae *E. coli* and *C. spiroforme*. These findings could describe a facilitating role of LBoV for the further invasion of other bacteria described in the enteric syndrome.

This research has demonstrated the frequent infection of RCoV and LBoV in commercial rabbit farms in Spain. The association of RCoV with enteric disease and the description of compatible lesions in its presence could indicate its primary role in causing illness. LBoV, on its part, shows considerably high prevalences, compatible HP findings, and statistical association with other pathogens with accredited roles in enteric syndrome. All these reasons clearly suggest the inclusion of both viruses as part of the digestive profile diagnostics.

Future research should focus on elucidating the pathogenic mechanisms of RCoV and LBoV, and evaluating the efficacy of control strategies such as regular or autogenous vaccines and biosecurity measures.

CONCLUSION

Molecular and histopathological evidence leads us to consider RCoV and LBoV as part of the infectious causes of enteric syndrome and include them in the diagnostic enteric profile for rabbits.

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WHAT'S IN THE FIELD? DESCRIPTION OF SOME ITALIAN MYXOMA VIRUSES AND IN-DEPTH MOLECULAR CHARACTERISATION OF THE "BORGHI" VACCINE STRAIN

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ABSTRACT

One of the best-studied examples of the coevolution of pathogen virulence and host resistance is represented by the Myxoma virus (MYXV) and the European rabbit (*Oryctolagus cuniculus*). In this susceptible host, MYXV causes myxomatosis, an endemic disease occurring in two clinical forms: the respiratory form (amyxomatous or 'atypical' myxomatosis) is more frequent in farmed rabbits, whereas the "typical form" causes a high mortality rate in wild rabbits. In countries where myxomatosis is endemic, its spread is commonly controlled thanks to vaccination with attenuated viral strains.

To identify gene variations of field and vaccine strains that may be responsible for changes in virulence, immunomodulation, immunoprotection, and host range determination, we sequenced the genome of the "Borghi" vaccine strain and 13 MYXV Italian field strains using NGS techniques. We did an in-depth description of the vaccine strain "Borghi". Also, we tried to characterise some field strains (referable to typical and atypical forms) circulating in different Italian regions in a time range from 2010 to date. Furthermore, among the characterised samples, one strain dates back to 1992. Like the Borghi strain, the MYXV-1992 strain seems a Californian-like MYXV. Many of the field strains were affected by several mutations that resulted in aminoacid substitution or deletion. Borghi strain revealed the complete deletion of 16 ORFs and truncation of almost 11 other ORFs. As already described in the Californian-like strains, the Borghi and MYXV-1992 strains show, immediately after the left TIR, a partial inverted duplication of some genes typically only located in the part preceding the right TIR.

Key words: MYXV 1, Italy 2, NGS 3, field strain 4, vaccine strain 5.

INTRODUCTION

Myxoma Virus (MYXV) (family Poxviridae; subfamily Chordopoxvirinae; genus Leporipoxvirus) originally evolved in the Americas. Indeed, two geographically distinct strains of MYXV have been identified: the South American virus, which circulates in *Sylvilagus brasiliensis*, and the Californian virus, which circulates in *Sylvilagus bachmani* (Kerr et al., 2013). Each virus is highly adapted to its host, causing a benign cutaneous fibroma at the inoculation site. Still, both types of MYXV infect the European rabbit (*Oryctolagus cuniculus*), causing myxomatosis, an endemic and highly lethal viral disease with high mortality rates (<80%) (Fenner, 1959). MYXV genome contains a large linear double-stranded DNA, consisting of 170 genes (158 ORFs), 12 of which diploid in terminal inverted repeats (TIRs) (Cameron et al., 1999). A characteristic brick-shaped virion contains the genome, and viral replication occurs exclusively in the cytoplasm of infected cells (Duteyrat et al., 2006). Different types of vaccines are used to protect farmed, laboratory or pet rabbits from myxomatosis. To date, in Italy, the registered vaccines are the trivalent *Nobivac Myxo-RHD Plus*® (Intervet International B.V., Boxmeer, The Netherlands) (Reemers et al., 2020) and two homologous vaccines, *Dervaximyxo SG 33*® (Boehringer Ingelheim, Animal Health Italy,

Milano, Italy), and *Cunivax Myxoma*® (Fatro, Ozzano Emilia (BO), Italy) based on the use of strains SG33 and Borghi, respectively.
This study aims to obtain the complete genome sequence of MYXV strains from different regions of Italy identified from 2010 to date. Among the selected samples, one strain dates back to 1992. Moreover, a deep study was conducted on the Borghi vaccine strain to understand its differences compared to the reference strain MYXV-Lausanne (KY548791, MYXV-Lu) and to field strains. This study will provide us with preliminary data on the evolutionary situation of MYXV in our country.

MATERIALS AND METHODS

Homogenate preparation, viral DNA extraction, molecular diagnosis

Tissue samples were homogenised in PBS 1X using the Tissue Homogenizer Precellys S24 (Bertin Corp, Rockville, USA), obtaining a sterile solution of 10% w/v. The viral DNA was extracted from 50µl of homogenate, using DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy) according to the manufacturer's instruction and indagated by PCR for a portion of approximately 470bp of the *M071R* conserved gene as previously described (Cavadini et al., 2010; WOAH 2021).

Whole genome sequencing, phylogenic analysis, recombinant detection

The sequencing library was obtained with the DNA Prep (M) Tagmentation Library Preparation kit (Illumina) and sequenced on the Illumina MiniSeq platform (2 x 150 bp). All bioinformatics analyses were conducted on the Galaxy platform (Galaxy community, 2022). The quality of the reads was assessed using FastQC (Galaxy Version 0.74+galaxy0) and trimmed using Trimmomatic (Galaxy Version 0.39+galaxy0). Reads were assembled *de novo* using the SPAdes assembler (Bankevich et al., 2012) (Galaxy Version 3.15.4+galaxy1), using default parameters. The contigs containing MYXV genomic DNA were identified using Bowtie 2 (Galaxy Version 2.5.0+galaxy0) and ordered into a single genome sequence against the Myxoma virus isolate Lausanne genome (KY548791, MYXV-Lu) using Minimap2 (Galaxy Version 2.26+galaxy0) and mapper (Galaxy Version 0.5.2). Genome annotation was transferred from the MYXV-Lu to the newly sequenced MYXV genomes using GATU (https://4virology.net/virology-ca-tools/gatu/). The obtained sequences had been aligned with a dataset including 18 sequences of MYXV retrieved from GenBank using MAFFT (Galaxy Version). Manual editing was performed using BioEdit software (version 7.0.) (Hall, 1999). The phylogeny was estimated using maximum likelihood (ML) analysis. Maximum Likelihood (ML) analysis was performed in MEGA v.6 (Kumar et al., 2016) using the GTR statistical model with gamma distribution (G). The robustness of the clusters was assessed by performing 500 bootstrap replicates, and branches with bootstrap values exceeding 70% were grouped together.

RESULTS

We sequenced the complete genomes of the Borghi vaccine strain and 13 MYXV field strains. Borghi is derived from the Californian MSD strain attenuated to the point of complete loss of pathogenicity (Cancellotti, 1985; Saito et al., 1964). The field strains selected for the study had come to our laboratory for diagnostic purposes following suspicion of myxomatosis (typical or atypical); they were all collected between 2010 and 2024, except for one isolate dated 1992. We annotated the various genomes using the GATU annotation transfer software from the Lausanne strain (KY548791, MYXV-Lu). Each genome was annotated with MYXV-Lu as a reference and other strains retrieved from GenBank, such as Californian-like strains MSW (KF148065) and MAV (KP723391), the latter is a vaccine strain (Braun et al. 2017). In this way, we compared the putative MYXV proteins of our strains, where present, with known ones.
The genomes of vaccine strain Borghi and field strain MYXV-1992 revealed the most differences among the sequenced strains. They revealed severe truncation and deletions of several ORFs. The Borghi genome resulted in 144,100 bp, and the MYXV-1992 strain genome 155,614 bp, i.e., the genomes were respectively 17.68 Kb and 6.2 kb shorter than MYXV-Lu's. In the Borghi strain, this was due to genome TIR left deletion spanning from part

of ORFs M006 to part of M010, and TIR right deletion spanning from an inner genomic region that includes some residual nucleotides of a putative non-coding *M154L* gene to a TIR genomic region coding for a putative truncated protein M006. Indeed, a large deletion was also present in the central part of the genome, spanning from residual nucleotides of *M134R* to residual nucleotides of *M141R* genes. As already described in the Californian-like strains, the Borghi and MYXV-1992 strains showed, immediately after the left TIR, a partial inverted duplication of some genes typically only located in the region close to the right TIR.

DISCUSSION

Compared with MYXV-Lu, the Borghi vaccine has only 142 ORFs, of which 11 encode for hypothetical truncated proteins. In Table 1, we compared putative Borghi and MYXV-1992 strains' immunomodulatory proteins with homologs in MYXV-Lu and the Californian-like vaccine strain MAV.

Many of the other field strains sequenced in this study were affected by several mutations that resulted in aminoacid substitutions or deletions. In general, all those strains are similar to MYXV-Lu, and in a phylogenetic analysis (data not shown), they were all allocated in the MYXV genome cluster, which groups together European and South American sequences and is distinct from the Australian sequences. Our samples differ in at least three distinct sub-clusters. One of the clusters is close to the MYXV-Lu-like sequences, another is close to a grade 5 Spanish strain-like sequences, and the other cluster groups only our strains and could be putatively named "Italian-like" cluster.

CONCLUSIONS

The results of this study provided us with preliminary data on the evolutionary situation of MYXV in our country. Knowing the variability and/or the evolution of Myxovirus strains in an open population of wild and domestic rabbits is of utmost importance in helping to define the best strategies of intervention and control of the disease and possibly to address the choice of optimal strains for the development of new attenuated vaccines.

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Table 1: Comparison between Borghi and MYXV-1992 strains putative immunomodulatory proteins with homologs in MYXV Lausanne and MAV

Gene	Predicted/Inferred function	MYXV-LU Protein Size	BORGHI protein predicted Size	% Protein Similarity to Lausanne strain		
Group 1: MYXV proteins with anti-apoptotic functions				BORGHI	MYXV-1992	MAV
a: Inhibition of pro-apoptotic molecules						
M002L/R	Tumor necrosis factor receptor (TNF-R) homolog	326	326	100	100	100
M011L	apoptosis regulator M11L	166	166	79.0	79.0	68.1
M146R	VACV N1L homolog/Bcl-2-like fold	108	108	89.8	89.8	100
b: Inhibition by protein-protein interactions by viral ankyrin repeat proteins						
M000.5L/R	E3 Ub ligase	72	72	100	100	100
M148R	Putative E3 Ub ligase	675	498	72.9	72.6	28.7
M149R	Putative E3 Ub ligase		262	52.9	52.9	-
M150R	E3 Ub ligase; NFκB inhibition	494	492	98.6	97.6	-
c: Inhibition of apoptosis by enhancing the degradation of cell proteins or down-regulating immune receptors.						
M004L/R	Apoptosis regulator	237	199	81.4	81.4	100.0
M143R	RING-E3 Ub ligase	234	234	99.1	97.9	97.4
M153R	E3 Ub ligase/MHC-1 down-regulation	206	191	91.3	-	-
d: Inhibition of apoptosis by blocking host Protein Kinase R (PKR)						
M029L	IFN resistance; VACV E3L homolog	115	115	100	100	100
M156R	Interferon resistance. eIF2α homolog	102	-	-	-	-
Group 2: MYXV serpins that inhibit cellular pro-inflammatory or pro-apoptotic proteases						
M008.1L/R	Secreted serpin	369	-	-	-	-
M151R	SERP-2	333	260	77.8	70.5	-
Group 3: MYXV proteins interfering with leukocyte chemotaxis						
M001L/R	Secreted chemokine binding protein	260	109	41.9	41.9	100
M007L	Gamma IFN receptor homolog	263	-	-	100	-
M007R	Gamma IFN receptor homolog	263	-	-	100	100
M104L	Potential immunomodulatory protein?	53	53	100	100	100
Group 4: MYXV proteins that interfere with leukocyte activation						
M013L	Pyrin domain/inflammasome	126	112	69.5	69.5	69.5
M121R	EV glycoprotein/NK receptor homolog	176	176	94.3	94.9	100.0
M122R	EV glycoprotein/NK receptor homolog	172	171	96.5	96.5	96.5
M128L	CD47 homolog	281	281	97.2	96.8	96.8
M154L	Down -regulation of NF-κB? VACV M2L orthologue	214	-	-	-	-
Group 5: MYXV proteins with sequence similarity to HIV proteins						
M129R	Predicted HIV gp120 homolog. function	136	136	94.1	96.3	93.4
M130R	Predicted HIV Tat homolog; virulence	122	122	98.4	81.9	81.9
Group 6: MYXV proteins with other immune function						
M010L	Epidermal growth factor-like protein	85	83	87.1	87.1	100.0
M135R	Immunomodulatory protein		-	-	87.8	100.0
M144R	VACV B5	300	298	88.7	89.3	99

SEEKING CORRELATES OF PROTECTIVE IMMUNITY FOLLOWING VACCINATION AGAINST MYXOMATOSIS

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ABSTRACT

Despite the use of vaccines, myxomatosis is a recurrent blight on Spanish rabbit farms. Routine serological monitoring procedures following vaccination are essential to help establish suitable control measures and provide information regarding immune status. Here we describe the development of serological tests that could be used for the detection of anti-MYXV antibodies based on recombinant proteins. A panel of 259 rabbit sera were characterized for the presence or absence of anti-myxoma virus (MYXV) antibodies using a commercial ELISA. The sera were then analyzed using optimized indirect ELISAs based on the recombinant MYXV proteins M022L and M115L. Results indicate that such tests may provide useful complementary analysis for the serological evaluation of vaccinated farm animals. The detection of specific anti-MYXV antibodies may help decipher the variability of individual rabbit immune responses and in-turn provide useful information on the identification of specific markers protective immunity.

Key words: Myxoma virus, myxomatosis, vaccine, rabbit, protection, immunity.

INTRODUCTION

Myxomatosis has been endemic in Spain since the 1950s and continues to be a high prevalence disease with high mortality in both industrial domestic and wild rabbits (Rosell et al., 2019). Caused by myxoma virus (MYXV) it is a deadly disease which causes respiratory or systemic infection and immune suppression (reviewed in Kerr et al., 2015). In wild rabbits the latest data suggest that 60% have antibodies against the causative agent (Camacho-Sillero et al., 2022) providing an uncontrollable natural reservoir. The disease remains a serious challenge to rabbit farmers despite the availability of effective live attenuated vaccines including homologous (attenuated MYXV strains) and heterologous (Rabbit (Shope) fibroma virus) vaccines and, the more recently developed (Barcena et al., 2000; Torres et al., 2001; Spibey et al., 2012), recombinant homologous bivalent strains (MYXV expressing rabbit hemorrhagic disease virus (RHDV) capsid protein).

Vaccines against MYXV show high efficacy, however, in spite of carrying out extensive control programs, myxomatosis is a recurrent blight on Spanish rabbit farms. Our previous analysis show that a high percentage of rabbits that receive a vaccine dose subcutaneously do not produce anti-MYXV antibodies and are not protected from disease. Even animals that do produce anti-MYXV Abs may be susceptible (Dalton et al., 2015). Monitoring the serological status of farmed rabbits after vaccination is a recommendable procedure when establishing appropriate control measures. Commercially available serological diagnostic tests (ELISA) use complete virus as antigen for antibody detection. The production of this antigen is expensive and requires specialized facilities, under adequate biological safety conditions. In this study we test the use of two recombinant viral proteins with antigenic character for the detection of specific anti-MYXV antibodies in rabbit sera and compare the results to a commercially available gold standard anti-MYXV antibody detection ELISA. The use of recombinant proteins as antigens would reduce biological safety risks and be cheaper to produce than conventional whole virus based ELISA. In addition, the identification of antibodies against specific MYXV proteins may provide biologically relevant information regarding immune status.

MATERIALS AND METHODS

Sera A total of 259 rabbit serum samples were tested by the commercial iELISA test (INgezim Mixomatosis, Ingenasa S.A). The outcomes derived from this analysis determined the serological status of each sample and categorized them into either positive or negative based on the presence or absence of antibodies against MYXV, respectively. The sera analyzed had been previously generated in experimental vaccinations and subsequent infections of rabbits with MYXV Lausanne. These experiments were approved by the local authority in accordance with the Spanish Government guidelines (Royal Decree 1201/2005) and the European Community Guide for the Care of Animals (Council Directive 86/609/EEC).

Production and purification of recombinant viral proteins Protein expression was carried out in Sf9 insect cells using recombinant baculoviruses generated to express the MYXV M022L and M115L genes following standard protocols as previously described (Dalton et al., 2021). Transfer vectors pTri-GST-m022l-His and pTri-m115l-His were used for the generation of recombinant baculovirus (rBaV) as described in Zhao et al., (2003). Recombinant viral proteins were purified from the soluble fraction of the cell lysate by affinity chromatography (GST and His-tag).

Indirect ELISA for antibody detection against antigenic proteins of MYXV Wells of 96-well plates were coated overnight with purified rM22 (rM22-iELISA) or rM115 (rM115-iELISA). An optimized procedure was developed for each ELISA using HRP conjugated anti-rabbit secondary Ab and development with TMB. The OD of each well was measured at 450 nm using a Varioskan® Flash plate reader (Thermo Scientific), driven by the SkanIt Software (Thermo Scientific).

The mean blank OD_{450} value was subtracted from the control and samples raw OD_{450}, and the resultant values were expressed as a relative OD percentage (OD %) using the formula:

$$OD\ \% = (OD.S - \bar{x}\,OD.NC)/(\bar{x}\,OD.PC - \bar{x}\,OD.NC) \times 100$$

where OD.S is the OD of any sera sample; $\bar{x}$ OD.NC is the mean OD of the negative control replicates; $\bar{x}$ OD.PC is the mean OD of the positive control replicates.

Statistical Analysis Statistical analysis was carried out at the University of Oviedo´s Statistical services. To optimal cut-off value for each recombinant iELISA was estimated using the Youden Index, maximizing the difference between the diagnostic sensitivity (Se) or true positive rate, and the 1-Specificity (Sp) or false positive rate. Through a receiver operating characteristic (ROC) curve analysis of the data.

RESULTS AND DISCUSSION

Analysis of sera using a commercial ELISA: A panel of 259 rabbit sera were analyzed for the presence of anti-MYXV antibodies using the gold standard indirect ELISA kit (Ingenzim). The results obtained using this test were the following: 155 sera were positive and 104 were negative. These sera had been previously generated in experimental vaccinations and subsequent infections of rabbits with MYXV Lausanne. In previous studies we have observed variation in the anti-MYXV antibody response following these different methods of vaccine application (Dalton et al., 2015), with immune response following SC vaccine application more variable than that observed after ID vaccine application. The sera used in our panel thus came from vaccinations which had been applied subcutaneously or intradermically. Of the 259 sera analyzed 132 sera corresponded to animals vaccinated via the SC route, while 38 the ID route and the remaining 89 were non-vaccinated (Figure 1A). The variation in OD responses following SC vaccination is observable in Figure 1B.

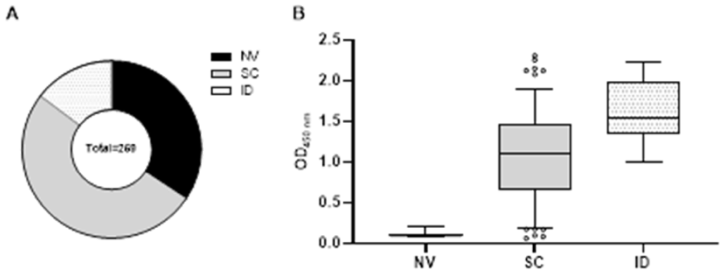

Figure 1 A Categories of the 259 rabbit sera in a donut chart based on their route of vaccination. Non-vaccinated (NV) (n=89) sera from non-vaccinated animals, SC (n=132) sera from animals vaccinated via subcutaneous and ID intradermal (n=38) routes. B) INgenizm test absorbance obtained for serum samples from NV and vaccinated (SC, ID) animals, expressed as OD_{450} values.

Indirect ELISAs using recombinant proteins: After optimizing the assay conditions for each of the recombinant proteins (data not shown), the same 259 rabbit sera were analyzed with the rM22-iELISA and the rM115-iELISA, respecting the particular conditions of each assay. The results obtained are compared with the commercial ELISA (Tables 1 and 2).

Table 1. Results of rM22 iELISA analysis of rabbit sera. The columns represented the two outcomes of the reference test (Ref. test) and the rows represented the outcomes of the iELISA-rM22 test. The table contains the frequency counts of samples corresponding to each combination of outcomes.

		Ref. test		
		Positive	**Negative**	**Total**
iELISA-rM22	**Positive**	117	14	131
	Negative	38	90	128
	Total	155	104	259

Table 2. Results of rM115 iELISA analysis of rabbit sera. The columns represented the two outcomes of the reference test (Ref. test) and the rows represented the outcomes of the iELISA-rM115 test. The table contains the frequency counts of samples corresponding to each combination of outcomes.

		Ref. test		
		Positive	**Negative**	**Total**
iELISA-rM115	**Positive**	149	30	179
	Negative	6	74	80
	Total	155	104	259

Results from the rM22-iELISA showed a fair correlation with the commercial gold standard test, with however, 14 negative sera gave false positive results. Of the 155 sera positive for global anti-MYXV Abs, 38 were negative for anti-M22 Abs. The rM115 ELISA showed a higher number of false positive results (30) while of the positive anti-MYXV sera, 6 were negative for Abs against M115. Analysis of these results considering the breakdown in to the different categories of sera, i.e. non-vaccinated, SC or ID vaccination allowed a more precise explication. While 1 serum from the ID group was negative for anti-M22 antibodies, 13 sera from SC vaccinated animals were negative for M22 antibodies. For the rM115-iELISA the SC group showed 5 false positive sera while 5 sera were negative for anti-M115 antibodies.

Comparative analysis our ELISAs and the commercial ELISA: The comparison of the results obtained from the ELISA tests based on recombinant proteins developed in our laboratory with those of using whole virus as antigen in the commercial system show promise, however, improvements are required. The relatively high number of false positives needs to be addressed before this system can be seen as an alternative to the gold standard that requires whole virus as antigen. We envisage the inclusion of more recombinant proteins as one option. MYXV virions are highly complex and the use of a small number of viral proteins may not be sufficient to adequately detect anti-MXYV antibodies. Our future studies will be focused on incorporating further antigens and testing combinations of these antigens to improve the potential of recombinant protein based ELISAs for the detection of MYXV antibodies.

Our study once again demonstrates that there is greater variation in the global Ab response when vaccination is applied via the SC route. The contents of individual sera vary greatly following vaccination by this route. We are currently studying the ability of recombinant antigens to discriminate sera based on antibodies to individual antigens in the hope of discerning the elements required for protective immunity.

CONCLUSIONS

ELISAs based on the recombinant proteins rM022L and rM115L could be used to carry out evaluations of the serological status of farm rabbits, but it is necessary to further optimize the tests to improve sensitivity and specificity. These ELISAs may be the basis of future tests that can detect correlates of protection.

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SURVEY ON VACCINATION PRACTICES IN RABBIT BREEDING IN FRANCE

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ABSTRACT

A survey on the vaccination of French rabbit herds was carried out in 2023 among 12.18% of farmers in 21 departments and 9 producer groups. It covered 75 questions relating to the diseases to be protected against, the vaccines, their administration, the handling of the animals, the equipment used, the vaccination method and working conditions. Several critical points emerged, and these will be the subject of instructions to farmers to help them improve their work.

Key words: Rabbit, *Oryctolagus cuniculus*, vaccination practices.

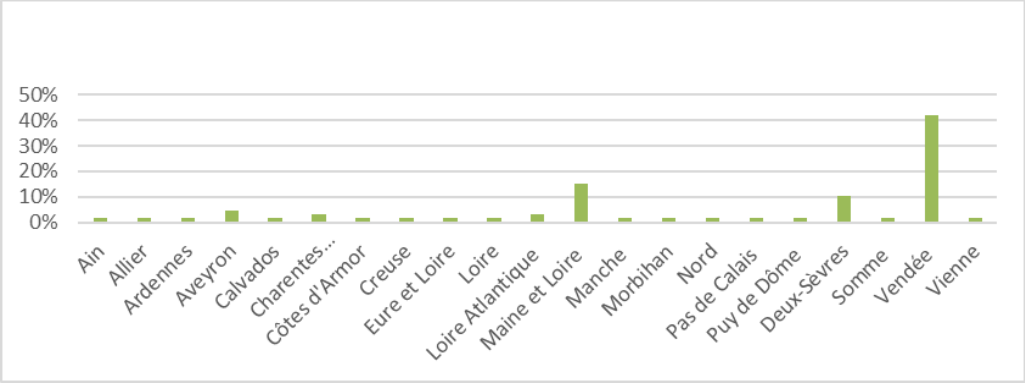
INTRODUCTION

When prescribing medication, as a veterinary surgeon your job is to ensure that the breeder complies with the instructions on the prescription. This is even truer for vaccines, where using the wrong dose or route of administration can have a major impact on vaccine uptake. We have therefore sought, by means of a survey based both on declarations and on observation of vaccination sites on farms, to understand how a vaccination session is carried out so that we can provide useful advice to rabbit farmers to help them progress in this task.

MATERIALS AND METHODS

French rabbit production ranks 3rd in the world behind China and Italy. France had 415 000 potentially vaccinated breeding rabbits in 2021 (ITAVI 2024). These rabbits are kept on 550 professional farms, mainly in the west and north of the country.
Our survey was carried out on 67 farms (12.18% of the total) spread over 21 departments and 9 producer groups.

Figure 1: percentage of breeders by department



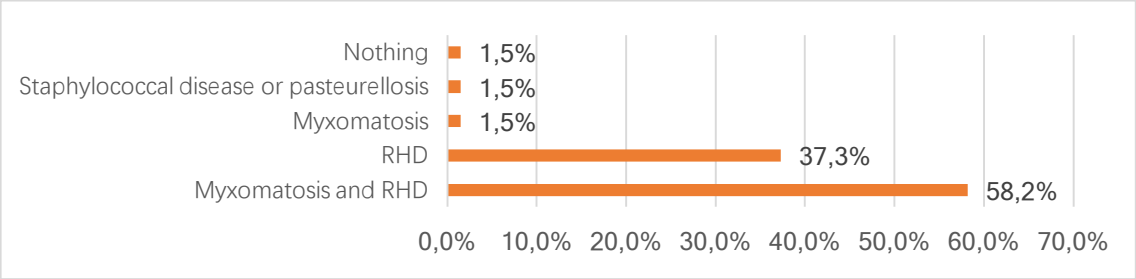
The survey covered several points divided into 75 questions. Firstly, the farmer was questioned by 5 interviewers working in pairs (1 veterinary surgeon and 1 non-veterinary interviewer trained in the survey) about the diseases he was vaccinating against, and his answer was correlated with the presence of a prescription confirming his statements. He was then asked how he prepared his vaccination session. He was then asked about the equipment used and the vaccines used, as well as their storage conditions. Finally, part of the questionnaire looked at how the tools and animals were handled, and what happened to

the waste. In half of the cases, the interviewers were present during the vaccination. For another half, the survey was purely declarative, as the visit could not coincide with a vaccination date.

RESULTS AND DISCUSSION

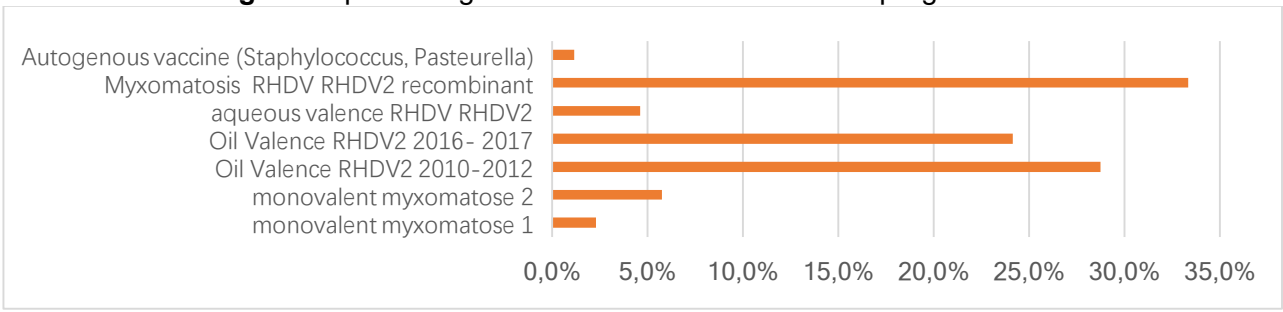
Disease and type of vaccine used.
In our survey of 67 farms, only 1.5% did not vaccinate, 1.5% vaccinated only against myxomatosis, one farm vaccinated against staphylococcal disease or pasteurellosis, 37.3% only against RHD and 58.2% against RHD and myxomatosis.

Figure 2: number of farmers by type of vaccination



The most widely used vaccine is a recombinant vaccine including myxomatosis, RHDV and RHDV2/ 2010-12. It is followed by oily vaccines against RHD including either the RHDV 2010/2012 strain or the more recent 2016/2017 strain.

Figure 3: percentage of use of each vaccine in the programs



99% of farmers told that they followed the prescribed dose when vaccinating their breeding rabbits. On the other hand, when it concerned to growing rabbits intended for meat production, it is common (30%) for the breeder to inject only between 50 and 80% of the prescribed dose for economical reasons.

Animal conditioning
Among the 67 breeders, 84% regularly deworm their rabbits using a medicine and a protocol recommended by the vet. 16% never deworm their rabbits. Fewer farmers administer a nutritional supplement (vitamin C, vitamin E) at the time of vaccination to help the rabbits take up the vaccine: 12% do so and 88% do not.

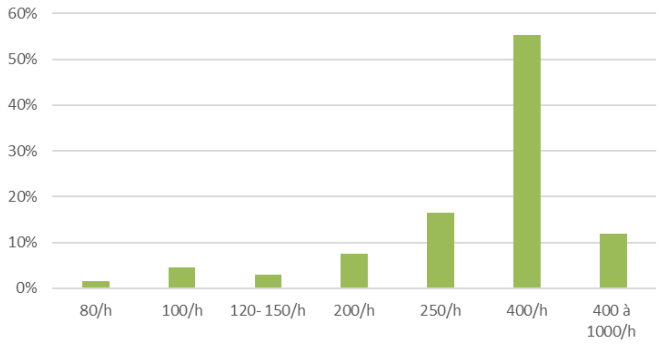
Equipment
All farmers do have a refrigerator, but only 76% use it only to store the farm's vaccines. 78% have a minimum/maximum thermometer, but only 5% check the values regularly.

The majority have automatic syringes (79%) with handles and vial holders (52%), straight metal syringes (9%) and often plastic syringes supplied by the vaccine manufacturer (18%). 21% of farmers use single-use syringes, and 21% of them reuse them from one site to the next after disinfecting them. The few farmers who need it (8%) have a device for intradermal injections, but note the difficulty of maintained it serviced, due to the lack of a repairer. The equipment is disinfected with alcohol.
Most farmers use single-use needles (73%) but inject several hundred rabbits with the same needle before throwing it away. The others (23%) use disinfected needles which they throw away "when they sting badly". The vast majority use needles that are 1 cm long (maximum 1.5 cm) and 1 mm wide, particularly for oily vaccines.

Vaccine administration
To do this, 94% of farmers tell they wash their hands (or use hydroalcoholic gel) before starting vaccination. 3% used gloves and 3% did not wash their hands.
No farmer reused an aqueous vaccine that had been reconstituted more than 4 hours previously, but 22% kept opened oily vaccines in the refrigerator with a view to using them 3 to 6 weeks later.
4% of farmers dilute aqueous vaccines with more solute than the manufacturer recommends. Most of them use water for injection or a sterile isotonic mixed solution.
67% inject the vaccine as soon as it comes out of the fridge. 9% warm the oily vaccine in a water bath to 25°C and 24% wait 15 to 30 minutes for the vaccine to reach room temperature before injecting it.
94% tell they test their equipment with a non-vaccine solution (usually water) every time they perform a vaccination session. However, this is not what we found during our observations.
Finally, 9% fitted their syringes with a device for disinfecting the needle before injection. No farmer disinfects the animals' skin before injection. Vaccines are always injected subcutaneously, in the dorsal neck area. The solution is constantly agitated during the work.

Vaccination work
In general, farmers vaccinate alone (81%), but in 15% of cases 2 people are involved (1 catches the rabbit, the other vaccinates it), or in 4% of cases 3 people are involved (no sharing of tasks). None of the farmers questioned used a vaccination company. However, this option exists and is used on very large farms that are short of manpower.
Two farmers told that they used this vaccination time to tattoo their rabbits at the same time, but the majority dedicated special time to this task.
87% of farmers did not take a break during vaccination, but 10% stopped every two hours, 1% every hour and 1% every 4 hours.
The work is considered uncomfortable by 30% of farmers, most of whom cite back problems, but also wrist problems and fatigue.
None of them consider this activity to be stressful, but we have witnessed some irritation during our observation visits, due to the fact that the animals themselves are not calm, that the farmer is not holding them properly, that fatigue is felt, and the gesture becomes less precise, and that the needle becomes painful. Except in the case of vaccination in a contaminated environment, where it is advisable to change needles for each adult rabbit and each cage of growing rabbits, one needle may be used for 10 to 500 rabbits, depending on the farm, with an average of 100 animals.
The rate of vaccination varies greatly, depending on the farmer and his physical condition or age, the layout of the farm (more or less easy to catch the rabbits) and the number of operators. 55.2% estimate that they vaccinate around 400 rabbits an hour. 16.4% vaccinate at a slower rate of around 250/h. 16.5% vaccinate between 80 and 200 rabbits per hour and 11.9% say they vaccinate more than 400 rabbits per hour (with a peak of 1000 rabbits per hour reported by one farmer).

Figure 4: vaccination speed (number of rabbits per hour)



Lastly, 100% of the farmers surveyed had their healthcare waste (needles, empty vials, etc.) disposed of by a specialized waste disposal service.

CONCLUSION

This survey of 12.18% of French rabbit farmers highlights a number of areas of improvement. These include improving the vaccination procedure to avoid fatigue or injury to operators, but also repeating instructions that have been given many times but not always followed, both on vaccine storage and on their use. As biosecurity instructions are not always followed properly, we will have to find solutions to make them more acceptable or better understood by farmers. This preliminary survey will enable us to work in this direction.

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INTRA-FARM DYNAMIC OF *PASTEURELLA MULTOCIDA* POPULATIONS IN COMMERCIAL RABBITRIES

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ABSTRACT

One of the major pathogen of rabbits is *Pasteurella multocida* (PM) that causes mainly respiratory disorders but that can be present in several anatomic districts as commensal. Indeed, the disease (pasteurellosis) can be predisposed by several factors such as overcrowding or non-optimal environmental conditions and, probably, other still unknown predisposing or pathogen-related elements. Pasteurellosis has a negative impact not only on the animal health but also on their performances, resulting in economic losses.

The control of the disease typically relies on antimicrobials administration that is widely discouraged due to the well-known antimicrobial-resistance issue. Vaccination can be considered an alternative to antimicrobials, but commercial vaccines are few and the effects are inconstant in different environments. In Italy veterinarians can prescribe auto-vaccines (tailor-made vaccines) produced with the inactivated strain isolated in the outbreak.

With this study, we investigated the dynamics of PM pathogenic populations within the individual farm, over the time. This information results useful to verify whether circulating strains are replaced over the time and, consequently, the tailor-made vaccines must be regularly updated with new strains.

To this purpose, 74 clinical strains of PM, isolated from eight meat rabbit farms, were genetically characterized for the following features: capsulotype, lipopolysaccharide gentotype (LPS) and some virulence genes (toxA, tbpA, hgbB and pfhA). Furthermore, multi-locus sequence typing (MLST) was performed in order to conduct an epidemiological analysis.

The results highlighted that strains of PM circulating within the farm were substantially stable when vaccination was regularly conducted or in farms with poor health problems related to pasteurellosis. On the other hand, an high variability was found in farms with an high daily mortality due to pasteurellosis (> 2‰) or where vaccination with autovaccines was adopted inconstantly. The genetic characterization evidenced the circulation of an epidemic strain LPS 3, capsulotype A or F belonging to the clonal complex ST9.

Due to the appearance of different LPS genotypes over the time, the regular characterization of the strains circulating is essential for the choice of the strains to be included in effective tailor-made vaccines.

Keywords: *Pasteurella multocida*, rabbit, genetic characterization, epidemiology

INTRODUCTION

Pasteurella multocida (PM) is a common commensal of the oropharyngeal tract of many vertebrate species. PM is also known as pathogen of several animal species, including rabbit, where it is responsible for different clinical manifestations: pneumonia, rhinitis, metritis, mastitis, cutaneous abscesses and septicemia. This leads to significant economic losses for farmers due to the decrease of the growth rates, increased mortality, worse feed conversion rate, antimicrobial treatments and environmental hygiene interventions. Stressful or immunodepressive factors may contribute to the presentation and severity of pasteurellosis, although, not all predisposing triggers are known (Harper, 2006; Boyce *et al*., 2010).

PM is a small (0.2 x 1-2 µm) Gram negative, non-motile, cocco-bacillus, with a worldwide distribution. It is aerobic or facultative anaerobic and grows in 24h in 5% sheep's blood agar.

It is classified into 5 different capsulotypes (A, B, D, E and F) according to the capsule composition and 16 different serotypes (1 to 16) based on the lipopolysaccharide (LPS) structure (Heddleston *et al*., 1972). Only 8 of these serovars express a complete external core of the LPS: serovars 1, 2/5, 3, 6, 8, 9, 12 and 16 express the parental structures, while

4, 7, 10, 11, 13, 14 and 15 express truncated LPS as result of mutations in the LPS-encoding locus (Harper *et al*., 2015). Only eight unique LPS outer core biosynthesis loci are found in the 16 Heddleston type strains, classified from L1 to L8. To date, only L6 and L3 have been found in clinical isolates of rabbit origin (Massacci *et al*., 2018). L3 may include 2 different Heddleston serotypes (3 and 4), whereas L6 may include 4 different serotypes (10, 11, 12, 15) (Harper *et al*., 2015).
Strain identification usually consist of the capsular serogroup (capsulotype) letter followed by the somatic serovar number (e.g., A:1, A:3, B:2, etc.).
The pathogenicity of PM is also related to various virulence factors (not all probably known) encoded by different genes. Among them, the detection of genes encoding hemoglobin-binding protein (hgbB), transferrin-binding protein (tbpA), filamentous hemagglutinin (pfhA), and dermonecrotic toxin (toxa) is typically explored to assess the pathogenic potential of the isolate (Ewers *et al*., 2006).
In recent years, an increase in the incidence of pasteurellosis in commercial farms has been observed in Italy, probably due to a decrease in the use of antimicrobials (IZSVe, unpublished data). *In vitro,* the isolates typically exhibit a broad susceptibility to a wide range of antimicrobials; however, the administration of antibiotics considered of first choice (sulphonamides and tetracyclines), usually give variable results *in vivo* with no results or possible relapses of the disease at the therapy suspension. This could be due to the difficult interpretation of the MIC results because of the lack of clinical breakpoints for rabbits. Moreover, achieving effective drug concentration against PM becomes challenging due to the pathogen's multi-site localization within the anatomical regions of the host. Additionally, in Italy, rabbit farms have been recently introduced into an antimicrobial usage classification systems and this stimulates the exploration of alternative strategies for the prevention of diseases. An interesting alternative to antimicrobials prescription is represented by autovaccines, produced in Italy by the IIZZSS (public institutes for animal health, food control and research), which however give inconstant results. This could be due to the antigenic variability of circulating strains compared with the strain used for the production of the autovaccine. To date, this intra-farm variability has not yet been investigated and this study aims to bridge this knowledge gap.

MATERIALS AND METHODS

In this study, we characterized 74 field strains of PM of eight different farms tracking their variability over time. The farms were located in four different provinces of the North of Italy: Verona (1), Padua (2), Cuneo (1) and Treviso (4). The farms were categorized based on the daily mortality due to pasteurellosis in “affected” farms (mortality > 2 ‰) or “non-affected” (mortality < 2 ‰). This survey spanned a maximum period of 21 years (15 strains) of a farm with regular vaccination and occasional pasteurellosis issues. In contrast, the shortest observation period was 2 years (4 strains) from a farm with no reported health problems.
Strains were isolated from gross lesions attributable to pasteurellosis, observed in commercial rabbits submitted for necropsy to the Veterinary Diagnostic Laboratory of Treviso (IZSVe).
The PM isolates were tested using PCRs to detect the genes coding for capsular antigens (Cps), virulence factors and lipopolysaccharide structures (LPS) (Townsend *et al.*, 2001; Atashpaz *et al*., 2009; Harper *et al*., 2015). Furthermore, multi-locus sequence typing (MLST) of 48 strains was performed in order to conduct an epidemiological analysis within and between farms, according to the pattern reported by RIRDC (https://pubmlst.org/pmultocida). The extracted DNA was used to amplify the loci of 7 housekeeping genes (*adk*, *est*, *gdh*, *mdh*, *pgi*, *pmi* and *zwf*) (Subaaharan *et al*., 2010). Based on the sequence type (ST) designation, each isolate was assigned to a clonal complexes (CC) which comprised strains that differed from a common possible progenitor for not more than 2 alleles.

RESULTS AND DISCUSSION

No strain harbored *tbpA* and *toxA* encoding genes. Only 2 LPS genotypes were detected: L3 (n. 54) and L6 (n. 20), according to previously results on strains of rabbit origin (Massacci *et al.*, 2018). L3 strains belonged to capsulotype A (n. 20) or F (n. 33) (one resulted not-typeable) and, interestingly, all belonged to ST grouped in the CC ST9. The majority of L3

strains (n.52) were *pfhA*-positive, 7 harbored also the *hgbB*-encoding gene and only 2 were exclusively *hgbB*-positive. L6 genotype were capsulotype D (n. 13) or A (n. 7) and all were *hgb*B-positive. Strains L6, capsulotype D belonged to the CC ST50 whereas L6 capsulotype A were included in the CC ST74. The dynamic of the strains within the farms is analyzed below.

Farm 1 was classified as "not-affected" and a prophylaxis against pasteurellosis with a tailor-made vaccine was regularly performed in does since 2003. This farm experienced sporadic pasteurellosis problems in 21 years maybe due to the emergence of strains D:6 *hgbB*-positive, ST50, which differed from the "endemic" strain A,F:3 *pfhA*-positive, CC ST9 frequently isolated over time (Table 1). Since different LPS-genotypes definitely corresponds to different serotype (Heddleston classification), animals vaccinated with L3-based vaccine are not protected against L6 strains.

Table 1 Genetic characterization of PM circulating in farm 1. Date is in month/year form.

Dates	12/03	08/06	04/11	03/13	05/15	11/15	08/16	01/18	01/20	10/20	01/22	12/22	01/23	09/23
LPS	L3	L3	L6	L3	L3	L3	L3	L6	L3	L3	L3	L3	L3;L3	L3
Cps	A:3	A:3	D:6	F:3	F:3	A:3	F:3	D:6	A:3	A:3	A:3	F:3	F:3;A:3	A:3
VFs	*pfhA*	*pfhA*	*hgbB*	*pfhA*	*pfhA*	*pfhA*	*pfhA*	*hgbB*	*pfhA*	*pfhA*	*pfhA*	*pfhA*	*pfhA*	*pfhA*
MLST (CC)	9	9	50	9	9	9	9	50	9	9	9	9	9	9

Farm 2 experienced pasteurellosis in the lactating rabbits, but not in does or fattening rabbits. The emergence of strains with different genetic characteristic was similar to farm 1 (Table 2). Interestingly, the characterization of more than one strain isolated at the same time in cohort animals revealed that strains with different LPS, capsulotype, ST and genetic markers of virulence are simultaneously involved in the same episode of pasteurellosis.

Table 2 Genetic characterizations of strains circulating in farm 2. Date is in month/year form.

Dates	03/16	05/16	11/16	02/17	04/18	03/19	06/19	11/19	01/20	03/21	02/22	09/23	02/24
LPS	L6	L3	L3	L3	L3	L3	L3; L6	L3	L6;L3	L3	L3	L6	L6
Cps	A:6	F:3	F:3	3 A:3	F:3	F:3	F:3;A:6	F:3	D:6;F:3	F:3	F:3	D:6	D:6
VFs	*hgbB*	*pfhA*	*pfhA*	*pfhA*	*pfhA*	*pfhA*	*pfhA;hgbB*	*pfhA*	*hgbB;pfhA*	*pfhA*	*pfhA*	*hgbB*	*hgbB*
MLST (CC)	74	9	9	nt*	9	9	9;74	9	9	9	9	50	-

*nt: not typable

Farm 3 reported recurrent episodes of pasteurellosis characterized by a cutaneous localization of the pathogen but the gene encoding for the dermonecrotic toxin (*toxA*) was never detected. Such evidence suggests a minor role of this virulence factor in the rabbit skin lesions. In this farm, there was a frequent alternation of strains with different genetic characteristics (LPS, capsulotype, markers of virulence and ST) (Table 3).

Table 3 Genetic characterization of strains circulating in farm 6. Date is in month/year form.

Dates	06/19	11/19	02/21	03/23	03/23	10/23	10/23	11/23
LPS	L3	L3	L3	L6;L3	L6;L3	L6	L3	L3
Cps	F:3	A:3	F:3	D:6;F:3	A:6;A:3	A:6	F:3	F:3
VFs	*pfhA,hgbB*	*pfhA*	*pfhA,hgbB*	*hgbB; pfhA*	*hgbB;pfhA,hgbB*	*hgbB*	*pfhA,hgbB*	*pfhA,hgbB*
MLST (CC)	9	9	9	-	-	-	-	-

Farms 4 and farm 5 reported respiratory disorders due to pasteurellosis in fattening rabbits. In 2021, farm 4 experienced the emergence three PM strains D:6 that, in April, were simultaneously responsible of disease with the strain F:3 (Table 4). This simultaneous presence of different strains resulted more evident in farm 5 (Table 5).

Table 4 Genetic characterization of strains circulating in farm 4. Date is in month/year form.

Dates	03/20	10/20	01/21	04/21	04/21	04/21	04/21	07/21	09/21	05/22	09/22
LPS	L3	L3	L6	L6	L6	L6	L3	L3	L3	L3	L3
Cps	A:3	A:3	D:6	D:6	D:6	D:6	F:3	nt*:3	F:3	F:3	A:3
VFs	*pfhA, hgbB*	*pfhA, hgbB*	*hgbB*	*hgbB*	*hgbB*	*hgbB*	*pfhA*	*pfhA*	*pfhA*	*pfhA*	*pfhA*

*nt: not typable

Table 5. Genetic characterization of strains circulating in farm 5. Date is in month/year form.

Dates	10/22	02/23	09/23	12/23	01/24	01/24	02/24
LPS	L3;L6	L6	L3	L3	L3	L3;L3	L6;L3
Cps	2 F:3;D:6	D:6	F:3	A:3	F:3	A:3;F:3	D:6;F:3
VFs	*pfhA; hgbB*	*hgbB*	*pfhA*	*pfhA*	*pfhA*	*pfhA*	*hgbB;pfhA*
MLST (CC)	9;50	50	9	-	9	9	-

Table 6. Genetic characterization of strains circulating in farm 6, 7 and 8. Date is in month/year form.

	Farm 6				**Farm 7**		**Farm 8**	
Dates	06/20	01/21	04/21	07/21	05/21	03/22	10/21	07/23
LPS	L3	L3	L3	L3	L6	L6;L3	L3	L3
Cps	F:3	F:3	F:3	F:3	A:6	2 A:6;F:3	A:3	A:3
VFs	*pfhA*	*pfhA, hgbB*	*pfhA*	*pfhA*	*hgbB*	*pfhA/hgbB; hgbB*	*hgbB*	*hgbB*
MLST (CC)	9	9	9	9	74	74; 9	-	-

Few strains were available from the last 3 farms, however different dynamics of the strain swapping can be noticed (Table 6). In farms 6 and 8 the strains were essentially conserved as far as the capsulotype and LPS is concerned. In farm 6 this persistence of the same strain or of strains genetically correlated is supported by the persistence of ST belonging to the same clonal complex (ST9). In farm 7, classified as “non-affected”, the few strains analyzed showed a high variability and, curiously, the prevalent sequence types belonged to the CC ST74. Farm 8 was the one with the only two L3 strains *hgbB*-positive identified.

Through MLST, 48 strains were analyzed. For three isolates of farm 2, two of which belonging to the same subject, it was not possible to identify the ST because a deleterious *mdh* allele was found which did not allow the allocation of an ST. Assignment to a specific clonal complex of identified STs was possible only for the other 45 strains. This analysis showed that 35 of 45 strains belonged to CC ST9, 6 to the CC ST50 and 5 to the CC ST74. A substantial majority of the CC ST9 was found.

CONCLUSION

The study showed that different strains may circulate in the same farm, at the same time and may be responsible of disease. Therefore, the characterization of strains employed for the set up of inactivated autovaccines results essential. The ST9 is confirmed to be an epidemic strain in Italian rabbitries.

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EVALUATION OF CLINICAL IMPROVEMENTS DUE TO AN AUTOVACCINE AGAINST PASTEURELLA MULTOCIDA IN RABBITS IN FIELD CONDITIONS

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ABSTRACT

In Italy, the Ministry of Health authorises the use of autogenous vaccines prepared with bacterial strains isolated in farms frequently affected by specific bacterial diseases. Despite their frequent use, in rabbit farms especially, the feedback of their efficacy is not available. The aim of this study was to evaluate the impact on clinical conditions of an autogenous vaccine against *Pasteurella multocida* (*P. multocida)* in field. A bivalent vaccine was prepared using *P. multocida* strains belonging to serogroups A and F responsible for recurrent pasteurellosis in a genetic centre of rabbits in southern Italy. The vaccine was administered twice at 15-day intervals, after four months, and twice a year at six-month intervals to all rabbits. The rabbits were monitored for two years after the first vaccination. The improvement of clinical conditions and the decrease of the mortality rate were found especially in the first-year post-vaccination. In addition, the number of does removed from breeding sector for pasteurellosis decreased. Nevertheless, *P. multocida* strains belonging to serogroup A were found at the end of the second-year post-vaccine. In addition, strains belonging to serogroup D were also identified in rabbits with pneumonia and metritis. These findings suggest that the vaccine is effective in the reduction of clinical symptoms but is not able to avoid the spreading of new *P. multocida* strains among the rabbit flocks.

Key words: *Pasteurella multocida,* rabbit, autovaccine, serogroup, virulence-associated genes

INTRODUCTION

Pasteurellosis causes economic losses in rabbit farms because of respiratory and reproductive syndromes, prevalently. *P. multocida* is currently classified into five capsular groups (A, B, D, E, and F) and 16 somatic serotypes (1 to 16). Capsular type A is most associated with pasteurellosis in rabbits, while capsular types D and F are less often involved in the disease. Also, type B has been detected in rabbitries in India (Katoch *et al*., 2015) and more recently in rabbits in Italy (Cucco *et al*., 2017). Poor management conditions such as prolonged exposure to ammonia, dust or inappropriate environmental temperature and levels of humidity may predispose the animals to the disease. Nevertheless, the pathogenicity of *P. multocida* is influenced by the presence of virulence factors (VFs), such as fimbriae, adhesion and colonization factors, hemoglobin-binding protein, transferrin-binding protein, iron and protein acquisition, Outer Membran Proteins (OMPs) and others (Ewers *et al*., 2006; Massacci *et al*., 2018). The treatment of choice for pasteurellosis is the use of antibiotics that does not result in the disease eradication and may increase the risk of antibiotic resistance. The disease control could be potentially achieved over time with management improvement and vaccines. Although a commercial vaccine against pasteurellosis is licensed for rabbits, farmers prefer the use of autogenous vaccines, which are authorized by Ministry of Health on farms with cyclical disease and are subjected to a detailed veterinary request. Despite their frequent use, poor feedback information of autovaccines efficacy is available. The aim of this study was to assess the effect of an autogenous vaccine containing *P. multocida* strains in field conditions.

MATERIALS AND METHODS

Animals and conditions in the farm

The study was carried out in the genetic centre of the Italian Rabbit Breeders Association (ANCI), in Volturara, FG, southern Italy, authorized by the Ministry of Agricultural, Food and Forestry Policies (MIPAF) for the maintenance of rabbit breeding farms. All rabbits were vaccinated for mixomatosis and rabbit haemorragic disease. Over a five-year period, a syndrome predominantly characterised by purulent conjunctivitis and rhinitis was observed cyclically. Accordingly, enrofloxacin was frequently used to reduce lesions and mortality. Severe respiratory symptoms, or reproductive disorders such as infertility and abortions led to the loss of 17.9% of rabbits during the year prior to vaccination. About 70 rabbits were analysed in the study period. Purulent pneumonia, metritis and otitis were found at the necroscopies. *P. multocida* was constantly isolated on blood agar.

Investigation of *P. multocida* strains

Thirty strains of *P. multocida* from 30 rabbits were investigated for serogroup using a multiplex PCR assay which allows both the species identification and capsular antigen typing (Townsend *et al*., 2001). Fourteen *P. multocida* strains isolated from 14 rabbits at the end of the second year after vaccination were also typed.

Vaccine preparation

Two strains of *P. multocida* belonging to serogroups A and F were selected to prepare the inactivated bivalent vaccine (AF). The bacterial strains were tested for capsule persistence using Anthony's stain (Anthony, 1931) prior to use. The bacterial suspension prepared in saline solution (0.9 %) using strain A and strain F (1:1) until a final bacterial density of 1.5 x 10^9 cells/ml was inactivated with formalin (0.4%) and diluted to achieve the desired cell density and fall within the maximum levels of formalin content allowed by the European Pharmacopoeia (0.05% of the final product). Inactivation control was performed by inoculating the bacterial suspension on Petri plates containing Blood Agar at 37 °C for 48 h. The vaccine was adjuvated with aluminium hydroxide (25%) and administered twice at 15-day intervals, after four months, and twice a year at six-month intervals to the rabbits of the farm.

Statistical Analysis

The data were analysed by univariate statistical analysis (Pearson's chi-square test and Fisher's exact test for independence). The odds ratio (OR) and 95% confidence interval (CI95%) were also calculated.

RESULTS AND DISCUSSION

Impact on clinical conditions

A significant decrease of mortality due both to respiratory and reproductive lesions was observed in the whole period, and in the first year after the vaccine administration especially. Also, the decrease of removals of does from the breeding sector was found (table 1), confirming that the inactivated vaccine is useful in mitigating the usual injuries associated with pasteurellosis (Mohamed *et al*., 2019). In addition, at the end of the vaccine monitoring period, a drastic reduction of antibiotic use was reported by the veterinarian of the farm. The use of the vaccine did not lead to the eradication of pasteurellosis in two years. It is likely that more time will be needed to achieve this result, but it is important that the use of vaccine is accompanied by the control of the predisposing factors the clinical conditions.

Laboratory investigation on *P. multocida* isolates

Eighteen and two *P. multocida* strains belonging to serogroup A were identified from pneumonia and metritis, respectively. In addition, six and four strains belonging to serogroup F from pneumonia and metritis, respectively, were found (Table 2). Among the strains investigated at the end of the second-year post-vaccine use, serogroups A and D were detected in both pneumonia and metritis while serogroup F was not identified. These findings could be related with lipopolysaccharides (LPS), which are the outer membrane components of the bacteria and the primary somatic markers (De Long *et al*., 1992) in the whole-cell lysate used in the vaccine. Although cross-reactions can also occur (Suckow *et al*., 2008; Lukas *et al*., 1987), capsular types can be linked to specific LPS genotypes (Massacci et al., 2018), that may induce specific antibodies directed to one serotype that react poorly, or not at all, with the lipopolysaccharides of other serotypes (Klaassen *et al*., 1985), probably causing the inability of the vaccine to prevent the introduction of new serotypes of *P. multocida*.

Table 2. Typing of *P. multocida* strains identified before and after vaccine use.

Reporting Period	Lesion	Serogroup (N° of strains belonging to)
Pre-vaccine use	Pneumonia	A (18)
		D (0)
		F (6)
	Metritis	A (2)
		D (0)
		F (4)
Post vaccine use	Pneumonia	A (8)
		D (1)
		F (0)
	Metritis	A (4)
		D (1)
		F (0)

CONCLUSIONS

The vaccination strategy improved the clinical condition of the rabbits, reducing the economic losses due to mortality, breeder rejections and antibiotic treatments. Therefore, a more frequent use of vaccines is particularly advisable in rabbit farms, because rabbit has been the species with the highest antimicrobial consumption among food-producing animals (Moulin and Chevance, 2015). Based on the results of this study, using an autogenous vaccine, it is important to monitor the strains spread in the rabbit population prior to the formulation of each new batch to ensure maximum efficacy of the vaccine.

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Table 1. Mortality recorded before and after vaccine use in all rabbits and mortality and loss of does removed from the breeding sector.

	Reporting period	Deaths from respiratory lesions (%)	OR	$CI_{95\%}$	p-value	Deaths from reproductive lesions (%)	OR	$CI_{95\%}$	p-value	Females removed from breeding sector (%)	OR	$CI_{95\%}$	p-value	Total (deaths + loss of does due to pasteurellosis) (%)	OR	$CI_{95\%}$	p-value
Does (4,020*)	Pre-vaccine year	100 (2.48)	Reference			90 (2.23)	Reference			530 (13.2)	Reference			720 (17.9)	Reference		
	1-year post-vaccine	77 (1.9)	0.77	0.57-1.03	0.009	65 (1.6)	0.72	0.52-0.99	0.007	420 (10.44)	0.77	0.67-0.88	<0.001	562 (13.9)	0.74	0.66-0.84	<0.001
	2-year post-vaccine	62 (1.54)	0.61	0.45-0.85		54 (1.34)	0.59	0.42-0.84		380 (9.45)	0.69	0.6-0.79		496 (12.3)	0.65	0.57-0.73	
All the other rabbits (26,230*)	Pre-vaccine year	436 (1.66)	Reference			333 (1.26)	Reference			-				769 (2.93)	Reference		
	1-year post-vaccine	310 (1.18)	0.71	0.62-0.83	<0.001	174 (0.66)	0.52	0.43-0.62	<0.001	-				484 (1.84)	0.62	0.55-0.7	<0.001
	2-year post-vaccine	285 (1.08)	0.65	0.56-0.76		132 (0.5)	0.39	0.32-0.48		-				417 (1.58)	0.53	0.47-0.6	
Total of rabbits (30,250*)	Pre-vaccine year	536 (1.77)	Reference			423 (1.39)	Reference			530 (1.75)	Reference			1489 (4.9)	Reference		
	1-year post-vaccine	387 (1.27)	0.72	0.63-0.82	<0.001	239 (0.79)	0.56	0.48-0.66	<0.001	420 (1.38)	0.79	0.69-0.9	0.039	1046 (3.45)	0.69	0.64-0.75	<0.001
	2-year post-vaccine	347 (1.14)	0.64	0.56-0.74		186 (0.61)	0.44	0.37-0.52		380 (1.25)	0.71	0.62-0.81		907 (2.9)	0.6	0.55-0.65	

OR: Odds ratio, $CI_{95\%}$: 95% Confidence Interval. Reference group is the pre-vaccine year.
*Other rabbits were introduced to replace the dead ones removed.

EXPLORING STRAIN AND ANTIMICROBIAL RESISTANCE DIVERSITY IN *STAPHYLOCOCCUS AUREUS* IN RABBIT FARMS

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ABSTRACT

In this study, 125 *S. aureus* samples were analyzed, identifying 22 different sequence types (STs) belonging to 11 clonal complexes (CCs). The most commonly isolated strains were from the ST3764, followed by ST2855 and ST121. Clonal complexes CC121 and CC96 were the most frequent in rabbits, while in humans, CC96 was the most frequent followed by CC121 and CC398. Concerning the farms, 17 out of 37 had workers sharing CCs and STs with rabbits, with two of them having identical average nucleotide identity (ANI) results between human and animal strains. The antimicrobial resistance study revealed there were no significant differences in five out of nine antimicrobial groups between both populations, suggesting gene transmission may have occurred. Similar results were observed with regards to the number of resistance genes, indicating potential gene transmissions in farm settings between different hosts.

Key words: *Staphylococcus aureus*, rabbits, humans, average nucleotide identity, antimicrobial resistance.

INTRODUCTION

Staphylococcus aureus is an opportunistic bacterial pathogen known for causing various diseases in rabbits, such as mastitis, pododermatitis, and abscesses; but it is considered a multi-host pathogen. In previous works, it has been described that humans play a central role in the transmission between species. Through changes in the genome, such as horizontal transfer or genetic diversification, *S. aureus* can adapt to a new host (Richardson *et al*. 2018).

It is able to rapidly acquire antimicrobial resistances, which represents a significant challenge to find effective treatments. Being a multi-resistant microorganism, the available therapeutic options are limited, and the rapid spread of these resistances, further increases this problem (Aires-de-Sousa 2017).

In this context, the present study focuses on examining the interaction between human and animal isolates obtained from different Spanish farms, identifying predominant strains and comparing their identity. Additionally, the presence of antimicrobial resistance genes is investigated, aiming to examine their transmission ability.

MATERIALS AND METHODS

Sample collection

Samples were obtained from humans and rabbits of 37 different Spanish farms. Nasal swabs were collected from 53 farmers and lesion swabs were collected from 72 animals. This samples were cultured in Columbia CNA (colistin-nalidixic acid) Agar with 5% Sheep Blood (Becton-Dickinson, Sparks, MD, USA) and incubated at 37°C for 24 hours aerobically.

Whole Genome Sequencing was conducted to obtain the MLST (Multilocus Sequence Typing) allelic profiles through computational analyses (PubMLST) (Jolley and Maiden 2010). To estimate global mutation distances and the average nucleotide identity (ANI), MASH was conducted (Ondov *et al*. 2016).

Statistical Analysis

The presence of virulence genes was evaluated using a generalized mixed model, with a binomial probability distribution for the response and a logit transformation [ln(μ/(1-μ))] as a link function (Proc Glimmix of SAS). The model included the interaction between the group of individuals (humans or animals) and the antimicrobial (aminoglycoside, beta-lactam, bleomycin, fosfomycin, lincosamide, macrolide, quaternary ammonium, tetracycline, trimethoprim) as fixed effect.

The number of virulence genes was evaluated using a generalized linear model (Proc GLM of SAS). The model included the interaction between the group of individuals (humans or animals) and the antimicrobial (aminoglycoside, beta-lactam, bleomycin, fosfomycin, lincosamide, macrolide, quaternary ammonium, tetracycline, trimethoprim) as fixed effect.

RESULTS AND DISCUSSION

Table 1: Number of isolated strains from humans and rabbits classified by clonal complex and sequence type

CC	ST	n	Carrier
5	5	1	H
	146	1	R
	8762	1	H
8	8	3	H
15	15	1	H
22	22	1	H
30	30	3	H
59	8755	1	H
96	8756	1	H
	8759	2	H / R
	8008	5	H / R
	96	6	H / R
	2855	27	H / R
121	8763	2	H / R
	7763	5	H / R
	121	18	H / R
	3764	29	H / R
130	4774	5	H / R
398	8753	2	H
	398	9	H
425	425	1	R
	8144	1	R

H: Human, R: Rabbit

Characterization of the isolates

Twenty-two different STs belonging to 11 CCs were identified amongst the 125 studied samples (Table 1). The most frequently isolated strains belonged to the ST3764, followed by the ST2855 and ST121.

Isolates belonging to the CC121 and CC96 were the most frequent in rabbits, as previously described in other works (Viana *et al*. 2007). In humans, the most frequent clonal complex was CC96, followed by CC121 and CC398. When comparing both results, it was observed that in 17 of the 37 farms (45.95%), workers shared CCs and STs with the rabbits. Regarding the analyses of the ANI, two of those farms shared identical results in strains isolated from humans and animals. This suggests there has been transmission between the studied populations.

Previous studies have described the adaptation of strains in different hosts, reviewed in Howden *et al*. (2023). Particularly in rabbits, Viana *et al*. (2015) described that the mutation in a single nucleotide was the key to the adaptation of the CC121 from humans to rabbits. In our study, further tests should be conducted to examine this strain interactions in detail.

Characterization of the antimicrobial resistances

A study of the presence and number of resistance genes in the sequenced strains was conducted.

Regarding the presence of resistance genes comparatively in both populations (Figure 1), it was observed that there were significant differences in 4 out of 9 antimicrobial groups (95% confidence interval and p-value 0.05). These genes can confer resistance to trimethoprim, tetracyclines, fosfomycin and ß-lactams.

The 5 remaining groups (aminoglycosides, bleomycin, lincomycin, macrolides and quaternary ammoniums) did not have significant differences. This finding may suggest gene transmissions could have occurred between both populations, which could be potentially transmitted to communities through mobile genetic elements (Partridge *et al*. 2018) and subsequently be a threat to public health.

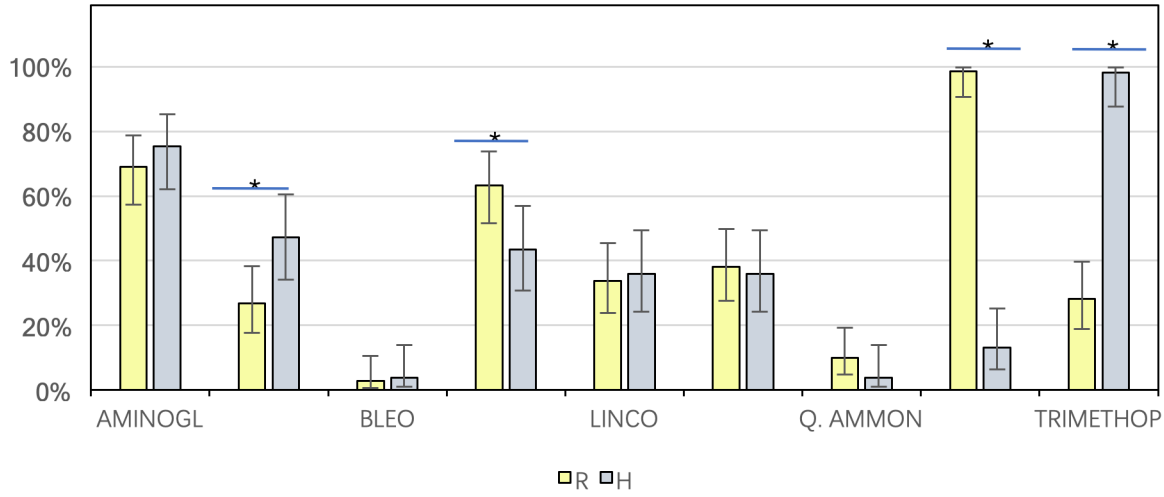

Figure 1: Presence (%) of resistance genes in animals and humans depending on the antimicrobial family analyzed

AMINOGL: Aminoglycosides; ßLACTAM: ß-lactams; BLEO: Bleomycin; FOSFOM: Fosfomycin; LINCO: Lincomycin; MACROL: Macrolides; Q. AMMON: Quaternary ammoniums; TETRAC: Tetracyclines; TRIMETHOP: Trimethoprim

Concerning the number of resistance genes in both populations, similar results were observed. When strains isolated from a population (animal or human) exhibited a higher resistance ratio, they also demonstrated a broader antimicrobial genetic profile. When no significant differences were found (95% confidence interval, p-value 0.05), similar number of resistance genes were observed. This reinforces the hypothesis that gene transmissions could be occurring in farm settings within different hosts.

CONCLUSIONS

Farmers and humans in close contact repeatedly share phylogenetically close strains. Moreover, this isolates occasionally have nearly identical average nucleotide identity (ANI).

Regarding antibiotic resistances, the observed results suggest a potential transmission of resistance genes between both populations.

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CLOSTRIDIOIDES DIFFICILE IN RABBITS: STUDY OF PRESENCE AND ASSOCIATED LESIONS

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ABSTRACT

The emergence of Clostridioides difficile as a pathogen of concern has led to significant shifts in its epidemiology, with strains like ribotypes 027 and 078 gaining attention due to their hypervirulent characteristics. Ribotype 078 is increasingly linked to community-acquired infections, particularly in animals like rabbits. This underscores the importance of monitoring and addressing the spread of C. difficile, especially ribotype 078, to mitigate infection risks in both human and animal populations. Despite extensive research in swine and other animals, studies on rabbits remain limited. Therefore, this study aimed to assess the presence of *C. difficile* in rabbit populations on meat production farms in Spain. Rectal swabs from healthy and diarrheic animals, along with intestinal contents from necropsies of diarrheic subjects, were collected. Microbiological analyses revealed low presence of *C. difficile*, with only 14 positive cases identified among 198 samples. Most isolates belonged to ribotype 126, indicating its significance in Spain's rabbit population. A significant association between *C. difficile* presence and health status was found, with all positive samples originating from animals with diarrhea. Histopathological examination of positive animals revealed generalized edema in the digestive system, further highlighting the pathogen's potential to cause harm. In conclusion, our data indicates a limited threat to the rabbit population, but confirms its ability to induce clinical manifestations. However, the capability of *C. difficile* to cause disease in the animals highlights the necessity to continue with surveillance and research efforts.

Key words: Epidemiology, One-Health, Microbiology, Pathology, *C. difficile*.

INTRODUCTION

Clostridioides difficile is an anaerobic bacterium that has traditionally been associated with nosocomial settings, particularly in conjunction with antimicrobial therapies. However, over the past two decades, a shift in its epidemiology has been observed, marked by the emergence of novel PCR-ribotypes and alterations in patient demographics (Lim et al., 2020). Two ribotypes, namely 027 and 078, have garnered significant attention due to their hypervirulent characteristics.

Ribotype 027 significance is related to the increased infectiousness and symptomatic disease rates compared to endemic strains, facilitating its rapid clonal dominance in healthcare settings (Yakob et al., 2015). Ribotype 078, on the other hand, has been increasingly associated with community-acquired infections, potentially indicating a zoonotic transmission route. This ribotype is also mostly isolated in animals.

This highlights the importance of monitoring and addressing the spread of ribotype 078 strains to mitigate the risk of infection in both human and animal populations (Cartman et al., 2010).

There is an increasing interest in understanding the significance of *C. difficile* in the context of animal health, particularly its implications for human health under a One Health framework. While research has primarily focused on swine, investigations into its relationship with human health extend to other animals such as horses, cattle, and companion animals like dogs and cats. However, studies concerning rabbits remain limited to date (Weese, 2020).

The primary aim of this study is to assess the presence of *C. difficile* in rabbit populations on meat production farms in Spain. Additionally, the study aims to investigate the associated implications and lesions caused by *C. difficile* infection in rabbits, both macroscopically and microscopically. Given the substantial impact of other clostridial infections and colibacillosis on rabbits, the presence of *Clostridium perfringens* and *Escherichia coli* was also investigated. Furthermore, the study investigates the significance of *C. difficile* in the health of both rabbits and humans, thereby contributing to our understanding of the potential zoonotic implications of this pathogen.

MATERIALS AND METHODS

Animals and experimental design

Two distinct types of samples were employed in this study: rectal swabs were collected from both healthy and diarrheic animals, while intestinal contents were extracted during necropsies of diarrheic subjects. A total of 160 swabs were taken, 47 from healthy animals, 97 from animals with clinical signs of diarrhea and 16 from animals with unknown health status. The animals originate from the Ebro Basin area in Spain, and a total of 29 different farms were included.

During this study, a total of 38 animals underwent necropsy procedures. Samples from various segments of the gastrointestinal tract, including the cecum, ileum, colon, and jejunum, were collected in sterile containers for subsequent microbiological analysis. For histopathological examination, tissue specimens were fixed in buffered formalin for 48 hours and then embedded in paraffin wax. Following embedding, the specimens were sectioned and blocked in paraffin. Histological preparations were sliced at 4 µm thickness and stained with hematoxylin and eosin for detailed examination.

Microbial Analyses

In this study, microbiological analysis were conducted on samples collected from various sections of the gastrointestinal tract of both live and necropsied animals. For each section, samples were plated on MacConkey agar and Tryptose Sulfite Cycloserine agar supplemented with egg yolk (TSC) for bacterial culture. Additionally, swabs were taken specifically for *C. difficile* analysis.
On MacConkey agar (Panreac), cultures were incubated aerobically at 37°C for 24 hours, and colonies compatible with *Escherichia coli* were identified using Gram staining and the indole test. Positive *E. coli* colonies underwent intimin PCR analysis. Similarly, TSC plates (Oxoid) were incubated anaerobically at 37°C, and black colonies with lecithinase activity were considered suspect for *C. perfringens*. Positive *C. perfringens* colonies were confirmed via Gram staining and *cpa* toxin testing using a modified multiplex PCR method.
For *C. difficile*, swabs were anaerobically incubated in brain-heart infusion broth (Oxoid) supplemented with fructose, sodium-taurocholate, and a *C. difficile* selective supplement (Oxoid), for a week at 37 º C. The resulting mixture was centrifuged after a 1h ethylic shock, and the pellets were plated on CLO agar (bioMérieux). Morphologically compatible colonies with *C. difficile* were selected and tested by multiplex PCR for the presence of toxins (A, B and binary) and *gluD* gene and ribotyping according to the Leeds-Leiden database and a protocol described elsewhere (Fawley et al., 2015).
E. coli and *C. perfringens* DNA was extracted by heat-lysis, whereas a Wizard Omega DNA purification Kit was used for *C. difficile* DNA extraction.

Statistical Analysis

The associations between pathogens, health status, and different pathogen types were examined using Fisher's exact test, with a confidence interval of 95%. Statistical analysis was conducted using R software, version 4.1.1.

RESULTS AND DISCUSSION

Presence of *C. difficile*

Given the study's sample size of 198 rabbits, the prevalence of *C. difficile* was found to be low, with only 14 positive cases identified. These isolates exhibited limited genetic diversity, with most of them belonging to ribotype 126 and sharing identical toxigenic profiles. Interestingly, non-toxigenic strains were encountered in our study, even if the animals presented disease symptoms.

While studies on *C. difficile* prevalence in meat rabbits are scarce, a notable Italian study in 2015 reported higher genetic diversity within the population (n = 1279), albeit with a similar positivity to ours. A possible explanation for our higher rate of isolation could be the fact that we encountered a possible outbreak during the study (table 1, 6 isolates belong to HH farm), although it could not be solely linked to the presence of *C. difficile*. The presence of ribotype 126 appears to be significant in rabbits in Spain. Although the study in Italy found the most isolated ribotype to be 078, our study found ribotype 126, which is closely genetically related (Drigo et al., 2015).

Table 1: Characteristics of *Clostridium difficile* positive rabbits and strain molecular data.

ID[1] (farm ID)	Age (days)	Toxin genes	RT	Antibiotic treatment[3]
R1 (HH)	45	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	Valnemulin and colistin (feed)
R2 (HH)	45	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	Valnemulin and colistin (feed)
R3 (HH)	45	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	Valnemulin and colistin (feed)
R6 (GM)	49	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	Tiamulin, colistin and bacitracin (feed); enrofloxacin (water)
R15 (VL)	42	-	204	NR
R19 (HH)	35	-	204	NR
R20 (HH)	50	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	NR
R21 (HH)	64	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	NR
R46 (CA)	67	-	051	Tiamulin, neomycin, lincomycin and spectinomycin (feed)
R16 (R)	50	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	NR
R17 (YB)	50	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	NR
R14 (H)	63	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	NR
R16.2 (H)	63	*tcdA*, *tcdB*, *cdtA*, *cdtB*	126	NR
TA36	Primiparous	*tcdA*, *tcdB*, *cdtA*, *cdtB*	ND	None

RT, PCR-ribotype; NR, data not recorded. [1]Sample identification; [2]antibiotic treatment at the time of sampling; ND, not-done

A statistically significant relationship between health status and *C. difficile* presence was noted, with all positive samples originating from animals with diarrhea ($p < 0.05$). This result suggests a possible association between health status and *C. difficile* presence, indicating its potential to induce clinical manifestations in rabbits.

Pathogen associations

Regarding lesions and pathogen isolation, the findings were consistent with expected clinical signs. In the context of diarrhea, where *E. coli, C. perfringens*, and *C. difficile* were collectively investigated, divergent lesion patterns highlighted the complex interactions between these pathogens and their hosts. Despite the low presence of *C. difficile*, its presence in rabbits with diarrhea suggests its relative importance compared to *E. coli* and *C. perfringens* in this species. However, further studies would be needed to elucidate such relationship.

Only one of the *C. difficile* positive isolates came from an animal with histology data (TA36). Macroscopically the animal showed mild signs of diarrhea, while the histology showed generalized edema in the digestive system.

Interestingly, all rabbits positive for epizootic rabbit enteropathy (ERE) tested negative for *C. difficile*. Likewise, no significant relationship was found between ERE and the presence of other two bacterial pathogens included in this study (data not shown). The role of pathogens in ERE remains uncertain, with multifactorial sources being a popular hypothesis, likely bacterial in nature given their infectious properties and associated dysbiosis (Puon Peláez et al., 2018).

CONCLUSIONS

In summary, our study reveals a low positivity for *C. difficile* in meat rabbits, indicating a limited threat to the population. However, the pathogen's capability to cause clinical disease is apparent, as it was associated with pathological manifestations in rabbits exhibiting diarrhea. The predominance of ribotype 126 is in accordance with its widespread nature in Spain. Additional investigations about the role of pathogens in conditions such as Epizootic Rabbit Enteropathy (ERE) are warranted, considering the uncertain etiology and multifactorial nature of the disease.

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DETERMINATION OF THE PRESENCE OF THE PSOROPTES CUNICULI MITE IN YOUNG RABBITS FROM THE SECOND WEEK OF LIFE

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ABSTRACT

The Psoroptic mite, also known as “auricular mite”, is a pathology very common in rabbit production centers as well as in companion rabbits. It is caused by an easily direct and indirect transmitted obligatory ectoparasite called *Psoróptes cuniculi*, which causes pruritus, excessive earwax production, crusty lesions inside the ear canal and along the auricular pavilion, allowing the proliferation of opportunistic bacteria. Furthermore, affects the animal welfare of the rabbits, as it causes discomfort, secondary lesions due to scratching, anorexia, and decreased the feed conversion efficiency, thus losses in production; also, unpleasant appearance due the presence of scabs and other lesions that may not be visually pleasing to producers, people who buy life rabbits or even owners of the pet rabbits. The objective of this work was to implement a low-cost method for early diagnosis of Psoroptic mite, which, unlike the traditional method, is not invasive and does not require the lesions to be evident, since the traditional diagnosis requires the veterinarian to observe the lesions and thus, to obtain a sample by removing the scabs for the identification of the mite under the microscope, in the same way, it is sought that the diagnosis through the endoscope otic camera avoids the increase of lesions by removal of scabs, bleeding and pain in the rabbit, and that can be applied in rabbit farms and veterinary clinics. In this work, 225 young California breed rabbits were analyzed from seven days of life to seventy-seven days old, they were divided into two groups: rabbits whose mothers at the time of birth had apparent lesions of Psoroptic mite, and the other group, rabbits whose mothers had not apparent lesions or other clinical signs of Psoroptic mite. The inside of both ear canals were observed weekly in every young rabbits, using an endoscope camera otic for human use, in search of the presence of the parasites and lesions that it causes; the otic endoscope was disinfected according to the producer’s indications to avoid acting as a vehicle for the transmission of the mite from rabbit to rabbit or from one ear canal to another. After observation for ten continuous weeks, the presence of parasites was determined in rabbits from nineteen days of life, with the increase of the infestation, and a progressive increase of lesions was found inside the auditory canal and later it was externalized along the pinna of the ear, in addition, it was possible to observe the evolution of the lesions caused by this mite through the collection of photographs and videos inside of the ear canal, where the characteristics of this parasite can be well appreciated for its identification. When analyzing data, it was found that the offspring of the does rabbit with apparent lesions of Psoroptic mite were infested at earlier ages, in contrast to the offspring of does rabbit that did not present lesions of Psoroptic mite at the time of birth, at the end of ten weeks of observations, all of the rabbit with lesions’ offspring were infested with the mite and presented lesions in different degrees, while some of the rabbits without lesions’ offspring did not present lesions at all throughout the 10 weeks. Since Psoroptic mite is a very common, resistant and recurrent pathology, it is of outmost importance to implement an efficient treatment and control system, which requires a diagnosis method to ensure the absence of parasite and not only external lesions, in addition to ensuring that new animals witch are introduced to a farm or attend a preventive medicine consultations, are free of the mite and thus can not be a focus infestation for rabbit production or other pet rabbits.

Key words: Psoroptic mite, Diagnosis, Rabbit production, Infestation, Endoscopy.

INTRODUCTION

The Psoroptic mange is caused by an obligate ectoparasite that affects rabbits and is transmitted by direct and indirect contact (Rodríguez *et al.*, 2015). The biological cycle develops relative quickly (21 days) however, the clinical signs depend on the degree of infestation (Borchet, 1981).

For the traditional diagnosis of this disease, an inspection of the skin is made in search of crusts and alopecic areas (Lleonart, 1995); for the identification of the agent, with the help of a tweezers a sample of crusty material is taken from the inside of the ears, counting and classification is made by morphological observation through a microscope; when the mites are scarce the presence of eggs in the cerumen is observed (Borchet, 1981; Lleonart, 1995).

The objective of this work was to implement a well-timed diagnostic techniques for psoroptic mange in production and pet rabbits that allows early detection.

MATERIALS AND METHODS

Animals and experimental design

A total of 225 California breed rabbits from multiparous does from 7 days life to 10 weeks of age were used, they were 12 litter whose mothers had no apparent Psoroptic mange lesions and 18 with the apparent lesions. These rabbits were observed weekly with the aid of a 5.5 mm diameter, 0° angle, 110 mm long Endoscope camera with a 150w cold light source connected by a cold light wire, with a human-use Android USB camera in searching for parasites and Psoroptic mange lesions.
The lesions were recorded at each observation, and photographic and video recordings were taken to observe the evolution of the disease.

Statistical Analysis

Descriptive statistics were obtained for the data collected.

RESULTS AND DISCUSSION

Young rabbits with wound of Psoroptes mite at different weeks of age.

In the present study the development of the lesions caused by the *Psoroptes cuniculi* mite was observed, the increase of the lesions was progressive along the postinfestation weeks, starting with the presence of the parasite (Figure 1 A), followed by the irritation in the dermis caused by the irritation in the dermis caused by the mite when feeding (Figure 1 B), subsequently the infestation and lesions increase inside the ear canal (Figure 1 C y D), eventually the production of cerumen and lesions begins to obstruct the entrance to the ear canal (Figure 1 E y F), finally the lesion begins to externalize appearing lesions in the auricular fold, to give rise to the presence of the characteristic crust (Figure 1 G y H).
In the results obtained the presence of lesions in rabbits from two weeks of life (Figure 2), Vázquez (2006) mentions that Psoroptic mange is a typical process of adult animals, which specifies the reproducers, which due to their zootechnical function are more long-lived (older than 4 months of age), compared to growing rabbits (younger to 70 days), however, in this studio it was observed that the presence of mite exists in young animals, although the lesions are not apparent to the first view (Figure 1).

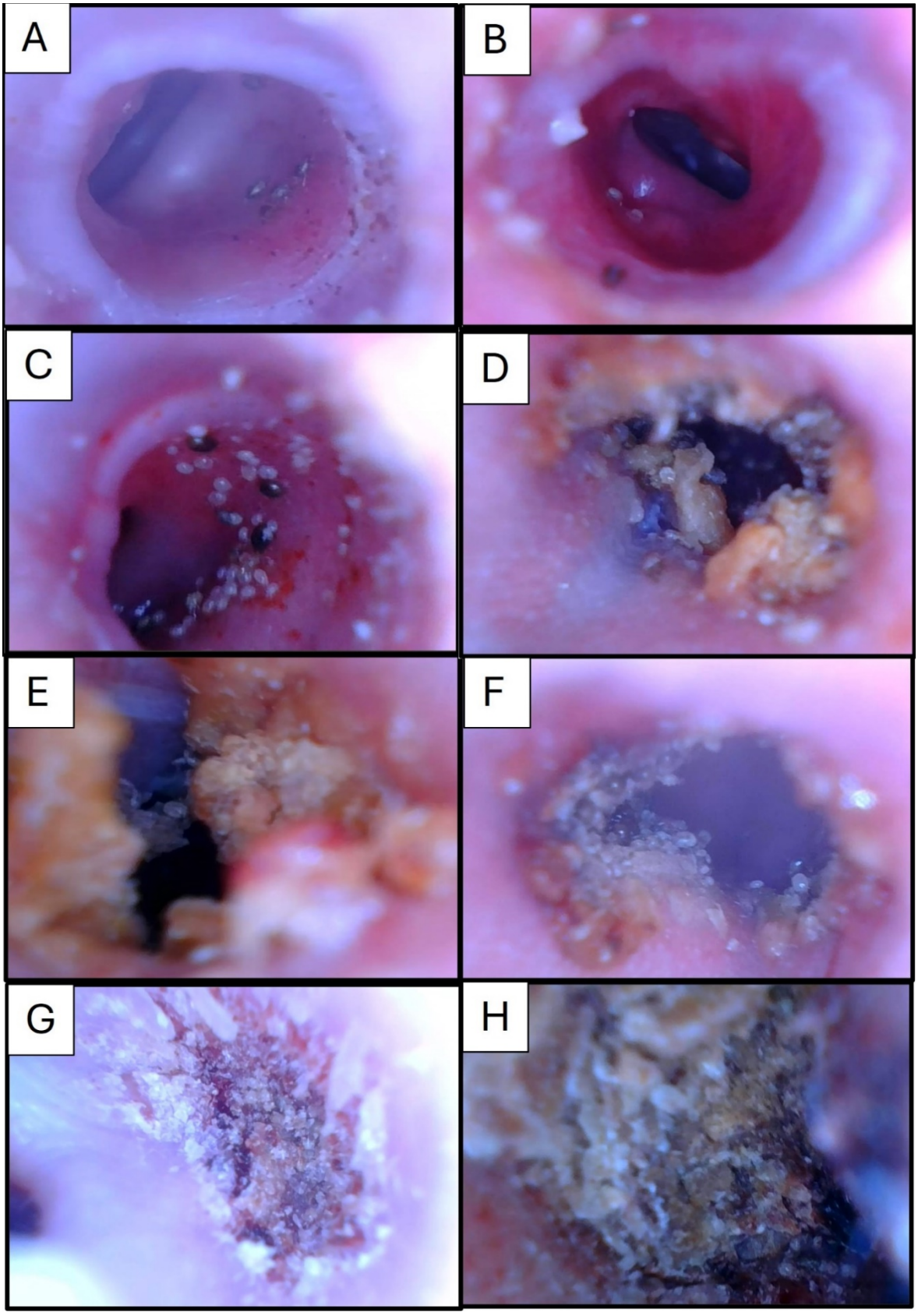
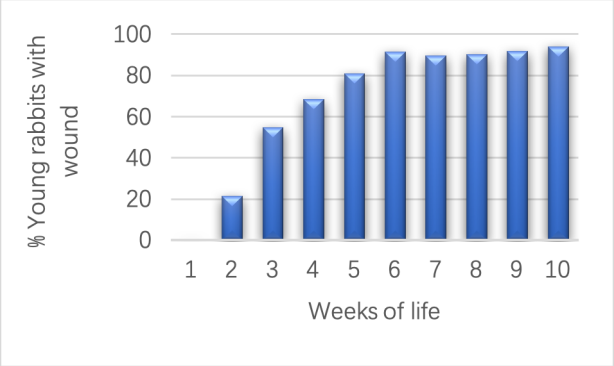


Figure 1: Evolution of Psoroptic mite lesions

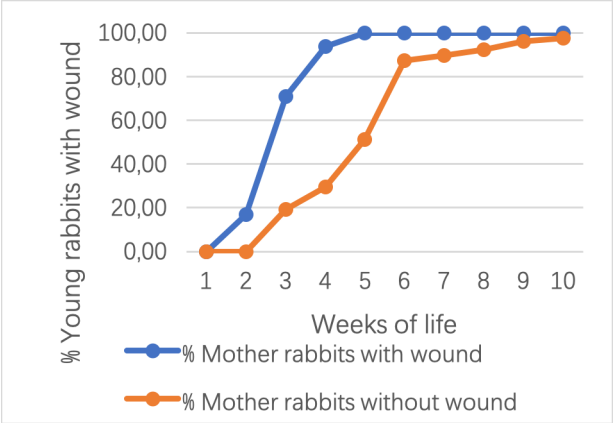
Figure 2: Young rabbits with wound



Percentage of the analyzed young rabbits were considered since, as in any production center, mortality was present due to causes unrelated to the study.
As Papeschi (2006) mentioned, direct contact with infested animals is an important aspect for the transmissions of the mite, and therefore, rabbits in contact with sick females present a greater probability of contagion, as was observed in the present study, rabbits from infected mothers presented lesions at earlier ages, in contrast to the rabbits whose mothers did not present lesions of Psoroptic mite (Figure 3).

Vázquez (2006) mentioned that after 2 to 3 weeks the proliferation of mites invade the entire ear, however, in the present study, the external lesions became visible between 5 to 7 weeks after the first appearance of the parasite, without invading the entire ear (Figure 1) being necessary the use of the endoscope camera to appreciate the lesions.

Figure 3: Young rabbits with wound



CONCLUSIONS

Psoroptes cuniculi was found in a rabbits as young as 19 days of age, including growing rabbits, which can have an impact on rabbit productions, so timely diagnosis and prevention are essential.

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Proyecto PIAPI 2011: Investigación del propóleo de abejas nativas (abejas sin aguijón) para su aplicación en Medicina Veterinaria y Humana

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ASSESSING THE CLINICAL EFFICACY OF SALINOMYCIN TO CONTROL EIMERIA MEDIA AND EIMERIA MAGNA IN RABBITS

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ABSTRACT

Registration of coccidiostats requires pre-clinical trials in rabbits showing efficacy in controlling recently isolated *Eimeria* strains. In most cases efficacy of these products can easily be demonstrated, based on mortality and performance parameters. Parasitological parameter endpoints are much more difficult to achieve as the prevalent *Eimeria* species, currently present in European rabbit farms, do not cause pathognomonic lesions and no scoring systems are described. In this study, a scoring system was used to demonstrate efficacy of salinomycin (Sacox®) after challenge with a mixed inoculum containing *E. magna* and *E. media*, two prevalent *Eimeria* species in commercial rabbitries in Europe.

Key words: *Eimeria media, Eimeria magna,* lesion scoring , salinomycin, coccidiosis, rabbits.

INTRODUCTION

Control and prevention of coccidiosis in rabbits is based on management measures and inclusion of coccidiostats in the feed. Rabbits can be infected by 11 different *Eimeria* species, differing in replication sites, pathogenicity and impact on health and performance. At present, only one coccidiostat is authorised as feed additive for the preventive use of coccidiosis in rabbits in the EU Coxiril® (active diclazuril). The use of a single coccidiostat in mono-programs will eventually result in drug resistance of coccidia (Peeters et al., 1987; 1988). The need for new products to enter the market is therefore critical. EU legislation (regulation 1831/2003) and EFSA (FEEDAP, 2018) give instructions and guidance to compile dossiers to demonstrate product quality, safety (for consumer, target animals and environment) and efficacy of coccidiostats. Efficacy must be proven in challenge studies, using recent mixed *Eimeria* isolates, targeting specific endpoints related to morbidity, coccidiosis related mortality, oocyst excretion, faecal scores and lesion scores. Performance data are considered as supportive data. This is where the problem arises for rabbits, as in most commercial rabbitries *E. media and E. magna* species are detected for which no scoring systems are described.

The objective of this study is to describe a lesion scoring system to demonstrate efficacy of coccidiostats to *Eimeria (E.). magna* and *E. media* in rabbits and its applicability in a trial with Sacox® (active salinomycin).

MATERIALS AND METHODS

Animals and experimental design

The inoculum used in this trial was collected in 2021 from a commercial rabbit farm in Italy. From at least 10 different pens in the farm, droppings were collected after weaning, pooled and transferred to the lab, where samples were first microscopically examined for identification of *Eimeria* species and determination of oocyst per gram (OPG). Oocysts were purified and sporulated in potassium dichromate, to compose an inoculum for virulence titration and challenge.

For the clinical trial, 240 Hyla Hybrid rabbits were received at the trial facility at weaning age of 35 days (Day-3). Rabbits were housed in floor pens and allocated at random into 3 groups (8 replicates, 10 rabbits per replicate): one uninfected control (UC), one infected control (IC) and one treated infected group, receiving salinomycin in the feed (targeted dose 20 ppm) from 3 days after the allocation (D0) till the end of the study (SAL) (see Table1).
Six days after allocation (D3) each rabbit from the IC and SAL group was orally challenged with 1ml of the inoculum that contained 74.100 *E. magna* and 129.200 *E. media* oocysts. The challenge dose was based on the results of a virulence titration trial which was performed prior to the study, with the aim to target a dose causing macroscopic lesions and impact on performance.
Mortality was recorded on a daily basis for the entire study period. Samples for oocyst counts by the McMaster method, were collected using a standardised protocol, on D0, D8, D10, D14, D17, D24, D31 and D42.
Two rabbits per replicate were euthanized and necropsied for lesion scoring on D7 and D10. The scoring system used was based on gross lesions observed after challenge with mixed inocula containing *Eimeria media* and *Eimeria magna (*unpublished data). Gross lesions were scored in duodenum (DD), jejunum & ileum (JI) and caecum (C). In DD and JI gross lesions were scored as follows: score 0: no gross lesions, score 1: mild congestion, watery contents, no thickening of the intestinal wall and score 2: severe congestion, thickening of the intestinal wall, white striations, watery content. Gross lesions of the caecum were classified in score 0: no gross lesions, score 1: mild congestion, some petechiae, content more fluid than normal and score 2: severe congestion, ballooning, thin wall, haemorrhages, very watery content. An average lesion score was calculated for the different sections scored and a total mean lesion score (TMLS), summing up the average scores for the 3 sections was calculated. Challenge dose and time of lesion scoring was based on the outcome of a virulence titration study performed on 24 Hyla Hybrid rabbits, prior to the study.
Rabbits were individually weighed on days D0, D7, D10, D17 and D42. Feed was weighed in and out on the same days, to calculate feed intake and feed conversion.

Table 1: Timing of critical study events

Study Day	Event
D-3	Arrival and allocation
D0	Start salinomycin in SAL group
D3	Coccidiosis challenge by oral gavage in IC and SAL group
D-3-D42	Mortality
D7 & D10	Lesion scoring
D0, D8, D10, D14, D17, D24, D31 and D42	Sampling for OPG
D0, D7, D10, D17 and D42	Weighing rabbits and feed

Statistical Analysis
Data were analysed with R (version 3.2.5 or higher). Data on body weight (BW) and daily weight gain at rabbit level (DWG) was analysed using linear mixed regression models (procedure lme of the package nlme) with treatment group as fixed effect and pen as random effect. Data on feed conversion ratio, intestinal lesion scores and (species-specific) OPG was analysed using linear regression models with treatment group as fixed effect (procedure lm of the core package). FCR was calculated based on pen level. In the acute phase of the infection (D4pi-D14 pi), when growth was halted, negative and/or high mortality occurred, FCR could not be calculated. A natural logarithmic transformation [Ln(x+1)] was performed on OPG data to obtain a normalized distribution. Mortality was analysed using cox proportional hazard models with treatment group as fixed effect. Residual plots were checked to evaluate model fit. In all models, groups were compared to the UC group as reference. Statistical significance was assessed at $p \leq 0.05$.

RESULTS AND DISCUSSION

Performance parameters

Individual challenge with the mixed inoculum (*E. media, E. magna*) resulted in a significant increase in mortality in the IC reaching 30% (versus 11.25% in UC, p-value <0.05). Daily weight gain and FCR were severely impacted by the challenge (see Table 2). The most severe impact was seen 4-14 days post infection (pi), where rabbits were losing weight (D4pi-D7pi) or growth was halted (D7pi-D14pi). For this period FCR could not be calculated as rabbits were losing weight, growth was halted and/or high mortality occurred. The impact of the infection on growth and FCR was visible till the end of the study, 39 days post infection. Feed supplementation of salinomycin (detected dose, 24 ppm- target dose 20 ppm) in the SAL group, significantly decreased mortality (15% versus 30% in IC, p-value<0.05). The mortality in the SAL group was not significantly different than in the UC group (15% versus 11.25%). Supplementation of salinomycin improved daily weight gain significantly in the first 14 days after infection and for the overall period after infection and improved FCR in the first week post infection and the overall trial period (see Table 2).

Table 2: Daily Weight Gain (DWG) and feed conversion ratio (FCR) in the different periods post infection (pi)

	D-3-D4pi		D4pi-D7pi		D7-D14pi		D14-39pi		D-3D39pi	
	DWG	FCR	DWG	FCR	DWG	FCR*	DWG	FCR	DWG	FCR
IC	16.74[b]	5.54[b]	-27.25[b]	NA	0.42[b]	NA	40.24	4.19	27.24[b]	6.72[b]
SAL	36.50[a]	3.06[a]	23.61[a]	NA	32.92[a]	NA	46.01	3.96	41.26[a]	3.90[a]
UC	34.95[a]	3.25[a]	14.35[a]	NA	55.84[a]	NA	49.81	3.81	43.59[a]	3.40[a]

Means with different letters in the same column differ significantly (DWG linear mixed regression models, FCR linear regression model)
NA: not applicable: FCR could not be calculated as rabbits were losing weight, growth was halted and/or high mortality occurred

Parasitological parameters

TMLS was significant higher on both scoring days in the IC in comparison to the UC (2.75 versus 0.8 on D4pi, p value<0.001; 3.53 versus 1.75 on D7pi, p value <0.05) (Table 3). The average score for the individual sections was significant higher for the IC in comparison to the UC in DD on D4 pi, for JI on day 7 pi and on both scoring days for the caecum (see Table3). Supplementation of SAL significantly reduced lesions in comparison to the IC on both scoring days for caecum and for JI on D7 pi. TMLS in the SAL group was significantly lower than the IC group on both scoring days (1.40 versus 2.75 on D4pi, p value<0.05; 1.25 versus 3.53 on D7pi, p value <0.001).

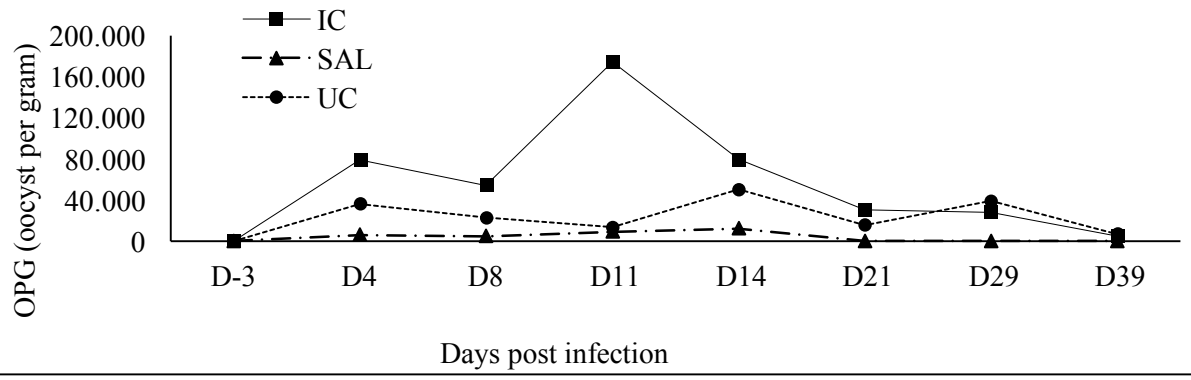
Table 3: Total mean lesion score (TMLS) and average lesion scores in duodenum (DD), jejunum and ileum (JI) and caecum (C) 4 and 7 days post infection (pi)

	TMLS		DD		JI		C	
	D4 pi	D7 pi	D4 pi	D7 pi	D4 pi	D7 pi	D4 pi	D7 pi
IC	2.75[b]	3.53[b]	0.95[b]	0.74	0.75	1.26[b]	1.05[b]	1.53[b]
SAL	1.40[a]	1.25[a]	0.60[b]	0.40	0.60	0.50[a]	0.20[a]	0.35[a]
UC	0.80[a]	1.75[a]	0.30[a]	0.50[b]	0.40	0.75[a]	0.10[a]	0.50[a]

Means with different letters in the same column differ significantly (linear regression model)

Oocyst excretion in the IC group reached a peak 11 days post infection (see Figure 1). Levels of oocysts were numerically higher in the IC group in comparison to the UC till 29 days post infection. As rabbits were housed in the same room, low levels of oocysts of *E. media and E. magna* were detected in the UC group. OPG levels in the SAL group were significantly decreased in comparison to the IC on all sampling points (p-value ≤0.05).

Figure 1: OPG after challenge in infected control (IC), salinomycin infected (SAL) and uninfected control (UC)



CONCLUSIONS

Individual challenge of a mixed inoculum containing *E. media* and *E. magna* significantly increased mortality and caused loss of weight in comparison to the UC group. Mortality in the UC and in the SAL group was mainly seen after allocation, due to enteropathy, probably caused by stress, while mortality in the IC increased till the end of the study. Supplementation of salinomycin in the feed at 20 ppm significantly decreased mortality and improved daily weight gain and FCR for the overall period in comparison to the uninfected control group. At 4 and 7 days after infection, the gastrointestinal tract was scored for gross lesions. Although the replication sites of *E. media* and *E. magna* are described to be located in the duodenum, jejunum and ileum, (Coudert *et al.*, 1995; Pakandl 2009) lesions were extending to the caecum. A possible explanation could be the high challenge dose used in this study. A significantly higher total mean lesion score was seen in the infected control group in comparison to the uninfected control and the salinomycin supplemented group. With the exception of one sampling point, supplementation of salinomycin decreased OPGs significantly. The scoring system described in the present study proved useful to evaluate severity of infection and therefore may become a valuable asset for veterinarians in the field and to achieve endpoints related to lesion scoring for demonstration of coccidiostat efficiency for inocula containing *E.media* and *E.magna.*

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INVESTIGATION ON ANTICOCCIDIAL DRUG RESISTANCE AGAINST DICLAZURIL OF RABBIT COCCIDIA IN SOME AREA OF CHINA

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ABSTRACT

Diclazuril is a widely utilized anticoccidial agent for controlling coccidiosis in livestock and poultry. To explore the potential development of drug resistance in rabbit coccidia, we conducted a survey encompassing eight regions, including Jiangsu, China. A total of 40 rabbit fecal samples were collected and examined for coccidia oocysts, revealing 27 positive samples under microscopic observation. PCR analysis confirmed infections with eight species of rabbit coccidia, notably *Eimeria magna.* In conjunction with an analysis of medication records from the respective rabbit farms, we observed an 83.3% infection rate of coccidiosis in rabbits treated with diclazuril. Drug resistance testing was performed in rabbits using the eight positive samples identified (five of which were treated with diclazuril). The findings indicated no significant disparity in body weight gain between the medicated and non-medicated groups. However, the oocyst shedding levels in the medicated groups either matched or even exceeded those of the non-medicated groups, suggesting a partial or complete loss of efficacy against these coccidia parasites. Further research endeavors are warranted to isolate and test drug-resistant coccidia species and elucidate the mechanisms underlying drug resistance.

Keywords: rabbit coccidia, anticoccidial drug resistance, diclazuril

INTRODUCTION

Rabbit coccidiosis, caused by parasitic infections of coccidia belonging to the genus *Eimeria*, is a highly prevalent parasitic disease, particularly among young rabbits under 3 months of age. The disease often results in significant mortality and imposes substantial losses on rabbit husbandry (Bhat *et al*., 2010). Symptoms of rabbit coccidiosis vary depending on the species of coccidia and the site of parasitic infection, demonstrating clinical sign of either intestinal or hepatic coccidiosis, or both (Dubey, 2019). All rabbit breeds are susceptible to coccidiosis, with newly weaned and young rabbits particularly vulnerable. In contrast, infected adult rabbits typically display milder symptoms and often become carriers of coccidia (Pilarczyk *et al*., 2020).

Currently, the primary strategy to prevent rabbit coccidiosis is the use of anticoccidial drugs. Polyether drugs such as salinomycin and monensin, as well as chemically synthesized drugs like diclazuril, dinitramide, sulfaquinoxaline, are commonly employed in rabbit farms (Gu, 2013). However, the improper use of anticoccidial drugs lead to the development of resistance in coccidia, as well as issues related to drug residues and toxicity (Sun *et al*., 2023). An investigation in China had suggested a diminishing therapeutic efficacy of diclazuril and several other anticoccidial drugs against rabbit coccidiosis, raising concerns about the emergence of drug resistance (Jing *et al*., 2012). Consequently, the investigation of diclazuril resistance in rabbit coccidia holds practical significance.

MATERIALS AND METHODS

Animals

The experimental rabbits are 30-35 day old New Zealand white rabbits provided by the Zhuozhou Experimental Station of Rabbits. A total of 51 rabbits were used for drug

resistance test. All rabbit involved in this study were experimented in strict accordance with the relevant rules and regulations of the Experimental Animal Welfare and Animal Experimentation Ethics Committee of China Agricultural University.

Fecal sample collection

Fecal samples were collected from rabbit farms located in 8 provinces in China. Approximately 300g of feces were randomly collected from rabbit populations. The sample information includes farm's location and details regarding rabbit breed, age, breeding methods, medication history, and other pertinent information. Samples were dispatched in low-temperature foam boxes with ice packs and promptly stored at 4°C upon receipt.

Experimental design

Coccidia oocysts in the fecal samples were checked quantified using the McMaster counting method, as described by Ahmed-Laloui *et al*. (2022). Coccidia oocysts were collected from fecal samples following the protocol outlined by Eckert *et al*. (1995). After sporulation, oocysts were subjected to microscopic examination of morphology using previous procedure (El-Shahawi *et al*., 2012). Species-specific primers targeting ITS-1 of *Eimeria* species were employed for the identification of rabbit coccidia (Oliveira *et al*., 2011).

Among the oocyst positive samples, 8 samples were selected for drug resistant test. Each sample was used for the infection of 2 groups of 3 rabbits (one group with supplementation of anticoccidial drug while another group without supplementation). Rabbits in the treatment group were given diclazuril in drinking water (0.5 mg/L) two days before infection with oocysts and continued to use it for 14 days after infection. A blank group consisting was also included. Except for the rabbits in the blank group, each rabbit received 5,000 oocysts. Body weight gain (day 0 to day 12 after infection) and oocyst output (day 5 to day 12 after infection) were compared between the medication and non-medication groups (Ogolla *et al*., 2018).

Data Analysis

The difference in susceptibility to diclazuril between the drug treatment and non-treatment groups was statistically analyzed using the chi-square test in SPSS 22.0.

RESULTS

The investigation of Coccidia infection in rabbit farms in some area of China

Between April 2023 and June 2023, a total of 40 rabbit fecal samples were collected from 9 rabbit farms across 8 provinces in China. In these samples, 27 (67.5%) were positive for oocysts. We use OPG (oocysts per gram) to estimate the intensity of infection ($OPG>10\times10^4$ is severe infection, $1\times10^4\leq OPG\leq10\times10^4$ is moderate infection, while $OPG<1\times10^4$ is mild infection (Hui *et al*., 2020)). Among the positive samples, 22 exhibited mild infection, while 5 displayed moderate infection. No severely infected samples were observed. Excluding Shanxi Province, all regions exhibited infection rates exceeding 50%. The analysis also revealed that young rabbits (1-3 months) exhibited the highest infection rate at 80%.

The utilization of anticoccidial drugs in these farms varied across the regions according to the simultaneously collected information of drug supplementation. Diclazuril was the most commonly used anti-coccidial drug with an infection of 83.3%, it accounted for 60% (3/5) of moderately infected samples and 77.3% (17/22) of mildly infected samples. Farms used robenidine hydrochloride with an infection rate of 71.3%, affecting 40% (2/5) of moderately infected samples and 13.6% (3/22) of mildly infected samples.

DNA extracted from the 8 oocyst-positive samples was used for PCR identification with species-specific primer pairs. *E. magna* was detected from 8 samples, while *E. intestinalis*、*E. media* and *E. vejdovskyi* were identified from 7 samples. Other *Eimeria* species identified include *E. exigua*, *E. coecicola*, *E. perforans* and *E. piriformis*. All these 8 samples were confirmed with mixed infection of coccidia, ranging from 4 species to 8 species(Table 1).

Table 1: Species Identification

Sample number	Species Identification
JS1	*Ema*、*Eme*、*Eco*、*Eex*、*Epe*、*Eve*、*Ein*
JS2	*Ema*、*Eme*、*Eco*、*Eex*、*Ein*
JS3	*Ema*、*Eme*、*Eex*、*Eve*、*Ein*
JL	*Ema*、*Eme*、*Eco*、*Epe*、*Epi*、*Eve*、*Ein*
ZJ	*Ema*、*Eme*、*Eco*、*Eve*、*Ein*
FJ	*Ema*、*Eme*、*Epi*、*Eve*
SC	*Ema*、*Eex*、*Eve*、*Ein*
AH	*Ema*、*Eme*、 *Eve*、*Ein*

The test of diclazuril resistance in rabbits

Body weight gain and total oocyst output were used as the indices for the test of diclazuril resistance in rabbits. There was no significant difference in body weight gain between the medicated and the non-medication group ($P > 0.05$) (Figure 1A).

There was no significant difference in total oocyst output between groups medicated or non-medicated for samples SC, FJ, JS2, and JL; while significant difference ($P < 0.05$) was detected between groups set for AH ($P < 0.001$) and JS1 ($P < 0.05$) (Figure 1B). In groups of ZJ and JS3 samples, medicated rabbits shed significant lower oocysts than those of non-medicated ones.

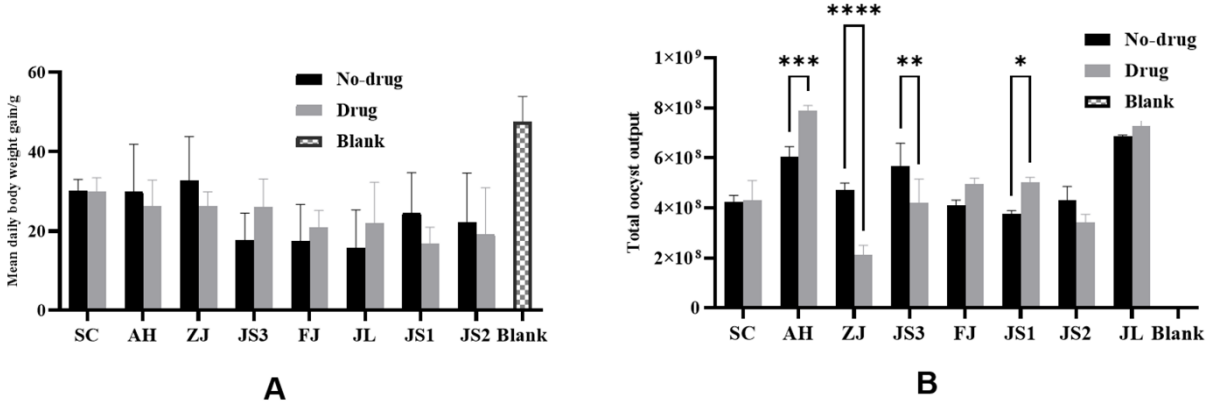

Figure 1 The daily body weight gain and total oocyst output of rabbits inoculated with 8 oocyst samples and medicated/non-medicated with diclazuril. Diclazuril medicated or non-medicated rabbits were inoculated with 8 samples of oocysts isolated from rabbit farms. Body weight (day 0 and day 12 after infection) was measured for each groups of rabbits, and feces was collected from each group (from day 5 to day 12 after infection) for oocyst counting. *, ** and *** stand for significant difference ($P < 0.05$), ($P < 0.01$) and ($P < 0.001$), respectively.

DISCUSSION

In summary, the results of the survey show that the infection rate of rabbit coccidiosis in rabbit farming in China is generally high, and they are all mixed infections with multiple types of rabbit coccidiosis.

Rabbit coccidiosis samples in various regions are treated with the anticoccidial drug Diclazuril in our trial. We calculated the amount of coccidia oocysts, and the result show that a large number of coccidia oocysts still excreted even using diclazuril. This study preliminarily confirmed the existence of the anticoccidial drug diclazuril resistance, rabbits cannot completely prevent *Eimeria* infection and are resistant to Diclazuril. We need to further isolate diclazuril-resistant strains and study the mechanisms of drug resistance.

ACKNOWLEDGMENTS

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CLINICAL AND PATHOLOGICAL FINDINGS AND MOLECULAR CHARACTERIZATION OF *TRICHOPHYTON MENTAGROPHYTES* IN AN ATYPICAL CASE OF DERMATOPHYTOSIS

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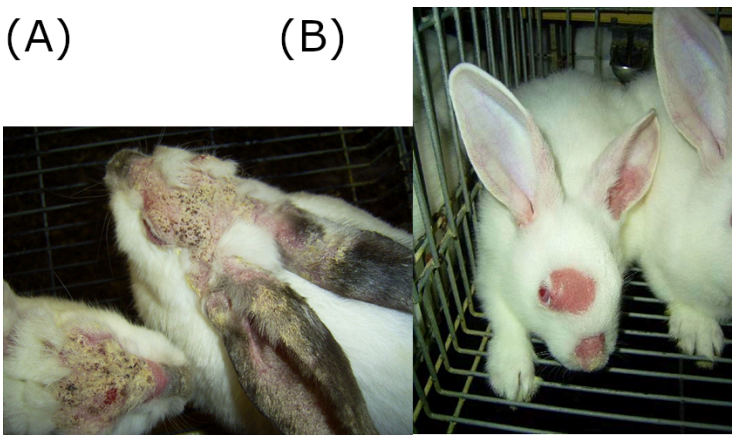
ABSTRACT

This study describes an atypical outbreak of dermatophytosis in a rabbit farm, focusing on epidemiological patterns, isolation, and characterization of the causal agent. Dermatophytosis mainly affects young rabbits, with the lesions manifesting as circular hair loss on the ears and head. *Trichophyton mentagrophytes* was identified as the causal agent and presented a self-limiting nature. Despite potential exposure, there was no evidence of disease transmission to farm personnel. Further investigation is warranted to understand the mechanisms underlying the self-limiting clinical presentation.

Key words: Dermatophytosis, *Trichophyton mentagrophytes*, pathology, skin diseases, zoonosis.

INTRODUCTION

Dermatophytosis or ringworm in rabbits is a zoonotic fungal infection caused mainly by *Trichophyton mentagrophytes* and, less frequently, by *Microsporum* ssp. *(Varga, 2014)*. However, other fungal species have also been isolated. Lesions are typically found on the skin of the head, especially around the eyes and nose, and on the legs, and rarely the nail beds, of young rabbits. They are characterized by areas of patchy alopecia and crusts, with erythema and pruritus observed occasionally. In does, lesions are often unnoticeable, and if visible, they are usually observed in the mammary area. The severity of the lesions depends on the species of fungus involved, with the most severe lesions being observed in rabbits infected by *Microsporum* spp. (Fig. 1).



This study aims to describe the clinical, lesional, microbiological, and molecular study of an atypical case of dermatophytosis due to *Trichophyton mentagrophytes* in a rabbit farm.

Figure 1: (A) *Microsporum canis*, (B) *Trichophyton mentagrophytes*

MATERIALS AND METHODS

Natural outbreak

In 2022, an unusual presentation of cutaneous lesions compatible with dermatophytosis was identified on a meat rabbit farm, that persists to this day. The farm raises Iberian hybrid rabbits and houses approximately 4000 breeding does. It includes three distinct production units, in which rabbits are raised with a difference of 15 days. Artificial insemination is performed at 11-day post-partum. The kits are separated from their mothers at 35 days of age and then relocated to new feedlot units, which houses approximately 8-10 kits.

Each unit is equipped with under-pressure mechanical ventilation systems, humidifiers in summer and heaters in winter, to maintain optimal environmental conditions. The units are routinely cleaned when rabbits are sent to the slaughterhouse, by using pressurized water and a rotating protocol of diverse disinfectants.

Fungal control is carried out routinely and includes the application desiccant on the covering material of the units before farrowing. Clinical evaluation is routinely performed during the lactation and fattening period and lesions compatible with dermatomycosis are monitored in does and kits, such as dermatitis, scales, hair loss, and congestion around the nipples (only in does).

Given the clinical suspicion of an atypical dermatophytosis, skin samples were taken for microbiological and histopathological studies. Molecular identification was carried out on the isolate.

Isolation and identification

Skin samples were taken by scraping the edge of the lesion with a sterile blade and then stamping it with adhesive tape. The sample was cultured in Sabouraud-Chloramphenicol (SC) agar, both at room temperature and 37 ºC, and incubated for 5 days. Lactophenol Cotton Blue Staining (LPCB) was performed in plate growth. Furthermore, VITEK2 technology (bioMérieux) was used to identify isolated organisms and molecular biology was performed to confirm the result.

DNA extraction from the culture involved creating suspensions using an isolation loop in a 1.5 ml tube containing 200 µl of PBS. These suspensions were then homogenized using a physical tissue homogenizer. The supernatant obtained from this homogenization process was utilized for DNA extraction using a genomic tissue DNA extraction kit (FavorPrep™ Tissue Genomic DNA Extraction Mini Kit, favorgene).

For fungal identification, the sequence of three previously described genes was used (Garzon, 2013; White et al., 1990): two ITS (nuclear ribosomal internal transcribed spacer) regions and the *β-tubulin* gene.

PCR amplification was carried out under the following conditions: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 40 seconds. A final extension step was performed at 72°C for 7 minutes. The PCR products were then analyzed by agarose gel electrophoresis (1.5%) to verify band integrity and size. Following gel electrophoresis, the PCR products were purified using the ExoProStar™ 1-Step kit (Cytiva). Subsequently, Sanger sequencing was outsourced to an external facility (Stabvida, Portugal). Both forward and reverse sequencing were performed to ensure accuracy. The obtained sequences were then assembled using BLAST to analyze and compare them with known sequences in the database.

RESULTS AND DISCUSSION

Clinical and pathological findings

Clinical signs were only observed in rabbits aged 40-45 days and older, with the onset of observations occurring at this age and persisting throughout the fattening period in isolated units.

Approximately 10 % of the units were affected and the number of affected rabbits within each unit was variable, with some units having all rabbits affected. Frequently, more than one rabbit was affected within the unit. Rabbits from units adjacent to affected rabbits remained

free of infection until slaughter, suggesting low transmissibility. Breeding animals were macroscopically examined, with no lesions observed.
The lesions corresponded to rounded, alopecic areas, isolated to confluent, located mainly on the ears and, less frequently, on the nose (Fig. 2). The lesions tended to remain unchanged and did not spread to other areas of the skin throughout the fattening period. No healing of lesions was observed in any case.
Interestingly, no infection among farm staff was observed during this period. which is an unexpected finding due to the elevated contagiousness of ringworm. It is worth to note that no especial control measures were taken to prevent transmission.

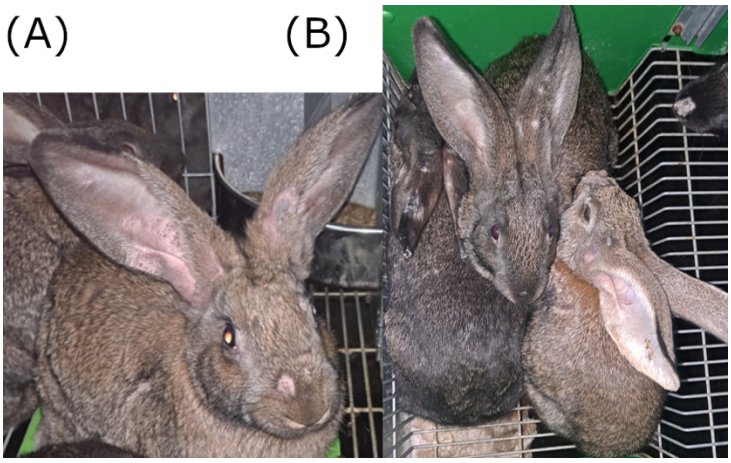


Figure 2: (A) Small lesions can be seen in the face and inner part of the ear. (B) A pair of animals with similar lesions.

Microscopically, a great number of fungal structures were observed within numerous hair follicles, and less often, within the superficial keratin (Fig. 3A). Numerous round microconidia and sporadic short septate hyphae were observed. Hyphae were better demonstrated with silver stain (Fig. 3B). Follicle loss and very mild multifocal dermatitis and perifolliculitis was also observed.

(A) (B)

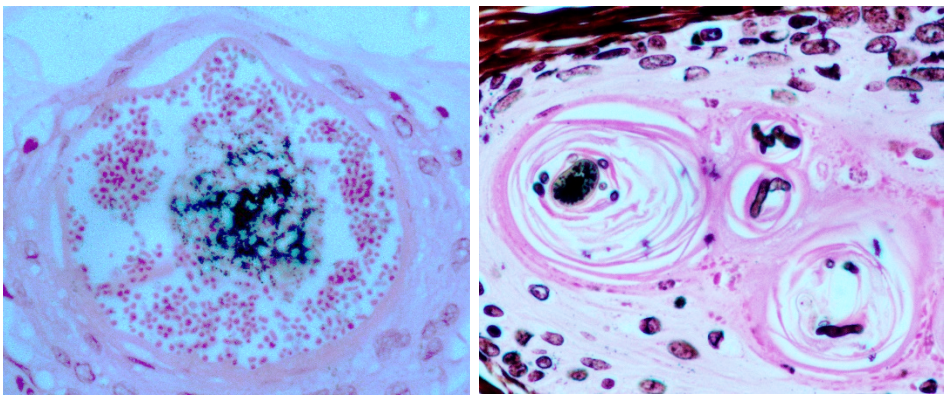


Figure 3. A. Numerous microconidia surrounding the keratin within a hair follicle. Hematoxylin and eosin. X 630. B. Small number of microconidia and small septate hyphae within hair follicles. Silver stain. X630.

Isolation and characterization

The SC culture initially revealed two types of fungi, one whitish and the other black. At 37°C, the whitish grew better, while at room temperature, the black was more apparent. Gross characteristics of the white fungus

Figure 4. *T. mentagrophytes* growth in SC culture medium.

suggested *Trichophyton*. It appeared white on the front of the culture, while the reverse side showed a brownish coloration (Fig. 4). Subsequently, the fungus was plated onto Dermatophyte Test Medium (DTM), where it grew without competitors.

VITEK2 further identified the white fungus as *Trichophyton* and the black fungus as *Ulocladium*.

LPCB staining of the *Trichophyton* revealed no macroconidia. We hypothesize that the absence of these structures may contribute to its self-limiting nature, although further research is necessary to verify this hypothesis.

Ulocladium is considered a saprophytic fungus and generally a contaminant (Kaur et al., 2010). In this study, it was considered unlikely to be the cause of the lesions.

Molecular studies indicated that the isolate corresponded with *Trichophyton mentagrophytes* (Table 1).

Table 1: score and hits of the three genes used for various *Trichophyton* species. Results with low scores have been omitted.

Species	ITS 1 score	ITS 1 hits	ITS 2 score	ITS 2 Hits	B-tubulin score	B-tubulin hits
Trichophyton mentagrophytes	436	68	544	52	743	82
Trichophyton vanbreuseghemii	-	-	-	-	715	1
Trichophyton interdigitale	436	20	544	44	715	95
Trichophyton violaceum	-	-	-	-	715	1
Trichophyton erinacei	436	1	-	-	715	1
Trichophyton benhamiae	436	1	-	-	710	1

CONCLUSIONS

In conclusion, this study presents an outbreak of dermatomycosis caused by *Trichophyton mentagrophytes* in a rabbit farm with an atypical epidemiological pattern, which includes the absence of lesions during the lactation period, its presence in isolated litters between 40 and 45 days of age and older, and the absence of transmission to farm personnel. Furthermore, the outbreak occurred in a breed of rabbit that is more susceptible to this infection than other breeds. The self-limiting nature of the disease was attributed to the absence of macroconidia formation, although it warrants further investigation.

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THE AHOL (ANIMAL HEALTH ONTOLOGY FOR LIVESTOCK) ONTOLOGY FOR INTEGRATING DATA ON THE MAIN DISEASES OF FARMED ANIMALS: AN EXAMPLE FOR THE RABBIT SECTOR

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ABSTRACT

Disease management in animal production is essential and requires comprehensive data integration. An ontology is a means of organizing knowledge and represents a set of concepts with logical relationships between them. The Animal Health Ontology for Livestock (AHOL), developed by INRAE, provides a standardized framework for organizing farm animal health traits. AHOL categorizes diseases, symptoms, affected species and pathogens, promoting interoperability and facilitating knowledge sharing. This article presents the development of AHOL and its possible application in the rabbit sector. AHOL offers a valuable resource accessible on its website with data on 60 rabbit diseases, their symptoms and pathogens: https://www.umrh.inrae.fr/ontologies/visualisation/public/ahol/diseases/show. Each of these elements is classified in a tree structure to facilitate the search and organization of information. Ongoing evolution and collaborative efforts are essential to the continuous improvement of AHOL and the extension of its application to animal health management. This tool can be used by professionals or students in animal health, as well as by all scientists or professionals working with farm animals.
Key words: Ontology, Disease, Rabbit, Database.

INTRODUCTION

Health management is crucial in animal production. It involves the identification of diseases and the development of study and methods to prevent, reduce, and cure diseases. Numerous studies and databases exist concerning animal health. However, an efficient disease management necessitates comprehensive data organization and integration. The Animal Health Ontology for Livestock (AHOL), a collaborative initiative led by INRAE with the participation of French Veterinary schools, addresses this need by providing a standardized framework for integrating and structuring information related to the health of farmed animals. An ontology is a way to organize knowledge and represents a set of concepts with the logical relations between these concepts (Ferret, 2021). As such, AHOL, categorizes and organizes information on diseases, symptoms, affected species, and pathogens in livestock. The overarching goal is to establish a common vocabulary that enhances interoperability, facilitates knowledge sharing, and promotes a unified understanding of animal health. The aim of this article is to present the development and application of the AHOL ontology for the rabbit farming sector. The rabbit farming sector is actively engaged in the reduction of antibiotic use and health management. Ontology is therefore a valuable tool to help professionals organize health information in a clear and easy way.

MATERIALS AND METHODS

Creation of the ontology

The Ontology AHOL was created in 2017 to complement the ATOL Ontology (Animal Trait Ontology for Livestock) created in 2009 and the EOL Onotology (Environment Ontology for Livestock) created in 2013. The EOL ontology describes the environmental conditions of domestic animal breeding. More specifically, it describes feeding, environment, farm

structure and breeding systems. ATOL is an ontology of all measurable or observable traits of livestock. It includes growth and meat production, reproduction, animal welfare trait, nutrition trait and other traits. As disease-related traits were not included, the new AHOL ontology was created to fill this gap for farm animals. Working groups for each animal species (or group of animal species) were created with experts from different fields (computer scientists, veterinarians, researchers, experimental farm managers). AHOL is structured into four sections: diseases, symptoms, pathogens and animal species. Diseases are classified into branches according to their type (transmissible, genetic, infectious, genetic), and each disease is linked to associated symptoms, affected species and relevant pathogens. This hierarchical structure enables a clear understanding of the relationships between the different elements of the ontology.

A template for collecting disease information

The experts defined the first diseases to be implemented (generally the main diseases present on farms or in INRAE's experimental facilities) and created a template to collect the main disease information (disease name, chronic or acute, pathogen, disease transmission, species affected, sex, age, physiological state, symptoms, effects on production characteristics). The disease template evolved over time, with the addition of new information (type of disease: infectious, nutritional, metabolic, genetic... to help classify the disease in the ontology; aggravating factors, disease evolution, affected breed, description of clinical signs). Finally, a more efficient approach was adopted. The template was simplified and only the main information was retained: disease name and definition, pathogen, disease transmission, affected species, symptoms. The list of rabbit diseases has been extended to provide a more comprehensive overview of rabbit diseases. Some diseases corresponding to syndromes (a combination of symptoms characterizing an abnormal state) have also been added.

Sources of information

AHOL data come from a variety of sources, including scientific publications, veterinary databases and expert opinions. For rabbits, the main sources of information were based on books (Boucher and Nouaille, 2013; Nowland and Brammer, 2015; Varga, 2014) and online veterinary manuals (MSD Veterinary Manual; Diseases of Research Animals DORA). Additional information was sought in specific scientific publications on the disease if necessary. After a first cycle of implementing the diseases in the ontology, the disease definitions were harmonized to include the main elements: type of disease, whether it is a zoonosis, pathogenesis or factors causing the disease, transmission, main symptoms and animal species affected.

Description of symptoms

Symptoms (defined as disorder or sign observable on clinical examination which is the manifestation of a disease and its evolution) have also been organized into branches and definitions have been included, based on other ontologies used in human medicine and animal anatomy (SNOMED CT,CSSO, SYMP ontologies available on https://bioportal.bioontology.org).

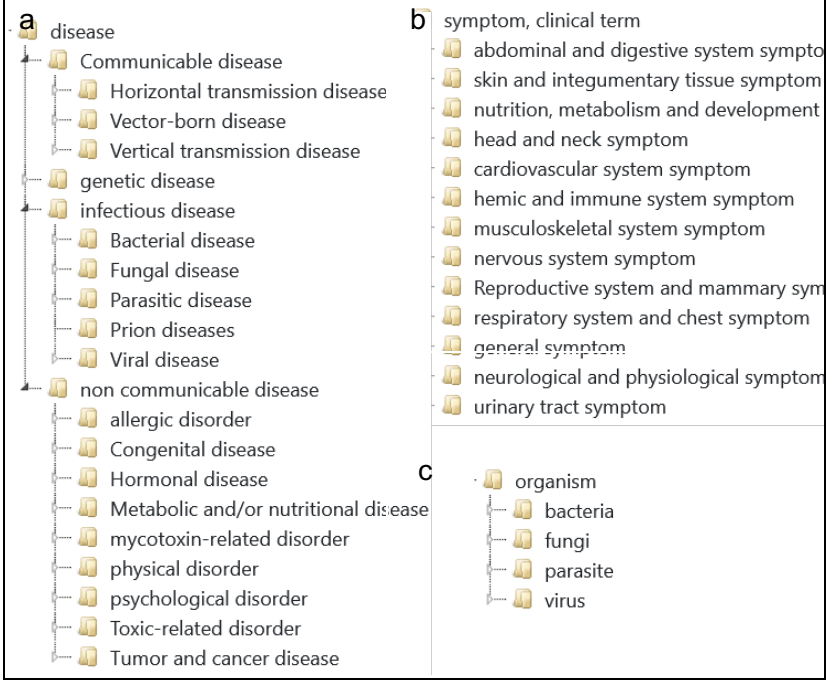
RESULTS AND DISCUSSION

Presentation of the ontology website

The AHOL ontology can be found here: https://www.umrh.inrae.fr/ontologies/visualisation/public/ahol/diseases/show. A search bar enables a quick search on a specific disease or on diseases affecting rabbits. At present, about 60 rabbit diseases are listed in the ontology.

Three ontology sections (Diseases, Pathogens, Symptoms) are accessible on the left-hand side of the screen. Each section is organized into branches and sub-branches (Figure 1).

Figure 1: Hierarchical organization of the ontology into branches for the a) diseases section, b) symptom sections, c) pathogens section of the AHOL Ontology.



Each disease is assigned to at least one branch. Many infectious diseases are found in two branches: an infectious disease can be a transmissible disease. For example, colibacillosis will be found in the communicable disease/ horizontal disease branch and in the infectious disease/ bacterial disease/Escherichia coli infection branch.

The symptoms section is organized into 13 branches according to the affected system (respiratory, digestive, etc.). The pathogen section is divided into four branches (bacteria, fungi, parasites, viruses). Clicking on a disease or symptom opens a sheet containing the name and the definitions in English and French. The disease sheet (example Figure 2) presents also the list of main symptoms, the pathogen(s) causing the disease and the animal species affected.

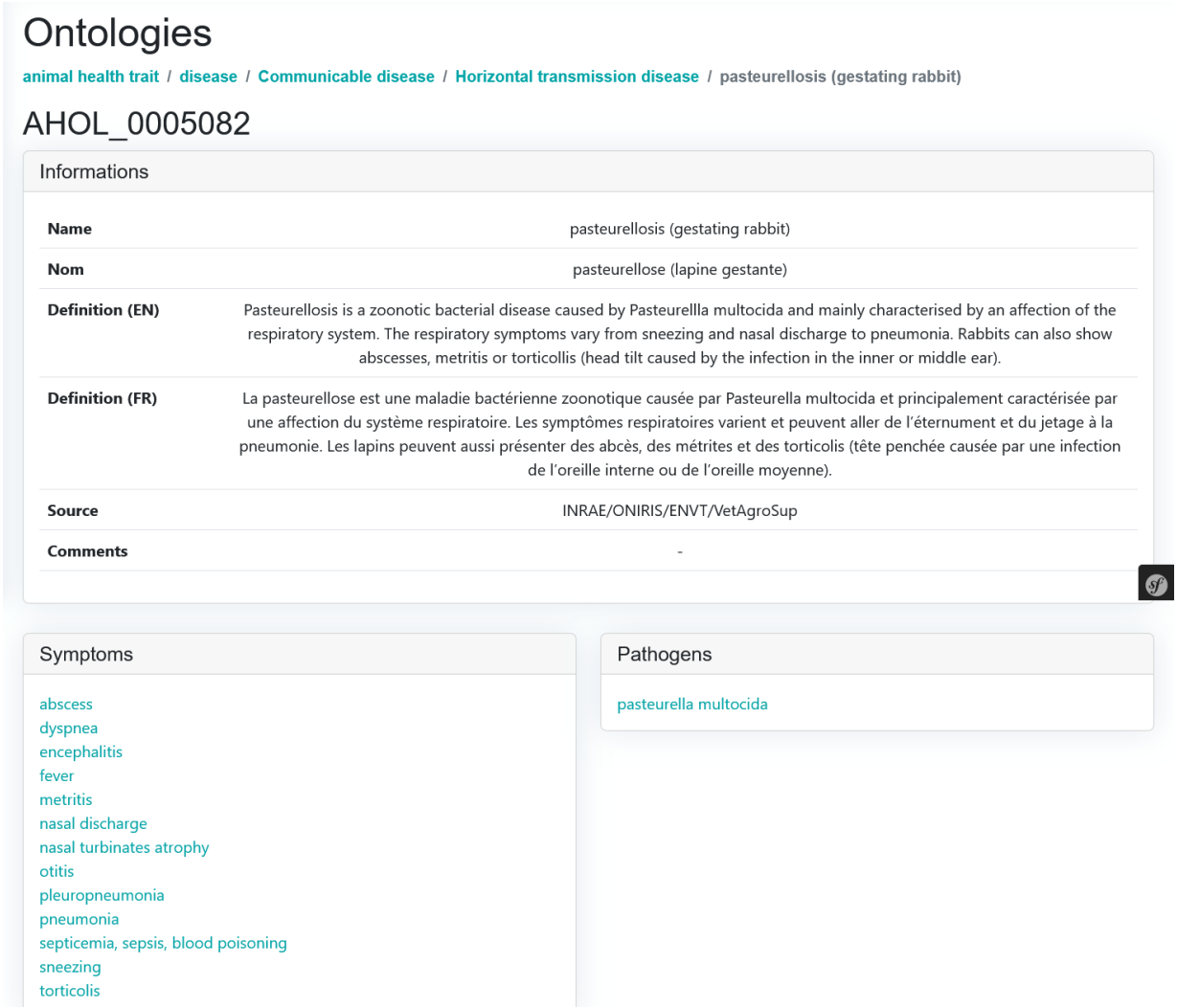
Ontology uses

AHOL helps standardize data, promote interoperability and foster a common understanding of diseases. The ontology's structured format enables efficient cross-referencing of information, facilitating access for researchers, veterinarians and other stakeholders. The ontology can be used by researchers to reference a common vocabulary, add metadata to scientific articles or databases deposited in public repositories, and for meta-analyses. The ontology can also be used as a pedagogical tool for animal health students. Indeed, AHOL provides a complete and organized summary containing only the main information. More generally, ontology can be used for animal health management, to explore the link between pathogen and symptoms, or between pathogens, or to compare diseases between animal species.

Adaptation and improvements

The AHOL ontology is still evolving, with many diseases still missing. Eventually, links could be built between different ontologies whose terms are interoperable, as for example with ATOL whose traits can be linked to symptoms in AHOL. An important development would be the ability to search for a symptom and obtain the corresponding diseases as a result. The rabbit diseases presented here mainly concern animals raised in a temperate European environment. The inclusion of diseases presents in other environments around the world would be necessary. The ontology is the result of a collaborative effort. If you have any suggestions for improving the ontology or implementing other rabbit diseases, please contact the corresponding author.

Figure 1: Screenshot of the Pasteurellosis disease sheet in the online AHOL ontology



CONCLUSIONS

The development and application of AHOL represents a crucial step towards a standardized, interoperable system for organizing farm animal health data. AHOL provides a comprehensive framework for organizing health information. It needs to be continually adapted and extended. AHOL can be a valuable resource for researchers, veterinarians, industry and educators.

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GENETIC VARIABILITY AND PHYLOGENETIC ANALYSIS OF RABBIT HEMORRHAGIC DISEASE VIRUS (RHDV) IN CHINA FROM 2020 TO 2022

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ABSTRACT

Rabbit hemorrhagic disease (RHD) induces lethal fulminant hepatitis in rabbits. GI.1 (RHDV) and GI.2 (RHDV2) genotypes responsible for RHD are reported. GI.2 was first detected in France in 2010 and rapidly spread worldwide. In 2020, GI.2 emerged in Sichuan province in China. To investigate the genetic variability and evolution of RHDV strains after RHDV2 arrival in China, 185 liver samples collected from 35 rabbit farms between 2020 and 2022 were tested by hemagglutination assay, and positive samples were collected. VP60 gene sequences were amplified and phylogenetic analysis of new isolates were conducted. Our results showed that within 3 years after emergence of RHDV2, GI.2 genotype had spread and became the dominant circulating strain, replacing GI.1 genotype in China. Considering the high risk of RHDV2 to the Chinese rabbit industry, ongoing surveillance and vaccine formulation update are imperative for control RHDV2 in China.

Keywords: Rabbit Hemorrhagic Disease Virus, Genetic variability, Phylogenetic analysis, China

INTRODUCTION

Rabbit hemorrhagic disease virus (RHDV), a single-stranded positive-sense RNA virus of the family *Caliciviridae*, genus *Lagovirus*, causes severe economic losses in rabbit industries. Two genotypes of RHDV (GI.1 and GI.2) are reported. GI.1 was first reported in China in 1984, contains the classical RHDV (GI.1b–GI.1d) and the antigenic variant RHDVa (GI.1a), while GI.2 (RHDV2) was first detected in France in 2010 (Le Gall-Reculé *et al*., 2013) and rapidly spread worldwide. GI.2 was reported to replace GI.1 in several European countries and Australia (Mahar *et al*., 2018), indicating it has some competitive advantages over GI.1 (Taggart *et al*., 2022).

In China, the first detection of GI.2 was reported in Sichuan province in 2020 (Hu *et al*., 2021). Previous works reported that the GI.1 vaccine did not confer proper protection against GI.2 (Abade Dos Santos *et al*., 2020), and the bivalent vaccine including both RHDV GI.1 and GI.2 was effective to achieve robust immunization against both genotypes (Dalton *et al*., 2021; O'Connor *et al*.,2022). Thus, continuous detection of the circulating genotypes of RHDV is important for vaccine formulation update and disease control. However, the field RHDV strains responsible for recent outbreaks after RHDV2 emergence in China have not yet been reported.

MATERIAL AND METHODS

Sampling

One hundred and eighty-five liver samples collected from 35 domestic rabbit farms in eight provinces in China between 2020 and 2022. The hemagglutination test was carried out and the positive samples were stored at −70°C for further analysis.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver tissues using Trizol RNAiso plus (Takara Bio, China). The vp60 gene sequence was amplified by RT-PCR using the Reverse Transcriptase XL (AMV) kit (Takara Bio) and the Ex Taq kit (Takara Bio). Specific primer pairs (RHDV-F: 5′-TATTCTGGGAACAACTCCAC-3′, RHDV-R: 5′-AACAGTCCGGTTGGATTTTG-3′ and RHDV2-F: 5′-CCCTGGAAGCAGTTCGTCAAAC-3′, RHDV2-R: 5′-GATGTCAACAAGGTCTGACAG-3′) designed in our study were used for identified the genotype GI.1 and GI.2 of RHDV. The amplified segments of GI.1 and GI.2 are 347 bp and 748 bp, respectively. Full-length VP60 genes (nt 5191–7144) were amplified using a pair of specific primers (sense, 5′-AAGAGRGTCGTCTCGGTRGTA-3′ and antisense 5′-GCACCTGCAAGTCCHARTCC-3′), which could amplify the VP60 of both RHDV and RHDV2. The PCR products were sequenced for further analysis.

Phylogenetic analysis

Phylogenetic analysis of full-length VP60 sequences was performed using MEGA 7 (version 7.0.26) with the maximum-likelihood approach based on GTR+G+I model. Reliability of the nodes was assessed with a bootstrap resampling procedure consisting of 1000 replicates of the ML trees.

RESULTS AND DISCUSSION

A total of thirty-five liver samples collected in different rabbit farms from eight provinces which including the major regions of rabbit breeding such as Sichuan, Shandong, and Henan provinces in China, were further analyzed by RT-PCR for identified the genotypes of RHDV. The results showed that three liver samples were double-positive of GI.1 and GI.2, indicating co-infection of the two genotypes in rabbits. Five liver samples were detected positive of GI.1, and the other twenty-seven liver samples were detected positive of GI.2. The proportion of GI.2 isolates in 2020, 2021 and 2022 were 64.3%, 76.9% and 100%, respectively (Table 1). These indicated that the GI.2 isolates were becoming the dominant circulating genotype of RHDV in 3 years after the emergence of original RHDV2 in China.

Table 1: The genotypes of RHDV isolates detected by RT-PCR collected from 2020 to 2022 in China

Year	2020	2021	2022
GI.1 isolates	3	2	0
GI.2 isolates	9	10	8
GI.1 and GI.2 co-infections	2	1	0
Proportion of GI.2	64.3%	76.9%	100%

The products of genotype identification PCR were sequenced and verified the present of both viruses in the co-infected sample. Then the full-length VP60 genes of the other thirty-two GI.1 and GI.2 isolates were amplified and sequenced, and phylogenetic analysis of VP60 sequences of single genotype of RHDV infection was performed. Corresponding to the RT-PCR results, five isolates fell into GI.1 strains, one of which clustered with GI.1c genotype, whereas the other four isolates clustered with GI.1a genotype. Twenty-seven isolates fell into GI.2 strains, indicating the RHDV2 was the most frequently epidemic genotype in China between 2020 and 2022 (Figure 1).

Importantly, the phylogenetic tree of the VP60 sequences revealed that all new RHDV2 isolates collected from 2020 to 2022 were clustered with the first Chinese GI.2 strain SC2020/0401 (MT586027) in a well-supported group (bootstrap value of 99, Figure 1). The VP60 nucleotide sequences of these new isolates were more than 98.3% identical to SC2020/0401. These indicated that the epidemic of these RHDV2 isolates might be the result of the transmission of SC2020/0401. Indeed, it had been identified that three of these GI.2 isolates (ON638913-ON638915) were recombinants in our previous work, and SC2020/0401 strain served as the major donor for the structural protein gene including VP60 (Hu *et al*., 2023). However, whether the other new GI.2 isolates were recombinant isolates

had not been identified. Furthermore, analysis of full genomic sequences of these new GI.2 isolates may improve our understanding of the evolutionary history and genetic relationships of RHDV in future.

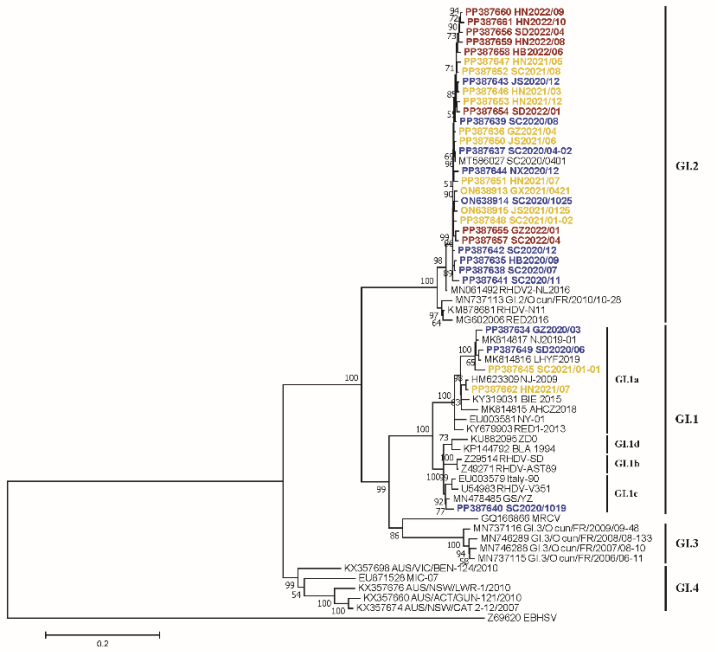

Figure 1: Maximum-likelihood (ML) phylogenetic trees for the complete nucleotide sequences of rabbit hemorrhagic disease virus (RHDV) capsid protein (VP60) genes. Bootstrap probability values above 50% with 1,000 replicates are indicated at the nodes. GenBank accession numbers of the sequences are indicated in the taxon names. The new isolates collected in 2020, 2021 and 2022 are marked in blue, yellow and red, respectively. European brown hare syndrome virus (EBHSV) strain GD (Z69620) was used as the outgroup to root the tree.

CONCLUSIONS

In summary, analysis of the new RHDV isolates collected from 2020 to 2022 in China suggests that within 3 years after RHDV2 arrival, GI.2 genotype had spread and became the dominant circulating strain, replacing GI.1 genotype in China. Thus, development of new genotype vaccine is important for control of the disease induced by RHDV2 in China.

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IMMUNOREACTIVITY ANALYSIS OF RHDV RDRP PROTEIN

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ABSTRACT

Rabbit hemorrhagic disease virus (RHDV) is a significant pathogen that causes severe and often fatal rabbit hemorrhagic disease (RHD) in both domestic and wild rabbits. RNA-dependent RNA polymerase (RdRp) is a key enzyme involved in viral replication. To investigate the immunoreactivity of RdRp protein, we expressed and purified the recombinant protein. And an indirect ELISA based RdRp protein was established to detect serum antibodies. The findings revealed that the fusion protein was successfully expressed in soluble with a correct molecular weight size. And RdRp protein could only be recognized and reacted with RHDV post-infection serum. It indicates that rabbits can induce a high level of RdRp antibody response after RHDV infection. RdRp protein could not be recognized by post-vaccine immunization. However, only 2 out of 10 serum samples from the post-immunization re-attacked infected rabbits could react with RdRp protein with low values. Immunoreactivity analysis of RdRp can be utilized to differentiate between RHDV infection and vaccine-induced immunity.

Keywords: Rabbit Hemorrhagic Disease Virus, RNA-dependent RNA polymerase (RdRp), Indirect ELISA (iELISA), Immunoreactivity

INTRODUCTION

Rabbit Hemorrhagic Disease Virus (RHDV) is a significant pathogen that induces hemorrhagic disease in rabbits[1].This acute and highly infectious disease manifests with rapid onset and transmission, characterized by respiratory hemorrhage, acute hepatic necrosis, edema in various parenchymal organs, bruising, and hemorrhage[2]. The morbidity and fatality rates associated with RHDV are notably high[3]. RHDV is divided into two genotypes GI.1 (RHDV1) [4]and GI.2 (RHDV2) [5]which exhibit low cross-reactivity. RHDV1 primarily affects rabbits over 2 months old, while RHDV2 can infect rabbits of all ages, particularly weaned rabbits, resulting in a high mortality rate.

RHDV viral particles contain a genomic RNA and a subgenomic RNA (about 2.2 Kb). The genomic RNA is 7437bp in length containing two open reading frames (ORFs). ORF1 encodes a polyprotein with approximately 257 KDa in size, which is cleaved by the virus-encoded protease after translation to form seven nonstructural proteins (p16, p23, 2C-like helicase (p37), p29, 3C-like protease (p15), VPg (p13), RNA-dependent RNA polymerase (p58), and coat protein VP1 (VP60)[6].VP60 is the main structural protein that constitutes the viral particle, which is a target protein for viral antigenic epitope studies and prevention of RHDV infection[7]. Here, in this study, recombinant RdRp protein was expressed and purified using a prokaryotic expression system. And an indirect ELISA assay was established to analyze the immunoreactivity of RdRp protein.

MATERIAL AND METHODS

Prokaryotic expression and purification of recombinant RdRp protein

In this study, based on the RdRp gene sequence of RHDV2-SC2020 strain (GenBank number: MT586027.1), the recombinant plasmid pMal-c6T-RHDV2-RdRp was constructed

by subcloning into pMal-c6T (New England Biolabs). The expression and purification of recombinant RdRp protein were carried out according to the procedures described above[8]. Recombinant plasmid pMal-c6T-RHDV2-RdRp was transformed into *Escherichia coli* TB1 competent. IPTG (Isopropylthio-β-galactoside) was added with a final concentration of 0.2 mM to induce protein expression at 27℃ for 6 h. The expression products were purified by amylose resin[9]. MBP (Maltose binding protein) tag was excised using TEV protease[10]. MBP tags and RdRp protein were separated by nickel column[11].

Establishment of Indirect RdRp-ELISA for detection antibodies against RHDV

The iELISA method was established according to the procedure described as following[12]. Specifically, the purified RHDV2-RdRp protein was used as the coated antigen. And the RHDV2-RdRp rabbit polyclonal antibody was prepared as positive reference serum. The optimal antigen concentration and serum dilution ratio for iELISA method were determined using checkerboard method. The antigen was diluted (31.25/62.5/125/250/500/1000 ng/well) and coated into a 96-well plate using 0.05M carbonate-bicarbonate buffer (CBS, pH 9.6) overnight at 4℃. Blocking with 5 % skimmed milk for 2h. 100μL of 1:50, 1:100, 1:200, 1:400 diluted positive and negative sera was added and incubated at 37℃ for 1h. After washing, 100μL goat anti-rabbit IgG-HRP (1:10000) was added and incubated at 37℃ for 1h. After substrate reaction, results were read by Microplate Reader. We used the established non-structural protein RdRp indirect ELISA to detect different types of serum samples. 9 serum samples were collected from rabbits at 0,14, 28 days post RHDV-NJ2009 strain challenge. A total of 26 serum samples were taken from rabbits at 14 days after immunization with RHDV2-SC strain subunit vaccine. 10 serum samples were collected from the rabbits which immunized with RHDV2-SC strain subunit vaccine for 14 days and then challenged by RHDV2-SC strain for 14 days. The pre-immunization and pre-attack healthy rabbit serum were used as a negative control.

RESULTS AND DISCUSSION

Expression and purification of recombinant RdRp protein

The recombinant RdRp protein was successfully expressed in soluble with the size of 98 KD. As identified by SDS-PAGE, RdRp protein was successfully separated with high purity with a single band size of about 57 KD (Figure 1).

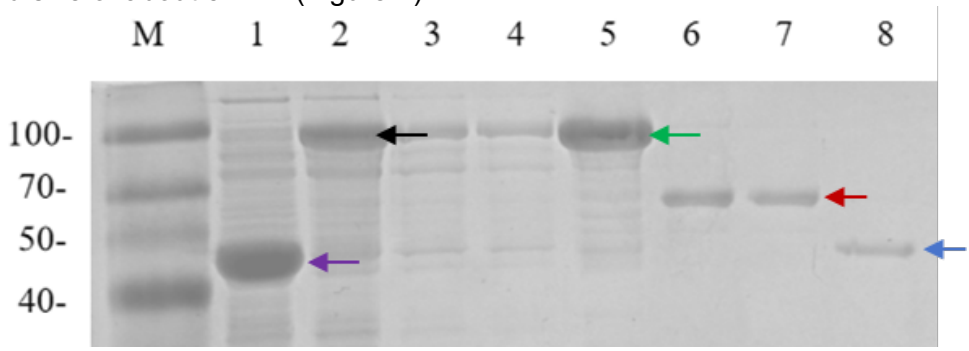

Figure 1. Prokaryotic expression and purification of recombinant RdRp protein. M: Marker;1. MBP tag expression control (Purple); 2. Pre-purification of recombinant RdRp protein (Black) ; 3. Flow-through; 4. Wash hybridization; 5. Purification of recombinant RdRp protein (Green); 6-7. Recombinant RdRp protein without MBP tag (Red); 8. MBP tag after cleaving (Blue)

Immunoreactivity analysis of RHDV RdRp protein

VP60, as the capsid protein of RHDV, can stimulate the body to produce an immune protective response, which is also a protective antigen for the design of RHDV subunit vaccine. Serum antibody tests based on VP60 protein are unable to differentiate between vaccine immunity and viral infection. As a positive control, VP60 protein could react with RHDV post-infection serum, post-vaccine immunization serum and post-immunization re-

attack serum samples. And RdRp protein could only be recognized and reacted with RHDV post-infection serum (Figure.2). However, only 2 out of 10 serum samples from the post-immunization re-attacked rabbits could react with RdRp protein with low values (Figure.3). This indicates that antibody against RdRp protein could be produced during infection of RHDV. Individual differences in the protective efficiency of the vaccine were observed, so that virus infection and transmission could still occur during the vaccine protection period.

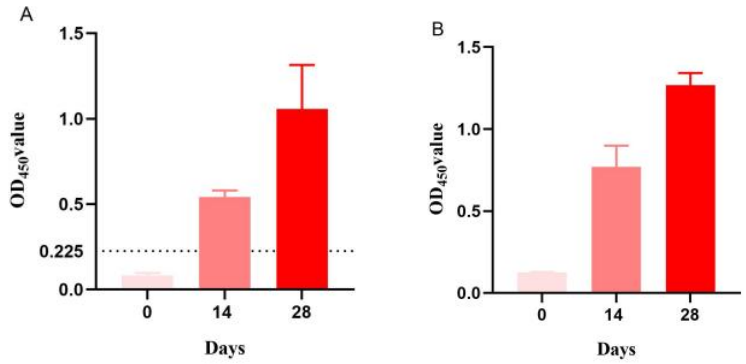

Figure 2. The immunoreactivity analysis of serum samples from rabbits which challenged with RHDV-NJ2009 strain. A. Immunoreactivity analysis of RdRp protein. B. Immunoreactivity analysis of VP60 protein

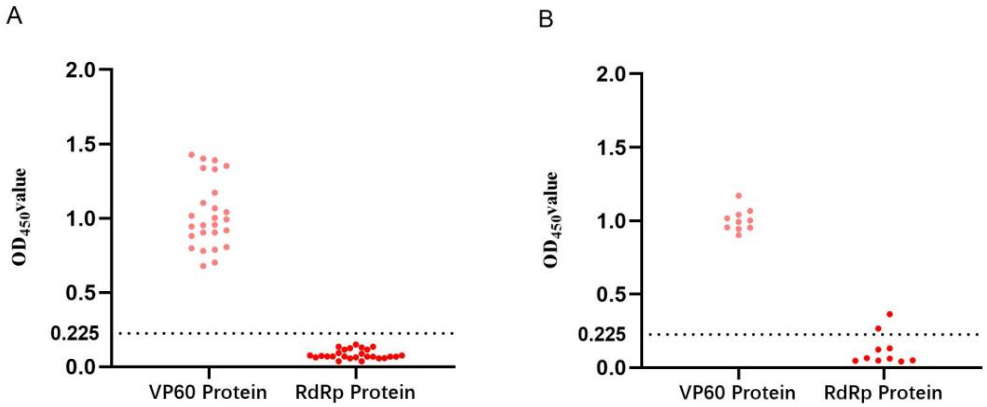

Figure 3. Immunoreactivity analysis of RHDV RdRp protein by indirect RdRp-ELISA. A. Immunoreactivity analysis of serum samples from rabbits at 14 days after immunization with RHDV2-SC strain subunit vaccine. B. Immunoreactivity analysis of serum samples from the rabbits which were immunized with RHDV2-SC strain subunit vaccine for 14 days and then challenged by RHDV2-SC strain for 14 days.

CONCLUSIONS

We established of indirect RdRp-ELISA for the detection of antibodies against RHDV. It suggests antibody responses against VP60 can be generated during both vaccine immunization and infection, whereas antibody responses against nonstructural protein RdRp are present only during viral infection and may be related to the level of viral replication.

ACKNOWLEDGEMENTS

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EFFICACY EVALUATION OF YURVAC® RHD AGAINST DIFFERENT STRAINS OF RABBIT HAEMORRHAGIC DISEASE

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ABSTRACT

Vaccination against Rabbit Haemorrhagic Disease (RHD) is the primary means of protection against this disease. For this reason, YURVAC® RHD, the new recombinant vaccine indicated for active immunisation of rabbits from 30 days of age onwards, has been developed to reduce mortality from Rabbit Haemorrhagic Disease (RHD) caused by the classical RHD virus (RHDV) and RHDV2, including highly virulent strains. The aim of this study was to demonstrate the efficacy of YURVAC® RHD against RHD. The active substance of the vaccine consists of a recombinant capsid protein of the RHD virus, corresponding to VP60. The antigen was obtained using recombinant DNA technology. All the efficacy studies followed the same structure: one group of animals vaccinated with YURVAC® RHD and a control group given PBS. In order to demonstrate both the onset and duration of immunity against each strain, all the animals were challenged at 7 or 14 days and 1-year post vaccination. In all cases, the animals were seronegative at the time of vaccination. The serological response was evaluated using the haemagglutination inhibition technique to detect antibodies against RHDV2. A duration of immunity of 1 year was confirmed for RHDV, RHDV2, and highly virulent RHDV2 strains. The onset of immunity was established at 7 days for RHDV2 and highly virulent RHDV2 strains, and at 14 days for RHDV. The results confirmed the efficacy of YURVAC® RHD against RHDV, RHDV2, and highly virulent strains of RHDV2.

Key words: YURVAC® RHD, rabbit haemorrhagic disease, vaccine, efficacy.

INTRODUCTION

Rabbit Haemorrhagic Disease (RHD) is a viral disease that can be caused primarily by two types of strains: classical strain (RHDV) and RHDV2. Moreover, it has been reported that the virulence of RHDV2 has increased in recent years (Capucci et al., 2017), posing a greater challenge in the prevention of this disease. The main measure for protection against Rabbit Haemorrhagic Disease is vaccination. The new recombinant vaccine YURVAC® RHD has been developed for this reason.

The objective of this study was to demonstrate the efficacy of YURVAC® RHD against RHD and to confirm the onset and duration of immunity against the classical strain (RHDV) and RHDV2, including highly virulent strains of RHDV2. The study design was based on the current edition of Ph. Eur. monograph no. 5.2.71. In the case of RHDV studies, the requirements established in specific monograph 2325 "Rabbit Haemorrhagic Disease Virus (Inactivated)" were followed.

MATERIALS AND METHODS

Animals and experimental design

YURVAC® RHD is a new recombinant vaccine intended for active immunisation of rabbits from 30 days of age onwards to reduce mortality caused by RHDV and RHDV2, including highly virulent strains. The active substance of the vaccine is the recombinant RHDV2 capsid protein, corresponding to the VP60, which auto-assembles into Virus Like Particles (VLPs). The antigen was obtained by means of recombinant DNA technology (yeast host-vector

system). All the efficacy studies followed the same structure: one group of animals vaccinated with YURVAC® RHD at 30 days of life and a control group given PBS, except for the onset of immunity study of the classical strain where the animals were vaccinated at 70 days of age. Number of animals and day of challenge are described in Table 1. All the animals were infected by the intramuscular route, as it is widely used in the published literature to perform experimental infection with both RHDV and RHDV2. and efficacy studies of other RHD vaccines.

Table 1. Number of animals and day of challenge of the different efficacy studies.

Efficacy study	Challenge strain	Day of challenge	Number of animals	
			YURVAC® RHD group	Control group
OOI	RHDV (Spain, 1997)	14 d.p.v.	11	11
	RHDV2 (Spain, 2013)	7 d.p.v.	22	21
	Highly virulent RHDV2 strain (France, 2020)	7 d.p.v.	20	20
DOI	RHDV (Spain, 1997)	365 d.p.v.	10	10
	RHDV2 (Spain, 2013)	365 d.p.v.	22	17
	Highly virulent RHDV2 strain (France, 2020)	365 d.p.v.	24	24

OOI: onset of immunity. DOI: duration of immunity. D.p.v.: days post-vaccination

Serological Analyses

The serological response was evaluated by haemagglutination inhibition assay (HAI) for the detection of antibodies against RHDV2[3]. The antigen used for the HAI technique is a strain heterologous to the vaccine used. It has been obtained after collecting and macerating livers from rabbits previously inoculated with this strain. This macerate is inactivated with 10% 0.1M BEI at 36-38ºC for 7 hours. Once inactivated, it is neutralized with 20% sodium thiosulfate, clarified and filtered.

Statistical Analysis

Mortality after challenge was recorded throughout the study, and the proportion of animals that died per group was analysed by Chi-square test.

RESULTS AND DISCUSSION

Figure 1 and Figure 2 show the results for the RHDV2 strains. In all cases, the mortality rate from the highly virulent RHDV2 strain was over 90%. These results confirm the higher virulence of this strain compared to the once isolated in Spain in 2013.

In all the efficacy studies, all the animals were seronegative at the time of vaccination. At the end of the studies, all the vaccinated animals were seropositive.

A duration of immunity of 1 year was confirmed for RHDV, RHDV2 and highly virulent strains of RHDV2. The onset of immunity was established as 7 days for RHDV2 and highly virulent strains of RHDV2, and 14 days for RHDV (Figure 1, Figure 2 and Figure 3). In all the cases, there were significant differences between the vaccinated and the control group.

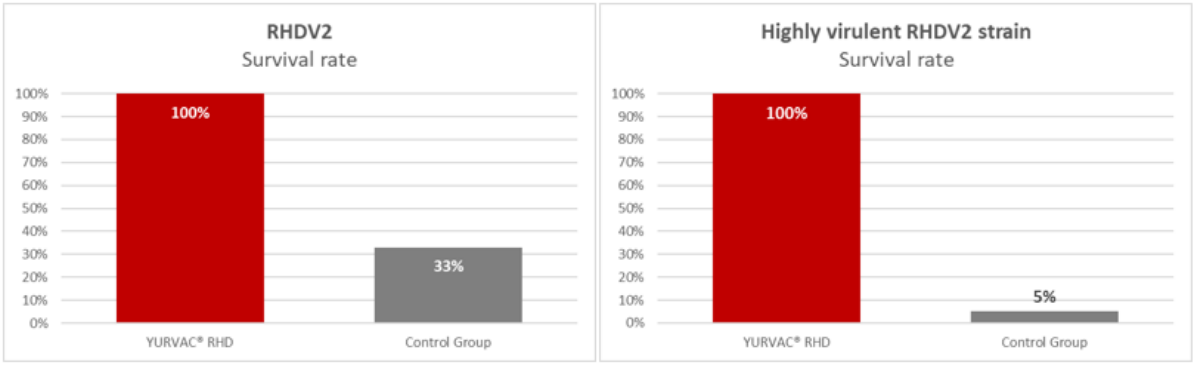

Figure 1. Survival rate after RHDV2 challenge one week post vaccination.

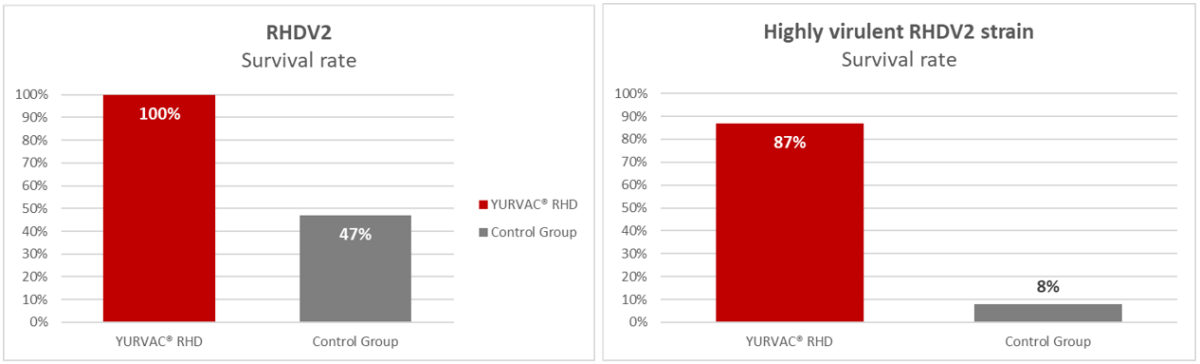

Figure 2. Survival rate after RHDV2 challenge one year post vaccination.

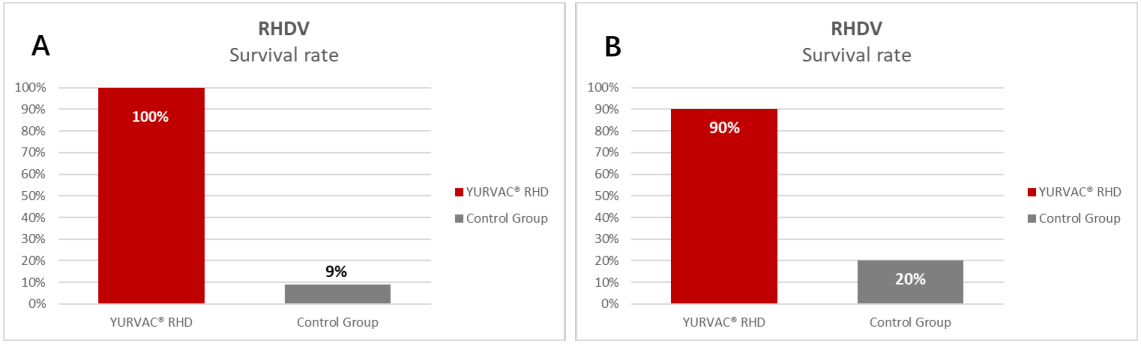

Figure 3. Survival rate after RHDV challenge two weeks post vaccination (A) and one year post vaccination (B).

CONCLUSIONS

The results confirmed the efficacy of YURVAC® RHD against the classical RHD strain (RHDV), RHDV2 and the highly virulent strains of RHDV2.

ACKNOWLEDGEMENTS

The Authors wish to thank the laboratory technicians, veterinarians, animal handlers and the R&D Department of HIPRA.

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Abrantes, J., Lopes, A. M. 2021. Review on the Methods Used for the detection and diagnosis of Rabbit Haemorrhagic Disease Virus (RHDV)

SAFETY EVALUATION OF YURVAC® RHD IN BREEDING DOES

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ABSTRACT Vaccination against Rabbit haemorrhagic disease (RHD) is the principal measure for protection against this disease[1] and its safety in breeding rabbits was tested in the present study. YURVAC® RHD is a new recombinant vaccine intended for the active immunisation of rabbits from the age of 30 days to reduce mortality caused by classical Rabbit Haemorrhagic Disease (RHDV) and the new virus RHDV2. The vaccination schedule includes one single dose administered via the subcutaneous route to rabbits from 30 days of age. In order to evaluate the safety of YURVAC® RHD in breeding does, as one of the most sensitive categories, pregnant does in the three thirds of gestation and lactating does were vaccinated with YURVAC® RHD or given a placebo and were monitored for the evaluation of general and local clinical signs, rectal temperature, reproductive parameters and body weight of the progeny. No general or local clinical signs were observed during the study. None of the mean rectal temperature increases exceeded 0.36ºC and none of the animals showed a temperature increase greater than 0.8ºC. No alteration of reproductive parameters relating to the vaccination were detected. No differences in kits' body weight were observed. In view of these results, YURVAC® RHD vaccine has proven to be safe when administered to breeding does.

Key words: RHD, RHDV2, RHDV, pregnant does, lactating does, safety, vaccine.

INTRODUCTION

YURVAC® RHD is a new recombinant vaccine against RHD that has been tested with the intention of demonstrating its safety in breeding does as one of the most sensitive categories of rabbits. Bearing in mind that in the event of an RHD outbreak it could be necessary to also vaccinate breeding females, it was considered essential to obtain further information on the safety of YURVAC® RHD when administered to pregnant and lactating females. To evaluate the safety of a single dose administration of YURVAC® RHD vaccine to breeding does, a test was performed with thirty-eight pregnant does divided between the three thirds of gestation, and sixteen lactating does. All the animals were healthy and free from antibodies against RHD[2].

The objective of the study was to evaluate the safety of a single dose administration of YURVAC® RHD vaccine to pregnant and lactating rabbits.

MATERIALS AND METHODS

Animals and experimental design

One group of 9 pregnant does in the first third of gestation (group A), another group of 8 pregnant does in the second third of gestation (group B), a group of 8 pregnant does in the last third of gestation (group C) and one group of 8 lactating does (group D) were vaccinated with YURVAC® RHD according to the established vaccination plan with a single 0.5 ml dose via the subcutaneous route. Another 3 groups of pregnant does (group E with 4 animals, group F with 5 animals and group G with 4 animals), in the three different thirds of gestation, and another group of 8 lactating does (group H), were given 0.5 ml of PBS with the same plan and route of administration. All the animals were seronegative on the day of vaccination. After the single dose administration, the group of pregnant animals was observed until parturition to examine any harmful effects during gestation or on the progeny. The reproductive parameters evaluated were litter size, number of alive kits born per female, new-borns malformations and abortions. Any abnormal local or systemic reactions were

adequately recorded for at least 14 days post parturition. The local reactions assessed were inflammation, nodules, ulcers, scabs or alopecia. The general clinical signs evaluated were depression, body condition, dyspnea, nasal discharge and ocular discharge. The effects on the progeny were recorded up to 3 days post parturition. In the case of the lactating groups, the animals were observed up to weaning to examine any harmful effects during lactation or on the progeny. The effects on the body weight of the progeny were recorded at the end of lactation.[3]

Serological Analyses

The serological response was evaluated by hemagglutination inhibition technique for the detection of antibodies against RHDV2 before vaccination and at the end of the study in order to detect the seroconversion of antibodies in the vaccinated group[4].

RESULTS AND DISCUSSION

None of the animals showed either local or general clinical signs during the follow-up period after the vaccination.

Regarding rectal temperatures, all were within normal ranges during the study. None of the mean rectal temperature increases exceeded 0.36ºC and none of the animals showed a temperature increase greater than 0.8ºC (Figures 1 to Figure 4). This proves that YURVAC® RHD did not affect rectal temperature when administered to pregnant does.

Different reproductive parameters were evaluated in this study. The litter sizes observed in the control and vaccinated groups were also within the normal kindling rates. The main causes for kit mortality during the study were parturition outside the nest box, abandonment of the litter or dystocia.

Abortion was only detected in one doe and was related to the manipulation of the does in late term gestation.

No statistically significant differences in mean body weight were detected between kits from vaccinated and non-vaccinated does from the lactating groups (Group D and Group H).

At the end of the study, the serological levels for RHDV2 antibodies were determined and all animals had seroconverted in the vaccinated group and all the animals in the control group remained seronegative, indicating an adequate vaccination administration to all the vaccinated animals.

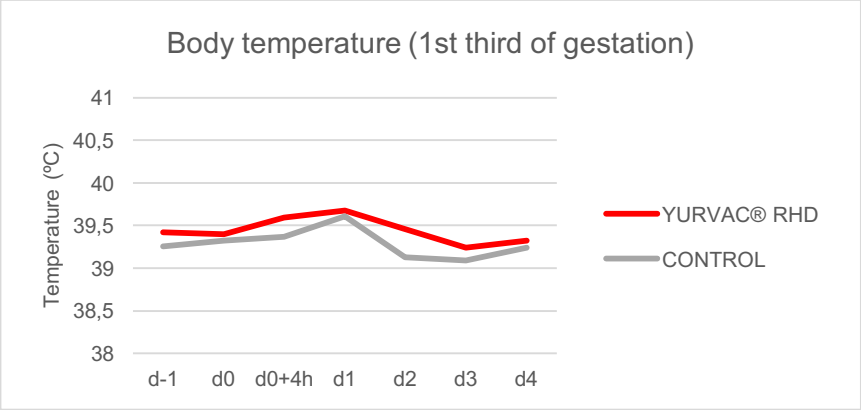

Figure 1. Average rectal temperature per group after vaccination (d0), during the first third of gestation (Group A and Group E).

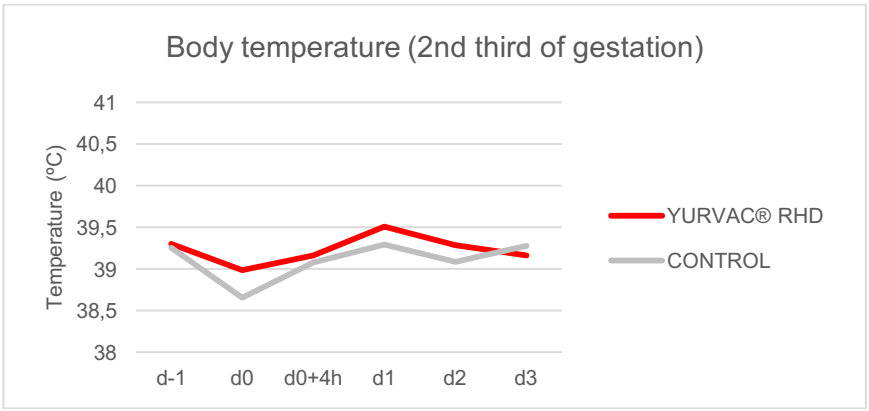

Figure 2. Average rectal temperature per group after vaccination (d0), during the second third of gestation (Group B and Group F).

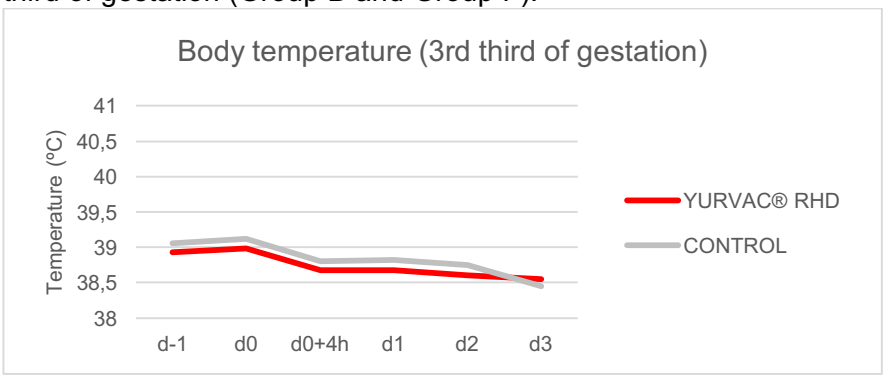

Figure 3. Average rectal temperature per group after vaccination (d0), during the third third of gestation (Group C and Group G).

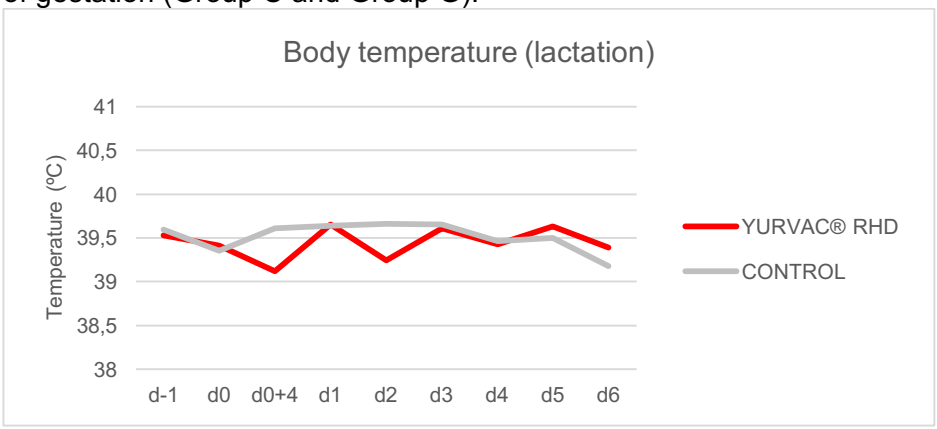

Figure 4. Average rectal temperature per group after vaccination (d0), during first lactation (Group D and Group H).

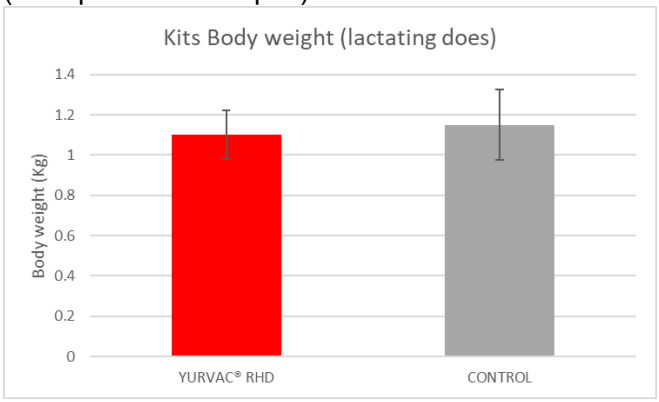

Figure 5. Average body weight from kits born from lactating does groups at the end of lactation (Group D and Group H).

CONCLUSIONS

These results confirm that the single dose administration of YURVAC® RHD to pregnant and lactating does via the subcutaneous route is considered safe in terms of local and general reactions, and rectal temperatures.

It was also confirmed that YURVAC® RHD does not affect the normal body weight of the offspring.

ACKNOWLEDGEMENTS

The Authors wish to thank the laboratory technicians, veterinarians and the animal handlers from the R&D Department of HIPRA.

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EVALUATION OF THE SAFETY AND IMMUNOGENICITY OF YURVAC® RHD IN RABBIT KITS UNDER FIELD CONDITIONS

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ABSTRACT

A clinical field trial was carried out with the main objective of evaluating the clinical safety and immunogenicity of YURVAC® RHD when administered to rabbits via the subcutaneous route under field conditions. The study was randomised, double-blinded and negative-controlled. YURVAC® RHD is a recombinant vaccine intended for the active immunisation of rabbits from the age of 30 days to reduce mortality caused by the classical Rabbit Haemorrhagic Disease Virus (RHDV) and the new virus (RHDV2), including highly virulent strains. A total of 5,789 kits from two different batches on two commercial rabbit farms located in France were included in the study. Each vaccination was performed at the age of 30 days after random distribution of the kits into two groups: YURVAC® RHD and a Negative Control Group. The animals were followed-up until the end of fattening. Several parameters were blindly evaluated during this period to assess the safety and immunogenicity of YURVAC® RHD. According to the results obtained, it can be concluded that YURVAC® RHD is a safe vaccine that induces an active immunisation of rabbits from the age of 30 days when administered via the subcutaneous route under field conditions.

Key words: Field trial, RHD, RHDV2, RHDV, vaccine, safety

INTRODUCTION

Rabbit Haemorrhagic Disease Virus (RHDV) is a calicivirus of the genus *Lagovirus* that causes Rabbit Haemorrhagic Disease (RHD) in adult European rabbits (*Oryctolagus cuniculus*). More recently, in 2010, a new virus was identified in France (RHDV2)[1]. This new virus showed a capsid protein sequence identity of about 80% with RHDV and was able to cause RHD in young rabbits.

YURVAC® RHD is a recombinant adjuvanted vaccine intended for the active immunisation of rabbits from the age of 30 days to reduce mortality caused by classical RHDV and variant RHDV2 strains, including highly virulent strains[2]. The vaccination schedule includes one single dose administered via the subcutaneous route to rabbits from 30 days of age.

The objective of this study was to assess whether YURVAC® RHD is safe and to assess its immunogenicity following the administration of a single dose to 30-day-old rabbits under field conditions.

MATERIALS AND METHODS

Animals and experimental design

The present study was performed on two commercial rabbit farms located in France. On both farms, the kits from each doe remained in the same cage where they were born until slaughter (all of them in the same house) while does were transferred to another cage in another house at weaning. All the breeding does were vaccinated against myxomatosis and the classical and variant strain of Rabbit Haemorrhagic Disease.

The Investigator randomly assigned the animals to a group code (A or B) but he did not attend the vaccination in order to preserve the blind. The Treatment Dispenser, the only person knowing the correspondence between the group code (A or B) and the treatment

group (Investigational group or Control group), was responsible for the product preparation and administration.

The assignment of each kit to the groups was done by stratification by doe, so that half of the kits in each cage were randomly assigned to group A and the other half to group B. All kits were identified with ear tags.

At 30 days of age (study day = D0) the investigational group was vaccinated with YURVAC® RHD (Group B) and the Control group (Group A) was mock-vaccinated with PBS. Both groups received a single subcutaneous dose of 0.5 ml of the corresponding product.

The animals were followed-up from study day D0 until slaughter (67-73 days of age). During the study, the animals were observed daily by the farmer and periodically by the investigator. Several safety parameters were recorded during the follow-up period in order to assess the clinical safety of YURVAC® RHD and blood samples from 25 animals/group were collected on study days D0, D7 and D28 for serological analysis in order to assess the immunogenicity of YURVAC® RHD when administered under field conditions.

Adverse Events, including the appearance of clinical signs related to other diseases, were also recorded during the study period.

The products were administered via the subcutaneous route to a total of 5,789 rabbits at 30 days of age. The distribution of the rabbit kits between groups and farms is detailed in the table 1.

Table 1: Distribution of the rabbit kits between groups and farms

		FARM 1	FARM 2	Total/group	TOTAL
Group A	Control Group	1,472	1,430	2,902	5,789
Group B	YURVAC® RHD	1,464	1,423	2,887	
Total included /farm		2,936	2,853		

Safety assessment variables

Adverse Reactions were considered to be those Adverse Events (AE) related to administration of the product. Systemic reactions were evaluated in a subgroup of 10 to 12 animals/group/farm on study days D0 (before vaccination), D0+4h, D1 and D2 (1 day and 2 days after vaccination)[3]. Local reactions at the injection site were evaluated in a subgroup of 10 to 12 animals/group/farm (the same as for systemic reactions) on study days D0 (just before vaccination), D0+4h, D1 and D2. Rectal temperature was evaluated in a subgroup of 10 to 12 animals/group/farm (the same as for systemic and local reactions) on study days D-1, D0 (just before vaccination), D0+4h, D1 and D2. A baseline value was calculated for each animal as the average temperature recorded on study days D-1 and D0. Body weight was evaluated in a subgroup of 100 to 109 animals/group/farm on D-1 (just before vaccination), on D28 and before going to slaughter.

Serological Analyses

Individual blood samples were collected at different time points (D0, D7 and D28) from 25 animals/group/farm in order to monitor the serological response to the vaccine and were evaluated by hemagglutination inhibition technique for the detection of antibodies against RHDV and RHDV2[4].

Statistical Analysis

Quantitative response variables were summarised for each level of explanatory variables using mean +/- standard deviation or standard error and sample size when necessary, whereas relative and absolute frequencies were used for qualitative variables.

The overall immunogenicity data (serological data) and body weight were analysed per day using a mixed model (lme procedure) of R. The model included the fixed effects of treatment, sampling time, and treatment x sampling time interaction and the random effect of each rabbit within the farm.

RESULTS AND DISCUSSION

None of the animals showed general clinical signs during the follow-up period after vaccination.

The presence of local reactions grade 1 (<2cm) was observed in 23% of the YURVAC® RHD animals one day after vaccination (D1), which was reduced to 14% 24 hours later (D2). One week after vaccination, local reactions had disappeared in all the animals. Regarding the rectal temperature of the animals, all were within physiological ranges during the study. None of the mean rectal temperature increases exceeded 0.44ºC and none of the animals showed a temperature increase greater than 1.15ºC (Figure 1). No differences in mean body weight were detected between groups at any time in the study (Table 2).

RHDV2 antibody titres before vaccination and one week after vaccination were similar between groups. On day 28, serological titres against RHDV2 were higher in the YURVAC® RHD group than in the control group, the differences being statistically significant (Table 3). The results observed confirmed the no interaction of maternally derived antibodies with the active immunization of the kits after vaccination.

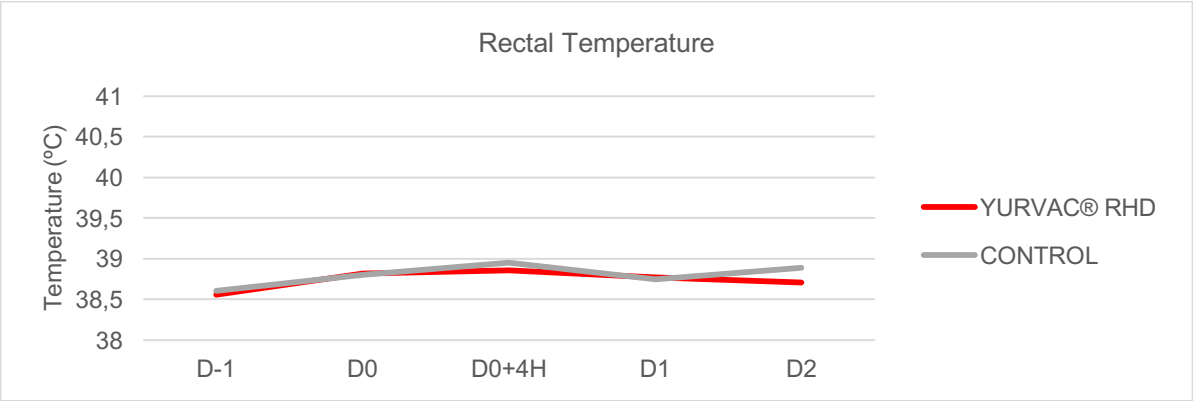

Figure 1. Overall average rectal temperature per group after vaccination (D0).

Table 2. Body weight progression (kg).

Group	BW day -1		BW day 28	
	Mean ± SD	p-value[1]	Mean ± SD	p-value[1]
YURVAC® RHD	0.67±0.14	0.731	1.96±0.29	0.876
Control	0.67±0.14		1.95±0.32	

[1]SMixed model described at section 4.8.5 pairwise-comparison was performed. As a result, Mann-Whitney test was performed. Results are significant if p<0.05.

Table 3. Serological response against RHDV2 on the day before vaccination (day 0), one week after vaccination (day 7) and on day 28. (log2 HAI/50µl Ac Against RHDV2) Animals with log2 HAI/50 µl ≥ 3 are considered seropositive.

Group	Day 0		Day 7		Day 28	
	Mean ± SD	**p-value**[1]	Mean ± SD	**p-value**[1]	Mean ± SD	**p-value**[1]
YURVAC® RHD	3.96±2.33	0.932	3.41±1.74	0.411	5.91±2.69	**<0.001**
Control	3.92±2.31		3.06±1.70		2.36±0.88	

[1]SMixed model described at section 4.8.5.2 pairwise-comparison was performed. As a result, Mann-Whitney test was performed. Results are significant if p<0.05.

CONCLUSIONS

According to the results obtained, it can be concluded that YURVAC® RHD is a safe vaccine in rabbits when administered via the subcutaneous route under field conditions. Vaccination of 30-day-old rabbit kits with maternal immunity with YURVAC® RHD induces an immunological response in terms of titration antibodies when administered via the subcutaneous route under field conditions.

ACKNOWLEDGEMENTS

The Authors wish to thank the external veterinarians, the animal handlers and the laboratory technicians from the R&D Department of HIPRA.

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FIELD EXPERIENCE WITH YURVAC® RHD DURING AN RHD OUTBREAK

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ABSTRACT

Rabbit Haemorrhagic Disease (RHD) poses a significant threat to the rabbit industry in Europe, with outbreaks having devastating consequences. Vaccination is a key preventive measure, but under certain socio-economic circumstances, fattening rabbits may not always be vaccinated, leading to emergency vaccination during outbreaks. The aim of this study was to compare the effectiveness of a new RHD vaccine with a commercial vaccine under such circumstances.

The study was conducted on a Spanish rabbit farm experiencing RHD problems. A total of four consecutive batches of rabbits were randomly allocated to receive either a commercial inactivated vaccine (Vaccine-A) or YURVAC® RHD. The mortality of each group was recorded up to the time of slaughter (56-64 days).

Statistical analysis using Chi-square with Yates's correction revealed significant differences between the two vaccines. Batch 1, vaccinated during an ongoing outbreak, exhibited the highest mortality. After the onset of immunity (7 days after vaccination at 40 days of age), mortality was significantly lower with YURVAC® RHD (4.10% vs. 8.14%, $p<0.05$).

Batches 2-4, receiving prophylactic vaccination, showed overall lower mortality (6.14-6.33% for Vaccine-A and 4.45-5.58% for YURVAC® RHD). Although no statistical difference was observed post onset of immunity, consistent trends suggested that preventive vaccination yielded better outcomes.

In conclusion, YURVAC® RHD demonstrated superior efficacy in controlling RHD outbreaks compared to Vaccine-A under real-world socio-economic conditions. These findings underscore the importance of tailored vaccination strategies for optimal disease management, thus endorsing preventive vaccination as a key tool in controlling Rabbit Haemorrhagic Disease in field conditions.

Key words: RHD, vaccine, rabbit, outbreak

INTRODUCTION

Rabbit Haemorrhagic Disease is endemic in Europe and outbreaks have devastating consequences in the rabbit industry (Dalton *et al.* 2014). Vaccination of the whole farm including breeding does and fattening rabbits is the most successful practice for prevention of the disease. Under certain socio-economic circumstances, farmers do not always vaccinate fattening rabbits; in this situation, vaccination is adopted as an emergency measure to control an outbreak. The objective of this study was to compare the effectiveness of a new RHD vaccine under the above-mentioned circumstances with another commercial vaccine.

MATERIALS AND METHODS

Animals and experimental design

The study was performed on a rabbit farm with 1,400 breeding does located in Spain. A total of 1,400 fattening rabbits were weaned per week at an age of 35 days. Breeding does were usually vaccinated against myxomatosis, RHD and RHDV2, whereas fattening rabbits were not usually vaccinated. The farm showed a willingness to start vaccinating rabbits, because of the detection of RHD problems. Haemorrhagic lesions were observed in the lungs and

livers of dead rabbits; additionally, the presence of RHDV2 was detected in liver samples by PCR. Mortality started 2 days before vaccination, affecting about 30 fattening rabbits each day. A total of four consecutive batches of rabbits were recruited for the study. Each batch was weaned 1 week apart. Rabbits from each batch were allocated randomly into two groups. One group was vaccinated with a commercial inactivated vaccine (Vaccine-A) against RHDV2 whereas the other was vaccinated with YURVAC® RHD, a recombinant vaccine against classic (RHDV) and variant strains (RHDV2), including highly virulent strains, in accordance with the respective manufacturer's instructions. Both vaccines were containing mineral oil adjuvants. The onset of immunity was considered to be 7 days post vaccination, as indicated in the SPC (Summary of Product Characteristics) for YURVAC® RHD. The mortality of the groups was recorded up to the time of slaughter which was between 56-64 days of life, depending on the batch.

Table 1: Allocation of the recruited animals. The numbers indicated in brackets show the number of animals present at the onset of immunity (from 7 days post-vaccination onwards).

Batch	Vaccine-A	YURVAC® RHD	Age at vaccination (days)	Age at the start of immunity (7 days after vaccination)
1	691 (676)	679 (659)	40	47
2	700 (691)	735 (727)	32	39
3	700 (692)	678 (669)	32	39
4	600 (595)	742 (740)	32	39

Statistical Analysis
The statistical comparison between groups was performed by Chi-square with Yate's correction using SPSS (IBM). The significance level was set at 5%.

RESULTS AND DISCUSSION

The disease was present to varying degrees across the batches. Batch number 1 was vaccinated once the disease was detected and thus later compared to the rest of the batches and additionally in the face of an ongoing outbreak in the batch. This is the probable reason why batch 1 had the highest level of disease compared to the rest of batches. The mortality observed through the entire cycle of this batch was 11.29 % and 8.41 % with Vaccine-A and YURVAC® RHD, respectively (Figure 1 A). These values were far beyond the average mortality recorded in the 4 batches before the outbreak, which was 5.25 ± 0.31 %, supporting the idea that batch 1 was experiencing the effect of the disease.
The mortality recorded after the onset of immunity of the vaccines (47 days of life) and up to slaughter age were 8.14 % and 4.10 % with Vaccine-A and YURVAC® RHD, respectively. The two vaccines showed a difference of almost 49.63 %, which was statistically significant ($p<0.05$) (Figure 2).
The mortality observed throughout the entire fattening cycle of batches 2-4 ranged between 6.14-6.33 % and 4.45-5.58 % with Vaccine-A and YURVAC® RHD, respectively (Figure 2 B). These values were closer to the normal mortality of the farm, supporting the idea that the prophylactic application of the vaccine probably had a better effect than vaccinating once the disease had already appeared. When the mortality recorded after the onset of immunity (39 days of life) was compared, the two vaccines showed some consistent differences across the batches although without a statistical difference ($p>0.05$) (Figure 2). This might suggest that the disease was still having an effect on mortality although to a much lesser degree; unfortunately, no further diagnosis was performed to confirm this hypothesis.

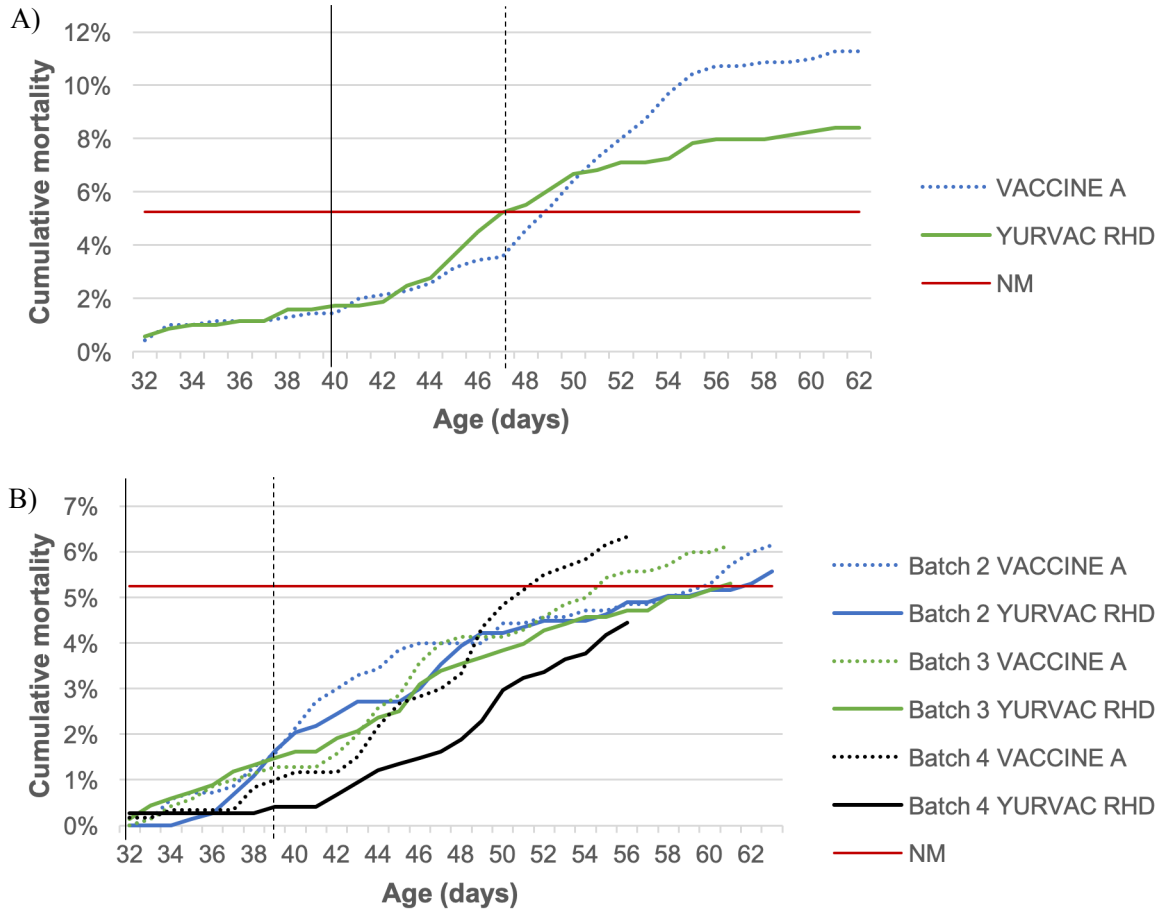

Figure 1: Cumulative mortality of rabbits throughout the study. A) Batch 1; B) Batches 2 to 4. The dashed vertical line indicates the onset of immunity of the vaccine which is considered to be 7 days post vaccination. The continuous vertical line indicates the age at vaccination which was 40 days for batch 1 and 32 days for batches 2-4. The Normal Mortality line (NM) indicates the average mortality observed in the batches before the outbreak.

The overall findings suggest that the two vaccines controlled the RHD outbreak differently. Additionally, they suggest that both vaccines can be used as emergency tool to control RHD outbreak. Despite this, they also support the recommendation for using vaccination as a preventive tool instead of using it once the disease has already appeared.

CONCLUSIONS

These results confirm in a case study that YURVAC® RHD is a suitable and distinctive solution to control RHD outbreaks in the field. The performance of YURVAC® RHD was better than that of Vaccine-A.

In conclusion, YURVAC® RHD demonstrated superior efficacy in controlling RHD outbreaks compared to Vaccine-A under real-world socio-economic conditions. The findings contribute valuable insights into the comparative efficacy of vaccines under real-world socio-economic conditions, emphasizing the importance of tailored vaccination strategies for optimal disease management.

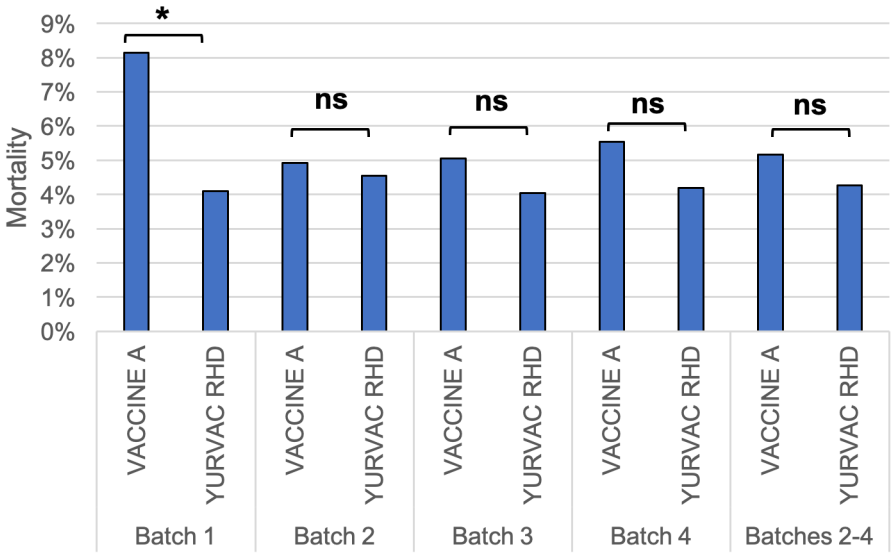

Figure 2: Mortality of fattening rabbits after the onset of the immunity of the vaccine. Asterisks indicate a statistically significant difference ($p<0.05$); ns: non-significant.

ACKNOWLEDGEMENTS

The authors wish to thank the R&D department for their support in the study.

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EFFICACY EVALUATION OF YURVAC® RHD AGAINST A CURRENT HIGHLY VIRULENT RHDV2 STRAIN

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ABSTRACT

YURVAC® RHD is a new recombinant vaccine intended for active immunization of rabbits from the age of 30 days to reduce mortality caused by classical Rabbit Haemorrhagic Disease (RHDV) and the new virus (RHDV2). In 2010, the RHDV2 virus emerged, spreading worldwide within a short period of time and showing higher prevalence than RHDV isolates in kits and adult rabbits[1]. But one of the most relevant characteristics of this pathogen is that it affects rabbit kits. Recently, an increase in the pathology of current strains was reported. For this reason, the efficacy of a dose of YURVAC® RHD was evaluated against a highly virulent currently circulating strain of RHDV2. The study was carried out with the subcutaneous administration of a single dose to 30-day-old rabbits. Efficacy was evaluated by an experimental challenge against a highly virulent RHDV2 strain isolated in The Netherlands in 2022, performed 14 days after vaccination. The results obtained in this trial fully support the efficacy claims of reducing mortality caused by highly virulent strains of RHDV2, demonstrating the efficacy of the vaccine.

Key words: RHDV, RHDV2, highly virulent, YURVAC® RHD.

INTRODUCTION

Vaccination against Rabbit Haemorrhagic Disease (RHD) is the principal measure to protect against this disease. YURVAC® RHD is a new recombinant vaccine intended for active immunization of rabbits from the age of 30 days to reduce mortality caused by RHDV and RHDV2. In fact, new strains of the RHDV2 have been detected recently and characterized as causing higher mortality in young rabbits and affecting older animals[2].
The aim of this study was to demonstrate the efficacy of YURVAC® RHD against one recent highly virulent strain of the variant of Rabbit Haemorrhagic Disease Virus (RHDV2) isolated in 2022 in The Netherlands.

MATERIALS AND METHODS

Animals and experimental design

Twenty-one rabbits of the minimum age recommended for vaccination (30 days) and free from antibodies against RHDV and RHDV2 were enrolled. The animals were divided into two groups; one group vaccinated with YURVAC® RHD (n=15) and one control group (n=6) which received a placebo PBS. All the animals were vaccinated via the recommended route of administration (subcutaneous) and according to the proposed schedule for vaccination (1 single dose). At day 14 post-vaccination, all the animals were challenged via the intramuscular route in order to assess the efficacy of the vaccine. This challenge route is widely used in the published literature to perform experimental infection with both RHDV and RHDV2 and efficacy studies of other RHD vaccines. The strain used in the efficacy study was isolated during an outbreak on a commercial rabbit farm in the Netherlands in 2022.
General clinical signs and mortality were recorded twice daily for 14 days after challenge, and liver samples were collected from dead animals in order to determine the presence of RHDV2 and to confirm the cause of death.

Laboratory analysis

The serological response was evaluated by haemagglutination inhibition technique (IHA) for the detection of antibodies against RHDV2[3]. The antigen used for the HAI technique is a strain heterologous to the vaccine used. It has been obtained after collecting and macerating livers from rabbits previously inoculated with this strain. This macerate is inactivated with 10% 0.1M BEI at 36-38ºC for 7 hours. Once inactivated, it is neutralized with 20% sodium thiosulfate, clarified and filtered.

Liver samples were analysed by haemagglutination technique to assess the presence of RHDV2 and to confirm the cause of death.

Statistical Analysis

Mortality after challenge was recorded throughout the study in both cases, and the proportion of animals that died per group was analysed by Chi-square test.

RESULTS AND DISCUSSION

All the rabbits included in the study were free of antibodies against RHDV and RHDV2 before vaccination. All the vaccinated animals seroconverted after vaccination.

The mortality results showed a 100% survival rate in the group vaccinated with YURVAC® RHD and a 0% survival rate in the control group, confirming the high virulence of the strain. All the deaths in the control group were reported between 24 and 72 hours after challenge. Statistically significant differences were observed between groups, thus confirming the efficacy of YURVAC® RHD against highly virulent strains (Figure 1).

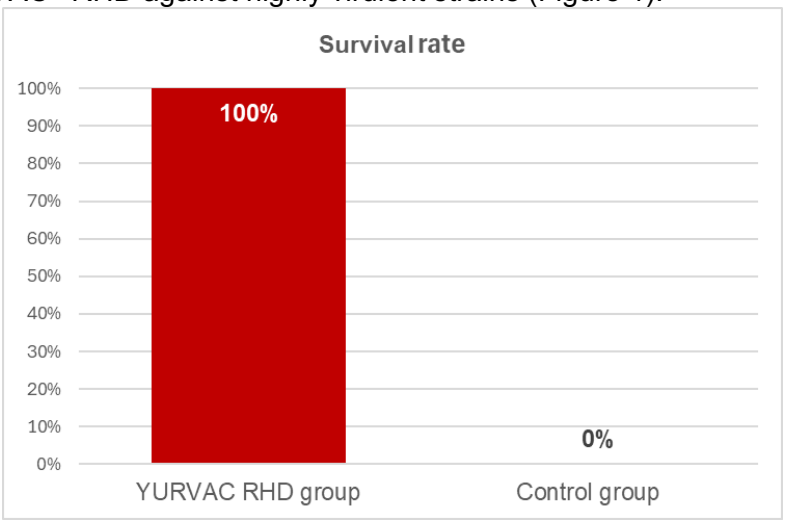

Figure 1. Percentage survival rate after challenge with highly virulent RHDV2 strains

Liver samples were collected and tested for the presence of RHDV2. In the control group, all deaths were confirmed as positive for RHDV2 in the liver, whereas no animals in the vaccinated group died.

The vaccinated group did not show any clinical signs throughout the study.

CONCLUSIONS

The results confirmed the efficacy of YURVAC® RHD against a highly virulent strain of RHDV2 isolated in The Netherlands in 2022.

ACKNOWLEDGEMENTS

The Authors wish to thank the laboratory technicians, veterinarians and animal handlers from the R&D Department of HIPRA.

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EFFICACY OF THE PASSIVE IMMUNITY AGAINST RHD IN RABBIT KITS WHEN BREEDING DOES ARE VACCINATED WITH YURVAC® RHD

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ABSTRACT

YURVAC® RHD is a recombinant vaccine intended for active immunisation of rabbits from the age of 30 days to reduce RHD mortality caused by the classical RHD virus and the RHDV2 strains, including highly virulent strains. The passive immunity against Rabbit Haemorrhagic Disease (RHD) of young rabbits acquired from does vaccinated with YURVAC® RHD was evaluated. The study was carried out by using the subcutaneous route and administering one single dose to does. Efficacy was evaluated by means of an experimental challenge with RHDV2, performed in the offspring of the vaccinated does, at 30 days of age. The results obtained in this trial fully support the efficacy of the passive immunity in reducing mortality caused by RHDV2 in the offspring of breeding does vaccinated with YURVAC® RHD.

Key words: RHD, RHDV, RHDV2, passive immunity, rabbit kits

INTRODUCTION

The classical strain of RHDV is a calicivirus of the genus Lagovirus that causes Rabbit Haemorrhagic Disease (RHD) in adult European rabbits (*Oryctolagus cuniculus*). Virions are small sized (35-40nm of diameter) and non-enveloped. The capsid, which protects the ssRNA + molecule, is composed of 90 dimers of the capsid protein called VP60. More recently, in 2010, an antigenically different virus was identified in France (RHDV2)[1,2]. This virus showed a capsid protein sequence identity of about 80% with RHDV and was able to cause RHD in vaccinated and young rabbits.

YURVAC® RHD is a recombinant vaccine intended for active immunisation of rabbits from the age of 30 days to reduce RHD mortality caused by the classical RHD virus and RHDV2 strains RHDV2, including highly virulent strains.

The aim of this study was to evaluate the efficacy of the passive immunity against RHD of young rabbits acquired from does vaccinated with YURVAC® RHD. The protection was demonstrated by an experimental challenge with the RHDV2 strain.

MATERIALS AND METHODS

Animals and experimental design

Fifteen primiparous pregnant does were included in the study. These rabbits were distributed into three groups: groups A and B were vaccinated subcutaneously with YURVAC® RHD following two different administration programmes (approximately 1 year and 2 months before the beginning of the study respectively) and group C (control group), which received a placebo (PBS). From these does, a total of 25 rabbit kits were selected from each group and were distributed into three corresponding groups; one group of 25 rabbit kits from breeding does vaccinated 300 days before the challenge phase of the trial (Group A), one group of 25 rabbit kits from breeding does vaccinated 84 days before the challenge phase of the trial (Group B) and one control group of 25 rabbit kits from non-vaccinated breeding does (Group

C). Rabbit kits were enrolled in the trial at 30 days of age (Day 0) and in order to demonstrate the protection of the progeny from passively acquired antibodies due to the YURVAC® RHD vaccine, all these 75 newborn rabbits were challenged via the intramuscular route with the RHDV2 strain (isolated in Spain in 2013) at 30 days of age. This challenge route is widely used in the published literature to perform experimental infection with both RHDV and RHDV2 and efficacy studies of other RHD vaccines.

General clinical signs and mortality were recorded twice daily for 14 days after challenge, and liver samples were collected from dead animals in order to determine the presence of RHDV2 and confirm the cause of death.

Serological Analyses
Blood samples were collected from breeding does and rabbit kits at different time points during the trial in order to assess the development of antibodies against RHDV2 using the haemagglutination inhibition technique. In breeding does, blood samples were collected on the day of vaccination and at parturition. In rabbit kits, blood samples were collected on the day of challenge (day 0) and 14 days after challenge to assess the serological response.

Statistical Analysis
For the vaccine to demonstrate the efficacy of the passive immunity received by the offspring, significant differences in mortality had to observed between rabbit kits from mock-vaccinated breeding does and rabbit kits from vaccinated breeding does. The results were analysed by Chi-square test.

RESULTS AND DISCUSSION

The results for mortality showed a 100% survival rate in groups A and B (rabbit kits from breeding does vaccinated 300 days and 2 months before challenge) and a 36% survival rate obtained in the control group. The total mortality rate observed in the control group was significantly higher ($p < 0.05$) than that obtained in the rabbit kits with maternal immunity, and no significant differences in mortality were observed between the rabbit kits born from vaccinated breeding does. All deaths in the control group were reported between 24 and 48 hours after challenge. (Figure 1).

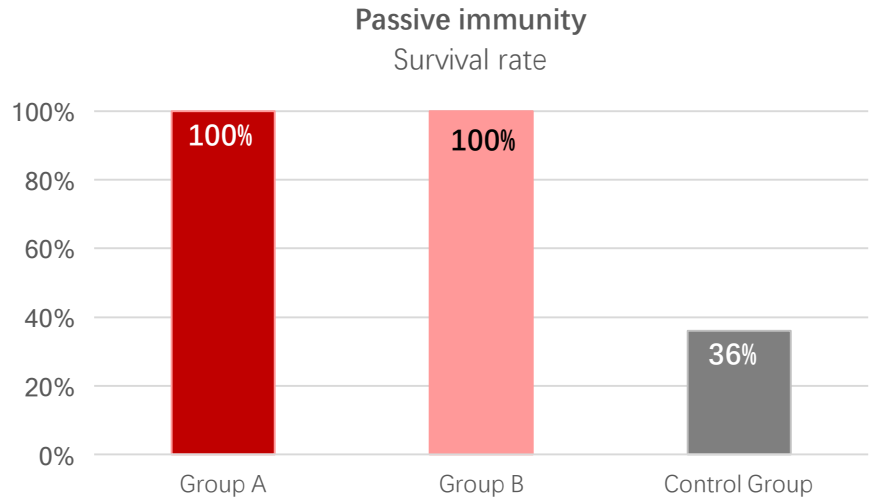

Figure 1. Percentage survival rate of the rabbit kits from breeding does vaccinated after challenge with the RHDV2 strain.

Liver samples were collected and tested for the presence of RHDV2. In the control group, all deaths were confirmed as positive for RHDV2 in the liver, whereas no animals in the passive immunity groups died.

No clinical signs were recorded during the challenge period in any of the animals.

All breeding does included in the study were free of antibodies against RHDV and RHDV2 before vaccination. All vaccinated breeding does were positive on the day of parturition. Blood samples were collected from all rabbit kits on the day of challenge and 14 days after challenge (only from surviving animals). It can be observed in the serological results obtained that the offspring from breeding does vaccinated with YURVAC® RHD presented antibodies against RHDV2 before challenge, whereas all the offspring from mock-vaccinated breeding does were seronegative against RHDV2 until the challenge. Serological titres detected in the survivors from the control group confirm that the challenge was properly performed. (Figure 2).

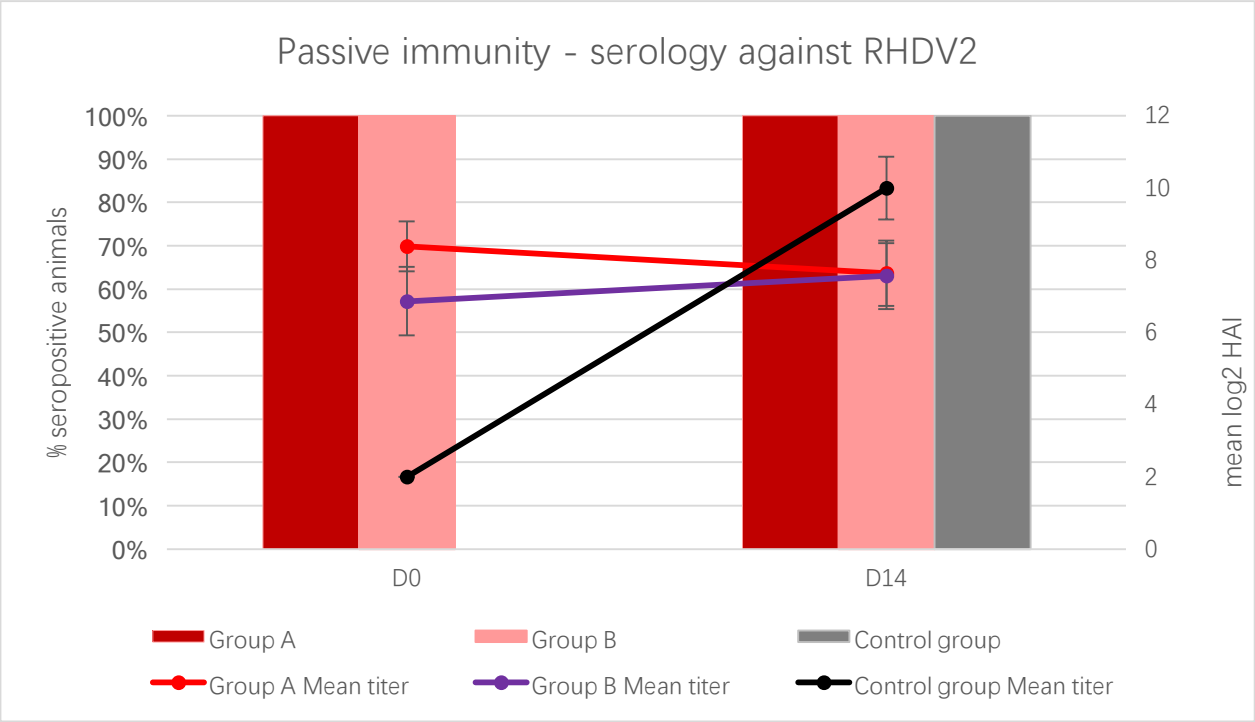

Figure 2. Serological response against RHDV2 of the offspring of vaccinated does and percentage positivity.

CONCLUSIONS

These results show that the vaccination of breeding does with YURVAC® RHD induces an immunological response in the offspring in terms of antibody titration and seropositive animals. In conclusion, the vaccination of breeding does with YURVAC® RHD induces a passive immunity against RHDV2 in their offspring, protecting non-vaccinated rabbit kits against RHDV2 at 30 days of age.

ACKNOWLEDGEMENTS

The Authors wish to thank the laboratory technicians, veterinarians, animal handlers and the R&D Department of HIPRA.

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FREQUENT MYXOMATOSIS VACCINATION WITH MIXOHIPRA® H DOES NOT REDUCE THE PRE-EXISTING ANTIBODY RESPONSE

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ABSTRACT

Myxomatosis is a prevalent disease in European regions such as Spain, necessitating year-round protection for adult rabbits. Although licensed live attenuated vaccines are recommended twice a year, there is a common practice of administering vaccines at reduced intervals to maintain herd immunity during high breeder rabbit preplacement rates. However, there are concerns about the potential decrease in myxomatosis virus (MYXV) antibodies due to induced tolerance or immunosuppression as a result of frequent vaccinations. The aim of this study, conducted on a rabbit farm in Spain, was to investigate these concerns by comparing antibody responses generated through different revaccination programmes. Replacement breeder rabbits were divided into three groups and revaccinated at varying intervals, with blood samples collected periodically up to 9 months post-vaccination. Antibody responses against MYXV were assessed using an indirect ELISA, and statistical analyses were performed to compare the results between the groups. The initial vaccination induced an antibody response in all groups. Comparisons of groups revaccinated at shorter intervals with the standard interval group revealed no statistically significant differences. This suggested that frequent revaccination did not decrease the existing antibody response, although no boost was observed. The findings suggest that frequent revaccination could be a strategy to maintain proper immunity on farms facing challenging disease and management situations, preventing the emergence of subpopulations of negative animals. Further investigations are needed to understand the boosting effects on vaccine protection and other components of the immune system, as the antibody response alone may not be a reliable correlate of protection. Indeed, humoral immunity is a very poor index of protection since most of the protection for Myxoma virus is due to cell immunity.

Key words: Myxomatosis, vaccine, rabbit

INTRODUCTION

Myxomatosis is endemic in European regions like Spain and thus it is important to keep adult rabbits protected throughout the year (Villafuerte *et al.,* 2017). Licensed live attenuated vaccines are indicated for twice yearly application, with revaccination every 6 months. Despite this, applying the vaccine at reduced revaccination intervals is a common practice adopted when there are concerns regarding challenging disease situations. The aim of this is to try to maintain or boost the immunity of the herd in the face of a high breeder rabbit replacement rate.
Despite the adoption of this practice, users are concerned that a high vaccination rate could decrease the production of myxomatosis virus (MYXV) antibodies due to an induced tolerance or immunosuppressive effect.

The objective of this study was to investigate this concern by comparing the antibody response generated by different revaccination programmes.

MATERIALS AND METHODS

Animals and experimental design

The study was performed on a rabbit farm located in Spain that had not experienced any cases of myxomatosis for over two years prior to the study. The farm routinely vaccinated animals against myxomatosis using a live vaccine administered intradermally. No screening was conducted before the trial began because it was not possible to differentiate between exposure to wild myxomatosis virus and exposure to the vaccine strain nor was it feasible to use sentinel animals. Replacement breeder rabbits of approximately 2 months of age were recruited for the study and randomly distributed into 3 groups of 77 animals each. All the animals were vaccinated with a first dose of MIXOHIPRA® H (HIPRA, Spain) and then revaccinated at either 6 months (Group A), 4 and 8 months (Group B), and finally 3 and 6 months later (Group C). The vaccine was administered by the intradermal route following the manufacturer's instructions. Blood samples (30 per group) were periodically collected up to 9 months post vaccination (mpv) based on the vaccination plan; specifically, group A was bled at 0, 1, 3, 4, 5, 6, 7, 8 and 9 mpv; group B at 0, 1, 4, 5, 8, 9 mpv; group C at 0, 1, 3, 4, 6, 7, 9 mpv.

Antibody response against MYXV

The purified sera samples were analysed by a commercial indirect ELISA against MYXV (HIPRA, Spain) to determine the presence and quantity of the antibody response against MYXV. The levels were indicated as relative index percent (IRPC). Positive and negative cut-offs were 2 and 1 IRPC respectively.

Statistical Analysis

Graph and statistical comparisons were performed by GraphPad (Prims). Kruskal-Wallis & Mann-Whitney U Tests were used to compare IRPC levels between groups. The significance level was set at 5%.

RESULTS AND DISCUSSION

The initial vaccination generated an antibody immune response against Myxomatosis in the three groups, as also previously described by other authors (Dalton *et al.*, 2015). When the antibody response of groups B and C, revaccinated at short intervals, was compared with the group re-vaccinated at the standard interval (Group A), no statistically significant difference was detected (Kruskal-Wallis & Mann-Whitney U Tests, $p>0.05$) (Figure 1).

This result suggested that the frequent administration of the vaccine did not decrease the pre-existing antibody response although no boost was observed either. Therefore, this finding suggested that frequent revaccination might be a solution to ensure the maintenance of a proper immunity on those farms facing challenging disease and management situations which might create subpopulations of negative animals.

Previous authors have described a similar finding where infection only slightly boosted the antibody response of rabbits vaccinated intradermally (Dalton *et al.*, 2015). The antibody response is not considered to be a reliable correlate of protection. The cellular immune response plays a crucial role in protecting against myxomatosis (Marlier, 2010). Therefore, the frequent administration of a live attenuated virus vaccine every 4 months is a common practice. This approach is not solely aimed at ensuring overall group protection during periods of high restocking rates but, more importantly, at sustaining a robust level of cell-mediated immunity. Therefore, further investigation is needed to elucidate the boosting effects on the extent of protection afforded by the vaccine or on other components of the immune system.

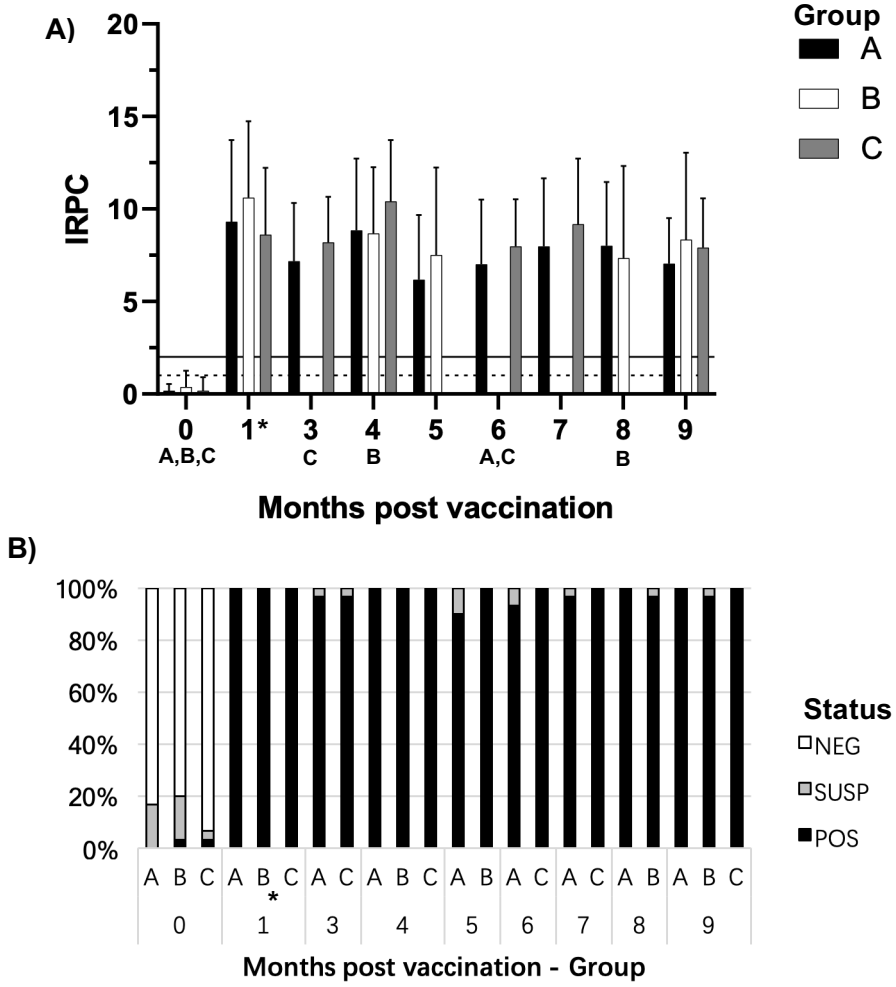

Figure 1: Antibody response against MYXV. Results are represented as A) average and standard deviation of IRPC values and B) percentage of positive samples. The continuous and dashed lines represent the positive and negative cut-offs respectively. *Only 10 samples per group were analysed. Groups A, B and C were revaccinated every 6, 4 and 3 months respectively (indicated with the respective group capital letter below the horizontal axis).

CONCLUSIONS

These results indicate that the frequent administration of MIXOHIPRA® H for revaccination of adult rabbits does not decrease the pre-existing antibody response. This practice might help to compensate for administration mistakes and to keep up with vaccination, thus avoiding generating subpopulations of negative animals in the case of farms with high replacement rates.

ACKNOWLEDGEMENTS

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ATYPICAL CLINICAL AND PATHOLOGICAL FINDINGS IN CASES OF MYXOMATOSIS AND RABBIT HEMORRHAGIC DISEASE

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ABSTRACT

Rabbit farming today faces significant economic challenges and infectious diseases play a relevant role. Myxomatosis and rabbit hemorrhagic disease (RHD) are two of the most important diseases due to their high mortality; however, both diseases can sometimes show similar clinical and pathologic findings. This study aims to show some atypical clinical signs and lesions that mimic both diseases and can delay the diagnosis. A large number of myxomatosis and RHD outbreaks were investigated over several years by routine farm visits, and clinical and lesion observations were documented. Both diseases are characterized by similar hemorrhagic lesions in various organs; however, RHD shows liver necrosis and hepatitis and myxomatosis cutaneous lesions. Although the clinical course is different, with RHD following a rapid course and myxomatosis a slower course, different pathological findings have recently emerged. Intestinal invaginations are commonly observed in RHD, but not in myxomatosis. They can be found in up to 35% of rabbits that die from RHD. Understanding these differences is essential for accurate diagnosis and effective management strategies in rabbit populations.

Key words: myxomatosis, rabbit hemorrhagic disease, diagnosis, pathology, atypical variants.

INTRODUCTION

Rabbit farming is currently facing significant economic challenges and control of infectious diseases is one of the most important. Myxomatosis and rabbit hemorrhagic disease (RHD) are important diseases that can cause significant losses, and early diagnosis remains one of the most effective methods for their control (Rosell et al., 2019). Myxomatosis has two clinical forms, the classic and the atypical, with the atypical being the most common today. Diagnosis depends on the expertise of veterinarians, who subsequently confirm the diseases at the laboratory. However, laboratory confirmation does not always arrive in time and clinical situations require more urgent actions.
Clinical signs in RHD and myxomatosis are usually different, with atypical myxomatosis typically progressing slowly over time and RHD progressing acutely, with minimal symptoms before death. However, both diseases can sometimes show similar clinical signs and lesions, highlighting the importance of an accurate diagnosis because their control is different. Moreover, atypical myxomatosis can sometimes be clinically confused with other diseases such as pasteurellosis, although gross lesions are very different (Rosell et al., 2019).

This work describes cases of myxomatosis and RHD with similar clinical and lesional findings and adds some clues for an adequate diagnosis.

MATERIALS AND METHODS

Animals and experimental design

Various outbreaks of myxomatosis and rabbit hemorrhagic disease in different farms were studied. Diagnosis of both diseases were done following epidemiological considerations, clinical assessment and necropsy findings. Additionally, RHD was confirmed by rapid testing and hemagglutination inhibition testing for RHDV2 (RHDV + RHDVb detection kit, certest, Spain). In myxomatosis, advanced cases were especially studied, and samples were taken from animals that died due to the disease, ensuring that lesions were not artifactually induced by euthanasia. Only cases of atypical myxomatosis were included in this study.
Samples were taken for histopathological studies. They were fixed in formalin for 24 hours, embedded in paraffin wax, sectioned at 4 µm and stained with hematoxylin-eosin.

RESULTS AND DISCUSSION

Skin lesions

Skin lesions are typically found in myxomatosis but are uncommon in RHD (Gay Gutiérrez, 2004). In one farm, advanced cases of myxomatosis (Fig. 1) were initially mistaken with pasteurellosis by the farmer and no measures were taken. Confusion with less severe diseases can lead to the spread of the virus, as sick animals are not eliminated. Additionally, rigorous disinfection and vaccination are recommended as control measures for myxomatosis (Rosell et al., 2019).

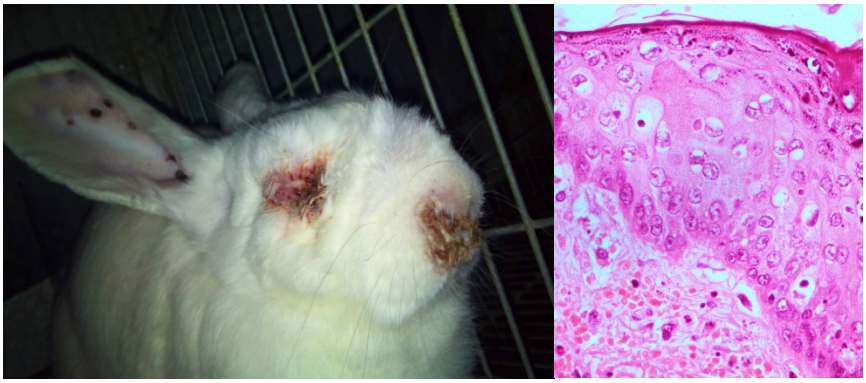


Figure 1: Doe affected with atypical myxomatosis. Myxomas can be found in the ear, conjunctivitis and bilateral purulent rhinitis, leonine head and dyspnea. Histology shows an eyelid with eosinophilic intracytoplasmic inclusion bodies and cells showing ballooning degeneration, characterized by cellular swelling with ground glass appearance of the cytoplasm.

Thoracic cavity lesions

In cases of advanced myxomatosis, despite the presence of dyspnea in the animals, the lungs often appear normal, which contradicts the expected symptoms. Microscopic evaluation, however, can show interstitial pneumonia (Fig 2A).

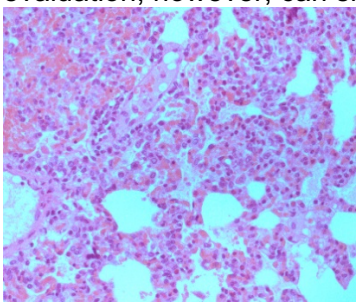


Figure 2A: Interstitial pneumonia

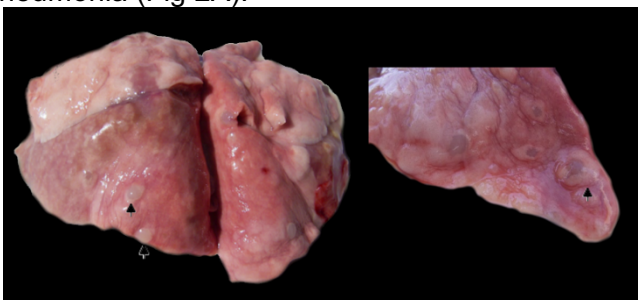


Figure 2B: Myxomatous nodules in the lungs, zones with cranioventral emphysema. No secondary infections were found.

Furthermore, in cases of very advanced myxomatosis, a rare but pathognomonic lesion may appear: small, whitish rounded nodules, known as myxomas (Fig. 2B). In the early phases of the disease this lesion is not seen, although visceral myxomas have been previously described (Rosell Pujol, 2000). Pulmonary hemorrhages are very common in both diseases. However, in RHD they affect the thymus, while in myxomatosis they do not (Fig. 3).

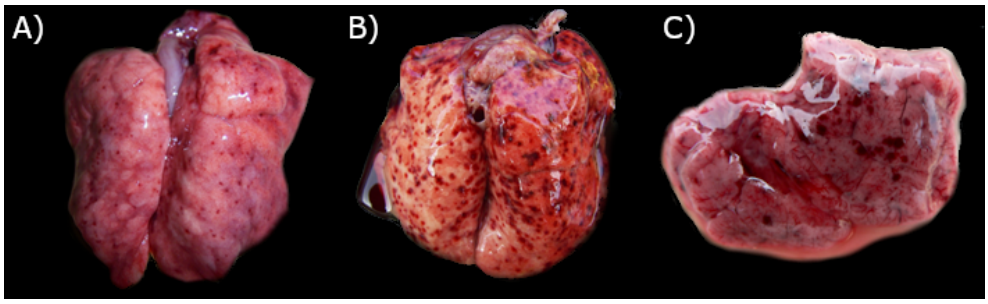


Figure 3: A) Myxomatosis: petechial hemorrhages in the lung. Severe and diffuse pulmonary emphysema and presence of nodules. B) RHDV2: multifocal hemorrhages in the lung. No differences between classical and variant forms have been found. C) RHD: Thymus with petechial hemorrhages. Not present in myxomatosis.

Abdominal lesions

In myxomatosis, hemorrhages and hyperemia are typically observed in the serosa of the fundus of the stomach and the small intestine. In addition, splenomegaly is also observed (Fig. 4). Enlarged spleen and intestinal vascular lesions have also been observed in RHDIn myxomatosis, vascular lesions tend to concentrate in areas rich in lymphoid tissue.

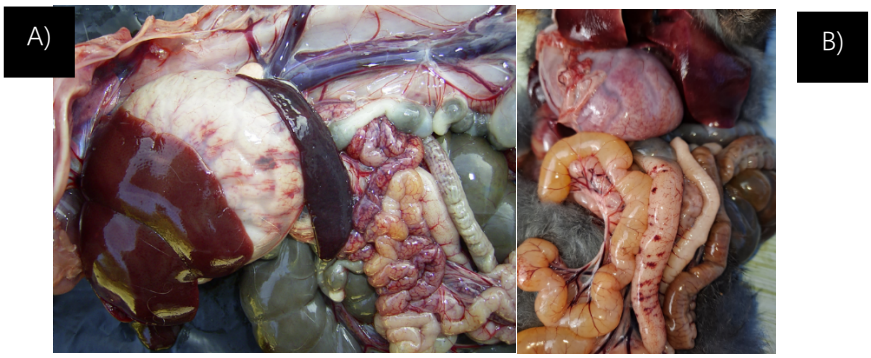
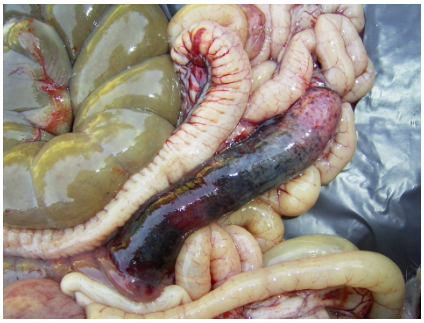


Figure 4:
Myxomatosis (advanced case): A) Splenomegaly and vascular changes are seen in the serosa of the stomach and in the small intestine. B) Petechiae in the small intestine and cecal appendix.



Occasionally, vascular changes in myxomatosis can be very severe showing widespread hemorrhages and significant thickening of the intestinal wall (Fig. 5). These severe lesions have never been observed in cases of RHD.

Figure 5: Myxomatosis. Severe swollen appendix with extensive coalescent hemorrhages and individual petechiae in its distal region.

As vascular changes can be similar in both diseases, the most important lesion to differentiate the two diseases is the hepatic degeneration characterized by a pale liver that is present in RHD but never in myxomatosis. Nevertheless, liver lesions can occasionally be subtle and difficult to evaluate macroscopically. In the kidneys, severe congestion with loss of detail between the cortex and medulla are typical of RHD, but kidney congestion can also be seen in cases of advanced myxomatosis (Fig. 6).

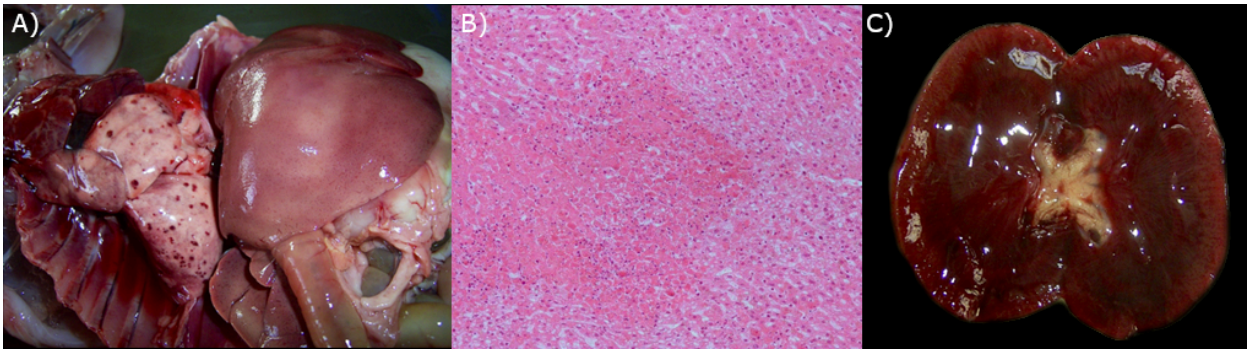


Figure 6: RHDV2. A) Hepatic degeneration, petechiae in the lung and thymus. B) Liver. Severe necrosis (H-E). C) Kidney. Severe congestion with loss of detail between the cortex and medulla.

Another interesting finding seen in RHD but not in myxomatosis is the presence of intestinal intussusceptions. Approximately 35% of rabbits that die from RHD have invaginations in the small intestine, either antemortem or postmortem (Fig. 7). Its origin is usually associated to the increased intestinal motility related to intestine irritation. However, it is important to note that this lesion is not pathognomonic of RHD, as other etiologies such as *E. coli* or *Salmonella* spp. can also induce it. In these cases, enteritis is typically observed and easily explains the intussusceptions; however, in RHD its pathogenesis is not clear.

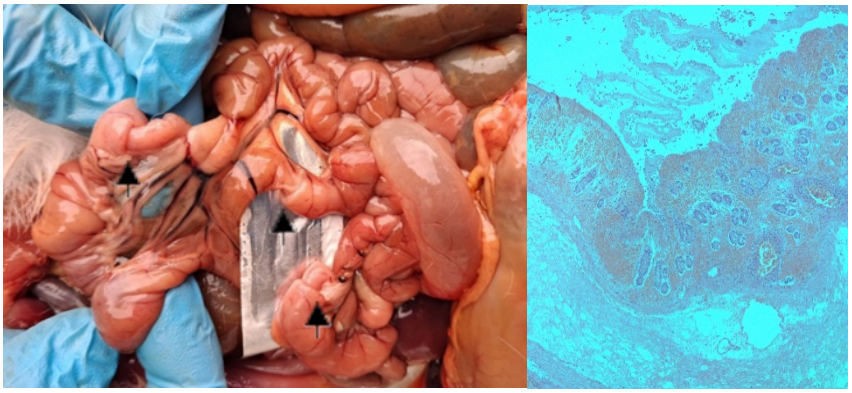


Figure 7: RHD. Rabbit of 60 days of age with 3 invaginations. Histology shows a postmortem intussusception in which hemorrhagic mucosa, submucosal edema and exudate in the lumen can be seen. Loss of the superficial epithelium is also observed.

CONCLUSIONS

Prompt intervention by farm veterinarians in cases of fatal diseases such as myxomatosis and RHD is encouraged. Mixomatosis has shown periodic changes in pathogenesis due to viral mutations, and atypical clinical and pathological findings can be observed. Therefore, it is important to recognize some atypical lesions to obtain a rapid identification. These novel findings could play a crucial role in improving the diagnosis of these disease and thus the economy of rabbit farmers.

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RECOMBINANT PRECOCIOUS LINE OF *EIMERIA MAGNA* EXPRESSING VP60-P2 FROM RHDV2 INDUCED HIGHER HUMORAL IMMUNE RESPONSE AGAINST EXOGENOUS PROTEIN IN RABBITS COMPARED WITH THE WILD-TYPE PARASITES

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ABSTRACT

Rabbit coccidiosis and Rabbit haemorrhagic disease (RHD) are two diseases that seriously affect the development of rabbit industry. Rabbit haemorrhagic disease virus 2 (RHDV2; GI.2) is a pathogenic lagovirus that emerged in 2010, and which now has a global distribution. RHDV2 can infect young rabbits and even classical RHDV-vaccinated rabbits. Thus the development of new vaccine against this emerging disease is urgent needed. In this study, we constructed a stable transgenic precocious line of *Eimeria magna* (EmagPWT) expressing the P2 subdomain of the capsid protein VP60 from RHDV2. Rabbits were immunized with either transgenic or wild-type oocysts. IgG antibody levels against either EmagWT sAg or VP60-P2(2) were detected. The results showed that after boost immunization with transgenic parasites, P2 antigen expressed by EmagP-VP60P2(2) could stimulate detectable IgG antibodies in rabbits.Our data are encouraging for development of live oocyst vaccine formulated with genetic modified precocious lines of *E. magna*.

Key words: *Eimeria magna*, Rabbit heamorrhagic disease virus 2, VP60-P2.

INTRODUCTION

At present, the rabbit industry mainly includes meat rabbit and fur rabbit breeding, the commercial scale has been further expanded. Rabbits can also be used as experimental animals or pets, the importance of rabbits is self-evident. Rabbit coccidiosis and Rabbit hemorrhagic disease can seriously affect the healthy development of rabbit industry. Once these diseases break out in the farm, they will cause huge economic losses.

Rabbit coccidiosis is caused by *Eimeria* spp., which parasitizes the digestive system of rabbits and can cause diarrhea, weight loss or even death. Although anticoccidial drugs can reduce clinical symptoms and oocysts excretion, there are also some drawbacks, such as drug resistance, which gradually decreases the effect of drugs, and the residue of drugs in animal products. Certain wild-type or attenuated *Eimeria* species could be formulated into live vaccines for the control of rabbit coccidiosis (Sadek *et al*.,2018; Fang *et al*., 2019). At present, with the development of gene editing technology, it is possible to construct transgenic *Eimeria* expressing exogenous antigens: transgenic *E. tenella*, *E. acervulina*, *E. necatrix*, *E. Magna* (Yan *et al*., 2009; Qin *et al*., 2014;Duan *et al*., 2019; Tao *et al*., 2017). Previous studies have shown that transgenic parasites expressing exogenous proteins can stimulate specific immune responses in their hosts, showing their great potential to be used as live recombinant vaccine vectors (Tao *et al*., 2017;Yu *et al*., 2024).

In the 1980s, there was an outbreak of a new viral disease in the European rabbit population, characterized by being extremely lethal and highly contagious in domestic rabbits, known as rabbit hemorrhagic disease. Today, the emergence of a new Lagovirus, RHDV type 2(RHDV2, genotype GI.2) was reported in France in 2010, dramatically changed the epidemiology of this disease (Mahar *et al*., 2018). Compared to RHDV1, RHDV2 causes infection in younger rabbits, infects a wider range of hosts, and has low cross-protection with other RHDV strains (Taggart *et al*., 2022). Molecular epidemiological studies have shown that RHDV2 has replaced RHDV1 as the current circulating strain in France, Spain, Portugal,

Australia and other countries (Dalton *et al*., 2014; Lopes *et al*., 2014). In China, RHDV2 was also detected since 2020 (Hu *et al*., 2021). Therefore, it is necessary to develop a vaccine that can control the emerged RHDV2 as soon as possible.

VP60, the capsid protein of RHDV, is the major structural and immunogenicity protein of RHDV. Studies have reported use of the VP60 protein in a subunit vaccine against RHDV (Parra and Prieto, 1990;Laurent *et al*., 1997). A large number of studies have shown that the recombinant expression of VP60 has good immunogenicity, which can not only stimulate a high specific immune response, but also protect against viral challenge. So it's a candidate component of a novel RHD vaccine (Mitro and Krauss, 1993). The P2 subdomain is the exposed region of capsid protein, which is responsible for binding to the host HBGA. The recombinant expression of P2 subdomain protein can stimulate high antibody titer (Wang *et al*., 2013).

In our study, the precocious line of *E.magna* with reduced pathogenicity and good immunogenicity was selected as a vaccine carrier to express the P2 subdomain of VP60 of RHDV2, in the hope that after immunizing rabbits with this transgenic *Eimeria*, the immune response against coccidia and exogenous antigen can be generated.

MATERIALS AND METHODS

Parasites and animals

The precocious line of *E. magna*(EmagPWT) selected from a local strain is used in this study (unpublished data). Transfection of EmagPWT sporozoites was conducted according to an established protocol (Yan *et al*., 2009). Procedures for the collection, purification, and sporulation of oocysts have been fully described (Long *et al*., 1976).

All rabbits used in this study were obtained from a rabbit farm of China Agricultural University Zhuozhou Experimental Station. Before infection with oocysts, they were kept coccidia-free in isolators and fed with coccidia-free pellet feed and water.

Experimental design

Eighteen 35-day-old coccidia-free rabbits were divided into three groups, i.e., control, EmagPWT and EmagP-VP60P2(2). Each rabbit was received 200μl PBS (Control group) or 3000 sporulated oocysts at day 0, and boost immunization was performed after 14 days with 200μl PBS(Control group) or 1×10^4 sporulated oocysts. Serum samples were collected before every immunization and 14 days after the secondary immunization. Fecal samples were collected 14 days after each immunization for oocyst counting (Fig.1A).

Serological ELISA

Serum samples from rabbits were analyzed by an indirect ELISA test using the recombinant VP60-P2 protein of RHDV and the soluble proteins from the EmagPWT oocyst as the antigen. The P2 protein was expressed in *Escherichia coli* BL21 (DE3) using the pMAL-c5x expression system, and the recombinant product was purified by dialysis. The extraction of soluble proteins from the oocyst with liquid nitrogen grinding method. The sAg of EmagPWT and purified VP60-P2 protein were used as the coating Ag to coat the 96-well microtiter plates (5 μg/mL, 2 μg/mL, respectively).

Statistical Analysis

All statistical analyses were performed in GraphPad Prism 8.0.1 software.

RESULTS AND DISCUSSION

Immune protection in rabbits immunized with the transgenic strain EmagP-VP60P2(2)

It can be seen from the results that there is no significant difference in daily body weight gain between the immunized groups and control group during the whole experiment, indicating that immunization with this dose of parasites not affect the normal growth of rabbits (Fig.1B). After the second immunization, the number of oocyst output was significantly reduced (Fig.1C), and the antibody level against *Eimeria* antigen was significantly increased (Fig.1D), indicating that a good level of protection against *Eimeria* had been generated in rabbits at this time. For exogenous antigen, there was no significant difference in antibody level after the prime immunization, but at 14 days after the boost immunization, the IgG antibody level

in the EmagP-VP60P2(2) group was higher than that in the control group and EmagPWT group (Fig.1E).

RHDV2 has gradually become the dominant epidemic strain, and it is necessary to develop a vaccine to control it. In this study, we expressed VP60-P2 of RHDV2 in the precocious line of *Eimeria magna*(EmagPWT) and observed that immunization of rabbits with EmagP-VP60P2(2) elicited an elevated level of humoral immune response against P2 protein compared with EmagPWT, which is similar to some previous studies related to transgenic *Eimeria* (Yu *et al*., 2024;Tang *et al*., 2016). However, the low IgG levels suggest that increasing the expression level of exogenous proteins in transgenic *Eimeria* is one of the methods to break through *Eimeria* as a vaccine carrier.

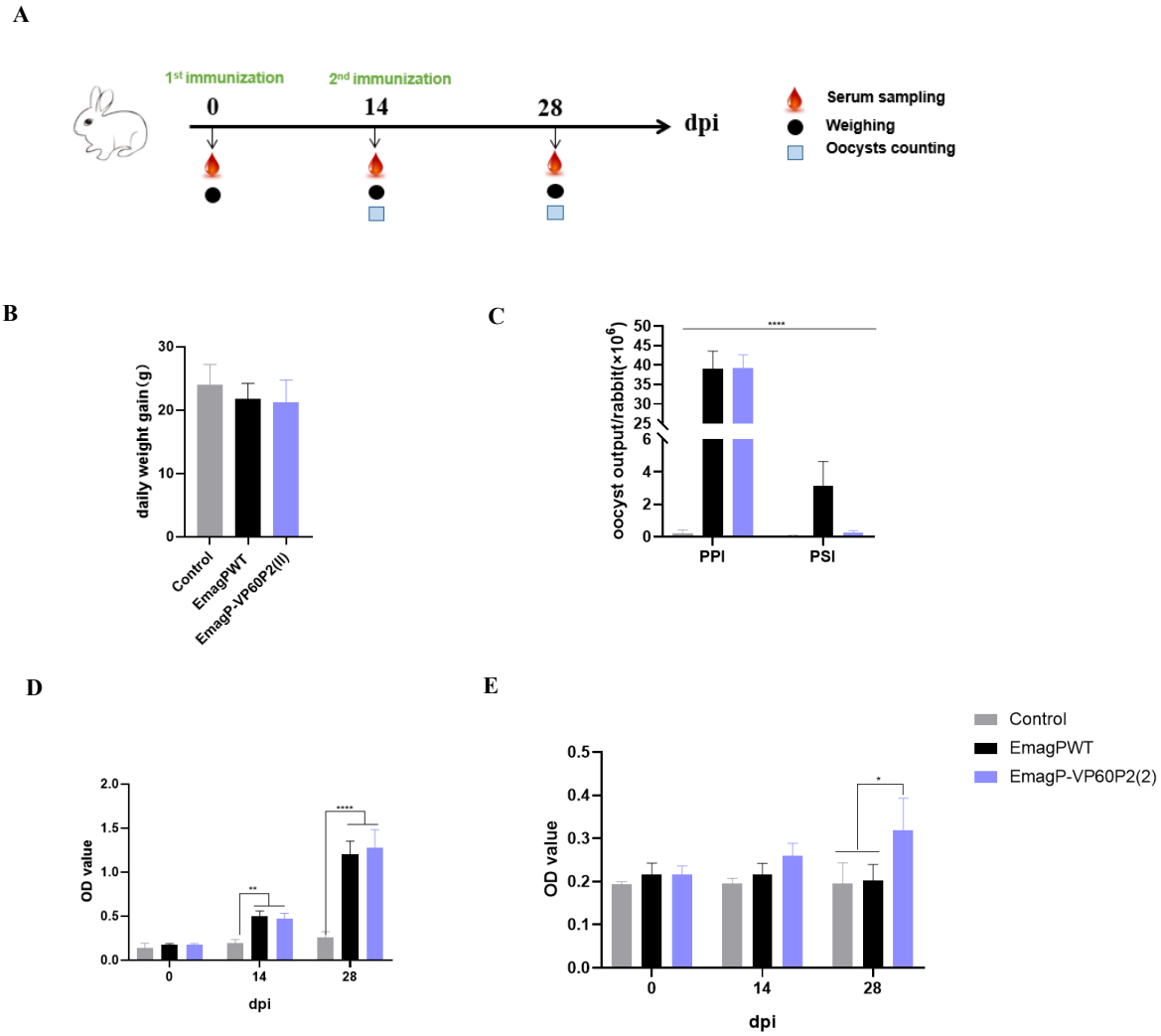

Figure 1 Protection level after immunization of of EmagP-VP60P2(2).(A) Diagram of immunological experiments. (B) Changes in daily weight gain throughout the experiment . (C) Oocyst output after the prime(PPI) and second immunization(PSI) .(D) IgG antibody levels against EamagWT sAg, detected by OD450. (E) IgG antibody levels against VP60-P2, detected by OD450. Statistical analysis was performed by one-way ANOVA, *p < 0.05, and **p < 0.01, *** p < 0.001, **** p < 0.0001.

CONCLUSIONS

In summary, we successfully constructed a transgenic precocious line of *E. magna* which expressing P2 subdomain of VP60 of RHDV2(Data not shown). The detection results of the

specific immune response stimulated by EmagP-VP60P2(2) showed that the specific humoral immune response level was up-regulated, but the antibody level was still low when compared with a commercial vaccine or the genetically engineered vaccine of RHD.

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A CLINICAL CASE: POTENTIAL CO-INFECTION OF *PASTEURELLA MULTOCIDA* AND RABBIT HEMORRHAGIC DISEASE VIRUS （RHDV and RHDV2） IN A RABBIT FARM

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ABSTRACT

Rabbit *Pasteurella multocida* (*P. multocida*) is a highly pathogenic bacterium responsible for causing rabbit pasteurellosis. Rabbit haemorrhagic disease virus (RHDV) consists of two different agents, RHDV1 (genotype GI.1) and RHDV2 (genotype GI.2). The presence of both *P. multocida* and RHDV, especially RHDV 2, poses significant threats to rabbit farming in China, leading to substantial economic losses. Recently, a significant number of deceased rabbits were discovered in a large-scale meat rabbit farm in Ningxia which is located in northwestern China. Consequently, the primary objective of this work was to ascertain the causative pathogens responsible for these mortalities. The results showed potential co-infections of *P. multocida* and RHDV1, as well as RHDV2, in the farm. These results were performed through anatomical examination, bacterial isolation, and PCR nucleic acid identification. In conclusion, it is crucial to implement continuous and comprehensive disease surveillance in rabbit farms, facilitating the prompt obtainment of recommendations for farmers and mitigating economic losses.

Key words: *Pasteurella multocida*; RHDV1; RHDV2; Co-infection; Rabbits

INTRODUCTION

Rabbit pasteurellosis poses a significant threat to rabbit farming and is caused by the bacterium *Pasteurella* (Massacci *et al*. 2018, Zhu *et al*. 2020). *P. multocida*, a commensal bacterium in the respiratory tract, belongs to the category of opportunistic pathogens (Peng *et al.* 2019) and is susceptible to co-infection with other pathogens, leading to various diseases in livestock animals and causing significant economic losses (Qiu *et al*. 2022). Rabbit haemorrhagic disease (RHD) has been reported since 1980s. Two different RHDV viruses have been identified so far. Previous studies have demonstrated that classic RHDV1 primarily induces clinical disease and mortality exclusively in adult rabbits, with minimal occurrence observed in young rabbits. Relevant attempts have been made to utilize RHDV1 as a biological approach for the management and reduction of the rabbit population in Australia (Abrantes *et al.* 2012). In contrast, RHDV2 exhibits the capability to cause clinical disease across all age groups of rabbits. (Le Gall-Reculé *et al*. 2013, Rouco *et al*. 2019). The presence of RHDV2 has been documented in meat rabbit breeding areas worldwide. Recently, a significant number of diseased and deceased rabbits were discovered in a large-scale meat rabbit farm in Ningxia which located in northwestern China. The farm annually yields over 1.3 million meat rabbits. The affected population included both adult rabbits and pre-weaned individuals, especially sucking rabbits exhibiting severe respiratory symptoms. The mortality of approximately 300 rabbit kits (aged 24-30 days) was observed within a population of over 900 suckling rabbit kits that had been vaccinated against RHDV and serotype A *P. multocida* on the farm. Therefore, objective of this study is to determine the causative pathogens responsible for these mortalities and investigate the prevalence of co-infection between *P. multocida* and RHDV.

MATERIALS AND METHODS

Samples collection and anatomical examination

The rabbits died not later than 24 hours were chosen for pathological anatomical examination, and aseptic collection of pathological tissue samples was conducted. In total, a cohort of six rabbits comprising five rabbit kits (aged 26-30 days) and one adult was selected. The pathological tissue samples were stored at -80℃ in a refrigerator for subsequent investigation.

P. multocida isolation identification and antimicrobial susceptibility test

Tryptic Soy Agar (Thermo Scientific, Waltham, USA) supplemented with 5% newborn calf serum (Every Green, Hangzhou, China) was used to culture bacteria from collected pathological tissue. According to the anatomical examination, it is possible that the sick rabbits were infected with *P. multocida*. Therefore, 40 single colonies were selected from TSA medium for individual bacterial colony PCR and serogroup identification as previously reported (using primers: KMT F: ATCCGCTATTTACCCAGTGG R: GCTGTAAACGAACTCGCCAC and A, B, D, E, F serogroup identification primers as described in a previous study) (Townsend et al. 2001). The minimum inhibitory concentration (MIC) of the strain was determined using the microbroth dilution method, following the guidelines set by CLSI 2023. A total of 17 antimicrobials were tested (Ciprofloxacin, Nalidixic acid, Ampicillin, Ceftazidime, Cefotaxime, Ampicillin/Sulbactam, Ceftazidime/avibactam, Meropenem, Ertapenem, Tetracycline, Tigecycline, Amikacin, Streptomycin, Azithromycin, Chloramphenicol, Colistin, Sulfamethoxazole), and resistance breakpoints were established based on CLSI recommendations.

RHDV 1 and RHDV 2 identification

The total RNA from pathological livers was extracted using the method described in previous study (Silvério *et al*. 2018). The cDNA was synthesized using the Polestar 1st cDNA Synthesis Kit (ToloBio, Shanghai, China), following the operational procedures outlined in the manufacturer's manual. The Green Taq Mix (Vazyme, Nanjing, China) was utilized for the amplification of the conserved region (vp60) of RHDV 1 and RHDV 2 (primer sequences: RHDV 1 F: CGTGCTTCAGTTTTGGTA, R: ATGAGTGCTGACGAGTAG; and RHDV 2 F: ACTACTAGCGTGGTCACCACC, R: TTGTTATAAACGCTCAGGACCAAC).

RESULTS AND DISCUSSION

Rabbits necropsy lesions

Six deceased rabbits were obtained from the farm, consisting of five rabbit kits and one adult. These carcasses were dissected, and necropsy examination revealed suspected mixed infection of *P. multocida* and rabbit haemorrhagic disease virus. The findings included splenic congestion and enlargement, fibrin exudate on the heart surface, dropsy of pericardium, as well as liver hemorrhage and necrosis. There were varying degrees of lesion severity observed among five pre-weaned rabbits. Fig1A performed dropsy of the pericardium. The splenic congestion and enlargement illustrated as Fig1B. These lesions indicate the potential presence of viral infection. The results depicted in Fig1C demonstrate the presence of fibrin exudate on the cardiac surface and pulmonary edema, which may be attributed to severe respiratory symptoms. Previous studies have demonstrated that *P. multocida* can induce severe pneumonia and edema in rabbits(Uenoyama *et al*. 2020, Yang *et al*. 2022). The presence of hemorrhage in the livers and lungs is evident, as depicted in Fig1D and Fig1E. These lesions may arise due to the co-infection of RHDV and *P. multocida*.

The isolates were classified as serotype D *P. multocida*

Forty single colonies were selected from the TSA medium for PCR amplification of the *KMT* gene in *P. multocida*, and 39 colonies shown positive Fig2A. Furthermore, 19 isolates were confirmed as D capsule serotype through PCR analysis Fig2B. Currently, serotype A *P. multocida* exhibits the highest prevalence, while there has been an observed increase in serotype D in recent years (Wang *et al.* 2019). In this study, the D serotype of *P. multocida* was isolated from the heart, lung, and liver of three rabbit kits. The farm administered only a type A *P. multocida* vaccine, excluding the administration of type D, which could potentially be associated with this outbreak.

Figure 1: Dissecting and examining lesions in deceased rabbits through necropsy. A: dropsy of pericardium; B: splenic congestion and enlargement; C: fibrin exudate on the heart; D: hemorrhage and necrosis in liver; E: hemorrhage spots in lung.

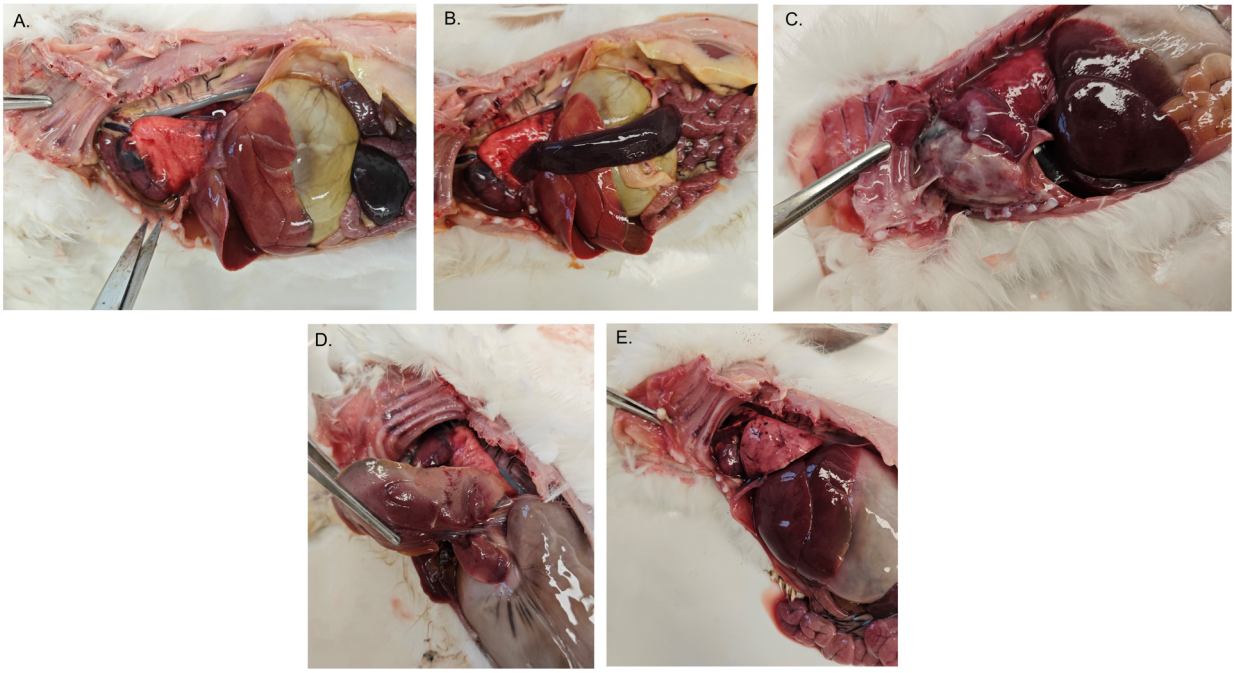
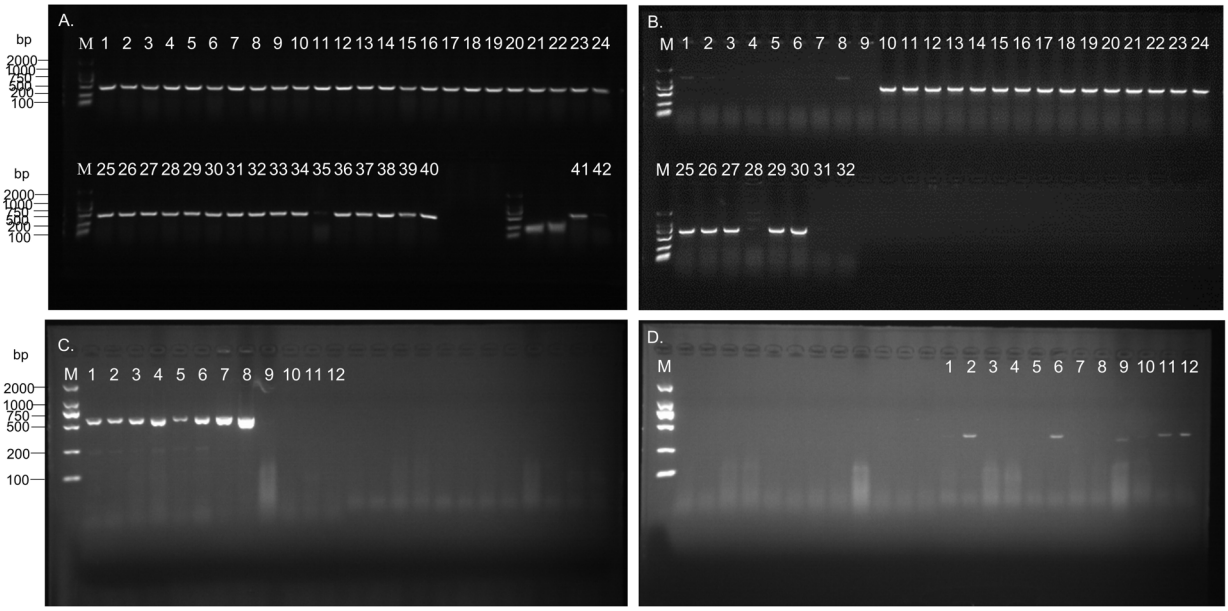


Figure 2: PCR amplification. A, 1-40: *P. multocida* individual bacterial colonies PCR, 41: positive control, 42: negative control; B, 1-29: D capsule serotype detection, 30: positive control, 31-32: negative control; C: RHDV 1 vp60 amplification, 1-6: cDNA from six livers, 7-8: RHDV1 positive control, 9-10: negative control, 11-12: RHDV2 positive control; D: RHDV 2 vp60 amplification, 1-12 were same as C.



Therefore, we should pay more attention on D serotype strains prevent. In addition, antimicrobial susceptibility test result shown isolate resistant to colistin (MIC 8 mg/L) and

Sulfamethoxazole (MIC 4/76 mg/L). The worthiness of further investigations is evident and calls for thorough excavation.

The presence of both RHDV1 and RHDV2 was identified in rabbit kits.

Relevant studies have previously demonstrated that RHDV 1 exhibits a higher mortality towards adult rabbits rather than young ones (Dalton *et al.* 2012). In this study, PCR amplification results showed that five rabbit kits harbored RHDV 1 vp60 (Fig2C, lanes 2-6), highlighting the potential lethality of RHDV 1 in young rabbits and emphasizing the importance of considering age distribution within populations died with RHDV 1. Six deceased rabbits were found positive for RHDV1 (Fig2C, lanes 1-6), with two of them testing positive for RHDV 2 (Fig2D, lane 2, lane 6). In conclusion, it is imperative to enhance efforts in preventing epidemics caused by RHDV 1 amidst the ongoing RHDV 2 pandemic, as there exists a potential for RHDV 1 to be lethal to suckling rabbits under specific circumstances. This report serves as a crucial alert, underscoring the necessity of comprehensive preventive measures against domestic rabbit diseases encompassing both *P. multocida* and RHDV1 and 2.

CONCLUSIONS

In summary, this report elucidates the potential simultaneous morbidity of RHDV 1, RHDV2, and *P. multocida* in a rabbit farm. The evident capacity of RHDV 1 to potentially cause morbidity in rabbit kits underscores the necessity for continuous and comprehensive disease surveillance on such farms.

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GENOME SEQUENCING AND ANALYSIS OF A RABBIT PASTEURELLA MULTOCIDA SEROGROUP F ISOLATE LH06 IN CHINA

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ABSTRACT

Pasteurella multocida, the most important pathogen of the rabbit respiratory tract, causes huge economic losses in the rabbit industry. Different serogroups of *P. multocida* cause disease in rabbits, but the mechanism has not been fully elucidated. The aim of this study was to sequence and analysis a serogroup F isolate LH06 to provide a better understanding of rabbit pasteurellosis at the genetic level. The isolate LH06 genome was sequenced and comparative genomic analysis was performed with reported rabbit high- and low-virulence rabbit-origin strains in China. Comparison of high- and low-virulence rabbit *P. multocida* genomes revealed 205 unique genes among the genomes of LH06, SD11, C51-17 compared with the genome of strain AH09. In particular, the 205 unique genes were identical to the 203 unique genespreviously reported in high-virulence strains, except for two genes, putP and yidD, which were predicted to be involved in amino acid transport and cell membrane formation, respectively. Comparative analysis of the rabbit origin strain LH06 and others isolated in China identified a number of genomic differences that may elucidate the ability of high-virulence strains in China to cause disease in the rabbit host.

Keywords: rabbit Pasteurella multocida, comparative genome analysis, unique gene, China

INTRODUCTION

Pasteurella multocida is a globally distributed, serious zoonotic pathogen that causes disease in many species of most domestic and wild mammals, birds, and even humans (Wilson and Ho, 2013). Five serogroups (A, B, D, E, and F) of *P. multocida* are differentiated on the basis of the capsule. It's worth noting that serovars A, B, D and F were all highly pathogenic in rabbit pasteurellosis (Cao et al., 2017; Katoch et al., 2015).

Since the sequencing of the Pm70 genome, the genome sequences of a large number of *P. multocida* strains have been sequenced and assembled, including those isolated from different species such as avian, swine, cattle, and so on (May et al., 2001). Meanwhile, many studies based on these genomes have been reported, and the pathogenic mechanism has been further elucidated. Bovine *P. multocida* genomes analysis was conducted, which reveal 96 unique genes shared by 12 bovine serogroup B strains genomes, which were predicted to play a role in the pathogenic mechanism (Moustafa et al., 2015). Another report of avian *P. multocida* genomes analysis showed that the virulent strain genome contained 336 unique genes related to the metabolism that could enhance the virulence (Johnson et al., 2013). At present, the virulence genes of *P. multocida* isolated from different hosts show a greater difference. Although there are some sequencing data of rabbit-origin *P. multocida* genomes, there are relatively few studies based on these genomes, resulting in a lack of understanding of the pathogenic mechanism. In our previous study, 203 unique genes were identified by genome comparative analysis with high- and low-virulence strains isolated from rabbits, and elaborated that these unique genes play an important role in the virulence of rabbit-origin *P. multocida* (Qiu et al*., 2022)*.

Therefore, the aim of this study was to sequence a new virulent *P. multocida* genome and perform genome comparative analysis, and to compare and confirm previously identified virulence genes to achieve validation of virulence genes in rabbit *P. multocida*.

MATERIAL AND METHODS

Bacterial strains

P.multocida serogroup F strain LH06 (GenBank accession no. CP143878), serogroup F strain SD11 (GenBank accession no. CP090520), serogroup A strain C51-17 (GenBank accession no. MAPQ00000000) were isolated from the lungs of rabbits with pneumonic pasteurellosis in China. serotype F strain AH09 (GenBank accession no. CP090521) was isolated from the nasal cavity of a rabbit with rhinitis pasteurellosis in China.

Genome sequncing, annotation, and comparative analysis

The whole genome of strain LH06 was sequenced and assembled on the Illumina sequencing platform by Genedenovo Biotechnology Co. Ltd (Guangzhou, China) using the PacBio system. Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline. The genome sequence of strain LH06 was deposited in GenBank under accession number CP143878. Clusters of orthologous groups of proteins (COGs) were assigned using the online tool EggNOG-Mapper. Protein cluster analysis was performed on the OrthoVenn2 web server. A phylogenetic tree was constructed using HarvestTools (version 1.2). Genome-linearised comparison was performed using the BRIG software (version 0.95).

RESULTS AND DISCUSSION

Genome annotation and phylogenetic analysis

The total genome of strain LH06 was approximately 2.24 Mbp in length, with a GC content of 40.24 %. The strain LH06 genome contained 2, 497 predicted functional genes. The predicted functional genes were blasted with Cluster of Orthologous Groups of proteins (COG) database and assigned to 22 COG categories. Of these, the function of 234 genes were unknown. The COG categories and genes were visualised as circle map in Figure 1A. Phylogenetic analysis of strain LH06 and other *P. multocida* genomes revealed that the strain LH06 and AH09 were closely related (Figure 1B).

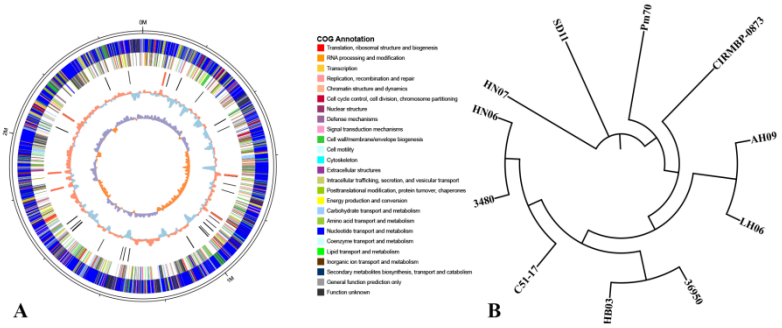

Figure 1: The genome annotation and phylogenetic analysis of the strain LH06. (A) Circular map of the genome of strain LH06 generated via Circos software. (B) Phylogenetic tree of strain LH06 in relation to other strains within the Pasteurellaceae family. Construction of the phylogenetic tree was based on genomes. All genomes were obtained from the NCBI database.

Whole-genome comparison and specific genes shared by highly virulent strains

A comparison of genome collinearity for strains LH06, SD11, AH09 and C51-17 was carried out using strain LH06 as the reference genome, the result suggested that both genomes were highly collinear. Another obvious data was that strain LH06 and AH09 had high homology for most of the genome area, and only one large genome region had low homology, which explained that both were more closely related in the phylogenetic tree (Figure 2 A).

Comparative genome analysis was performed on 4 rabbit-origin *P. multocida* strains that had been previously sequenced, including strains LH06, C51-17, SD11 and AH09. The results showed that 205 protein clusters were shared by 3 highly virulent strains (Figure 2 B). The 205 unique genes were shared by strains LH06, SD11 and C51-17, which was similar to the previously reported 203 unique genes. Then, the 2 clusters of unique genes were compared and found that the 205 unique genes were completely similar to the 203 unique genes reported in the previous study, except for 2 additional genes (Figure 2 C). The gene putP (locus_tag:V3C05_02440) was predicted to be involved in proline transport, and was assigned to category E. The product of gene yidD (locus_tag:V3C05_07485) may be responsible for membrane protein insertion efficiency, which was assigned to category S (Figure 2 D).

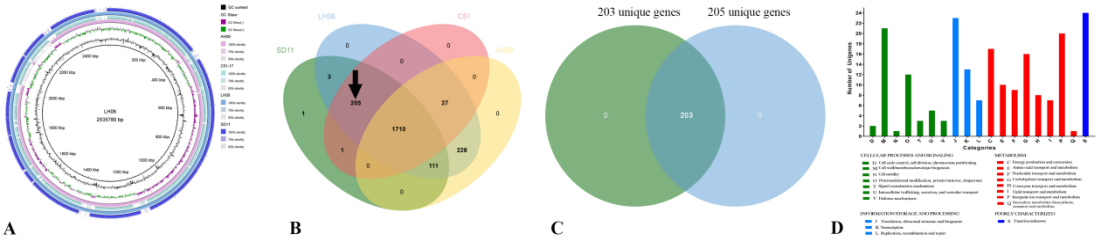

Figure 2: Comparative genome analysis of four *P. multocida* strains. (A) Genome collinearity comparative analysis. (B) Venn diagram showing different protein clusters in the genomes of *P. multocida* strains isolated from rabbits. The numbers represent the number of protein clusters at the intersection. The overlapping sections indicate shared numbers of proteins. (C) Compared the 205 unique genes in this study to the previous reported 203 unique genes. (D) Functional categories of 205 unique genes of strain LH06 based on COG.

CONCLUSIONS

P. multocida serogroups (A, B, D,and F) strains are frequently associated with atrophic rhinitis, pneumonia, and haemorrhagic septicemia in rabbits, and the pathogenic mechanism remains unclear. In our results, the rabbit-origin strain *P. multocida* strain LH06 shared 205 unique genes with the virulent strains SD11 and C51-17, which was very similar to our previous report. Through the two studies of comparative analysis of the rabbit-origin *P. multocida* genomes, we could confirm that these unique genes shared by rabbit-origin virulent strains were also found in rabbit-origin strain LH06.

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CHARACTERIZATION AND IMMUNOLOGICAL EFFECT OF OUTER MEMBRANE VESICLES FROM *PASTEURELLA MULTOCIDA*

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ABSTRACT

Pasteurella multocida is an important bacterial pathogen that can cause diseases in both animals and humans, resulting in substantial economic repercussions within the rabbit industry. The prevention of *P. multocida* infection through immunization is impeded by the absence of a safe and effective vaccine. Outer membrane vesicles (OMVs) secreted by Gram-negative bacteria are spherical vesicular structures that can induce antibacterial immune responses within the host. In this study, OMVs were isolated from *P. multocida*, and their morphology, protein constitution, and anti-infection efficacy in vivo were studied. Transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) revealed that the OMVs exhibited typical spherical and bilayered lipid vesicular architecture, exhibiting an average diameter of approximately 147.5 nm. The yield of OMVs was 2.6×10^{11} particles/mL. Proteomic analysis revealed the high abundance of membrane-associated proteins within OMVs, with the capability to instigate the host's immune response. Our results also showed that *P. multocida* OMVs could significantly increase the levels of anti-*P. multocida* and anti-OMV IgG antibodies in vivo. These results supported the prospective applicability of *P. multocida* OMVs as a platform in the context of vaccine development.

Keywords: *Pasteurella multocida*, Outer membrane vesicles, Proteomics, Immune response.

INTRODUCTION

Louis Pasteur first identified the role of *Pasteurella multocida* as a pathogen in the 1880s. *P. multocida* is highly infectious and can cause significant economic losses in rabbitries. However, there is currently a lack of commercial vaccines in China to protect rabbits against this bacterium. Therefore, it is crucial to develop a safe and efficient vaccine against *P. multocida*.

Bacterial OMVs have gained attention as potential vaccines. OMVs enhance bacterial interactions and pathogenesis, and they contain various components such as lipids, proteins, and PAMPs. The nanoscale structure of OMVs and their immunostimulatory properties make them promising candidates for bacterial infection prevention and treatment. We isolated OMVs from *P. multocida*, characterized their morphology, studied their protein composition, and investigated their immune function in rabbits.

MATERIALS AND METHODS

Bacterial culture and isolation of OMVs

P. multocida was cultured in Martin broth at 37℃ until reaching the stationary phase. The cultured mixture was then centrifuged and filtered to remove bacteria and debris. The supernatant was concentrated and ultracentrifuged to isolate the OMVs. The OMVs were resuspended in PBS and stored at -80℃ for future use.

Particle size, concentration, zeta potential and TEM

The particle size, concentration, and potential of the OMVs were measured using NTA (Zetaview, Particle Metrix, Germany). TEM analysis was conducted by isolating OMVs and

depositing them onto a 200-mesh copper grid. The samples were stained with uranyl acetate and imaged using a Tecnai™ G2 Spirit BioTWIN electron microscope operating at 80 kV.

Proteomic analysis of OMVs
SDS-PAGE analysis was performed on protein samples from both the entire-cell lysates and OMVs. Qualitative proteome analysis of OMVs was carried out by Novogene Biotech (Beijing, China). The samples were subjected to trypsin digestion and subsequent LC-MS/MS analysis.

Experimental animals and immunization
45-day-old female New Zealand White rabbits were randomly assigned to three groups, include alum-OMV group, OMV group and blank control group (BC). They were subcutaneously immunized on days 0, 7, and 14 with OMV, a mixture of OMVs and alum (1:1), or PBS. Blood samples were collected on days 14, 28, and 42 for antibody analysis (n=5). All experiments followed ethical guidelines (Protocol No. 1935). Antibodies against OMV or *P. multocida* protein in post-immunization rabbit serum were detected using ELISA. The reaction was performed in a 96-well microtiter plate. Each well was coated with 100 µL of antigen extracted by sonication, diluted in 0.05 M carbonate buffer (pH 9.6), and then incubated at 37°C for 2 hours, followed by overnight incubation at 4°C. After washing three times with washing buffer (PBST), the plates were incubated at 37°C for 2 hours with 200 µL of blocking buffer per well. 100 µL of mouse serum diluted 10,000-fold in skim milk was added to each well after three washes, and incubated at 37°C for 1 hour. After washes with PBST, 100 µL of HRP-conjugated goat anti-rabbit IgG diluted 5,000-fold in skim milk was added to each well, followed by incubation at 37°C for 1 hour. Finally, 100 µL of TMB substrate was added to each well and incubated at 37°C in the dark for 10 minutes. The color reaction was stopped by 100 µL of 0.5 M H_2SO_4, and the OD value at 450 nm wavelength was measured using an automated ELISA plate reader. Results were expressed as optical density (OD).

Statistical analysis
Data were analyzed using GraphPad Prism software. Results are presented as mean ± standard error (SE) deviation. Statistical significance was determined using t-tests and one-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Characterization of OMVs from *P. multocida*
OMVs were isolated from *P. multocida* cultures using ultrafiltration concentration technique. TEM showed irregular spherical, bilayer lipid vesicles (20-300 nm) (

a, b). NTA analysis showed an average diameter of 147.5 nm (

c). The concentration of OMVs was 2.6×10^{11} particles/mL (

c) and the zeta potential of -28.86 mV (

d). According to the NTA analysis, an estimated 2.7 OMVs were released per bacterium (

e).

Proteomic analysis of *P. multocida* OMVs
The results demonstrated that the produced OMVs were free from bacterial contamination and contained a diverse array of immunogenic proteins. (

f). Proteomic analysis identified 429 proteins in *P. multocida* OMVs. Key protein data is listed

in Table 1. Our findings show numerous antigenic proteins linked to virulence and infection in *P. multocida* OMVs, indicating their potential as vaccine targets.

OMV induced the Antigen-Specific antibodies

Both Alum-OMV and OMV induced higher OMV-specific and *P. multocida*-specific IgG in rabbit post-immunization. (

Table **1.** List the important *P. multocida* OMV proteins identified in order of decreasing PSMs

Accession number	Protein name	Molecular mass (kDa)	Gene	Subcellular localization
I2BGA6	Outer membrane protein A	38	ompA	Outer membrane
A0A2J9QJ69	Outer membrane protein A	38	ompA	Outer membrane
V4NAK3	Peptide ABC transporter substrate-binding protein	61.6	P1062_02050 60	Periplasm
A0A126QGC9	Tol-Pal system protein TolB	45.9	tolB	Periplasm
A5H9S0	Lipoprotein E	37.5	plpE	--
A0A8E2A638	Sugar ABC transporter substrate-binding protein	33.8	A0R67_09330	Periplasm
A0A191VYV1	Outer membrane protein assembly factor BamA	87.7	bamA	Outer membrane
A0A379BDM0	Hemolysin activation/secretion protein-1	63.5	lspB1_1	Periplasm
A0MCG2	Outer membrane protein	37.2	ompH	Outer membrane
A0A1E3XLL0	Membrane-bound lytic murein transglycosylase C	40.2	mltC	Periplasm
V4PX95	Sialidase	93.3	P1062_02071 65	Extracell
A0A1E3XJI9	TolC family protein	50.6	BGK37_07070	Outer membrane
A0A379BCW7	Filamentous hemagglutinin protein	234.5	pfhB1_1	Extracell
Q9CLZ8	Outer membrane protein assembly factor BamC	37.4	bamC	Outer membrane
A0A379BCX4	Protein PfhB2	49.2	pfhB2_2	--
A0A291ID33	Sialidase protein (Fragment)	32.2	nanH	Extracell
A0A1E3XJ59	MipA/OmpV family protein	28.6	BGK37_06210	Outer membrane
A0A379B9M3	Porin, opacity type	21.7	NCTC10722_ 00237	Outer membrane
J5MYK7	Lipoprotein	30.2	AAUPMB_174 30	Inner membrane
A1Z0J3	OmpW	21.9	--	cell outer membrane

Figure. 1 Physical characteristics of OMVs derived from *P. multocida*

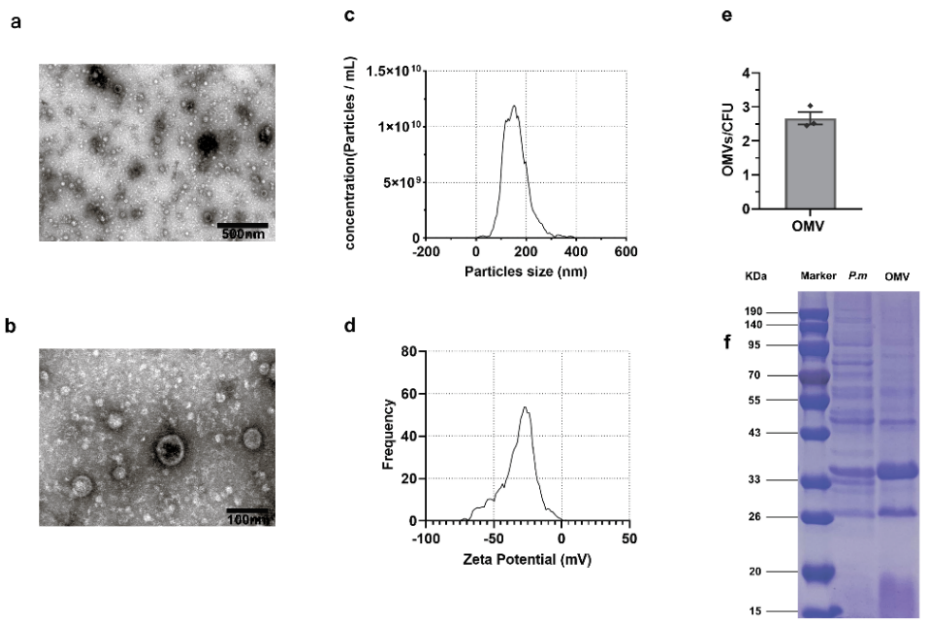

Figure. a,

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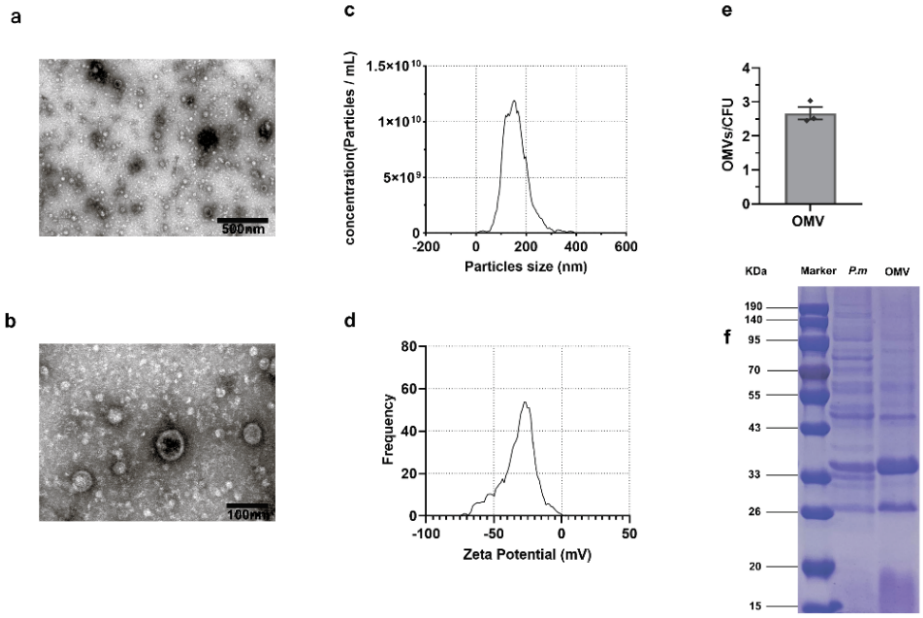

Figure. b). Our findings support *P. multocida* OMVs as promising vaccine candidates, capable of effectively stimulating humoral immune responses against *P. multocida* infection.

Table 1. List the important *P. multocida* OMV proteins identified in order of decreasing PSMs

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Figure. 1 Physical characteristics of OMVs derived from *P. multocida*

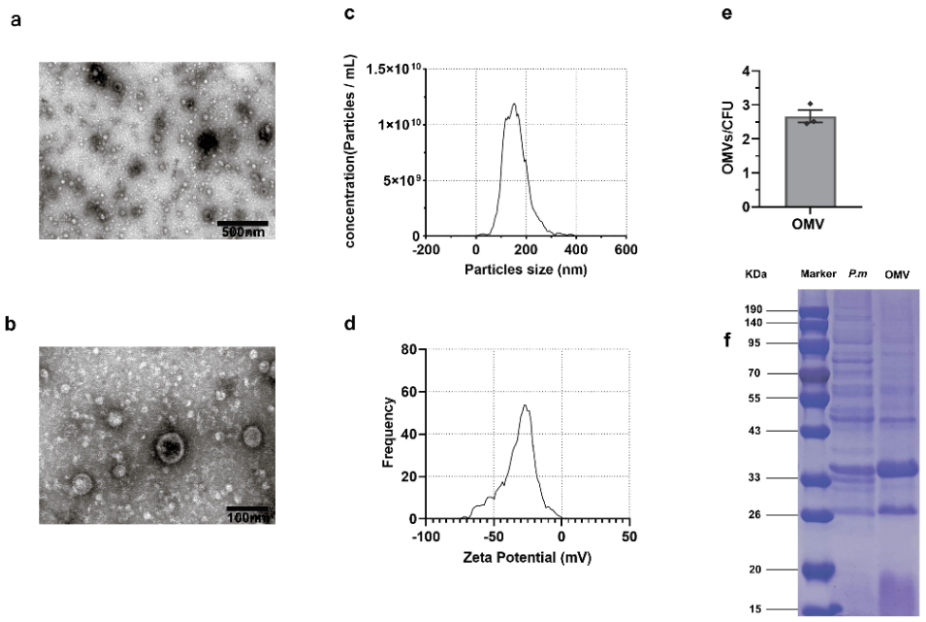
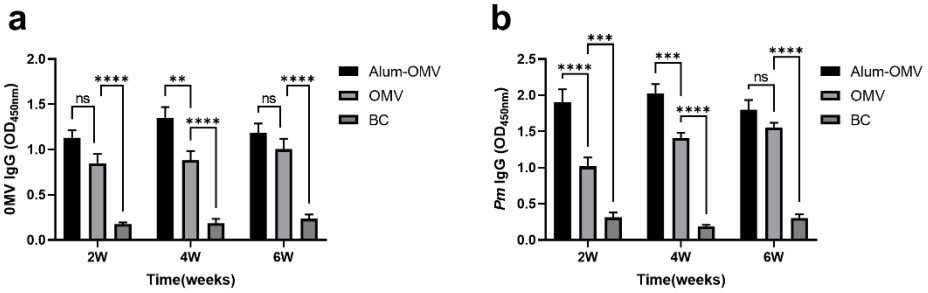


Figure. 2 OMV-specific (a) and *P. multocida*-specific (b) IgG produced by Alum-OMV and OMV immunized rabbits



CONCLUSIONS

In summary, our study provides a molecular basis for considering *P. multocida* OMVs as promising subunit vaccine antigen candidates and demonstrates their effective stimulation of immunity *in vivo*, thus promoting the development of a novel vaccine against *P. multocida* infection.

ACKNOWLEDGEMENTS

This work was supported by "Pioneer" and "Leading Goose" R&D Program of Zhejiang (Grant No. 2023C02047), National Natural Science Foundation of China (Grant No. 32002323, and No. 32102714), China Agriculture Research System of MOF and MARA (Grant No. CARS-43-C-2), and the Key Research and Development Program of Zhejiang Province (No. 2021C02007).

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A VEGETABLE OIL ADJUVANT ENHANCED THE IMMUNE RESPONSE INDUCED BY *BORDETELLA BRONCHISEPTICA* VACCINE IN RABBITS

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ABSTRACT

The rabbit industry in China is facing a significant threat from infectious respiratory diseases caused by Bordetella bronchiseptica (Bb). Unfortunately, the situation is exacerbated by the absence of any licensed Bb vaccines against this particular pathogen.The current study aimed to assess the potential of an inactivated Bb antigen, formulated with a vegetable oil adjuvant containing soybean oil (SO) and tea saponins (TS), as a vaccine against Bb infection in rabbits. Since mineral oil (ISA 206) is a common adjuvant used in animal vaccines, it was used as a control adjuvant in this study. To compare the adjuvant effects of SO containing TS and mineral oil on Bb vaccine: Six-week-old male New Zealand rabbits were s.c. injected twice (two weeks interval) with 1 mL of Bb antigen alone, or SO+TS-Bb vaccine or mineral oil-Bb vaccine. Blood samples were collected at different times before or after second immunization. Elisa method was adopted to detect IgG level in the serum, agglutination test was used to detect IgG titer. Our results showed that IgG level and IgG titer in mineral oil-emulsified or in the SO-TS emulsified Bb antigen have significant differences ($P< 0.05$) compared to Bb antigen alone. Additionally, IgG level and IgG titer in SO+TS-emulsified Bb antigen is much higher than that in mineral oil-emulsified Bb antigen. Moreover, SO+TS-Bb vaccine effectively protected rabbits from Bb infection. Overall, the presented findings suggest that the SO+TS-Bb vaccine effectively improves the immune response and confers robust protection against Bb infection in rabbits. Therefore, the SO+TS-Bb vaccine holds great promise as a potential vaccine candidate for the prevention of Bb infection.

Key words: *Bordetella bronchiseptica*, Tea saponins, Vegetable oil adjuvant, Vaccine.

INTRODUCTION

Bordetella bronchiseptica (Bb) is seriously endangering the development of the rabbit industry in China. Unfortunately, vaccines against Bb for use in rabbits have still not yet available, so it is urgent to develop an effective vaccine to control Bb infections and to prevent the potential animal-human transmission events. To date, Bb vaccine research mainly focuses on sub-unit vaccines or inactivated vaccines in which mineral oil based adjuvant is common additive ingredients to enhance vaccine efficacy (Xiao *et al.,* 2016; Zhang *et al.,* 2019). While, side effects, such as abscesses, cysts or necrosis, after the injection of mineral oil greatly limit its wide application. In addition, due to the poor metabolism of mineral oil in the animal body, it may remain in the meat that is intended for human consumption (Chen *et al.,* 2016). Since rabbits are farmed for not only meat but also fur, which is an important economic trait, selecting/developing adjuvants for rabbit vaccines should be performed in a careful manner.

Tea saponins (TS) as one of its biological active compound possess a range of pharmacological activities including anti-tumor, anti-oxidant and anti-inflammatory effects. Recently, saponins isolated from Tea have been found to be an immune-stimulating agent in animal vaccines(Chi *et al.,* 2017). Compared to mineral oils, vegetable oils are edible and much safer. Our previous study showed that vegetable oils soybean oil (SO) containing ginseng saponins had adjuvant effect on foot-and-mouth disease (FMD) vaccines (Cui *et al.,* 2019; Cui *et al.,* 2020). Therefore, we hypothesized that a potent adjuvant effect on the Bb

vaccine may be obtained when a vegetable oil is supplemented with TS. In the present study, soybean oil (SO) containing TS was evaluated for antigen effectiveness in Bb vaccination in rabbit.

MATERIALS AND METHODS

Animals and experimental design

Six-week-old New Zealand rabbits (Animal Center Co., Ltd., Zhejiang, China) were used for all experiments.The animals were acclimatized for one week prior to use and kept in cages at a controlled temperature (20 ± 5°C) and humidity (50% ± 10%) on a 12 h light–dark cycle and had free access to water. All animal experiments complied with the guidelines of the Animal Welfare and Use Committee of Zhejiang Academy of Agricultural Sciences (ZAAS, Hangzhou, China).

To investigate the combined adjuvant effects of SO and TS on the Bb vaccine. Rabbits (n = 9/group) were s.c. injected twice with 1 mL of inactivated Bb antigen (0.9×10^{10} CFU/mL)(Antigen group), or Bb antigen adjuvanted with SO+TS or mineral oil at a 2-week interval. Two mL blood was drawn for serum collection. Serum samples were collected 1 week before, booster day, and 1, 2, 3 weeks after the booster to measure Bb-specific IgG and IgG titer. Then, the rabbits (12-week-old) were challenged by ear vein injection with live Bb (2.0×10^{10} CFU/rabbit), the animal survival rate was monitored over the next 7 days.

Analysis of Bb-Specific Antibodis

Briefly, the whole inactivated Bb protein fraction was added to a 96-well microtiter plate (2 μg/mL, 100 μL/well) and incubated at 4℃overnight. Then, the plates were washed 3-5 times with phosphate-buffered saline containing 0.05% Tween-20 (PBST), and the wells were blocked with 5% skim milk and incubated at 37℃ for 2 h. After washing, 100 μL of the serum sample (1:1000) was added to each well and incubated at 37℃ for 1 h. Then, 100 μL of goat anti-rabbit IgG (1:10,000) (Abcam, Cambridge, UK) was added and incubated at 37℃ for 1 h. After washing 3–5 times, tetramethyl-benzidine (TMB) reagent (100 μL/well) was added to the plates and incubated at 37℃ for 10-15 min in the dark. The reaction was terminated by adding 50 μL/well 2 M H_2SO_4, and the absorbance was measured by a microplate reader at 450 nm (Thermo-Multiskan FC, Shanghai, China). For IgG titer, when adding serum to be tested, 1:20 dilution of serum was added to the first column of each plate, then diluted with PBS containing 5% skimmed milk, incubated at 37℃ for 1 h, and other steps were same with IgG analysis.

Statistical Analysis

GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA) was used for data analysis. Multiple comparisons were conducted by two-way ANOVA, followed by Tukey's multiple comparisons test, or by one-way ANOVA, followed by Tukey's multiple comparisons test. The results are expressed as the mean ± SE, and $p < 0.05$ was considered statistically significant.The log-rank test was performed for data of survival.

RESULTS AND DISCUSSION

Effect of SO+TS adjuvant on the humoral immune response of Bb vaccine in rabbits

The humoral immune response of IgG can provide protection against Bb infection (Cui *et al.,* 2022). Our results showed that IgG level and IgG titer in the two adjuvant groups have significant differences compared to the Bb antigen group ($P< 0.05$) (**Figure1 and Figure2**). For the IgG level, one week before booster immunization, there was no antibody was induced among each group(-1 week). On the booster immunization day (0 week), there was no significant difference between the mineral oil adjuvant group and the Antigen group, while the higher antibody and IgG titer were found in the vegetable oil SO+TS adjuvant group ($P< 0.05$). One week after the second immunization, antibody in the two adjuvant groups were

significantly higher than that in Antigen group ($P< 0.05$), no significant difference between the two adjuvant groups (1 week). Two and three weeks after the second immunization, IgG and IgG titer induced by the addition of SO+TS adjuvant were the highest in the three groups ($P< 0.05$), the mineral oil adjuvant group with higher antibody level than in Antigen group ($P< 0.05$). In our study, significantly higher Bb-specific antibody and IgG titer were detected in both adjuvant groups, while highest antibody level was found in SO+TS adjuvant group, which suggests that SO+TS exhibited adjuvant activity and enhanced the Bb vaccine humoral immune response in rabbits.

Figure 1: Serum Bb-specific IgG

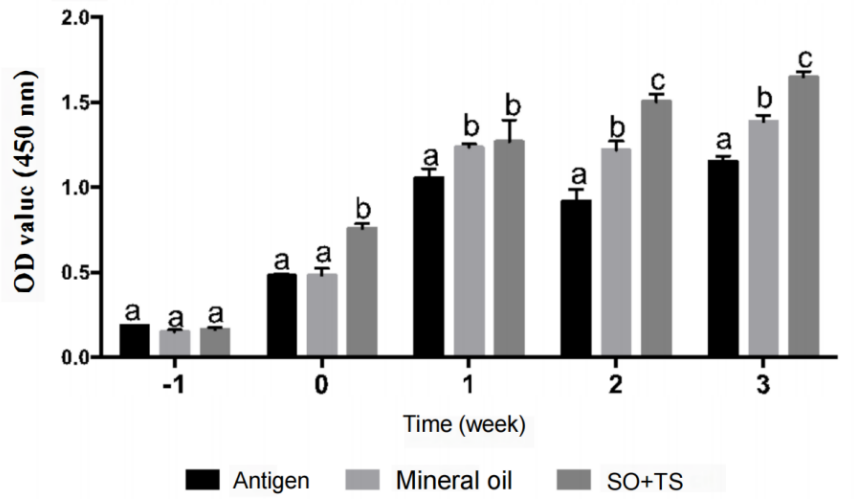
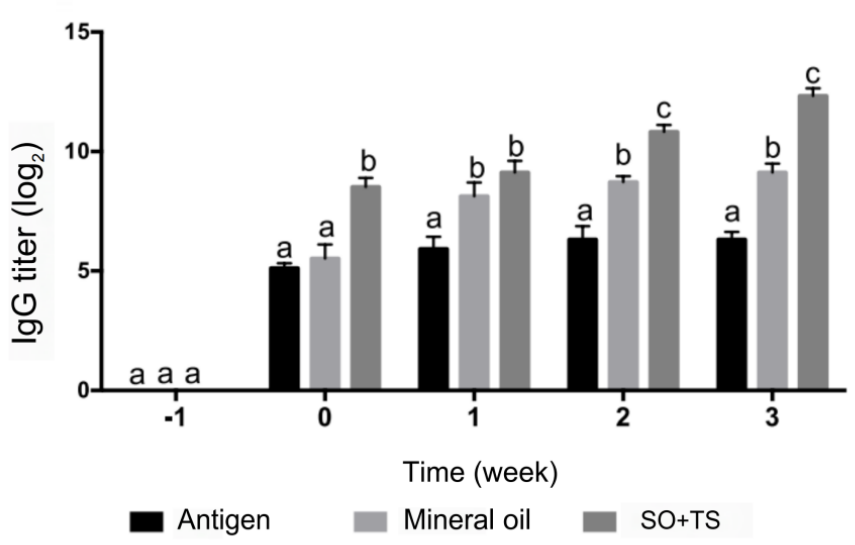


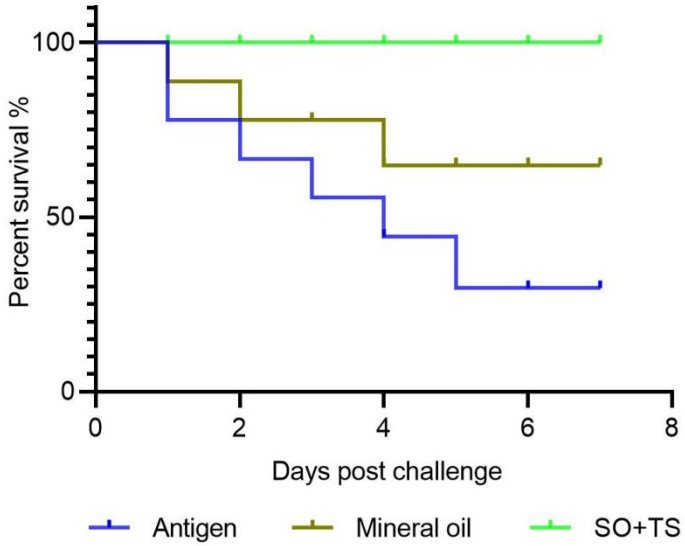
Figure 2: Serum Bb-specific IgG titer



Effect of SO+TS-Bb vaccine in protecting rabbits from Bb challenge

A suitable vaccine should effectively protect animals from pathogen infection (Cui *et al.,* 2022). In this study, we evaluated the protect effect of Bb vaccine that combined with different adjuvants, the rabbit survival rate was observed 7 days post-challenge. As presented in **Figure3**, rabbits immunized with the mineral oil-Bb vaccine were better protected (64.8%survival) than those in Bb antigen group (29.6% survival), while the best survival rate was observed in the SO+TS-Bb vaccine group (100% survival) after challenge.

Figure 3: Survival rate



CONCLUSIONS

In summary, the Bb vaccine adjuvanted with SO+TS effectively induced immune responses against Bb infection in rabbits. Thus the vegetable-derived SO+TS-adjuvanted Bb vaccine should be considered a promising candidate vaccine for preventing Bb infection.

ACKNOWLEDGEMENTS

The project was supported by the National Natural Science Foundation of China (grant number 32102714), the China Agriculture Research System of MOF and MARA (No. CARS-43-C-2), and“Pioneer” and “Leading Goose” R&D Program of Zhejiang (No. 2023C02047).

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PANGENOME AND PHILOGENY IN *STAPHYLOCOCCUS AUREUS* STRAINS FROM RABBITS

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ABSTRACT

The widespread presence of *Staphylococcus aureus* poses a substantial global challenge, resulting in significant economic losses and health issues for rabbit producers worldwide. This study investigates the clonal diversity and genomic characteristics of *S. aureus* strains from Spanish rabbits, comparing them with international isolates, emphasizing specific lineages' prevalence. Results highlight diverse clonal complexes (CC) in each country, showcasing the genetic variability of *S. aureus*. Pangenomic analysis reveals a rich genomic landscape, featuring 2,158 core genes and 4,058 total genes, with exclusive genes in each country indicating regional genomic variations. A chronological phylogenetic analysis uncovers the divergence between CC96 and CC121 around 1980, with subsequent differentiation events aligning with the intensification of rabbit farming. This study provides valuable insights into *S. aureus* evolution, emphasizing the ecological impact of livestock development on bacterial diversification within specific niches over time. Understanding these dynamics is crucial for comprehending the bacterium's adaptive strategies and potential clinical implications in diverse populations.

Key words: Phylogeny, Pangenome, *Staphylococcus aureus*, Clonal Complex, Rabbit.

INTRODUCTION

Rabbit farming has evolved significantly since the 1950s, adopting an industrial approach to obtaining meat, fur, research and companionship, among others (González Redondo & Caravaca Rodríguez, 2007). However, a widespread challenge emerges with the presence of *Staphylococcus aureus*, which mainly causes skin infections, respiratory disorders, and systemic diseases, posing a considerable problem for producers worldwide (Barthe *et al*., 2009). Despite preventive measures, *S. aureus* infections persist, requiring culling and disinfection. Further studies are needed to understand the evolution of these recent epidemic strains (Haag *et al*., 2019). The aim of the study is to investigate the clonal diversity and genomic characteristics of *S. aureus* strains from Spanish rabbits, comparing them with international and pet rabbits isolates, with emphasis on the prevalence of specific lineages and possible differences at the genomic level. Additionally, it examines exclusive genes to understand adaptive strategies and their functional roles in microbial diversity. Finally, it is intended to elucidate the temporal evolution of *S. aureus*, particularly between Clonal Complex 96 (CC96) and CC121.

MATERIALS AND METHODS

Sample collection

In this study, a comprehensive analysis was conducted on a set of 260 *S. aureus* sequences isolated from rabbits. The dataset comprises 202 strains sourced from the CEU Cardenal Herrera university stock, with 6 strains from Portugal and 254 from Spain, reflecting farm rabbits. Additionally, the dataset encompasses 45 strains from Hungary (PRJEB37661), reflecting farm rabbits. In contrast, 10 from the United Kingdom (Holmes *et al*., 2016), 1 from the United States (SRR14933443), and 1 from Germany (SRR10426515) are associated with pet rabbits.

Bioinformatic Analyses

Preprocessing was applied to raw sequences using FastP v0.23.2 (Chen *et al*., 2018) and FastQC. Assembly was performed using Shovill 0.7.1, followed by quality assessment using Quast. Genomic annotation was executed using Prokka 1.14.6 (Seemann, 2014). Pan-genome analysis employed Panaroo (Tonkin-Hill *et al*., 2020). The phylogenetic tree was constructed with IqTree (Nguyen *et al*., 2015) using the GTR+F+I+R2 substitution model with 2000 bootstrap replicates, and tree dating was incorporated using Bactdating (Didelot *et al*., 2018).

Statistical Analysis

Statistical analyses were conducted using ANOVA and Tukey's Test, complemented by the Monte Carlo Markov Chain approach for a more comprehensive assessment. All statistical analyses were performed using R version 4.3.3.(R Core Team, 2023).

RESULTS AND DISCUSSION

Global Variation in *S. aureus* Clonal Complexes: Insights from Six Nations

In Hungary, 16 samples revealed predominant ST5993 (prevalent in 35% of samples) and 15 samples of CC121 (distributed in 33% of samples). The US sample exhibited ST133. In Spain, 103 samples highlighted CC121 (dominant in 52.8% of samples) and CC96 (distributed in 30.25% of samples). For the UK, CC30 (predominant in 30% of samples) and ST425 (present in 20% of samples) were identified. German sample displayed ST130. In Portugal, CC96 (abundant in 66.7% of samples), CC398 and ST130 were observed. These findings underscore the genetic diversity of *S. aureus* across these nations, offering insights into clonal prevalence and potential clinical implications (Figure 1).

The clonal distribution analysis reveals significant differences between farm rabbits and pet rabbits. Farm rabbits predominantly exhibit CC121 and CC96, while pet rabbits primarily have strains associated with CC30, which is the Clonal Complex with the highest number of hosts. Additionally, only one strain from CC121 was detected in pet rabbits. (Hagg *et al*.,2019).

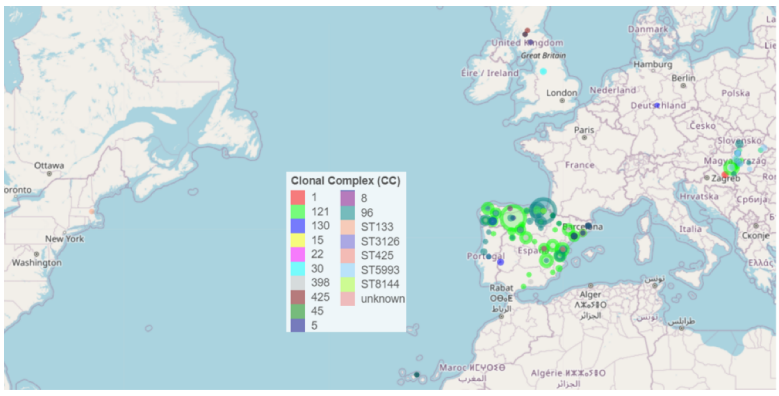

Figure 1. Geographic Distribution of Sequenced Strains of *S. aureus* in Rabbit Populations. Each colored circle represents a detected CC with the circle's radius proportional to the number of samples belonging to that

Genomic Diversity and Exclusive Gene Profiles of *Staphylococcus aureus* Across Global Populations: Insights from Pangenomic Analysis and Country-Specific Genes.

Examining the global pangenome of *S. aureus* across Hungary, USA, Spain, the UK, Germany, and Portugal revealed distinctive genomic characteristics. The pangenome comprises 2,158 core genes essential across all populations, with an additional 37 soft core genes that exhibit some variability. Moreover, 701 shell genes contribute to the flexible genome, showing intermediate prevalence, while 1,162 cloud genes represent the accessory genome, varying widely among populations, reflecting a greater variability than established by Fitzgerald *et al*. (2001). In total, the pangenome encompasses 4,058 genes. Insights from the pangenomic analysis highlight the genetic diversity and adaptability of *S. aureus*. Consideration of genetic variations is crucial for a comprehensive understanding of its evolutionary dynamics and potential clinical implications. Additionally, the identification of

exclusive genes in each country enriches our understanding of *S. aureus* genomic diversity (Table 1), providing valuable information for discerning adaptive strategies and factors influencing clinical outcomes or epidemiological patterns.

.

Table 1: Exclusive Genes in *S. aureus* Pangenome Across Countries

	USA	Germany	UK	Hungary	Spain	Portugal
Uncharacterized protein	16	6	120	8	244	2
Characterized protein	1	0	18	0	36	0
Total	17	6	138	8	280	2

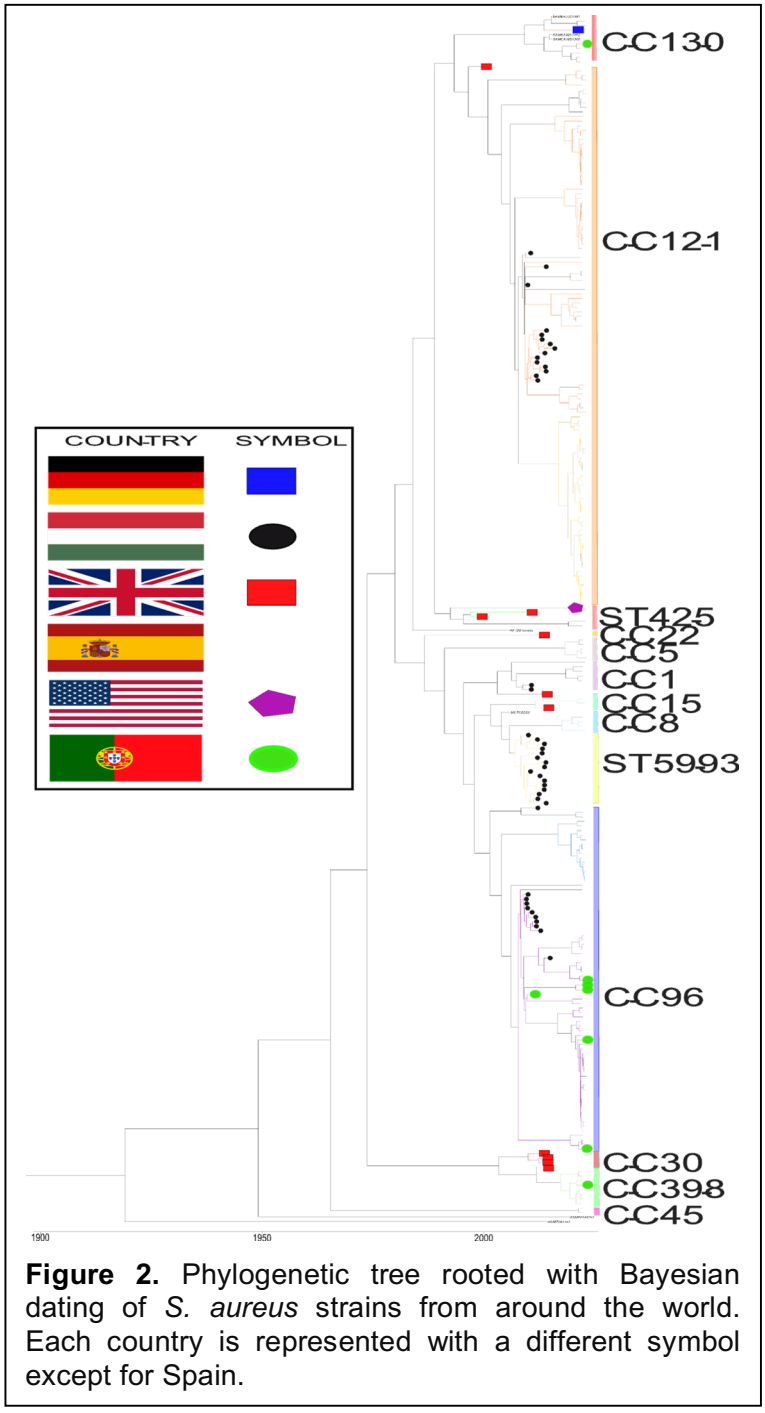

Figure 2. Phylogenetic tree rooted with Bayesian dating of *S. aureus* strains from around the world. Each country is represented with a different symbol except for Spain.

Evolving Landscape: Chronological Insights into *Staphylococcus aureus* Phylogeny and Ecological Shifts in Response to Rabbit Intensification.

A chronological exploration of *S. aureus* phylogeny unveils pivotal milestones and ecological transformations (Figure 2).

The divergence between CC96 and CC121 is estimated to have occurred around 1980. Significantly, a notable surge in *S. aureus* differentiation events in rabbits emerges from the 1980s onwards, coinciding with the intensification of rabbit farming and the adaptation of ST121 strains from human to rabbit (Viana et al., 2015). The divergence within CC96 took place between 1998 and 1999, followed by CC121 in the early 2000s, coinciding with the appearance of a very virulent staphylococcal outbreak in Spain. Closeness of branches within the same ST indicates horizontal gene transfer facilitated by mobile genetic elements, serving as a key mechanism for differentiation events (Khedkar *et al.*, 2022). This temporal phylogenetic analysis provides valuable insights into the evolutionary trajectory and adaptive responses of *S. aureus*, highlighting the profound ecological impact of livestock development on bacterial diversification within specific niches over time.

CONCLUSIONS

The global study on *S. aureus* reveals diverse clonal patterns, emphasizing complex epidemiology. Pangenomic analysis shows significant genetic diversity, with exclusive genes in each country indicating regional variations. A temporal phylogenetic analysis highlights divergence between CC96 and CC121 around 1980, with increased events in the 1990s linked to rabbit farming. In summary, the findings offer concise insights into global clonal diversity, pangenomic composition, and temporal evolution, providing a foundation for understanding adaptive strategies and ecological dynamics with clinical implications.

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MOBILE GENETIC ELEMENTS IN *STAPHYLOCOCCUS AUREUS* STRAINS FROM FARM RABBITS

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ABSTRACT

Staphylococcus aureus colonizes human and animal skin and mucous membranes. Recent observations indicate an increase in the frequency and severity of staphylococcal infections in rabbit farms, implicating a potential shift in strains that could be driven by mobile genetic elements (MGEs). This study has analyzed the MGE of 242 clinical isolates from rabbits. *Staphylococcus aureus* pathogenicity islands (SaPIs) were present in only a small percentage of isolates, suggesting that they could not be a determinant factor to staphylococcal virulence. Conversely, a high percentage of strains harbored bacteriophages (83.5%), with CC96 strains typically harboring more bacteriophages per strain than CC121 strains. The different types of phage integrases exhibited different prevalence, with Sa1 being the most frequent one, especially in CC121. In contrast, Sa6 and Sa7 integrases were frequent (32%), especially in CC121 and CC96, respectively. Although Sa2 was commonly found in CC96, it was also widespread across all the rest of the clonal complexes, excluding CC130. Plasmids were found in 71,9% of the isolates, with CC96 strains harboring more plasmids per strain than CC121 strains. Notably, plasmid replicon types (reps) displayed a diverse distribution among clonal complexes, with some reps being more prevalent across multiple complexes than others. In conclusion, plasmids and bacteriophages were frequently found in rabbit isolates and some results suggested a possible mobilization between them. Further study is needed to understand their role in staphylococcal pathogenicity.

Key words: *S. aureus*, bacteriophage, plasmid, SaPI, rabbit.

INTRODUCTION

Staphylococcus aureus, a pathogen known for its commensal and opportunistic nature, resides on the skin and mucous membranes of both humans and animals (Haag et al., 2019). Notably, in rabbits, it has demonstrated that a single nucleotide mutation facilitated the transmission of the ST121 strain from humans to rabbits (Viana et al., 2015). Analysis of accessory genomes revealed that strains from humans contained species-specific mobile genetic elements (MGEs) that were absent in rabbits' strains. This lineage represents one of the prevalent clones in rabbit farms, alongside CC96 (Vancraeynest et al., 2006). Over the past few years, however, an increase in the frequency and severity of staphylococcal infections has been observed in rabbit farms, with strains resistant to various antibiotics (Pérez-Fuentes et al., 2017). Mobile genetic elements are believed to play a significant role in this evolutionary process of *S. aureus*, potentially facilitating the emergence of strains that are more virulent or better adapted to their host environments. Consequently, the primary objective of this study is to identify and thoroughly examine the MGEs responsible for driving the evolution of different clones in rabbit farms.

MATERIALS AND METHODS

Sequencing and basic genetic characterization of isolates

Using an Illumina Hiseq XTen kit, 242 clinical isolates from farm rabbits were sequenced (2 x 150 bp paired end). The isolates were collected from 110 farms across 45 different Spanish provinces. Sequence assembly was performed by Unicycler (Wick et al., 2017) and typing by multilocus sequence typing (MLST).

Identification of mobile genetic elements (MGEs):
The study focused on the most relevant MGEs: plasmids, bacteriophages and SaPIs. The detection of plasmids in the sequences was carried out using PlasmidFinder (Carattoli et al., 2014). The number of plasmids was counted as the number of rep sequences detected. Integrase detection was used to identify bacteriophages and SaPIs in the genome, using the BLAST tool to explore similarities between the previously described sequences of the integrases and the complete genomes of the strains (Ingmer et al., 2019).

Statistical analysis:
The number of integrasas and plasmids were evaluated using a general linear model (Proc GLM of SAS, 2009) including CC as fixed effect. LSmeans were estimated and Tuckey correction was applied for the multiple comparisons.

RESULTS AND DISCUSSION

SaPIs and bacteriophages
Traditionally, it was believed that *S. aureus* strains from rabbits did not carry MGE (Viana et al., 2015). In this study, *S. aureus* pathogenicity islands (SaPIs), they were present in only 5% of the isolates (12/242). However, none of these strains belonged to either of the two predominant clonal complexes, CC121 and CC96, which constitute approximately 80% of isolates obtained from staphylococcal lesions in farms (Pérez-Fuentes et al., 2017). Consequently, SaPIs do not seem to play an important role in the pathogenesis of infections in rabbits. On the other hand, 83.5% of the strains contained bacteriophages, being more common to have two per genome (Figure 1). In contrast to CC121, all CC96 strains had bacteriophages, and on average, each CC96 strain harbored a higher number of these compared to CC121 (Figure 2). The CC had a significant effect on the number of bacteriophagues (P<0.001). There were significant differences in the number of integrases, with CC425 having more integrases than CC5 and CC45 (*p*-value < 0.05).

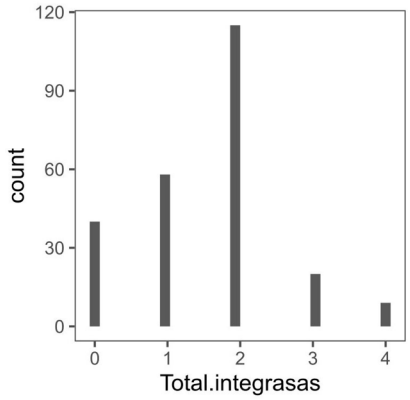

Figure 1: Nº of bacteriophages per strain

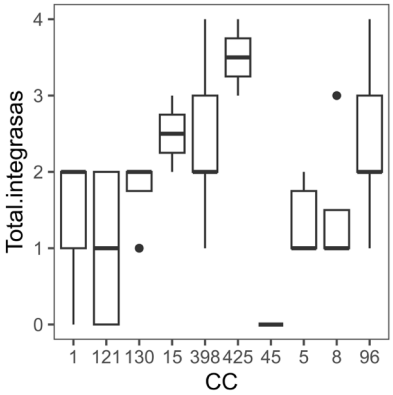

Figure 2: Nº of bacteriophages per CC

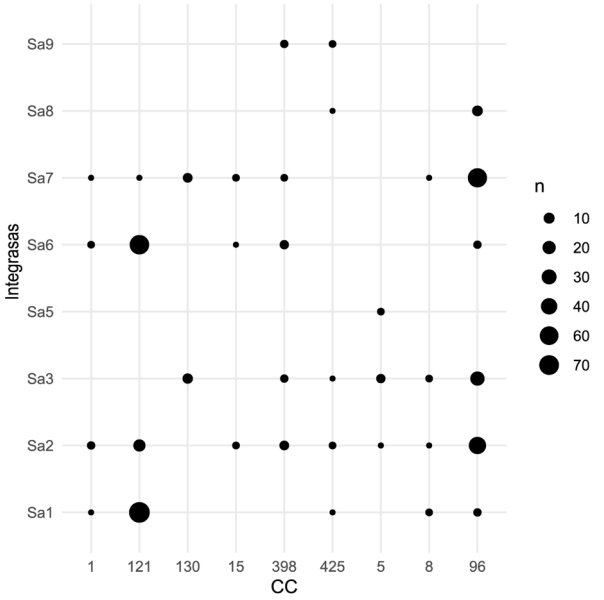

Figure 3: Type of bacteriophage integrases and counting per CC

Bacteriophage integrases can be classified into 12 types (Ingmer et al., 2019). In our study, the most frequent was Sa1 found in 36% of the isolates (87/242), while Sa2, Sa6 and Sa7 were found in the same proportion, 32% each (78/242). Integrases Sa4, Sa10, Sa11 and Sa12 were not found in any of the isolates. The distribution of the type of integrase according to the clonal complex was studied (Figure 3). Despite Sa1 being the most frequent integrase, it was found mainly in CC121, while in the rest of the clonal complexes it was infrequent or nonexistent. Similarly, Sa6 integrase was associated with CC121 and Sa7 with CC96. In contrast, Sa2 was frequent in CC96 but was also found in all the rest of the clonal complexes found in rabbits besides CC130. Comparing the two main clones in rabbits, CC121 and CC96, the most frequent integrases in each of them are different. Therefore, it would indicate that their bacteriophage integrase profile would be dependent on the clonal complex. However, some types of integrases were observed in several clonal complexes, which could indicate a potential mobilization between them.

Plasmids

Plasmids were present in 71.9% of the strains, with one plasmid per strain being more common (Figure 4). The result of the comparison of CC121 and CC96 is similar to what was observed in bacteriophages, as all CC96 strains had plasmids and, on average, each CC96 strain harbored a higher number of plasmids in its genome than CC121 strains (P<0.001) (Figure 5). In general, the CC had a significant effect on the number of plasmids (P<0.001). For example, CC5 presented a significantly higher number of plasmids than all the CCs, with the exception of CC1 and CC15 (P<0.001).

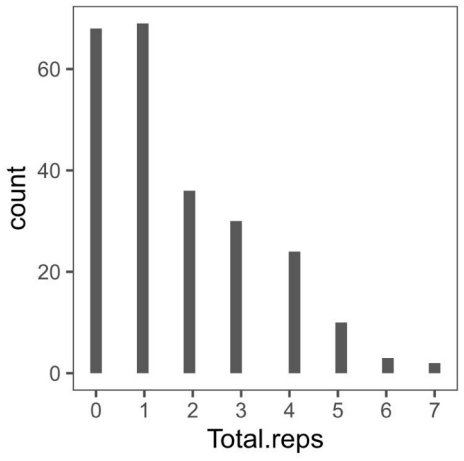

Figure 4: Nº of plasmids per strain

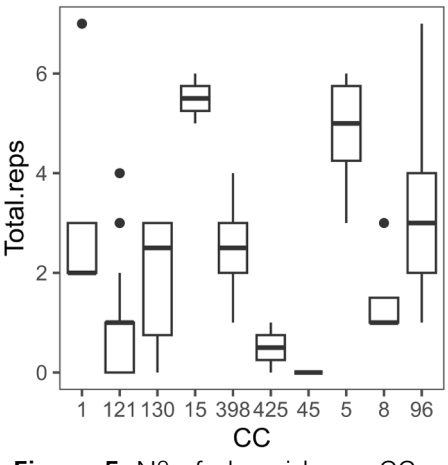

Figure 5: Nº of plasmids per CC

Plasmids can be classified into rep types (Carattoli et al., 2014). The most frequent was rep7c_1 found in 31% of the isolates (75/242). Other frequently found reps were rep7a_16, rep10_3 and rep22_1b with a 28,9% (70/242), 23,1% (56/242) and 21,5% (52/242) respectively. The distribution of rep sequences and its count was studied according to the

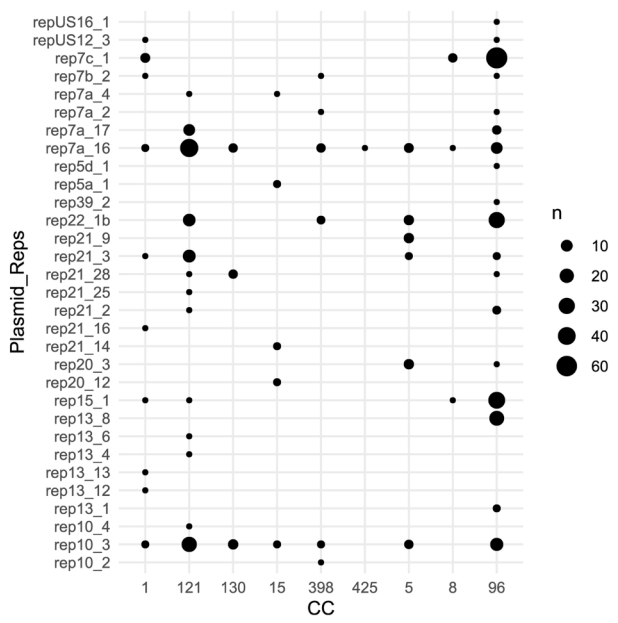

Figure 6: Type of plasmid reps and counting per CC

clonal complex (Figure 6). Unlike integrases, many of the reps found in CC121 were also found in CC96. However, the reps found in CC96 were much more diverse compared to any other CC. Of the 34 rep sequences found, 44.1 % (15/34) of them were found in more than one clonal complex. For example, rep10_3_ and rep7a_16 were found in 7 of the 10 clonal complexes and rep15_1, rep21_3 and rep22_1b were found in 4 of the 10 clonal complexes. Such distribution could indicate a mobilization of plasmids between different strains.

CONCLUSIONS

Plasmids and bacteriophages were frequently found in rabbit isolates and, according to their classification, some of their types were found in different clonal complexes, which could indicate a potential mobilization between them. Therefore, it is necessary to further study and characterize these MGEs and their genetic content to know the role they play in rabbit strains

ACKNOWLEDGEMENTS

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BACTERIOPHAGE COMBINATIONS SIGNIFICANTLY REDUCE *E. COLI* INFECTIONIN RABBITS

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ABSTRACT

Enteropathogenic *E. coli* (EPEC), as the main pathogen causing diarrhea in rabbits, is highly contagious and severely restricts the healthy development of rabbit industry. In this study, an atypical EPEC strain ZJER1 and five of its specific bacteriophages were isolated, named ZJRP1, ZJRP2, ZJRP3, ZJRP4 and ZJRP5 respectively. The anti-ZJER1 effects of ZJRP5 and ZJRP1-5 (equal ratio combination of bacteriophages ZJRP1, ZJRP2, ZJRP3, ZJRP4 and ZJRP5) were compared. The weight gain of rabbits in group ZJRP5 and ZJRP1-5 were significantly higher than that in challenge group ($P < 0.05$). Mental status and diarrhea rate of the rabbits were continuously observed and recorded for 5 days, and the results showed that the rabbits in the blank control group and ZJRP1-5 group had normal appetite and exercised freely; 3 rabbits in challenge group had diarrhea, and 1 rabbit died; 1 rabbit in the ZJRP5 group had diarrhea. The results of the ileal section showed that ZJER1 infection caused intestinal epithelial villi damage in challenge group, while it remained intact in ZJRP5 and ZJRP1-5 group. In summary, ZJRP5 and ZJRP1-5 isolated in this study can resist ZJER1 infection in rabbits, and the antibacterial effect of ZJRP1-5 is better than ZJRP5. Therefore, phage therapy has broad application prospects in the treatment of rabbit colibacillosis.

Key words: bacteriophage, combination, *E. coli* infection

INTRODUCTION

Colibacillosis, caused by pathogenic *Escherichia coli* and characterized by diarrhea or systematic infection, is a common bacteriosis and generates huge economic losses in the animal husbandry industry. Rabbits have suffered from colibacillosis caused by enteropathogenic *E. coli* (EPEC) since the beginning of the 1980s. Antibiotics and chemical drugs are the main treatment methods for colibacillosis, but with the rapid rise of multi-drug-resistant bacteria, researchers have to investigate alternative treatment methods for colibacillosis, and one promising solution is the use of bacteriophages (Chan. et al., 2013, Lin D.M., et al., 2017, Zhao J., et al., 2017.). Bacteriophages are bacterial viruses, with the ability to lyse and destroy bacterial cells (Gordillo A. et al., 2019). There are a number of advantages to using phages therapeutically, such as strong bactericidal effect, high target specificity, low toxicity, minimal disruption of the normal microflora (Golkar Z. et al., 2014.). Several studies demonstrated that a single orally administrated bacteriophage or bacteriophage cocktail reduced the levels of pathogenic bacteria in the gastrointestinal tract of sheep or mice experimentally infected with *E. coli* and decreased the rate of diarrhea and the mortality of poultry in farms (Al-Mamun A. et al., 2013., Wang L.L., et al. 2017, Yilmaz E.G., et al., 2024).

We recently isolated an EPEC strain and five of its specific bacteriophages. We compared the efficacy of bacteriophage and bacteriophage combinations on reducing *E.coli* infection in rabbits.

MATERIALS AND METHODS

Isolation and morphological characterization of bacteriophages

In this study, one *E. coli* strain was isolated and identified from the liver of a dead rabbit which came from a diarrhoea-prevalent commercial rabbit farm in Zhejiang Province of

China, which was named as ZJER1. Manure samples of rabbits for isolation of phages were collected from ten rabbit farms in Zhejiang Province. Five bacteriophages, named as ZJRP1, ZJRP2, ZJRP3, ZJRP4 and ZJRP5, were later isolated by the host bacteria ZJER1 from the fecal samples. Morphological characteristics of the phages were observed by transmission electron microscope (TEM). Bacteriophages were deposited on copper grids with carbon-coated films and stained with 2% uranyl acetate (pH 4.0). Phages were observed using TEM between 12,000× and 80,000× magnification (Thermo Fisher Scientific, MA, USA).

Experimental trials

The five bacteriophages were used either singularly (ZJRP5) or in a pool (ZJRP1-5). 35-day-old New Zealand rabbits were divided into 4 groups, namely group A (blank control group), B (challenge group), C (oral administration of ZJRP5) and D (oral administration of ZJRP1-5). Rabbits in group B, C and D were orally challenged with 50 mL of *E. coli* ZJER1 (4×10^8CFU/mL) and in the meantime administrated of ZJRP5 or ZJRP1-5 by gavage every 8 h for a total of 3 times. The colonies of E. coli were washed with phosphate-buffered saline (PBS). The washed pellets of bacteria were suspended in PBS to match McFarland tube no. 0.5 to achieve a concentration of approximately 2×10^9 PFU/ml. One before infection, 3 days and 5 days after the infection, all rabbits were weighed. Mental status and diarrhea rate of the experiment rabbits were continuously observed and recorded for 5 days. The rabbits were sacrificed 5 days after oral infected with *E. coli* in order to investigate whether ZJER1 could adhere to the villi of the small intestine. Formalin-fixed ileum specimens were embedded and cut into sections. Sections were stained with hematoxylin and eosin and viewed under the microscope (Thermo Fisher Scientific, MA, USA).

Statistical Analysis

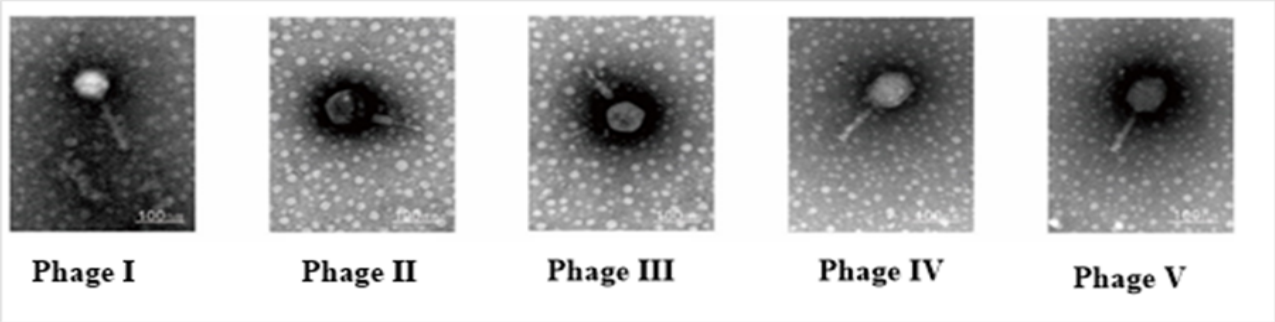
Statistical differences between groups were determined using IBM SPASS statistics 19.0 (SPASS Software).

RESULTS AND DISCUSSION

Isolation and Morphological characterization phages

One pathogenic *E. coli* strain was isolated and identified, which was named as ZJER1. Five bacteriophages, named as ZJRP1, ZJRP2, ZJRP3, ZJRP4 and ZJRP5, were isolated from the host bacteria ZJER1. The phages then observed under TEM, the bacteriophages were composed of hexagonal head, contractible tail sheath and tail tube. All the five strains belonged to *Myococcyphage* family.

Figure 1: Transmission electron microscope image of bacteriophage ZRP1-5



Effects of phage against *E. coli* in rabbits

It can be seen from Table 1 that the weight of rabbits of the Group B decreases and the weight gain is negative ($P<0.05$) from 0 to 3 days; The weight gain of the Groups C and D is not significant compared with that of the Group A; However, the weight gain of the Groups C and D is significantly different from that of the Group A from 0 to 5 days ($P<0.05$); There was no significant difference in weight gain between the Groups D and C. Within 5 days of the

experiment, the rabbits in the Group A had normal appetite and exercised freely, with no clinical symptoms such as diarrhea; A total of 3 rabbits in the Group B had diarrhea, and 1 rabbit died; 1 rabbit in the Group C had diarrhea, while the rest of the rabbits had normal appetite and exercised freely, with no clinical symptoms such as the diarrhea; And the rabbits in Group D had normal appetite and exercised freely, with no clinical symptoms such as the diarrhea. Related data are shown in Table 2. The results of the ileal section showed that ZJE1 infection caused intestinal epithelial villi damage in group B, while it remained intact in group C and D (Figures 2).

Table 1 Changes of weight gain in rabbits treated with phage after ZR1 infection

		Group A	Group B	Group C	Group D
0-3 days	weight-gain（g）	73±3[a]	-3.8±3[b]	54±5	65±5
0-5 days	weight-gain（g）	95±13[a]	14.2±6[b]	118±20[a]	142±18[b]

Means with different letters on the same row differ significantly.

Table 2 Effects of phage against *E. coli* in rabbits

Group	Total	Dose（CFU/mL）	Number of diarrhea	Deaths of rabbit
A	6	4×10^8	0	0
B	6	4×10^8	3	1
C	6	4×10^8	1	0
D	6	4×10^8	0	0

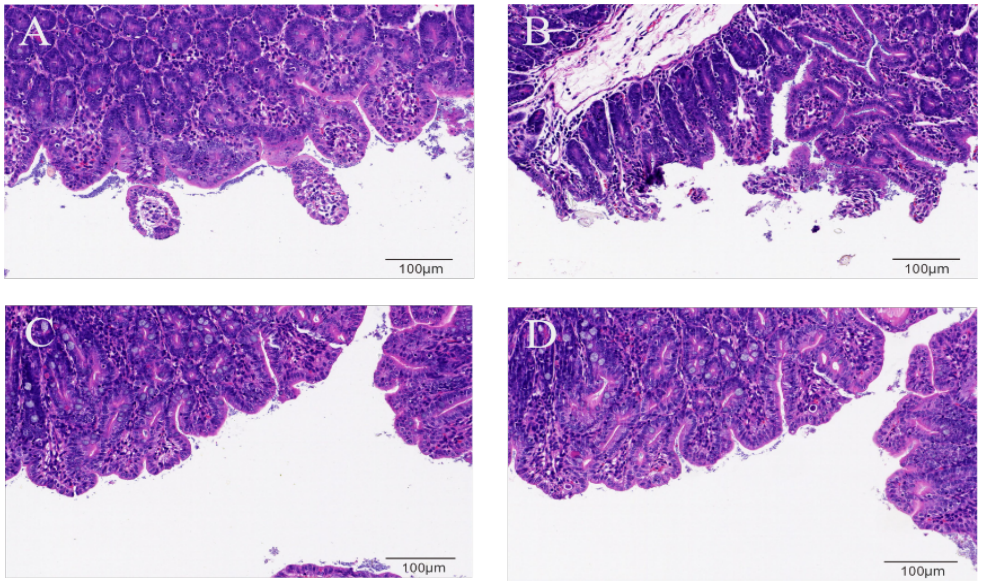


Fig. 2 Tissue section of ileum of infected animal
A: blank control group, B: challenge group, C: oral administration of ZJRP5, D: oral administration of ZJRP1-5.

CONCLUSIONS

In summary, ZJRP5 and ZJRP1-5 isolated in this study can resist *Escherichia coli* infection in rabbits, and the antibacterial effect of ZJRP1-5 is better than that of ZJRP5. Therefore, phage therapy has broad application prospects in the treatment of rabbit colibacillosis.

ACKNOWLEDGEMENTS

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A CASE REPORT OF TYZZER'S DISEASE IN COMMERCIAL RABBIT DOES

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ABSTRACT

Clostridium piliforme (Cp), a pleomorphic spore-forming obligate intracellular bacterium, is the causative agent of Tyzzer's disease. The pathology affects multiple species, including rabbits, in which the disease is sporadic in recently weaned animals.
The present work describes a case report of Tyzzer's disease in a commercial rabbit farm presenting does mortality and late gestation abortions. Animals underwent necropsy and further microbiological, parasitological and histopathological analysis were performed. Anatomopathological lesions were suggestive of Tyzzer's disease and the presence of Cp was confirmed by PCR. Parasitological analysis resulted negative and bacteriological examination of intestines revealed high load of *Escherichia coli* and *Clostridium perfringens* that were considered secondary pathogens. *Chlamydophila* sp and *Toxoplasma gondii* infections were excluded by PCR as causative agents of abortions. To the authors' knowledge, this is one of the first reports of Tyzzer's disease occurred only in the reproductive sector of a rabbit farm. The constant reduction of the antimicrobial use observed in recent years, could make some neglected diseases emerge again. For this reason, it is important to be able to suspect such uncommon pathologies based on the macroscopic findings.

Key words: *Clostridium piliforme*, Tyzzer's disease, rabbit does

INTRODUCTION

Ernst Tyzzer first described the disease in 1917 in a case of fatal diarrhea in a group of Japanese waltzing mice. He observed miliary necrotic foci of the liver and supposed the causative agent might be the pleomorphic, gram-negative, motile, spore-forming bacillus he saw in cells bordering the areas of necrosis (Tyzzer, 1917). After 16S RNA sequencing, the obligate intracellular bacterium was named *Clostridium piliforme* (Cp) (Duncan *et al.*, 1993). Ever since the disease was reported in multiple animal species (Percy, D. H., & Barthold, S. W., 2013).
To date Tyzzer's disease in commercial rabbits has been rarely reported and the epidemiology poorly investigated. The infection spreads via oro-faecal route and the clinical picture is characterized by anorexia, profuse watery diarrhoea and mortality, especially in recently weaned rabbits (Tyzzer, 1917; Swerczek, 1976).
The diagnosis can be presumed during necropsy in case of multiple tiny (1-2 mm diameter) necrotic foci on liver, together with oedema and fibrotic plaque formation in the ileum, caecum and proximal part of the colon mucosa (Allen *et al.*, 1965; Peeters *et al.*, 1985; Ganaway *et al.*, 1971).
Presumptive diagnosis needs to be confirmed by bacterial observation in histopathological lesions and/or by PCR. Cp cultivation is not routinely performed in veterinary microbiology laboratories because of the impossibility to grow the pathogen in standard media. Indeed, Cp can be isolated only in cell-cultures and embryonated eggs (Ganaway *et al.*, 1971).

To control the disease, an early diagnosis, the elimination of sick animals, prolonged antibiotic treatments of the remaining animals and a proper cleaning-and-disinfection programme are necessary (Peeters *et al.*, 1985).
The present report describes a case of Tyzzer's disease occurred in gestating does in a commercial rabbitry.

MATERIALS AND METHODS

Case history and clinical findings

The commercial rabbitry was in Treviso, North-eastern Italy, and it was organized in 4 barns: a house for 3000 commercial hybrid does with forced ventilation and 3 plein-air barns for the fattening period. Pregnant and young does were housed in wire cages with droppings pits underneath, and were fed ad libitum with a commercial feed. The water system of the house was connected to the municipal aqueduct. Rabbits were weaned at approximately five weeks of age.
Starting from 20th December 2022 does started dying (6.5% mortality) with profuse diarrhoea and the farmer reported multiple abortions 7-3 days before parturition. The clinical picture appeared in animals irrespective of the parity order. Mortality and abortions were registered daily by the farmer.

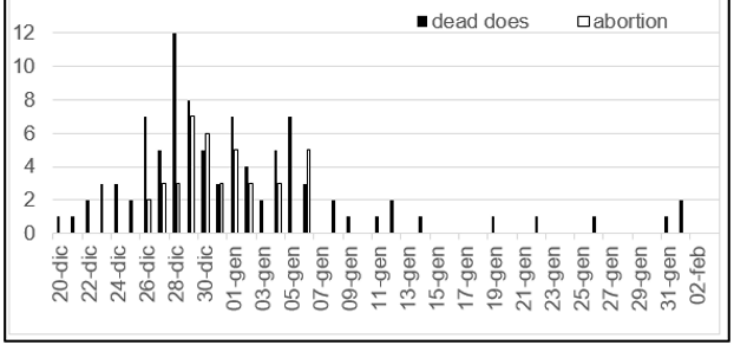
Post mortem examination

Three does (1-3) that died spontaneously underwent necropsy in the Microbiology and Veterinary Diagnostic Laboratory of Treviso (IZSVe) and pathology examination was performed according to the standard protocol. Pathological samples were collected in order to perform bacteriological, parasitological and histopathological examinations.

Microbiological and parasitological examinations

Intestinal contents (1-3) were aseptically collected and streaked on Eosin Methylene Blue (EMB) and Perfringens Agar Base (PAB) Petri dishes. Plates were incubated for 24-48 hours at 37°C in aerobic and anaerobic conditions, respectively. Bacteria species were identified by means of traditional microbiology techniques and by MALDI-TOF Mass Spectrometry (MALDI-TOF MS, Brucker, Germany). In addition, smears of intestinal contents were Gram-stained in order to evaluate the presence of *Clostridium* (*Cl.*) *spiroforme*. Samples of caecum walls (1-3) and livers (1-3) were tested by means of a PCR for the presence of *Cl. piliforme* (Feldman *et al.*, 2006).
Samples of spleen (n.3) and uterus (1 and 3) were tested for *Toxoplasma gondii* and *Chlamydophila* sp by means of PCR protocols (Ho *et al.* 1996; Ehricht *et al.* 2006).
Parasitological examination was performed individually through the microscopic observation of fecal smears and, in a representative pooled sample, by means of both McMaster egg counting technique and immunofluorescent essay for the detection of *Cryptosporidium sp* and *Giardia sp* (Garcia *et al.* 1987).

Figure 1. N° of dead does and abortions from the 20th December to February the 2nd in the farm



Histopathology

At necropsy, samples of caeca (1 and 3), kidney (1) and spleen (3) were immediately fixed in 10% formalin neutral-buffered formalin for 48 h and routinely processed for histological investigations. Sections were stained with haematoxylin and eosin (HE).

RESULTS

Case history and clinical findings

Mortality and abortion trends are represented in figure 1.

Post-mortem examination

External examination of the animals revealed mild fecal staining of the perianal area.
Does 1 and 2 had serous effusion in peritoneal cavity (approximately 5 mL) with multiple tiny necrotic foci on the liver. Severe oedema of the caecum and mesentery was a consistent feature in all animals. Doe 1 presented diffused areas of yellow-greyish necrosis in the caecal mucosa, while the others had fluid and haemorrhagic intestinal content.
Does 1 and 3 showed sub-capsular haemorrhagic petechiae on the kidneys' surface and splenic enlargement, respectively. The uteruses had necrotic placenta fragments and blood clots in the lumen.

Microbiological and parasitological examination

The results of the bacteriological and parasitological examinations are resumed in table 1.
All caecal walls (1-3) and the liver of doe 2 resulted positive for *Cp*.
The research of intestinal parasites, *Toxoplasma gondii* and *Chlamydophila* sp resulted negative.

Table 1: Results of the diagnostic examinations

	Doe 1	**Doe 2**	**Doe 3**
Bacteriological examination	*E. coli* +++ *Cl. perfringens* ++	*Citrobacter sediakii* ++ *Cl. perfringens* ++	*E. coli* +++ *Cl. perfringens* +++
Parasitological examination	Negative	Negative	Negative
Cl. spiroforme	Negative	Negative	Negative
Cl. piliforme	Positive (caecal wall)	Positive (caecal wall and liver)	Positive (caecal wall)
Cryptosporidium sp & *Giardia* sp	Negative		
Toxoplasma gondii	Negative	//	Negative
Chlamydophila sp	Negative	//	Negative

Note. The number of "+" refers to the bacterial load on the plates

Histopathology

In subjects 1 and 3 a severe, multifocal to diffuse, fibrino-necrotic heterophilic typhilitis with areas of erosion of the mucosa was observed. The lamina propria and the submucosa showed diffuse oedema with moderate scattered infiltrates of mixed leukocytes. Moreover, in the subject 3, foci of necrosis of the muscularis mucosae with mild heterophilic infiltrates were observed. Necrotic material admixed with heterophils and cellular debris were present in the lumen.
No significant histopathological lesions were observed in the kidney (1) and spleen (3).

DISCUSSION

Tyzzer's disease is a sporadic finding that has been reported primarily in recently weaned rabbits. The present case-report describes one of the first outbreaks occurred in does, to the best knowledge of the authors.
Although the clinical signs and anatomopathological lesions align with those documented in literature, it is crucial to note that these findings, if considered individually, may potentially result in a misleading diagnosis. Indeed, disseminated hepatic necrotic foci were present in 2 out of 3 does, might also be linked to other bacterial diseases (eg. *Salmonella* sp, *Staphylococcus aureus, Pasteurella multocida*). In addition, the severe oedema of the caecal wall be the consequence of the action of the binary-toxin produced by *C. spiroforme*, (Allen *et al.*, 1965; Cutlip *et al.*, 1971).
Even though bacteriological examination of intestines revealed high load of *E. coli* and *C. perfringens*, histopathology did not show any Gram-negative bacteria attached to the luminal border of enterocytes as described for some strains of enteropathogenic *E. coli* in rabbits,

causing caecal oedema and epithelial desquamation (Cantey and Blake, 1977; Peeters *et al.*,1984). Therefore, such bacteria proliferation was considered a secondary reaction.
The enteric syndrome caused by Cp was the leading cause of the abortions (indirectly), because nor the lesions nor microbiological tests revealed the presence of any causative agent of reproductive disease.
Since there is not availability of antibiotics registered for the disease, the therapy was set up with oxytetracycline as described by Peeters *et al.*, 1985 and it started on January 9th 2023 in drinking water (500mg/g; 5 days) followed by 5 cycles of antibiotic treatment in feed (1200ppm/q). However, the treatment started when mortality and abortions naturally decreased, so it is not possible to evaluate the efficacy of oxytetracycline in reducing clinical features.
Remarkably, in the subsequent weeks, certain does exhibited diffuse subcutaneous and abdominal oedema without enteric symptoms. Despite Cp was not detected in these animals, the peculiar manifestation in a farm with a history of Tyzzer's disease hints at a possible association with this occurrence.
The constant reduction of antimicrobial use promoted in EU in recent years may cause the appearance of neglected and rare diseases such as Tyzzer's. For this reason, it is important to recognise the macroscopic findings and confirm the diagnosis as soon as possible by means of histopathological examinations and specific biomolecular analysis.

CONCLUSIONS

Tyzzer's disease may affect does and the diagnosis of in rabbits is still rather difficult and might be misled if few animals are submitted for necropsy because the lesions are inconstant and might be attributed to different pathogens. Veterinarians and pathologists should be aware about the possibility to encounter the disease and the recognition of anatomopathological lesions followed by appropriate diagnostic examinations (PCR and histopathology) are necessary to diagnose Tyzzer's disease.

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EFFECT OF *PILIOSTIGMA THONNINGII* LEAVES ON YOUNG RABBIT DIGESTIVE HEALTH AND GROWING PERFORMANCE

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ABSTRACT

The aim of this study was to evaluate the effect of *Piliostigma thonningii* leaves on the digestive health of growing rabbits. 60 rabbits weaned at 30 days of age were used. They were divided into 4 groups of 15 rabbits. All animals were feed the same food from 30d to 84d. However, the rabbits from group FL, ML and DL received respectively a supplement of fresh leaves, macerated leaves and decocted leaves. The last group (C) was the control. The animals were weighed weekly. Droppings were collected and analyzed to assess the effect of the treatment on coccidia. Blood was collected at 58 days to observe the hematological and biochemical parameters of the treated rabbits. Results showed that growth was the same in all groups. Final weight was 1800g. Weight gain average was 18 g/d. The number of coccidia oocysts decreased significantly in the treated group ($P<0.01$). The average number of oocysts was 3811 in the group treated with the leaves. This number was 5.8 higher in group C. Hematological and biochemical parameters of the treated rabbits showed a significant improvement compared to the control. The FL and ML groups showed better results than the DL group. Treated groups obtained a reduction in the total cholesterol, HDL cholesterol and Triglyceride levels of the treated rabbits between 7 and 30 g/L, 2 and 8 g/L, 1.11 and 1.44 g/L respectively ($P<0,01$). *P. thonningii* leaves can be used as an antiparasitic in rabbit breeding to improve the digestive health of growing rabbits.

Keywords: Rabbit, coccidian, blood parameters, *Piliostigma thonningii*

INTRODUCTION

Digestive disorders are common in growing rabbits. These disorders lead to a drop in productivity and economic losses. They are mainly food related. They appear in rabbits during the post-weaning period.

Young animals are therefore exposed to gastrointestinal conditions which cause diarrhea and mortality. The prevention of these digestive disorders relies on the use of antibiotics and other drugs. These drugs can cause resistance in certain cases. Probiotics, prebiotics and phytobiotics are the main alternatives to the use of drugs (Kimsé *et al*; 2012).

Phytobiotics are medicinal plants used as a source of bioactive substances favorable to digestion. They have anti-inflammatory, anti-oxidant, anti-microbial and anti-parasitic properties. Recent studies have shown that *Piliostigma thonningii* possesses several of these properties (Ouattara *et al.,* 2020). Indeed, this plant is commonly used according to ethnobotanists to combat certain severe digestive disorders. The leaves are widely used in the north of Ivory Coast thanks to their anti-fungal properties. Macerated leaves are used to effectively combat diarrhea and verminosis (Kumwimba *et al*., 2017).

MATERIALS AND METHODS

Animals and experimental design

Sixty young rabbit (*Oryctolagus cuniculus*) hybrid from a cross between New Zealand and Californian, weaned at 30 days of age were used. Animals were caged individually ((0.6 x 0.5 x 0.25 m3).The average weight of the rabbits was 730g. They were divided into 4 groups of 15 rabbits. All animals in the 4 groups were feed with 110 g pelleted diet (basal diet) from 30d to 84d (Table 1). Water access was unlimited. However, the rabbits from group FL

Table 1. Chemical composition of diet (according by company)

Nutrients	%
Dry matter	16.6
Fats	3.9
Cellulosic material	13.2
Crude protein	17.2
Ash	9.4
Calcium	0.9
Phosphor	0.9
Sodium	0.3
Vitamin A	7500UI/kg
Vitamin D3	3000UI/kg
Vitamin E	15mg/kg

supplement of fresh leaves in cage (5g/d). Groups ML and DL were received respectively a macerated leaves and decocted leaves in drinking water (3g/L). The last group was the control. The animals were weighed weekly. The weighing were carried out individually.

Blood Sample Collection

A blood sample was performed at fasting morning in each young rabbit. It was carried out according to the method described by Bléyéré *et al.* (2013). After a careful and rigorous restraint of young rabbit, blood sampling was carried out on one of ears. Drip puncture was made into marginal vein of ear during 58th day of investigation. Venous blood was collected in a tube with anticoagulant (EDTA). It was used for to define all haematological parameters (Red blood cells, Hemoglobin, Hematocrit, Mean corpuscular volume, Mean corpuscular hemoglobin, Mean corpuscular hemoglobin concentration, White bood cell, Neutrophils, Eosinophils, Basophils, Lymphocytes, Monocytes and Thrombocytes) by the Sysmex PLC Xt 2000i.

Droppings collection and parasitology analyses

Droppings were collected early in the morning before 8 a.m. on mosquito nets previously placed under each cage. This method was described by Dakoury *et al*. (2020). The samples taken under each cage were cleared of all debris then homogenized in batches.

The presence of the parasite and the coccidian load (number of oocysts per gram of feces = OPG) were determined using the McMaster method. The samples were analyzed using a LEICA DM300 brand binocular microscope set at G×100 magnification for counting and diagnosis and at G×400 magnification for identification.

Statistical Analyses

The effect *of P. thonningii* leaves on growth, number of coccidian, blood and biochemical parameters were evaluated by the one-way ANOVA analysis method. GraphPad Prism software 8.0.1 was used. Difference was significant if P-value was less than or equal to 0.05. Tukey's multiple comparison test was used to structure the means when a significant difference was observed.

RESULTS AND DISCUSSION

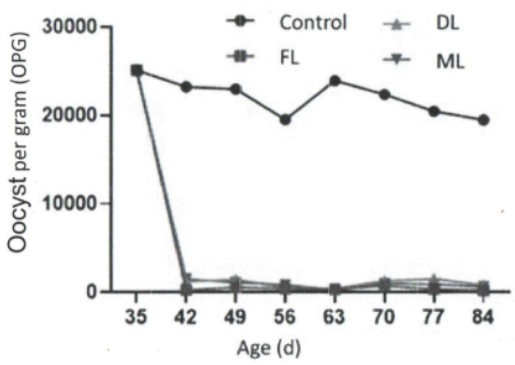
Effects of *Piliostigma thonningii* leaves on weight

Table 2. Effects *P. thonningii* fresh leaves, decoction and macerated on growing performances

	Treatment				*Probability*	
	C	FL	DL	ML	*CV*	*P-value*
Weight (g)						
35 d	771.5	726.7	690.8	727.5	*0.02*	*0.93*
49 d	1045	980.2	907.7	917.3	*0.08*	*0.60*
63 d	1389	1338	1208	1270	*0.13*	*0.40*
84 d	1781	1805	1690	1749	*0.05*	*0.76*
Daily gain (g/d)						
35-49j	19.5	18.2	18.5	17.6	0.03	*0.73*
49-63j	24.6	25.6	21.5	25.2	0.12	*0.15*
64-84j	18.7	22.2	23	22.8	0.05	*0.28*

FL: supplement of fresh leaves, ML: macerated leave; DL decocted leaves; C: control

The effect of *P. thonningii* leaves growth in all forms did not show any negative effect on rabbits (Table 2). However, the growth was low compared to that obtained in the literature (Harouz et al., 2018). This may be linked to the genetic strain used from intuitive crossbreeding and the quality of the commercial food in Ivory Coast (Sangaré *et al*., 2022).



Effect of *Piliostigma thonningii* leaves on coccidian

P. thonningii eliminated coccidia in rabbits (Figure 1). This plant has already been used against digestive parasites. The leaves have been used most often to combat diarrhea in humans or ruminants (Kumwimba *et al.,* 2017).

Figure 2. Effects *P. thonningii* fresh leaves, decoction and macerated on coccidian

FL: supplement of fresh leaves, ML: macerated leave; DL decocted leaves; C: control

Effect of *Piliostigma thonningii* leaves on blood parameters

The average level of white blood cells, red blood cells, hemoglobin and hematocrits of rabbits treated with *P. thonningii* leaves were not significantly different compared to those of rabbits (controls) except the treatment of fresh leaves (Table 3). The concentration of different hematological components is influenced by diet and antinutritional elements. The essential role of red blood cells is the transport of oxygen and carbon dioxide (Alagbe *et al*., 2019). The increase in hematocrit shows better transport and leads, therefore primary and secondary polycythemia.

Table 3. Effects *P. thonningii* fresh leaves, decoction and macerated on blood parameters

	Treatment				*Probability*	
	C	FL	DL	ML	*CV*	*P-value*
WB (103/µL)	7.32	5.78	7.38	7.08	*0.35*	*0.07*
RB (106/µL)	5.69[a]	4.65[b]	5.29[a]	5.45[a]	*0.59*	*<0.01*
HG (g/dL)	11.4[a]	9.56[b]	10.66[a]	11.06[a]	*0.68*	*<0.01*
HC (%)	45.32[a]	39.8[c]	43.8[b]	42.02[b]	*0.48*	*0.01*
PLT (103/µL)	237.6[b]	453[a]	395.6[a]	129[c]	*0.74*	*<0.01*
NEUT (103/µL)	2.68[b]	2.35[b]	3.23[a]	0.81[c]	*0.92*	*<0.01*
LYMP (103/µL)	3.97[a]	2.92[b]	3.55[a]	2.06[b]	*0.47*	*0.02*
MONO (103/µL)	0.61	0.47	0.55	0.38	*0.47*	*0.29*
BASO (103/µL)	0.06	0.04	0.05	0.02	*0.37*	*0.06*

FL: supplement of fresh leaves, ML: macerated leave; DL decocted leaves; C: control, RB: Red blood cell, WB: White blood cell; HG: Hemoglobin; HC: Hematocrit; PLT: Blood platelet; NEUT: neutrophil; LYMP: Lymphocyte; MONO: Monocyte; BASO: Basophile

Hepatorenal toxicity was studied by measuring certain biochemical parameters such as ASAT, ALAT, urea and creatinine. ASAT and ALAT enzyme concentration decreased significantly in the treated animals compared to the control between 13.5 and 37.4 and 13.8 respectively ($P<0.05$, Table 4). ALAT and ASAT levels rise rapidly when the liver is damaged for various reasons including hepatic cell necrosis, hepatitis, cirrhosis as well as the hepatotoxicity of certain drugs (Pratt *and* Kaplan, 2000). The reduction in circulating lipids (total cholesterol, triglycerides and HDL cholesterol) is significantly beneficial in the treated batches. It is possible that the extract has the ability to facilitate the transport of cholesterol and triglycerides from the blood to the tissues. The results of creatinine, uric acid decreased. This demonstrates that *P. thonningii* did not have a negative effect on the animal's kidneys (Ighodaro *and* Omole, 2012).

Tableau 4. Effects *P. thonningii* fresh leaves, decoction and macerated on blood biochemical parameter

	Treatment				*Probability*	
	C	FL	DL	ML	*CV*	*P-value*
Cholesterol T (g/l)	0.87 [a]	0.57 [b]	0.80 [a]	0.67 [b]	*0.48*	*0.01*
Cholesterol HDL (g/l)	0.22 [a]	0.14 [b]	0.20 [a]	0.21 [a]	*0.77*	*<0.01*
Triglyceride (g/l)	3.68 [a]	2.26 [c]	2.57 [b]	2.24 [c]	*0.93*	*<0.01*
Hepatic activity						
ASAT (UI/L)	65.03 [b]	84.38 [a]	51.57 [c]	27.59 [d]	*3.03*	*0.03*
ALAT (UI/L)	40.32 [a]	26.52 [b]	40.32 [a]	26.52 [b]	*1.71*	*0.02*
Renal parameters						
Créatinine (mg/L)	16.09 [a]	13.31 [b]	16.52 [a]	9.85 [c]	0.44	0.02
Acide Urique (mg/L)	65.46 [C]	45.95 [d]	75.33 [b]	85.39 [a]	0.60	0.02
Urée (mg/L)	0.32 [b]	0.33 [b]	0.41 [a]	0.25 [c]	0.24	0.21

FL: supplement of fresh leaves, ML: macerated leave; DL decocted leaves; C: control, ASAT: Aspartate aminotransferase; ALAT: Alanine aminotransferase.

CONCLUSION

Piliostigma thonningii leaves strengthened the immune system of rabbits. in terms of blood parameters. They have very significant antiparasitic activity. The leaves of P thonningii would thus contribute to improving health. However, additional studies on blood parameters are necessary to identify the most effective form in rabbits.

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EFFECTS OF THE AQUEOUS EXTRACT OF PILIOSTIGMA THONNINGII (SCHUMACH.) MILNE-REDH. (FABACEAE) OF THE LEAVES ON DIGESTIVE HELTH IN RABBITS

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ABSTRACT

The aim of this study was to evaluate the potential of Piliostigma thonningii as an anticoccidial agent in growing rabbits. Local breed rabbits (24) approximately five weeks old with an average weight of 500 ± 2.5 g were used. Particular attention was paid to Piliostigma thonningii for its nutritional and pharmacological properties. A phytochemical and antiparasitic study was therefore carried out for this purpose. Phytochemical screening and assaying with the decoctate and macerate of this plant species revealed the presence of large groups of chemical compounds with antiparasitic activity, such as total polyphenols (360.21 ± 11.96 mgEq gallic acid/g for the decoctate ; 207.80 ± 15.5 mgEq gallic acid/g for the macerate, flavonoids (33.0 3± 7.3 mg Eq quercetin/kg), for the decoctate; 9.80 ± 1.5 mg Eq quercetin/kg), for the macerate, tannins (51.21 ± 21 mgEq catechins/g for the decoctate; 42.63 ± 0.82 mgEq catechins/g), for the macerate. A parasitological study using the MacMaster method showed that treatments R1 and R2 were 97% effective against coccidia oocysts. This study therefore justifies the traditional use of Piliostigma thonningii leaves to treat gastrointestinal parasites in rabbits.

Key words: *Piliostigma thonningii*, phytochemistry, anticoccidial, rabbit, Ivory Coast

INTRODUCTION

Digestive disorders are common in growing rabbits, leading to reduced productivity and economic losses. These digestive disorders take different forms, from reduced food consumption to weight loss, diarrhea and even mortality (INRA, 2018). Their origin is complex, resulting from environmental, dietary and/or pathological factors such as colibacillosis, coccidiosis and epizootic enteropathy Regarding food-related digestive disorders, they often affect young rabbits aged 4 to 9 weeks (Gidenne *et al*., 2019).

The prevention of these digestive disorders generally relies on the use of antibiotics and synthetic anticoccidial drugs. These products are often very expensive and ineffective. However, due to the ban on the use of certain drugs in animal feed, natural alternatives are sought, including phytobiotics, probiotics and prebiotics. Phytobiotics are medicinal plant extracts rich in bioactive compounds favorable to digestion, with anti-inflammatory, antioxidant, antimicrobial and antiparasitic properties.Among phytobiotics, *Piliostigma thonningii*, a plant of the Fabaceae family, has attracted particular interest due to its medicinal properties in humans (Ouattara *et al*., 2020) and animals (Kiéma *et al*., 2019). The aim of this study is to evaluate the potential of *Piliostigma thonningii* leaves as an anticoccidial agent.

MATERIALS AND METHODS

Animal and plant materiel

24 local-breed rabbits, approximately 5 weeks old and weighing an average of 500 ± 2.5 g, were used in the study. They were housed in wire cages (80 cm x 45 cm x 35 cm), with a feeder and a drinker. The plant material consisted of leaves of *Piliostigma thonningii*, which were collected in the Marahoué region in west-central Côte d'Ivoire.

Matériel d'extractions et des tests phytochimique
The extraction equipment includes an accurate electronic balance, a variety of glassware, an oven and a spectrometer. The solvents used are water, methanol, ethanol, dichloromethane and chloroform. Reagents include folinic acid, hydrochloric acid, ferrous chloride, Degendrof's reagent, gallic acid and quercetin as a standard for phytochemical tests.

Preparation of aqueous extracts (decocted and macerated) for phytochemical screening
500g of plant powder was decocted with 500 mL of distilled water, brought to the boil on a hot plate for 15 minutes, then filtered. Similarly, 500g of plant powder was macerated with 500 mL of distilled water, then mixed and filtered. The extracts were then dried at 45°C for 48 hours. They were stored at room temperature in sample dishes for future use.

Analyses quantitative
Total polyphenol levels were determined using the Folin-Ciocalteu colorimetric method (Heilerová et al., 2003; Singleton and Rossi, 1965), condensed tannins in the various extracts were determined using the method described by Heimler et al. (2006) and flavonoids were determined using the method used by Arvouet-Grand et al. (1994).

Experimental device and treatment administration
Two treatments were implemented, namely the treatment with the decoction and the treatment with the macerated leaves of *Piliostigma thonningii*.The animals were weighed and randomly divided into three homogeneous groups of 8 rabbits.
Treatment 1: Granulated concentrate with 5 g of decocted extract of Piliostigma thonningii leaves in 1 liter of water for drinking water.
Treatment 2: Granulated concentrate with for drinking water 5 g of the macerated extract of Piliostigma thonningii leaves in 1 liter of water.
Treatment 0: Granulated concentrate with tap water for drinking water

Coprological analysis: Faecal samples were collected from each cage at different times (D0, D7, D14, D28) and sent to the Botany and Plant Diversity Laboratory. To estimate the number of coccidial oocysts, 3 grams of faeces were diluted in 45 millilitres of water, filtered and then centrifuged at 2,000 rpm for 5 minutes. The sediment obtained was homogenised with 5 millilitres of flotation liquid (NaCl: density = 1.20). From this liquid, 0.15 millilitres was taken and placed in each chamber of the McMaster slide. After 10 minutes' rest, the coccidial oocysts were observed under a light microscope at 100x magnification and counted by following the lines engraved in the two chambers of the McMaster slide. The number obtained was multiplied by the dilution factor (100) to obtain the number of oocysts per gram (OPG). The overall prevalence of coccidiosis was calculated by dividing the number of positive faecal samples by the total number of samples collected.

Statistical analysis
Data were entered using the Microsoft Office Excel 2010 spreadsheet. Statistical analyzes were performed using GraphPad Prism 8.0.1 software. The effects, weight gain, consumption index, on were tested by analysis of variance (ANOVA) using the Tukey HSD model. Differences are considered significant at the 0.05 threshold.

RESULT AND DISCUSSION

Contents of polyphenols, total flavonoids and condensed tannins of *Piliostigma thonningii* extracts
The results of the quantitative determination of polyphenols, total flavonoids and condensed tannins are presented in Table 1. This table clearly shows the presence of these compounds in the aqueous extracts of Piliostigma thonningii, but with a higher quantity in the decocted extract. It therefore emerges from this analysis that the aqueous extract of *Piliostigma thonningii* has a high content of total polyphenols. The quantitative analyzes of total phenols,

flavonoids and tannins are determined from the linear regression equations of each calibration curve

Table 1: average values of some chemical compounds according to the extracts.

Extract type	Decocted	Maerated	P-value
Chemical compounds			
Total flavonoids	33,03172 ± 7,37	9,80 ± 1,50	0,0001
Total polyphenols	360,218 ± 11,96	207,80 ± 15,58	0,0001
Tannins	51,21 ± 2,90	42,63 ± 0,82	0,0001

Piliostigma thonningii leaf extracts have an important source of polyphenols. Concentration 360.218 ± 11.96 mgEq gallic acid/g for the decoction; 207.80 ± 15.58 mgEq gallic acid/g for the macerated. Studies by (Brunet *et al*., 2008) showed that flavonoids have anti-inflammatory, analgesic, anti-plasmidic, antibacterial, anthelmintic, hepatoprotective and antiviral properties.The content found is 33.03 ± 7.3 mg Eq quercetin/kg for the discount; 9.80±1.50 mg Eq quercetin/kg for the macerated. The biological properties of tannins arise from their physicochemical properties. Tannins have activities such as: inhibition of enzymes; antioxidant activity (Lim *et al.,* 2007); antimicrobial, antiviral preventive effects against cardiovascular disease, antiseptic activity; antifungal activity. The tannin content determined during this study in *Piliostigma thonningii* leaf extracts are: 51.21 ± 2.90; 42.63± 0.82 mgEq catechins/g for decocted and macerated.This variability of results found at the level of these large groups of bioactive molecules can be explained by the different environmental conditions, the different countries and the different harvest seasons the genetics of the plant, the method drying, the stage of maturity of the leaf and the different extraction methods used.

Effect of aqueous extracts of *Piliostigma thonningii* leaf on the reduction rate of OPG

Coprology carried out before treatment at D0, showed that coccidia oocyst excretion varied from 5820 to 4880 OPG (Table 2). After 7 days of treatment (D7), the level of excretion of coccidial oocysts decreased considerably ($P<0.05$) in the treated batches. This coproscopy remained positive in the control batch throughout the study. These results show that macerated extract (R2) of Piliostigma thonningii leaves reduced the excretion of coccidial oocysts in treated rabbits.

Table 2: Average number of coccidial oocysts per gram of faeces before and after treatment.

Time	Lots		
	R0	R1	R2
J0	4047,5	4873,5	5815
J7	3620	192	240
J14	4809,6	203	323
J21	5209,2	17	49
J28	4725	21	12

These results showed that the treatment of the animals led to a reduction in the infestation with reduction rates in the fecal excretion of coccidiosis oocysts 94% from D7. This reduction was associated with an increase in weight in treated batches compared to controls.

According to the classification of MCKenna (1990), and the recommendations of the World Association for the Advancement of Veterinary Parasitology, which sets the only effectiveness at 90% of the rate of reduction of fecal excretion, we can deduce that the macerated extract have a significant effectiveness $P = 0.001$ in reducing the rate of fecal

excretion of coccidia oocysts. The reduction in parasitic load confirms that the leaves of *Piliostigma thonningii* have anthelmintic and anticoccidial activity in rabbits.Therefore, *Piliostigma thonningii* leaf extract can be recommended in the treatment of gastrointestinal parasites in rabbits to improve the nutritional and health status of these animals.

CONCLUSION

The results of this study show that *Piliostigma thonningii* leaves are rich in total polyphenols, flavonoids and tannins. These large chemical compounds gave these leaves an interesting antiparasitic activity. These results therefore show the enormous potential of *Piliostigma thonningii* leaves in the treatment of gastrointestinal parasites in rabbits growing in their drinking water. And why not in their food rations. The valorization of *Piliostigma thonningii* leaves would thus contribute to improving the health of rabbits at a lower cost and would also make it possible to combat the use of antibiotics in rabbit farms.

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13TH WORLD RABBIT CONGRESS
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QUALITY OF PRODUCTS

RABBIT MEAT QUALITY WITH AN APPROACH TO ITS PROCESSING WITH THE USE OF SPICES OR SPECIFIC INGREDIENTS AS ANTIOXIDANTS

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ABSTRACT

Due to its low lipid content and high amounts of essential amino acids, rabbit meat is known for having excellent nutritional qualities, being very digestible and ideal for use in healthy diets. Unfortunately, rabbit meat has one of the highest lipid peroxidation susceptibilities, which restricts its use in processed food. Additionally, rabbit meat has some technological peculiarities that make it more challenging to incorporate into processed meat products, such as fragile bones, poor juiciness, and a marginal anise-like flavour. But there may still be a demand for rabbit meat today. It might also be suggested as a substitute for traditional meat-based products, which are typically made with beef and pig, particularly for young children and elderly. Additionally, customers may well receive ready-to-cook products, such as meatballs and burgers, and also new market trends could emerge. The addition of spices may help improve the flavour and appeal of processed products made from rabbit meat while also increasing the antioxidant content and boosting nutritious value and shelf life. Furthermore, the use of natural antioxidants (spices) responds to the growing attention shown by consumers and food industries to avoid the use of synthetic molecules that decrease the products' appeal.

Key words: processing, oxidation, antioxidant, spices, burger.

INTRODUCTION

Rabbit meat is known for being lean and low in fat, making it a healthier alternative to other meats. It is also rich in protein, vitamins, and minerals, providing essential nutrients for a balanced diet. Rabbit meat has a mild, delicate flavour that can be easily incorporated into various dishes. It can be roasted, grilled, stewed, or used in sausages and pâtés, offering a versatile option for culinary enthusiasts. Rabbit meat represents a traditional meal in several different Mediterranean countries with typical popular dishes that are part of the national cuisine of Italy, France, and Spain. Actually, rabbit meat consumption in the Mediterranean region is mainly limited to Algeria, Cyprus, Egypt, France, Italy, Malta, Portugal, and Spain and it is only partially present in some other European countries (Belgium, Czech Republic, Germany, and Luxembourg) (Cullere and Dalle Zotte, 2018; Trocino *et al.*, 2019).

More appealing and practical product formulations have been introduced to the market in recent years by the rabbit industries, particularly for customers who are pressed for time while preparing meals (Petracci *et al.*, 2018b, 2018a; Petracci and Cavani, 2013).

Because of their widespread use and broad public approval, ready-to-cook meat products account for a significant portion of the food manufacturing process. Due to their simplicity of preparation and consumption, patties, burgers, and frankfurters are nowadays among the most popular meat products. The well-known grinding procedure causes muscle structure to be disrupted, which makes the food matrix less stable and more susceptible to enzymatic and chemical oxidation processes as well as enhanced microbial growth (Emswiler *et al.*, 1976; Mancini and Hunt, 2005). One of the main ways that the quality of meat and meat products deteriorates is due to oxidative reactions, which reduce the meat's taste, colour, and nutritional content and shorten its shelf life (Kanner, 1994). Unfortunately, rabbit meat

has one of the highest lipid peroxidation susceptibilities, which restricts its use in processed food. Last but not least, compared to chicken and pig, rabbit meat has several technological oddities. These include weak bones, low juiciness, noticeable fibrousness, and a faint anise-like flavour. These suggest that home cooking and processing methods should be used instead of factory processing (Ariño *et al.*, 2007; Dalle Zotte *et al.*, 2016). Notably in the last years some research groups pointed out the possibility to overcome bone fragility and process rabbit carcasses with the production of mechanically deboned meat (McNitt *et al.*, 2003; Negatu *et al.*, 2006; Paula *et al.*, 2020; Petracci and Cavani, 2013). Nowadays, processed rabbit meat products are sold in very small quantities. Examples include infant meals, fresh sausages, filled buns, and hamburgers. Conversely, it is difficult to increase the present range of ready-to-cook items without sacrificing the recognisability of rabbit meat, also in terms of nutritional value and relative cost (Petracci *et al.*, 2018b; Trocino *et al.*, 2019). Over the past several decades, the addition of antioxidant molecules as food additives was deeply studied to protect processed meat products against oxidation and inhibit microbial development (Dalle Zotte *et al.*, 2016; Decker and Park, 2010; Falowo *et al.*, 2014). Customers started to favour goods with natural antioxidants after the controversy over the potential health risks of synthetic antioxidant molecules. This led the food sector to consistently develop the newest natural food additives (Brewer, 2011; Jiang and Xiong, 2016; Shahidi and Ambigaipalan, 2015).

Meat products added with plant-derived antioxidants may be accepted by customers due to their natural origins. Many spices, essential oils, extracts, powders, and other plant by-products have been studied in the past few decades to find out how well they work and what influence they have on meat products (Burt, 2004; Shah *et al.*, 2014; Shahidi and Zhong, 2010).

In this context, this paper aims to provide a state of the art of literature dealing with rabbit meat processed products with the addition of antioxidant ingredients in order to increase and preserve the nutritional value and quality of this meat.

PRODUCTS CHARACTERISTICS

Processing products with spices or specific ingredients as antioxidants

Table 1 reports the research studies dealing with rabbit products added with specific ingredients as antioxidants. All the studies have taken into consideration the processing of rabbit meat with the formulation of patties or burgers. Different rabbit cuts were used in the production of the meat goods, with the majority of them using hind legs (7/13) followed by loins (3/13), whole carcass (2/13), loins (2/13) and only one using both hind legs and loins. Some results from the same research study were published in different articles; these reports were reported in this review as results of companion studies.

The studies evaluate the effect of different natural ingredients such as extracts - fermented rooibos (*Aspalathus linearis*) extract and liquorice (*Glycyrrhiza glabra*) extract; powders - garlic (*Allium sativum*) powder, ramsons (*Allium ursinum*) powder, turmeric (*Curcuma longa*) powder, purslane (*Portulaca oleracea*) powder, and ginger (*Zingiber officinale*) powder; and essential oil - *Zanthoxylum bungeanum* (Sichuan pepper) essential oil. Some of the research studies tested also combinations of spices together.

All of them compared the effects of the spices to control products made with only meat and in some cases positive controls were made with the use of synthetic antioxidants, i.e. BHT (butylated hydroxytoluene) or ascorbic acid. Only three studies in companion tested also the effects of a pro-oxidant ingredient such as salt.

Formulated meat products differ in shape (most of them moulded in burgers-like shapes, with different diameters and heights) and weights (namely 20-30-50-100 grams). Shelf-life of the products was tested in relation to storage time at different days of conservation at 4 °C. In some cases, both raw and cooked samples were tested. Details of the rabbit meat products are reported in Table 1.

pH, water holding capacity and related traits

Results on meat products' pH revealed heterogeneous results. Indeed, some spices delayed or stopped the normal increase in pH during storage such as fermented rooibos extract, purslane powder and Sichuan pepper essential oil (Cullere *et al.*, 2019; Wang *et al.*, 2022, 2021a). On the contrary garlic and ramson powders mix reported higher values of pH than control samples in both raw and cooked samples (Śmiecińska *et al.*, 2022). Other spices did not affect the pH of the burgers (turmeric, ginger, garlic powders) (Mancini *et al.*, 2020a, 2019, 2017b, 2015). These effects, in some cases, might be mainly attributable to the organic acids contained in some spices, in other cases to the inhibition of microbial proliferation in the minced meat.

No statistically significant changes were related to the addition of antioxidant ingredients into the meat products in terms of drip loss. Probably this lack of modification must be ascribed to the low amount of spices used that did not change the chemical composition of the products. On the other hand, when the patties were cooked some ingredients showed to increase the water holding capacity. Indeed, purslane, turmeric and garlic powders showed to decrease the water loss during cooking in relation to the control (Mancini *et al.*, 2019, 2015; Wang *et al.*, 2021a). Due to their chemical properties spices could link water and fat during the cooking and improve the water holding capacity, leading to lower losses during cooking. Notably, no general conclusion can be formulated as several articles reported a lack of variation in the presence of spices and plant products.

Colour

All the articles reported changes in meat products' colour in relation to the antioxidant ingredients used. Rabbit meat, due to its pale pink colour, can be easily affected by the natural colour of the ingredients used in a formulation. The colour of the patties and burgers showed to be deeply affected by the dark-brick red colour of the rooibos extract, as well as the strong bright yellow-orange colour of turmeric powder (Cullere *et al.*, 2019; Mancini *et al.*, 2015). Interestingly also the pale yellowish colours of ramsons and garlic powders induced some changes in rabbit burger ones (Mancini *et al.*, 2020a, 2019; Śmiecińska *et al.*, 2022). In addition, ingredients showed the capacity to delay the discolouration effect typical of minced meat products over the storage time. This effect was reported in both strongly coloured burgers, which maintained a vivid colour during time, as in products that even preserved a colour similar to the control at the beginning of the trials. These results suggested that these ingredients inhibited the protein and lipid oxidation that caused the change in colour as lipid oxidation could produce reactive oxygen species and various aldehydes with powerful biological activities, these components could promote protein and heme iron oxidation. The effects on the colour of a specific ingredient could also vary in raw and cooked samples. Indeed, during the cooking session, the coloured burgers showed the tendency to maintain a colour more similar to the raw ones than what was shown by the control samples.

Fatty acids profile

Due to the low amount of the ingredients added into the formulations the profile of the rabbit meat fatty acids (FA) was only partially affected. Indeed, quite all the articles reviewed stated that the use of the spices was performed to protect the rabbit fatty acids profile. The effects on the lipid oxidation are reported in the following section. Mancini *et al.* (2015) reported some changes in the FA profile of burgers added with turmeric powder (3.5% w/w) showing that the burgers with turmeric powder gained higher values of C18:3n-3, C20:2n-6 and C20:3n-3 than the C burgers. In addition, turmeric powder increased the proportions of arachidonic acid (C20:4n-6), EPA (C20:5n-3) and DHA (C22:6n-3). These modifications in FA profile characterised the burgers with turmeric inducing higher amounts of total n-3 FAs and PUFAs than the other formulations. Similarly, ginger powder, a plant of the Zingiberaceae family such as turmeric, was shown to induce modification into the FA profile of the burgers (1% and 2% w/w) (Mancini *et al.*, 2017a). In this article, C burgers showed higher content of C16:0 and SFA than ginger burgers. Furthermore, burgers formulated with 2% w/w of ginger powder were characterised by a higher proportion of PUFA due to the

contents of n-6 FAs and n-3 FAs. As reported above the differences observed in burgers depended primarily on PUFA differences, mainly in C18:2n-6, C18:3n-3, C20:5n-3 and C22:6n-3. Similarly, after cooking ginger powder showed to have an effect on the FA profile of the burgers. As reported for the raw samples the SFA content was lower than the control burgers as well a strong modification was reported in the MUFA, PUFAn-3, and PUFAn-6 (Mancini *et al.*, 2017a).

Antioxidant capacity vs lipid and protein oxidation

The antioxidant capacity of rabbit meat products formulated with different ingredients was reported to be affected in relation to the chemical composition and the quantity of the spice employed. Dal Bosco *et al.* (2019) revealed minor or no changes in tocopherol and tocotrienol isoforms and retinol with liquorice addition into the burgers (0.25% w/w liquorice extract). Noteworthy, following these slight modifications in antioxidant composition the TBARS revealed a decrease in lipid oxidation during storage time. Mancini *et al.* (2015, 2016)added turmeric powder at 3.5% w/w in rabbit burgers reporting an increased antioxidant capacity of the burgers determined as ABTS, DPPH and FRAP. This potential was showed also over time during a storage period. In raw burgers, the TBARS was not affected by the turmeric addition, and an increase in lipid oxidation was reported over time (Mancini *et al.*, 2015). Interestingly after cooking the burgers formulated with turmeric powder showed a reduced tendency to be prone to lipid oxidation, resulting in lower TBARS than control burgers at both the tested times (Mancini *et al.*, 2016). Similarly, Mancini *et al.* (2017a) reported an increase in antioxidant capacity (ABTS, DPPH and FRAP) of raw burgers added with 1 and 2% w/w of ginger powder. In this article was also revealed a correlation between ABTS and FRAP values with the quantity of ginger powder employed. The same trend was also reported in cooked burgers, highlighting that the antioxidant capacity was maintained in the final product. The increased capacity to contrast oxidation showed by the employed probes was confirmed by TBARS quantification in both raw and cooked burgers. Indeed, ginger powder in both concentrations kept the TBARS by the burgers added with ginger lower than the control burgers. (Mancini *et al.* (2019) indicated that garlic powder 0.25% w/w induced an increase only in the DPPH values of raw samples. Indeed, the use of this spice did not show a strong effect in enhancing the antioxidant capacity of the burgers. Following this slight modification in antioxidant capacity, the effect of garlic powder in reducing TBARS was shown only in raw samples where the spice was mixed with salt (pro-oxidant ingredient). This lack of effect was confirmed also during storage time, in both raw and cooked samples (Mancini *et al.*, 2020b). This lack of effect could be surely linked to the scarce addition of antioxidants derived from garlic powder, as reported for thiols, tocopherols and tocotrienols content in burgers (Mancini *et al.*, 2020b). Only a small level of protection on proteins was revealed by carbonyls determination that showed a partial effect of garlic in control protein oxidation after cooking but only in relation to salt addition. Indeed, burgers with garlic and salt mixed showed protein oxidation similar to the control ones and burgers with added garlic. (Cullere *et al.*, (2019) reported that fermented rooibos extract inhibits the formation of total volatile basic nitrogen (TVBN) at the beginning of the trial, this capacity was then lost after 6 days of storage. On the other hand, fermented rooibos extract displayed a positive impact in relation to lipids oxidation as the peroxide value was maintained below the control sample level. In this contest, the flavonoids present in rooibos extract showed their antioxidant effect, and it could be scribed mostly to the presence of aspalathin, quercetin and nothofagin. Similarly, an inhibitory effect on protein oxidation, expressed as carbonyl and total sulfhydryl contents, might be attributed to the presence of phenol, flavonoid and alkaloids in purslane powder solution as reported by (Wang *et al.*, 2021b). Purslane powder showed also to have a positive effect on lipid oxidation (peroxide value). These effects were also shown during storage time, with lower TVBN values of purslane burgers with respect to control burgers, and delayed lipid oxidation until day 10 of storage (Wang *et al.*, 2021a). Also, the Sichuan pepper essential oil showed similar trends with TVBN, TBARS and peroxide values of treated burgers lower than control

burgers and showed the capacity to inhibit the degradation of sulfhydryl groups and the formation of carbonyls (Wang *et al.*, 2022).
Even a negative effect of the addition of natural plant spices is reported. Śmiecińska *et al.* (2022) reported that burgers added with ramson powder (0.35% w/w) and garlic powder mixed with ramson powder (0.35% + 0.35% w/w) had higher TBARS values than control burgers, in cooked samples; also, garlic powder burgers (0.35% w/w) were worse than control in the raw samples. No suggestions on the reason for these changes were furnished by the authors.

Microbiological profile
Concerning the ingredients employed and the microbiological analyses conducted the effects on the control of microbiological spoilage could differ largely. Indeed, Śmiecińska *et al.* (2022) reported a lack of effectiveness of garlic and ramson powders on the growth of Enterobacteriaceae, *Pseudomonas* spp., lactic acid bacteria and total aerobic psychrotrophic bacteria. Similarly, Mancini *et al.* (2015) reported a lack of effect of turmeric powder on the total aerobic count, *E. coli*, Enterobacteriaceae and Staphylococci. On the other hand, Wang *et al.* (2021a) reported that purslane powder slightly delayed the total aerobic counts' growth during 12 days of storage as well as the essential oil of Sichuan pepper (Wang *et al.*, 2022). Also, ginger powder (at 2% w/w) showed a slight effect in the control of the proliferation of total aerobic count, Enterobacteriaceae, *Pseudomonas* spp., and Staphylococci (Mancini *et al.*, 2017b).

Sensory characteristics
Spices and other ingredients can strongly affect the sensory characteristics of meat products. Indeed, most of these plant elements were employed historically to cover unpleasant colour, odour and flavour. All the articles reviewed reported strong modification of the sensory appeal of the rabbit meat burgers and patties (Cullere *et al.*, 2019; Mancini *et al.*, 2020b, 2017b; Śmiecińska *et al.*, 2022; Wang *et al.*, 2022, 2021a). These changes could be related to the strong colour of the spices, their aroma, their tendency to increase texture (due to their capacity to bind water), persistence, and how they are perceived in relation to rabbit flavour and odour characteristics. Moreover, also their modifications in terms of flavour and colour during the cooking are crucial for increasing the perception of the consumers. Most of the tested spices induced an increase in odour and flavour during the storage time if compared to the control burgers. These results highlight the capacity of the spices to inhibit the proliferation of microorganisms that cause off-flavour and off-odour but also their capacity to cover some defects.

CONCLUSIONS

Rabbit meat products, mostly ready-to-cook products, represent one of the options to increase rabbit consumption. Rabbit meat due to its chemical composition and flavour is not suitable for the formulation of meat products that do not require the addition of other ingredients. Natural antioxidants were studied as ingredients in rabbit meat products in order to increase the shelf life of the goods and follow the requests of the consumers to have more tasty products. More studies are needed in order to increase the knowledge of rabbit meat preparations and to produce useful data for the producers. Studies on the consumers' perception of rabbit meat products are also needed, taking into account the culinary traditions of different countries. Indeed, on one hand, different spices could be tested, considering their features, such as their antioxidant capacity, on the other hand, their effects on the consumers' perception and sensory characteristics are not secondary.

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Table 1: Framework of the research study on rabbit meat products formulated with antioxidant ingredients.

Reference	Formulation	Product	Meat	Temp	Storage time (days)	pH	Water Holding Capacity	Colour	FA	Antioxidant capacity	Oxidation	Micro	Sens
Cullere *et al.* (2019)	C control (only meat) R1 fermented rooibos extract 0.5% w/w R2 fermented rooibos extract 1% w/w R3 fermented rooibos extract 2% w/w	patty 50 g	hind legs and loins	4 °C	0-1-3-6	Raw C=R1> R2=R3	Drip loss NS	Raw L* linearly decreased with R inclusion a* increased in R2 and R3 b* increased in R1, R2 and R3	Cooked some small differences in FA profiles in relation to the formulation R showed a protective effect on the FA		Raw TVBN T0 C≥R1≥R2=R3; T6 R2≥R3≥C=R1 Cooked Peroxide reduced by R		Cooked colour more intense in relation to rooibos incorporation R3 patties reached the highest flavour overall intensity score R3 and recorded the highest rooibos flavour perception and the lowest rabbit flavour R2 and R1 showed an increasing off-flavours perception C and R1 reported similar sensory features
Dal Bosco *et al.* (2019)	Meat derived from feeding animals with control vs liquorice diets Cc control-1 (meat from the control feeding group) Cl control-2 (meat from the liquorice feeding group) Lc liquorice extract at 0.25% w/w (meat from the control feeding group) Ll liquorice extract at 0.25% w/w (meat from the liquorice feeding group)	burger 20g	hind legs	4°C					some small differences in FA profiles in relation to the formulation liquorice protected rabbit meat FA profile	minor o no changes in tocopherol isoforms, tocotrienols isoforms and retinol	TBARS liquorice extract controlled the oxidation over time		
Śmiecińska *et al.* (2022)	C control (only meat) G garlic powder 0.35% w/w R ramsons powder 0.35% w/w GR garlic powder 0.35% w/w and ramsons powder 0.35% w/w	burger 100g	hind legs	4°C	0-7	Raw GR≥G=R≥C Cooked GR>G=R=C	Cooking loss NS	Raw L* C>G>R=GR a* C>G>R>GR b* C>R≥G≥GR Cooked L* C=G>R=GR a* C=G>R=GR b* GR≥R≥G≥C			Raw TBARS GR>R≥G≥C Cooked TBARS GR>R>G=C	No effect of G, R and GR on Enterobacteriacea, *Pseudomonas* spp., lactic acid bacteria and total aerobic psychrotrophic bacteria	Raw G, R and GR had no influence on the presence of off-odours Cooked R and GR received the highest scores
Wang *et al.* (2021b)	C control (meat added with 10 mL of distilled water) BHT 10 ml BHT solution final concentration of 0.02% w/w PE1 10 mL purslane powder solution final ncentrations of 0.1% w/w PE2 10 mL purslane powder solution final	patty (burger) 20g	*Longissimus thoracis et lumborums*	4°C	0-2-4-6-8-10-12						Positive effect of PE on carbonyl content, total sulfhydryl content, and peroxide value		

	ncentrations of 0.3% w/w PE3 purslane powder dissolved in distilled water (10 mL) final concentrations of 0.5% (w/w)												
Wang *et al.* (2021a)	C control (meat added with 10 mL of distilled water) BHT 10 ml BHT solution final concentration of 0.02% w/w PE1 10 mL purslane powder solution final concentrations of 0.1% w/w PE2 10 mL purslane powder solution final concentrations of 0.3% w/w PE3 purslane powder dissolved in distilled water (10 mL) final concentrations of 0.5% (w/w)	patty (burger) 20g	*Longissimus thoracis et lumborums*	4°C	0-2-4-6-8-10-12	C>PE1, PE2,PE3,BHT with differences in relation to storage time	Cooking loss C>BHT=PE1>PE2=PE3 with differences in relation to storage time	L* and a* decreased with PE b* increased by PE				TVBN PE delayed the protein oxidation TBARS PE delayed the lipid oxidation until day 10	PE slightly delayed the total bacterial counts growth	PE increase the preference by the panelists and the panelists' evaluations of the texture
Wang *et al.* (2022)	C control (only meat) BHT 0.02% w/w ZBMEO 0.1% w/w ZBMEO 0.3% w/w ZBMEO 0.5% w/w	patty (burger) 30g 30 mm diameter, 20 mm height	*Longissimus thoracis et lumborums*	4°C	0-2-4-6-8-10-12	ZBMEO delayed the increase in pH during storage		L* and a* decreased with ZBMEO b* increased by ZBMEO				TVBN, TBARS and peroxide value delayed by ZBMEO Inhibit degradation of sulfhydryl groups and formation of carbonyls	ZBMEO slightly delayed the total aerobic counts growth	ZBMEO delayed the effects of storage on odour, colour, flavour, texture, and overall acceptance
Mancini *et al.* (2015)	C control (only meat) AA ascorbic acid 0.1% w/w TU turmeric powder 3.5% w/w	burger 50g	hind legs	4°C	0-7	NS	Drip loss NS Cooking loss Time 0 C>TU	a* and b* increased with TU	TU induced positive modifications in FA profile	ABTS, DPPH and FRAP increased by TU	TBARS NS	No effect of TU on total aerobic count, *E. coli*, Enterobacteriaceae and Staphylococci	
Mancini *et al.* (2016)	C control (only meat) AA ascorbic acid 0.1% w/w TU turmeric powder 3.5% w/w	burger 50g	hind legs	4°C	0-7					ABTS, DPPH and FRAP increased by TU	TBARS C>TU at both the testing time		
Mancini *et al.* (2017a)	C control (only meat) GI1 ginger powder 1% w/w GI2 ginger powder 2% w/w	burger 100 g 85 mm diameter	whole carcass	4°C	1-4-7				GI induced modifications both in raw and cooked burgers	Raw and Cooked ABTS and FRAP G2>G1>C DPPH G2=G1>C	Raw and Cooked TBARS C>G1=G2		
Mancini *et al.* (2017b)	C control (only meat) GI1 ginger powder 1% w/w GI2 ginger powder 2% w/w	burger 100 g 85 mm diameter	whole carcass	4°C	1-4-7	NS	Drip loss NS Cooking loss NS	Raw L* and a* decreased by GI b* increased by GI Cooked L* decreased by GI a* and b* increased by GI				TU slightly delayed the total aerobic count, Enterobacteriaceae, *Pseudomonas* spp., and Staphylococci	G affected positively the juiciness and the global score
Mancini *et al.* (2019)	C control (only meat) G garlic powder 0.25%	burger 100 g	hind legs			NS	Cooking loss	Raw L* C=G (effect of salt		Raw and ABTS and FRAP NS	Raw TBARS S≥GS≥G=C		

	w/w S salt 1% w/w GS garlic powder 0.25% w/w and salt 1% w/w	100 mm diameter					C≥G≥S= GS	in GS) a* decreased by G b* increased by G Cooked L* and a* C=G (effect of salt in GS) b* increased by G		DPPH G≥GS≥S=C Cooked ABTS, DPPH and FRAP NS	Cooked TBARS NS		
Mancini *et al.* (2020b)	C control (only meat) G garlic powder 0.25% w/w S salt 1% w/w GS garlic powder 0.25% w/w and salt 1% w/w	burger 100 g 100 mm diameter	hind legs	4°C	0-4-7				some small differences in FA profiles in relation to the formulation	Raw and Cooked ABTS, DPPH and FRAP NS low variations in thiols, tocopherols and tocotrienols	Raw TBARS and Carbonyls S=GS>G=C Cooked TBARS S=GS>G=C Carbonyls S>GS=G=C		G affected colour, aroma and flavour intensities, hardness, juiciness and global evaluation
Mancini *et al.* (2020a)	C control (only meat) G garlic powder 0.25% w/w S salt 1% w/w GS garlic powder 0.25% w/w and salt 1% w/w	burger 100 g 100 mm diameter	hind legs	4°C	0-4-7	NS	Drip loss NS Cooking loss NS	Raw L* C=G (effect of salt in GS) a* decreased by G b* increased by G Cooked NS				No effect of G on Enterobacteriaceae, *Pseudomonas* spp., lactic acid bacteria, yeast and moulds, total aerobic mesophilic, and total aerobic psychrotrophic bacteria	

EFFECT OF EXTRUDED LINSEED AND ALGAE PADINA PAVONICA'S DIETARY SUPPLEMENTATION ON MEAT FATTY ACID PROFILE IN FATTENING RABBITS

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ABSTRACT

Dietary supplementation with n-3 polyunsaturated fatty acid (n-3 PUFA) in livestock animals is an effective strategy to produce functional foods. This study aimed at investigating the effect of dietary supplementation with extruded linseed alone and in combination with the algae *Padina pavonica*, which has boosting effect on DHA (22:6 n-3) assimilation, on the fatty acid profile of fattening rabbit meat. At weaning age (35 days), 120 rabbits were divided into three groups according to the experimental diet (n=40 rabbits/diet) provided *ad libitum* until the slaughter age (82 days): commercial control diet (CNT), CNT diet supplemented with 5% extruded linseed (L5%), and CNT diet supplemented with 3.5% extruded linseed and 0.2% *Padina pavonica* algae (LPP). At slaughter, the *Longissimus thoracis et lumborum* (LTL) from 20 randomly selected carcasses per group (n=60) was collected, and proximate chemical composition and fatty acid profile analyses were performed. No significant differences were found in the carcass characteristics and proximate composition of LTL. Compared to the CNT group, both L5% and LPP LTL showed higher concentrations of n3 PUFAs, with better n6/n3 ratio, and improved atherogenic and thrombogenic indexes. LPP showed higher n-3 very long chian fatty acids, specifically DHA compared to the L5% group. Results from the L5% group were consistent with the literature, whereas there are no previous studies on the benefits of combining extruded linseed and algae. Therefore, these findings show, for the first time, the benefits on the rabbit meat quality of combining extruded linseed and the algae *Padina pavonica* in the diet of fattening rabbits.

Key words: *Oryctolagus cuniculus*, omega-3 polyunsaturated fatty acids, extruded linseed, algae, meat quality.

INTRODUCTION

The imbalance in the n-6/n-3 fatty acids ratio and the low absolute mass of essential fatty acids consumed in the modern diet have been linked to the increased occurrence of

cardiovascular diseases in developed countries. To reduce the health risk of unbalanced diets, research is engaging in the development of novel functional foods. In this regard, n-3 polyunsaturated fatty acids (n-3 PUFAs) dietary supplementation in livestock animals has proven to be an effective strategy (*i.e.*, meat, eggs, milk; Lewis et al. 2000). This dietary supplementation can also improve the productive and reproductive traits as well as the health and welfare of rabbits (Agradi et al., 2023). In rabbit farming, some experiments have already been conducted using various sources of n-3 PUFA, with encouraging results (Agradi et al., 2023). However, several critical issues still need to be resolved such as the dose of n-3 PUFAs sources to be used and the effect of combining it with other sources, such as *Padina pavonica* algae, which has boosting effect on DHA (22:6 n-3) assimilation, on rabbit meat fatty acid composition. In this sense, our study aimed at investigating the effect of dietary supplementation with extruded linseed alone and in combination with the *Padina pavonica* algae on the fatty acid profile of rabbit meat.

MATERIALS AND METHODS

Animals and experimental design

The rabbits were raised in two commercial farms located in Central Italy. The experimental protocol was approved by the Ethical Committee of the Department of Veterinary Medicine of the University of Milano (OPBA_18_2021). At weaning age (35 days), 120 New Zealand White rabbits (n=60/farm) were divided into three groups according to the isoenergetic experimental diet fed (n=40/group): commercial control diet (CNT), CNT diet supplemented with 5% of extruded linseed (L5%), and CNT diet supplemented with 3.5% of extruded linseed and 0.2% *Padina pavonica* algae (LPP) (chemical composition and fatty acid profile were determined for all the diets, data not shown). Animals were individually housed in conventional cages (L×W×H: 75×35×25 cm) under controlled environmental conditions. All animals were slaughtered at 82 days of age. Only 60 carcasses (n=20 randomly selected carcasses/group) were transported to the CREA-ZA laboratory and dissected following the Worlds Rabbit Science Association recommendations (Blasco and Ouhayoun, 1996). The weights and lengths of the three regions (fore legs, trunk, and hind part) were determined, along with the tissue composition of the right thigh.

Chemical Analyses

Twenty four hours after slaughtering, the *Longissimus thoracis et lumborum* (LTL) was excised from each chilled carcass (4°C), and the proximate chemical composition (dry matter, crude protein, total fat and ash) of LTL (AOAC, 2016) was performed. After chloroform:methanol (2:1 vol:vol) fat extraction and methylation with 2 N methanolic potassium hydroxide, the fatty acid methyl esters (FAME) were quantified using a gas-chromatography (GC 6890N Agilent, Inc., Santa Clara, CA, USA) instrument equipped with flame ionization detector and a CP-Sil88 fused silica capillary column. The GC-FID conditions and other details were described in Failla et al. 2021. Fatty acids methyl esters were identified by comparing the peaks retention time of each compound with standard peaks from Supelco mix 37 (Sigma-Aldrich Merck, Darmstadt, Germany); FAME and the different classes of fatty acids as amount of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, are expressed as % of total FAME. From some fatty acid were calculated the atherogenicity index (AI) and thrombogenicity index (TI) following Ulbricht and Southgate (1991) indications.

Statistical Analysis

Data were analyzed with one-way ANOVA using Welch's F when the assumption of homogeneity of variances was violated. Sidak correction was used for multiple comparisons. Kruskal-Wallis tests were used when the transformation did not improve the data distribution. Statistical analyses were performed with SPSS Statistics version 25 (IBM, SPSS Inc., Chicago, IL, USA). Statistical significances were declared at an alpha value of 0.05.

RESULTS AND DISCUSSION

No significant differences were found in the carcass characteristics, tissue composition, and proximate chemical composition of LTL (data not shown). Previous studies confirm the

limited effect of isoenergetic diets supplemented with different n-3 PUFAs sources on carcass characteristics (Agradi et al., 2023). Conversely, the extruded linseed and algae supplementations modified the fatty acid composition of the LTL (Table 1). This demonstrates the effectiveness of incorporating extruded linseed and *Padina pavonica* algae into the diet of fattening rabbits to produce a functional food.

Table 1: Mean contents and standard errors of means (SEM) of fatty acids in *Longissimus thoracis et lumborum*.

Fatty acids (% of total FAME)	Groups						*P*-value
	CNT		L5%		LPP		
	Mean	SEM	Mean	SEM	Mean	SEM	
14:0	1.35b	0.05	1.25ab	0.05	1.15a	0.04	0.009
16:0	24.24b	0.22	23.53a	0.21	23.48a	0.13	0.010
18:0	8.13	0.17	8.04	0.12	8.32	0.11	0.364
18:1 cis-9 OA	18.74a	0.19	19.65b	0.20	19.14ab	0.21	0.009
18:2 n-6 LA	28.06b	0.31	24.06a	0.27	24.00a	0.20	<0.001
18:3 n-3 ALA#	2.05a	0.05	6.71c	0.33	5.33b	0.24	<0.001
20:4 n-6	6.43b	0.25	5.01a	0.24	6.07b	0.20	<0.001
20:5 n-3 EPA#	0.19a	0.02	0.55b	0.03	0.61b	0.02	<0.001
22:5 n-3 DPA#	1.16a	0.06	2.37b	0.11	2.70b	0.08	<0.001
22:6 n-3 DHA	0.24a	0.01	0.44b	0.03	0.52c	0.03	<0.001
SFA	35.87b	0.19	34.83a	0.25	35.02a	0.17	0.001
MUFA	24.23	0.31	24.49	0.24	24.10	0.25	0.572
n-6	35.19c	0.28	29.72a	0.22	30.78b	0.23	<0.001
n-3*	3.66a	0.12	10.11b	0.22	9.21b	0.17	<0.001
PUFA	39.07a	0.31	40.07b	0.31	40.22b	0.22	0.011
n-6/n-3*	9.77c	0.27	2.97b	0.07	3.37a	0.07	<0.001
VLCFA n-3#	1.61a	0.09	3.40b	0.16	3.88c	0.12	<0.001
AI	0.30b	0.00	0.29a	0.00	0.28a	0.00	<0.001
TI*	1.18b	0.02	0.72a	0.01	0.76a	0.01	<0.001

Values followed by the same letter(s) are not significantly different at p≤ 0.05. # Welch's F statistic. * Non-parametric statistics. For brevity, means and SEM are presented but the data were analyzed with nonparametric techniques. FAME=fatty acid methyl ester; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; VLCFA = very long chain PUFA; AI = index of atherogenicity; TI = index of thrombogenicity.

The SFA, particularly C14:0 and C16:0, which are considered unhealthy fatty acids, were significantly higher in the CNT group compared to the others (P<0.05). No significant differences were observed in MUFA except for OA (18:1 cis-9), which constitutes the principal MUFA. It exhibited a higher percentage in the L5% an LPP groups.

The higher intake of ALA (18:3 n-3) in the L5% and LPP group compared to the CNT group primarily resulted in an increase of this fatty acid in their meat (P<0.001). Additionally, due to the ability of rabbit tissues to desaturate and elongate the ALA into n-3 VLCFA, also EPA (20:5 n-3), DPA (22:5n-3) and DHA (22:6 n-3) significantly increased in the meat of rabbits fed with L5% and LPP diets (P<0.001). As a consequence, the n-6/n-3 ratio, as well as the AI and TI indexes also showed significant improvements, when compared to the CNT group (P<0.001). Our result confirms the added value that linseed supplementation brings to rabbit meat fatty acid composition, as evidenced by previous studies (Matics et al. 2017; Tariq et al. 2017).

Regarding the incorporation of *Pavina pavonica* algae into the diet, this is the first experiment to utilize it in fattening rabbits. Other few studies have examined diets with *Spirulina* or *Schizochytrium* spp. to modify the fatty acid profile on rabbit meat but the reported results have been contrasting (Mordenti et al. 2010; Peiretti and Meineri 2011). Here, the combination of *Pavina pavonica* algae with extruded linseed further increased the content of n-3 PUFA in comparison with CNT group (P<0.001). Furthermore, in LPP group the concentration of n-3 VLCFA (i.e., EPA, DPA, and DHA) in the LTL was even higher than in the L5% group (P<0.001). The beneficial properties of n-3 VLCFA against cardiovascular, neurodegenerative, and other chronic diseases are well documented (Saini et al. 2021). In

human medicine, it has been proven that algae oils (as fish oil) are more valuable in improving the body status of EPA plus DHA rather than linseed oil, probably because of the rate-limiting step of bioconversion of ALA to its reaction intermediates (Saini et al. 2021). For these reasons, rabbit meat produced with the supplementation of extruded linseed with the addition of *Padina pavonica* algae in the diet should be considere as a feasible strategy to increase n-3 fatty acid and improve both n6/n3 ratio and the AI and TI indices. This product could offer functional compounds, particularly beneficial for people suffering from cardiovascular diseases, while also providing high biological value proteins and low cholesterol contents, typical of rabbit meat (Dalle Zotte and Szendro 2011).

CONCLUSIONS

Based on the obtained results and the significant increase in ALA, EPA, DPA, and DHA, we can conclude that the dietary incorporation of linseed derivatives and *Padina pavonica* algae for fattening rabbits is an effective strategy to produce a functional food that may contribute to the preservation of human health.

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IMPACT OF DIETARY INCLUSION LEVELS OF CAMELINA CAKE (*Camelina sativa* (L.) CRANTZ) ON MEAT QUALITY IN GROWING-FATTENING RABBITS

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ABSTRACT

The present research tested the feasibility of a dietary incorporation with different levels of *Camelina sativa* (CS) cake in the diet of growing rabbits as a partial or total replacement of rapeseed meal. To this purpose, a n=168 32-days-old hybrid Hyplus rabbits were divided into 3 treatments (n=56 per treatment) and fed with one of the following experimental pelleted diets: Control diet (10% rapeseed cake as major source of crude protein), CS5 diet (5% rapeseed cake and 5% CS cake), and CS10 diet (10% CS cake). At 74 days of age, rabbits were electrically stunned and slaughtered. Then, from n=14 randomly selected rabbit carcasses/treatment, n=1 hind leg was excised, deboned, ground, and freeze-dried in order to determine hind leg proximate composition and fatty acid (FA) profile. Data were analysed by a one-way ANOVA that considered the dietary treatment as fixed effect. Results showed the dietary inclusion of CS did not affect the proximate composition of rabbit hind leg, except for moisture content which was higher in Control meat than in CS10 one, with CS5 treatment having an intermediate result (P=0.0359). Differently the dietary treatment affected meat FA profile: the proportion of total Polyunsaturated FA (PUFA) linearly increased along with CS inclusion level (P<0.0001), to the detriment of both total Saturated FA (SFA) (P=0.0006) and Monounsaturated FA (MUFA) (P<0.0001). The PUFA increase was attributable to both *n*-6 (P=0.0088) and *n*-3 (P<0.0001) fractions. Results of the present experiment indicated that the replacement of 10% rapeseed cake with CS cake can be specifically recommended to increase PUFA proportion of rabbit meat, especially the beneficial *n*-3 fraction.

Key words: *Camelina sativa*; Fattening rabbits; Meat quality; Proximate composition; Fatty acids.

INTRODUCTION

The unsustainable production of feedstuffs for food-producing animals and their high cost are amongst the most challenging issues affecting the supply of animal products to the growing global population. In fact, production of conventional feed ingredients generates an ever-increasing pressure on the natural resources that leads to a loss of animal biodiversity, land degradation, and contributes to water shortages, ultimately challenging ecosystems and exacerbating the food-feed competition too (Makkar, 2018). In this context, the identification of human-inedible resources of feed and pursuing sustainable production systems is pivotal. *Camelina sativa* (CS) is an annual brassicaceae native of Europe, increasingly cultivated for its oil rich in *n*-3 fatty acids and low requirements of agricultural inputs: CS tolerates low temperatures, drought, and adversities, including certain insects and diseases (Zanetti *et al.,* 2021). The seeds present an attractive nutritional composition, being rich in oil (30 – 49%) and proteins (24 – 31%). While the oil has an interesting market thanks to its several applications (Mondor and Hernández-Álvarez, 2022), the cake represents the by-product, yet

rich in protein and with an interesting residual oil amount. Unfortunately, CS seed, and especially cake, also contains anti-nutritional compounds, mainly glucosinolates. The latter is an aspect that needs improvement to make CS cake a promising alternative for the animal production sector (Colombini *et al.,* 2014). The available research testing the possible utilisation of CS cake as feedstuff for food-producing animals is limited to few studies, mostly conducted on fish (Mondor and Hernández-Álvarez, 2022) and poultry species (Singh *et al.,* 2023a, b). In the sole study carried out on a limited number of growing rabbits (Peiretti *et al.,* 2007) it was concluded that CS seed can be included at levels of up to 15% without detrimental effects on growth performances and carcass traits and with an improved meat lipid profile. However, given the paucity of information on the topic, further research to establish an ideal dietary inclusion level of CS cake into rabbit diet in relation to meat quality outcomes is required. Therefore, the present research studied the inclusion of CS cake into the diet of growing-fattening rabbits at two distinct levels (5% and 10%) to investigate the impact on meat quality traits.

MATERIALS AND METHODS

Animals and experimental design

The *in vivo* part of the present study was conducted at the Rabbit Unit of the Institute of Animal Science (Prague, Czech Republic), upon approval of the Ethics Committee (E3/2023). At weaning (32 days), a total of n=168 hybrid Hyplus rabbits were housed by four into wire-net cages (80 x 60 x 45 cm). During fattening period (42 days), rabbits were fed with one of the following three pelleted diets (n=56 rabbits/treatment): Control (10% rapeseed cake), CS5 (5% rapeseed cake and 5% CS cake), and CS10 (10% CS cake). The CS cake was obtained by an improved variety, selected to have a reduced content of glucosinolates (Alan, CNR Milano, Italy). The experimental diets were formulated to have similar dietary levels of crude protein (CP), neutral detergent fibre (NDF), acid-detergent fibre (ADF), and acid detergent lignin (ADL) and to comply with dietary recommendations for growing-fattening rabbits (de Blas and Mateos, 2020). Diets and water were offered *ad libitum* to the animals throughout the experimental period. At 74 days of age, n=14 rabbits/treatment (one male/each cage) was selected and slaughtered following the recommendations of the World Rabbit Science Association (Blasco and Ouhayoun, 1996). After carcass chilling (24h at + 4 °C), one hind leg/carcass was ground, freeze-dried and packaged. Sampled were transported to the MAPS Department of Animal Medicine, Production and Health – MAPS of the University of Padova (Italy), where the chemical analyses of diets and meat were conducted.

Chemical analyses of experimental diets and hind leg rabbit meat

Hind leg meat samples were analysed following the AOAC (2000) procedures to determine dry matter (DM: method no. 934.01), CP (method no. 2001.11), and ash (method no. 967.05) contents. Ether extract was determined after acid hydrolysis (EC, 1998). Lipid extraction of the experimental diets and the hind leg meat was performed by modified accelerated solvent extraction (M-ASE). For experimental diets, extraction was conducted using the solvent petroleum ether, while for rabbit meat was used a binary solvent mixture of chloroform/methanol at a 1:2 ratio, according to the method of Dalle Zotte *et al.* (2022). Main FA classes were expressed as % of the total detected FA methyl esters (FAME).

Statistical Analysis

Data were analysed by a one-way analysis of variance (ANOVA) that considered the experimental diet as fixed effects (PROC GLM; SAS, 2008). Least square means were obtained using the Bonferroni test, and the significance was calculated at $P<0.05$ level.

RESULTS AND DISCUSSION

Proximate composition of rabbit hind leg meat

The result presented in Table 1 showed that the proximate composition of meat belonging to the CS cake-containing diets did not significantly differ ($P>0.05$) from that of the Control one,

with the sole exception of moisture, which showed an indirect trend to that of CS cake inclusion level (P<0.05). These results are thus coherent with the findings of Peiretti *et al.* (2007), where the proximate composition of rabbit *longissimus dorsi* meat was not significantly affected by 10 and 15 % dietary inclusion of CS seed. Another study testing fermented rapeseed meal in rabbit diets observed no effect on both *longissimus dorsi* and *biceps femoris* proximate composition (Nowakowicz-Dębek *et al.,* 2021).

Table 1: Effect of the dietary inclusion of 0%, 5%, and 10% *Camelina sativa* cake on the proximate composition of rabbit hind leg meat (g/100 g meat).

	Treatments			RSD[1]	P value
	Control	CS5	CS10		
N.	14	14	14		
Moisture	72.7[a]	71.9[ab]	71.8[b]	0.96	0.0359
Protein	20.2	20.6	20.5	0.13	0.0582
Lipids	5.63	6.00	6.39	0.99	0.1430
Ash	1.09	1.11	1.20	0.46	0.1380

[a,b]Values with different superscripts within a row differ significantly P<0.05.
[1]RSD: Residual standard deviation.

Fatty acid profile of experimental diets and rabbit hind leg meat

The main FA classes of rabbits' hind leg meat were significantly affected by the dietary treatment (Table 2). As expected, observing the FA composition of the diets, incorporating CS cake significantly increased the PUFA proportion of meat, which is explained by rabbit metabolism reflecting the dietary FA in meat, like other monogastric species (Dalle Zotte, 2002; Singh *et al.,* 2023a, b). Specifically, a 5% inclusion of CS cake increased the PUFA fraction (P<0.0001) and reduced the MUFA (P<0.0001) proportion. A higher inclusion level (10%), instead augmented PUFA fraction (P<0.0001) to the detriment of both SFA (P=0.0006) and MUFA (P<0.0001) proportions. The PUFA increase was linked to both *n*-6 (P=0.0088) and *n*-3 (P<0.0001) FA, though the latter group was the one directly influenced by the presence of CS cake, notably rich in linolenic acid (C18:3 *n*-3; Zanetti *et al.,* 2021).

Table 2: Main FA classes (% of total FAME) of the three experimental diets and the hind leg meat obtained from rabbits fed with the inclusion of 0%, 5%, and 10% of *Camelina sativa* cake.

	Diets			Treatments			RSD[1]	P-value
	Control	CS5	CS10	Control	CS5	CS10		
N.	2	2	2	14	14	14		
SFA	17.7	14.7	14.9	34.3[A]	34.1[A]	32.8[B]	1.03	0.0006
MUFA	40.4	31.2	29.0	36.1[A]	33.5[B]	31.5[C]	1.26	<0.0001
PUFA	32.3	44.9	47.0	24.6[C]	27.2[B]	30.6[A]	1.43	<0.0001
n-6	27.2	35.1	32.4	20.7[b]	21.8[a]	22.0[a]	1.12	0.0088
n-3	5.11	9.79	14.7	3.91[C]	5.49[B]	8.64[A]	0.41	<0.0001
n-6/*n*-3	5.32	3.58	2.21	5.29[A]	3.97[B]	2.55[C]	0.19	<0.0001

[a,b]Values with different superscripts within a row differ significantly P<0.05. [A,B]Values with different superscripts within a row differ significantly P<0.001. [1]RSD: Residual standard deviation.

The observed changes in the FA profile of meat led to the progressive reduction of the *n*-6/*n*-3 ratio (P<0.0001) with increasing the incorporation of CS cake. A lowered *n*-6/*n*-3 ratio represents an important trait for rabbit meat since can help consumers to lower their dietary *n*-6/*n*-3 intake. The latter is a recognised key nutritional target, given the implications on long-chain *n*-3 FA (specifically EPA and DHA) and the *n*-6/*n*-3 (< 6) in maintaining cardiovascular and cerebral health (Massaro et al., 2008). The improvements of meat FA attributable to the dietary incorporation of CS cake were achieved without increasing the lipid content of meat (Table 1). Rabbit meat already has a favourable FA profile for human nutrition (Dalle Zotte, 2002) and present results indicated that the dietary inclusion of CS cake, rich in linolenic

acid, is an effective tool to further enhance rabbit meat healthiness working in the direction of sustainability of the production system.

CONCLUSIONS

From this study, it emerged that the inclusion of CS cake in the diets of growing-fattening rabbits (both 5 and 10% incorporation levels) had no impact on the proximate composition of rabbit hind leg meat. Conversely, the diet-related effect was significant in enhancing meat lipid profile thanks to a remarkable improvement in the proportion of *n*-3 FA, especially when using the 10% CS cake incorporation level. Further insights into the chemical and sensory characteristics of meat are required to have a complete view on the impact related to the dietary inclusion of CS in rabbit diets.

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NIX COLOUR SENSOR AS POTENTIAL TOOL FOR EVALUATING COLOUR IN RABBIT MEAT

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ABSTRACT

The present study aimed to test an innovative sensor for the instrumental evaluation of colour in rabbit meat. Once the anatomical part representative of the carcass and likely having different energetic metabolism were identified (i.e., loin and hind leg meat), these were stored up to 12 days and used to measure colour by using a tristimulus colorimeter and a colour sensor, a less expensive and user-friendly device. For this purpose, 36 rabbit carcasses (Martini group hybrid, 11-week-old, average live wt 2.7 kg) were considered and dissected to obtain the main muscles used to measure pH 24 h *post-mortem* (pHu). Then, 15 loins and hind legs were selected, packed in ordinary atmosphere, and stored for 12 days at 4°C. Data concerning pHu were analysed by one-way ANOVA, while colour data obtained throughout storage period were analysed by repeated measures ANOVA. When significant, means were separated by Tukey-HSD ($p<0.05$). The lowest pHu values were measured in the *Longissimus thoracis et lumborum* muscle, with special reference to the median and posterior part. These can be attributed to a higher proportion of type IIB fibers with glycolytic metabolism. Different colour values were found when comparing the data obtained with tristimulus colorimeter and colour sensor. These may be due to the light emitting source which is included in the devices ultimately leading to a different scattering of the incident light as a result of its interaction with the meat surface and/or structure. In conclusion, these results indicated that the Nix colour sensor can be a viable alternative to conventional chroma meters for both research and industrial purposes. In addition, updated reference values for pHu for the main muscles of rabbit carcass were provided.

Key words: rabbit meat, pH, colour, chroma meter, colour sensor.

INTRODUCTION

Over the past years, few studies have been performed to characterize the main quality parameters of rabbit meat, including ultimate pH (pHu) and colour (Ouhayoun & Dalle Zotte, 1993; Hulot & Ouhayoun, 1999). However, the majority of them may be outdated and likely do not mirror pHu and colour values observed in the modern genotypes which are used for meat production purposes. In detail, the pH, measured 45 min and 24 h *post mortem*, is an important indicator of meat quality (Ouhayoun & Dalle Zotte, 1996). Similarly, meat colour is a crucial factor influencing consumers' acceptability and willingness to pay (Dalle Zotte, 2022; Faustman *et al*., 2022). However, since colour perception is subjective, it is necessary to adopt methods to objectively measure it (Tapp III *et al.*, 2011; King *et al.*, 2023). Currently, many options are available for instrumental colour analysis such as chroma meters and spectrophotometers. Each device offers a variety of options that allow users to choose among several colour systems (Hunter, CIE and Tristimulus), illuminants (A, C, D65 and Ultralume), observers (2° and 10°), and aperture size (0.64-3.2 cm) (Mancini & Hunt, 2005; King *et al.*, 2023). Tristimulus colorimeters (i.e., Minolta and HunterLab) are the most widely used instruments in food colour research in light of their sensitivity and accuracy, and their use has been recommended also in rabbit meat (Ouhayoun & Dalle Zotte, 1996). However, the high cost and some limitations (i.e., large size could make it more difficult to maneuver in a meat processing plant setting) can restrict their use. Therefore, less expensive and more user-friendly devices could represent an alternative for both researchers and quality-

assurance specialists interested in objectively evaluating meat colour (Dang *et al.*, 2021). Among them, it is worth mentioning the colour sensors easily operating via mobile apps, whose application on beef and poultry meat has been recently tested and validated (Schelkopf *et al*., 2021; Che *et al.*, 2023).

The present study aimed to validate the possibility to adopt an innovative sensor (Nix Spectro 2 Pro Color Sensor) to evaluate the colour changes occurring during refrigerated storage in glycolytic and oxidative rabbit muscles.

MATERIALS AND METHODS

Samples collection and analyses

Thirty-six rabbit carcasses belonging to 3 homogeneous batches (12/batch) (Martini group hybrid - 11 weeks-old, average live weight of 2.7 kg) were collected from a commercial processing plant and stored at 4±1°C until 24 hours post-mortem. Then, carcasses were cut to excise 8 muscles (*Agitator caudae*, *Semimembranosus*, *Tensor faciae latae*, *Vastus lateralis*, *Biceps femoris*, *Gastrocnemius ext*., *Semitendinum*, *Tricipitis brachii,* and *Longissimus thoracis et lumborum* that was subsequently divided into its anterior, median, and posterior parts) which were used to assess pHu according to the iodoacetate method proposed by Jeacocke (1977). In addition, a total of 15 loins (*m. Longissimus thoracis et lumborum* – LTL) and 15 hind legs (*m. Biceps femoris* - BF), belonging to 5 carcasses/batch, were packed in ordinary atmosphere, and stored at 4±1°C for 12 days. Colour was measured on median part of LTL and BF after 0, 3, 5, 7, and 12 days of refrigerated storage by using a Chroma Meter CR-400 (Minolta Corp., Milan, Italy) and a Nix Colour Sensor (Model Spectro 2, Nix Sensor Ltd., Hamilton, ON, Canada). For both devices, measurements were collected by using illuminant source C and observation angle 2°.

Statistical Analysis

The statistical analyses were performed using STATISTICA 10 (StatSoft). Data concerning pHu were analyzed using the one-way ANOVA to evaluate eventual differences which may be ascribed to muscles metabolism. In addition, repeated measures ANOVA was applied using data obtained with Minolta and Nix systems to evaluate the colour changes occurring during refrigerated storage. When significant means were subsequently separated using Tukey-HSD ($P<0.05$).

RESULTS AND DISCUSSION

Characterization of pHu muscles differing in energy metabolism

The results concerning pHu values are reported in Figure 1. The lowest pHu values were measured in the *LTL* muscle, with special reference to the median and posterior parts (5.90 and 5.88).

These results can be likely attributed to a higher proportion of type IIB fibers having glycolytic metabolism, and thus resulting in lower pHu, within this muscle (Hulot & Ouhayoun, 1999). On the other hand, it is worth mentioning that the anterior part of the *LTL* muscle as well as all the others considered in the present study exhibited significantly higher ($P < 0.001$) pHu values. These may be considered as an indicator of a more oxidative metabolism. Considering the data in absolute terms, the pHu values measured in the present study are slightly different (higher) from those available in the literature (Ouhayoun *et al.*, 1993; Hulot & Ouhayoun, 1999). This discrepancy may be associated to a different genetic background of the modern rabbit hybrids or to differences in the management practices ultimately affecting the glycolytic potential.

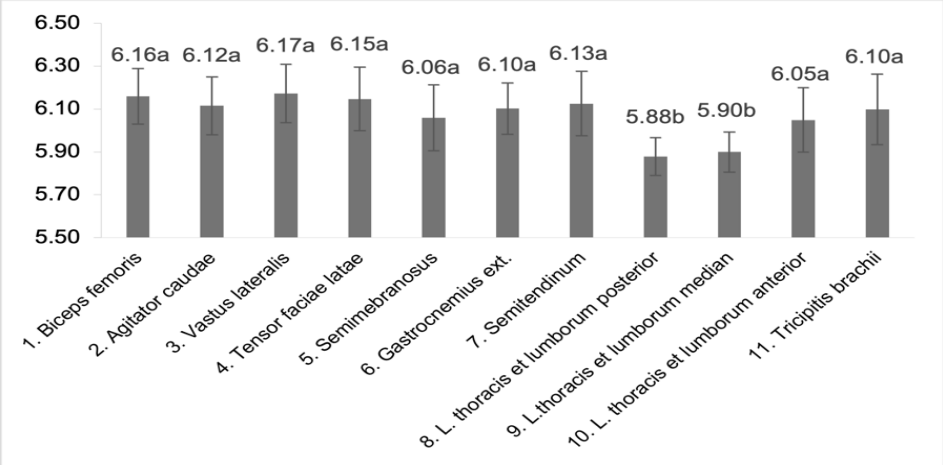

Figure 1: Mean values (± standard deviation) of pHu measured in different muscles (n=36). a,b = mean values followed by different letters significantly differ among the muscles (P<0.05).

Assessment of colour evolution during refrigerated storage by innovative Colour Sensor

Five carcasses/batch (n=15) were selected to excise loin and hind leg meat and, based on the results obtained for pHu, colour was measured by using Chroma Meter and Colour Sensor over 12 days of refrigerated storage (0, 3, 5, 7, and 12 d) on median part of LTL and BF muscles (Tables 1 and 2) which exhibited very different average pHu values (5.90 *vs.* 6.16, Figure 1).

Table 1: Colour changes measured by Chroma Meter (Minolta) and Colour Sensor (Nix) of median part of *L. thoracis et lumborum* muscle during refrigerated storage at 4±1°C for 12 days.

	Chroma Meter			Colour Sensor		
day	L*	a*	b*	L*	a*	b*
0	53.0b±1.9	2.1±0.7	0.7b±1.0	38.7c±2.5	-2.0b±0.5	0.7±1.9
3	55.3ab±2.1	2.2±0.8	3.1a±1.5	39.4bc±1.4	-1.2a±0.5	0.5±1.3
5	56.4a±2.7	2.1±0.9	3.3a±1.1	40.8abc±2.6	-1.4a±0.5	1.0±1.9
7	55.8ab±3.2	1.8±0.8	3.1a±1.5	41.5ab±3.2	-1.8ab±0.5	0.8±2.1
12	55.2ab±3.9	1.8±0.9	3.5a±1.6	42.2a±0.5	-1.8ab±0.5	1.3±1.7
p-value	0.019	0.588	<0.001	0.001	<0.001	0.797

a,b = For each parameter, means value (± standard deviation) followed by different letters significantly differ over the different storage times considered (Tukey-HSD, P < 0.05).

Table 2: Colour changes measured by tristimulus Chroma Meter (Minolta) and Colour Sensor (Nix) of *Biceps femoris* muscle during refrigerated storage at 4±1°C for 12 days.

	Chroma Meter			Colour Sensor		
day	L*	a*	b*	L*	a*	b*
0	55.8±2.9	2.5±0.6	0.1a±1.3	41.0b±2.3	-1.2b±0.7	0.3a±1.4
3	57.5±3.0	2.6±0.6	-1.3b±1.3	42.4ab±2.3	-0.4a±0.5	-3.1b±1.9
5	57.4±3.0	2.6±0.5	-1.7b±1.0	42.7ab±1.4	-0.5a±0.3	-4.0b±1.7
7	57.1±3.1	2.7±0.5	-1.1ab±1.2	42.7ab±1.5	-0.7a±0.3	-2.6b±1.4
12	57.4±3.0	2.6±0.7	-1.4b±1.3	43.0a±2.0	-0.6a±0.2	-3.1b±1.5
p-value	0.475	0.968	0.002	0.063	<0.001	<0.001

a,b = For each parameter, mean values (± standard deviation) followed by different letters significantly differ over the different storage times considered (Tukey-HSD, P < 0.05).

Overall, when comparing the data obtained with tristimulus Chroma Meter and Colour Sensor, different values were found. These may be attributed to the light emitting source

which is included in the devices ultimately leading to a different scattering of the incident light as a result of its interaction with the meat surface and/or structure. As for loin meat (Table 1), an increase in lightness (L*) was observed over the storage time. In detail, a significant ($P<0.05$) increase in L* measured with tristimulus colorimeter was found from 0 to 5 d, whereas it progressively increased ($P<0.01$) from 0 to 12 d when measured with Colour Sensor. Significant ($P<0.001$) differences over the storage time were also detected by yellowness (b*) measured with tristimulus Chroma Meter and redness (a*) assessed with Colour Sensor.

With regard to hind leg meat (Table 2), measuring colour with Chroma Meter did not allow us to detect any significant change for lightness and redness, while only yellowness significantly ($P<0.05$) increased over the time. On the other hand, significant differences in all the colour parameters were found when the Colour Sensor was used. In detail, lightness tended ($P=0.06$) to progressively increase during storage time, while redness and yellowness significantly ($P<0.001$) decreased from 0 to 3 d of storage and then remained steady until the of shelf-life. This allows to hypothesize a higher sensitivity of the colour sensor in detecting absolute differences existing over the refrigerated storage in rabbit meat.

CONCLUSIONS

This study suggests that the Nix Spectro 2 Colour Sensor provides an opportunity for a less expensive, more mobile, and multipurpose device for objectively measuring rabbit meat colour for both research and industrial purposes. Although Nix Colour Sensor is not equivalent to Minolta Chroma Meter, our results indicated that it guarantees higher sensitivity for assessing rabbit meat colour changes during refrigerated storage. In addition, updated reference values for ultimate pH in different muscles of the rabbit carcass were provided.

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CONSUMER PERCEPTION OF THE HEALTHINESS OF RABBIT MEAT. PRELIMINARY RESULTS

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ABSTRACT

The aim of the study was to assess respondents' opinions on healthiness of mats derived from different animal species in several countries. A total of 1116 questionnaires from Spain, Italy, Poland, Hungary and China were analyzed. According to the perception of all respondents, the ranking on the healthiness of different meats is as follows: fish (39.9%), rabbit (20.8%), chicken (15.1%), beef (13.4%), pork (4.9%) lamb (4.3%), and horse (1.6%). In specific countries, variations in rankings were observed. In Italy, Poland and Hungary fish claimed the top position, while in Spain, rabbit meat was ranked the highest. Conversely, in China, fish and beef were considered the healthiest options. Gender showed a negligible difference, while rabbit meat received the lowest rating among younger respondents (<29 years old). The findings suggest that respondents' opinions on the healthiness of meats could be leveraged for the development of marketing strategies with the meat industry.

Key words: Meat, Animal species, Healthiness, Consumer attitudes, Countries.

INTRODUCTION

Meat is a rich source of protein and is of great importance as a key provider of essential micronutrients such as iron, selenium, vitamins A, B12, and folic acid. These micronutrients are either absent in plant-based foods or have limited bioavailability (de Castro Cardoso Pereira and dos Reis Baltazar Vicente, 2013; Biesalski, 2005). Consumption of meat-based food is particularly important for pregnant and breastfeeding women, children and the elderly (Hill, 2002; Biesalski, 2005). There are many arguments for and against the consumption of animal products, especially meat. One is whether meat is healthy food or not. Consumption of a healthful diet is associated with increased nutritional well-being, healthier lifestyle choices and sometimes an increased perception of overall well-being (Diehr and Beresford, 2003). Interest in health and healthy eating may also vary significantly between countries due to cultural differences, among many other aspects. Consumers' knowledge and opinions are shaped by many factors such as traditions, income, and prices - to name a few -, which contribute to their decisions of meat from different animal species on the basis of healthiness. The collective opinions of individuals often reveal broad preferences within a country or region.

The aim of the study was to assess respondents' opinions on healthiness of various meats from different animal species, with a particular focus on rabbit meat in Spain, Italy, Poland, China and Hungary.

MATERIALS AND METHODS

Sampling Methods

Respondents from different countries were asked in an online multifaceted questionnaire, translated into the corresponding language. In the present study, we focus on the evaluation of a specific question: "*Which animal species' meat do you consider to be the healthiest?*" In addition to rabbit meat, options included beef, chicken, fish, horse meat and lamb. Among the non-probability sampling techniques, snowball sampling of data collection was used (Goodman, 1961). A total of 229 Spanish, 242 Italian, 198 Polish, 420 Hungarian and 201

Chinese respondents answered the questionnaire, however, the results reflect the responses of 1,116 respondents, as those who do not consume meat were excluded from the assessment.

Table 1: The distribution of the sample, % (n=1116)

Background information	Countries				
	Spain	Italy	Poland	Hungary	China
Gender					
Female	105	82	93	217	99
Male	85	95	68	185	88
Age					
18-29	24	33	54	85	182
30-39	47	28	30	108	3
40-49	46	50	41	109	0
50-59	58	54	23	53	2
60+	15	12	13	47	0

Statistical Analysis

Only questionnaires without errors were evaluated with one-way ANOVA using SPSS software. Frequency distributions, cross-tabulations and significance analysis (Chi^2-probe) were performed.

RESULTS AND DISCUSSION

Approximately 40% of respondents considered fish to be the best choice for health, followed by rabbit meat (20.8%), chicken (15.1%) and beef (13.4%). Pork and lamb accounted for less than 5% each, and horse meat just over 1%.

The healthiness of meat varies from species to species. Despite the concern that marine fish may contain some toxic metals (Bosch *et al.,* 2016; Sumner and Ross, 2002), consumers have an overwhelmingly positive perception of fish, with the concept of wholesomeness prevailing (Olsen, 2003). Consumers typically prioritize the benefits of fish consumption over the potential risks (Pieniak *et al.,* 2008). Also, rabbit meat, especially for consumers in Mediterranean countries, is recognized for its healthy and nutritious properties (Dalle Zotte and Szendrő, 2011). Likewise, respondents consistently express a favorable perception of poultry, and rank it high in terms of healthiness. Poultry's enduring popularity is linked to its positive and health-conscious image, as shown in studies by Verbeke and Viaene (1999), which found it to be a healthier choice than pork and beef. In general, beef is the subject of conflicting opinions. Beef consumption is generally not excessively high in most countries, and the perceived risk is likely to be driven by negative reports rather than widespread consumption. Pork scored relatively low. Despite consume' opinion, pork does not seem to deserve the worst rating for fat content, cholesterol content or fatty acid profile. Interestingly, Verbeke *et al.* (1999) found a more significant disparity between pork and poultry than between beef and pork. In the case of lamb, factors such as breed, feeding system, and age at slaughtering play a crucial role, potentially more so than in other farmed animal species. Traditionally, lamb has been considered as a healthier choice compared to beef due to its lower fat content (Kubberød *et al.,* 2002). Last but not least horses are not typically associated with meat production, and horse meat is the least known type of meat. The likely low score of respondents is partly due to a lack of knowledge.

Effect of nationality

Table 1 illustrates the healthiness scores for meat from various animal species in Spain, Italy, Poland, Hungary and China. In Hungary, fish ranked first, with a share of around 50%, followed by Italy and Poland at around 40%, but even Spanish and Chinese respondents considered it relatively healthy (30.5% each). In Spain, rabbit meat took the top spot. Spanish population has a tradition of consuming rabbit meat and is convinced of its healthiness (Trocino *et al*., 2019). Surprisingly, Polish respondents considered rabbit meat almost as healthy as fish. This positive sentiment was mirrored by Italians, who ranked rabbit

meat second, with more than 20%. In contrast, despite being the world's leading producer of rabbit meat and actively promoting its consumption (Trocino *et al.,* 2019), China showed a more negative perception, with respondents giving rabbit meat a notably low healthy score. In Hungary, rabbit meat was the third healthiest meat type.

Table 2: Frequency of meat of different animal species based on the healthy score in different countries, % (n=1116)

c	Countries					SE
Meat	Spain	Italy	Poland	Hungary	China	
Fish	30.5_{a}	$39.5_{a, b}$	36.6_{a}	50.1_{b}	30.5_{a}	0.010
Rabbit	35.8_{c}	$22.6_{b, c}$	34.2_{c}	14.5_{b}	5.9_{a}	0.011
Chicken	$18.9_{a, c, d, e}$	$16.4_{c, e}$	5.0_{b}	$18.7_{d, e}$	$10.7_{a, b, c, d, e}$	0.004
Beef	$7.4_{b, c}$	$11.9_{b, c}$	16.8_{c}	7.7_{b}	30.5_{a}	0.015
Pork	2.6_{b}	2.8_{b}	1.9_{b}	5.0_{b}	11.8_{a}	0.006
Lamb	1.6_{b}	$4.5_{a, b}$	$3.1_{a, b}$	3.2_{b}	10.2_{a}	0.006
Horse	3.2_{a}	2.3_{a}	2.5_{a}	0.7_{a}	0.5_{a}	0.012

$_{a-f}$ Percentage data with different letters on the same row differ significantly ($p < 0.05$).

Effect of gender

The relationship between meat and masculinity has a long historical background, and eating meat is identified as a characteristic of the ideal man (Rothgerber, 2013). It is noteworthy that the health justification for meat consumption is generally higher for men than for women (Vukasovič, 2011). In our survey, there was no significant difference between the genders of respondents in the countries surveyed (20.8% of women and 20.7% of men considered rabbit meat the healthiest type of meat).

Effect of age

The effect of consumers' age on the healthy scores of different meat types among the countries was significant for rabbit meat. The youngest consumer group scored lower than individuals in other age groups. This difference might be due to the fact that younger respondents believe that rabbits are pets, which leads to a less favorable perception of their meat (González-Redondo and Contreras-Chacón, 2012).

Table 3. The healthy score of rabbit meat depending on the age of respondents, % (n=1116).

	Age, years				
Meat	<29	30-39	40-49	50-59	>60
Rabbit	15.1_{a}	$24.1_{a, b}$	$21.5_{a, b}$	26.3_{b}	$23.0_{a, b}$

$_{a,b}$ Percentage data with different letters in the same row differ significantly ($p < 0.05$).

CONCLUSIONS

Our overall findings show that white meat, particularly fish, is generally considered the healthiest choice. However, in regions with a strong tradition of rabbit meat consumption, it has become the preferred choice. The older generation prefered rabbit meat as healthier. Given the difference in preferences based on gender, age, as well as other factors, a targeted marketing strategy should take into account as many influencing factors as possible. While campaigns to promote rabbit meat consumption may differ from country to country, the results of this study offer a basis for emphasizing the importance of tailoring strategies to specific target groups.

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EFFECT OF HOUSING SYSTEM ON LOCAL RABBIT PERFORMANCE, CARCASS AND MEAT QUALITY

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ABSTRACT

This study evaluated the productive performance, carcass traits, and meat quality of 300 Grigio di Carmagnola's rabbits (GC) at different slaughter ages (120d and 150d) and reared under different housing systems (HSs). compared HS were: Single cage (S; N = 100 rabbits; 600 x 250 x 330 mm; 16-17 animals/m2), Group farming (G; N = 100 rabbits; 8 rabbits/collective cage; 2000 x 1000 x 1000 mm; density <40 Kg/m2), and Mixed pilot system (M; N = 100 rabbits), in which animals have been bred in G till sexual maturity, then moved into S till slaughters. Due to emergency slaughtering (at 100d old) of G rabbits, we had to continue experimental protocol with S and M GC's rabbits. The housing system did not affect slaughter performance and meat quality traits. Conversely, age had a significant impact on live weight, average daily feed intake, and feed conversion ratio. Additionally, carcass data showed significant effects of age on slaughter weight, ready-to-cook weight, dressing-out, carcass yield and head proportion. The findings indicate changes in slaughter performance and carcass quality with increasing age, attributed to the maturation stage of the rabbits. Accordingly, with age increased meat dry matter and fat contents. Microbial contamination revealed a tendency for higher bacterial counts in S rabbits, suggesting potential differences in meat hygiene. Concluding, the M pilot system showed potential benefits, conciliating performance and welfare of G and S breedings, in the best system with respect to physiological phase.

Key words: Housing system, Local rabbit, Performance, Carcass, Meat, Quality.

INTRODUCTION

The scientific literature provides several insights concerning the fact that the different housing systems play a key role on performance, qualitative traits of meat and welfare in rabbits (Mugnai et al., 2014). Rabbit production has to adapt to the new welfare standards which take into account rabbit welfare, like the use of pens with different dimensions for growing rabbits instead of current single or bicellular cages according to the new European regulation. The achievement of these targets means more expenses for farmers (Szendro et al., 2020) and this economic effort is much more addressable for commercial rabbits, usually fast-growing lines (mean slaughter age at 70-77 days), than for breeders of local population's, usually medium growing lines (mean slaughter age 120-150 days). Conversely, local population are worth to be preserved due to their higher capability to resist, given their genetic peculiarities (Notter, 1999), to the new challenges of climate change (Hoffman, 2010) and, non-negligible detail, because of their role, as niche product, in the culinary local traditions. In Piedmont Region, North West Italy, there is a local population, namely Grigio di Carmagnola (Carmagnola is a city 25 km far from Torino), its coat is gray, with darker shades and it is a typical medium to slow growing line rabbit, with live slaughter weight ranging from 3.5 to 4.5 Kg between 120 and 150 days.

We aimed at studying the effects of three different housing systems (Single cage (S), Group colony (G) and Mixed pilot system (M)) and two slaughter ages (120 and 150 days) on live and slaughter performance, and meat quality of 300 GC rabbits.

MATERIALS AND METHODS

Trials were performed at Department of Veterinary Science facilities, during the period from March to July 2022 and were approved by the Bioethics Committee of the University of Turin (Prot. n. 0245520).

Animals and experimental design

A total of 300 weaned rabbits (35d old), from Carmagnola's rabbit has been randomly distributed between three housing systems (HS): Single cage (S; N = 100 rabbits; 600 x 250 x 330 mm; 16-17 animals/m2), Group farming (G; N = 100 rabbits; 8 rabbits/collective cage; 2000 x 1000 x 1000 mm; density <40 Kg/m2), Mixed pilot system (M;N = 100 rabbits), in which animals have been bred in the same condition as G group till sexual maturity (80d old), then moved into S till slaughters (120-150d). During the trial, at 100d of life, to minimize the suffering of the rabbits we had to change experimental design. In fact, after puberty (70-80d of age), the increasing rate of aggressive behaviors and fights between G rabbits produced a severe reduction of rabbits' health conditions and, at the same time a progressive increase of rabbit's morbidity and mortality. It's evident that the consequences of G rearing, at this physiological phase, are in contrast with animal health and welfare.

Productive and carcass measurements

Live weight (LW) of rabbits was recorded weekly; the average daily gain (ADG) was individually calculated. Average daily feed intake (ADFI) and mortality rate were recorded weekly, and the feed conversion ratio (FCR) was calculated as g feed /g gain. At 120 and 150d, rabbits (20/group/age) from S and M groups, after fasting for 12 hours were slaughtered (Blasco and Ouhayoun, 1996). Carcasses (including the head but without thoracic cage organs, liver, kidneys and perirenal fat) were weighed to obtain the ready to cook carcass weight for dressing-out percentage calculations. Dressing-out percentage (%) was calculated by dividing the hot carcass weight (RTC) by the live weight at slaughter of age. The cold carcass (CC) weight was recorded after chilling the carcasses at 2-4°C for 24 h and used in the calculation of the reference yield.

Chemical Analyses of meat

At commercial ages (120 and 150 days of age), 20 rabbits' group were slaughtered and after 24H at 4°C, ultimate pH of *Longissimus lumborum* was measured, after that muscles were dissected, empty packaged and stored at -20°C till analysis. Chemical analysis of meat was performed by an accredited laboratory (DGRL G065753 del 04.05.2017; GRUPPO MAURIZI srl, Via Pellaro, 22, 00178 Roma, Italy), following the Rapports ISTISAN 1996/34, while total protein was calculated following the ISO 1871:2009.

Hygienic traits of meat

In order to assess production process hygiene levels, a trained operator sampled 20 rabbit's carcass per group at 120 and 150 days of age, using a sponge moisted with 10 ml of buffered peptone water. ISO procedures were used for Total mesophilic aerobic bacteria (TMAB) and Enterobacteriaceae counts (ISO 4833–1:2013 and ISO 21528–2:2017, respectively). To assess the contamination of the left thigh muscle a sample (20gr) was aseptically collected for the isolation of: *Salmonella spp*. (ISO 6579–1:2017), *E. coli* spp. (ISO 16649–12:2001) and *L. monocytogenes* (ISO 11290-2). The results are expressed in CFU/cm^2.

Statistical Analysis

A two-way analysis of variance (ANOVA) was performed to assess the impacts of the housing system (HS) and age (A), as well as their interactions (HSxA). Subsequently, multiple comparisons of the means were conducted using the Duncan test to calculate the least significant difference. Statistical analyses were performed using R software, specifically Version 3.1.2 (R Core Team, 2014). Non-parametric tests were done on mortality rate with proc CATMOD and significance was evaluated by χ values. Significance was set at $P \leq 0.05$

RESULTS AND DISCUSSION

Effect of housing system on performance, carcass and meat quality of rabbit at commercial slaughtering

Regarding slaughter performance (table 1), neither the housing system nor its interaction with age showed significant effects. However, an age effect was observed in rabbits' live weight (LW) (2904 g vs. 3413 g at 120 and 150 days, respectively; P<0.001), average daily feed intake (ADFI) (110.41 g vs. 125.98 g for 120 and 150 days, respectively; P<0.001), and feed conversion ratio (FCR) (4.86 vs. 6.47 at 120 and 150 days, respectively; P<0.01). Our findings align with the established relationship between age and live weight, where larger rabbits typically consume more feed, leading to higher ADFI. Despite maintaining consistent ADG in our study, feed conversion efficiency declined with increasing age, this decline is commonly attributed to increased fat deposition, as supported by following results.

Table 1: Effect of housing system (HS), age (A) and their interaction on productive performance of rabbits at commercial slaughters

	HS		A		P			MSE	
	S	M	120	150	HS	A	HSxA	HS	A
Rabbits, no.	20	20	20	20	-	-	-	-	-
LW (g)	3270	3348	2904^{A}	3413^{B}	0.11	0.00	0.16	33.29	30.42
ADFI (g/d)	111.66	114.73	110.41^{A}	125.98^{B}	0.24	0.00	0.08	1.63	1.63
ADG (g/d)	22.16	23.69	21.08	21.78	0.18	0.55	0.96	0.84	0.84
FCR	5.39	5.94	4.86^{a}	6.47^{b}	0.28	0.01	0.09	0.13	0.13
Mortality* (%)	2.5	3.5	4.5	1.5	0.71	0.22	0.48	1.99	2.76

^{a,b}: means with different letters on the same row differ significantly for P≤0.05. $^{A, B, C}$: means with different letters on the same row differ significantly for P≤0.001. *: χ value.

Age had a significant effect on: slaughter weight (SW) (3011 g vs. 3418 g at 120 and 150 days, respectively; P<0.05), ready-to-cook weight (RTC) (2186 g vs. 2405 g at 120 and 150 days, respectively; P<0.05), cold carcass (CC) (2158 g vs. 235 g at 120 and 150 days, respectively; P<0.05), carcass yield (72 vs 69% at 120 and 150d, respectively; P<0.05), dressing-out percentage (73% vs. 70% for 120 and 150 days, respectively; P<0.05), and head proportion (8.5% vs. 9.4% at 120 and 150 days, respectively; P<0.001) (table 2). As age increased, slaughter performance and carcass quality changed, with increased SW, RTC, CC, head and intravisceral fat proportion and decreased yield and dressing percentages, due to the maintenance in the proportions of gastrointestinal tract, legs, kidneys, liver, and feet with older age (Szendrő et al. 2002). Also, the contribution of the head increased significantly with age, which reflects its later maturation relative to the body as a whole; accordingly, growth of the lipid portion of the carcass is reflected in the increase in the fat percentage of meat (Pascual et al., 2008). Age increased (P<0.001) also the DM (26.7 vs 30.2% at 120 and 150d, respectively) and fat content (2.17 vs 4.10 %, at 120 and 150d, respectively) of meat. These results are in line with Cavani et al. (2000), who reported the decrease of moisture and the increase of fat content of meat with the advancement of age. *Salmonella* spp., E. *coli* Beta Glucuronidase + and *Listeria monocytogenes* were not detected in any sample. Microbiological contamination of rabbits didn't show significant defences between rabbits even if Losacco et al. (2024) found that rabbits reared in free-range systems showed an improvement of the meat hygienic status.

CONCLUSIONS

Despite age-related changes, well known in the literature, in slaughter performance and carcass and meat quality, these findings underscore the importance of considering age-related changes with respect to the production systems. In fact, even if new welfare standards recommend G rearing for growing rabbits, this system becomes incompatible with maintenance of animal health and welfare for long cycles of production (> 70-80d old).

Thanks' to the fact that housing system didn't show any significant effect on performance, carcass traits and meat quality (chemical and microbiological) of rabbits at commercial slaughters, M system could be considered a satisfactory compromise between rabbit's

welfare (rearing according to the rabbit physiological phase, data presented in WRC2024) and performance (same as those in S).

Table 2: Effect of housing system (HS), age (A) and their interaction on carcass traits and meat quality (chemical and microbiological) of rabbits at commercial slaughters

	HS		A		P			MSE	
	S	M	120	150	HS	A	HSxA	HS	A
Rabbits, no.	20	20	20	20	-	-	-	-	-
SW (g)	3291	3327	3011^{b}	3418^{a}	0.23	0.02	0.93	77.04	86.13
RTC (g)	2336	2353	2186^{b}	2405^{a}	0.35	0.04	0.19	51.78	57.89
CC (g)	2298	2331	2158^{b}	2354^{a}	0.21	0.03	0.32	49.27	55.09
Yield (%)	70	70	72^{a}	69^{b}	0.55	0.01	0.69	0.98	1.02
Dressing-out (%)	71	71	73^{a}	70^{b}	0.18	0.02	0.23	1.78	0.97
Empty intestine (%)	12.1	13.0	12.3	12.8	0.90	0.27	0.13	0.28	0.31
Legs (%)	2.2	2.2	2.1	2.3	0.88	0.12	0.60	0.06	0.07
Head (%)	9.0	8.9	8.5^{B}	9.4^{A}	0.24	0.00	0.66	0.17	0.19
Kidneys (%)	0.7	0.8	0.7	0.8	0.31	0.49	0.45	0.02	0.02
Liver (%)	3.9	3.4	3.8	3.9	0.23	0.21	0.11	0.09	0.10
Heart (%)	0.3	0.4	0.4	0.3	0.18	0.35	0.12	0.01	0.01
Intravisceral Fat (%)	2.4	1.8	1.9	2.2	0.35	0.14	0.20	0.12	0.14
DM (%)	28.7	27.2	26.7^{B}	30.2^{A}	0.06	0.00	0.54	1.08	1.08
Ash (%)	1.55	1.49	1.72	1.33	0.87	0.31	0.96	0.26	0.33
Protein (%)	23.2	23.3	24.4	22.2	0.93	0.14	0.85	0.65	0.72
Fat (%)	2.33	2.07	2.17^{B}	4.10^{A}	0.61	0.00	0.11	0.26	0.67
pH^{u}	6.1	5.9	6.1	6.0	0.81	0.31	0.12	0.06	0.06
CBMT	3.28	2.26	2.49	2.97	0.06	0.37	0.36	0.27	0.17
Enterobatteriacee	1.04	0.73	0.82	0.95	0.15	0.55	0.64	0.25	0.21

^{a,b}: means with different letters on the same row differ significantly for P≤0.05. $^{A, B}$: means with different letters on the same row differ significantly for P≤0.001. *: χ value.

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INFLUENCE OF CAGE OR GROUND PEN HOUSING ON CARCASS TRAITS AND MEAT QUALITY OF RABBIT

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ABSTRACT

To meet new societal expectations, an alternative housing system to the cage, called CUNILOFT®, has been developed by Mixscience to allow rabbits to be kept in groups on the ground, while maintaining good working conditions for the breeder. The objective of this study was to evaluate the influence of this alternative housing system on carcass traits and meat quality of rabbit. Tree consecutive trials were conducted with a control group of 288 rabbits (C-Cage group) located in 48 standard cages (18.1 rabbits/m²) and successively, a total of 564, 432 and 486 rabbits (P-Pen group) housed in 3 collective ground pens group (12.9 rabbits/m²; 10.9 rabbits/m² 11.9 rabbits/m²). We measured carcass performances, cutting pieces quantity and meat quality. Final live weight was moderately affected in P-Pen group (-1.5%, P<0.001). A lower carcass yield was accompanied by a lower adiposity score (-6.8% in P-Pen group, P<0.001). However fore part, saddle and hind legs yields were not influenced by the type of housing. Pen reared rabbits showed a tendency for a higher femur bone weight (+2.3%, P<0.10) probably due to a higher calcification as already assumed by literature (Dalle Zotte *et al.*, 2009). This assumption is consistent with maximum force of hind leg bone observed in P-Pen group (+7.1%, P<0.01). This hence probably contributed to lead to a lower hind leg meat-to-bone ratio (-4.2%, P<0.05. Colour measurements did not put forward any differences between groups. Water loss during cooking was higher for the P-pen group (+17.6%, p<0.05). A blind tasting revealed a statistical tendency toward a better appreciation (+6%, P<0.10) of the meat of rabbits reared in pens. However, the other tasting criteria were not statistically different although taste and texture appreciation score were numerically higher in P-Pen group.

Key words: Ground pen housing, carcass yield, meat quality, Cuniloft®

INTRODUCTION

Developments in society and the latest scientific advances which aim at better characterizing animal welfare, are leading us to reconsider the cage-rearing system, still commonly used in France for raising rabbits. Regulations will evolve soon as the European Commission (2021) has responded positively to a citizens' initiative entitled "End the Cage Age", and is committed to put an end to the use of cages, in favor of alternative housing.

Taking into account the considerations highlighted by the European Food Safety Authority (EFSA, 2020), namely that to improve the animal welfare, it is necessary to increase and improve the space and structure, Mixscience has developed an alternative housing system to the cage. This system was developed to enable rabbits to be raised in collective groups of 100 to 300, allowing them more movement than in a cage (Gohier *et al.*, 2023), thus encouraging the expression of their natural behaviors.
Some studies revealed a carcass and meat impacts of stocking density and collective pens systems in medium groups (≤80 rabbits) (Metzger *et al.,* 2003). The aim of the present study was to identify how the rabbits housed in collective pens in large groups (144 to 188 rabbits) affect the quality of carcass and meat.

MATERIALS AND METHODS

The trial was conducted in 3 successive bands on a commercial breeding from February to July.

The Cuniloft® system developed by Mixscience, designed to maintain good working conditions for the breeder (working time and ergonomy), consists here of a module of 3 pens within a building. In each pen, burrow and platforms offered the rabbits the possibility of taking refuge.
852, 720 and 774 rabbits were divided successively into two groups at weaning (36 days old), according to their weight. A first group (P-Pen group) made up of 3 collective ground pens with respectively 188, 144 or 162 rabbits per pen (i.e. a density 12.9, 10.9, 11.9 rabbits/m² equivalent to an available area of 774, 917 or 843 cm²/rabbit). A second group (C-Cage group) of 48 standard cages with 6 rabbits per cage (i.e. 288 rabbits and a density of 18.1 rabbits/m², equivalent to an available area of 551cm²/rabbit). The rabbits were reared until 70 days.
Feed consumption could not be measured precisely as it was an automatic screw distribution system. Both groups of rabbits were fed a single fibrous fattening feed throughout the fattening period. Rationing was "hourly" using the Durefix® method: the animals were given an unlimited quantity of feed, distributed at fixed daily times. The rabbits also had unlimited access to water.
The farm has an on-site slaughterhouse. The day before slaughter, the rabbits were weighed and 240, 160 o 170 (successively for 3 trials) were selected on the average of their live weight group and buckled. These identified rabbits are monitored to measure carcass quantity before and after chilling (2h at 1.5°c), adiposity (using INRAE measuring grid: score 1 to 5) and cutting pieces quantities (front with head, saddle and hind legs). Additional measurements were taken on legs, colorimetry (L*a*b scale from Honikel, 1998) with a Minolta chromameter, bone quality, cooking water losses (after 20 min cooking at 85°c), and a blind organoleptic tasting test organized with a specialized laboratory (65 adult consumers : 18% men and 82% women) : Consumers were asked to evaluate the 2 rabbit leg products in a sequential monadic design, according to a balanced presentation plan (some consumers had P-Pen meat as their 1st product, others C-Cage meat first).

Statistical Analysis
The slaughter results were performed with R software (version 4.4.0), using a mixed linear model with the housing effect as a fixed factor and the trial effect as a random factor.
Sensory analysis was processed using Student T-tests on paired data.

RESULTS AND DISCUSSION

Effect of housing system on final live weight, carcass yield and adiposity
Final live weight was lower in P-Pen group (-1.5%, P<0.001). Summer period accompanied by was obtained by Maertens and Ven Herck (2000), and by Matics *et al* (2019).
Significant results were observed on hot and cold carcass yield which is different from Combes *et al.* (2010) and Pinheiro *et al.* (2011) observations. In our trials, the lower carcass yields may be explained by the lower adiposity scores (-6.8% in P-Pen group, P<0.001). Combes *et al.* (2010) reported a significant lower adiposity in large pens.
We did not observe any difference in fore part, saddle and hind legs yields contrary to numerous studies such as Combes *et al.* (2010) or Pinheiro *et al.* (2011) who found more development of hind parts. The explanation may be due to the higher variability of part yield datas in our samples and the lower global carcass yield average observed in our trial compared to other trials influenced by the slaughtering method.
A tendency for a higher femur bone weight was measured in P-Pen group (+2.3%, P<0.10) probably due to a higher calcification as already assumed by literature (Dalle Zotte *et al.*, 2009). This assumption is consistent with maximum force of hindleg bone observed in P-Pen group (+7.1%, P<0.01). All these results are similar with those obtained by Martrenchar *et al.* (2001), who indicate that the bones of rabbits raised in pens are heavier, with a larger diameter and less elastic than those raised in cages. This hence probably contributed to lead to a lower hind leg meat-to-bone ratio (-4.2%, P<0.05), in accordance with the observations of Dalle Zotte *et al.* (2009) and Matics *et al.* (2014).

Colour measurements did not put forward any differences between groups. Water loss during cooking was higher for the P-pen group (+0.68pts, p<0.05). Combes *et al.* (2010) found similar results for water loss but different conclusion regarding yellowness of meat which was varying for pen reared rabbits compared to cage reared rabbits.

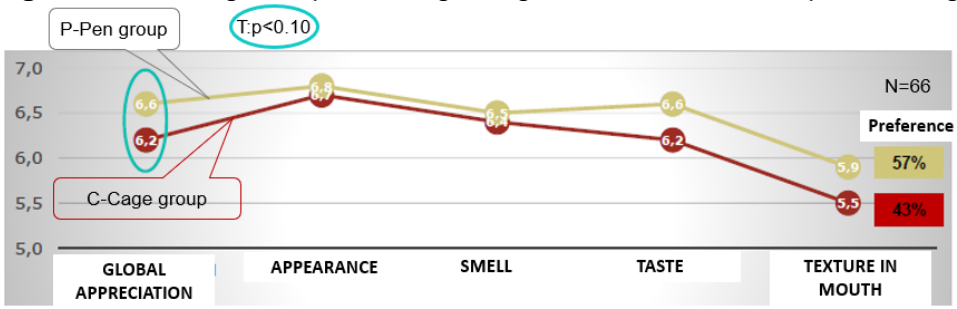
Table 1: Effect of the housing system on rabbit slaughter traits, cooking loss and rheological traits in hind leg

	Trial 1		Trial 2		Trial 3		Mean difference (Pen - Cage)	Housing Effect *(P value)*	RCV (%)
	Cage (C)	Pen (P)	Cage (C)	Pen (P)	Cage (C)	Pen (P)			
Rabbits, no.	60	180	40	120	50	120			
Slaughter weight 70d (g)	2 624	2 510	2 523	2 482	2 207	2 236	-35.6	***	4.3
Hot carcass weight (g)	1 454	1 374	1 386	1 328	1 159	1 178	-32.9	***	6.6
Hot carcass yield (%)	55.4	54.8	54.9	53.5	52.5	52.7	-0.5	**	4.3
Chilled carcass weight (g)	1 417	1 340	1 354	1 296	1 133	1 147	-33.0	***	6.6
Cold carcass yield (%)	54.0	53.4	53.6	52.5	51.3	51.3	-0.5	**	4.3
Carcass adiposity (rating 1 to 5)	2.98	2.59	2.80	2.63	1.52	1.46	-0.17	***	29.0
Rabbits, no.	30	90	40	40	-	-			
Liver (g)	63.7	65.0	61.4	59.4	-	-	0.8	ns	11.0
Fore part (%)	37.4	37.7	37.6	36.6	-	-	-0.2	ns	5.3
Hind leg (%)	31.9	32.0	31.6	32.2	-	-	0.3	ns	5.9
Saddle (%)	26.1	25.6	26.6	26.5	-	-	-0.5	ns	6.2
Hind leg bone (g)	11.4	11.6	10.8	11.1	-	-	0.3	T	6.8
Hind leg meat-to-bone ratio	18.3	17.3	18.6	18.3	-	-	-0.8	*	8.5
Rabbits, no.	30	30	20	20	-	-			
Hind leg cooking loss (g/100g)	3.87	4.55	-	-	-	-	0.68	**	19.9
Breaking force (N) of hindleg bone	258.9	255.7	215.1	229.1	-	-	3.7	ns	26.0
Maximum force (N) of hindleg bone	292.8	311.4	259.1	280.4	-	-	19.7	**	12.8
Stiffness (N/mm) of hindleg bone	225.6	235.3	201.2	206.7	-	-	8.0	ns	12.3
Colour of carcass surface over Bicep Femoris muscle									
L* (lightness)	47.9	48.1	-	-	-	-	0.2	ns	5.4
a* (redness)	0.18	-0.04	-	-	-	-	-0.22	ns	23.3
b* (yellowness)	4.8	4.7	-	-	-	-	-0.1	ns	21.7

RCV, %: residual coefficient of variation
ns, non-significant; T P<0.10 ; *P <0.05; **P <0.01; ***P <0.001.

Organoleptic taste trial. A statistical tendency would suggest that P-Pen meat was more appreciated (+6%, P<0.10) by consumers (Figure 1). However, the other tasting criteria were not statistically different.

Figure 1: Blind organoleptic tasting of legs of rabbits reared in pens or cages



CONCLUSIONS

This study showed that under the conditions of our trials, raising rabbits in ground pens (CUNILOFT® system) have moderately affected carcass traits compared to cage housing system. Nevertheless, this study demonstrated that rabbit meat tended to be more appreciated by consumers when reared in ground pens system.

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EFFECT OF EARLY CASTRATION ON ANGORA HAIR PRODUCTION WEIGHT

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ABSTRACT

The study involved 9 male French Angora rabbits neutered at the age of 40 days. They were reared and compared within siblings made up of 6 whole brother rabbits and 6 sisters. At the same time, 66 uncastrated males and 20 females were used as controls. Adult sterilised rabbits are heavier than uncastrated males but their genitalia remain small. Their fleece is similar in quality and quantity to that of females. The hair is longer than that of males and the mass of hair produced is only 11.4% less than that of females but 16.2% greater than that of whole males. This technique could make it possible in the future to find an economic interest in male Angora rabbits, which are sometimes considered to be non-valuable on farms.

Key words: Rabbit, *Oryctolagus cuniculus*, Angora, French Angora, wool, fur, animal welfare.

INTRODUCTION

It is recognised that the production of French Angora hair is quantitatively and qualitatively better in females (Rougeot and Thébault 1984). As a result, in many breeding farms, non-breeding males are not kept by breeders. Today, the civil society would like “non-breeding males” (male chicks from laying hen flocks, male calves from dairy cow lines or young rabbits etc.) to be bred in the same way as their collateral (Prospecierara 2024, BVA et al. 2020, Ministère de l’Agriculture 2022, Alteroche 2022). It was against this backdrop that we experimented with surgical sterilisation of male French Angora rabbits to see whether this technique could improve the quality and quantity of hair to restore profitability to male Angora rabbit breeders.

MATERIALS AND METHODS

Animals

These were male French Angora rabbits, 40 days old on the day of the operation (FFC 2015). They were born on two selection farms. We bred together 6 siblings composed of 9 neutered males, 6 whole males and 6 females. The animals operated on were not fasted prior to the operation.

At the same time, because we have no published standard, we collected hair production data from 66 whole males from the main farm over the first three harvests. We also collected hair production data from 20 females from the same farm over the first three harvests.

Anaesthetic protocol

The chosen protocol provides sufficient anaesthesia for more than 30 minutes.

Premedication was with Glycopyrrolate (atropine was not chosen because of the potential presence of atropinase in rabbits) at 0.1mg/Kg PV administered intramuscularly.

Anaesthesia was achieved using a mixture of ketamine hydrochloride administered at 15mg/Kg PV (live weight) and medetomidine hydrochloride at 0.1mg/Kg PV injected mixed intra-muscularly.

Prior to surgery, each sleeping rabbit received a tracer subcutaneous injection of intratesticular lidocaine hydrochloride at a rate of 0.1 ml per purse.

After surgery, each operated rabbit received a subcutaneous injection of meloxicam at 0.5mg/kg to manage any pain.

The rabbit wakes up under a heat lamp after an injection of atipamezole at 1 mg/Kg PV as soon as the operation is complete (Coquelle 2022, Boucher and Nouaille 2013, Wengers 2012).

Surgical technique

Surgical sterilisation of young male rabbits involves removal of the testicles. In this case, it was performed via the scrotal approach. After triple disinfection using alcohol and an aqueous solution of povidone-iodine, which also enables the hairs to be pressed against the skin, an incision is made in the skin of the scrotum and then in the vagina. Each testicle is removed and the testicular cords and vas deferens are cauterised. The wounds heal in the second stage.

Harvesting

The hair is harvested every 95 days. A first harvest takes place at around 70 days of age but it is of little importance in terms of quality and quantity and we removed it from the processed data to take into consideration only the 3 following harvests called harvests 1, 2 and 3. Before combing, which consists of removing the hair, the rabbit is fed a dose of Lagodendron (crushed seeds from an African mimosa) to facilitate hair loss. The animal is weighed at the time of shearing and the entire fleece harvested is also weighed (Allain et al 1999).

RESULTS AND DISCUSSION

Following other authors, we have observed that at harvest 3, sterilised rabbits have a mass close to that of the females in their family (Jehl et al 1998, Rougeot and Thébault 1984). They are heavier than whole males (but the values are not significantly different at $P<0.05$). Their hair is softer and 3 cm longer than that of whole males and is of similar quality to that of females. The paucity of data available does not allow a proper statistical analysis and we reserve the data on coat quality for a future study including more castrated animals. Their external genitalia remain small. The mass of hair produced and harvested increases with each harvest. It is always greater than that of the males in the siblings, but also greater than that of the males on the farm in general. From the second harvest onwards, it can be compared with that of females.

The low number of sterilised rabbits and the high standard deviation of the values in relation to the number of whole rabbits does not allow for very significant discrimination and does not reflect what we observe in the field. It would undoubtedly be necessary to repeat the statistical analysis with a larger number of sterilised rabbits.

Table1: Rabbit average weight over the first 3 harvests for parent rabbits.

sex	Rabbit weight 1st harvest (g)	Rabbit weight 2nd harvest (g)	Rabbit weight 3rd harvest (g)
Female siblings	2904 ± 285 a	3825 ± 51 a	4073 ± 78 a
Male siblings	2891 ± 245 a	3697 ± 76 a	3925 ± 81 a
Sterilised males	2794 ± 194 a	3755 ± 52 a	4068 ± 97 a

Data represent mean± se. Within a column, data followed by the same letter are not significantly different at *P<0.05* (Kruskal-Wallis test for harvest 1, anova for harvests 2 and 3).

Early surgical sterilisation of male Angora rabbits has the advantage of being better tolerated by the animal when performed (0.3% of fatal accidents out of nearly 3,000 cases recorded in another study carried out by the authors) (Lee et al 2018, Boucher et al. 2024).

The resulting hormonal change allows the production of more hair, which is also of better quality. It is accepted that the hair yield of a male is 40% lower than that of a female (Allain et al 1994, Rochambeau and Thébault 1989). Under the conditions of the experiment, the hair

yield of sterilised male rabbits was 11.4% lower than that of females in the comparison group, but 16.2% higher than that of whole males.
Sterilisation also means that male rabbits can live together without fighting. This is one way of meeting the need for social interaction in adult rabbits reared in a restricted environment.

Table 2: Comparison of hair mass for the three groups of rabbits over the three harvest, for both parent and non-parent rabbits

	Hair mass harvest 1 (g/rabbit)	Hair mass harvest 2 (g/rabbit)	Hair mass harvest 3 (g/rabbit)	Hair mass, average for harvests 1, 2 and 3 (g/rabbit)
Female siblings and not sibling	162 ± 6 a	247 ± 9 a	283 ± 8 a	231 ± 7 a
Male siblings or not simbling	152 ± 3 a	191 ± 2 b	208 ± 3 b	184 ± 2 b
Sterilised males	168 ± 13 a	198 ± 14 b	252 ± 9 a	204 ± 9 ab

Data represent mean± se. Within a column, data followed by the same letter are not significantly different at *P<0.05* (Kruskal-Wallis test followed by Dunn test with Bonferroni adjustment).

CONCLUSION

Surgical sterilisation of male Angora rabbits produces more hair. It also means that these rabbits can be reared in siblings without fighting. In our study, the hair yield of a sterilised male rabbit was 11.7% lower than that of a female. Knowing that a sterilised rabbit makes better use of its feed and therefore costs less to produce than a whole male, its production is perfectly compatible with the economics of an angora hair production workshop. In this way, we can hope that we will no longer have to sacrifice supernumerary male angora rabbits, which until now have been considered as non-valuable.

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MANUFACTURED NUTRITIONAL BENEFITS OF A RANGE OF DELICATESSEN PRODUCTS FROM RABBIT MEAT FED A DIET ENRICHED WITH DHA OF ALGAL ORIGIN

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ABSTRACT

Previous studies have highlighted the possibility to produce DHA enriched rabbit meat and its benefits to compensate the DHA deficit in human diets. As the level of consumption of rabbit meat is low in many countries, this work proposes rabbit meat delicatessen adapted to new trends in alimentary habits as snacks and take-away food tailored for a social and user-friendly context: preserved rabbit meat, rabbit meat spread, rabbit meat dry stick, rabbit meat jerky, the latter particularly adapted to the Chinese market. These products are derived exclusively from meat of rabbits consuming algal DHA enriched feed. Preserved rabbit meats have high levels of DHA in absolute terms (220 to 240 mg/100g) and in relative ones (4.4 % of the fatty acids FA) and they present many possible claims. Rabbit meat spread and rabbit meat dry stick also provide high absolute levels of DHA (140 to 200 mg/100g) but much lower relative ones (0.6 to 0.7% of FA), limiting the possibility of such claims. DHA enriched rabbit meat jerkies contain 100 mg of DHA/100 g representing 1.3 % of FA. All these products bring an interesting complement to the menus focusing on increasing DHA intake, particularly for a high-need people such as breastfeeding women, children, elderly, athletes and people with chronic diseases.

Key words: DHA, Rabbit, Meat, Delicatessen food

INTRODUCTION

The enrichment of rabbit meat with DHA (docosahexaenoic acid - C22:6w3) by incorporating microalgae (*Schizochytrium*) in the rabbit feed has already been reviewed a number of times (Colin *et al*., 2023). The increase of this nutrient intake reduces the risk of degenerative diseases (Krolak-Salmon, 2020), of age-related macular degeneration (Merle, 2012) and of cardiovascular diseases when combined with EPA (Eicosapentaenoic acid - C20:5w3) (Delarue, 2018). However, the world population (with few exceptions) is deficient in DHA (Colin *et al*., 2023). Menus combining DHA-enriched rabbit meat, eggs and chicken with some, albeit limited, fish can cover the population's DHA requirements without changing dietary habits (Colin *et al*., 2023). However, the low consumption of rabbits in most countries limits strongly its contribution to cover the world's DHA requirement. The intake of DHA enriched rabbit meat can be increased by proposing it as "meat delicatessen" adapted to modern ways of consumption: take-away food, snacks, tailored for a social and user-friendly context.

This publication presents different products adapted to the European market as preserved, spread and sticks rabbit meat (Palacios *et al*., 2023) and to the Chinese one (Jerky). A particularly interesting habit of rabbit meat consumption takes places in Sichuan (central China): the rabbit meat is presented in small pieces of rabbit meat jerky eaten several times a day as snack. Some results of this presentation have already been published (Palacios *et al*., 2023).

MATERIALS AND METHODS

Animals and manufacturing of rabbit meat products

This work is based on the development of a range of exclusively rabbit-meat based delicatessen products made from meat enriched in DHA through the feed. 4 types of products have been manufactured that way between 2013 and 2020.

- Preserved rabbit meat in 2013 and 2019, 2 recipes: natural and with seaweed (Same receipt that the natural one but with incorporation of Wakamé sea weed),
- Spread rabbit meat in 2019,
- Sticks rabbit meat in 2020, specially designed for on-the-go consumption,
- Jerky rabbit meat adapted to the Chinese market in 2014.

To design this DHA enriched rabbit meat jerky, 5 samples of Rabbit meat jerky meat have been collected in shops of Chengdu (China) and analyzed. Afterwards, a product has been formulated.

These products were manufactured in 2 specialized industrial units respecting the good practices of the charcuterie manufacturing (Palacios *et al*., 2023) and following 2 constraints:

- To use rabbit meat and fat as the only meat products.
- With no added chemical additives.

The 520 rabbits of this study were bred and slaughtered in a commercial farm and slaughtering plant respecting the Welfare legislation. They received a feed containing on average 0.8 % of *Schizochytrium* bringing 0.16 % of DHA. The of DHA content in the meat was between 160 and 220 mg/100g (vs. less than 20 mg/100g for a standard rabbit) (Colin *et al*., 2023).

Analysis of rabbit meat products chemical composition and fatty acids composition

The products were analyzed in pool on 4 to 6 samples to determine:

- The basic chemical composition: Moisture (Method NF V 04 401), proteins (Dumas Method), fat (Method NF V 04 401), mineral matter (Method NF V 04 401).
- Fatty acid profile (NF ISO 12966-4).

The ability to make a claim on the nutritional and health levels of these products has been considered in the context of the EU legislation for the different products.

RESULTS AND DISCUSSION

Preserved DHA enriched rabbit meat

The preserved rabbit meat is characterized by a high-level DHA, in terms of absolute content (135 mg /100 g of product) and relative one (4.4% for DHA) (Table 1). Small quantities of EPA are present (18 mg /100 g of product). These analyses confirm the potential contribution of the preserved rabbit meat to cover the human needs in omega 3 and DHA requirements. The levels of DHA + EPA exceeding 80 mg /100 kcal for the 2 recipes enables us to make the following nutritional claim (R-EC N°1924/2006) “sources of omega 3 or rich in omega 3” and to use the functional claims (R-EU N°432/2012) “help to reduce cardiovascular, cerebral and visual risks”.

DHA enriched Rabbit meat spread and rabbit DHA enriched meat stick

Spread and stick rabbit meat are much fatter than preserved rabbit meat: around 30% fat *vs.* 6.0 to 6.5% (Table 1).The relative levels of DHA in spread rabbit meat and in rabbit meat stick (% of the FA) are 1.3 to 1.8 time lower than in preserved rabbit meat (0.6 to 0.7% *vs.* 4.5%), probably due to the important use of visceral fat, containing less omega 3 than structural fats (Colin and Teillet, 2009). However, due of their high fat content, the absolute content of DHA is quite high (140 to 200 mg/100g of product). They can also help to cover human's DHA requirements as complement of a DHA-enriched menus. The DHA enriched rabbit meat spread can also claim on “the reduction of the cerebral and visual risks”. But, the absence of significant levels of EPA doesn’t not allow claiming on the cardiovascular risks.

Table 1: Nutritional characteristics of the different DHA enriched rabbit meat delicatessen

	Natural rabbit meat preserved DHA enriched	Seaweed rabbit meat preserved DHA enriched	Rabbit meat spread DHA enriched	Rabbit meat stick DHA enriched	Rabbit meat jerky DHA enriched	P value
Chemical composition						
Moisture (%)	72.1	70.2	48.4	24.9	49.9	<0.001
Protein (%)	17.5	17.7	18.4	35.5	31.7	<0.001
Fat (%)	6.3	6.0	31.8	30.2	9.0	<0.001
Energy (kcal/100g)	138	14	360	427	235	<0.001
Omega 3 content						
Omega 3 (mg/100g)	886	846	2414	2092	748	<0.001
ALA (% FA)	10.8	10.4	7.7	8.0	7.4	<0.001
ALA (mg/100g)	605	604	2185	1915	602	<0.001
DHA (% FA)	4.5	4.3	0.77	0.60	1.3	<0.001
DHA (mg/100g)	243	227	199	147	105	<0.001
EPA (% FA)	0.3	0.4	0.0	0.00	0.05	<0.001
EPA (mg/100g)	11.0	17.2	0.0	0.00	5	<0.001
DHA + EPA (mg/100g)	254	239	199	147	110	<0.001
DHA + EPA (mg/100kcal)	184	171	55.3	34.0	46.8	<0.001

FA : Fatty acid, ALA : Alpha-linolenic acid (C18:3ω3), EPA : Eicosapentaenoic acid (C20:5ω3), DHA : Docosahexaenoic acid (C22:6ω3)

DHA enriched rabbit meat Jerky

Compared with the ones collected in China, the DHA enriched rabbit meat jerkies are lower in protein (31.7 % *vs.* 37.3 %), higher in moisture (49.9 % *vs.* 41.4 %) and leaner (9.0 % *vs.* 24.7 %) (Table 2). The omega 3 and ALA level of the DHA enriched rabbit meat jerkies in percentage of FA are respectively 2.2 and 1.8 times higher than for the ones collected on the market. However, due to the high level of fat in the Chinese jerkies, these have high absolute levels of ALA, without specific enrichment, probably as consequence of utilization of fresh grass and alfalfa in the feeding of the rabbits (Gigaud and Combes, 2008).but DHA is totally absent. The level of DHA in the rabbit meat jerky DHA enriched is 1.3 % of the FA. It supplies 105 mg/100g of DHA and 44.7 mg /100 kcal, so as to be able to make the claim “source of omega 3” and consequently that it “contributes to the reduction of the cerebral and visual risks”. In conclusion, it is possible to increase strongly the nutritional value of the rabbit meat jerky very popular in some regions of China by incorporation of algal DHA in the rabbit feed.

CONCLUSIONS

In conclusion, delicatessen rabbit meats manufactured with algal DHA enriched rabbit meat provide a significant intake of DHA in a form tailored for a social and user-friendly context and consequently complement the “DHA oriented menus”. The recommendation of a weekly consumption of 50 grams of them (Colin *et al*., 2023) means an intake 120 mg of DHA, i.e. half of the daily requirement. Regarding the DHA enriched rabbit meat jerky, the consumption of 100 g/week (about 3 pieces/day) brings an intake 100 mg/week of DHA. Such products can increase the rabbit meat consumption and open new markets for rabbit meat, particularly for consumers sensitive to the intake of DHA, such as breastfeeding

women, young children, students, elderlies, athletes and people suffering from chronic illnesses. This approach is facilitated by the possibility of health and nutritional claims, as in the EU legislation.

Table 2: Nutritional characteristics of rabbit meat jerkies collected on the Chinese market and manufactured with DHA enriched rabbit meat

	Jerky rabbit meat CHINESE MARKET	**Rabbit meat jerky DHA enriched**	**DHA enriched % Chinese market**	**P value**
Chemical composition				
Moisture (%)	41.4	49.9	+20.5	<0.001
Protein (%)	37.3	31.7	-15.0	<0.001
Fat (%)	24,7	9.00	-63.6	<0.001
Energy (kcal/100g)	395	235	-40.5	<0.001
Omega 3 content				
Omega 3 (mg/100g)	888	748	-15.8	<0.001
ALA (% FA)	4.17	7.4	+77.5	<0.001
ALA (mg/100g)	876	602	-31.3	<0.001
DHA (% FA)	0.00	1.30	<100	<0.001
DHA (mg/100g)	0.00	105	<100	<0.001
DHA (mg/100kcal)	0.00	44.7	<100	<0.001
EPA (% FA)	0.00	0.05	<100	<0.001
EPA (mg/100g)	0.00	5.00	<100	<0.001
DHA + EPA (mg/100g)	0.00	110	<100	<0.001
DHA + EPA (mg/100kcal)	0.00	46.8	<100	<0.001

FA : Fatty acid, ALA : Alpha-linolenic acid (C18:3ω3), EPA : Eicosapentaénoic acid (C20:5ω3), DHA : Docosahexaenoic acid (C22:6ω3)

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EFFECT OF EXTRUDED LINSEED INTEGRATION IN RABBITS' DIET, ON MEAT'S FATTY ACID PROFILE AND MICROBIOLOGICAL QUALITY

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ABSTRACT

The effects of extruded linseeds (LN) intake on fatty acid profile and microbiological quality of rabbits' meat were investigated. A total of 69 rabbits were weaned at 35 days of age, and equally divided into three experimental groups: Control group (C), receiving a commercial diet; LN2.5 group, were fed diet containing 2.5% LN and LN5 group where rabbits were given 5% LN. At the end of the fattening period (70 days), rabbits were slaughtered. Extruded linseeds' used had a significant effect ($p<0.05$) on meat fatty acids profiles; rabbits from LN5 group showed had higher polyunsaturated fatty acids (PUFAs) n-3 content ($P<0.001$), particularly linolenic acid C18:3n3, and lower SFA ($P<0.05$) compared to LN2.5 and C groups. Hence, LN5 and LN2.5 groups had lower ($P<0.001$) n-6/n-3 ratio. For microbial count meat was safe up to 14 days of storage without exceeding the threshold. LN5 group showed significantly lower total viable counts (TVC) and *Escherichia Coli* (*E.coli)* count, throughout storage period, thus LN supplementation inhibited microbial growth. Therefore, LN could be used in rabbit feeding in order to improve meat products' nutritional value, with better fatty acids content and microbiological quality.

Key words: Rabbit, linseed, meat, fatty acids, microbiological quality

INTRODUCTION

Worldwide, there is an increased demand for safer meat product with higher nutritional values, and recently there has been increasing interest in PUFA-enriched meats due to their beneficial impact on heart health and potential chemo-protective purposes (Huang and Ziboh, 2001). Rabbit meat is characterised by low lipid and cholesterol levels and high polyunsaturated fatty acid (PUFA) content (Dalle Zotte, 2002; Hernandez, 2008); therefore, rabbit meat consumption could be healthy for consumers. Numerous researches have been undertaken aiming to produce dietetic rabbit meat, with a better nutritional value through dietary supplementation. The most used ingredient to increase PUFA level and to balance the n-6/n-3 ratio of feed and animal products is linseed. Many studies have proposed the dietary use of linseed to obtain meat with raised n-3 PUFA in rabbits (Dal Bosco *et al.*, 2014; Peiretti and Meineri, 2010). The purpose of this study, therefore, was to investigate the effect of the dietary extruded linseed supplementation on meat quality (fatty acids profile and microbiological quality) in rabbit.

MATERIALS AND METHODS

Animals and experimental design

Sixty-nine New Zealand white rabbits weaned at 35 days of age, were assigned to three homogeneous groups of 23 rabbits and were given three different diets: Control (C) diet with no extruded linseed (LN) added; LN2.5 diet containing commercial diet with 2.5% of LN supplementation and LN5 where animal were fed a commercial diet with 5% of LN supplementation. For each group, rabbits were housed in eight cages: seven cages containing three and one cage containing two rabbits. Cages were wired (L×W×H: 390 × 370 × 290 mm) and the feeding program provided a daily administration of pelleted feed. Water and feed were supplied *ad libitum.* The temperature in the rabbitry was 20 ± 3 °C and the lighting schedules were 14 to 16 hours of light per day. At the end of the trial (70 days), rabbits were slaughtered and the dissection procedures were performed according to the recommendation of the World Rabbit Science Association (Blasco and Ouhayoun, 1996). For each treatment, the *Longissimus thoracis et lumborum* (LTL) were excised from both sides of carcasses, trimmed of all external fat and epimysium connective tissue, then, meat samples were stringed and stored in a permeable plastic box at 4°C for 14 days, for microbiological analysis. Ground meat mixes from thorax, loin, forearm and leg frozen at −80 °C for analyses of fatty acid (FA) profile.

Microbiological analysis

The microbiological parameters were measured on days 0, 4, 7 and 14 of storage. Plate Count Agar (PCA), Violet red bile lactose (VRBL), Sorbitol MacConkey agar (SMA), Hektoen Enteric agar (HEA) and Chapman agar (CA) were used to enumerate total viable counts (TVC), *Escherichia Coli, Pseudomonas, Enterobacteriaceae* and *Staphylococcus aureuscounts,* respectively. The microbial counts were expressed as log10 of colony forming units/g (log CFU/g).

Fatty acids measurement

For fatty acid methyl esters (FAME) quantification, meat lipids were extracted in duplicate according to Folch *et al.* (1957) method and the methyl fatty acid esters (FAME) were then quantified using a gas-chromatograph (GC 6890N Agilent, Inc., Santa Clara, CA, USA) with C19:0 as an Internal standard. Fatty acids methyl esters were identified by comparing the peaks retention time of each compound with standard peaks and were expressed as a percentage of the total FAME (% of total FAME).

Statistical analysis

The effect of dietary supplementation on rabbits' meat FA profile was processed by a one-way analysis of variance ANOVA method, using the GLM procedure (SAS, 2003). Rabbits' meat microbiological quality was assessed using the mixed procedure considering dietary treatment as fixed effect and time as a repeated measure. The individual rabbit was used as the experimental unit. Differences among groups were tested by the Tukey test. A P-value of $P<0.05$ was considered significant for all measurements.

RESULTS AND DISCUSSION

Effect of extruded linseed (LN) incorporation on rabbit's meat microbiological quality

The results of microbiological counts of meat samples are reported in Table 1. The results show that the microbial population significantly ($p<0.001$) increased during the storage days. Meat samples from all groups had lower microbiological counts at day 0 and increased with storage time from day 4 to 14. No microbial growth was detected for *E.coli* until the end of storage period where the lowest count was recorded in LN5. In fact, LN use, at a dose of 5%, significantly reduced *E.coli* growth. *E. coli* presence is generally an indicator of faecal contamination resulting mostly from defects occurring during skinning and evisceration. *For, Staphylococcus aureus* growth, meat was safe up to 4 days, then the count increased to reach values higher than 2 log CFU/g, a limit established by European legislation (EC, 2005), however, LN groups had lower count than C. *Pseudomonas* and *Enterobacteria count* were

not affected by LN dietary incorporation. Our results are in agreement with previous study in which supplementation in animal diet did not affect microbiological quality of animal food products (Ranucci *et al.* 2015).Total viable count increased with storage time (p<0.001), from 1.36 on day 0 in LN5, to reach the highest value of 3.40 log CFU/g on day 14, recorded in C group. For all groups, the count remained lower then the threshold of 5.69 log CFU/g (Regulation EC 2073/2005).In meat processing, contamination of the product depends on several factors such as the slaughter methods used, a bad carcass and meat handling, the equipment and facilities used or to the personnel involved in the operation (Cerveny *et al.,* 2009).

Table 1 : Effect of extruded linseed (LN) dietary incorporation on rabbit's meat microbiological quality during storage (Log CFU/g)

	Treatment	Storage day				p-time	p-LN
		0	4	7	14		
E.Coli	C	0	0	0	1.90	***	*
	LN2.5	0	0	0	1.97^{a}		
	LN5	0	0	0	1.31^{b}		
Staphylococcus aureus	C	1.96^{b}	2.21^{a}	3.21^{a}	3.20^{ab}	***	*
	LN2.5	1.63^{b}	2.08^{b}	2.92^{b}	3.13^{a}		
	LN5	1.52^{a}	1.86^{c}	2.62^{c}	3.10^{ab}		
Pseudomonas	C	0	1.81	2.24	2.34	***	ns
	LN2.5	0	1.68	2.30	2.37		
	LN5	0	1.19	2.23	2.44		
Enterobacteria	C	0	0	1.56	3.51	***	ns
	LN2.5	0	0	1.41	3.03		
	LN5	0	0	1.32	2.55		
TVC	C	2.05^{a}	2.00^{a}	2.33^{a}	3.40^{a}	***	*
	LN2.5	1.49^{b}	2.08^{a}	2.14^{b}	3.32^{ab}		
	LN5	1.36^{b}	1.89^{b}	2.17^{b}	3.17^{b}		

C: Standard diet; LN2.5: Standard diet +2.5%LN; LN5: Standard diet +5% LN , TVC: total viable counts ; E. Coli : Escherichia coli ; ns: no significant difference (P >0.05); *: P<0.05 ; ; **: P<0.01; ***: P<0.001 ; p-time : effect of storage day ; p-LN : effect of extruded linseed supplementation

Effect of extruded linseed (LN) incorporation on rabbit's meat fatty acids profile

The effect of LN on the fatty acid composition of rabbit meat is presented in Table 2. Total SFA content was significantly higher in C group compared to LN groups (p<0.05). This results may be due to higher individual SFA content recorded in C group, especially, palmitic acid (C16:0) which was the major SFA found. The total MUFA and PUFA were not affected by dietary supplementation. In contrast, a study about the effect of linseed incorporation in rabbit diet on fatty acid proportions showed that MUFA and PUFA were similar for all diets (Mattioli *et al*., 2020). Individual PUFA n-3 content was significantly (p<0.001) affected by linseed supplementation, mostly linolenic acid C18:3n3 where the highest value was recorded in LN5 group. In fact, several studies have shown that the use of linseed as dietary supplementation can increase α-linolenic acid (C18:3 n-3) content in rabbit meat (Dal Bosco *et al.,* 2004; Peiretti, and Meineri, 2010). For linoleic acid C18:2n6, even though no significant difference was recorded, C18:2n6 concentration was lower in LN5 group. Therefore, these results may explain the decreased n-6/n3 ratio, particularly in LN5. Thus, linseed may be a used as dietary supplementation in order to improve rabbit meat fatty acid profile by increasing PUFA n-3 levels.

CONCLUSIONS

The present study showed that extruded linseed supplementation improved rabbit's meat fatty acid profile by increasing PUFA n-3 content; hence, it inhibited TVC and *E.coli* growth during refrigerated storage leading to longer shelf life. In conclusion, LN supplementation, up to 5%, in rabbit diet could be an interesting alternative to improve meat nutritional and microbiological quality and increase its shelf life.

Table 2 : Effect of extruded linseed (LN) dietary incorporation on rabbit's meat fatty acids profile (% of total FAME)

	Treatment			
Item	C	LN2.5	LN5	P-values
∑SFA	35.78[a]	35.19[ab]	34.05[b]	*
∑MUFA	22.98	22.13	23.73	ns
∑PUFA	37.44	38.68	38.91	ns
C18:3n3 ALA	2.18[b]	3.17[ab]	6.05[a]	***
C18:2n6 LA	28.01	28.75	26.97	Ns
∑n3 PUFA	3.25[c]	4.36[b]	7.31[a]	***
∑n6/∑n3	10.41[a]	8.09[b]	4.36[c]	***

C: Standard diet; LN2.5: Standard diet +2.5%LN; LN5: Standard diet +5% LN; ns: no significant difference (P >0.05); *: P<0.05; **: P<0.01; ***: P<0.001

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LOW-INPUT DIET FOR FATTENING RABBITS: INDEXING OF THE MEAT FATTY ACID PROFILE

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ABSTRACT

The aim of this study, once the positive effect of a significant dietary increase in dehydrated alfalfa was verified, was to comprehensively indexing the fatty acid profile of rabbit meat. At 30 days of age, two hundred Martini hybrid rabbits were divided into two homogeneous groups and fed commercial pelleted feed (Control group) and pelleted feed based on very high level alfalfa (Alfalfa group). Rabbits were slaughtered at 80 days of age and fatty acid profile and the total lipids contenet were assessed on the *Longissimus lumborum* muscle.The meat lipid content showed significant decrease in the Alfalfa group and consequently a lower amount of SFA (expressed in mg/100 g of meat), however with a significant increases in n-3 and reductions in n-6 fatty acids. The comparison of the nutritional indexes showed that n-6/n-3 ratio as well as Linoleic/Linolenic acid and Hypocholesterolemic/hypercholesterolemic ratio, were able to discriminate the Alfalfa group, whereas PUFA/SFA, Unsaturation index, Atherogenicity and Thrombogenicity index and Δ5+Δ6-desaturase activity did not detect significant differences between the groups. The Healthy Fatty Indexes 1 (HFI1) showed a significant higher in Alfalfa group respect to the Control one.

Key words: rabbit, alfalfa, fatty acids, indexing

INTRODUCTION

This preliminary study is part of a PRIN National Project on the sustainability of the rabbit supply chain which aims to find management, environmental, feeding and genetic solutions to the serious period of crisis that the sector is going through in Italy. Regarding feeding aspects, in recent decades there has been a strong reduction in the use of forage in rabbit diets (down to less than 20%) in order to enhance productive performance and reduce feeding cost. This scenario is currently difficult to understand considering the problems of sustainability of animal production and in particular for the rabbit, that being a strictly herbivore, can digest fibres not competing with human nutrition and providing meat with high nutritional value. Therefore, diets high in forage content should be carefully examined to reduce the dependence of the rabbit's diet on grains and protein crops. Among these, alfalfa is taking on an important role in rabbit nutrition, considering that its lipids consisting of mono- and digalactosidiglycerides, contain approximately 80% of linolenic acid (Van der Veen and Olcott, 1964), the precursor of PUFA n-3 long chain, whose role in human health is scientifically proven (Inner and Calder, 2020).
For this reason, it is necessary to carry out a precise assessment of the fatty acid profile to classify foods according to their nutritional/functional properties. The aim of this study, once the positive effect of a significant increase in dehydrated alfalfa was verified, was to comprehensively indexing the fatty acid profile of rabbit meat.

MATERIALS AND METHODS

Animals and experimental design

The trial was carried out on Valleuno rabbit farm (San Leo, Italy). At the weaning (30 days of age) two hundred Martini hybrid rabbits were divided into two homogeneous groups and fed

commercial pelleted feed (Control group; 20% of dehydrated alfalfa) or experimental pelleted feed based on 80% of alfalfa (Alfalfa group), both for the post-weaning and fattening periods. Thus, both post-weaning and fattening diets based on alfalfa were differed by their high crude fibre content, compared to the control diet that had a higher percentage of ether extract (data not shown).
Naturally, such large change in formulation ingredients determined different chemical characteristics of feeds.
At 80 days of age, rabbits were slaughtered in in a slaughterhouse approved by the EU, 12 h after feed withdrawal. The rabbits were sacrificed by severing the carotid arteries and jugular veins following electro-stunning and the carcasses were prepared according to the methods described by Blasco and Ouhayoun (1993) and immediately transferred to the Laboratory of the Department of Agricultural, Food and Environmental Science (Perugia). Following carcass chilling (24 h at + 4 °C), the two *Longissimus lumborum* muscles were removed and carefully freed from connective and adipose tissues.

Chemical Analyses
The measurement of intramuscular fat content was based on the methods of Folch et al. (1957). Total lipids were extracted in duplicate from 5 g of each homogenised sample and calculated gravimetrically. The fatty acid composition was determined by gas chromatography. The separation of fatty acid methyl esters (FAME) was performed with an Agilent capillary column (30 m × 0.25 mm I.D, CPS Analitica, Milan, Italy) coated with a DB-Wax stationary phase (film thickness of 0.25 µm). Individual fatty acid methyl esters were identified based on the retention time of Henicosanoic acid (C21:0) methyl ester added before extraction as an internal standard. Fatty acids proportion (%) and the FA quantification based on the conversion on fat quantity (Weihrauch et al., 1977) was reported. The average amount of each fatty acid was used to calculate the sum of the saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA).

Indexes
All the indexes considered in this study, with the relevant bibliographical reference, are summarized in Table 1.

Table 1. Fatty acid indexes compared

Index	FA expression	References
PUFA/SFA	%	Many Authors
n-6/n-3 ratio	%	Simopoulus, 2008
Linoleic/Linolenic ratio	%	Undurti, 2006
Unsaturation Index (UI)	%	Shahidi and Zhong, 2010
Index of Atherogenicity (IA)	%	Ulbricht and Southgate, 1991
Index of Thrombogenicity (IT)	%	Ulbricht and Southgate, 1991
Hypocholesterolemic/Hypercholesterolemic (HH)	%	Santos-Silva et al., 2002
Δ5-desaturase + Δ6-desaturase	%	Sirri et al., 2011
QuantiN-3 index	mg/100 g	Dal Bosco et al., 2021
Healthy fatty index 1	mg/100 g	Dal Bosco et al., 2021
Healthy fatty index 2	mg/100 g	Dal Bosco et al., 2021

Statistical Analysis
The statistical analysis was carried out with the ANOVA model where the fixed effect evaluated was the diet. For the multiple comparison, the Bonferroni ad hoc test was used, with an alpha value of 5%.

RESULTS AND DISCUSSION

The lipid content and the classes of fatty acid profile expressed in percentage and weight (mg/100 g meat) of the *Longissimus lumborum* muscle are presented in Table 2. The lipid content of the two experimental groups showed significant decrease in the Alfalfa group. This result is linked to the different levels of fat of diets (3.5 and 3.8 % respectively in the Control post-weaning and fattening diets; 1.65 and 1.85 % respectively in the Alfalfa post-weaning and fattening diets) Unexpectedly, this last group showed a higher of SFA with a reduction of MUFA and PUFA even if, when expressed in mg/100 g, the reduction in lipid content determined an inverse trend. In short, the whole fatty acid profile was affected by dietary supplementation, although the n-3 and n-6 series were the most affected, as previously observed in our studies (Dal Bosco et al., 2012; 2014; 2015; Mattioli et al., 2019); Indeed, independently from the expression in % or in weight, the alfalfa group showed significant increases in n-3 (almost double) and reductions in n-6 fatty acids.

Table 2. Lipid contents (g/100 g meat), fatty acid classes (g/100 g fatty acids and mg/100 g meat) and main indexes of *Longissimus dorsi* muscle of rabbit

	Control	**Alfalfa**	**SEM**	**Control**	**Alfalfa**	**SEM**
Lipids (g/100 g meat)	1.28^{b}	0.99^{a}	0.12			
	% of total FA			mg/100 g meat		
Total SFA	34.91^{a}	38.52^{b}	4.54	345.00^{b}	318.80^{a}	75.14
Total MUFA	26.15^{a}	24.14^{b}	4.70	258.43	200.15	40.23
Total n-6	32.27^{B}	27.10^{A}	3.37	318.91^{B}	224.69^{A}	39.42
Total n-3	2.65^{A}	5.01^{B}	1.54	26.19	41.54	3.98
Total PUFA	35.13^{a}	32.12^{b}	3.04	347.17^{b}	266.31^{a}	49.87
PUFA/SFA	1.01	0.83	0.12			
n-6/n-3	12.18^{B}	5.41^{A}	2.14			
LA/ALA	22.87^{B}	5.88^{A}	3.01			
Unsaturation Index	108.15	101.14	12.02			
Index of Atherogenicity	0.52	0.63	0.18			
Index of Thrombogenicity	0.92	0.91	0.20			
Hypocholesterolemic/ Hypercholesterolemic	2.17^{B}	1.74^{A}	0.29			
Δ5+Δ6-desaturase	0.19	0.19	0.08			
Quanti n-3 index				2.25^{A}	5.06^{B}	1.78
Healthy fatty index 1				171.95^{A}	198.93^{B}	27.96
Healthy fatty index 2				3.52	3.36	0.48

N = 20 per group; a..b: P<0.05; A..B: P< 0.001;.

The comparison of the nutritional indexes showed that n-6/n-3 ratio as well as linoleic/linolenic acid and Hypocholesterolemic/Hypercholesterolemic, were able to discriminate the Alfalfa group (P<0.001); whereas PUFA/SFA, Unsaturation index, Atherogenicity and Thrombogenicity index and Δ5+Δ6-desaturase activity did not detect significant differences between the groups.

The last three indexes would have in mind the connection of the fatty acid profile with the total lipid content, in the belief that these nutritional traits cannot be evaluated separately (see Dal Bosco et al., 2021). In particular, the quantiN-3 index relates the PUFA n-3 expressed in weight (mg/100 g) of meat with the quantity (g/100 g) of fat.

The Alfalfa group showed a value more than double compared to the Control one (P<0.001), highlighting the health properties that the increase in dietary levels of dehydrated alfalfa had on the rabbit meat.

The Healthy Fatty Indexes (1 and 2), which careful differentiate the various classes of fatty acids (by unsaturation and by the position of the double bonds), partly following the indications of Ulbricht and Southgate (1991), but relating to the quantity of lipids of meat (HFI1). In HFI2, the various classes of fatty acids were considered by increasing or decreasing their relative content expressed in weight according to their health impact,

deduced from the consolidated scientific literature. These two indexes showed different trends and in particular, the HFI2, in its complexity, flattened the differences between the two groups in a somewhat unexpected way, considering the large difference in beneficial fatty acids present in the Alfalfa group. On the contrary, HFI1 was able to significantly discriminate ($P<0.001$) the two dietary treatments, showing a much higher, and therefore healthy, value in the meat of rabbits of Alfalfa group.

The aim of this study was only to stimulate the attention of the scientific community on the complexity of fatty acid indexing to assess the nutritional or health potential of rabbit meat. Without prejudice to the great opportunity that the massive use of alfalfa could represent from a sustainability point of view, there is no doubt that from a nutritional point of view there are many advantages, even if not all the indexes are aligned; this necessarily will bring to make further reflections, which will be the subject of future research.

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POST-MORTEM CHILLING OF CARCASSES FOR 18 HOURS PRIOR TO FAST FREEZING ENHANCES THE TENDERNESS OF BOTUCATU RABBIT MEAT

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ABSTRACT

The objective of this study was to evaluate the influence of carcasses' chilling time prior to fast freezing at -18ºC on tenderness of the thawed meat from male and female Botucatu rabbits. Shear force, myofibrillar fragmentation index and sarcomere length were analyzed in the *longissimus lumborum* muscle. The results indicated that *rigor mortis* process began 1.5 hours *post-mortem*, with its establishment between 4.5 hours and 6 hours and resolution with 6 hours of chilling. Shear force was greater ($P<0.05$) for not chilled carcasses, indicating greater hardness of the samples from time 0h compared to the other chilling times. Sarcomere length was shorter ($P<0.05$) from 18h *post-mortem* onwards. Myofibrillar fragmentation index increased ($P<0.05$) with chilling time. None of the tenderness parameters evaluated were influenced ($P>0.05$) by the sex of the animals. It is concluded, therefore, that chilling the carcasses for 18 hours *post-mortem* before fast freezing them provides great tenderness to the meat of Botucatu rabbits.

Key words: Meat quality, Myofibrillar fragmentation index, Rabbit production, Sarcomere length, Shear force.

INTRODUCTION

Rabbit carcasses chilled at temperatures close to the freezing point in the first hours *post-mortem* may lead to quality loss due to cold shortening (Jolley *et al*. 2007), especially when frozen before *rigor mortis* establishment, as result of the phenomenon already known as thaw rigor (Lawrie, 2007). These extreme temperatures rightly after slaughter promote different acidification rates and muscle contraction, which can compromise the myofibrillar structures in such a way that the tenderness of the meat might be irreversibly altered (Hite, 2021).

Beside the carcass's storage processes, the emergence of new genetic lineages has also demanded more studies about rabbit meat quality. This is the case of the Botucatu genetic group, composed by medium-sized animals selected for litter size and growth. Originated from Norfolk 2000 hybrid rabbits (White New Zealand vs California vs Blanc de Bouscat) (Moura *et al*., 2000, 2001), Botucatu rabbits have been recently implemented on rabbit farming in Brazil, presenting good results in terms of feed efficiency and productive performance, however studies focused on their meat quality are needed (Bianospino *et al*., 2006).

Given this scenario, the present study aimed to determine the ideal chilling time used in the first 24 hours *post-mortem* for male and female Botucatu rabbit carcasses prior to fast freezing at -18ºC in the industry, through the evaluation of physicochemical properties related to the tenderness of the *longissimus lumborum*.

MATERIALS AND METHODS

Animals and experimental design

160 carcasses of male and female rabbits (n=80/sex) of Botucatu lineage [slaughtered at 90 days of age in a commercial slaughterhouse, with average live weight of 2.97kg] were used. The animals were raised in the Experimental Rabbit Farming at UNESP, Jaboticabal, Brazil, under the same environmental conditions, food and health management, with approval of Commission of Ethics in the Use of Animals (CEUA) of the above Institution (protocol no. 1924/22). All rabbits were subjected to 12-hours pre-slaughter fasting (Cavani and Petracci, 2004) and stunned by electronarcosis (110V, 60Hz, 1.40A, within 3 seconds), following the recommendations given by the Regulation (CE) No. 1099/2009 (Council Regulation, 2009).

The hot carcasses were stored in a cold room under the air circulation speed of 0.3 to 1.0 m/s and 2 ± 2ºC, which were subjected to eight different chilling times (T): 0h, 1.5h, 3h, 4.5h, 6h, 12h, 18h and 24h (n=20 carcasses/time) + fast freezing (-18ºC). The carcasses from time 0h were immediately frozen, without prior chilling in the cold room. It was decided to fast freeze all carcasses at the end of cooling with the purpose of following the practices adopted by the Brazilian industry. After 24 hours of fast freezing the last group, all carcasses were transported to the Laboratory of Analysis of Animal Products of the Department of Agricultural and Environmental Biotechnology at UNESP, where remained frozen for 48 hours until they were thawed in air-conditioned chambers, under the temperature of 4ºC for 24 hours. Then, deboning was carried out, under 10ºC, in order to collect the *longissimus lumborum* muscle, to sequentially proceed with the analyzes of shear force, fragmentation index myofibrillar and sarcomere length.

Shear Force

Cooled cooked samples were used, cut into sections of approximately 1cm^2, which were positioned with the fibers oriented in a perpendicular direction to the Warner-Bratzler blaze, coupled to the Texture Analyzer TA-XT2i texturometer, and subjected to cutting in triplicate, which expressed the force required to shear the samples in Kgf, according to the method described by Lyon *et al*. (1998).

Myofibrillar Fragmentation Index

Determined in triplicate using the methodology proposed by Culler *et al*. (1978) and complemented with the use of the biuret technique (Gornal *et al*., 1949), used to determine the concentration of proteins in the myofibrillar suspension originating from the muscle samples, on its cranial and distal portion, respectively, using the formula MFI= optical density x 200.

Sarcomere Length

Determined by Cross *et al*. (1981). 0.5 g of subsamples from distal and cranial portions of the muscle were homogenized in Ultra-Turrax with 30 mL of potassium chloride 0.08 mol/L and potassium iodide 0.08 mol/L, at speed of 15000 rpm, for 30 seconds. A drop of the homogenate was transferred to a slide and covered with a coverslip. 12 readings/slide were taken in an optical microscope using phase contrast at 1000x magnification (100x objective, 10x eyepiece). Sarcomere length was expressed in µm.

Statistical Analysis

A completely randomized design distributed in 2 x 8 factorial scheme (sex vs carcass chilling time) with 10 replications was assigned. The results were analyzed with Proc GLM from SAS statistical program (SAS Institute Inc, 2002 – 2003, Cary, NC, USA). The data were tested by analysis of variance (ANOVA) and the means compared by Tukey test, with significance level set at 5%.

RESULTS AND DISCUSSION

It was observed effect of carcass chilling time (P<0.0001) on shear force, myofibrillar fragmentation index and sarcomere length. However, these variables were not influenced by the sex of the animals (P>0.05).

The shear force was greater in carcasses that were not chilled (1.930 Kgf), indicating greater hardness for those samples in relation to the chilled ones (1.298 – 1.495 Kgf). The myofibrillar fragmentation index gradually increased as the carcass chilling time progressed (61.29 – 83.99). The sarcomere length pattern was inversely proportional to that found for MFI, with the highest values registered in samples from time 0h (2.40 µm), gradually reducing in size as the carcass chilling time progressed, demonstrating maximum contraction at 18h and 24h *post-mortem* (1.87 and 1.82 µm).

Table 1: Means (± SEM) of shear force (SF), myofibrillar fragmentation index (MFI) and sarcomere length (SL) for the *longissimus lumborum* muscle of Botucatu rabbits as function of sex and carcass chilling time during the first 24 hours *post-mortem*.

	SF (Kgf)	MFI	SL (µm)
Sex (S)			
Female	1.457 ± 0.05	71.28 ± 1.38	2.08 ± 0.02
Male	1.443 ± 0.04	74.39 ± 1.54	2.10 ± 0.01
Carcass chilling time (T)			
0h	1.930 ± 0.09^{A}	61.29 ± 3.23^{D}	2.40 ± 0.03^{A}
1.5h	1.472 ± 0.07^{B}	66.60 ± 1.74^{CD}	2.22 ± 0.03^{B}
3h	1.298 ± 0.07^{B}	66.37 ± 3.14^{CD}	2.16 ± 0.02^{BC}
4.5h	1.378 ± 0.07^{B}	70.54 ± 2.31^{BCD}	2.12 ± 0.03^{BCD}
6h	1.445 ± 0.09^{B}	74.35 ± 2.19^{ABC}	2.10 ± 0.02^{CD}
12h	1.296 ± 0.07^{B}	77.72 ± 2.29^{AB}	2.04 ± 0.02^{D}
18h	1.495 ± 0.08^{B}	81.83 ± 2.25^{A}	1.87 ± 0.02^{E}
24h	1.302 ± 0.08^{B}	83.99 ± 2.17^{A}	1.82 ± 0.02^{E}
P - value			
P (S)	0.8436	0.0709	0.3621
P (T)	< 0.0001	< 0.0001	< 0.0001
P (S x T)	0.3116	0.1766	0.3009
CV (%)	2.98	14.84	13.33

$^{A-E}$ Means followed by different letters in the columns differ from each other using the Tukey test (P<0.05); SEM: standard error of the mean; CV: coefficient of variation; S: sex; T: carcass chilling time.

Shear force is an important indicator of meat tenderness (Xiao *et al*., 2020); just as sarcomere length is an indicator of muscle contraction, that also reflects the meat tenderness (Hopkins *et al*., 2011). During the development of *rigor mortis*, the sarcomere retracts and subsequently elongates on *post-rigor* as result of its resolution, while shear force varies according to these changes in sarcomere size (Xiao *et al*., 2020). Generally, increase of shear force occurs concomitantly with shrinkage of sarcomere length (Wheeler and Koohmaraie, 1994). Lan *et al*. (2016), when evaluating the tenderness of rabbit hindlegs, also found that shear force tended to decrease with cold storage time. However, the destruction of the myofibrillar structure under the action of endogenous enzymes can overcome the toughness induced by sarcomere shortening, producing meat with lower shear force during storage (Wheeler and Koohmaraie, 1994), as observed.

Myofibrillar fragmentation index is more related to the level of muscle maturation or protein degradation (Birkhold and Sams, 1995) mediated by the action of myofibrillar proteolysis of intermediate filaments and the enzymatic activity of calpains and cathepsins. Proteins such as desmin, troponin T and tinin degrade and cause weakening of myofibrils and subsequent tenderization of the meat (Carrillo-Lopez *et al*., 2021). Besides, the hypothesis that the loss of resistance of the cell membrane caused by the formation of ice crystals may also have

contributed to the reduction in force required to shear the samples cannot be ruled out (Leygonie *et al*., 2012).

Regardless the mechanism of action, this index predicts at least 50% of the variation in meat tenderness (Hopkins *et al*., 2000). The higher this index, the greater the meat tenderness. Culler et al. (1978), ensure that meat with myofibrillar fragmentation index above 60 has satisfactory texture. Based on that, the results demonstrated that *longissimus lumborum* of Botucatu rabbits subjected to up to 24 hours of air chilling, followed by frozen storage, is tender, although higher shear force has been observed for carcasses frozen in the pre-rigor phase.

CONCLUSIONS

It is recommended that carcasses of Botucatu rabbits are not immediately frozen at -18ºC after slaughter, without first being cooled for 18 hours at 4ºC, in order to ensure complete establishment of *rigor mortis* and its resolution, giving the rabbit meat greater tenderness.

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EFFECT OF FREQUENCY AND INTENSITY OF ULTRASOUND ON RABBIT MEAT

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ABSTRACT

High-Intensity Ultrasound (HIU) applied to rabbit meat increases tenderness and generates positive changes in colour. The objective was to evaluate the effect of different frequencies and intensities of HIU on the physicochemical (PQ) and sensory (SEN) profile of rabbit meat. thirteen rabbit carcasses were used, they were cut into half carcasses. HIU was applied to each half carcass (20 min) with the following treatments: 37kHz,150 W (T1), 40 kHz,11W/cm ^{2} (T2), 25 kHz,540 W (T3) and 50kHz, 200 W (T4) and the control treatment (without HUI). The PQ variables evaluated were pH, color (L*, a*, b* and C*), hue angle (HUE), water holding capacity (WHC), shear force (SF). Besides, a sensory tenderness classification test was carried out. Statistical analysis was performed in a completely randomized design using GLM and means with Tukey's test ($P < 0.05$). Significant differences were observed in HUE angle, yellowness, WHC and SF. All HIU treatments generated decreases in SF and WHC. T4 generated a greater decrease in SF and WHC. T1 generated the smallest decrease in WHC and SF. The panelists selected T4 as the sample with the highest perception of tenderness. The power and intensity of the treatments affect the changes observed in the PQ of rabbit meat when applying HIU in short times (20 minutes).

Keywords: cavitation, quality, muscle, tenderness

INTRODUCTION

The application of HIU has a high potential to be used in rabbit meat. Effects have been observed in the reduction of water retention capacity (WHC), decrease in pH, reduction in shear stress (SF) and changes in color parameters and decrease in marinating times (Reyes Villagrana et al., 2020; Carrillo López et al., 2021; Gómez Salazar et al., 2021). This technique is effective in increasing softness by causing disruption of the muscle structure and modifying the collagen structure (Alarcón-Rojo et al., 2019). In addition, it can improve the technological properties of rabbit meat, without compromising other quality parameters. Therefore, the objective was to determine the effect of different frequencies and intensities of HIU on the physicochemical (PQ) and sensory (SEN) profile of rabbit meat.

MATERIALS AND METHODS

The study was carried out on rabbit meat from the French Loop breed (75 d of age). The rabbits were raised under the same production and feeding conditions in a livestock production unit, located in the municipality of Delicias, Chihuahua, Mexico. The slaughter was carried out in accordance with current Mexican regulations. The carcasses (thirteen carcasses) were refrigerated 24 h post-mortem and then frozen (-18 °C) for transport (1 h), maintaining a constant temperature of -15 to -5 °C. In the laboratory, carcasses were identified and cut longitudinally along the spine. Half carcasses were obtained, individually

weighed (675.16 ± 5.4 g/half carcass) and individually packed in polypropylene vacuum bags. The half carcasses of each breed were distributed in the four ultrasonic treatments and one control treatment, each with five replicates. The application of the treatments was carried out in bath-type ultrasonic equipment with different frequencies and intensities [T1 (37 kHz, 150 W, Elmasonic ® S60H); T2 (40 kHz, 11 W/cm 2 , Branson ® 5510); T3 (25 kHz, 540 W, Elmasonic xtra ST ® 500H) and a probe type equipment [T4 (50 kHz, 200 W, Hielscher ® UP400St)] and a time of 20 min . The control half carcasses were kept in the same conditions (without HIU to simulate ultrasonic conditions). The diffusion medium for HIU and control was deionized H 2 O water (3 to 5 °C). For the physicochemical profile (PQ), the variables of: pH, color (CIEL*a*b*), Chroma, tone angle (HUE), water retention capacity (WHC) and shear stress (SF) were evaluated. A sensory test (SEN) was carried out to organize the perception of tenderness of the meat. Nine semi-trained panelists participated. The data were analyzed with the statistical package SPSS version 17 (IBM SPSS 2008) in a completely randomized design and using the general linear model (GLM) procedure. Comparing means with Tukey test ($P < 0.05$).

RESULTS AND DISCUSSION

HIU exerted a significant effect for the variables b*, Chrome, WHC and SF. The HIU generated significant decreases (P= 0.023) of b* in all treatments. T1 was the treatment that had the greatest decrease compared to the control (CON= 5.01 ± 1.91 T1= 3.63 ± 1.91). The color of the meat is dependent on the levels of myoglobin oxidation-reduction, so that at higher rates of this process the b* values increase (Peña - González et al., 2017). These decreases are positive since the increase in this coordinate (yellowness) is an undesirable quality in rabbit meat (Reyes-Villagrana et al., 2020). In Chrome the HIU generated increases in T2, T3 and T4 (P= 0.049). And it decreased in T1 (P= 0.049) The changes in color saturation in the sonicated samples are related to the instability of the hemopigments due to the effect of HIU, generating more orange tones in the meat (Alarcón-Rojo et al., 2019). In WHC, treatment with HIU generated significant decreases in all treatments (P= 0.002). T4 was the treatment that decreased the most (CON= 77.97 ± 9.4; T4= 69.58 ± 9.4). This effect is due to the fact that acoustic cavitation exerts pressures and decompressions that generate the opening of microchannels in the cell wall, through which there is a constant flow of water (Caraveo et al., 2022). In SF, treatment with HIU generated significant decreases in all treatments (P= 0.002). T4 was the lowest SF value (CON= 10.74 ± 1.11; T4= 8.11 ± 1.11). The effect of acoustic cavitation generates a double effect in which there is the breakdown and denaturation of collagen macromolecules, coupled with the breakdown of muscle tissue. This effect generates the migration of minerals and substances such as enzymes contained in lysosomes that accelerate the proteolysis process of the structural proteins of the muscle (González-González et al., 2017). This effect may be related to the result of the sensory ordering test in T4 (39 points out of 45 total), the sample with the highest perception of cuteness was found.

CONCLUSIONS

HIU exerts a positive effect on the PQ and SEN of rabbit meat, increasing tenderness in all the treatments analyzed. The HIU does not affect the quality of the color parameters of rabbit meat. But, it increases the WHC which can affect the juiciness of the meat. T4 recorded the best values regarding the measurement and perception of tenderness. HIU is an emerging technology that changes parameters of rabbit meat without affecting its quality.

Table 1. Physicochemical parameters of rabbit meat under application of different high intensity ultrasound treatments. Mean ± S.E.

	TREATMENTS					
VARIABLES	**CONTROL**	**T1 (37kHz 150 W)**	**T2 (40 kHz 11W/cm^2)**	**T3 (25 kHz 540 W)**	**T4 (50kHz 200 W)**	**P < 0.05**
			PROFILE COLOR			
L*	45.68 ± 1.13	47.63 ± 1.13	50.25 ± 1.13	49.97 ± 1.13	48.71 ± 1.13	0.53
a*	8.22 ± 2.23	5.53 ± 2.23	9.09 ± 2.23	12.25 ± 2.23	7.86 ± 2.23	0.15
b*	5.01 ± 1.91	3.63 ± 1.91	3.83 ± 1.91	3.81 ± 1.91	3.98 ± 1.91	**0.023***
HUE	70.37 ± 3.01	76.33 ± 3.01	65.27 ± 3.01	57.62 ± 3.01	65.03 ± 3.01	0.14
C*	11.23 ± 3.38	7.166 ± 3.38	12.92 ± 3.38	20.06 ± 3.38	11.84 ± 3.38	**0.049***
pH	5.70 ± 0.14	5.6 ± 0.14	5.79 ± 0.14	5.56 ± 0.14	5.81 ± 0.14	0.11
WHC (%)	77.97 ± 9.4	70.16 ± 9.4	71.46 ± 9.4	73.65 ± 9.4	69.58 ± 9.4	**0.002***
SF (N)	10.74 ± 1.11	9.61 ± 1.11	9.14 ± 1.11	8.98 ± 1.11	8.11 ± 1.11	**0.041***

P < 0.05, NS= No significant statistical differences were observed (P > 0.05), HIU= Treatment with high intensity ultrasound, L= Luminosity, a*= Redness, b*= Yellowness, HUE= Hue angle, C*= Chrome WHC= Water holding capacity, SF= Shear force

Table 2. Sensorial tenderness test of rabbit meat under application of different high intensity ultrasound treatments. Mean ± S.E.

	TREATMENTS				
JUDGE	**CONTROL**	**T1 (37kHz,150 W)**	**T2 (40 kHz,11W/cm^2**	**T3 (25 kHz,540 W)**	**T4 (50kHz, 200 W)**
1	3	2	1	5	4
2	2	1	3	4	5
3	1	2	3	5	4
4	1	2	4	3	5
5	3	4	2	1	5
6	1	3	2	5	4
7	1	3	2	5	4
8	2	4	3	1	5
9	2	5	1	4	3
SUMATORY	**$^{**}16_c$**	26_b	21_b	33_b	**$^{**}39_a$**

**P < 0.05, 1= least tenderness, 5= most tenderness.

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EFFECT OF SEX AND AGE ON THE CHEMICAL COMPOSITION OF THE *LONGISSIMUS THORACIS ET LUMBORUM* MUSCLE IN BOTUCATU RABBITS

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ABSTRACT

This study aimed to evaluate the effect of sex and age on the chemical composition of the Longissimus thoracis et lumborum muscle in Botucatu rabbits. Ten carcasses from each experimental group were used (females and males aged 3 and 12 months old). The chemical composition (moisture, lipids, protein and mineral matter) was evaluated. A completely randomized experimental design was used in a 2x2 factorial scheme, consisting of rabbits of two ages (3 and 12 months old) vs 2 sexes (females and males), with 10 replications for each treatment. The data obtained were subjected to analysis of variance and the means compared using the Tukey test (P<0.05). The sex factor influenced (P<0.05) the chemical composition of the meat, in which young females presented meat with a higher (P<0.05) concentration of lipids compared to meat from males of the same age, however, opposite results were obtained when evaluating the meat of rabbits at 12 month-old. Regarding chemical composition, meat from 12-month-old rabbits had higher (P<0.05) percentages of protein and lower (P<0.05) percentages of mineral matter when compared to meat from young rabbits. Age influences the chemical composition of rabbit meat, with meat from 12-month-old rabbits having higher percentages of protein and lower percentages of moisture compared to meat from young rabbits. Furthermore, the influence of sex must be highlighted. especially in the chemical composition of meat in rabbits

Key words: loin, *Oryctolagus cuniculus*, protein, rabbit meat farming.

INTRODUCTION

Beef rabbit farming has been expanding in recent years in several countries, reaching a global production of 861,739.35 tons of meat in 2021 (FAOSTAT, 2021). Among the main advantages of meat rabbit production are the characteristics specific to the species, such as productive characteristics (rapid growth and rusticity) (Haryati *et al*., 2021), reproductive characteristics (precocity and prolificacy) (Abubakar *et al*., 2015), associated with the use of intensive breeding systems, which optimize the production of offspring and reduce the interval between births, weaning offspring in less time, with a greater number of offspring per year (Olivares Pineda *et al.,* 2009).

However, in these intensive breeding systems, the productive lifespan of these females is increasingly shorter due to their greater use in the herd in the shortest possible time, resulting in high replacement rates of breeding females and males that have completed their productive cycle. Replacement rates of up to 120% can be observed (Ramón and Rafel, 2002), with most of these rabbits destined for slaughter along with young rabbits or sometimes being sold as pets (Castro-Gutierrez and Martinez-Castro, 2010).

Although some work has been carried out evaluating the effect of age on the quality of rabbit meat (Cavani *et a*l., 2000; Gasperlin *et al.,* 2006; De Souza *et al*., 2022), there is little information on the quality of the meat of breeding rabbits.

Thus, this study aimed to evaluate the effect of sex and age on the chemical composition of the *Longissimus thoracis et lumborum* muscle in breeding rabbits of the Botucatu line.

MATERIALS AND METHODS

Animals and experimental design

Rabbits from the Botucatu line were used, which were continuously mated and selected for culling at 12-month-old, when they were then slaughtered together with the F1 generation (three-month-old).

Forty rabbits from the Botucatu line were used for the experiment, from the same origin and which underwent the same management conditions, distributed into 4 experimental groups (T1: three month old female rabbits; T2: 12 month old female rabbits; T3: three month old male rabbits and T4: 12 month old male rabbits). Each treatment consisted of 10 replications, with the experimental unit being the rabbit itself, which were slaughtered in a commercial rabbit slaughterhouse inspected by the Federal Inspection Service (SIF).

After slaughter, the carcasses remained in a cold room (4ºC) for 24 hours and subsequently passed through a freezing tunnel (-18ºC). They were then transported in a truck with temperature control (-18ºC) to the Animal Food Analysis Laboratory (LaOra), in the Department of Agricultural and Environmental Biotechnology at FCAV/UNESP, Campus Jaboticabal, for carrying out meat analysis

After deboning, samples of *Longissimus thoracis et lumborum* were weighed, separated individually, vacuum packed and frozen in order to preserve their cellular structures, so that the following analyzes could be carried out in due course.

Chemical Composition

It was determined through analyzes of percentage moisture (method 950.46), protein (method 977.14) and ash (method 920.153), according to procedures recommended by the Association of Official Analytical Chemists (AOAC, 2011), and the concentration of total lipids (Bligh and Dyer, 1959).

Statistical Analysis

A completely randomized experimental design was used in a 2x2 factorial scheme, consisting of rabbits of two ages (3 and 12 months old) vs 2 sexes (females and males), with 10 replications for each treatment. Data were analyzed Proc GLM of the Statistical Analysis System (SAS, 2002). The results were subjected to analysis of variance and compared using the Tukey test with significance defined at $P<0.05$.

RESULTS AND DISCUSSION

There was an interaction ($P<0.05$) between sex and age on the chemical composition (moisture, lipids, protein and mineral matter) of the *Longissimus thoracis et lumborum* muscle of female and male Botucatu rabbits aged 3 and 12 months old (Table 1).

The chemical composition values obtained in the present work are within the normal standards already described for rabbits (Salvini *et al.,* 1998).

With the increase in the age of the males, there was a reduction in the percentages of meat moisture, which corroborates the literature (Cavani *et al.,* 2000; Hernández *et al.*, 2004), possibly due to the greater deposition of fat in the meat as it increases the age of the animal, causing the concentration of other components (Gondret *et al*., 1998; Hernández *et al*., 1998; Cavani *et al.,* 2000), with an inversely proportional relationship between the percentages of lipids and meat moisture (Fraga *et al*., 1978; Szendrö *et al.,* 1998). In the case of females, an increase ($P<0.05$) in the percentages of meat moisture was observed as age increased, this may be related to the decrease in lipid percentages in older females, as there is an inversely proportional relationship between moisture percentages and lipid percentages. This decrease in lipid percentages in relation to age is possibly caused by the use of the female's energy reserves during pregnancy (mainly in the last 10 days before birth) and lactation (in milk production), causing a decrease in lipid concentrations. in meat (Xiccato *et al*., 2004; Taghouti *et al*., 2021).

Table 1: Means (± SEM) of the interaction of the values of the percentage of moisture, lipids, protein and mineral matter of the *Longissimus thoracis et lumborum* muscle of female and male Botucatu rabbits aged 3 and 12 months old.

Age of the animal (A) (n=10)	Sex of the animal (S) (n=10)		P-value		
	Female	Male			
Moisture (%)			P (S)	P(A)	P(SxA)
3-month-old	69.10± 0.30^{Bb}	70.90± 0.30^{Aa}	0.613	0.212	<0.001
12-month-old	71.15± 0.32^{Aa}	69.68± 0.36^{Bb}			
Lipíds (%)			P (S)	P(A)	P(SxA)
3-month-old	4.31± 0.31^{Aa}	2.47± 0.33^{Ab}	0.006	<0.001	0.019
12-month-old	2.16± 0.31^{Ba}	1.99± 0.36^{Aa}			
Protein (%)			P (S)	P(A)	P(SxA)
3-month-old	22.45± 0.25^{Aa}	21.75± 0.25^{Ba}	0.882	0.013	0.025
12-month-old	22.52± 0.27^{Aa}	23.14± 0.32^{Aa}			
Mineral Matter (%)			P (S)	P(A)	P(SxA)
3-month-old	1.68± 0.09^{Aa}	1.17± 0.09^{Ab}	0.021	0.636	0.010
12-month-old	1.37± 0.08^{Ba}	1.40± 0.10^{Aa}			

$^{A-B,\ a-b}$ Means followed by different letters in columns (uppercase) and rows (lowercase) differ from each other using the Tukey test (P<0,05).

It was observed that 3-month-old rabbits, the meat of females presented higher (P<0.05) lipid concentrations (2.16%) when compared to males (2.47%), possibly due to the action of sex hormones in the metabolism of fatty acids, such as estrogen, which influences lipid absorption and lipogenesis (North *et al.,* 2019).

It was observed that the meat of 12-month-old males had higher (P=0.025) protein percentages (23.14%), when compared to 3-month-old males (21.75%), which is consistent with the results obtained by Gondret *et al.* (1998) possibly due to the greater deposition of protein in the meat as the animal matures.

It can be observed that the meat of 3-month-old females presented higher (P=0.01) percentages of mineral matter (1.68%) when compared to males of the same age (1.17%), which it was also observed by Gašperlin *et al.* (2006). On the other hand, at 12 months of age there were no differences between the sexes regarding the percentages of mineral matter. It is interesting to highlight that there was a decrease (P<0.05) in the percentages of mineral matter in the meat of 12-month-old females, when compared to the meat of 3-month-old females, corroborating Polak *et al*. (2006).

CONCLUSIONS

Age influences the chemical composition of rabbit meat, 12-month-old rabbits presented meat with higher percentages of protein and fat and lower percentages of moisture compared to meat from 3-month-old rabbits. Furthermore, the influence of sex on the chemical composition of meat in rabbits must be highlighted.

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STUDY ON THE EFFECT OF FREEZE-DRYING TREATMENT ON RABBIT HAIR PERFORMANCE

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ABSTRACT

Rabbit hair fibers have many excellent qualities and are a class of luxury textile materials. However, rabbit hair textiles are prone to shedding, which hinders the development and application of rabbit hair textiles. To improve the anti shedding and dyeing properties of rabbit hair fabric, freeze-drying method is used to treat the rabbit hair fabric. Freeze drying technology is a commonly used modern experimental technique, which freezes water trapped in the material into ice and directly sublimates it into vapor to remove it, thus retaining the pore in the material without changing it's morphology. Furthermore, this study also performed a combined treatment of silk fibroin and freeze-drying on rabbit hair fabrics. Through X-ray diffraction analysis, as well as testing of the relevant properties of rabbit hair fibers and textiles, the effects of freeze-drying treatment on rabbit hair fibers and the role of silk fibroin were explored. Research has found that freeze-drying treatment increases the diameter of rabbit hair fibers, decreases crystallinity, and increases the surface friction coefficient of fibers. It is believed that this is due to the phase change of water inside the rabbit hair fibers from liquid to solid, resulting in an increase in water volume and an increase in the distance between the protein macromolecular chains of the rabbit hair fibers, leading to a loose structure of the rabbit hair fibers. Therefore, both freeze-drying treatments, including individual freeze-drying and a combination of silk fibroin and freeze-drying, showed an improvement in anti shedding performance and an increase in dyeing percentage of rabbit hair fabrics. Furthermore, due to the deposition of silk fibroin inside and on the surface of the fibers, the anti shedding and dyeing performance of rabbit hair was further enhanced, The application potential of silk protein and freezing treatment in functional finishing of rabbit hair textiles, especially in anti shedding finishing, has been demonstrated.

Key words: rabbit hair; silk protein; staining performance; warmth preservation; ultraviolet resistance; freeze-drying

INTRODUCTION

Rabbit is a kind of expensive textile raw material and textiles made from rabbit hair is believed have the performance of cashmere fabrics (Onal et al., 2007; Oglakcioglu et al., 2009), but avoid the serious damage to the grassland caused by raising goats0. However, due to the smooth surface of rabbit hair fiber, as well as the small number of curls and friction coefficient, the holding force between the fibers in rabbit hair yarn is small, leading to a serious hair-shedding of rabbit hair fabric (Li et al., 2022; Wang et al., 2021). In addition, the high content of medullary layer in rabbit hair fiber result in a low dyeing percentage, which resulting in a significant color difference in the conditions of rabbit hair/wool blend fabric (a very popular textile product) dyeing, causing limited use of rabbit hair. In this study, the green clean freeze-drying technology, combined with the application of filin protein, was used to reduce hair-shedding and increase the dyeing properties of rabbit hair fabric, which is conducive to the development and utilization of rabbit hair textile products.

MATERIALS AND METHODS

Materials

Fabrics: rabbit hair fabric, rabbit hair fiber (provided by Mengyin Yida Rabbit Industry Co., LTD., China).

Reagents: anhydrous ethanol (Tianjin Fengchuan Chemical Reagent Technology Co., LTD.), anhydrous calcium chloride (Tianjin Bodi Chemical Co., 22 LTD.), fibroin protein liquid (self-made), Acid red 2F dye, Acid yellow 2F dye, Acid blue 2F dye.

Instruments: Fourier transform infrared spectrometer (Nicolet S500 3040404), X-ray diffraction instrument (D8 DISCOVER 03030502), thermal field emission scanning electron microscope (Gemini SEM 50003040702), Textile Heat Transfer Performance Tester (YG606E-II), vacuum low temperature dryer (FD-1A-50), friction fastness tester (SDLATLAS), M228C soap washing fastness tester (SDLATLAS)

Methods

2.2.1 Freeze-drying treatment of rabbit wool fabric

Rabbit hair fabric was impregnated in distilled water and absorbed water to saturation, then the fabric was be frozen at-15℃ for 15 h followed by freeze-drying at-50℃ for more than 24 h until the fabric is dried.

2.2.2 Silk protein combined freeze-drying treatment of rabbit fabric

Rabbit fabric was firstly impregnated in 60℃ silk fibroin solution for 30 min, and then be frozen at -15℃ for 15 h followed by freeze-drying at -50℃ for more than 24 h until the fabric is dried.

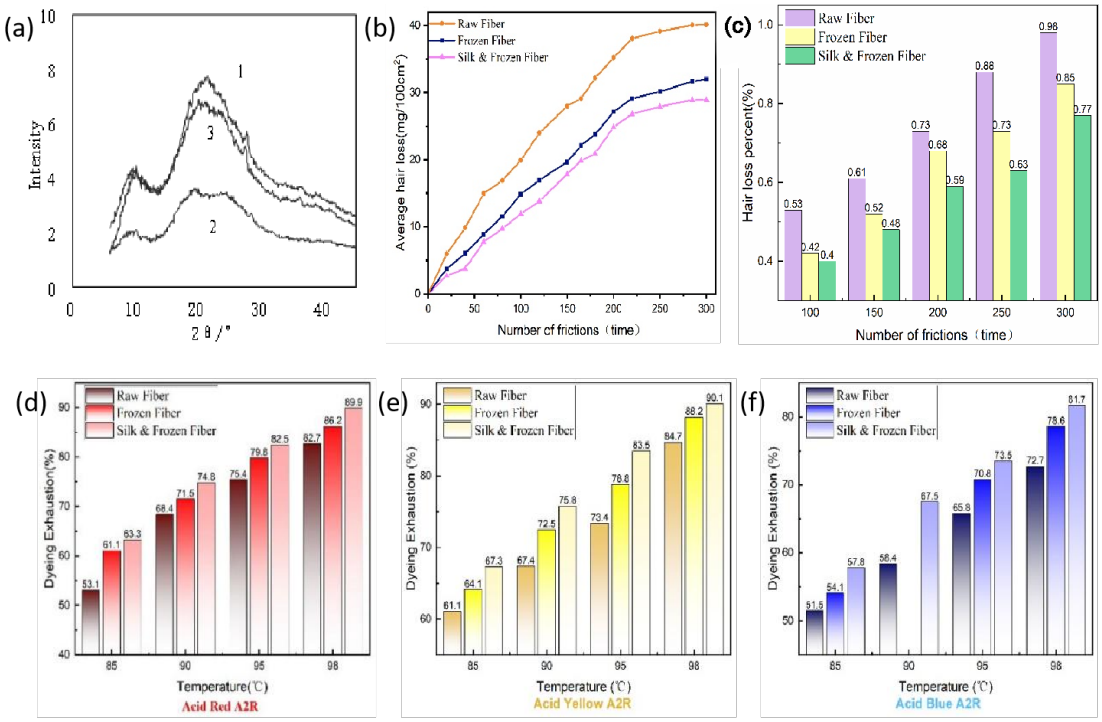
2.2.3 Dyeing of rabbit hair fabric with acidic dyes.

Dyeing prescription:

Dye (o.w .f) 3 % ;pH Value 3~4;bath ratio of 1:50

Dyeing process: Prepare the dye solution and heat it to 50℃. Place the fabric to be dyed into the dye solution, heat the dye solution to the specified temperature, and dye at this temperature for 60 min. Then, remove the fabric and wash it with water to remove any floating colors, and dry the fabric.

RESULTS AND DISCUSSION



Infrared analysis and XRD analysis

The XRD spectrum (Fig.a)showed that the position of X-ray diffraction peak did not change for the three samples, but intensity of the diffraction peak for both frozen treated samples (Frozen fibers and Silk & Frozen fibers) decreased obviously especially for Frozen fibers, showing a decrease in crystallinity of the treated rabbit hair fibers. It is believed that the water molecules will penetrate into the fiber during the immersion process, the volume expansion of water during phase changed from liquid water to solid ice the fiber changed the

microstructure of the fiber, and the crystal structure of rabbit hair fiber is destroyed with distance of molecule chain of rabbit hair protein enlarged. Silk protein macromolecules are mainly deposited on the surface and amorphous region of fibers, which limits the swelling effect of water molecules on rabbit hair fiber. Therefore, the supramolecular structure changes of rabbit hair fiber after fibroin protein treatment are not as obvious as direct cryodrying.

Hair loss nature test

The amount of fiber falling during the friction of rabbit hair fabric with 300 times friction was measured (Fig.b&c).The test results showed that the freeze-drying treatment and the combined treatment of filin and freeze-drying significantly decreased the amount of air loss per unit area of the fabric (mg/100cm^2) and hair loss percentage , indicating that the anti-shedding performance of the treated rabbit hair fabric was improved. Among them, the combined treatment of filin and freeze drying improved the performance of rabbit hair fabric more obviously. Frozen-drying treatment made the fiber swell, increased the scale warping Angle, leading the friction force between the fibers increased, and makes the fiber not easy to slip away from the yarn. The presence of filin protein not only further increases the surface roughness of rabbit hair fiber, increases the friction between fibers, but also has a certain adhesion effect, increases the adhesion between fibers, so that the fiber is less likely to slip.

Dyeing performance analysis

Rabbit hair, like wool, needs to be dyed at 100℃,because the scales can be fully opened at this time, the dye can smoothly enter the fiber inside, obtaining a high percentage of dye. However, high temperature dyeing not only damages the fiber, affects the bad quality of the fabric, but also causes the increase of energy consumption. Therefore, low temperature dyeing of wool has been research focus of textile area. This study found that the combination of freezing and filin freezing effectively improved the dyeing performance of rabbit hair. Not only did the dyeing percentage and K / S value of rabbit hair significantly increase in conventional 100℃ staining, but also the low temperature dyeing performance. Especially for Silk&Frozen fiber, the dyeing percentage in 95℃ was close to the dyeing percentage of 100℃ of untreated samples. The reason is that after freeze-drying treatment, the fiber structure becomes loose and the crystallinity is reduced, which is conducive to the easily diffusion of dye in to fibers. Moreover, the silk protein increased the affinity of the fiber for the dye because of strong attracting force between silk protein and dye molecules.

CONCLUSIONS

After two ways of freeze-drying treatment, the anti-hair loss performance and dyeing performance of rabbit hair fabric are significantly improved. It is believed that freeze-drying treatment can reduce the crystallinity of rabbit hair fiber, make the fiber structure more loose, fiber surface roughness increases, thus increasing the holding force between fibers, and make the dye more easy to enter the fiber. Although the deposition of silk protein reduces the swelling degree of rabbit hair fiber, it increases the surface roughness and the affinity of the dye, thus improving the anti-hair loss and dyeing performance of rabbit hair fabric more significantly. Freeze drying and silk protein treatment do not use environmentally harmful chemicals, and is a clean dyeing and finishing technology with application potential.

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ANTI-HAIR LOSS FINISHING OF RABBIT HAIR FABRIC AND ITS EFFECT EVALUATION BASED ON IMAGE PROCESSING

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ABSTRACT

Abstract: Rabbit hair fabric is prone to hair loss during use due to the small curl of rabbit hair fiber and smooth surface. Therefore, anti-shedding finishing of rabbit hair fabric is very important for the application of rabbit hair fiber in textile field, however, the lack of effective methods to evaluate the shedding behavior of rabbit hair fabrics also leads to no way to evaluate the effect of anti-shedding finishing. In this paper, the rabbit hair fabric was reacted with acrylic resin modified waterborne polyurethane and protease respectively, and the effects of different treatments on the hair lossproperties of rabbit hair fabric were investigated by image processing method by using DigiEye color measurement system. It has been found that the acrylic resin modified waterborne polyurethane treatment can effectively improve the anti-hair loss performance of rabbit hair knitted fabrics, because the polymers of the finishing agent are deposited between the rabbit hair fibers and form a network crosslink, which limits the slippage of rabbit hair fibers. The protease treatment improves the anti-hair loss performance of rabbit hair fabric, but the improvement effect is limited.

Keywords：rabbit hair fabrics；anti-hair loss treatment; foam processing

INTRODUCTION

Rabbit hair, a luxurious natural fiber, shares similarities with wool as both are animal hair fibers primarily composed of protein. Renowned for its whiteness, fluffiness, and soft touch, rabbit hair fiber also exhibits a gentle luster that adds to its appeal[6, 5]. But the tendency of rabbit hair to shed during wear and washing not only compromises the fabric's aesthetic and textural qualities but also poses inconvenience to users and diminishes consumer interest in purchasing such products. In China, where the angora rabbit breeding industry is well-established, addressing the issue of shedding is vital for sustaining and enhancing the marketability of rabbit hair textiles.

However, the evaluation of anti-shedding treatments for rabbit hair fabrics is hindered by the lack of straightforward, effective, and objective methods to assess the shedding behavior of the fabric[3]. To address this gap, the present study introduces a novel approach to evaluating the shedding properties of rabbit hair fabrics using the DigiEye color measurement system. This method leverages image processing techniques to provide a more objective, efficient, and accurate assessment of fabric shedding, thereby facilitating the development of effective anti-shedding treatments.

MATERIALS AND METHODS

Materials

Fabrics: Pure rabbit hair knit, cashmere knit, wool knit, and acrylic knit

Reagents: Sodium dodecyl sulfate, end-capped water-based polyurethane, water-based polyacrylic acid ester, TCEP, and alkaline protease.

Instruments: DigiEye Digital Eye Image Color Management System, DK-5E Needle-type Dip Coating and Baking Machine

Methods

2.2.1 Modification of Rabbit hair Fabric with Polyurethane Finishing

The preparation parameters for the finishing working solutions are detailed in Table 1. These solutions were applied to the rabbit hair fabric using dip-coating and foam-finishing methods, respectively.

Table 1: Preparation parameters of finishing fluid

Working fluid type	Working fluid 1#	Working fluid 2#
component	15% PU + 5% PA (diluted to 100% with water, pH adjusted to 7.0-8.0 with sodium carbonate)	20% PU + 5% PA (diluted to 100% with water, pH adjusted to 7.0-8.0 with sodium carbonate)

Protease Treatment of Rabbit hair Fabric
The rabbit hair fabric was subjected to treatments using protease alone and a combined treatment of protease with TCEP.
Adhesive Tape Hair Image Collection and DE Value Testing Method:
The fabric shedding status was quantitatively assessed using the DE value testing function of the DigiEye digital color measurement system[4].
Color Clustering Analysis:
The adhesive tape samples with fibers were analyzed using the Colour Clustering function of the DigiEye digital color measurement system.

RESULTS AND DISCUSSION

Analysis of Shedding Causes in Rabbit hair Fabric

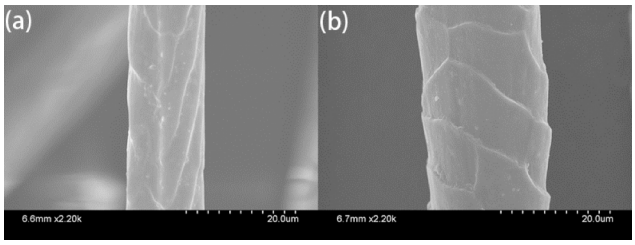

Figure 1: SEM photos of rabbit hair fiber (a) and wool fiber (b)

The surface morphology of rabbit hair and wool fibers was observed using scanning electron microscopy (SEM), and the surface friction properties and fiber curliness of both rabbit hair and wool were tested.
Compared to the fine wool fibers, rabbit hair has a more smooth external scale, resulting in a reduced friction coefficient[7, 2]. Additionally, rabbit hair exhibits a lower degree of curliness compared to wool.

Table.2 Comparison of friction effect and crimp between rabbit hair and wool

Sample Type	Fiber Fineness(μm)	Friction Effect		Curvature (curls/cm)
		static state	trends	
Rabbit hair	13.6	0.133,00	0.125,27	2-3
Wool	18.7	0.142,39	0.166,35	4-6

Characterization of Fabric Shedding Properties by Image Processing Method

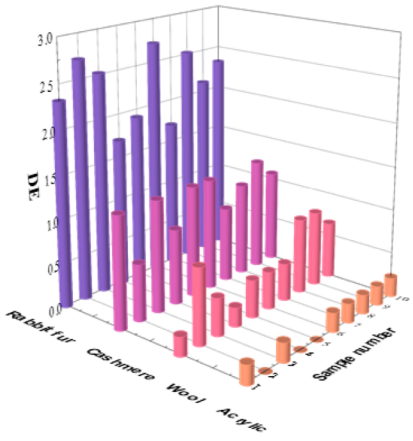

Figure.2 DE values of different fabrics

Four groups of knitted fabrics, each composed of rabbit hair, cashmere, wool, and acrylic respectively, were tested with different colors (excluding white) and different structural compositions. White adhesive tape was used to test these four groups of fabrics, and the obtained DE values are shown in Figure 2.
The shedding propensity of fabrics correlates with the DE value, where a higher DE value indicates greater shedding. As depicted in Figure 2, the DE value ranking is as follows: rabbit hair > cashmere > wool > acrylic. This ranking aligns with conventional understanding. Further validation was provided by color clustering analysis, which quantifies the

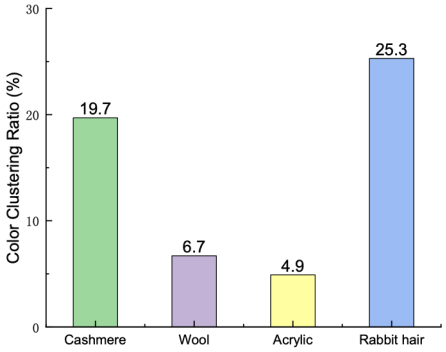

Figure.3 Color clustering test

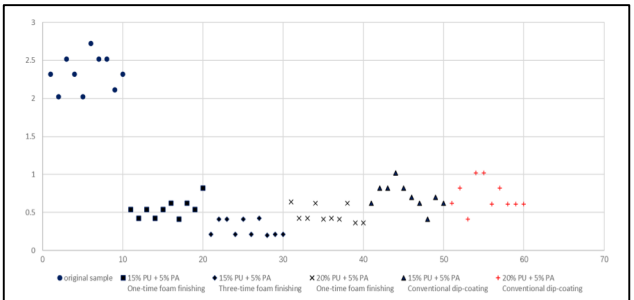

Figure.4 DE values of rabbit hair samples finished with modified polyurethane

percentage of fiber-colored pixels in the adhesive tape's digital image. A higher Clustering value signifies increased shedding. Consistent with the DE value results, Figure 3 demonstrates that rabbit hair displayed the highest Clustering value.

3.3 Evaluation of Anti-Shedding Effects with Different Finishing Treatments

3.3.1 Anti-Shedding Finishing with Modified Polyurethane

Thermoreactive water-based polyurethane, supplemented with acrylic resin, was utilized as the main component of the finishing agent to enhance the anti-shedding properties of rabbit hair knitted fabric[8]. To assess the impact of different finishing methods on the anti-shedding effect, both conventional dip-coating and foam finishing techniques were applied. The adhesive tape test was employed to evaluate the shedding behavior of the treated samples, and the corresponding DE values were measured. The results are presented in Figure 4.

As observed in Figure 4, all treated samples exhibited a significant reduction in DE values. This indicates that the modified polyurethane finishing effectively improved the anti-shedding performance of rabbit hair fabrics.

Impact of Enzyme Treatment on Anti-Shedding Performance

Figure.5 DE values of samples with enzymes treatment

Enzyme treatment has been reported to improve the anti=shedding issue of rabbit hair fabric. This study conducted enzyme treatment alone and in combination with TCEP on rabbit hair knitted fabric to reduce shedding.

Figure 5 illustrates that compared to the control, the DE value of samples treated solely with protease decreased, indicating a reduction in shedding. However, the DE value of the fabric treated only with enzymes remained between 1 and 1.5, suggesting a still considerable shedding issue.

DISCUSSION

Addressing the causes of shedding in rabbit hair, this study utilized a reduced application of protease and a combined treatment with aqueous polyurethane and acrylic resin for anti-shedding finishing of rabbit hair fabrics. A novel image-based evaluation method for fabric shedding properties was tested using the DigiEye color measurement system. It was observed that protease treatment marginally improved the anti-shedding performance of rabbit hair fabrics, while the acrylic resin-modified aqueous polyurethane finishing notably enhanced the anti-shedding properties. The foam finishing method, compared to

conventional dip-nip techniques, not only demonstrated a more significant anti-shedding effect but also offered advantages in energy conservation and emission reduction, thereby making it the recommended finishing technique.

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REPRODUCTION

EXPLORING RABBIT SEMEN CRYOPRESERVATION AND THE CRUCIAL ROLE OF CRYOBANK: UNVEILING ADVANTAGES AND PROMISING PERSPECTIVES

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ABSTRACT

Over the years, efforts have been made to establish a reference protocol for the first Italian rabbit semen bank. Here, we will provide an overview of our group's key discoveries, which have led us to identify an effective freezing protocol for rabbit semen. However, a drawback of our protocol involves diluting the semen with the freezing media at fixed dilution ratios, resulting in significant variability in the sperm number per straw. Addressing this limitation underscores the necessity to standardize the freezing protocol by investigating the effect of sperm concentrations within straws on the reproductive performances of cryopreserved rabbit semen. In this regard, in an *in vivo* trial, 192 multiparous receptive rabbit does were randomly assigned to six treatment groups, including a control group. The remaining groups were inseminated with doses containing 15, 25, 35, 55, and 75 × 10^6 sperm, respectively. Sperm concentrations of 25, 35, and 55 × 10^6/straw exhibited higher reproductive performances compared to other tested concentrations and were akin to fresh semen. Through the adoption of freezing protocols with standardized sperm concentrations, cryobanks can surely preserve rabbit population genetic resources, offering invaluable support for future breeding and research efforts.

Key words: rabbit, cryopreservation, cryobank, sperm concentration.

INTRODUCTION

Rabbits have always been indispensable for human life because they are valuable for agriculture, for biomedical research and because of their docile nature they make popular pets. The rabbit's short life cycle, brief gestation period, prolificacy, and high feed conversion capacity (Lebas et al., 1997) make it theoretically ideal for meat production. Breeding rabbits for human consumption is largely dependent on artificial insemination (AI) programs. In rabbit farms, AI predominantly employs fresh or cooled semen, as opposed to frozen semen, due to the observed compromised fertility following thawing and/or for highly variable results (Mocè and Vicente, 2009; Lavara et al., 2017; Kubovicova et al., 2021).

Frozen rabbit semen is crucial for international export, research, and conservation of genetic resources (endangered breeds or high-value males). Semen cryopreservation, which is part of the ex-situ *in vitro* strategy, is a valuable tool for safeguarding animal genetics via cryobanks, particularly in rabbits, offering a cost effective alternative to embryo preservation (Mocé and Vicente, 2009). However, the limited survival rate of rabbit sperm following cryopreservation represents a significant challenge that hinders the widespread adoption of frozen semen in AI programs. It also poses as a barrier to the effective preservation of genetic resources through the establishment of a sperm cryobank. Several studies have investigated methods to improve the cryopreservation protocol of rabbit sperm, examining factors including freezing methods and the types and concentrations of cryoprotectants (Mocé and Vicente, 2009; Nishijima et al., 2021; Viudes-de-Castro and Vicente, 2023). Despite substantial technical enhancements made in recent years (Kubovicova et al., 2021; Viudes-de-Castro and Vicente, 2023), the lack of a firmly established standard freezing

procedure for rabbits persists due to inconsistent and divergent outcomes observed across various studies.

One factor that may account for the inconsistent outcomes in rabbit sperm cryopreservation could be the variability in sperm quality (Kubovicova et al., 2021). Another significant aspect contributing to the fluctuation in results is the unpredictability of the final sperm concentration within the straws, as freezing protocols typically involve diluting the semen with freezing media at fixed dilution ratios. Additionally, it's acknowledged that the success of freezing rabbit sperm differs among various breeds (Kulikovà et al., 2017) and among donors (Mocé et al., 2005).

Over the years, we have also dedicated significant efforts to developing a reference semen freezing protocol for establishing the first Italian bank that is a milestone within "MiPAAF" project aiming to conserve and promote Italian rabbit breeds.

Here, we provide an overview of our group's research, which has led to the development of an effective freezing protocol. On one hand, this protocol has enabled us to achieve reproductive performances comparable to those observed with fresh semen (Iaffaldano et al., 2012, 2014; Di Iorio et al., 2018, 2020). On the other hand, we encountered a significant variability in the number of sperm present in each straw, due to the use of a fixed dilution ratio. Consequently, our focus has shifted towards determining the precise sperm concentration in straws, with the aim of standardizing rabbit cryopreservation protocols and reducing result variability.

In our recent study, Di Iorio et al. (2024) specifically examined the influence of different sperm concentrations within straws on *in vitro* semen quality during the cryopreservation process. Therefore, in order to offer a more comprehensive insight into the impact of sperm concentration, our present report also includes additional investigations that evaluate the effects of different concentrations (15, 25, 35, 55, and 75 × 10^6) within straws of cryopreserved semen on reproductive performances in rabbit.

MATERIALS AND METHODS

Animals and experimental design

In the last decade, our research endeavors have focused on establishing an effective semen freezing protocol for rabbits. This comprehensive investigation entailed evaluating the impact of various permeable and non-permeable cryoprotectants, determining their optimal combinations, equilibrium time, and cooling time prior to freezing was reported in Table 2 (Iaffaldano et al., 2012, 2014; Di Iorio et al., 2018, 2020).

While the cryopreservation protocol resulting from these studies yielded satisfactory outcomes, standardization was necessary due to the variability in the number of spermatozoa within each straw caused by the utilization of a fixed dilution ratio.

Consequently, Di Iorio et al. (2024) investigated the impact of sperm concentration/straws on *in vitro* sperm quality of the main step of the cryopreservation procedure (table 3). To conclude our study, an AI trial was conducted herein to evaluate the *in vivo* effectiveness of semen cryopreserved at various sperm concentrations per straw (15, 25, 35, 55, and 75 × 10^6) on reproductive performances. The semen doses utilized in the *in vivo* trial were realised in our previous research (Di Iorio et al., 2024).

In this regard, in Di Iorio et al. (2024) 80 adult rabbit bucks (7-9 months old) of the Bianca Italiana breed, from the Central Breeding Farm of Italian Rabbit Breeders Association (ANCI-AIA, Volturara Appula (FG), Italy) were used. The rabbits were individually housed in flat-deck cages and provided with a 16 h light/8 h dark photoperiod. A commercial standard breeder diet and free access to water were provided. Ejaculates were collected using a pre-heated artificial vagina. Ejaculates were pooled to avoid the effects of individual differences among males, in total, eight pooled semen samples were used. Semen samples were transported from the farm to the laboratory within 30 minutes, at a temperature of

approximately 30°C in a polystyrene box. Upon arrival at the laboratory, a portion of each sample was promptly assessed for fresh semen quality, which included testing sperm motility, membrane integrity, and concentration. For each fresh semen sample pool, the sperm concentration was assessed through spectrophotometric analysis. This involved measuring the optical density at 530 nm of samples diluted 1:200 in a 0.9% NaCl solution. Sperm concentration was calculated through interpolation on a previously calibrated calibration curve and expressed in millions/mL. Each semen pool was split into five aliquots. The aliquots were prediluted with Tris-citrate-glucose extender (TCG; 250 mmol/L Tris-hydroxymethylaminomethane, 88 mmol/L citric acid, and 47 mmol/L glucose, pH 6.8) and then with TCG supplemented with cryoprotectants (CPAs) (freezing extender) until reaching the pre-determined concentrations to assure the following final number of spermatozoa inside the straws: 15, 25, 35, 55, and 75 × 10^6 sperm/straw, respectively as reported by Di Iorio et al. (2024) (table 1). Thus, each semen sample was processed and cryopreserved using the freezing technique as outlined by Iaffaldano et al. (2012). To prepare for freezing, the semen samples that were prediluted with TGC, were cooled at 5°C for 90 min (Di Iorio et al., 2018). After cooling, they were diluted to a ratio of 1:1 (v:v) with a freezing extender composed of TCG containing 16% dimethylsulfoxide (DMSO), as a permeable CPA, and 0.1 mol/L sucrose, as a non-permeable CPA. The diluted semen was aspirated into 0.25 mL plastic straws, equilibrated at 5°C for 45 min (equilibration time) and frozen by exposure to liquid nitrogen vapor 5 cm above the liquid nitrogen surface (temperature was approximately -125/-130°C) for 10 min. Finally, the straws were plunged into liquid nitrogen for storage at -196°C.

Table 1: Dilution process of fresh rabbit semen (example of initial concentration: 720 × 10^6 spermatozoa mL^{-1}) with TCG and freezing extender to achieve final sperm concentrations in the straws of 15, 25, 35, 55 and 75 × 10^6.

	First dilution with TCG			Second dilution with F.E.				
Initial fresh sperm concentration (× 10^6 mL^{-1})	Sperm concentration after pre-dilution with TCG (× 10^6 mL^{-1})	Dilution rate	Semen volume (mL)	TCG volume (mL)	F.E. volume (mL)	Final volume	Sperm concentration after dilution with F.E. 1:1 (× 10^6 mL^{-1})	**Sperm concentration per straw (× 10^6)**
720	120	6.00	0.42	2.08	2.50	5.00	60	15
720	200	3.60	0.69	1.81	2.50	5.00	100	25
720	280	2.57	0.97	1.53	2.50	5.00	140	35
720	440	1.64	1.53	0.97	2.50	5.00	220	55
720	600	1.20	2.08	0.42	2.50	5.00	300	75

TCG: tris, citric acid, glucose; F.E.: freezing extender composed of TCG, 16% DMSO, and 0.1 M sucrose.

Evaluation of *in vitro* sperm quality

The semen quality was immediately evaluated after dilution of fresh semen and thawing. The frozen semen doses were thawed in a water bath heated to 50°C for a duration of 10 seconds.

Sperm motility was assessed using a computer-aided sperm analysis system linked to a phase-contrast microscope (Nikon Eclipse model 50i; negative contrast, Firenze, Italy) with Sperm Class Analyzer (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain) (Rusco et al., 2022). Samples of semen were diluted in 0.9% NaCl to achieve a sperm concentration of 50×10^6/mL. After a 5-minute incubation at 37°C, 3 µL of the prepared sample were placed on a prewarmed 20-micron Leja slide (Leja Standard Count, Nieuw Vennep, The Netherlands) and examined under the microscope at 100× total magnification. Various parameters were recorded, including total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), and straightness (STR, %). A minimum of 500 spermatozoa per sample were examined across three distinct microscopic fields.

Sperm membrane integrity (SMI) assessment was conducted using the Muse® Cell Analyzer (Luminex Corporation, Austin, TX, USA) according to the manufacturer's guidelines. Semen samples were initially diluted in Phosphate-buffered saline (PBS) to achieve a concentration ranging from 1×10^5 to 1×10^6 spermatozoa/mL. A 20 µL portion of this suspension was mixed with 780 µL (dilution factor 1:40) of a Muse Count and Viability Kit® in an Eppendorf tube (Luminex Corporation). After 5 minutes of incubation at room temperature in the absence of light, the Eppendorf tubes were subjected to flow cytometry analysis. The software generated two dot plots: one for nucleated cells, which helps in the distinction of cells with a nucleus from debris and non-nucleated cells, and another for viability, which identifies viable (non-staining living cells) from non-viable (staining dead or dying cells) based on membrane integrity.

In vivo experiment to test the effect of different sperm concentrations

Regarding the AI trial 192 multiparous (31 days postpartum) receptive rabbit does were randomly assigned to 6 treatment groups: a control group (inseminated with 0.5 ml of fresh semen diluted 1:10), the other groups were inseminated with 15, 25, 35, 55, and 75×10^6 spz/dose, respectively. Each group consisted of 32 rabbits. Before insemination all does were synchronized following biostimulation protocol based on flushing and changing cages (3 days before insemination) and increasing the photoperiod from 16 to 24 h of light (2 days before insemination). At the moment of insemination, each female received an intramuscular injection of buserelin acetate at a dosage of 1 microgram per doe to induce ovulation.

Fertility rate (number of pregnant does/number of inseminations), kindling rate (number of does giving births/number of inseminations), prolificacy (total born/kindling) and number of kids born alive (total live-born/kindling) were considered when assessing the reproductive performance. The fertility rate was determined by abdominal palpation performed on each doe 17 days after AI, while the other factors were registered at parturition.

Statistical Analysis

A comparison of the reproductive performance (fertility rate, kindling, prolificacy, and number of kids born alive) of the does which received the six different insemination treatments was carried out by an analysis of variance, followed by Duncan's comparison test. All statistical tests were performed using the software package SPSS (IBM SPSS Statistics 23.0 for Windows, 2020; SPSS, Chicago, IL, USA). Significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Over the years, our research group has achieved satisfactory results in developing an effective freezing protocol for rabbits, substantiated by the findings, and validated through both *in vitro* and *in vivo* assessments of frozen semen, as depicted in Table 2. Many variables of a rabbit semen freezing protocols were studied such as cryoprotectant (CPA) and its concentration, freezing extender and initial cooling time (Iaffaldano et al., 2012, 2014; Kuliková et al., 2015; Di Iorio et al., 2018, 2020). Summing up the results obtained in the above-mentioned work, the protocol involving the dilution of semen with freezing media at fixed dilution ratios allowed us to reach similar reproductive performances with frozen semen as those recorded with fresh semen. During the cryopreservation process, the spermatozoa undergo various types of stress caused by ice formation, chemical toxicity, and oxidative stress, which mainly injures cytoplasm membrane, consequently leading to a lower post-thawed quality and fertility (Khan et al., 2021). To shedding light on molecular damage mechanisms impacting post-thaw sperm quality, recently, we studied the proteome of fresh and frozen rabbit semen to identify proteins altered during cryopreservation. The protein alterations make sperm more susceptible to stressors during and after cryopreservation, leading to disturbances in the fertilization process (Rusco et al., 2022).

Intracellular ice crystals formed during cryopreservation can lead to cell destruction. This can be prevented by dehydrating cells using a permeable CPA in the freezing solution (Iaffaldano et al., 2012). Permeable CPAs may also harm sperm by destabilizing membranes and

denaturing proteins and enzymes, with toxicity linked to CPA concentration and exposure time. Including non-permeable CPAs in the freezing medium which mitigates cryodamage caused by permeating CPAs, reducing the required amount (Swain and Smith, 2010). Hence, selecting the appropriate CPA was crucial for developing an effective freezing protocol for rabbit semen.

The most widely used CPAs to preserve rabbit semen are a combination of permeable compounds such as DMSO or acetamide and non-permeable compounds such as lactose, sucrose, raffinose, trehalose or egg yolk (Mocé and Vicente, 2009; Viudes-de-Castro and Vicente, 2023).

The choice of CPA is certainly among the most important factors for an effective rabbit semen freezing protocol. Our previous findings indicate that the permeable CPA used and its concentration, the speed of cryopreservation, and the presence and particular combination of nonpermeating CPA affected the cryosurvival of rabbit semen (Iaffaldano et al., 2012; Rosato and Iaffaldano, 2013). The main points established were:

-DMSO and CPAs containing amide groups resulted in better cryosurvival rates of rabbit sperm than glycerol (Rosato and Iaffaldano, 2013) in accordance with other authors (Kashiwazaki et al., 2006). On the contrary methanol, ethylene glycol, and propylene glycol showed an immediate toxic effect after 5 minutes of incubation with sperm even at low concentrations (Rosato and Iaffaldano, 2013);

-CPA concentration and equilibrium time affected the survival rate of rabbit sperm after cryopreservation and that DMSO was better than DMA at preserving the quality and fertility of rabbit sperm (Iaffaldano et al., 2012; Rosato and Iaffaldano, 2013). In accordance with Holt 2000 that reported that the toxicity of penetrating CPAs increases with its concentration, exposure temperature, and exposure time.

The reason why rabbit sperm cells were able to better withstand DMSO than DMA during the cryopreservation process is unknown, but several explanations can be proposed. DMA and DMSO share many common physical-chemical properties, but they differ in molecular weight and molecular structure. These differences may influence permeability through the cell membrane, which could explain why DMSO provides better cryoprotective protection (Iaffaldano et al., 2012). Subsequently, our efforts focused on selecting the best non-permeable CPA. Through testing different concentrations of lipoproteins (LDL) in comparison with sucrose and egg yolk, we found that sucrose was the most suitable non-permeable CPA for freezing rabbit semen (Iaffaldano et al., 2014). These finding underscore that sucrose's effectiveness as a non-permeable CPA for rabbit sperm cryopreservation, attributed to its dehydration ability at high subfreezing temperatures, inhibiting ice crystal growth, and allowing rapid cooling. Sucrose also has a higher glass transition temperature, enabling long-term storage at high subzero and suprazero temperatures (Woelders et al., 1997; Gómez-Fernández et al., 2012).

By investigating the effect of initial cooling time during semen cryopreservation process we showed that 90 min of initial cooling (at 5°C) before freezing resulted in better post-thaw semen quality and reproductive performances compared to the semen cooled for 45 min. The longer cooling time prepares the sperm more effectively for the addition of the CPAs (Di Iorio et al., 2018). In addition, we also compared a Tris-citrate-glucose (TCG) extender with a commercial one (Cortalap®) regarding the *in vitro* freezability and fertilizing ability of cryopreserved rabbit semen. No significant differences in reproductive performances were found, showing that Cortalap® could be a valid alternative to TCG. Being a ready to use extender, it implies a reduction in time, mistakes, and microbial contaminations during its preparation (Di Iorio et al., 2020).

Integrating the outcomes of these studies, we devised an optimal protocol for freezing semen, which encompassed cooling sperm at 5°C for 90 minutes, diluting it with a freezing extender (TCG containing 16% of DMSO and 0.1 M of sucrose), 45-minute equilibration time at 5°C, and exposing it to liquid nitrogen vapor at 5 cm above the nitrogen. This protocol

based on a fixed semen extender dilution rate of 1:1, enabled us to achieve reproductive outcomes with frozen semen that were comparable to those observed with fresh semen (Iaffaldano et al., 2012, 2014; Di Iorio et al., 2018, 2020). Our protocol's major drawback was the wide variability in sperm numbers per straw. Additionally, it should also be noted that most of the protocols developed by other authors are based on fixed sperm to extender ratios, ranging from 1:1 to 1:10 (Mocé and Vicente, 2009; Mocé et al., 2014; Viudes de Castro 2014, 2021; Nishijima et al., 2015; Lavara et al., 2017; Domingo et al., 2019; Fadl et al., 2019; Küçük et al., 2021; Mohammed et al., 2022).

Therefore, identifying the optimal sperm concentration within the straw is a crucial aspect that has received little attention to date. This goal is essential for standardizing rabbit cryopreservation protocols, reducing result variability, and enhancing freezing techniques. For example, the dairy bull industry is considered a model in the context of sperm cryopreservation due to its highly standardized protocols, which involve consistent and specified sperm concentrations in each straw.

Our *in vitro* study demonstrated that sperm concentration plays a significant role in specific phases of the cryopreservation process (dilution of fresh semen, cooling, equilibration, immediately after and 30 minutes post-thawing) (Di Iorio et al., 2024).

Our findings highlighted the significant impact of sperm concentration per straw on key stages of the cryopreservation process, particularly during equilibration and post-freezing phases. Additionally, the final sperm concentration in the straws influences the post-thaw motility of cryopreserved rabbit sperm. We observed that mid-range sperm concentrations (25 and 35 × 10^6/straw) showed greater sperm quality immediately after thawing compared to other concentrations (table 3). Importantly after a 30-minute post-thawing period, semen quality no longer exhibited a discernible influence on sperm concentration (Di Iorio et al., 2024).

Table 2: An overview of the *in vitro* quality and reproductive performances outcomes with cryopreserved rabbit semen, obtained by our research group over the last decade.

Reference	Breed	Freezing protocol			Post-thaw *in vitro* quality		Reproductive performances			
		Cooling	*Freezing extender composition*	*Dilution rate*	*Sperm viability (%)*	*Motility (%)*	*Fertility (%)*		*Prolificacy (mean ± SEM)*	
							Fresh	*Frozen*	*Fresh*	*Frozen*
Iaffaldano et al. 2012	Hybrid of Centro genetica Martini	90 min at 5°C	TCG 12% DMA 2% sucrose	1:1 (v:v)	36.1 ± 1.6	TM: 30.9 ± 2.5 PM: 23.5 ± 1.3	81.6	47.4	8.6 ± 0.3	6.7 ± 0.4
			TCG 16% DMSO 2% sucrose		47.1 ± 1.8	TM: 42.6 ± 2.1 PM: 35.4 ± 1.9		79.8		7.7 ± 0.3
Rosato and Iaffaldano 2013	Hybrid of Centro genetica Martini	90 min at 5°C	TCG 10% DMSO 0.5% BSA 0.1 M trehalose	1:1 (v:v)	40.0 ± 1.1	TM: 44.4 ± 1.0	84.0	52.0	8.8 ± 2.1	7.4 ± 2.8
			TCG 10% DMSO 0.5% BSA 0.1 M sucrose		36.5 ± 1.2	TM: 41.1 ± 1.8		77.0		8.1 ± 2.5
Iaffaldano et al. 2014	Bianca Italiana breed	90 min at 5°C	TCG 16% DMSO 0.1M sucrose	1:1 (v:v)	43.5 ± 1.0	TM: 38.9 ± 1.4 PM: 29.7 ± 1.7	93.3	86.7	10.1 ± 0.5	9.2 ± 0.5
			TCG 16% DMSO 10 % LDL		39.9 ± 1.1	TM: 35.4 ± 0.9 PM: 27.9 ± 1.1		66.7		8.2 ± 0.6
Di Iorio et al. 2018	Bianca Italiana breed	45 min at 5°C	TCG 16% DMSO 0.1 M sucrose	1:1 (v:v)	36.8 ± 1.8	TM: 30.5 ± 1.0 PM: 22.4 ± 0.7	74.0	64.0	8.7 ± 0.6	8.2 ± 0.6
		90 min at 5°C			42.5 ± 1.0	TM: 37.3 ± 1.1 PM: 27.8 ± 1.1		76.0		8.3 ± 0.6
Di Iorio et al. 2020	Bianca Italiana breed	90 min at 5°C	TCG 16% DMSO 0.1M sucrose	1:1 (v:v)	44.7 ± 2.0	TM: 36.8 ± 1.5 PM: 30.2 ± 1.9	80.0	86.7	7.7 ± 0.7	7.4 ± 0.8
			Cortalap® 16% DMSO 0.1M sucrose		52.5 ± 1.8	TM: 43.4 ± 1.4 PM: 36.5 ± 1.1		76.7		9.4 ± 0.6

TCG: Tris-citrate-glucose; DMSO: dimethylsulfoxide; DMA: dimethylacetamide; LDL: low-density lipoproteins; BSA: bovine serum albumin; TM: total motility; PM: progressive motility; data are presented as mean ± SEM.

Table 3: Effect of different straw sperm concentrations on quality of freshly diluted and thawed rabbit semen (data extracted from Di Iorio et al., 2024).

Treatments		Sperm variables							
	Sperm concentration	SMI (%)	TM (%)	PM (%)	VCL (µm/sec)	VAP (µm/sec)	VSL (µm/sec)	STR (%)	LIN (%)
Fresh	**15**	89.6 ± 0.7^{a}	92.2 ± 0.8^{a}	66.1 ± 1.3^{b}	73.0 ± 2.5^{a}	40.8 ± 2.4^{a}	27.1 ± 2.2^{a}	61.8 ± 1.4^{a}	36.9 ± 2.4^{ab}
	25	89.9 ± 0.7^{a}	91.4 ± 0.9^{a}	67.2 ± 1.0^{ab}	75.4 ± 1.9^{a}	38.2 ± 1.8^{a}	24.4 ± 1.3^{a}	60.9 ± 1.2^{a}	32.7 ± 1.5^{b}
	35	90.2 ± 0.7^{a}	91.9 ± 0.9^{a}	69.4 ± 0.9^{ab}	76.3 ± 1.5^{a}	40.1 ± 1.9^{a}	26.2 ± 1.5^{a}	61.0 ± 0.9^{a}	33.2 ± 1.4^{ab}
	55	90.8 ± 0.8^{a}	91.4 ± 1.0^{a}	68.1 ± 1.6^{ab}	75.1 ± 2.8^{a}	45.5 ± 4.1^{a}	31.2 ± 3.9^{a}	62.9 ± 1.7^{a}	39.4 ± 3.1^{a}
	75	91.3 ± 0.5^{a}	91.8 ± 0.8^{a}	70.5 ± 1.7^{a}	75.9 ± 2.1^{a}	41.6 ± 1.8^{a}	26.7 ± 1.8^{a}	61.7 ± 1.5^{a}	35.6 ± 1.8^{ab}
Frozen	**15**	75.5 ± 1.1^{a}	42.2 ± 1.6^{bc}	14.9 ± 1.1^{bc}	41.9 ± 1.3^{c}	20.0 ± 0.5^{b}	10.1 ± 0.4^{b}	45.5 ± 0.9^{c}	22.6 ± 0.7^{c}
	25	77.1 ± 1.0^{a}	49.9 ± 2.5^{a}	19.7 ± 1.4^{a}	44.6 ± 1.0^{bc}	21.5 ± 0.4^{ab}	11.3 ± 0.5^{ab}	49.0 ± 1.0^{b}	24.7 ± 0.8^{ab}
	35	75.8 ± 1.7^{a}	46.2 ± 3.0^{ab}	19.7 ± 1.8^{a}	47.7 ± 1.4^{ab}	22.7 ± 0.7^{a}	12.2 ± 0.4^{a}	50.0 ± 1.0^{ab}	24.8 ± 0.8^{ab}
	55	75.4 ± 1.7^{a}	38.8 ± 1.7^{c}	17.0 ± 0.9^{ab}	49.2 ± 1.6^{a}	22.8 ± 0.8^{a}	12.6 ± 0.5^{a}	50.5 ± 0.6^{ab}	23.9 ± 0.6^{bc}
	75	72.5 ± 1.8^{a}	31.5 ± 2.0^{d}	12.5 ± 1.1^{c}	46.1 ± 1.8^{abc}	22.7 ± 0.8^{a}	12.6 ± 0.6^{a}	51.8 ± 0.9^{a}	26.5 ± 0.7^{a}

Different superscripts $^{(a,b,c,d)}$ within the same column indicate a significant effect of sperm concentration in fresh and frozen semen.

Preliminary *in vivo* results obtained by testing different sperm concentrations/straw

Regarding the reproductive performances of post thawed semen that are presented for the first time here (Fig. 1 and 2) no significant differences for fertility and kindling rate among fresh and frozen semen with 25, 35, 55 × 10^6 concentrations were registered. The concentration of 75 ×10^6 showed the worst value of fertility ($P<0.05$) compared to fresh semen and the concentrations of 25, 35, 55 × 10^6 respectively, whilst the kindling rate resulted as significant only in respect to fresh semen. No significant difference was found among fresh and frozen semen for all concentrations tested on total born and live born. The lowest reproductive outcomes were observed with 75 × 10^6 compared to other concentrations aligning with *in vitro* findings (Di Iorio et al., 2024), possibly due to a dose-dependent reduction in cryoprotective effectiveness as noted by Contri et al. (2012). Additionally, it has been suggested by other authors, such as Lahnsteiner (2000), that increased sperm numbers per straw could lead to cellular compression due to limited intercellular space, consequently reducing post-thaw sperm quality. Based on these findings, our freezing protocol using concentrations of 25, 35 and 55 × 10^6 per straw resulted in minimized damage during freezing, leading to optimal reproductive performances. Furthermore, determining the optimal freezing concentration for rabbit semen marks a significant advancement, addressing an aspect previously overlooked in this field.

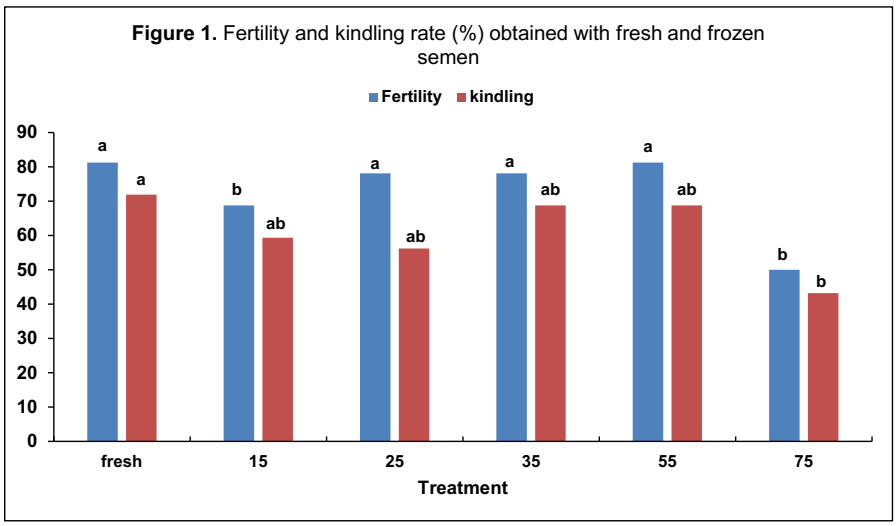

Figure 1. Fertility and kindling rate (%) obtained with fresh and frozen semen

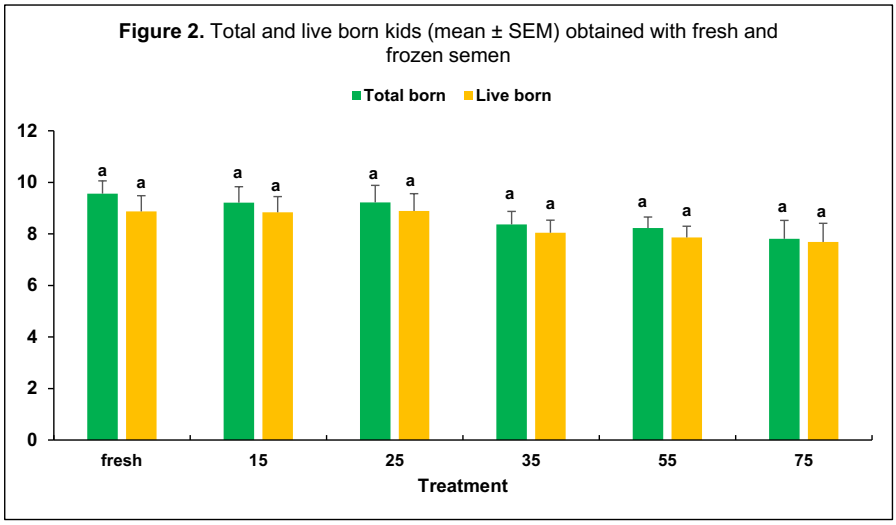

Figure 2. Total and live born kids (mean ± SEM) obtained with fresh and frozen semen

Different lowercase letters indicate a significant difference ($P < 0.05$).

Developing an efficient freezing protocol was crucial for establishing the first Italian rabbit cryobank, which promotes ex-situ conservation strategies for safeguarding rabbit breeds. Currently, the cryobank contains 3846 semen doses from 43 rabbit breeds.

This diversified repository not only serves as a valuable genetic resource but also provides a platform for research and breeding programs focused on preserving rabbit diversity and genetic heritage.

The significance of semen cryobanks extends beyond the storage, as they offer numerous advantages. One notable benefit is their role as a backup for populations preserved *in vivo*, providing a safeguard against potential genetic issues like inbreeding and genetic drift. This capability ensures the preservation of genetic diversity and offers the potential to reconstruct breeds in the event of extinction or a significant reduction in the population, thereby contributing to the conservation of rabbit biodiversity on a broader scale. In the context of cryobank purposes, sperm concentration controls are crucial for safeguarding the genetic integrity and viability of stored genetic material. Testing lower sperm concentrations also aligns with the potential of freezing individual donor sperm for cryobanking. Given that a single donor of these breeds may yield lower sperm concentrations, exploring this range is important for a comprehensive assessment.

CONCLUSIONS

These findings provide valuable insights for improving rabbit semen freezing techniques. Standardizing sperm concentration in each straw is essential to minimize result variability and accurately determine the number of sperm received by each doe during the AI procedures, guaranteeing the reproducibility and accuracy of the AI technique, which is particularly crucial for successful rabbit population breeding management. Sperm concentrations of 25, 35, and 55 × 10^6/straw demonstrated higher reproductive performance compared to other concentrations tested and was similar to fresh semen.

Through the adoption of freezing protocols with standardized sperm concentrations, cryobanks can confidently preserve rabbit population genetic resources. Additionally, rabbit breeding facilities could start to take advantage of the opportunities by extensive use of doses of frozen semen.

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EFFECT OF FEED SUPPLEMENTATION WITH PLANT-DERIVED OMEGA 3 ON THE PRODUCTIVE AND REPRODUCTIVE PERFORMANCE OF FEMALE RABBITS

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ABSTRACT

Rabbit farming faces significant challenges due to high mortality and culling rates among females, mainly attributed to intensive reproductive rhythms, which lead to a negative energy balance and reduced fertility. Recent studies are therefore focusing on improving both the productive and reproductive performance of rabbits while also promoting their welfare. In this context, this study aims to investigate the combined effects of extruded linseed and algae *Padina pavonica* extract as dietary sources of omega-3 on female rabbits' productive and reproductive performance. Thirty-six nulliparous New Zealand White female rabbits were divided into three experimental groups (n=12) according to different diets: commercial diet (CNT group), commercial diet enriched with 5% extruded linseed alone (L5% group), and in combination with 0.2% algae *Padina pavonica* extract (L5%PP group). The rabbits were monitored from artificial insemination until weaning of the rabbit kits, and different productive and reproductive parameters were evaluated. Our results indicate that dietary enrichment with extruded linseed and alga *Padina pavonica* did not affect feed intake or live weight of female rabbits, suggesting the absence of adverse effects and validating their palatability. Additionally, no significant differences were observed in litter size, litter weight or milk yield. Interestingly, perinatal and pre-weaning mortalities were significantly lower in litters born to females of both the supplemented groups.

Key words: Algae, Fertility, Linseed, Omega-3, *Oryctolagus cuniculus*

INTRODUCTION

Rabbit farming relies on frequent breeding, and its success critically depends on the reproductive performance of female rabbits. Nevertheless, in female rabbits, intensive reproductive rhythms often induce a negative energy balance, ultimately leading to a reduction in their fertility. Primiparous rabbit does are particularly vulnerable to this negative energy balance, as their feed intake capacity and growth are not fully developed (Castellini et al., 2010). Furthermore, the energy deficit is closely associated with the reduced lifespan of reproductive females, as the primary reasons for culling in rabbit breeding are infertility and

poor body conditions (Menchetti et al., 2019). Therefore, recent studies prioritize enhancing both productive and reproductive performance as well as the welfare of this species (Menchetti et al., 2019). To achieve these goals, the scientific community is investigating the potential benefits of incorporating various nutraceuticals into the rabbits' diet. Among the investigated nutraceuticals, those containing high levels of omega-3 polyunsaturated fatty acids (n-3 PUFA) have demonstrated the most interesting results due to their numerous physiological functions (Castellini et al., 2010). This study investigates the combined effects of extruded linseed and algae *Padina pavonica* extract, as sources of n-3 PUFA, on the reproductive and productive performance of nulliparous female rabbits.

MATERIALS AND METHODS

Animals and experimental design

Thirty-six nulliparous New Zealand White female rabbits, aged 4 months, were individually housed in conventional cages (L×W×H: 75×38×25 cm) under controlled environmental conditions in a commercial farm located in Central Italy. The rabbits were randomly assigned to three groups (n=12/group) according to different diets: commercial diet (CNT group), commercial diet enriched with 5% extruded linseed alone (L5% group), and in combination with 0.2% algae *Padina pavonica* extract (L5%PP group). Following a nutritional adaptation period of 60 days, the female rabbits were artificially inseminated (AI) using fresh heterospermic semen. Ovulation was induced by intramuscular injection of 10 µg of synthetic GnRH immediately before AI. Twelve days after AI, pregnancy was diagnosed by abdominal palpation. During the experimental period (AI until weaning), the feed intake was registered daily, while live weight (LW) was recorded weekly until day 21 *post-partum*. The following reproductive and productive parameters were evaluated: receptivity, fertility, litter size at birth, litter size at weaning, litter weight at birth, litter weight at weaning, perinatal and pre-weaning mortality, as well as milk yield.

Chemical Analyses

The experimental diets were isoenergetic and formulated according to current recommendations for female breeding rabbits. The proximate chemical composition of the diets (Table 1) was determined following the AOAC methods (AOAC, 2016).

Table 1: Chemical composition of the experimental diets.

	Diets[1]		
g per 100 g of dry matter	CNT	L5%	L5%PP
Dry Matter	89.34	89.49	89.94
Crude Protein	17.72	18.33	18.59
Ether Extract	3.95	6.21	5.22
Ash	7.99	7.69	8.21
NDF	43.24	39.65	42.40
ADF	27.90	25.72	25.53
ADL	7.76	7.83	7.22

[1]CNT: Commercial control diet; L5%: CNT diet supplemented with 5% extruded linseed; L5%PP: CNT diet supplemented with 5% extruded linseed and 0.2% algae *Padina pavonica* extract. NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin, following Van Soest and Wine, 1967.

The fatty acid profile of raw materials (extruded linseed and algae *Padina pavonica*) and diets (Table 2) was analyzed using GC-FID (Varian 4500). Fatty acid methyl esters were used to identify the different fatty acids. The algae *Padina pavonica* revealed a greater prevalence of saturated fatty acids primarily attributed to palmitic (C16:0) and oleic (C18:1) fatty acids. Conversely, extruded linseed exhibited higher concentration of α-linolenic acid (ALA). In terms of the fatty acid profiles of the experimental diets, both L5% and L5%PP diets exhibited higher concentrations of n-3 PUFA compared to the CNT diet. Notably, the main PUFA fraction in the CNT diet was omega-6, primarily composed of linoleic acid (LA). The L5%PP diet exhibited minimal differences in fatty acid composition compared to the L5%

diet, mainly observed in the presence of long-chain fatty acids (0.13% EPA, 0.05% DPA, and 0.06% DHA).

Table 2: Fatty acids profile (% of total fatty acids) of raw materials (extruded linseed and algae *Padina pavonica*) and diets.

Fatty acid (% of total fatty acids)	Raw material		Diets[1]		
	Linseed	Algae	CNT	L5%	L5%PP
C16:0	5.97	27.95	13.92	11.14	11.65
C18:0	4.54	12.52	3.07	3.82	3.48
C18:1	20.93	25.84	25.39	23.89	23.98
C18:2cis n-6, LA	15.11	9.72	47.63	32.41	33.53
C18:3 n-3, α-ALA	52.19	8.98	6.53	23.25	22.63
C20:5n-3, EPA	0.00	0.03	0.00	0.00	0.13
C22:5n-3, DPA	0.00	0.00	0.00	0.00	0.05
C22:6n-3, DHA	0.00	7.63	0.00	0.00	0.06

LA: linoleic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid. [1]CNT: Commercial control diet; L5%: CNT diet supplemented with 5% extruded linseed; L5%PP: CNT diet supplemented with 5% extruded linseed and 0.2% algae Padina pavonica extract.

Statistical Analysis

Krusjal Wallis test was used to compare feed intake among groups, while Linear Mixed models were used to analyse LW, milk yield, and litter size, including days after AI or after parturition as repeated factors. The models evaluated the main effects of time, group, and their interaction. The number of rabbit kits was included in the models as a covariate when appropriate. Sidak adjustment was used to conduct multiple comparisons. Perinatal and pre-weaning mortality were analysed by Generalized Linear models using Poisson distribution and Log link function. The receptivity and fertility were analysed by Chi-square, Fisher exact, and z-tests. Statistical analyses were performed with SPSS Statistics (IBM, SPSS Inc., Chicago, IL, USA). Differences were declared assuming an alpha value of 0.05.

RESULTS AND DISCUSSION

To the best of our knowledge, this is the first study to examine the combined effects of extruded linseed and the extract of *Padina pavonica* algae, as sources of n-3 PUFA, on the reproductive and productive performance of nulliparous female rabbits. Feed intake did not differ among groups during pregnancy, suggesting that both linseed and algae did not negatively affect the diets' palatablity. The LW of female rabbits was affected only by time (P<0.001). Specifically, during pregnancy, LW progressively increased, while during lactation it decreased within one week after parturition (data not shown). Concerning the rabbit's sexual receptivity, while the L5% and L5%PP groups demonstrated a higher percentage of does with red vulvas (75%) than the CNT group (58.8%), analysis revealed only trend towards significance (p=0.086). A comparable outcome was observed regarding the rabbits' fertility, as both L5% and L5%PP experimental groups exhibited higher pregnancy rates (83.3%) compared to the CNT group (66.7%), even though statistical analysis could not find significant differences among groups (p=0.447). As regards the productive parameters, no significant differences were observed in litter size among groups, neither at birth nor weaning (Table 3). Moreover, litter weight was influenced by litter size (p<0.01) but not by the group. Our results are consistent with previous studies (Rebollar et al., 2014) which found no significant effects of n-3 PUFA supplementation on both litter size and weight at birth and weaning. However, our study revealed that both supplemented groups had significantly lower perinatal (P<0.001) and pre-weaning (P<0.05) mortality (Table 3). It is suggested that dietary n-3 PUFA supplementation during pregnancy promotes early neuronal development and regulates neurochemical aspects related to stress response, growth, and cognitive functions, being very important for the vitality of newborns, especially in the first days of life (Bernardi et al., 2012). Newborn mammals can also benefit from n-3 PUFA supplementation through milk consumption, as the fatty acid composition of milk typically reflects the composition of the

mother's diet (Rodríguez et al., 2018). Although milk composition was not assessed in our study, milk yield increased until day 18 *post-partum* (P<0.001) and was influenced by litter size (P<0.001) but without differences among groups.

Table 3: Productive performance of the experimental groups.

Parameter	Experimental groups[1]			P value
	CNT	L5%	L5%PP	
Litter size at birth (total n)	7±1	8±1	7±1	0.576
Litter size at weaning (n)	6±1	7±1	7±1	0.507
Litter weight at birth (g)*†	427±25	440±20	468±19	0.394
Litter weight at weaning (g)*†	4987±199	4814±188	4965±177	0.784
Perinatal mortality (%)*	6.67c±0.86	2.78b±0.56	0.00a±0.00	<0.001
Pre-weaning mortality (%)*	5.56c±0.79	0.00a±0.00	3.33b±0.53	0.016
Milk yield (g)*	161.9±2.8	158.6±2.6	159.90±2.5	0.679

Data are expressed as mean ± standard errors.* Estimated. † Litter size was included as covariate in the models. Values followed by the same letter in each row do not differ significantly (P<0.05). [1]CNT: Commercial control diet; L5%: CNT diet supplemented with 5% extruded linseed; L5%PP: CNT diet supplemented with 5% extruded linseed and 0.2% algae Padina pavonica extract.

CONCLUSIONS

Our data suggest that dietary supplementation with n-3 PUFA in nulliparous rabbit does: (1) did not significantly affect feed intake or live weight, suggesting no negative effects on the diets' palatability; (2) showed a tendency to enhance receptivity and fertility, although a larger sample size is needed to statistically confirm this trend; (3) reduced perinatal and pre-weaning mortality in rabbit kits. Based on these findings, dietary n-3 PUFA supplementation with extruded linseed and *Padina pavonica* algae appears to be a promising strategy for improving the productive and reproductive performance of nulliparous female rabbits.

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EFFECT OF LINSEED AND ALGAE DIETARY SUPPLEMENTATION ON QUALITATIVE CHARACTERISTICS OF RABBIT SEMEN

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ABSTRACT

Forty-five New Zealand White rabbit bucks were used for the experimental trial (15 rabbit/groups). Three different diets were administered *ad libitum* for 110 days: standard diet (C), the same diet with 5% of linseed (L), and another one with 5% linseed + 0.2% of *Padina pavonica* algae extract (LPP). The semen was collected twice per week and volume, concentration, motility, vitality, capacitation and acrosome reaction were evaluated. The oxidative status and the fatty acids were also assessed by spectrophotometer and chromatography analysis, respectively. The aim of research is to test the use of an algae (*Padina pavonica*) extract as an enhancer of long-chain fatty acids-metabolism in combination with a limited amount of linseed. The results showed that the use of flaxseed (5%) combined with 0.2% algae extract (LPP) did not have a significant effect on the main kinetic traits and fatty acid profile of sperm, compared to L group, whereas, after 8 wk. of administration, there was an increase in the quantity of n-3 PUFA compared to the control group.

Keywords: Rabbits, Polyunsatured Fatty Acids, *Padina Pavonica*, Lineseed, Reproduction, Meat Quality.

INTRODUCTION

Linseed supplementation is considered a useful strategy for improving the reproductive parameters of male and female rabbits. Literature reported that about 10% dietary supplementation of linseed administered for 110 days modifies the fatty acid profile and the semen quality of rabbit bucks (Castellini et al., 2021). However, this level of inclusion is not always economically sustainable, thus also other dietary strategies should be proposed. In this context, the purpose of this research is to test the use of an algae (*Padina pavonica*) extract as an enhancer of long-chain fatty acids metabolism in combination with a little amount of linseed (5%) with the aim to improve the semen characteristics of rabbits without increase a lot the cost of feed.

MATERIALS AND METHODS

Animals and experimental design

At the Department of Agricultural, Environmental and Food Science of Perugia University rabbitry, forty-five New Zealand White rabbit bucks (6 months of age) were divided into 3 experimental groups (Table 1): Control group (C) fed a standard diet, Linseed group (L) fed a standard diet enriched with 5% of linseed and the flax-algae group (LPP) fed standard diet plus *Padina Pavonica* algae at 0.2% and linseed at 5%. The diets were formulated to be isoenergetic, and isoproteic (Martini s.r.l) and were administered for a total of 120 days (60d of adaptation + 60d experiment). Semen collection was performed twice every week.

Table1: Formulation and composition (%, UI, Kcal) of diets

	C	L	LPP
Ingredients			
Wheat bran	25.1	24.8	24.9
Barley	13.3	13.0	13.0
Sunflower seed	12.0	11.7	11.5
Alfalfa meal	10.8	13.0	13.0
Sunflower husks	10.0	10.0	10.0
Beet pulp	7.5	5.7	5.5
Extruded linseed	-	5.0	5.0
Full-fat soybean	5.0	2.9	3.1
Wheat straw	4.2	2.0	2.0
Molasses cane	3.0	3.0	3.0
Wheat	2.5	2.5	2.5
Grape seed meal	2.3	1.7	1.7
Soya hulls	0	1.7	1.7
Calcium carbonate	1.6	1.5	1.4
Soybean oil	0.78	-	-
Sodium chloride	0.40	0.40	0.40
Palm oil	0.33	-	-
Carboxymethylcellulose	0.30	0.30	0.30
Mineral-vitamin premix[1]	0.25	0.25	0.25
Alga PP	-	-	0.20
Lysine HCl	0.16	0.17	0.17
Liquid acidifier[2]	0.15	0.15	0.15
Magnesium oxide	0.1	0.1	0.1
Methionin	0.06	0.07	0.07
Choline	0.05	0.05	0.05
Vitamin E 50%	-	0.03	0.03
L Threonine	0.03	0.01	0.01
DL Methionine	0.03	-	-

	C	L	LPP
C. Protein	16.5	16.5	16.5
Lipids	3.62	3.93	3.93
Fiber	17.16	16.79	16.79
Ash	7.99	8.09	8.09
DE (kcal)	2350	2350	2350

Semen sampling and qualitative characteristics evaluation

The semen was collected with an artificial vagina in different glass tubes for each rabbit and then stored in eppendorf. The samples were immediately transported to the laboratory of the University of Perugia where qualitative analyses were performed. The sperm kinetic traits were evaluated with CASA System (ISAS©, Valencia, Spain).

Chemical Analyses

The oxidative status and fatty acid profile of rabbit semen were assessed by spectrophotometer (Ke et al, 1997) and chromatography (Folch et al, 1961) analysis respectively, while the tocols profile of semen and blood plasma (tocols) was assessed by HPLC (Hewavitharana et al, 2004) at 0, 4 and 8 weeks of dietary administration.

Statistical Analysis

The statistical analysis was done with a mixed linear model considering the repeated effect of buck and the effects of the time of collection, diet and their interaction. The post-hoc Bonferroni test were used for defining the significance of the differences ($p<0.05$).

RESULTS AND DISCUSSION

Table 2 shows the main traits of rabbit semen. All the effects have been reported (diet, time and their interaction), however, only the effect of diet will be discussed in detail. There are no differences between experimental groups regarding volume, live and dead cells, static sperm, non progressive motility, curvilinear velocity (VCL), oscillation index (WOB) and the movements of the sperm head (ALH).

Semen concentration and progressive motility were higher in L and LPP groups as well as other kinetic parameters (linearity-LIN, straight trajectory-STR, and flagellum beat cross frequency -BCF). The L group also increases the BCF which could indicate a pre-capacitative status of sperm cells.

The reasons for this light improvement can be traced back to a probable modofied asset of PUFA profile which affects the lipid structure of the membranes (Mourvaki et al. 2010) and at the same time improves their elasticity and motility.

Table 2. Semen quality evaluations of rabbit fed experimental diets

Parameters	Unit	C	L	LPP	RMSE	*P*		
						Diet	Time	DxT
Volume	ml	0.30	0.30	0.38	0.13	0.115	0.018	0.249
Concentration	x 10^6/ml	509.36a	576.05b	595.08b	3.93	0.046	0.385	0.408
Live cells	%	78.21	80.00	78.70	1.06	0.686	<0.001	0.318
Dead cells	“	21.78	20.00	21.29	1.06	0.686	<0.001	0.318
Static	“	25.00b	18.85a	20.00a	0.11	0.087	0.002	0.004
Motile non progressive	“	64.00	60.00	63.00	0.11	0.384	0.007	<0.001
Motile progressive	“	11.00a	20.00b	16.50ab	0.1	0.001	0.008	<0.001
VCL	mm/s	239.10	242.16	230.98	1.93	0.724	<0.001	0.035
LIN	%	19.83a	24.8b	21.81ab	0.67	<0.001	0.011	0.003
STR	%	42.38a	52.27b	44.77a	0.95	0.01	0.01	<0.001
WOB	%	47.70	47.39	49.06	0.64	0.118	<0.001	0.058
ALH	mm	3.22	3.47	3.12	0.24	0.013	0.714	0.245
BCF	Hz	16.39a	25.34b	18.52ab	0.85	<0.001	<0.001	<0.001

On the same row different letters (a, b) means P<0.05 for the effect of diet

The fatty acid profile of semen (Table 3) was not affected by DietXTime interaction.
Instead, we had a significant difference related to the dietary effect on quantitative of docosahexaenoic acid (DHA-C22:6n-3) which is higher in the LPP group after 8 weeks of administration, than others. While the time effect affects the quantitative of Polyunsaturated fatty acids (PUFA) and PUFA n-3, always from 8 weeks of administration.

Table 3. Oxidative status (TBARS, nmol MDA/mL) and fatty acid profile (% total fatty acids) of rabbit semen at 0, 4 and 8 weeks of dietary administration.

	0			4 wk			8 wk				*P*		
	C	L	LPP	C	L	LPP	C	L	LPP	RMSE	Diet	Time	DxT
TBARS	4.46	14.15	13.16	4.07	12.43	12.36	8.74	12.06	17.40	1.00	0.969	0.820	0.388
C18:2n-6	8.06	7.69	4.96	7.91	7.02	7.89	7.58	7.51	8.76	0.59	0.245	0.650	0.125
C18:3n-3	0.15	0.34	0.18	0.00	0.26	0.25	0.00	0.17	0.28	0.82	0.136	0.162	0.131
C20:3n-3	1.07	0.77	0.79	0.66	0.78	0.33	0.64	0.99	0.99	0.27	0.255	0.482	0.319
C20:4n-6	1.29	0.96	0.96	1.08	0.84	0.49	1.10	1.28	1.20	0.20	0.654	0.720	0.356
C20:5n-3	1.07	0.88	0.95	0.83	0.66	0.64	0.23	0.50	0.23	0.59	0.181	0.259	0.079
C22:5n-6	19.66	17.7	17.84	16.04	16.95	11.84	23.75	23.36	23.55	0.92	0.182	0.593	0.399
C22:5n-3	2.22	1.51	1.57	1.82	1.76	1.20	2.07	2.68	2.25	0.56	0.697	0.610	0.544
C22:6n-3	0.65c	0.51c	0.79c	0.80c	0.62c	0.31b	0.10a	0.18a	0.39b	0.18	0.004	0.243	0.118
SFA	46.7	47.71	50.68	47.12	51.88	52.23	49.1	48.63	46.42	0.26	0.275	0.278	0.987
MUFA	3.85	8.02	8.79	7.73	7.22	16.63	5.86	5.55	8.12	0.25	0.407	0.468	0,228
PUFA	34.17	30.36	28.04	29.14	28.89	22.95	35.47	36.67	37.66	0.27	0.906	0.002	0.390
PUFAn-3	2.94	2.50	2.71	2.29	2.32	1.53	0.97	1.84	1.89	0.97	0.668	0.043	0.220
PUFAn-6	31.23	27.86	25.33	26.85	26.57	21.42	34.50	34.83	35.76	0.36	0.345	0.189	0.394

on the same row different letters (a,..c) means P<0.05 for Diet

The tocol profile (Table 4) showed a different trend in semen and blood. In semen the highest value was found in L groups than C and LPP after 4 wk., with a subsequent reduction at 8 wk., whereas in blood the highest value was found in LPP one, followed by L and C.

Table 4. Total Tocol content (nmol/mL) of rabbit semen and blood after 0, 4 and 8 weeks of administration.

	wk 0			wk 4			wk 8				*P*		
	C	L	LPP	C	L	LPP	C	L	LPP	RMSE	Diet	Time	DxT
Tocols semen	1.01a	1.39a	1.39a	1.22a	1.89b	1.38a	1.69b	0.88a	0.83a	0.356	0.025	0.056	<.001
Tocols blood	0.72b	0.74b	0.80b	0.40a	1.18b	2.60c	0.84b	0.30a	0.77b	0.14	<.001	<.001	<.001

on the same row different letters (a, b) means P<0.05 for Diet

CONCLUSIONS

In this study, the effect of two sources of PUFA (linseed combined or not with algae extract) in feeding of rabbit bucks were studied. The results showed that the use of low amount of linseed (5%) combined or not with 0.2 % of algae extract (due to the economic sustainability of the supplementation) did not improve the most relevant semen traits.
With respect to other studies, which used higher amount of linseed, nor the kinetic traits nor the fatty acid profile of semen were greatly affected by treatment. Accordingly, the expected enhancing effect of algae on rabbit buck metabolism was not recorded. Probably, higher amount of linseed and algae extract are required, or a prolonged time of administration.
Further studies to deepen the algae linseed interaction on rabbit metabolic response (blood, organs) are needed.

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EFFECT OF DIFFERENT GnRH ANALOGUE TREATMENTS ON REPRODUCTION OF LIGHT AND FAST-STIMULATED RABBIT DOES

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ABSTRACT

This study evaluated the effect of intramuscular (i.m.) or intravaginal (i.vag.) GnRH-analogue treatment used for ovulation induction on the reproduction of rabbits differently synchronized for estrus by light program (L) or by short fast-refeeding plus light program (FL) before insemination (AI). For estrus synchronization, the 1-14 days controlled nursing rabbits were subjected to light stimulation (L) or fast-refeeding plus light stimulation (FL) before AI (on day 11). On day 8 before AI the daily 9 h and 50 lux LED lighting was increased to 16 h and 100 lux that was gradually set back until day 5 after AI. The L rabbits were fed ad libitum. The FL rabbits received the same diet but they had a 24 h water-only fast and 48–50 h ad libitum re-feeding before AI. Two-third of rabbit does were induced to ovulate by i.m. administration of 0.2 mL GnRH analogue (Receptal®, 0.84 µg buserelin acetate/doe) and one-third by i.vag. via 0.5 mL semen extender (MRAbit®, 25 µg GnRH analogue [des-Gly10, D-Ala6]-LHRH ethyl amid/doe). Receptivity rates of the i.m. GnRH analogue treated rabbits were lower (L: 43 vs 54% and FL: 40 vs 53%; P=0.05) but their pregnancy rates were higher (L: 93 vs 85% and FL: 93 vs 87%; P=0.05). Kindling rates were also higher with the i.m. GnRH-analogue treatments (L: 88 vs 81% and FL: 92 vs 79%; P=0.05). The number of live born kits per litter were similar (L: 9.84 vs 9.60 and FL: 9.33 vs 9.51; P=0.39). Productivity at birth (number of live born kits per 100 AI) of the i.m. GnRH analogue treated L (869 vs 774) and FL (861 vs 747) rabbits were 11% and 13% higher.

Key words: Receptivity, Ovulation, Lighting, Nursing, Nutrition

INTRODUCTION

Synchronization of estrus and induction of ovulation is necessary for successful artificial insemination (AI). Instead of hormone treatments given intramuscularly, the methods without injection are more advantageous from the point of view of animal welfare and work organization (Dal Bosco *et al.*, 2011). The dominant players in rabbit meat producing sector are the large-scale farms, where breeding is done by AI. The development of technological elements is necessary to preserve the efficiency, marketability and animal welfare. In rabbit doe, the hormone injections required for AI can be replaced by hormone-free estrus synchronization methods (Villamayor *et al.*, 2021; Chen *et al.*, 2023) and/or induce ovulation with a GnRH analogue administered with a sperm diluent (Gogol, 2016; Hassanein *et al.*, 2021; Mattioli *et al.*, 2021; Rebollar *et al.*, 2012 and 2023; Viudes-de-Castro *et al.*, 2023).

Regarding alternative estrus synchronization, we compared the effect of light program and short fast-refeeding plus light program used as biostimulation before AI on reproduction (Eiben *et al.*, 2021). At that time, there was no aim to study the effect of the ovulation induction method, although we also used intravaginal GnRH analogue treatment in some of the rabbits. There are few results where both estrus and ovulation induction happened without intramuscular injection.

This study focused on the effect of the intramuscular or intravaginal GnRH-analogue treatment used for ovulation induction on the reproduction of rabbits differently synchronized for estrus, i.e. by light program or by light program plus short fast-refeeding before AI.

MATERIALS AND METHODS

Animals and nursing

The study was carried out at rabbit farm of S&K-Lap Ltd in Galgamácsa. Multiparous Hycole rabbits with litters standardized to ten kits were divided into two equal groups based on doe live weight at kindling. Artificial insemination (AI) was done on January 2015 with two consecutive cycles (n=294 and 294). Controlled nursing was used by opening the metal-sheet nest door from 9 a.m. to 10 a.m. from postpartum day 1 to 14 and free nursing thereafter.

Estrus synchronization by light program (L)

There was no hormonal estrus synchronization but from day 8 before AI the duration and intensity of daily lighting were increased in both groups as light stimulation. In the buildings, the dimmable cold-white multichip four-die LED lamps (15x20 cm) provided the daily 9 h and 50 lux basic illumination. On day 8 before AI, the 9 h L (8 a.m. to 5 p.m.) was increased by 7 hours to 16 h L (6 a.m. to 10 p.m.). The lighting was reduced by 2 hours on days 3 and 4 after AI (14L, 6 a.m. to 8 p.m. and 12L, 8 a.m. to 8 p.m.) and by 3 hours on day 5 after AI, returning to the 9 h (8 a.m. to 5 p.m.) daily lighting. To increase light intensity, the LEDs were set to 100 lux from day 8 before AI to day 3 after AI. On day 4 after AI the light intensity was reduced to 80-90 lux and then back to 50 lux.

Estrus synchronization by fast-refeeding plus light program (FL)

The rabbits were fed the same diet (10.0 MJ/Kg DE, 17.5% CP, 3.80% EE, 14.9% CF, 7.7% ash) and *ad libitum* in only-light stimulated group (L). In group stimulated with fast-refeeding plus lighting (FL) the does were subjected to a 24 h water-only fast between days 8 and 9 and a 48-50 h *ad libitum* re-feeding before AI. The automatic feeder was closed at Monday night. At 8 a.m. on Tuesday morning about 380-400 g feed remained in the collective feeder supplying four does. The feeder was reopened at 2 p.m. on Wednesday and thereafter there was *ad libitum* feeding (AI on Friday between 10 and 12 a.m.).

Ovulation induction and AI

AI was done on postpartum day 11 with heterospermic pooled semen from Hycole bucks (0.5 mL/doe) within three hours after nursing.

Two-third of the rabbit does were induced to ovulate by **i.m**. administration of 0.2 mL GnRH analogue (Receptal®, 0.84 µg buserelin acetate/doe) and one-third by **intravaginal** via 0.5 mL semen extender (MRAbit®, 25 µg GnRH analogue [des-Gly10, D-Ala6]-LHRH ethyl amid/doe).

At AI the does with red / violet and turgid vulvas were judged to be receptive.

Pregnancy was checked on day 14 after AI by abdominal palpation. In air conditioned (18-20°C) building the rabbits were housed in wire-net breeding cages (80 x 53 cm with 90 cm height) equipped with a plastic mat, an elevated platform (40 x 53 cm) at 25 cm height, a gnawing stick and an outer nest (23 x 53 cm) with metal sheet walls.

The effects of intramuscular or intravaginal GnRH treatment combined with light program or fast-refeeding plus light program on receptivity, pregnancy and kindling rates were evaluated by the chi-squared test and on the number of kits born by ANOVA using the Statgraphics 6.0 (1992) statistical software. Productivity was calculated as the number of live born kits per 100 AI.

RESULTS AND DISCUSSION

Sexual receptivity

Compared to the i.m. GnRH analogue treated FL rabbits (47% or 54%), a higher proportion of i.vag. treated FL or L rabbits had red-violet or turgid vulva, respectively (60% or 66%), resulting in their higher receptivity (40% vs 54% or 53%, Table 1, P=0.05). This is important to mention because the intravaginal GnRH treatment is less effective (Quintela *et al*., 2023; Viudes-de-Castro and Vicente, 2023). Earlier we confirmed that good reproduction can be expected from estrus rabbits (Eiben *et al*., 2014) and it is particularly important when ovulation is induced by intravaginal GnRH treatment.

Pregnancy and kindling rates

The pregnancy rate was higher (P=0.05) in the i.m. FL rabbits (93%) than in the i.vag. L does (84%) despite the lower receptivity of the i.m. FL females. Kindling rate was affected by the ovulation induction method. It was significantly higher with the i.m. GnRH treatment but only in the case of the FL rabbits (92% vs 79%, P=0.05).

Litter size

The number of born kits (10.7-11.2) and live born kits (9.33-9.84) per litter were not influenced (P>0.05) by the treatments (Table 1).

Productivity

Compared to the i.m. L rabbits (869), productivity at birth was near similar in the i.m. FL rabbits (861) but it was lower by 11 and 14% (774 and 747) in the i.vag. L and FL rabbits (Table 1).

Table 1: Effect of intramuscular or intravaginal GnRH analogue treatment on the reproduction of rabbits synchronized for estrus by light (L) or fast-refeeding plus light (FL) program

Ovulation induction	Intramuscular (i.m.)		Intravaginal (i.vag.)		
Estrus synchronization	L	FL	L	FL	Prob.
	n=196	n=196	n=98	n=98	
Red-violet vulva, %	54.6^{ab}	46.9^{b}	58.2^{ab}	60.2^{a}	0.05
Turgid vulva, %	60.7^{ab}	53.6^{b}	66.3^{a}	63.3^{ab}	0.05
Sexual receptivity, %	42.9^{ab}	39.8^{b}	54.1^{a}	53.1^{a}	0.05
Pregnancy rate, %	92.9^{ab}	93.4^{b}	84.7^{a}	86.7^{ab}	0.05
Kindling rate, %	88.3^{a}	92.3^{a}	80.6^{ab}	78.6^{b}	0.05
No. of born kits per litter	11.1±0.2	10.7±0.2	10.9±0.3	11.2±0.3	0.23
No. of live born kits	9.84±0.2	9.33±0.2	9.60±0.3	9.51±0.3	0.39
Productivity at birth	869	861	774	747	-

The productivity compared to the current farm practice (light-programmed estrus synchronization and i.m. GnRH analogue to induce ovulation) was reduced with intravaginal ovulation inducing GnRH-analogue treatment, but its extent and reason depended on how to synchronize estrus.

Estrus synchronization with FL tended to decrease sexual receptivity but the pregnancy rates seemed to improve. In the study of Daoud *et al*. (2012) feed restriction with refeeding increased the number of quality oocytes and GDF-9 gene expression responsible for fertility supporting our results. However, the number of live born kits per litter seemed to decrease in FL rabbits. This “dual” stimulation could impair prenatal life. Feeding can affect embryonic development (Lorenzo *et al*., 2014).

The intravaginal ovulation induction in the L rabbits led to 7.7% lower kindling rate but it was 13.7% lower in the FL rabbits. The number of kits per litter seemed to decrease in the L rabbits but increase in the FL does. One reason for these differences can be that feed restriction can alter the sensitivity to hormones and hormonal effects (Harrath *et al*, 2017; Sirotkin *et al*., 2017).

CONCLUSIONS

From an animal welfare point of view, the advantage of the examined intravaginal ovulation induction in stimulated rabbits is that no injection was used either to synchronize estrus or to induce ovulation. However, with the method, the productivity at birth decreased by 11-14%. Further research is needed to reveal possible causes such as doe's condition and physiological-hormonal state (Martínez-Paredes *et al*. 2022; Peiró *et al*., 2023) or type and form of the GnRH analogue used (Dal Bosco *et al*., 2014).

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ENHANCING OVULATION RESPONSE WITH CANNULA USE IN RABBIT ARTIFICIAL INSEMINATION

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ABSTRACT

When Artificial Insemination (AI) is carried out in rabbits, ovulation is induced by GnRH analogues. However, it has been observed that some females are able to ovulate in response to physical stimuli such as that produced when the insemination cannula is introduced. The aim of this study was to find out how the mechanical stimulation produced by the introduction of the cannula affects fertility at birth, ovulation rate and litter size. The results showed that introducing the insemination cannula twice 5 min apart was able to stimulate the nervous/mechanical pathway to trigger ovulation in at least 73% of cases. Significantly more efficient than when only one stimulation was performed. We see that when inseminating using this double stimulation technique, we obtained fertility results close to the two control groups that were administered buserelin acetate intramuscularly and vaginally (0.68 ± 0.062 vs 0.77 ± 0.077 for the former and 0.78 ± 0.086). In addition, no differences in litter size were observed. On the other hand, the group that was administered 3.5 ug of buserelin acetate trapped in chitosan-dextran sulphate nanoparticles in the insemination diluent obtained the same results as the control group that was administered 1 ug of buserelin acetate intramuscularly. It can therefore be concluded that the introduction of the cannula is responsible for a high percentage of nulliparous rabbits ovulating without the use of exogenous endocrine factors. It is possible that by perfecting management techniques and insemination methods and probably the type of cannula, it will be possible in the future to eliminate or reduce the group of inseminated rabbits that have to be stimulated to ovulate by GnRH analogues, improving animal welfare and increasing the biosecurity of artificial insemination.

Key words: ovulation induction, insemination cannula, encapsulated hormone, rabbit.

INTRODUCTION

Rabbits are an ovulation-induced species and therefore need the stimulus produced by coitus to release GnRH and cause an LH surge to trigger ovulation (Bakker and Baum, 2000). Rabbits exhibit heightened sensitive to physical stimuli compared to other ovulation-induced species. Adequate genital stimulation or other mating-related cues are imperative for triggering ovulation in this species. Recent studies conducted by Viudes de Castro *et al.* (2017) and Garcia-Garcia *et al.* (2018) have shown that ovulation can be mechanically stimulated by the introduction of a commercial cannula in at least 50% of females.This process simulates the nervous/mechanical stimulation that occurs during coitus. This is consistent with the results of Rebollar *et al.* (2012) who observed that females subjected to epidural anaesthesia, thereby blocking nerve stimulation, did not undergo ovulation in the absence of vaginal stimulation, emphasising the crucial significance of physical stimulation in the ovulation process of rabbits. Maranesi *et al.* (2018 and 2021) propose that the mechanical stimulation that occurs during coitus or the introduction of an insemination cannula into the vaginal/uterocervical regions, in conjuntion with the paracrine pathway of endocrine factors present in the seminal plasma at the uterus/uterine cervix level, represent two interrelated pathways that facilitate ovulation in the doe.

In AI procedure, where coital stimulation is absent, ovulation is induced through the parenteral application of GnRH analogues, usually administered intramuscularly or subcutaneously. Despite the evident advantages of intravaginal administration of GnRH analogues as a minimally invasive approach for inducing ovulation in females, achieving fertility results comparable to those of intramuscular administration necessitates higher hormone due to the elevated enzyme levels present in the seminal plasma of the rabbit and the limited permeability of the vaginal mucosa (Viudes de Castro *et al.*, 2014). Protection of the analogue by nanoencapsulation may help overcome enzymatic degradation and enhance its bioavailability (Viudes de Castro *et al.*, 2023).

The aim of this study was to determine how the mechanical stimulation produced by the introduction of the cannula affects ovulation rate, fertility at birth and litter size. Two control groups were included: one receiving an intramuscular GnRH analogue, and the other receiving an encapsulated GnRH analogue within the seminal dose.

MATERIALS AND METHODS

Animals and experimental design

A total of 210 females and 20 males of New Zealand origin (line A) were used. In a first experiment carried out at the experimental farm of the “Universidad Politecnica de Valencia”, (animal experimentation authorization code of the project procedures 2021-VSC-PEA-0270), one hundred nulliparous receptive females were used. To achieve receptivity synchronization, females were administered 10-15 IU of eCG intramuscularly two days before ovulation induction. Three experimental groups were established based on the method used for ovulation induction. A control group was injected intramuscularly with 1ug of buseriline acetate, and on the other hand, two groups were intended to induce ovulation by inserting a silicone cannula with a maximum thickness of 7 mm and a depth of insertion of 15 cm, once (single stimulation) or twice, with a separation of 5 min (double stimulation). After 12 days, the induction capacity and ovulation rate were assessed by laparascopy, observing the presence of corpora lutea in the ovaries.

In a second experiment carried out at the experimental farm of the “Centro de Investigación y Tecnología Animal” of Segorbe (animal experimentation authorization code of the project procedures 2023-VSC-PEA-0219), a total of 110 nulliparus rececptive females were inseminated using 3 different methods to induce the ovulation. Seminal doses of 0.5 ml of diluted semen were made from a mixture of ejaculates from at least 10 males with more than 70% sperm motility and less than 20% abnormal spermatozoa. The heterosperm mixture was diluted with Tris-citric-glucose (Viudes de Castro *et al.*, 1997) to a concentration of about 40-50 million/ml. In the first group (control) ovulation was induced through intramuscular injection of 1ug of busereline acetate. The second group, however, received 3.5ug of busereline acetate encapsulated in chitosan-based nanoparticles, incorporated into the extender, as described by Viudes de Castro *et al.* (2023). The third group did not utilize any GnRH analogue, instead, ovulation was induced through a dual stimulation approach using the insemination cannula. Initially, the cannula was introduced to elicit mechanical stimulation, followed by a subsequent introduction of the cannula and deposition of the insemination dose after a 5-minute interval. Fertility rate and litter size at birth were recorded.

Statistical Analysis

Data were analysed with the statistical package SPSS 23.0 (IBM Corp. Released 2015.IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). Analysis of the effects of the induction ovulation method on ovulation induction frequency and fertility at birth were carried out using a probit link with binomial error distribution, while ovulation rate and litter size were analysed by analysis of variance.

RESULTS AND DISCUSSION

The results of the first experiment are shown in Table 1. There was no significant difference in the ability to induce ovulation when double stimulation with the cannula was performed with respect to the control group (0.73 ± 0.078 and 0.82 ± 0.065, respectively, Table 1). There were significant differences in ovulation rate between single stimulation (10.9 ± 0.70) and control and double stimulation (13.1 ± 0.59 and 13.3 ± 0.64 respectively, Table 1). In the second experiment, no significant differences were observed among experimental groups (Table 2). Reproductive performance of nulliparous does in terms of fertility at birth and litter size were similar, reaching a 78%, 77% and 68% for control, encapsulated GnRH extender and double stimulation groups, respectively. The results observed in the group treated with encapsulated GnRH align with the recent findings of Viudes de Castro *et al.* (2017 and 2023). In their studies, comparable outcomes were noted for fertility at birth and litter size.

Table 1: Effect of stimulation method on ovulation induction frequency and ovulation rate.

	N	Ovulation frequency LSM± SE	Ovulation rate LSM± SE
Control	34	0.82 ± 0.065[a]	13.1 ± 0.59[a]
Single stimulation	33	0.55 ± 0.087[b]	10.9 ±0.70[b]
Double stimulation	33	0.73 ± 0.078[ab]	13.3 ± 0.64[a]

N: number of does.
LSM±SE: Least Square Mean ± standard error.
a,b Values with different superscript in the same column differ significantly (P<0,05).

Table 2: Effect of ovulation induction method on fertility and litter size at birth

	N	Fertility rate at birth LSM± SE	Litter size LSM± SE	Alive at birth LSM± SE
Control	30	0.77 ± 0.077	9.8 ± 0.51	9.1 ± 0.57
aGnRH extender	23	0.79 ± 0.086	10.3 ±0.63	9.6 ±0.71
Double stimulation	57	0.68 ± 0.062	9.7 ± 0.39	8.9 ±0.44

N: number of does.
LSM±SE: Least Square Mean ± standard error.

The stimulation generated by the cannula insertion seems to mimic the stimulus induced by the male during mating. In some cases, seems to be sufficient to initiate the cascade of signals leading to ovulation, but it does not have the same efficiency as when GnRH analogues are administered. This could be due to the fact that the repetition of the stimulation makes it easier for one of the two insertions to trigger the ovulation chain, or it could suggest that achieving a higher level of stimulation, whether in terms of duration or intensity, is necessary to increase the efficiency of the cannula-induced stimulation and approach the efficiency of the techniques that use GnRH analogues.

CONCLUSIONS

The findings unequivocally illustrate that physical stimulation efficiently induces ovulation. Consequently, we hypothesize that by optimizing animal management practices and designing an insemination cannula with appropriate dimensions and materials, we can faithfully replicate the stimulatory mechanisms inherent in natural mating. This optimization, in turn, holds the potential to efficiently trigger ovulation during the insemination process, thereby eliminating the need for exogenous endocrine agents to induce ovulation or facilitating a substantial reduction in their concentration. At the same time, it shows that

encapsulating the hormone can substantially reduce the amount of GnRH analogues administered vaginally, bringing the amounts more in line with those used in alternative administration routes, thus solving the main drawback of this technique. All this would mean an improvement in animal welfare as well as facilitating handling and increasing the biosecurity of artificial insemination.

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POSITIVE EFFECTS OF KISSPEPTIN ON RABBIT OVULATION

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ABSTRACT

The regulation of reproduction in mammals involves different factors, all converging via the hypothalamus. Among crucial neurons implicated in this system, the neurons that produce Kisspeptin (Kp) stimulate the release of gonadotropin-releasing hormone (GnRH) by hypothalamic neurons, leading to secretion of pituitary gonadotrophins (LH and FSH) and sexual steroids in the gonads to induce gametogenesis. Different works have used Kp and their analogues to induce ovulation in various mammalian species. This study aimed to assess, for the first time, the impact of an intravenous administration of Kp10 on ovulation induction in rabbits does that were hormonally synchronised and then assigned to three experimental groups: intravenous (i.v.) injected 0.5 ml saline solution (SS), intramuscular (i.m.) administered 20 µg gonadorelin (GnRH) and i.v. injected 250 µg Kp10 (Kiss-1 (112-121)). The presence or absence of corpora lutea (CL) on the ovarian surface determined the ovulation rate (OR), corpora lutea (CL) were counted, and serum progesterone (P4) concentrations were assessed in plasma blood samples on Day 0 and 7 post-treatment. SS does did not ovulate. The same OR rate (87.5%) and a similar number of CL in the GnRH and Kp10 groups were observed (14.6 ± 1.4 and 12.9 ± 1.4 ng/ml, respectively; p=0.4089). However, mean plasma P4 concentration was higher in ovulated does treated with GnRH than in those with Kp10 (25.12 ± 4.17 and 8.35 ± 3.49 ng/ml, respectively; p<0.0211), suggesting possible differences in the functionality or development of CL induced by these treatments. These preliminary results confirm the potential of Kp10 as a key neuropeptide modulating the rabbit hypothalamus and provide new insights to regulate reproduction management in this species.

Key words: Corpora lutea, Gonadotropin-releasing hormone, Neuropeptide modulation, Ovulation induction, Progesterone.

INTRODUCTION

The control of reproductive functions in mammals involves a variety of factors such as age, physiological status, environment conditions, stressors, pheromones, and biological rhythms (Scott et al., 2019). All these elements are integrated via the hypothalamus-pituitary-gonadal (HPG) axis through neurons that receive all this information. Among these, Kisspeptin (Kp) neurons produce a biologically inactive prepro-kisspeptin (145 amino acids). Then, four biologically active peptides are generated after the cleavage of prepro-Kp within the cell: Kp54, Kp14, Kp13, Kp10 (Tena-Sempere, 2006). These peptides have a preserved C-terminal region that contains an Arg-Phe-NH2 (RF-NH2) motif, which enables the high-affinity binding to and full activation of KISS1r receptor (West et al., 1998). Kp peptides/KISS1r system controls the HPG axis, modulating GnRH/LH secretion and gonadal function (Gottsch et al., 2004) and is an important key to inducing hormonal conditions favourable to reproduction. In addition, Kp/KISS1r system is expressed in other organs, especially in the ovary of different species (reviewed by Hu et al., 2018), and seems to play a role in ovulation. In many mammal females with spontaneous ovulation, such as sheep

(Wang et al., 2012), sow (Qin et al., 2022), mare (Magee et al., 2009), and cow (Ezzat et al., 2009), the administration of Kp in different routes and dosages can finely modulate pituitary (LH/FSH) and ovary (progesterone) secretions pointing out the possibility of managing livestock reproduction as Beltramo and Decourt (2018) have described. Thus, different dosages, administration routes and synthetic analogues have been tested in livestock species. However, it has not yet been verified in rabbit does, whose ovulation is induced, needing the administration of GnRH analogues during artificial insemination. Therefore, the main objective of this study was to evaluate the effect of an intravenous (i.v.) administration of Kp10 on ovulation induction in rabbits by measuring the ovulation rate, the number of CL, and plasma P4 concentrations.

MATERIALS AND METHODS

Animals and experimental design

New Zealand White x California multiparous rabbit females (4 kg), bred in the experimental farm at Technical University of Madrid were used under controlled conditions: photoperiod of 16 HL/8HD, 20-25 °C, and 60-75% relative humidity. Rabbits does had *ad libitum* access to food and water. All experimental procedures were approved by the Animal Ethics Committee of the Community of Madrid (PROEX 237.8/23) following the Spanish guidelines for the care and use of animals in research (BOE, RD 53/2013). All of them were synchronised with 25 I.U. of equine chorionic gonadotropin (Serigán, Lab. Ovejero, León, Spain) 48 h before the experiment.

A total of 24 multiparous females were assigned to three experimental groups as follows:

i) SS (n= 8): i.v. administration of 0.5 mL of saline solution (SS)
ii) GnRH (n= 8): i.m. administration of 20 µg of gonadorelin (Cystoreline, Ceva, Spain)
iii) Kp10 (n= 8): i.v. administration of 250 µg of Kisspeptin-10 (also known as Kiss-1 (112-121)/ Metastin (45-54), 445888, Sigma-Aldrich)

In order to determine the dose of Kp10 tested, a literature review was conducted, taking into account the species, route of administration, and the body weight of the treated animals (Wang et al., 2012; Hashizume et al., 2010; Caraty et al., 2007).

Blood samples were obtained on day 0 (D0, day of administration of treatments) and day 7 (D7) from the central ear artery in heparinised tubes (EDTA), centrifuged at 700 g for 15 min at 4°C, and plasma samples were stored at -20 °C until analysis. On D7, rabbits were sacrificed in compliance with RD52/2013, and the ovaries were recovered. The presence or absence of CL on the surface of both ovaries was assessed under a magnifying glass from each doe to determine the ovulation rate (OR) [(number of rabbits with CL/ total rabbits) x100] and the number of CL per doe.

Hormonal Analyses

Concentration analysis of P4 on D0 and D7 was carried out using a competition ELISA kit (Progesterone ELISA, Demeditec Diagnostics GmbH Kiel, Germany). The sensitivity was 0.045 ng/mL, and intra-assay coefficient of variation was 3.73%. Absorbance was measured in a microplate spectrophotometer (Epoch, Bio-Tek Instruments, Inc., VT, USA) at 450 nm with subtraction at 630 nm, and P4 concentrations were calculated by extrapolation of a logistic four-parameter sigmoidal standard curve developed by GraphPad Instat software (Version 5.01, San Diego, CA, EEUU).

Statistical Analysis

Differences on plasma P4 concentrations between experimental groups on D0 was performed by means a one-way ANOVA using Proc GLM. The same procedure was utilised to analyse P4 concentrations on Day 7 and the number of CL only in ovulated does. Also, ovulation rates were compared by a $\chi 2$ test (SAS/STAT, Cary, USA). Results are shown as lsmeans ± standard error.

RESULTS AND DISCUSSION

Effect of Kisspeptin on the number of CL and the percentage of ovulated females

As expected, on D7, no CLs were found in the ovaries of the rabbit females treated with SS, resulting in an ovulation rate of 0% (Table 1). The number of CL detected per rabbit doe after the Kp10 treatment was similar to the GnRH group (P=0.4089), so the OR was the same in both groups. These preliminary results indicate, for the first time, that i.v. administration of Kp10 is able to induce ovulation and produce a similar number of CL as the i.m. gonadoreline in rabbit does.

Table 1. Ovulation rate and number of corpora lutea (CL) on D7 of rabbit does treated with saline (SS), 20 µg of gonadoreline (GnRH) and 250 µg of Kisspeptin-10 (Kp10).

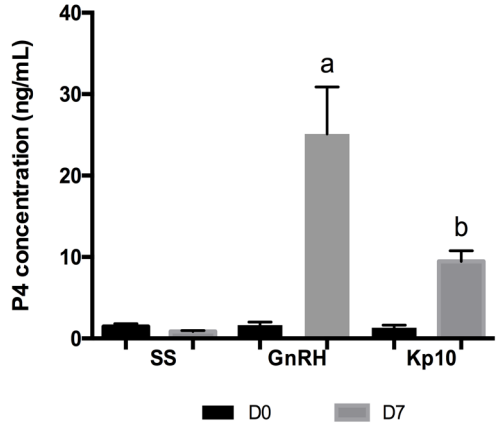
	SS	GnRH	Kp10	χ2	P>f
n	8	8	8		
CL[1]	-	14.6 ± 1.4 (7)	12.9 ± 1.4 (7)	-	0.4089
Ovulation rate (%)	0 (0/8)[b]	87.5 (7/8)[a]	87.5 (7/8)[a]	16.8	0.0002

[1] Mean± s.e.m. of CL in ovulated females with different letters on the same row differ significantly (χ2 test).

Effect of Kisspeptin on P4 concentration in rabbit does.

As shown in Fig. 1, mean plasma P4 concentration on D0 was similar between groups (P= 0.2884) with a mean of 1.57 ± 0.33 ng/ml. On D7, SS does did not ovulate, showing the lowest mean plasma P4 concentration (0.86 ± 3.49 ng/ml). On the other hand, the highest P4 levels were observed in ovulated does from GnRH followed by the Kp10 group (25.12 ± 4.17 and 8.35 ± 3.49 ng/ml, respectively; p< 0.0211). For the first time, this result provides evidence that Kp10 has a direct stimulating effect on rabbit female ovulation. Kp10 or its analogues have been shown to increase GnRH secretion and consequently LH secretion in many species as bulls and cows (Ezzat et al., 2009; Kadokawa et al., 2008); goats (Hashizume et al., 2010); sheep (Caraty et al., 2007); sows (Lents et al., 2008); mares (Magee et al., 2009); and fish (Rabouti et al., 2022). In addition, the presence of CL and high concentrations of P4 have been observed in ewes after a constant i.v. infusion of mouse Kp10 after 2-3 days (Caraty et al., 2007). The increase of P4 was similar in breeding and non-breeding season ewes treated with a Kp analogue (C6) by i.m. via (Decourt et al., 2016) as well as in Alpine goats (Decourt et al., 2019). Also, bovine Kp10 i.v. in prepubertal heifers induced the development of CL in 28% of the females, accompanied by increased serum P4 (Santos et al., 2014). In the current study, the different P4 concentrations observed between GnRH and Kp10 groups could indicate that the CL resulting from the Kp10 administration may not be equally functional as those from the GnRH group or, at least, some delay in their development could exist. This delay could be because the gonadoreline directly elicited the pituitary LH secretion, but Kp10 had to act first in the hypothalamus, resulting in possible retarded pituitary and ovarian responses.

Figure 1. Mean P4 concentrations of rabbit does treated with saline (SS), 20 µg of Gonadoreline (GnRH) and 250 µg of Kisspeptine-10 (Kp10) on Day 0 and Day 7. Letters indicate differences between GnRH and Kp ovulated females (P=0.0211).



CONCLUSIONS

This work suggests, for the first time, that an i.v. administration of Kp10 directly affects rabbit HPG axis, inducing ovulation with the same number of CL, incrementing plasma P4 concentrations. However, further experiments to confirm CL functionality, pregnancy success and other administration routes for field applications in a single i.m. or subcutaneous injection to induce ovulation are required in this species.

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COMPARISON OF DIFFERENT METHODS TO ASSESS RABBIT SPERM VIABILITY

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ABSTRACT

Assessment of sperm of any species may include various tests that allow predicting the reproductive capacity of males. Several tests for sperm quality assessment have been described; however, sperm viability is always carried out. For this, there are tests based on plasma membrane integrity (eosin/nigrosine, SYBR14/Propidium Iodide (PI)), or plasma membrane functionality (hypo-osmotic swelling test (HOST)). To decide which of these tests would be employed many factors should be considered: simplicity to prepare and interpret the test, time consuming, cost, use of any special microscope (fluorescence, phase contrast), purpose of the assessment (research, artificial insemination, reproductive control of the males), etc. There is not a single protocol, for each test, that may be employed in the sperm across species. Thus, the objective of this work was to compare different methods for the determination of rabbit sperm viability (eosin/nigrosine, HOST, NucleoCounter™ and SYBR14/PI), and the modification of one of these tests (SYBR14/PI with the addition of 0.4% glutaraldehyde). For the development of this work, ejaculates were collected from 6 Chinchilla males of proven fertility, twice a week; gel plug was removed, and each ejaculate was diluted (1:1 v/v) in Tris-Citric Acid-Glucose extender. A quick macroscopic and microscopic evaluation was made, and a pool was formed with those ejaculates that had at least 70% of progressive motility (7 pools, 4 ejaculates, at least, in each one). Subsequently, the pools were adjusted to $400x10^6$ sperm per milliliter and the previously mentioned tests were performed. Descriptive statistics were obtained from the data collected. Furthermore, after transformation to the arcsine for normalization, analysis of variance (ANOVA) was carried out on the percentage of viability obtained by the different tests, to determine if there were differences between them. There was a significant difference ($P<0.05$) between viability with NucleoCounter™ (69.9 ± 9.39) and that with SYBR14/PI (81.9± 5.86), however, there were no significant differences between eosin/nigrosine (76.1 ±3.84), HOST (74.6 ± 3.17) and SYBR14/PI with the addition of 0.4% glutaraldehyde (79.3 ± 7.73). Regarding comparison between the use of the SYBR14/PI technique previously described and the one modified (addition of 0.4% glutaraldehyde), there was no significant difference between them (81.9 ± 5.86% vs. 79.3 ± 7.73%). It is concluded assessment of rabbit sperm viability with eosin/nigrosine, hypo-osmotic swelling test, NucleoCounter™, SYBR14/PI and SYBR14/PI (added glutaraldehyde at 0.4%) produced similar results, thus any of them could be used interchangeably depending on the availability of materials to carry out the tests or the personal expertise. On the other hand, addition of 0.4% glutaraldehyde to the previously described SYBR14/PI test, produced no differences in viability.

Key words: Semen, Eosin/Nigrosine, HOST, SYBR14/PI, NucleoCounter™

INTRODUCTION

There are various sperm parameters whose assessment is essential when sperm is intended to be used in artificial insemination, research, or the reproductive control of the males. There are several tests that try to predict the reproductive capacity of males based on different aspects of sperm integrity and physiology; however, one of the main parameters to be

evaluated is sperm viability (Boiti *et al*., 2005). For its determination there are various tests such as eosin/nigrosine, hypo-osmotic swelling test, SYBR14/PI, special devices (NucleoCounter™) and some others. The differences between these are mainly the ease of assembly, reading and even the cost; therefore, it is important to have the possibility of carrying out any of them to provide reliable results. Therefore, the objective of this work was to compare different tests for determining rabbit sperm viability, and the modification of one of these (WHO, 2021).

MATERIALS AND METHODS

Animals and experimental design

Six Chinchilla male rabbits with proven fertility aged from 14 to 16 months were housed in the Modulo de Cunicultura of the Facultad de Estudios Superiores Cuautitlán, UNAM, Mexico, which has a natural environment with regulated ventilation by curtains. Males were housed in individual cages and fed with pelleted food *ad libitum*. Semen collection was carried out twice a week, macroscopic evaluation was done considering colour, volume, consistency, and the presence of foreign particles, the gel plug was removed, and each ejaculate was diluted (1:1 v/v) in Tris-Citric Acid-Glucose extender (TCG; 250mM, 88mM, 47mM), and transported, within 45 min, to the Animal Reproduction Laboratory of the Multidisciplinary Research Unit of the same Faculty. A pool was formed with those ejaculates that were not contaminated with urine, blood, or foreign particles and that had at least 70% progressive motility (PM). Each pool was adjusted to 400 $x10^6$ sperm per mililiter. Assessment of PM was performed visually, plasma membrane functionality with the hypo-osmotic swelling test (HOST) and viability with eosin/nigrosine (EN), NucleoCounter™ (NC), fluorescent stains SYBR14/PI (SYBR), and SYBR added 0.4% glutaraldehyde (SYBR+G) (Figure 1).

Sperm assessment

For the evaluation of PM, a 1:10 dilution (v/v sperm from the pool/TCG medium) was prepared, an equilibration time of 10 minutes was allowed and a drop from that mix was placed on a tempered slide, covered with a coverslip, and viewed in a phase contrast microscope (Leica DMIL) at 10x. Then, percentage of sperm moving progressively was subjectively determined (Martínez-Rodríguez *et al*., 2020).

For the evaluation of viability by EN, a drop of the 1:10 dilution and a drop of eosin/nigrosine at 37°C were placed on a tempered slide, mixed, left 30 seconds and then spread and quickly dried with a fan; 200 cells were observed under a microscope (Leica DMIL) at 100x, those not stained were classified as live, while those stained pink/purple were considered dead (Martínez-Rodríguez *et al*., 2020).

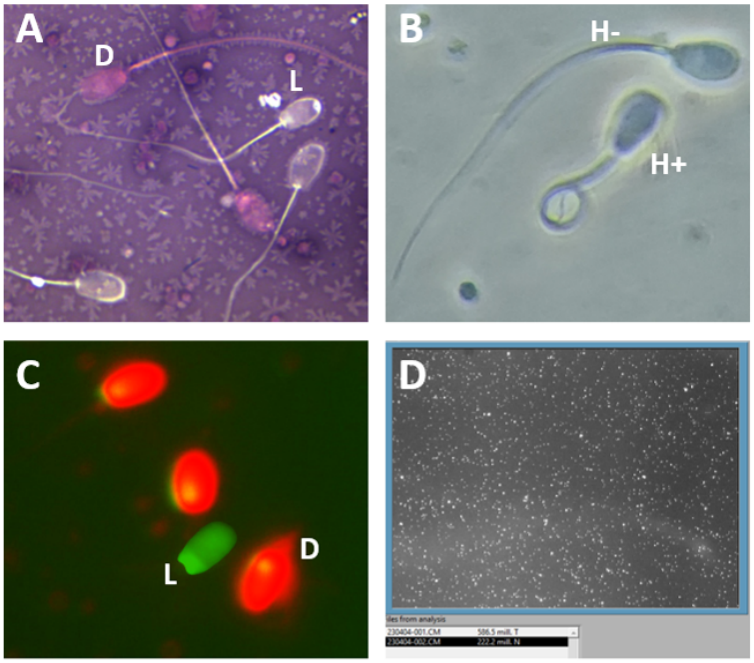
For the evaluation of HOST, the method described by Rosato and Iaffaldano (2011) was used with some modifications described in González-Ruiz *et al*. (2023). Briefly, in an Eppendorf tube, 50 µL of the diluted semen was mixed with 200 µL of distilled water, incubated for 5 minutes at 37°C and subsequently 22 µL of 0.4% glutaraldehyde was added. A drop of that mix was placed on a tempered slide, covered with a coverslip, and observed at 100x under a phase contrast microscope (Leica DMIL). 200 cells were observed, coiled tails were considered HOST+ (functional plasma membrane), while straight tails were considered HOST- (non-functional plasma membrane).

For the evaluation of NC a modification to the bovine sperm assessment protocol was done; two readings were taken using Propidium Iodide-loaded cartridges. For the first reading, 25 µL of semen were placed in 1000 µL of RS-100 (lysis solution), mixed perfectly and the cartridge was filled according to the equipment instructions. For the second reading, 25 µL of semen were placed in 1000 µL of TCG medium, mixed perfectly and the cartridge was filled according to the equipment instructions. Next, the percentage of viability was obtained by difference.

For the evaluation with SYBR, the methodology described by Rosato and Iaffaldano (2011) was used; 50 µL of TCG + 1 µL of SYBR14 (diluted 1:100 in DMSO) were placed in an Eppendorf tube, then 5 µL of diluted sperm were added. This mix was allowed to incubate for

10 minutes at 37°C; next, 5 µL of PI (diluted 1:100 in TCG) were added and incubated for 5 minutes at 37°C. A drop of sperm added SYBR14/PI was placed on a tempered slide, covered with a coverslip and observed at 100x under a fluorescent light microscope (Leica DMLS); 200 cells were observed, those showing green fluorescence were considered live, while those showing red fluorescence were considered dead. For the evaluation with SYBR+G, the same technique previously described was performed but after addition of SYBR14/PI a drop (22 µL) of 0.4% glutaraldehyde was added.

Figure 1. Reference images from the tests



A. Viability with EN; L: live (white), D: death (purple). B. Membrane functionality with HOST; H+: Positive HOST (Live), H-: Negative HOST (Death). C. Viability with SYBR; L: live (green), D: death (red). D. NucleoCounter™ screen showing viability

Statistical Analysis

Descriptive statistics of each pool were obtained; in addition, after arcsine transformation for normalization, analysis of variance (ANOVA) was performed on the data obtained with the different methodologies, to compare viability percentages.

RESULTS AND DISCUSSION

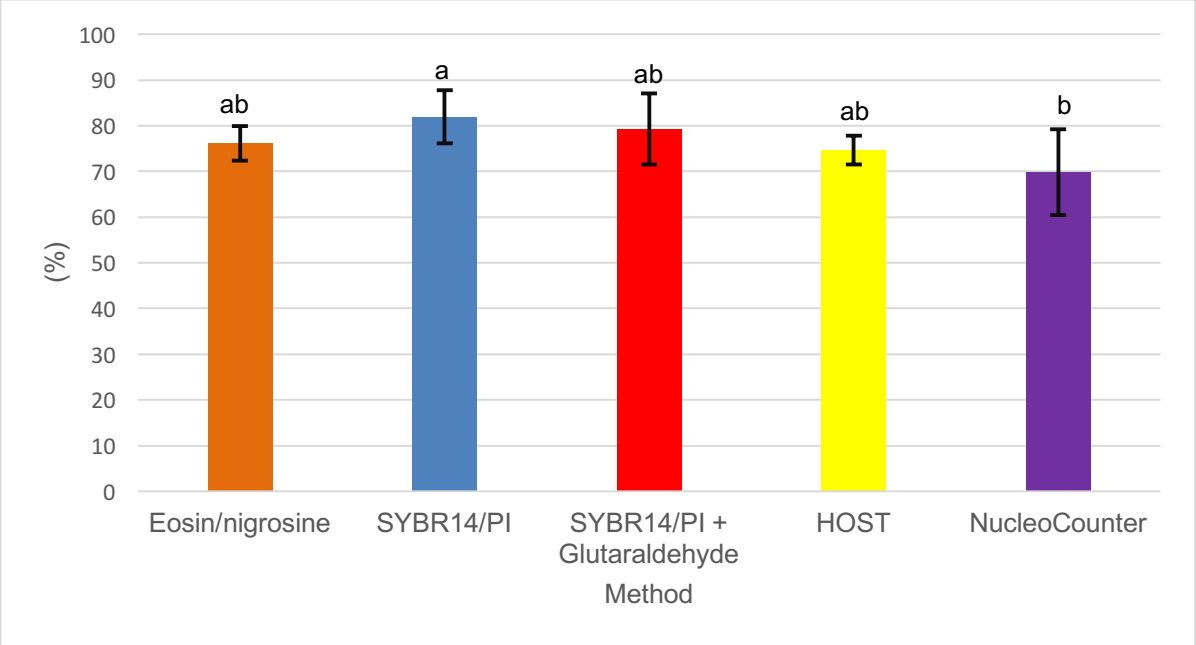
Sperm viability of rabbit Chinchilla males was similar to that described by Salcedo-Baca *et al*. (2004) in Mexico. A difference between viability obtained by NucleoCounter™ and that by SYBR14/PI was found (Figure 2); however, no differences amongst the other methodologies were found.

Evaluation of viability with eosin/nigrosine, SYBR14/PI and NucleoCounter is based on membrane integrity, while HOST indicates the functional status of the membrane, so although they all give indications of viability, their basis may be different. Some authors (Schrader *et al*., 1987) indicate that while some tests reveal the integrity of the plasma membrane, others such as HOST evaluate some physiological aspects of the sperm; for this, they recommend performing both tests. On the other hand, NucleoCounter™ produced the lowest percentage of viability; it could be, this device has not been set up to evaluate rabbit semen, thus configuration employed for other species may be inappropriate.

When SYBR14/PI technique described by Rosato and Iaffaldano (2011) was performed in our laboratory, the sperm continued being motile, which made difficult to count them under the microscope, for this reason a modification was made; a routine step in other tests is the addition of glutaraldehyde at 0.4% to prevent cell movement (Martínez-Rodríguez *et al*., 2020), so it was decided to add it and evaluate whether there was any difference between

adding it or not. A comparison between both methods revealed no significant difference, so it is possible to add a drop (22µl) of 0.4% glutaraldehyde to facilitate the classification (green/red) of the sperm.

Figure 2. Viability comparison with the different tests



They were evaluated 7 pools, formed by approximately 4 ejaculates each one (Means±S.D.). Different literals show significative differences (P<0.05)

CONCLUSIONS

It is concluded that all the tests performed in this work (Eosin/Nigrosine, SYBR14/PI, SYBR14/PI + Glutaraldehyde, HOST and NucleoCounter™) are useful for the assessment of rabbit sperm viability.

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TEAT NUMBER AND ITS IMPORTANCE IN RABBIT. MINI REVIEW

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ABSTRACT

Rabbits usually have 8, 9 or 10 teats. Most breeds have a similar litter size, but hybrids have more than 10 kits per litter. The relationship between the number of teats and the number of rabbits at birth is weak. Mortality, the number of reared rabbits, especially when more than 10 rabbits are born, depends more closely on the teat number. Although the h^2 value of the teat number is weak or moderate, the mating of rabbits with different teat numbers proves that this trait is highly inherited. There is a significant difference in the distribution of offspring born from the mating of rabbits (sire x dam) with 8 x 8 and 10 x 10 teats. The results of selection experiments prove that it is possible to quickly increase the proportion of rabbits with 10 teats in a breed. However, rabbits with 11 or 12 nipples are rare. It is recommended that the teat number be included in the selection criteria for the maternal lines. It would be worth investigating what is the barrier to more rabbits being born with 11 or more teats.

Key words: rabbit, teat number, distribution, prolificacy, selection

INTRODUCTION

In the rodents (order Rodentia), it has been observed that half or at most as many offspring are born as the number of teats of their mother (Gilbert, 1986). The connection called "one half role" is valid for European wild rabbit, but not for the domesticated rabbit. Most rabbit pure breeds have litter sizes between 7 and 9, while hybrids have 11-12 kits.

According to Hudson and Distel (1983), the pure nursing time is less than 2 minutes. Although the kits find the teats in a few seconds with the help of pheromonal guidance. During the time of nursing, they change teats several times (they are not attached to a teat like piglets). In such a short time, the kits have to suck the amount of milk they need, which is 20-25% of their body weight. The number of teats therefore plays a major role in ensuring that each kit gets milk in such a short time.

RESULTS AND DISCUSSION

Teat number and prolificacy

More than 30 years ago, some interesting results were published regarding the teat numbers in rabbit, and since then new results have been published with varying intensity. At the World Rabbit Congress, Szendrő and Holdas (1984) showed the distribution of the teat number in 3 lines. The proportion of rabbits with 8 teats varied between 40% and 67%, and that of rabbits with 10 teats between 11% and 29%. Four years later, according to the results of Rochambeau *et al*. (1988), this ratio improved in two INRA lines, 30% and 23% of rabbits had 8 teats, and 37% and 51% of rabbits had 10 teats. It is important to note that increasing the litter size played an important role in the development and selection of both lines. In other words, by increasing the litter size, the proportion of rabbits with 10 teats and the average number of teats also increased. This was confirmed by Mocé *et al*. (2000) in a divergent selection experiment on uterine capacity over 10 generations. In the High and Low lines, the proportion of rabbits with 8 teats was 7% and 25%, and those with 10 teats were 62% and 43%, respectively. These results also confirm that selection for prolificacy effects on teat number.

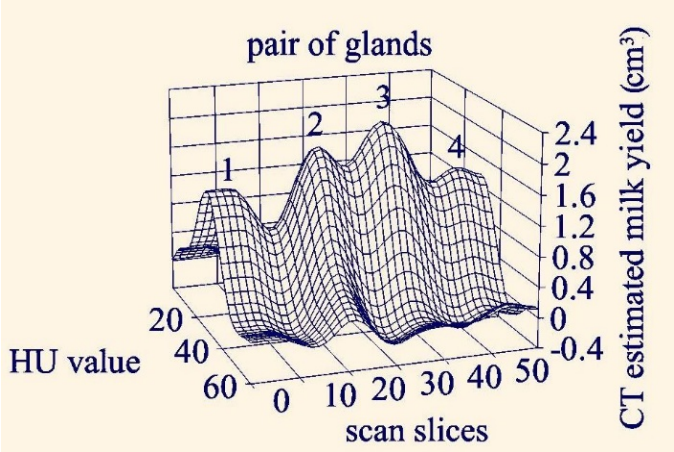
This relationship is also true in the opposite way. There is a weak correlation between the teat number and the number of rabbits born (Szendrő and Holdas, 1984; Rochambeau *et al*.,

1988). The mortality of suckling rabbits was more influenced by the teat number. Compared to does with 8 teats, the mortality of kits was 3-7% lower in does with 10 teats (Szendrő and Kampits, 1985; Rochambeau *et al.,* 1988; Pascual, 1993). This is especially true if the litter size is more than 10 (Rochambeau et al., 1988). The difference between the two groups (8 and 10 teats) in the litter size at 3 weeks was 0.63 on average (0.21-1.25 kits; Szendrő and Holdas, 1984; Rochambeau *et al*., 1988; Szendrő *et al*., 1992).

The relationship between teat number and milk production is uncertain (Rochambeau *et al*., 1988; Szendrő *et al*., 1992). However, does with higher teat numbers produce more milk, but this is partly or entirely due to the correlation between litter size and milk yield.

The milking of pairs of teats (mammary glands) is different. The milk production of the first and last pairs is lower than that of the middle pairs (Petersen *et al*., 1989). This is well demonstrated by Donkó *et al*. (2008) who created a 3D diagram using CT images taken before and after nursing (Figure 1),. Since the kits change teats during nursing, if there are enough teats, each rabbit can suckle for a similar amount of time. Despite this, the larger rabbits grow faster, presumably because they suck more intensively.

Figure 1: CT estimated milk yield in a doe at the second week of the lactation, depending on the location of the pair of glands (Donkó *et al*., 2008)



Morphology

The distance between teat pairs depends on the number of teats. From the direction of head to tail, the distance between the teat pairs on does with 8 teats was 64.3, 69.7 and 101.3 mm, and on does with 10 teats it was 58.8, 55.4, 70.7 and 66.6 mm, respectively, and the distance between the first and last pair was 235 mm and 252 mm, respectively (Szendrő and Kampits, 1985). Virág et al. (1991) performed measurements on newborn rabbits. In rabbits with 8 and 10 teats, the average distance between the teat pairs was 19.9 and 16.2 mm. If the rabbits had more teats, the distance between the teat pairs decreased, which was especially significant among the last pairs.

Mohamed (2012) measured the body length of rabbits and found only a 4-5 mm difference between rabbits with 8 and 10 teats at 18-22 weeks of age. So, it seems that, regardless of the number of teats, the volume of the total mammary gland does not or hardly change, only the number of outlet openings (teats) changes. The offspring of rabbits with 10 teats therefore do not get more milk in total, but each kit has a greater chance of getting milk during the short nursing period, even if it is less per kit.

Heredity

Only a few values of estimated heritability were reported for the teat number in rabbits, in addition moderate h^2 values were reported for sows (based on 57 papers, most often

between 0.20 and 0.45). Contradictory results were reported examining rabbits originating from different mating combinations. If the dam had 8, 9 or 10 teats, the proportion of offspring with 10 teats was 17%, 41% and 43%, respectively (Rochambeau *et al*., 1988). The same values in Pascual's (1993) study were 18%, 34% and 44%, respectively. The difference was even more spectacular if, in addition to the dams, the teat number of the sires was also known. Figure 2 shows the distribution of the teat number of kits born from matings of 8 x 8 and 10 x 10 combinations (sire x dam) (Szendrő *et al*., 1992). Compared to the 10 x 10 mating, the 8 x 8 mating produced 3.4 times more kits with 8 teats, while compared to the 8 x 8 mating, the 10 x 10 mating produced 3.1 times more kits with 10 teats. These results demonstrate that teat number is a highly heritable anatomical trait.

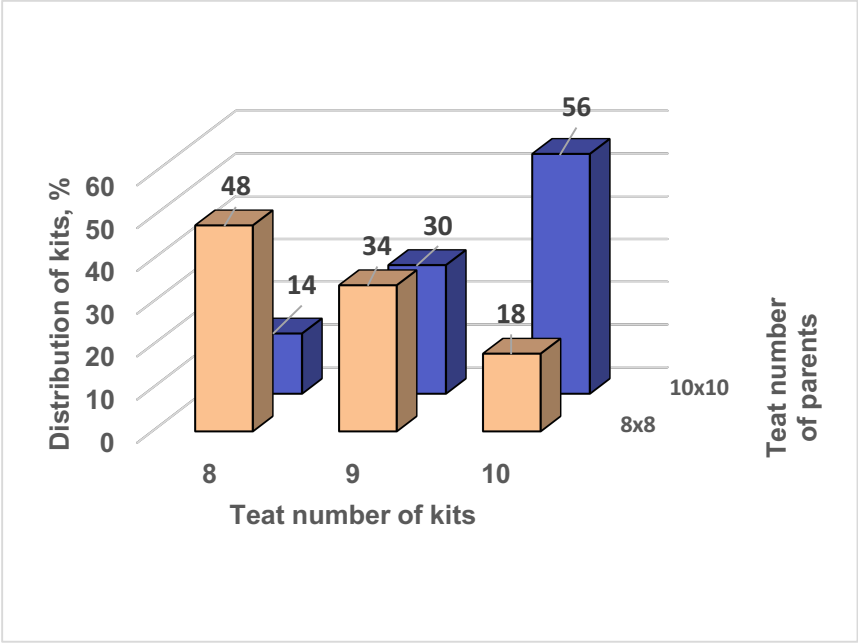


Figure 2: Distribution of teat number on kits depending on the teat number on their parents (Szendrő et al., 1992)

Selection

Two experiments were conducted in which rabbits were selected based on the teat number. In the experiment of Virág *et al*. (1991) which lasted only two years and selected female and male rabbits based on the number of teats counted on newborn rabbits. Despite the short time, the proportion of rabbits with 8 teats decreased from 59% to 29%, and the proportion of rabbits with 10 teats increased from 14% to 34%. Baselga *et al*. (2021) reported the results of a study between 2004 and 2012. While the proportion of rabbits with 8 teats decreased from 66% to 21%, those with 10 teats increased from 15% to 46%, and even some rabbits with 11 (0.2%) and 12 teats (0.1%) they found. According to Diamond (1987), the litter size can be increased by selection, but the teat number is a fairly constant species characteristic. This appears to be the reason why so few rabbits are born with 11 or more teats.

CONCLUSIONS

The teat number in rabbits plays a role in increasing the number of rabbits raised, especially when the litter size is more than 10. After sucking (full belly, tight skin), teats can be counted in the newborn rabbits, in both sexes. This is a highly heritable trait, the proportion of rabbits with 10 teats can be increased quickly. At the same time, we do not know the reason why there are very few rabbits with 11 or 12 teats even in the case of selection. Knowing and solving this question can be an interesting research topic, why the teat number is fairly a constant species characteristic trait

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ULTRASOUND-ASSISTED LIQUEFACTION OF RABBIT SEMEN: EVALUATING THE IMPACT ON SPERM QUALITY

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ABSTRACT

The precision of semen analysis is critical for reproductive research and practice in rabbit industry. However, the Computer-Assisted Semen Analysis (CASA) system faces significant challenges in analyzing sperm cells due to the presence of gel or lipid droplets. This study investigates the efficacy of ultrasound sonication as a novel approach to liquefy these obstructive droplets, aiming to improve semen analysis accuracy. Semen samples from six rabbits were pooled and divided into two aliquots: one untreated and the other diluted with a Tris-citric acid-glucose (TCG) solution. Each aliquot was further subdivided into control and experimental groups, where the latter were subjected to ultrasound sonication under varying conditions (30-1, 30-2, 60-2, 120-2 seconds-frequency; T1 to T4). Post-sonication, samples were incubated at 36°C, and sperm quality parameters, including viability, membrane integrity, acrosome reaction rate (ARR), motility, particle size, and viscosity, were assessed at 30 and 90 minutes. The results indicate that ultrasound sonication, particularly at short durations, numerically increases sperm motility without compromising sperm viability and membrane integrity. Similarly, viscosity and particle size decreased significantly in T3 and T4 groups ($P<0.05$). However, prolonged exposure to high-frequency ultrasound was found to increase ARR, suggesting potential detrimental effects on sperm quality. The findings advocate for tailored sonication protocols to avoid adverse effects and improve the reliability of CASA.

Key words: Rabbit, Sperm cells, Ultrasound, Frequency, Motility.

INTRODUCTION

Rabbit semen presents unique challenges for spermatological analysis, characterized by its low volume and the presence of lipid droplets compared to other livestock species. The accessory sex glands in rabbits produce significant amounts of protective gel for sperm cell protection and contain unknown decapacitating factors. This gel, frequently observed post-collection, is lipid-rich, and over time, these droplets can obscure sperm cells, thereby complicating microscopic examination (Castellini et al., 2006). Such precular nature of rabbit semen can significantly impede the clarity of microscopic analysis. Gross semen examination and simple tests for plasma membrane integrity are feasible, but droplets can challenge the detailed kinematic analysis of semen evaluation. While various separation methods, such as Percoll column and centrifugation, have been employed to enhance microscopic clarity (Aksoy et al., 2010), these approaches often detrimentally affect semen storage, sperm plasma membrane integrity, and the acrosome reaction (El-Bahrawy et al., 2017). The Computer-Assisted Semen Analysis (CASA) system, a cornerstone for assessing sperm kinematics, encounters limitations when lipid droplets approximate the size of sperm heads, leading to potential misidentification and inaccurate sperm count during analysis. Various updates in softwares and use of artificial intelligence might overcome such difficulty but simple CASA system and ImageJ might not exclude these droplets unless manual interventation is made. Although these adjustments can extend the duration of analysis (Pérez-Sánchez, 1996).

Lipids, primarily produced by the prostate gland, serve a protective role for sperm cells against various stresses and external environmental factors (Mourvaki et al., 2010). Thus, identifying methods to effectively reduce or liquefy lipid droplet size without compromising sperm health is of paramount importance. We hypothesize that applying high-energy, low-frequency ultrasound waves can effectively reduce lipid droplet size through resonance, without adversely affecting sperm cell integrity. This investigation aims to elucidate the impact of ultrasound treatment on sperm health parameters, including motility, viability, acrosome reaction, and kinematics, with the objective of enhancing semen analysis accuracy and reliability in rabbit spermatology.

MATERIALS AND METHODS

Animals and experimental design

The study was conducted at the Department of Reproduction and AI, Adnan Menderes University, with approval from the local Ethical Committee in compliance with institutional animal care guidelines. Six New Zealand albino bucks, aged 1.5 to 2.5 years, served as semen donors, housed individually with ad libitum access to feed and water under natural lighting. Only semen samples exhibiting >70% motility were pooled for experiments. The ultrasound sonication procedure was standardized using 1.5ml Eppendorf tubes in 8.89-cm water bath within an Elma ultrasonic unit (D-78224 Sigen/Htw, Germany) at 35 kHz, ensuring uniform wave distribution. Treatment durations were 30 sec (1x or 2x; T1 and T2), 60 sec (2x; T3), and 120 sec (2x; T4). Semen was divided into neat and 1:5 diluted aliquots with TCG, further subdivided into control and sonicated groups (T1 to T4), and incubated at 36°C for evaluation at 30 and 90 minutes.

Evaluation of Semen Quality Parameters

Viability was assessed using eosin-nigrosine staining, with a minimum of 200 sperms counted under 400× magnification. Membrane integrity was evaluated via the hypo-osmotic swelling (HOS) test, distinguishing sperms with coiled (intact) vs. straight (compromised) tails. Acrosome reaction was assessed by FITC-PNA staining, with fluorescent microscopy (Olympus BX53) at 1000× magnification, identifying acrosome-reacted vs. intact cells among 200 sperms per sample.

Particle Size, Viscosity and CASA Analysis

Particle size was measured pre- and post-sonication using ImageJ. An Elma ultrasonic unit (D-78224 Sigen/Htw, Germany) operating at an acoustic (nominal) frequency of 35 kHz (input power 230 V) and integrated with a mechanical timer was used for rabbit semen liquefaction. Viscosity of semen was assessed using Brookfield DV-E digital viscometer, LVDV-E, Brookfield Engineering Laboratories Inc., Middleboro, MA. While total and progressive sperm motility were assessed under a phase-contrast microscope at 37°C, analyzing at least 500 sperms across five fields.

Statistical Analysis

Data were analyzed using R, employing GLM for the effects of treatment, time, and their interaction, with ANOVA and Tukey's post-hoc test comparing groups. Spearman's correlation assessed the relationship between ultrasound exposure and semen characteristics, setting significance at $p<0.05$.

RESULTS AND DISCUSSION

Our study reveals significant insights into the optimization of sonication treatments for a particular focus on the balance between enhancing semen analysis and preserving the fundamental aspects of sperm health. The motility analysis, as summarized in the table 1, reveals a clear decrement in both total and progressive motility across higher sonication treatments (T2, T3, T4), particularly evident in diluted semen samples ($P<0.05$)). These findings emphasize the nuanced effects of sonication on sperm motility, further delineating the fine line between beneficial and detrimental sonication applications. Our findings indicate that sperm motility, ARR, HOST and viability in neat semen remains largely unaffected by mild sonication (T1), with percentages closely mirroring those of the control group at both 30 and 90 minutes of incubation respectively (figure 1).

Table 1: The effect of sonication on motility of rabbit sperm cells following 30 and 90 mins of incubation at 36°C. Different superscripts a, b, c, d indicates significant differences in same row P<0.05.

Parameters	Time	Groups				
		Control	T1	T2	T3	T4
Total motility Neat (%)	30	75.4±4.65[a]	78.2±5.85[a]	58.6±3.91[b]	45.4±3.37[c]	20±2.53[d]
	90	57±2.12[a]	62.2±3.42[a]	45.8±2.01[b]	14±2.88[c]	0
Total motility Diluted (%)	30	71.6±3.32[a]	76.8±3.47[a]	39.2±2.43[b]	4.4±1.50[c]	0
	90	49.6±4.46[a]	52.8±3.73[a]	15.8±1.42[b]	0	0
Progressive Motility Neat (%)	30	54.8±5.11[a]	63.2±3.48[a]	22.2±2.74[b]	14.8±2.15[c]	7.4± 1.80[d]
	90	26±2.19[a]	34.4±2.4[b]	25.4±1.80[ac]	5±1.18[d]	0
Progressive Motility Diluted (%)	30	46.4±2.90[a]	49.2±2.63[a]	11.6±1.32[b]	4.2±1.06[c]	0
	90	22.6±2.42[a]	28.4±2.97[a]	8±1.67[b]	0	0

Means with different letters on the same row differ significantly (Bonferroni test).

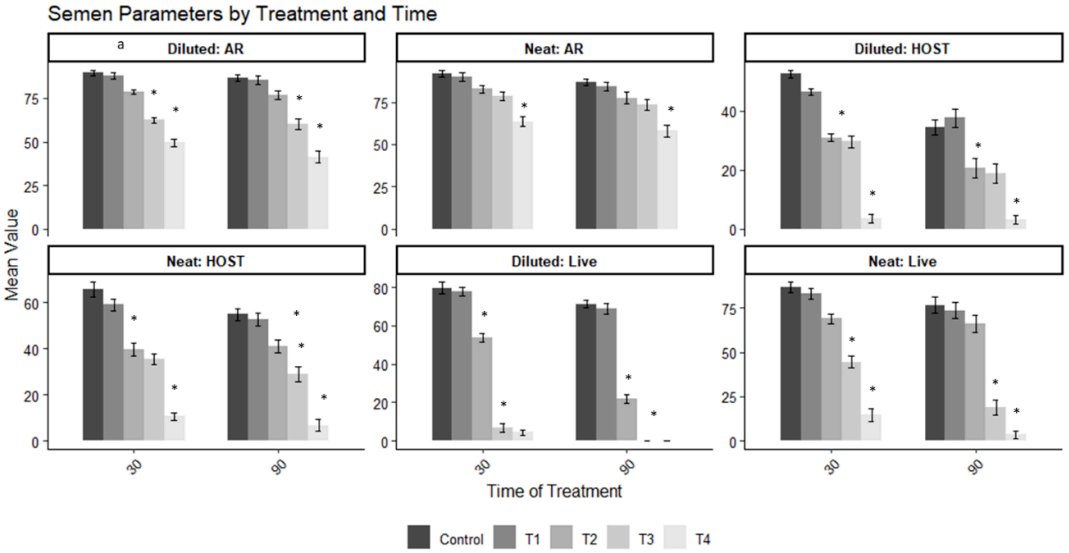

Fig 1. The effect of sonication on Livability, HOST and AR rate of rabbit sperm cells following 30 and 90 mins of incubation at 36°C. Asterisk indicates significant differences.

Low-intensity sonication does not compromise the vital function of sperm cells but more intense sonication treatments (60-2 and 120-2) drastically declined parameters to as low as zero %, respectively (P<0.05). Diluted semen samples exhibit a similar pattern of sperm motility, ARR, HOST and viability, where T1 maintains viability akin to control samples, but T2, T3, and T4 treatments significantly reduced semen quality parameters underscoring the sensitivity of sperm cells to higher sonication intensities. This deterioration in membrane integrity with higher sonication levels highlights the critical threshold beyond which sonication becomes harmful to sperm cells, as explained in a study by Maxwell et al., 1996, where higher dilution rate can have harmful effect on sperm membrane. Higher sonication intensities may induce stress responses detrimental to sperm functionality, potentially affecting fertility outcomes (Rateb, 2016).

Our study's results agree with literature on the seminal role of plasma in maintaining sperm viability and motility, as well as the challenges posed by the high lipid content in rabbit semen (Domingo et al., 2018). Different factors like measuring chamber (Massanyi et al., 2008), type of software, dilution of semen and number of particles in semen can influence reliability of measurement of spermatozoa parameters.Sonication's capability to significantly reduce particle size in both neat and diluted semen samples (P<0.05) underscores its potential to improve the homogeneity of semen (figure 2), as T2 and T3 decreases viscosity (P<0.05) thereby facilitating more consistent cryopreservation outcomes (El-Bahrawy et al., 2017). The dispersion of lipid droplets can directly address the complexity of high lipid content characteristic of rabbit semen. These lipids, essential for the protection of spermatozoa, often

aggregate, posing substantial obstacles. In antoher study Maxwell et al., 1999 explained how addition of seminal plasma can change sperm quality in different procedure of cryobioloy, like in semen analysis and preservation by reducing the efficacy of sperm assessment and cryopreservation techniques (Aksoy et al. 2010).

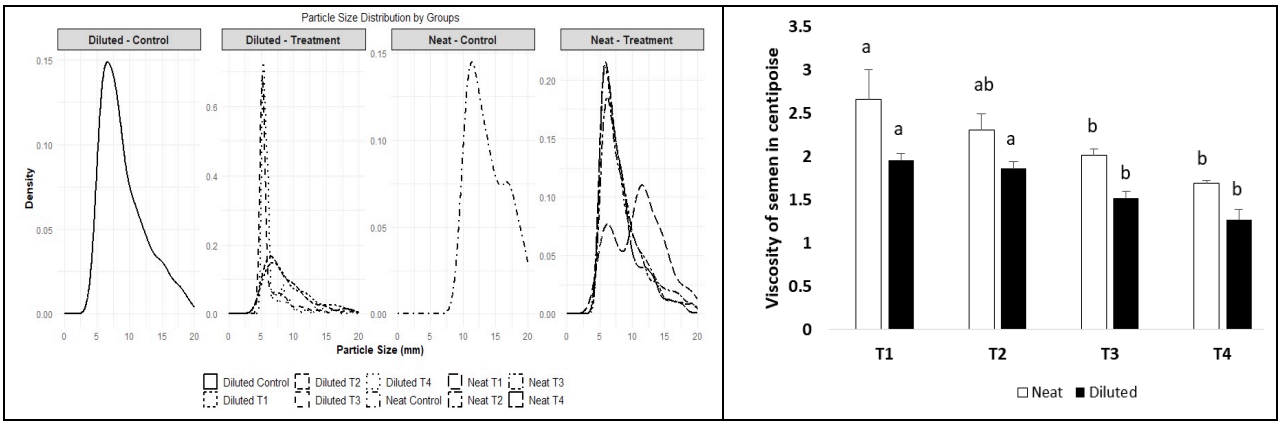

Fig 2. The effect of sonication on particle size, density and viscosity in rabbit semen.

Notably, treatments T1 and T2 appear to be minimally disruptive, maintaining semen quality parameters while rapidly reducing viscosity and enhancing image clarity for (CASA) (Rateb et al., 2016). By enhancing the homogeneity of semen samples, sonication can ensure more uniform cooling and warming rates during cryopreservation, a critical factor in maintaining sperm viability post-thaw.

CONCLUSIONS

Sonication not only presents a solution to lipid aggregation challenges in semen analysis but also heralds a new era in cryobiology and semen equilibration techniques. The balance between sonication intensity and sperm health preservation is crucial, warranting further exploration as a tool in reproductive biotechnology. In conclusion, the promising outcomes of T1 sonication advocate for its adoption in CASA system, and can be a guiding light for other reproductive technologies.

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EFFECT OF THE ADDITION OF MELATONIN DURING THE CRYOPRESERVATION OF RABBIT SEMEN IN CENTRAL MEXICO

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ABSTRACT

The artificial insemination on rabbit production is a practice that has been increasing in recent years, however, to obtain high percentages of fertility and prolificacy, it can only be carried out with fresh or refrigerated, for short periods of time, semen. Currently, different from other species, there are no standardized protocols for rabbit semen cryopreservation that allow to obtain high motility and viability levels upon thawing; therefore, artificial insemination on rabbit production is not carried out with semen subjected to this process. One of the main problems that sperm face during cryopreservation is the formation of free radicals, which causes cell damage when they are produced on excess. At this regard, nowadays exists a tendency to add to cryopreservation media molecules with antioxidant effects. One of these molecules is melatonin, which addition during sperm cryopreservation has been tested in various species, including the rabbit. The objective of this work was to evaluate the effect of the addition of different melatonin concentrations during sperm cryopreservation in rabbit of central Mexico. Ejaculates were collected from 6 Chinchilla males; a pool was formed with those ejaculates that presented at least 70% of progressive motility (18 pools in total). The pools were evaluated microscopically (mass and progressive motility subjectively, sperm viability and morphology with eosin/nigrosine stain, membrane functionality with the hypo-osmotic swelling test (HOST), acrosome integrity with phase contrast microscopy and membrane integrity with the fluorescent stain SYBR14/PI). A two-step freezing protocol was performed with INRA-82 as extender and dimethyl sulfoxide and dimethylformamide at 4% each one as cryoprotectants. Aliquots were taken to form six concentrations of melatonin (0.0, 0.1, 0.75, 1.5, 2.25 and 3.0 mM), packaged in 0.25 ml straws and cooled to 5°C, subsequently exposed to nitrogen vapors to finally be immersed in liquid nitrogen. Thawed sperm were assessed as previously described in the fresh pools; in addition, another test of viability (NucleoCounter^{TM}) was done. Descriptive statistics were obtained from the data collected in fresh and thawed semen. Furthermore, after transformation to the arcsine for normalization, analysis of variance (ANOVA) was carried out on the thawed semen data, to compare the effect of the different concentrations of melatonin added during cryopreservation. In fresh semen, it was determined that the progressive motility´s mean was 79.4% ± 4.16, while viability´s was 80.5% ± 5.15. Progressive motility upon thawing in the different treatments was found in the range from 7.4 to 9.4%, on the other hand, viability remained between 32.4 and 35.7%. No significant differences were found between any of the melatonin concentrations employed ($P>0.05$). In conclusion, melatonin addition during rabbit sperm cryopreservation has no effect on sperm motility, viability, acrosomal integrity, membrane functionality, membrane integrity nor morphology upon thawing in central Mexico.

Key words: Cryopreservation, Spermatozoa, Melatonin, Antioxidant.

INTRODUCTION

Rabbit production is defined as the branch of livestock farming that is responsible for the reproduction, breeding and fattening of rabbits (González-Ruiz *et al.,* 2023). It is a widely spread activity in some countries, however, in some others, such as Mexico, it is a little spread activity or carried out in backyard production systems for self-consumption. In recent years, there have been made efforts to make rabbit production more efficient, for this reason, artificial insemination has been implemented since this technique allows to inseminate various does with one ejaculate (Kubovicova *et al*., 2021). However, unlike other species, artificial insemination with cryopreserved semen is not carried out in rabbit production on a recurring way since protocols that allow high levels of fertility after cryopreservation have not been standardized. One of the main problems that sperm face during cryopreservation is the formation of free radicals due to their metabolism, reason for which currently the addition of substances with antioxidant effects during cryopreservation has been tested. One of these substances is melatonin, which addition during semen cryopreservation has already been tested in various species, including the rabbit (Medrano *et al.,* 2017; Zhu *et al.,* 2019). The objective of this work was to evaluate the effect of the addition of melatonin in different concentrations during the cryopreservation of rabbit semen in central Mexico. For this, mass and progressive motility, viability, acrosome integrity, morphology, and functionality of sperm membrane of ejaculates from chinchilla rabbits were assessed.

MATERIALS AND METHODS

Animals and experimental design

Six chinchilla rabbits, 12 to 14 months old, were used, housed in the "Módulo de Cunicultura" of the Facultad de Estudios Superiores Cuautitlán, UNAM, Mexico, which has a natural environment with regulated ventilation with curtains. They were housed in individual cages and fed with pelleted food ad libitum. Semen collections were carried out twice a week, macroscopic evaluation of the ejaculates was carried out (color, volume, consistency, presence of foreign particles), the gel plug was removed, they were diluted in a transport medium (1:1 v/v, INRA-82 without cryoprotectants, skim milk or egg yolk) and transported to the Animal Reproduction Laboratory of the Multidisciplinary Research Unit of the same Faculty, within 45 min.

After a period of tempering at room temperature (10 minutes at about 24°C), mass motility was assessed, subsequently the ejaculates were centrifuged at 1500 rpm for 5 minutes and the cell pellet was resuspended in INRA-82 extender without cryoprotectants.

The mass (MM) and progressive motility (PM) of each ejaculate were evaluated and those that had at least 70% of progressive motility were used to form a pool (5 ejaculates per pool/ 18 in total); those ejaculates with the presence of foreign bodies or that were contaminated with blood or urine were discarded. Each pool was adjusted to 400 $x10^6$ sperm per ml with INRA-82 extender without cryoprotectants (Fraction A). The pool was evaluated microscopically: mass motility (MM) and progressive motility (PM), concentration, morphoanomalies (MA) and viability (EN), acrosome integrity (AI), and membrane functionality (HOST).

Subsequently, INRA-82 extender (Fraction B) containing 4% Dymethylsulfoxide (DMSO) and 4% Dimethilformamide (DMF) was added in 3 parts with intervals of 10 minutes until the pool was adjusted to 200 $x10^6$ sperm per ml. Aliquots were taken to add melatonin (MLT) in different concentrations (0.0, 0.1, 0.75, 1.5, 2.25 and 3.0 mM) at room temperature (23°C), an equilibrium time of 10 minutes was allowed, then diluted sperm were packaged in 0.25 ml straws that were cooled up to 5°C in approximately 3 hours. Finally, straws were exposed to nitrogen vapors for 15 minutes (4.5 cm above liquid nitrogen level) and immersed and stored, at least two weeks, in liquid nitrogen.

Three straws per treatment were thawed (30 seconds in a water bath at 37 °C), ten min later MM and PM of each straw were assessed, and a pool was formed with the 2 best straws per treatment. Subsequently, viability was assessed with fluorescence (SYBR14/PI), viability with the use of NucleoCounter (NC), MM, PM, EN, MA, HOST, and the AI of each treatment.

Quality assessment

The evaluation of mass and progressive motility, viability with eosin/nigrosine, membrane functionality, concentration and membrane integrity were carried out according to the methodology reported in González-Ruiz *et al.* (2023). For the evaluation of SYBR14/PI, the technique reported by Garner and Johnson (1995) was used.

For the evaluation of NC, a modification to the bovine sperm assessment protocol was done; two readings were taken with Propidium Iodide cartridges. For the first reading, 25 µL of semen were placed in 1000 µL of RS-100 (lysis solution), mixed perfectly and the cartridge was filled according to the equipment instructions. For the second reading, 25 µL of semen was placed in 1000 µL of Tris-Citric Acid-Glucose extender (TCG), mixed perfectly and the cartridge was filled according to the equipment instructions. Next, the percentage of viability in the ejaculates was obtained by difference.

Statistical Analysis

Descriptive statistics were obtained from the data collected in fresh and thawed semen. Furthermore, after transformation to the arcsine for normalization, analysis of variance (ANOVA) was carried out on the thawed data, to compare the effect of the different concentrations of melatonin added during cryopreservation on semen quality.

RESULTS AND DISCUSSION

Parameters obtained in fresh semen (Figure 1) were similar to those reported by other authors who evaluated Chinchilla rabbit semen in Mexico (Salcedo-Baca *et al*., 2004).

In our work three different tests were carried out for sperm viability (EN, HOST and SYBR14/PI), the first 2 were very similar among them, however, the third test was different from them; this can possibly be explained by the fact that it was used a protocol that is usually applied to various species, but not specifically in rabbits.

Figure 1. Fresh semen characteristics

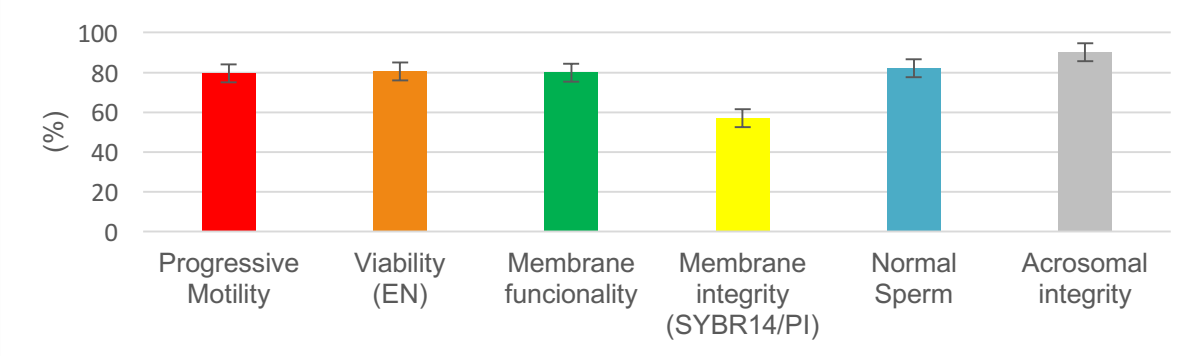


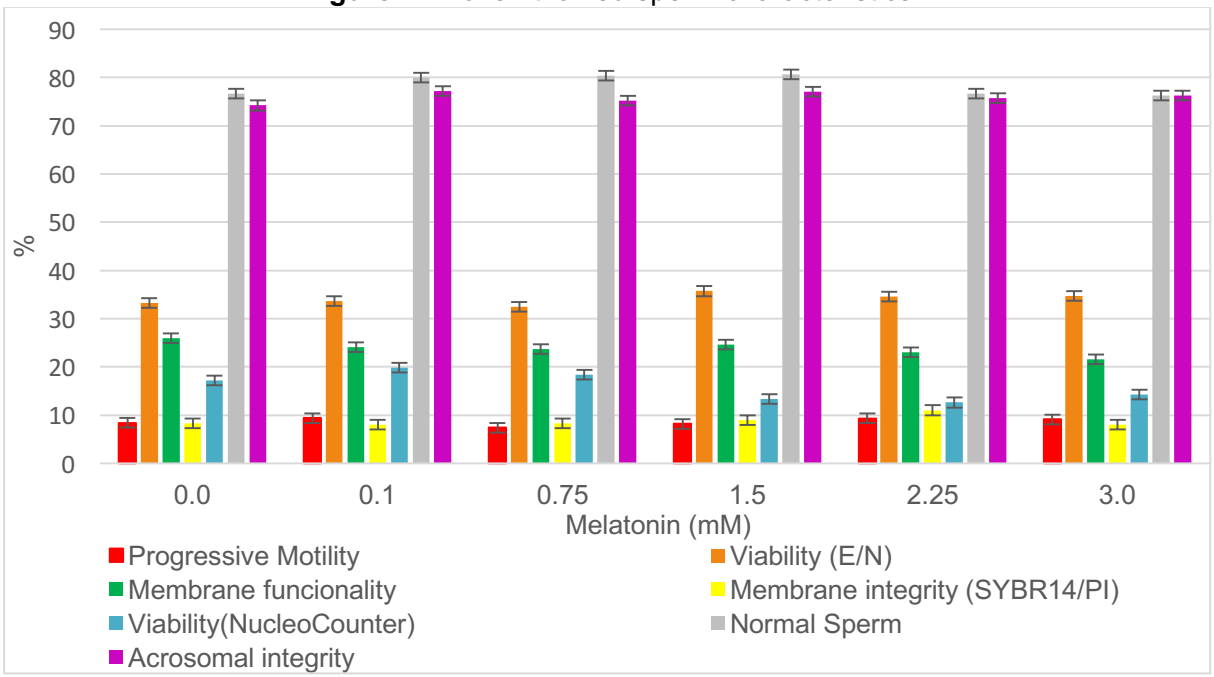
Figure 1. Fresh sperm characteristics (18 pools, 5 ejaculates in each one). Values are Means ± S.E.M.)

Regarding the assessment of thawed semen, there were no differences between MLT treatments (Figure 2). In contrast, Zhu *et al*. (2019) obtained higher values of progressive motility and membrane integrity than us (50% vs. 9.4% of PM; 45% vs. 8% of membrane integrity) when added 0.1 mM of MLT. However, acrosome integrity was similar in both works (80% vs. 77.2%). These differences could be due to various factors such as the extender (TCG vs. INRA-82), cryoprotectants (DMSO 4% vs. DMSO 4%+DMF4%), breed of rabbits (unspecified vs. Chinchilla), and the place where the work was carried out (China vs. Mexico). On the other hand, the difference between viability with SYBR14/PI and EN could be due to the methodology employed which is not specific to rabbits (SYBR14/PI).

Other work in which 1.0 mM MLT was added to rabbit freezing medium (Fadl *et al*., 2020), reported higher values of progressive motility, viability, and membrane functionality than ours (48.8% vs 7.4% of PM; 57.1% vs 32.4% of EN; 54 vs 23.7% of HOST). However, normal

sperm were similar (79.1% vs 80.4%), but AI was smaller (66.4% vs 75.2 %). Since the extender and cryoprotectants were the same in both works, differences could be due to factors such as breed (New Zealand vs. Chinchilla), location (Egypt vs. Mexico) and environmental conditions such as photoperiod (16 h light vs 12 h light) and temperature.

Figure 2. Frozen thawed sperm characteristics



They were evaluated 18 pools (5 ejaculates in each one). Values are Means ± S.E.M. No significative differences were found between MLT treatments in any variable (P>0.05)

CONCLUSIONS

The addition of melatonin in different concentrations (0.0, 0.1, 0.75, 1.5, 2.25 and 3.0 mM), to sperm of Chinchilla rabbit had no effect on sperm quality after freeze thawing in central Mexico.

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STUDY OF THE QUALITY OF REFRIGERATED SEMEN SAMPLES IN TWO LINES DIVERGENTLY SELECTED FOR LITTER SIZE VARIABILITY

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ABSTRACT

The aim of this study was to analyse the quality of refrigerated semen for 24 hours in males selected divergently for litter size variability. The homogenous line (L) is selected to decreased litter size variability and the heterogenous line (H) is selected to increased litter size variability. Samples of 46 males were collected from June to October using artificial vagina. Later, samples were refrigerated at 4 ºC for 24 hours for further evaluation. A total of 120 sperm samples were analysed for motility and kinetic parameters. Motility was assessed using the CASA-Mot system. Sperm kinematic parameters assessed by CASA included: curvilinear velocity (VCL, µm/s); straight line speed (VSL, µm/s); average trajectory velocity (VAP, µm/s), linearity (LIN: VSL/VCL × 100, %), straightness (STR: VSL/VAP × 100, %). Total motility and progressive motility were calculated. Bayesian methodology was used for statistical analysis. The homogeneous line showed higher percentage of progressive motility (D_{L-H} = 2.5%; P = 93%), VSL (D_{L-H} = 5.3 µm /s; P = 90%), LIN (D_{L-H} = 1.8; P = 98%) and STR (D_{L-H} = 1.8; P = 96%) than heterogeneous line. Similar results were obtained for total motility, VCL and VAP in both lines. The higher stress level of the heterogeneous line males could influence the poorer quality of the movement of the refrigerated semen samples. In conclusion, kinetic parameters in refrigerated semen were better in the homogeneous line than in the heterogeneous line.

Key words: Maternal line, Sperm motility, Rabbit, Resilience.

INTRODUCTION

Artificial insemination (AI) is a highly efficient assisted reproductive technology that has become common practice in commercial rabbit farms (Viudes de Castro and Vicente, 2023). AI is used both with semen from paternal males to produce the broodstock and with maternal males to produce the crossbred female. Usually, fresh or refrigerated semen is used for AI, because frozen semen does not achieve competitive fertility rates. Therefore, the profitability of AI centres depends on the quality of fresh and refrigerated semen (Bielsa et al., 2022).

A divergent selection experiment for litter size variability is being carried out at the Miguel Hernández University. The homogenous line is selected by decrease litter size variability and the heterogenous line is selected by increase litter size variability (Blasco et al., 2017). Lines differ not only in variability of litter size but also in average of litter size, showing homogeneous line higher litter size than heterogenous line (Argente et al., 2017; Agea et al., 2019). In previous studies, we have found that females from the heterogenous line have a higher stress response and lower disease resistance than those from the homogeneous line (Argente et al., 2019). Thus, homogenous lines would be more resilient than the heterogenous lines (Beloumi et al., 2020).

Males of homogenous line could be used as a maternal line in AI centres. Thus, knowledge of semen quality is essential for the dissemination of this line. The aim of this study is to analyse the quality parameters of refrigerated semen for 24 hours in males of homogeneous and heterogeneous lines for litter size variability.

MATERIALS AND METHODS

Animals and experimental design

All experimental procedures were approved by the Committee of Ethics and Animal Welfare of the Miguel Hernández University with code VSC PEA 0226 type 2.

A total of 46 males were used, 23 from the heterogeneous line and 23 from the homogeneous line at the Miguel Hernandez University. Males belonged from 17th generation of selection. Males were bred and kept in individual cages (37.5 cm × 33 cm × 90 cm) under standard conditions (photoperiod 16 h:8 h). Animals were fed with a standard feed (16.3% crude protein, 15% crude fibre and 3.2% ether extract, 8.9% ash, 0.56% phosphor and 0.23% sodium). Males were trained to ride in artificial vagina for 1 month from 4.5 months of age. Samples were collected from June to October every 14 days. An overall of 126 semen samples were collected by artificial vagina tempered to 45°C. The gel, if present, was immediately removed. Samples were diluted with Tris-acid citric and glucose to a standard concentration of 25 millions of spermatozoa/mL. Samples were refrigerated at 4 ºC and they were evaluated 24 hours after. Six contaminated samples were discarded. Therefore, 120 samples were analysed for kinetic traits.

Sperm Analyses

Motility was assessed using the CASA-Mot system new generation with artificial intelligence to recognize rabbit sperm by images (AI Station v1.2; Spain). Captures were taken at 100 frames per second with a digital camera (FLYR HS640m, AI Station, Spain) mounted on a microscope (UOP/PROISER R+D) using a 10x negative phase contrast objective (AN 0.25). A total of 500 spermatozoa per sample were analyzed. Sperm kinematic parameters assessed by CASA included: curvilinear velocity (VCL, µm/s) measured in point-to-point reconstitution of the sperm trajectory; straight line speed (VSL, µm/s), defined by the straight line between the first and last point on the track; average trajectory velocity (VAP, µm/s), linearity (LIN: VSL/VCL × 100, %), straightness (STR: VSL/VAP × 100, %). In addition, two progression ratios, expressed as percentages, were calculated: total motility and progressive motility.

Statistical Analysis

The model included the effects of line (homogeneous and heterogeneous line), month (June-July and September-October), random effect of male and the error.

All analyses were performed using Bayesian methodology (Blasco, 2017). Bounded uniform priors were used for all effects with the exception of the male permanent effect, considered normally distributed with mean 0 and variance σ^2_m. Residuals were a priori normally distributed with mean 0 and variance σ^2_e and uncorrelated with the male effect. The priors for the variances were also bounded uniform. Features of the marginal posterior distributions for all unknowns were estimated using Gibbs sampling. The Rabbit program developed by the Institute for Animal Science and Technology (Valencia, Spain) was used for all procedures. We used a chain of 60,000 samples, with a burn-in period of 10,000. Only one out of every 10 samples were saved for inferences. Convergence was tested using the Z criterion of Geweke and Monte Carlo sampling errors were computed using time-series procedures.

RESULTS AND DISCUSSION

Table 1 shows the features of marginal posterior distributions of the differences between lines for semen quality traits. Total motility is about 68%. The value is similar to those found

by Safaa et al. (2008a) with fresh semen and for both maternal and paternal lines. However, the rest of the sperm kinetic traits do not agree with those presented for fresh (Safaa et al., 2008b) and refrigerated semen (López-Gatius et al., 2005). This discrepancy could be due to the different in vitro analysis methodology used.

The homogeneous line showed higher percentage of progressive motility (D_{L-H} = 2.5%; P = 93%), VSL (D_{L-H} = 5.3 µm/s; P = 90%), LIN (D_{L-H} = 1.8; P = 98%) and STR (D_{L-H} = 1.8; P = 96%) than the
heterogeneous line. Similar results were obtained for total motility, VCL and VAP in both lines. The homogeneous line would show better kinetic properties of the spermatozoa than the heterogeneous line, also the higher LIN and STR would indicate lower circular trajectories of the spermatozoa. Previous studies with fresh samples indicated a higher percentage of live sperm in the homogeneous line (88.8%) than in the heterogeneous line (79.6%, P = 95%; Baeza et al., 2023).

We know that males of the heterogeneous line have higher temperature measured by infrared thermography than the homogeneous line (Serrano-Jara et al., 2024) and therefore a higher level of stress. Sperm quality is affected by stressful situations, especially related to thermal stress (Oladimeji et al., 2022). So, these preliminary results would indicate the higher level of stress in the heterogeneous line males, both when resting and after applying a stress stimulus (Serrano-Jara et al., 2024), could affect the quality of spermatozoa.

Table 1: Effect of genetic line on the quality parameters of refrigerated spermatozoa

Trait	Lines[1]		D_{L-H}	$HPD_{95\%}$	P (%)
	Homogeneous	Heterogeneous			
Total Motility (%)	68.0	67.0	0.1	-6.2; 7.9	61
Progressive Motility (%)	68.0	65.5	2.5	-0.8; 5.8	93
VCL (µm /s)	261.5	260.6	0.9	-20.1; 22.8	53
VSL (µm /s)	65.9	60.6	5.3	-3.2; 12.7	90
VAP (µm/s)	118.9	113.9	5.0	-7.6; 17.6	79
LIN (%)	25.3	23.5	1.8	0.0; 3.5	98
STR (%)	60.8	59.0	1.8	-0.22; 3.7	96

[1]Median. D_{L-H}: Difference between the Homogenous and the Heterogenous lines. HPD95%: Highest density region at 95%. P: Probability of D_{L-H} being >0.

CONCLUSIONS

In conclusion, sperm showed lineal and straight linear velocity trajectory with higher progressivity in in the homogeneous line than in the heterogeneous line when refrigerated during 24h. Further studies are required to substantiate these findings on sperm quality, and their justification with studies on testosterone and cortisol levels.

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SEMEN CHARACTERISTICS OF NEW ZEALAND WHITE RABBIT BUCKS FED DIETS CONTAINING BANANA CO-PRODUCTS

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ABSTRACT

Seminal characteristics of rabbit bucks are directly dependent on the nutrition, which can quantitatively and qualitatively influence sperm production and quality. In this sense, some alternative foods can add extranutritional components, with bioactive properties, capable of mitigating oxidative stress in semen and increasing sperm count. The aim of the work was to evaluate seminal parameters and sperm quality of New Zealand White rabbit bucks fed diets containing dehydrated banana leaves and banana peels. Thirty New Zealand White rabbit bucks were used, aged between 1 and 3 years and live weight between 3.5 and 5.0 kg. The experimental design was completely randomized, with three treatments and ten replicates, with one animal per experimental unit. The treatments consisted of a basal diet and two test diets, with 10% inclusion of dehydrated banana leaf (DBL) or dehydrated banana peel (DBP). Two ejaculate collections were carried out per rabbit, the first collection at the beginning of the experiment and the second 55 days after provision of the experimental diets. An artificial vagina made of a plastic tube 8 cm long and 4 cm in diameter was used, internally coated with a non-lubricated condom, supplied with previously heated water (38 to 39°C). Volume of semen was determined, as well as pH, color, motility score and oxidative status (malondialdehyde – MDA). Sperm count, total and progressive sperm motility and linear sperm velocity were determined in the Computer-Assisted Sperm Analysis (CASA). Diets containing DBL or DBP resulted in a lower concentration of MDA ($P>0.05$) in the semen of rabbits at 55 days of evaluation, when compared to the basal diet. Similarly, the concentration of MDA was lower ($P<0.05$) in the semen evaluated at 55 days than in the semen evaluated on the first day of the test, for rabbits that consumed diets containing DBL and DBP. Similarly, diets containing DBL or DBP resulted in an increase of almost 50% in the sperm count performed at 55 days compared to collection on the first day. The inclusion of 10% of dehydrated banana leaf or dehydrated banana peel in the diet of New Zealand White rabbit bucks increases sperm concentration and reduces semen lipid peroxidation.

Key words: Rabbit farming, Reproduction, Sperm.

INTRODUCTION

Rabbit farming in Brazil is still a discrete activity, but it has great potential for growth. In addition to the concern with meat production, its mandatory to improve reproductive indices of rabbit bucks, such as semen characteristics, which are fundamental for the assessment of puberty, sexual maturity and reproductive capacity (Campos et al., 2012). However, seminal characteristics are directly dependent not only on genetics, age and health status, but also on the nutrition of males, which can quantitatively and qualitatively influence sperm production and quality (IRRG, 2005).

In this sense, some alternative foods can add extranutritional components, with bioactive properties, capable of mitigating oxidative stress in semen and increasing the viable sperm count. Banana leaf (*Musa paradisiaca*) and banana peel have a high concentration of polyphenols, which at moderate levels have nutraceutical, antimicrobial, anti-inflammatory and antioxidant properties (Qamar and Shaikh, 2018). Considering that spermatogenesis in

rabbits lasts approximately 44 days (França and Russel, 1998), it is likely that providing moderate levels of polyphenol-rich products during this period will modulate this process.
The aim of the work was to evaluate seminal parameters and sperm quality of New Zealand White rabbit bucks fed diets containing dehydrated banana leaves and banana peels.

MATERIALS AND METHODS

Animals, experimental design and treatments

The experiment was carried out at the State University of Maringá, Brazil, in the Rabbit Farming Sector, between October and November 2021 (temperature: 26,1±3,8ºC; relative humidity: 72,7 ±5,4%). All experimental procedures were approved by the University's Committee for Ethical Conduct in the Use of Animals in Experimentation (CEUA/UEM) (Protocol no. 7526270720).
Thirty New Zealand White rabbit bucks were used, aged between 1 and 3 years and live weight between 3.5 and 5.0 kg. The experimental design was completely randomized, with three treatments and ten replicates, with one animal per experimental unit. Treatments were a basal diet and two test diets, with 10% inclusion of dehydrated banana leaf (DBL) or dehydrated banana peel (DBP). Basal diet was formulated with alfalfa hay, tifton 85 hay, wheat bran, corn, soybean meal, amino acids, minerals, vitamins and additives, according to the requirements of adult rabbits (De Blas and Mateos, 2010). Animals received water and food *ad libitum* for 55 days.
The rabbit bucks were housed individually in experimental cages (0.6 x 0.8 x 0.4 m), equipped with automatic drinker (nipple) and semi-automatic metal feeder. The banana leaves came from young trees (1 to 2 years old) from the Experimental Farm of the University, and the ripe banana peels were collected immediately after disposal at the Popular Restaurant of Maringá, Brazil. The fractions were individually disintegrated in an electric organic waste crusher, and subsequently dehydrated in the sun until they reach a minimum dry matter of 90%. After obtaining the dehydrated material, the DBL and DBP were crushed in a knife-type mill (sieve of 2.5 mm holes), which resulted in an average geometric diameter of 1022 µm for DBL and 1048 µm for DBP.

Semen collection and analyses

Two ejaculate collections were carried out per rabbit, the first collection at the beginning of the experiment and the second 55 days after provision of the experimental diets. An artificial vagina made of a plastic tube 8 cm long and 4 cm in diameter was used, internally coated with a non-lubricated condom (Scapinello, 1997), supplied with previously heated water at a temperature of 38 to 39°C (Campos et al., 2012). All collections were carried out in the morning, inside the breeders' own cages, in which five trained females were used as dummy. The collection container was previously prepared by adapting a sterilized and dry Eppendorf tube (2 mL). The gelatinous fraction present in the semen was removed with the aid of sterilized surgical forceps. The samples were diluted to a 1:2 concentration with Tris-Gem diluent (300 mOsm/g, pH 7.1).
Immediately after semen collection, ejaculate volume was determined. pH was measured using a Merck® pH indicator strip. The color was measured and classified into scores: 1- watery white, 2- milky white, 3- creamy white, 4- yellow, 5- reddish. Semen motility was evaluated subjectively using an electron microscope with a 40x objective and given an arbitrary score from 0 to 5 based on the following assessment: 0 (0%, no discernable motility); 1 (1 to 20% of sperm exhibiting slight undulating movement); 2 (20 to 40% of sperm showing undulating movement); 3 (40 to 60% of sperm showing progressive motility); 4 (60 to 80% of sperm showing progressive motility); and 5 (80 to 100% of sperm in vigorous and progressive movement). Semen samples were centrifuged at 2500 g for 20 min. The seminal plasma (supernatant) was stored at -80°C for subsequent analysis. Lipid peroxidation in seminal plasma was measured by thiobarbituric acid reactive substance (TBARS) as described by Richard et al. (1992), and the values were expressed in nmol of malondialdehyde (MDA) per mL. Semen samples with diluent (37 ºC) were sent to the Reproduction Laboratory University, for evaluation in the “Computer-Assisted Sperm

Analysis – CASA (ISAS®, Proiser RD, Paterna, Spain). The following parameters were recorded: sperm count, total and progressive sperm motility (%) and linear sperm velocity (µm/s).

Statistical Analysis

Analysis of variance (ANOVA) was performed using the GLM procedure of SAS statistical software. Tukey test was applied to compare means between treatments on the same day (P<0.05). For comparison between days, the treatments were subjected to the F Test (P<0.05).

RESULTS AND DISCUSSION

There was no difference between treatments or between evaluation days (P>0.05) for ejaculate volume, pH, color and motility score (Table 1). However, diets containing DBL or DBP resulted in a lower concentration of MDA (P>0.05) in the semen of rabbits at 55 days of evaluation, when compared to the basal diet. Similarly, the concentration of MDA was lower (P<0.05) in the semen evaluated at 55 days than in the semen evaluated on the first day of the test, for rabbits that consumed diets containing DBL and DBP. This result indicates a reduction in seminal lipid peroxidation due to the treatments evaluated.

Table 1. Semen characteristics of New Zealand White rabbit bucks fed basal diet (BASAL), with 10% of dehydrated banana leaf (DBL) or with 10% of dehydrated banana peel (DBP), for 55 days

Variables	Day 1			SEM[1]	P-value	Day 55			SEM	P-value[2]	P-value (Day1 *vs* Day55)[3]		
	BASAL	DBL	DBP			BASAL	DBL	DBP			BASAL	DBL	DBP
Bucks, no.	10	10	10	-	-	10	10	10	-	-	-	-	-
Volume (mL)	0.51	0.53	0.47	0.06	0.94	0.49	0.51	0.50	0.03	0.88	0.89	0.91	0.50
pH	8.00	7.80	8.02	0.15	0.85	7.60	7.80	7.60	0.16	0.86	0.35	0.99	0.35
Color	2.20	2.00	2.30	0.04	0.44	2.40	2.10	2.30	0.03	0.46	0.49	0.35	0.99
Motility score	3.20	3.80	3.44	0.24	0.62	3.33	3.82	3.64	0.13	0.49	0.88	0.99	0.68
MDA (nmol/mL)	2.58	2.91^{A}	2.86^{A}	1.20	0.51	2.76^{a}	1.89^{bB}	2.08^{bB}	1.41	0.02	0.55	0.02	0.04

1- Standard error of mean.
2- Different lowercase letters between treatments of Day 55 differ from each other (Tukey, P<0.05).
3- Different uppercase letters between Day 1 and Day 55, for the same treatment, differ from each other (F test, P<0.05).

There was no difference among treatments or evaluation days (P>0.05) for total sperm motility, progressive sperm motility and straight-line velocity (Table 2). However, diets containing DBL or DBP increased almost 50% sperm count performed at 55 days compared to collection on the first day. This result indicates a positive effect of the foods evaluated on spermatogenesis. Probably the positive effect may be due to the direct effect of antioxidants present in DBP on stored sperm, maybe due to the blood compounds effects (blood-testis barrier modulation).

Sperm, like other living cells, produce reactive oxygen species (ROS) that have important functions for reproduction, as in small concentrations they mediate normal sperm functions, such as capacitation, hyperactivation, acrosomal reaction and fusion of the sperm with the oocyte (Agarwal et al., 2005). Mammalian sperm have plasma membranes rich in polyunsaturated fatty acids, which make them more fluid and, at the same time, very susceptible to peroxidative damage that causes loss of their functions and DNA integrity (Silva and Gadella, 2006). Therefore, the addition of ingredients rich in polyphenols to the diet of rabbits, as banana co-products (Sundaram et al., 2011), can reduce the concentration of malonaldehyde, indicating an improvement in the antioxidant defense of sperm cells.

Table 2. Sperm concentration and motility[1] in the semen of New Zealand White rabbit fed basal diet (BASAL), with 10% of dehydrated banana leaf (DBL) or with 10% of dehydrated banana peel (DBP), for 55 days.

Variables[2]	Day 1			SEM[3]	P-value	Day 55			SEM	P-value	P-value (Day1 *vs* Day55)[4]		
	BASAL	DBL	DBP			BASAL	DBL	DBP			BASAL	DBL	DBP
Bucks, no.	10	10	10	-	-	10	10	10	-	-	-	-	-
SC (10^6 spz/mL)	306.8	315.6^B	305.6^B	37.2	0.51	305.3	411.6^A	401.2^A	43.2	0.88	0.76	0.02	0.04
TSM (%)	69.38	75.35	64.25	5.51	0.74	68.05	82.22	78.55	6.27	0.86	0.95	0.47	0.33
PSM (%)	61.60	62.94	56.74	5.20	0.89	58.13	71.02	67.01	6.21	0.46	0.86	0.45	0.45
SLV (µm/sec)	39.55	37.04	38.82	4.31	0.97	41.89	51.83	45.17	4.45	0.76	0.88	0.13	0.55

1- Variables determined in the computer-assisted sperm analysis system – CASA (ISAS®, Proiser RD, Paterna, Spain).
2- SC: sperm count; TSM: total sperm motility; PSM: progressive sperm motility; SLV: straight-line velocity.
3- Standard error of mean.
4- Different uppercase letters between Day 1 and Day 55, for the same treatment, differ from each other (F test, P<0.05).

In addition to the improvement in the antioxidant status of the semen, the addition of banana leaves and peels to the rabbits' diets increased the sperm count of rabbits 55 days after starting to provide the diets (Table 2), indicating a significant improvement in spermatogenesis. The process of spermatogenesis occurs in the testicles, which are made up of numerous seminiferous tubules and are composed of spermatogenic cells (spermatogonia, spermatocytes and spermatids), which are sent through the common ducts, carrying sperm to the outside (Neto et al., 2005). Although it is an organic function that occurs constantly in male rabbits after puberty, the process depends on the supply of organic nutrients, minerals, vitamins and energy, as well as specific enzymes and coenzymes, intensifying cellular respiration and generating a considerable amount of ROS (Agarwal et al., 2005). Thus, the presence of bioactive compounds in diet can modulate this process, due to natural antioxidant defense of spermatogenesis, reducing the number of sperm cells degenerated by free radicals, rising up sperm count.

CONCLUSIONS

The inclusion of 10% of dehydrated banana leaf or dehydrated banana peel in the diet of New Zealand White rabbit bucks increases sperm concentration and reduces semen lipid peroxidation.

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SEMEN QUALITY POST-THAWING: THE USEFULLNESS OF SPERM WASHING PROCEDURE FOR RABBIT SEMEN

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ABSTRACT

The study aimed to investigate the quality of cryopreserved rabbit semen without and with sperm washing by centrifugation in extender after thawing. Twenty-three cryopreserved semen samples were submitted to a repeated measures design with a specific focus on evaluating the impact of the sperm washing procedure on cryopreserved semen quality. Two protocols were compared in post-thawing semen, PROTO1: without sperm washing and PROTO2: with sperm washing by centrifugation in extender. The average percentage of progressive sperm cells was 7.79% for PROTO1 and significantly higher at 18.38% for PROTO2 ($p<0.05$, *test-t*). This represented a 136% improvement in progressive motility of sperm cells. Analyzes were performed using the Computer Assisted Sperm Analysis (CASA). In conclusion, results presented in this study substantiate the value of incorporating an extra post-thawing step referred to as the "sperm washing" procedure to enhance sperm progressive motility.

Key words: cryoprotectant, progressive motility, rabbit, semen, sperm washing.

INTRODUCTION

Artificial insemination is widely used in rabbit farms. Fresh semen is predominantly used instead of frozen semen due to the low survival rate of rabbit sperm post-thawing. This limitation hinders the effective preservation of genetic resources through the establishment of a sperm Cryobank (Di Iorio et al., 2023, Viudes-de-Castro et al., 2023, Nishijima et al.,2021).

Several studies have investigated improvements of rabbit sperm cryopreservation by refining freezing techniques and cryoprotectant formulations. It is known that cooling, freezing and thawing processes are important stages in determining the survival rate of sperm cells (Akarsu et al., 2023). However, inconsistent outcomes across studies have prevented the establishment of a standardized freezing and thawing protocol (Di Iorio et al., 2023). Cryopreservation agent added to semen during slow cooling causes toxic stress due to prolonged exposure (Akarsu et al., 2023). Possibly, one of the factors for the low post-thawing sperm survival rate is due to such toxic stress caused by component(s) present in the cryoprotectant agents. Therefore, it is crucial to focus on improving the thawing protocols for the survival rate of cryopreserved rabbit sperm. Adding a washing step after thawing could help improve survival by removing toxic cryoprotectant agents.

This study aimed to assess whether the sperm washing procedure sustains better progressive motility of individual sperm cells post-thawing.

MATERIALS AND METHODS

Animals and experimental design

In this study, semen was collected from 23 New Zealand White type rabbits (1 to 2 years old). Semen was frozen using a cryopreservation medium containing acetamide and egg-yolk as main components. Bucks were individually housed in cages; controlled maintenance feed diet and *ad libitum* water were provided. Photoperiod was 12h light /12h dark.

For the analysis, a total of 23 straws containing 23 different frozen semen samples were subjected to two sperm quality assessment protocols (PROTO1 and PROTO2). The frozen semen-containing straws were thawed in water bath at 38°C for 60 seconds. For each of the 23 straws, thawed semen was four-folds diluted in extender medium (1.5 ml extender medium + 0.5 ml semen), split into two samples and tested using a Computer Assisted Sperm Analysis (CASA) for total count (million/ml) and progressive motility (% sperm cells). For PROTO1, the diluted semen sample was analyzed in CASA without washing. For PROTO2, the diluted semen straw was centrifuged for 10 minutes at 1800 rpm, supernatant was removed, and the sperm pellet was resuspended with extender medium to obtain the same concentration as for PROTO1. Centrifuge 5430, rotor F-35-6-30 was used.

Semen Analysis

Total sperm count in million/ml and progressive motility in percentage were measured using CASA system configured specifically for rabbit semen using the CASA software. At least 500 sperm cells, or 5 fields for each semen sample were observed for the calculation of the parameters analyzed in this study.

Statistical Analysis

Thawed semen samples were submitted to a repeated measures design and compared between the two protocols using the t-test at 5% significance, in the R software (R, 4.2.2). Before the analysis, the data were verified for normal distribution using Shapiro-Wilk test. Results are reported as Means ± Standard Deviation. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Effect of the sperm washing procedure on the quality of post-thaw semen

The t-test analysis at 5% significance presented in Table 1 revealed a significant difference in percentage of progressive sperm cells ($p \leq 0.05$), while there was no significant difference in the total sperm-count ($p > 0.05$).

Table 1. Progressive sperm cells (%), and the total sperm-count in million/mL without and with the sperm washing protocol, submitted to a t-test at 5% significance.

Parameter	PROTO1: Without sperm washing	PROTO2: With sperm washing	P-value
Progressive, %	7.79 ± 6.95[b]	18.38 ± 9.60[a]	0.0001**
Total count, M/ml	79.69 ± 25.51	69.54 ± 30.03	0.2234

[a,b] Means ± standard deviation (SD) differed significantly at $p \leq 0.05$** according to t-test analysis at 5% significance. M/ml= millions of sperm cells per milliliter of semen.

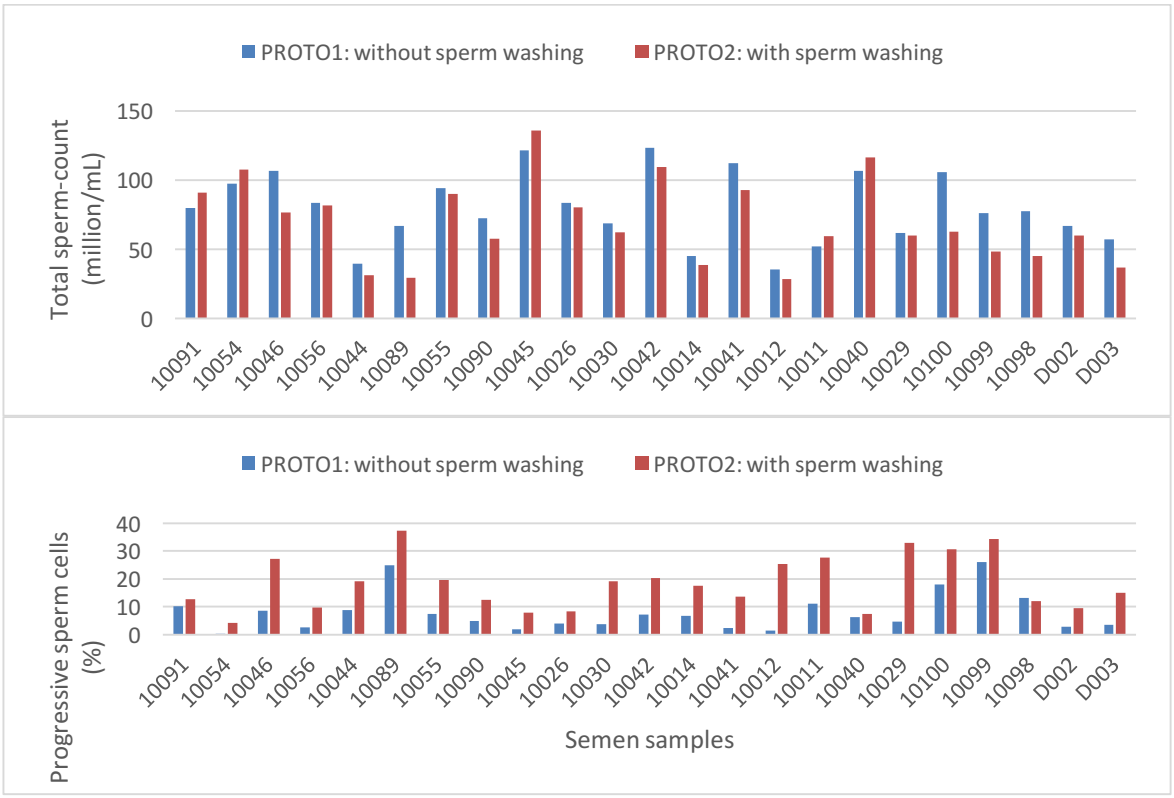
The average percentage of progressive sperm cells was 7.79% for PROTO1 (without sperm washing) and significantly higher at 18.38% for PROTO2 (with sperm washing). This represented a mean 136% improvement in progressive motility of sperm cells compared to the progressive motility of sperm cells without the additional sperm washing step.

Figure 1 illustrated the results for all 23 semen samples, presenting a bar-chart displaying the total sperm-count in million/mL (top chart) and the percentage of progressive sperm cells (bottom chart) both without and with the sperm washing procedure.

An apparent difference was observed between the total sperm-count without sperm washing (79.69 ± 25.51 SD) and with sperm washing (69.54 ± 30.03 SD), but this difference was not found to be statistically significant ($p=0.2234$). The presence of a large variation between samples, as indicated by the standard deviation, may be one of the factors contributing to the lack of significant difference in the total-sperm count. The absence of outliers in the dataset indicated that the data is well-distributed. Given that semen is a biological sample, it was acceptable to observe greater variation among semen samples during the analysis.

Additionally, taking into account the considerable increase in the number of progressive sperm cells, this apparent (non-significant) loss of total sperm cell count can be considered to be of no consequence.

Figure 1. Total sperm-count in million/mL (top figure) and the percentage of progressive sperm cells (bottom figure) without and with sperm washing protocol for each individual semen batches.



Seventeen out of 23 semen samples showed an improvement of over 100% in progressive sperm cells with sperm washing compared to their state without washing. Moreover, five semen samples demonstrated improvements ranging from 16.2% to 69.9% in progressive sperm cells when subjected to the washing protocol.

A substantial change was observed in semen sample (10054). Without the sperm washing, this batch presented progressive motility of only 0.16%. With the washing protocol, this same semen sample exhibited a progressive motility of 4.14%.

One semen sample (10098) exhibited a decrease in progressive sperm cells of 9.1% but was not considered relevant enough to negatively impact this study. This semen sample initially had a progressive sperm cell of 13.06%, which decreased to 11.87%. This sample was included in the statistical analysis, but it did not impact the overall outcome.

Despite the fact that the total sperm-count did not show statistical difference, between protocols, ($p > 0.05$), loss of cells during the centrifugation might still occur and should be monitored and taken into account. Results demonstrated that centrifugation for 10 minutes at 1800 rpm was sufficient for the sperm washing protocol, with no significant number of sperm cells remaining in the supernatant. In order to achieve a more consistent understanding on the effect of sperm washing, further investigations are planned to determine its fertilizing capability in artificial insemination.

CONCLUSIONS

In conclusion, the findings presented in this study substantiate the value of incorporating a sperm washing step into the thawing protocol. This additional step suggests the importance of removing certain chemical components from the cryopreservation medium, thus fostering enhanced sperm progressive motility.

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EMBRYONIC DEVELOPMENT AND PLASMA FATTY ACID PROFILE AT EARLY GESTATION IN TWO LINES DIVERGENTLY SELECTED FOR LITTER SIZE VARIABILITY

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ABSTRACT

A divergent selection experiment for litter size variability in rabbits was carried out at the Miguel Hernández University of Elche for sixteen generations of selection. The aim of this study is to analyse the plasma fatty acid profile at 72 hours of gestation in females of the heterogeneous and the homogeneous lines for litter size variability, as well as its relationship with embryonic development. A blood sample was taken at 72 hours of 5th mating in 6 non-lactating females of the heterogeneous line and in 12 non-lactating females of the homogeneous line. The blood samples were centrifuged and the plasma was stored at -80°C for later analysis of the fatty acid profile. Gas chromatography was used in the identification and quantification of fatty acids in plasma. At 72 hours of gestation, ovulation rate and number of embryos were recorded. The embryos were classified under a stereo microscope according to their development in early morulae, compact morulae or blastocyst. Bayesian methodology was used for statistical analysis. Ovulation rate was similar between the lines. However, embryonic survival was lower in the heterogeneous line than in the homogeneous line (-0.11, P =70%), as a consequence of reduced embryonic development. Regarding fatty acids, the heterogeneous line showed higher concentration of pentadecanoic (C15:0), iso-pentadecanoic (iso C15:0), margaric (C17:0), stearic (C18:0), myristoleic (C14:1), palmitoleic (C16:1), linoleic (C18:2 n-6), gamma-linolenic (gamma C18:3 n-6), and arachidonic (C20:4 n-6) (P $\geq$ 95%). In conclusion, the fatty acid profile seems to be related to the development and survival of the embryo at 72 hours of gestation.

Key world: fatty acids, embryo, morulae, litter size variability, resilience.

INTRODUCTION

Fatty acids play an important role in embryonic development with the formation of cell membranes and energy supply during cell division, being key to successful gestation (Tsujii et al., 2009; Valckx et al., 2014). Several studies have found a negative effect of long-chain saturated fatty acids such as palmitic (C16:0), stearic (C18:0) and tricosanoic (C23:0) on oocyte maturation, fertilization and pre-implantation embryonic development (Mirabi et al., 2017; Zeng et al., 2023). Regarding polyunsaturated fatty acids (PUFAs), especially a negative effect on embryonic development has been described for linoleic (C18:2 n-6), alpha linoleic (C18:3 n-3), eicosapentaenoic (C20:5 n-3), docosahexaenoic (C22:6 n-3) and arachidonic (C20:4 n-6) (Jungheim et al., 2011; McKeegan and Sturmey, 2011; Schindler et al., 2020; González-Brusi et al., 2023).

A divergent selection experiment for litter size variability is being carried out at the Miguel Hernández University of Elche. In previous studies, we have found that selection has been successful (Blasco et al., 2017). In addition, females from the heterogeneous line have higher stress response and lower disease resistance than those from the homogeneous line (Argente et al., 2019). Stress can cause metabolic changes leading to variations in the fatty acid profile (McKeegan and Sturmey, 2011) with negative effects on oocyte growth and early embryonic development (Zeng et al., 2023). A higher sensitivity to stress in the heterogeneous line would agree to its smaller litter size (Blasco et al., 2017), as a consequence of a less advanced embryonic development in the first hours of gestation (Calle et al., 2017).

The aim of this study is to analyse the plasma fatty acid profile at 72 hours of gestation in females of the heterogenous and the homogeneous lines for litter size, as well as its relationship with embryonic development.

MATERIALS AND METHODS

Animals and experimental design

Females used in this study come from the 16th generation of a divergent selection experiment for litter size variability, see more details about selection procedure in Blasco et al. (2017). All animals were reared in the farm of the Universidad Miguel Hernández de Elche (Spain). The rabbits were fed *ad libitum* with a standard commercial diet (Cunilactal®, Nanta S.A., Las Palas, Spain). Females were housed in individual cages under a constant photoperiod of 16 h continuous light: 8 h continuous darkness, and with controlled ventilation.

One blood sample was taken at 72 hours of 5th mating in 6 non-lactating females of the heterogeneous line and in 12 non-lactating females of the homogeneous line. Samples were collected into tubes containing K3-EDTA. The blood samples were centrifuged and the plasma was stored at -80°C for later analysis of the fatty acid profile. Next, females were euthanised and their reproductive tract was removed. The ovulation rate (OR) was estimated as the number of corpora lutea. The numbers of normal embryos (NE), abnormal embryos and oocytes were counted after collection by perfusion of each oviduct and uterine horn. At 72 hours of gestation, normal embryos were classified as early morulae (EM), compacted morulae (CM) or blastocysts (B) using a binocular stereoscopy microscope. The number of EM, CM and B, were expressed as a percentage of normal embryos. The early embryonic survival (EES) was estimated as NE divided by OR.

All experimental procedures involving animals were approved by the Miguel Hernández University of Elche Research Ethics Committee (2023-VSC-PEA-0079), in accordance with Council Directives 98/58/EC and 2010/63/EU.

Chemical Analyses

A 200 µl of plasma sample was taken in a scrum cap glass tube. The plasma sample was processed following the method by Shirai et al. (2005). The fatty acids were measured using a gas chromatograph (GC-17A, Shimadzu, Kyoto, Japan) with Flame ionization detector (FID), equipped with a CP-Sil 88 for FAME capillary column (100 m x 0.25 mm x 0.36 mm; 0.20 µm film thickness; Agilent technologies, Madrid, Spain). The fatty acids were identified by comparing the retention times with FAME MIX standard (CRM47885, Supelco, Spain).

Statistical Analysis

The model used to analyse plasma fatty acids profile, OR, NE, EES, %EM, %CM and %B included the effect of line (homogeneous and heterogeneous line). All analyses were performed using Bayesian methodology. Bounded uniform priors were used for effect line. Residuals were a priori normally distributed with mean **0** and variance $\mathbf{I}\sigma_e^2$. The priors for the variance were also bounded uniform. Marginal posterior distributions of the differences between lines were estimated for all unknowns using Gibbs sampling with the program Rabbit developed by the Institute for Animal Science and Technology (Valencia, Spain).

RESULTS AND DISCUSSION

Ovulation rate was similar between the heterogeneous and homogeneous lines as in the previous study by Calle et al. (2017) (see Table 1). The heterogeneous line showed almost one less embryo at 72 hours of gestation than the homogeneous line (P=80%). In addition, this line had a higher percentage of early morulae (P=99%) and a lower percentage of blastocysts (P=97%) than the homogeneous one. This result agrees with a less advanced embryo development in the females of the heterogeneous line that would penalize the embryonic survival in the first 72 hours of gestation (P=90%).

Table 1: Ovulation rate (OR), number of normal embryos (NE), early embryonic survival (EES), and percentage of early morulae (%EM), compacted morulae (%CM) and blastocysts (%B) at 72 hours of gestation in the heterogeneous and homogeneous lines for litter size variability.

	Heterogeneous line (n=6)		Homogeneous line (n=12)				
	Mean	SD	Mean	SD	D	HPD95%	P
OR	10.0	0.88	9.99	0.61	0.02	-2.09, 2.21	50
NE	7.23	1.16	8.13	0.82	-0.92	-3.79, 0.90	80
EES	0.69	0.08	0.80	0.06	-0.11	-0.30, 0.03	90
%EM	36.6	9.07	6.97	5.91	29.2	5.97, 53.2	99
%CM	80.7	13.7	84.1	9.79	-3.44	-36.6, 31.4	57
%B	1.24	11.9	27.2	7.22	-26.8	-54.0, 0.89	97

SD: Standard deviation. D: median of the difference between heterogeneous and homogeneous lines. HPD95%: highest posterior density region at 95%. P: probability of the difference being > 0 when D> 0, and probability of the difference being < 0 when D < 0.

Table 2: Plasma fatty acids profile at 72 hours of gestation in the heterogeneous and homogeneous lines for litter size.

	Heterogeneous line (n=6) (ng/ml)		Homogeneous line (n=12) (ng/ml)				
	Mean	SD	Mean	SD	D	HPD95%	P
C12:0	446	257	71.1	165.5	367.8	-290, 1078	87
C14:0	2992	1597	793	1032	2256	-1865, 6495	85
C15:0	1371	465	461	309	917	-196, 2070	95
ISO C15:0	201	59	39.1	38.0	162	22.3, 313	98
C16:0	56883	18097	23939	12489	33130	-14554, 75745	91
C17:0	2266	733	617	486	1633	-256, 3454	96
ISO C17:0	1174	468	317	304	854	-322, 2039	92
C18:0	31234	8474	10935	5946	20381	-458, 41710	97
C20:0	398	151	117	97	280	-85.8, 691	92
C23:0	481	699	409	429	56.9	-1927, 1761	53
C14:1	116	35	21.9	23.2	93.6	8.78, 186	97
C16:1	1804	492	710	331	1098	-123, 2270	96
C18:1 t9	501	405	320	241	170	-898, 1240	62
C18:1 n-9	58383	20668	16835	13520	41578	-11013, 95271	94
C18:2 n-9	109	29	54.9	20.0	54.7	-17.9 122	94
C18:2 n-6	90327	27378	30140	19294	60173	-5911, 129218	96
C18:3 n-6 gamma	148	47	41.9	30.7	107	-22.5, 215	96
C18:3 n-3 alfa	4134	1535	1321	1040	2814	-822, 6855	92
C20:3 n-3	983	3607	4789	2373	-4023	-12479, 5626	80
C20:3 n-6	2040	741	836	512	1193	-728, 3036	91
C20:4 n-6	4519	1297	1664	880	2866	-380, 6043	96
C20:5 n-3	158	72	119	49	35.1	-147, 209	65
C22:6 n-3	77.3	32.1	24.9	22.3	52.2	-33.7, 126	90

SD: Standard deviation. D: median of the difference between heterogeneous and homogeneous lines. HPD95%: highest posterior density region at 95%. P: probability of the difference being > 0 when D> 0, and probability of the difference being < 0 when D < 0.

Regarding fatty acid profile at 72 hours of gestation (Table 2), females of the heterogeneous line showed higher plasma levels than those of homogeneous line in several long chain saturated fatty acids, such as pentadecanoic (C15:0), iso-pentadecanoic (iso C15:0), margaric (C17:0) and stearic (C18:0) ($P \geq 95\%$). Besides, plasma levels were larger in the heterogeneous line than in the homogeneous one for monounsaturated fatty acids, such as myristoleic (C14:1) and palmitoleic (C16:1), as well as for polyunsaturated fatty acids, such as linoleic (C18:2 n-6), gamma-linolenic (gamma C18:3 n-6) and arachidonic (C20:4 n-6) ($P \geq 95\%$). These findings are consistent with the hypothesis that increased susceptibility to stress increases plasma fatty acid levels.

Several studies have found a negative effect of long-chain saturated fatty acids, such as palmitic (C16:0), stearic (C18:0) and tricosanoic (C23:0), on oocyte maturation, fertilisation and pre-implantation embryo development (Mirabi et al., 2017; Zeng et al., 2023). Regarding polyunsaturated fatty acids, linoleic (C18:2 n-6) and arachidonic (C20:4 n-6) seem to have a controversial role, promoting early embryonic development and impair embryo growth from day 7 onwards of gestation (Mirabi et al., 2017; González-Brus et al., 2023). A higher plasma concentration of long-chain saturated fatty acids, as well as linoleic (C18:2 n-6) and arachidonic (C20:4 n-6) in females of the heterogeneous line would agree with a less advanced development of their embryos at 72 hours of gestation.

CONCLUSIONS

The heterogeneous line had a lower early embryonic survival as a consequence of a lower embryonic development at 72 hours of gestation. A higher concentration of long-chain saturated and polyunsaturated fatty acids in heterogeneous line would support its lower embryonic development and higher embryo loss rate compared to line homogeneous line.

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EFFECTS OF DIETARY ASTAXANTHIN ON REPRODUCTIVE PERFORMANCE AND ANTIOXIDATIVE CAPACITY OF FEMALE RABBITS UNDER HEAT STRESS

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ABSTRACT

Heat stress is a major environmental threat to the reproductive performance of female rabbits. The aim of this study was to investigate the effects of dietary astaxanthin (ASTA) on reproductive performance and blood antioxidative capacity of female rabbits under heat stress condition. A total of 240 New Zealand White female rabbits with similar body weight were randomly assigned into four dietary treatments (60 rabbits each). Rabbit received a basal diet supplemented with 0 (ASTA0), 5 (ASTA5), 15 (ASTA15), and 25 (ASTA25) mg ASTA/kg diet. The temperature, relative humidity, and temperature humidity index (THI) were documented. Reproductive performance and antioxidative capacity were determined. THI showed that ratios of heat stress condition, extreme heat stress, severe heat stress, and moderate heat stress were 63%, 28%, 17%, and 18%, respectively. Rabbits added with 25 mg ASTA/kg had higher ($P < 0.05$) pregnancy rate, farrowing rate, litter weight at weaning, and average weight of kit at weaning. Moreover, the malondialdehyde (MDA) concentration in plasma was reduced ($P < 0.05$) by 15 and 25 mg ASTA addition. Furthermore, activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) were enhanced by 25 mg ASTA addition. Conclusively, supplementing ASTA up to 25 mg/kg diet for female rabbits is indorsed to improve reproductive performance and antioxidative capacity under heat stress.

Key words: Astaxanthin, Heat stress, Reproductive performance, Antioxidative capacity

INTRODUCTION

Global warming has been recognized as a major threat to the productivity of livestock species. High environmental temperature accompanied by high humidity disturb a balance between heat production and emission of animals, which result in heat stress, a main environmental stressor that cause substantial economic loss in livestock industry (Ebeid *et al*., 2023). In this sense, heat stress exerts detrimental effects on growth performance, immunity and reproduction of livestock by disordering physiological and metabolic activities (Khalid *et al*., 2022). Owing to a few functional sweat glands and thick fur, rabbits are more susceptible to heat stress when compared with other livestock species (Amber *et al*., 2021). Growing evidence demonstrated that heat stress induced reproductive efficiency (reduced litter size, litter weight and milk roduction) in rabbits is mainly attributed to oxidative stress resulting from heat stress during pregnancy and lactation (Amber *et al*., 2021). It has been reported that dietary antioxidants are considered as effectively nutritional strategies to mitigate the negative effects of heat stress on reproduction.

Astaxanthin (ASTA) is a red-colored xanthophyll carotenoid with varied biological/physiological activities, such as scavenging reactive oxidative species (ROS), repressing inflammation, and reducing the proliferation of cancer cells. Owing to its outstanding antioxidative capacity, ASTA has been found to protect organism against oxidative stress damage (Higuera-Ciapara *et al*., 2006). Moreover, ASTA has been reported to promote growth performance, reproductive performance and antioxidative capacity of animals (He *et al*., 2023). However, there is little information on whether ASTA could improve

the reproductive performance and antioxidative capacity of female rabbits under heat stress conditions.

Therefore, the present study aims to investigate effects of dietary ASTA on reproductive performance and antioxidative capacity of female rabbits reared under heat stress conditions.

MATERIALS AND METHODS

Animals and experimental design

This study was carried out at Institute of Animal Science, Jiangsu Academy of Agricultural Sciences during the hot summer months from July to September in 2021. All experimental procedures were approved by the Institutional Animal Care and Use Committee of China Agricultural University. A total of 240 New Zealand White female rabbits with similar body weight (3.93 ± 0.05 kg) e used in this study. After 7 days' adaption, all rabbits were randomly assigned into four groups (60 rabbits each) and fed a basal diet supplemented with 0 (ASTA0), 5 (ASTA5), 15 (ASTA15), and 25 (ASTA25) mg ASTA/kg diet. All rabbits were fed with commercial pelleted feed containing 9.28 MJ/Kg digestible energy, 18.69% crude protein, 16.13% crude fiber, 3.37% ether extract, and 8.72% ash. Rabbits were fed experimental diets from 6 days before artificial insemination and last for 35 days after postpartum. The rabbits had free access to feed and water. Rabbits were performed with a 42 days breeding cycle.

Heat stress assessment

The temperature and relative humidity were auto-measured once every hour during trial period. Temperature humidity index (THI) was documented according to the equation described by a previous study (Marai *et al*., 2001).

Reproductive performance

Reproductive performance including pregnancy rate, farrowing rate, litter size, litter weight, and individual kit weight during the experimental period were determined.

Blood sampling and antioxidant indices analysis

Six female rabbits from each group were randomly selected to collect blood samples via auricular veins. Then, blood samples were centrifuged at 3000 g for 15 min to obtain plasma. Plasma was assayed for malondialdehyde (MDA) concentration, and activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and catalase (CAT) by using commercial assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Statistical analysis

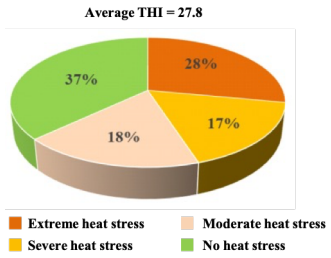
All data were expressed as means ± SEs and statistically analyzed using SPSS 20.0 software (SAS Inc., Chicago, IL). Statistical significance among groups was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Temperature humidity index during trial period

As shown in Figure 1, the average THI was 28.7 (Left), which is higher than 27.8 (No heat stress). Furthermore, heat stress assessment pie chart (Right) showed that the ratio of heat stress was 63%, ratio of extreme heat stress was 28%, ratio of severe heat stress was 17%, and ratio of moderate heat stress was 18%. The heat stress condition was similar with a pervious study (Chen *et al*., 2023). These THI values indicated heat stress conditions for rabbits.

Figure 1: Temperature humidity index curve and heat stress assessment pie chart



Effect of dietary ASTA on reproductive performance of female rabbits under heat stress The results (Table 1) showed that female rabbits fed a diet supplemented with 25 mg ASTA/kg diet had higher ($P<0.05$) pregnancy rate, farrowing rate, litter weight at weaning, and average weight of kit at weaning when compared with ASTA0 group. In agreement with our results, previous study found that ASTA supplementation improved quality of embryos in endometriosis patients undergoing assisted reproduction (Rostami *et al*., 2023) and enhanced numbers of corpora lutea, implantation sites and fetuses and litter size (Hansen *et al*., 2001). No significant differences were observed among ASTA treated groups ($P>0.05$). In addition, dietary ASTA supplementation had no significant differences ($P>0.05$) on born kits per litter, live born kits per litter, average weight of litter at birth, average weight of alive kits at born, and average litter size at weaning when compared with rabbits fed without ASTA. These results implicated that dietary ASTA exerted improvement on reproductive performance of female rabbits.

Table 1: Effects of astaxanthin on reproductive performance of female rabbits under heat stress conditions

Parameters	Groups			
	ASTA0	ASTA5	ASTA15	ASTA25
Pregnancy rate, %	55.9^{b}	69.5^{ab}	71.7^{ab}	76.7^{a}
Farrowing rate,%	47.5^{b}	59.3^{ab}	61.7^{ab}	66.7^{a}
Born kits per litter	7.9±0.45	7.2±0.43	7.1±0.36	7.5±0.40
live born kits per litter	7.1±0.44	5.9±0.37	6.3±0.39	6.8±0.44
Average weight of litter at birth (g)	330.2±18.60	285.3±16.83	291.1±14.46	328.6±20.57
Average weight of alive kits at birth (g)	47.6±1.51	49.0±1.80	48.5±1.64	49.2±1.15
Average litter size at weaning	3.3±0.53	4.2±0.45	3.8±0.48	4.6±0.40
Litter weight at weaning (g)	2194.8±346.91^{b}	2731.9±293.18^{ab}	2331.2±296.64^{ab}	3112.8±296.64^{a}
Average weight of kit at weaning (g)	415.9±60.91^{b}	535.2±49.08^{ab}	436.4±51.12^{ab}	565.4±44.38^{a}

Means with different letters on the same row differ significantly.

Effect of dietary ASTA on antioxidative capacity of female rabbits under heat stress In addition, antioxidant indices were revealed in Figure 2. Heat stress induces mitochondrial dysfunction which lead to an overproduction of ROS (Amber *et al*., 2021). ASTA, the king of antioxidants, has a strong antioxidative capacity to combat ROS, inhibit lipid peroxidation and maintain redox homeostasis (Higuera-Ciapara *et al*., 2006). Rabbits that received 15 and 25 mg ASTA/kg diet had lower ($P<0.05$) MDA concentration, and higher ($P<0.05$) activities of SOD in plasma, as compared with ASTA0 group. Moreover, rabbits that received 25 mg ASTA/kg diet had higher ($P<0.05$) GSH-PX activities when compared with ASTA0 group. Growing evidence revealed that ASTA could enhance activities of SOD and GSH-PX (Rostami *et al*., 2023). Rabbits that received 5 and 15 mg ASTA/kg diet had no significances ($P>0.05$) on GSH-PX activities. Additionally, no difference ($P>0.05$) of CAT activities was observed among groups. These data suggested that dietary ASTA at dosage of 25 mg/kg diet enhanced antioxidative capacity of female rabbits.

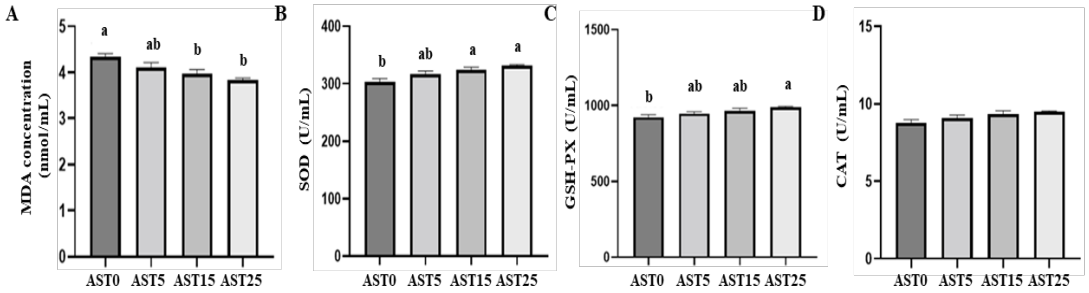


Figure 2: Effects of dietary astaxanthin on antioxidant indices of female rabbits under heat stress condition.

CONCLUSIONS

In conclusions, THI revealed that female rabbits were exposed to heat stress condition. Results documented that dietary ASTA supplementation improved pregnancy rate, farrowing rate, litter weight at weaning, and average weight of kit at weaning. In addition, ASTA reduced MDA concentration and enhaced activities of SOD and GSH-PX in plasma.

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USE OF DAPI STAINING TO ASSESS MEMBRANE INTEGRITY BY FLOW CYTOMETRY IN RABBIT SPERMATOZOA.

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ABSTRACT

Flow cytometry has become a useful tool for semen quality analysis, but it can be difficult to find the appropriate combinations of fluorochromes to evaluate jointly several parameters. One of the reasons for this is the limitation of the number of free channels, limiting the possibilities for other fluorochromes with the same emission spectrum. This is what happens when using propidium iodide (PI), a non-permeable fluorochrome that is commonly used in combination with other stains. In the present study, we propose 4',6'-diamidino-2-phenylindole (DAPI), a semi-permeable and cheap fluorochrome, as the go-to protocol for evaluating sperm viability by flow cytometry, since it will allow the rest of the channels to remain free for other parameters. For the study, 60 rabbit semen samples were used. Every sample was divided in two subsets and stained with both DAPI (blue) and the PI (red) and sperm viability was checked. The results obtained with the two different protocols were equivalent (around 73 % of viability). The correlation between them was higher than 76 % ($p<0.01$). In conclusion, we confirmed that a DAPI is an appropriate blue fluorochrome to assess cell viability in refrigerated rabbit sperm.

Key words: flow cytometry, semen, viability, DAPI, rabbit.

INTRODUCTION

Understanding the values of sperm viability is mandatory for the evaluation of seminal quality in any species. This gains a special importance when working with semen obtained in a different location, since time from recovery, transport or refrigerating conditions can cause a significant increase in the mortality rate. The settlement of flow cytometry as an everyday technique has allowed the improvement of the efficiency of semen evaluation. Not only it has reduced the subjectivity associated to microscopic assessment, but also it has increased exponentially the number of cells evaluated (Martínez-Pastor et al., 2010). Furthermore, the use of the flow cytometer makes possible the simultaneous analysis of different parameters, which simplifies noticeably the researcher tasks. In order to do this, we're in need of fluorochromes whose emission spectrum don't overlap. When working with spermatozoa, this is not always easy, since most of the fluorochromes used for evaluating these cells emit between the green and the red channel, which reduces the number of possible combinations (Egyptien et al., 2023).

Traditionally, sperm viability has been assessed using propidium iodide (PI), a non-permeable fluorochrome that binds to the nucleic acids in cells whose membranes have lost integrity, marking them in red (peak of emission = 618 nm). This fluorochrome is usually applied in combination with Hoechst33342, that binds with the nucleus of every cell, both dead and live. The emission channel of Hoechst33342 is blue so, by using this combined staining, only the green channel is left free for another parameter (Martínez-Pastor et al., 2010). IP is also commonly combined with SYBR®14, that permeates through live cells and stain them in green. This, added to what has previously been explained, can complicate the

researcher's work, and stay in the way of taking profit of the full potential of the cytometer (Egyptien et al., 2023).

On the other hand, 4′,6-diamidino-2-phenylindole (DAPI) is a fluorochrome that, same as IP, binds to the DNA of cell whose membrane integrity has been compromised, marking them in blue instead of red (peak of emission = 461 nm). This, in addition to its low cost, give the researcher the advantage of leaving both green and red channels free for other fluorochromes, allowing them to analyze three different parameters at the same time. Nevertheless, DAPI is a semi-permeable fluorochrome, which means that some live cells might be stained if the concentration of the fluorochrome or the incubation time is inappropriate.

The objective of this study is to evaluate if the viability rate in rabbit spermatozoa measured with DAPI is equivalent to the commonly used combined staining and suggest its use as an alternative to the IP method.

MATERIALS AND METHODS

All experimental procedures were approved by the Committee of Ethics and Animal Welfare of the Miguel Hernández University with code VSC PEA 0226 type 2.

Animals and Semen Collection

Rabbit bucks used for de essay were housed at the farm of the University Miguel Hernández of Elche, Spain. Semen collection was developed following the usual protocol. Briefly, artificial vaginas were prepare using hot water (45 °C) and females were presented to de bucks in order to encourage ejaculation. Individual samples were diluted with Tris-acid citric and glucose and were kept cooled at 4 ºC until further analysis at Universitat de València.

Sample Preparation

Each sample was divided in two aliquots in order to check two different stainings. Three µL of semen were stained either with DAPI (100 µg/µL) or PI (0.5 mg/µL) + Hoechst33342 and PBS was added until a volume of 200 µL was reached. DAPI samples were incubated at 37 ºC for 15 min and PI samples for 5 min.

Flow Cytometry Essay

Cytometry assessments were developed in the core facility of cell culture and flow cytometry at the Central Service for Experimental Research (SCSIE) of Universitat de València (Valencia). Samples were analyzed using a BD LSRFortessa flow cytometer equipped with five lasers (UV wavelengths at 355 nm, violet at 405 nm, blue at 488 nm, yellow-green at 561 nm and red at 640 nm) and controlled using FACSDiva 8 software. Signals were amplified logarithmically, and photomultiplier settings were adjusted to each staining. A minimum of 10,000 cells per replicate were recorded.

Statistical Analysis

All analysis were performed using the SPSS software (IBM® SPSS® 28.0.1.1 for Windows; IBM corp., Armonk, NY, USA). Both methods were compared using a bivariate correlation and the value R^2 was calculated according to the linear fit for the scatter plot.

RESULTS AND DISCUSSION

A total of 60 samples were analyzed. The average viability rate of samples stained with the staining IP was 73.18%, while the one observed with DAPI was 73.28% (Table 1). This difference, however, was not statistically significant. In fact, Pearson's correlation showed a match of 76.6% of both techniques, this being significant at the 0.01 level (Table 2). The R^2 value equaled 0.60 (Figure 1), which proved said positive relationships between both stainings.

Table 1: Descriptive Statistics of DAPI and IP stains

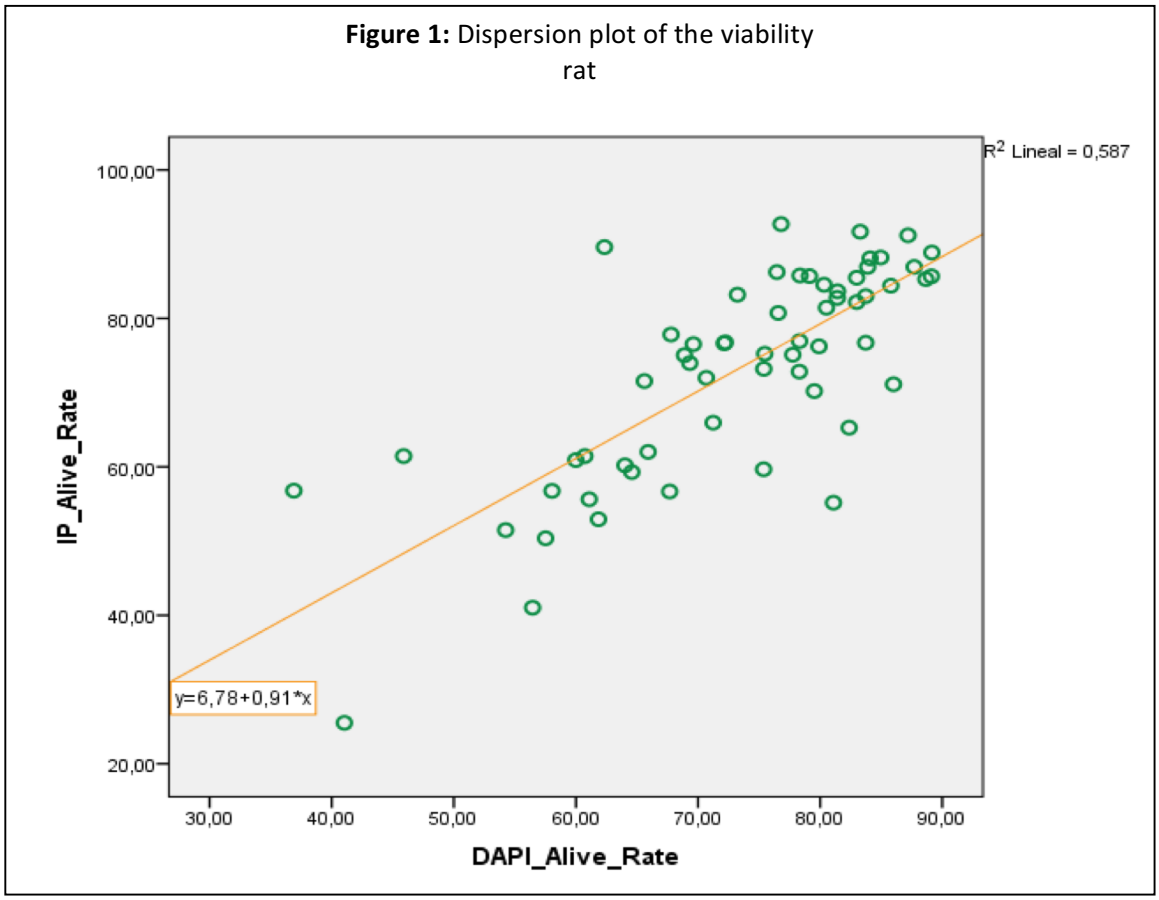
	Mean (%)	Std. Deviation (%)	N
IP_Alive_Rate	73.18	14.06	60
DAPI_Alive_Rate	73.28	11.89	60

Table 2: Correlation between DAPI and IP stains

		IP_Alive_Rate
DAPI_Alive_Rate	Pearson Correlation	0.766**
	Sig. (2-tailed)	0.000
	N	60

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 1: Dispersion plot of the viability rat



CONCLUSIONS

In sight of this results, we can conclude that DAPI is a perfect tool for evaluating sperm viability in refrigerated samples in rabbit, being as efficient as IP. The use of DAPI allows the combination with other fluorochromes emitting the red spectrum like mitochondrial polarization.

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GERMPLASM COLLECTION OF ITALIAN RABBIT BREEDS: BIODIVERSITY CONSERVATION THROUGH THE ESTABLISHMENT OF A SEMEN CRYOBANK

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ABSTRACT

The conservation of genetic diversity in rabbits is of crucial importance, considering the reduction of local breeds due to intensive production based on the use of a few commercial hybrids. The establishment of cryobanks for genetic resources provides a link between *in situ* and *ex situ* techniques to improve the efficiency of conservation programmes. The aim of this work is the establishment of a cryobank for collecting male gametes, aimed at safeguarding the genetic heritage of Italian rabbit breeds. The cryobank is located at the Genetic Centre in Volturara Appula (Foggia, Italy) and, currently contains 3846 doses of semen from 1106 individuals belonging to 43 breeds. The semen doses in the cryobank can be used for the reconstruction of rare genetic lines and the backup of *in vivo* preserved populations. Future efforts should prioritize breeds that are at risk of extinction and increase the population at the Genetic Centre to store more frozen semen doses in the cryobank.

Keywords: cryobank, biodiversity, conservation program, Italian rabbit breeds, cryopreservation.

INTRODUCTION

Due to the ever-increasing intensification of production systems and the use of commercial hybrids with high production capacity, over the years, the role of rabbit breeds selected for meat production, has dramatically decreased (Jochová et al., 2017). This has led to an erosion of genetic variability, making animals less flexible in their responses to sudden environmental variations and severely limiting their ability to adapt (Carneiro et al., 2011). In addition, most of the historical breeds are included in the national programs of genetic resources conservation according to the FAO guidelines (FAO, 2007). The value of these breeds has encouraged many conservation programs based on *in situ* and *ex situ* strategies at global and European levels. The semen cryopreservation is currently an optimal strategy within *ex situ in vitro* conservation program of the genetic resources of rabbit species. One of the main objectives of a germplasm repository is the possibility of recreating breeds or reproductive lines in case of disaster losses (Comizzoli et al., 2010). Furthermore, cryopreserved material can support *in vivo* conservation by reducing inbreeding and genetic drift in small managed populations, combining live animals and cryopreserved germplasm as a powerful tool for conservation (Meuwissen, 1999).

In this context, ANCI (Italian Association of Rabbit Breeders) undertook a series of activities, supported by the "Cun-Fu 2, project, funded by MASAF, Measure 10.2 Biodiversity—Rabbit sector" aimed at promoting and supporting the conservation of the genetic resources of the Italian rabbit while simultaneously enhancing its productive and reproductive performance. Notably among these activities is the establishment of a cryobank for collecting male gametes, aimed at safeguarding the genetic heritage of Italian rabbit breeds. Consequently, with the collaboration of the University of Molise, ANCI implemented a cryobank focused on cryopreserving semen from a diverse range of rabbit breeds, with particular attention to those at risk of extinction across the entire national.

MATERIALS AND METHODS

Animals, semen collection and assessment

The donors had a known genealogy as they were registered in the national genealogical registry, and they were also selected on specific morphological characteristics assessed by breed experts of the ANCI group in accordance with the indications reported in the breed standard of the rabbit population register (https://www.anci-aia.it/registro-anagrafico/#razze).
Semen was collected using a pre-heated artificial vagina. Semen collection took place both at trade fairs and at many different registered farms, involving the entire national territory. In accordance with the guidelines of the FAO and the OIE (World Organization for Animal Health), semen samples were obtained from healthy animals that had undergone vaccination against M.E.V. (Viral Haemorrhagic Disease) and Myxomatosis. Semen sampling, analysis, and freezing activities were conducted using a mobile laboratory located in a clean area. This ensured that technical operators could carry out their work accurately.
The semen of each donor was subjected to a qualitative evaluation, which allowed the identification and selection of semen samples suitable for freezing. This was assessed based on macroscopic parameters such as volume, colour, density and the presence of contaminants, and microscopic parameters related to the total and progressive motility of the semen. Volume was measured using a graduated test tube. Ejaculates with an ivory-white colour were used for the subsequent microscopic evaluation stage. Motility was assessed subjectively by assigning a percentage score. For this purpose, 5 µL of semen diluted in physiological solution (NaCl 0.9%), were observed with a portable microscope at 400 × total magnification. Spermatozoa had to have at least 65% total motility and 50% progressive motility to be considered suitable for the cryopreservation.

Semen cryopreservation

The cryopreservation protocol utilized to establish the semen cryobank was developed by team from the University of Molise prior to the commencement of the project. This protocol was identified by evaluating the factors involved in the freezing process, including the type of permeant and non-permeant cryoprotectants, equilibration time and cooling duration (Iaffaldano et al., 2012, 2014; Di Iorio et al., 2018). The protocol included the following steps: the semen was cooled at 5°C for 90 min. Then, the cooled samples were diluted to a ratio of 1:1 (v/v) with a freezing extender composed of TCG diluent (47 mM glucose, 250 mM Tris, 88 mM citric acid) containing 16% of dimethyl sulfoxide (v/v) and, 0.1 mol/L sucrose (w/v). The processed semen was subsequently loaded in 0.25 mL straws, equilibrated at 5°C for 45 min and frozen by the exposure to the liquid nitrogen vapor 5 cm above the liquid nitrogen surface for 10 min. Finally, the straws were plunged into liquid nitrogen for the storage at -196°C.
To ensure traceability, each semen dose, packed in straw was identified by an alpha-numeric code including the breed and donor subject information. The cryobank is located at the Genetic Centre in Volturara Appula (Foggia, Italy) under the direct control of the ANCI staff that is responsible for maintaining and monitoring the semen doses.

RESULTS AND DISCUSSION

During the project timeframe frozen semen doses from Italian rabbit breeds were stored in the cryobank. Currently, cryobank consists of 3,846 semen samples (Table 1) originating from 1,108 individuals across 43 breeds listed in the rabbit population register, as well as from 3 breeds recorded in the rabbit studbook, as detailed in Table 1. The breeds represented in the cryobank encompass both autochthonous genetic and cosmopolitan breeds.
According to FAO guidelines, the minimum number of subjects required for the establishment of a semen cryobank is 25. For the majority of breeds, this target was met, with more than 25 donors participating. However, for certain breeds with a smaller population, the total number of subjects fell below this threshold. Specifically: Ariete Inglese (9), Pezzata Tricolore (5), Alaska (3), Martora (4), Avana (8), Cincillà Piccolo (2), Russo (12), Angora (12), Volpe (0), Satin (8), Pezzata Piccola (6), and Leprino di Viterbo (7).

CONCLUSIONS

The results achieved so far are very satisfactory; however, cryopreservation of semen from Italian rabbit breeds is still ongoing. The establishment of the Italian semen cryobank of autochthonous rabbit breeds stands as a valuable resource for safeguarding and conserving biodiversity within the Italian rabbit breed populations still prevalent in agricultural systems. The semen cryobank will facilitate the implementation of integrated schemes, combining *in vivo* and *in vitro* methods, to address genetic issues and mitigate the risk of breed extinction.

In fact, in the near future, it is anticipated that the ANCI team at the Volturara Appula Genetic Centre (Italy) will prioritize enhancing the populations of breeds categorized as critically endangered or at risk of extinction according to the FAO classification (2003), while simultaneously conserving their semen doses within the cryobank.

Table 1. *Overall summary of semen doses stored in the cryobank*

Breed	Number of donors	Doses number (straw 0,25 ml)
Gigante	30	125
Gigante Bianco	25	173
Gigante Pezzato	27	122
Ariete	25	80
Argentata di Champagne	33	104
Cincillà Grande	26	52
Ariete Inglese*	9	17
Blu di Vienna*	32	123
Fulva di Borgogna*	34	195
Hotot*	10	18
Bianca di Nuova Zelanda*	29	98
Argentata Grande	17	48
Californiana*	32	87
Rossa della Nuova Zelanda*	36	85
Bianca di Vienna*	17	71
Giapponese*	26	66
Pezzata Tricolore	5	20
Alaska*	3	12
Lepre	28	86
Turingia*	28	55
Giarra Bianca	28	55
Ariete Piccolo	25	78
Martora	4	10
Fata di Marburgo	13	45
Oro di Sassonia	30	90
Fata Perlata	28	110
Pezzata Inglese*	25	69
Lince	17	66
Argentata Piccola	33	84
Avana	8	21
Olandese*	25	63
Cincillà Piccolo	2	5
Focata	25	57
Russo	12	15
Ariete Nano	32	73
Ermellino	27	43
Nani Colorati	25	52
Angora	12	21
Volpe	0	0
Rex	25	142
Satin	8	18
Pezzata Piccola	6	17
Leprino di Viterbo	7	24
Bianca Italiana	81	434
Macchiata Italiana	74	348
Argentata Italiana	62	269

**Cosmopolitan breeds*

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ANTIMICROBIAL RESISTANCE IN THE PRODUCTION OF RABBIT SEMINAL DOSES

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ABSTRACT

The transmission of infections in rabbit artificial insemination traditionally relies on antibiotics added to semen diluents, with penicillin and streptomycin being the conventional combination. *Staphylococcus aureus* (*S. aureus*) is a major concern due to its association with various rabbit diseases and its ability to develop resistance to antibiotics. In this context, the aim of this study is to investigate the presence of *S. aureus* in rabbit semen and assess its resistance to commonly used antibiotics. To this end, semen samples from 21 healthy New Zealand buck rabbits were examined. *Staphylococcus* was isolated in 52.4% of the samples, with 45.5% identified as *S. aureus*. All of which showed resistance, with 60% being multidrug-resistant. Tetracycline exhibited the highest resistance frequency (100%), followed by chloramphenicol (75%). Other antibiotics, including those categorized as Highly Important Antimicrobials, also showed resistance. The findings highlight a potential risk of antibiotic failure against *S. aureus* in semen diluents, increasing the risk of bacterial transmission and resistance spread. The study emphasizes the need to explore alternative approaches to mitigate this risk.

Key words: *Staphylococcus aureus*, Multidrug-resistance, Semen diluents

INTRODUCTION

Traditionally, the transmission of infections in rabbit artificial insemination has been controlled through the addition of antibiotic or mixtures of antibiotics to the semen diluents, being penicillin and streptomycin, the traditional combination added (Viudes-de-Castro *et al.,* 2021; Viudes-de-Castro and Vicente, 2023). While penicillin (β-lactam antibiotic) is predominantly used against Gram-positive bacteria, streptomycin (aminoglycoside antibiotic), is used against Gram-negative bacteria (Tufa *et al.,* 2023). Among the main bacteria present are *Staphylococcus, Enterobacter*, *Pseudomonas* or *Enterococcus*, among others (Rouillon *et al.,* 2022). In this sense, *Staphylococcus aureus* (*S. aureus*) stands out as one of the most significant causes of nosocomial- and community-acquired bacterial infections in rabbits. This Gram-positive bacterium is notable for its ability to invade and act as an etiological agent in various rabbit diseases, including subcutaneous abscesses, mastitis, pododermatitis and septicemia. Furthermore, *S. aureus* has the capacity to develop resistance to all clinically available classes of antibiotics, and resistance can emerge through de novo mutations, promoting the transmission of antimicrobial resistance (Silva *et al.,* 2023). Penicillin was the first antibiotic in mass produced. Although initially it was highly effective for treatment of *S. aureus* infections, today over 80% of *S. aureus* strains isolated are penicillin resistant (Vestergaard *et al.,* 2019). Hence, the aim of this study is to investigate the presence of *S. aureus* in rabbit semen and assess its resistance to commonly employed antibiotics in seminal doses.

MATERIALS AND METHODS

The experiment was carried out with bucks from one Spanish commercial rabbit line (Line A) based on New Zealand White rabbits. All animals were housed at the Universitat Politècnica de València (Valencia, Spain) experimental farm in flat deck indoor cages (75 × 50 × 30 cm), with free access to water and commercial pelleted diets (Cunilactal, Nanta). Animals were housed under a photoperiod of 16L:8D, while the room temperature was maintained between 14°C and 28°C. On the same day in October 2023, semen samples were obtained from 21 healthy two-year-old bucks with proven fertility. To this end, one ejaculate per buck was collected using an artificial vagina.

For *S. aureus* isolation, semen samples were pre-enriched (buffered peptone water 1:10 vol/vol) and incubated at 37 ± 1 ºC for 24 hours. Subsequently, the suspension was streaked on non-specific agar Columbia CNA agar with 5% Sheep Blood. Finally, colonies exhibiting suspected characteristics of *Staphylococcus*, along with a positive catalase test result, were confirmed to be *S. aureus* by API-STAPH test.

Antimicrobial resistance (AMR) test was carried out by Minimum Inhibition Concentration (MIC) assay (Sensititre plate GPALL1F, ThermoFisher Scientific™). Finally, the results were interpreted in accordance with the established breakpoints 2023 of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), available on the European Society of Clinical Microbiology and Infectious Diseases website (https://www.eucast.org/ast_of_bacteria/calibration_and_validation).However, certain MIC values are not currently available in EUCAST. Consequently, the recommendations outlined in M100 (CLSI, 2023a) and VET01-S2 (CLSI, 2023b) by the Clinical and Laboratory Standards Institute (CLSI) were followed. Finally, the assessment of Methicillin-resistant *Staphylococcus aureus* (MRSA) strains was studied by monitoring the AMR observed against cefoxitin. Moreover, multidrug-resistance (MDR) was defined as acquired resistance to at least one agent in three or more antimicrobial classes (Magiorakos *et al.,* 2012).

A Generalised Linear Model using the probit link function, which assumed a binomial distribution for AMR patterns in *S. aureus* was performed. A p-value ≤ 0.05 was considered to indicate a statistically significant difference. Data are shown as least squares means with standard error of the least squares means. Statistical analysis was conducted using the R software packages EMMs [Searle], car [Fox], and multicompView (wrave).

RESULTS AND DISCUSSION

Out of the 21 samples, *Staphylococcus* was isolated in 52.4% (n=11). Among the isolated *Staphylococcus*, 45.5% (n=5) were identified as *S. aureus*. Bacteria are usually present in semen and their collection process is not entirely sterile. This result is in line with previous authors (Rouillon *et al.,* 2022) and it was expected because *S. aureus* is one of the most prevalent nosocomial bacteria, being the aim of obtaining sterile semen almost unachievable.

In the present study, all the isolates were resistant and 60% were MDR (n=3). The highest frequency of AMR was found to tetracycline (100 %, n=5), followed by chloramphenicol (75±21.7 %, n=4) (p-value < 0.05). Both antibiotics are categorized as Highly Important Antimicrobials (HIAs) on the recent World Health Organization (WHO) Medically Important Antimicrobial List. While its usage is permitted for both humans and animals, it is imperative to establish controls, given that this antibiotic is utilized for treating zoonotic diseases or severe infections where limited antibiotic alternatives are available. Resistance was also observed for penicillin, vancomycin, cefoxitin, ciprofloxacin, clindamycin, chloramphenicol, erythromycin, gentamycin, levofloxacin, linezolid, moxifloxacin, quinupristin/dalfopristin, tigecycline, trimethoprim-sulfamethoxazole (25.0±21.7 %, n=1) (Figure 1). It's worth noting the resistance to gentamicin. This antibiotic or mixtures of antibiotics are frequently added to

semen diluents, so an increase in resistance could lead to failure in bacterial elimination and facilitate transmission to females. It's also noteworthy the resistance to penicillins. As highlighted by Chai and colleagues (2021), even though these animals have not been administered antibiotics, resistance to penicillin could come from rabbit handlers, as it is widely used in human medicine. Finally, D-test was positive in 25.0% (n=1) of *S. aureus* strains isolated, which assesses inducible resistance to clindamycin. This antibiotic has served as a rescue treatment for infections caused by resistant *S. aureus*, notable for its 90% oral bioavailability, cost-effectiveness compared to other antibiotic alternatives, and its beneficial role in inhibiting the production of certain toxins and virulence factors (Montoya *et al.,* 2009).

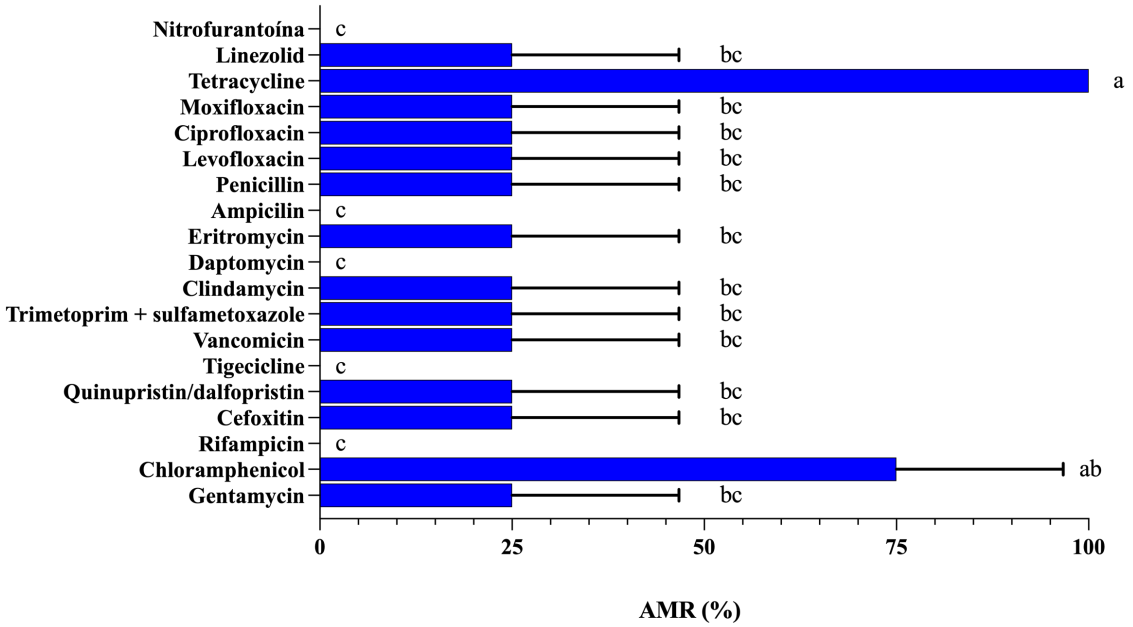

Figure 1. Antimicrobial Resistance pattern of *Staphylococcus aureus* strains isolated from rabbit semen. AMR: Antimicrobial Resistance.

The results of this study demonstrate a potential risk of antibiotic failure against *S. aureus*, as resistances are observed to the antibiotics commonly used in semen diluents, increasing the likelihood of bacterial transmission. Furthermore, the presence of these bacteria in semen poses a significant risk of resistance spread, with many classified as HIAs by the WHO. Therefore, it is crucial not only to further understand the AMR levels in semen, both in *S. aureus* and other bacteria present, but also to emphasize the search for new alternatives.

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CLONING OF GONADOTROPIN-INHIBITORY HORMONE RECEPTOR GENE *NPFFR1* IN RABBIT (*ORYCTOLAGUS CUNICULUS*) AND ITS EXPRESSION ANALYSIS IN HYPOTHALAMIC-PITUITARY-TESTICULAR AXIS

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ABSTRACT

Neuropeptide FF receptor 1 (NPFFR1) is the major receptor for gonadotropin inhibitory hormone (GnIH), which plays a pivotal role in regulating the reproductive physiology of animals. To preliminarily understand the expression and function of *NPFFR1* gene in rabbits (*Oryctolagus cuniculus*), we isolated and characterised the cDNA of the hypothalamic *NPFFR1* gene and studied the mRNA expression pattern using qPCR. The full-length cDNA of the rabbit *NPFFR1* gene is 3,712 base pairs and encodes a deduced 432-amino acid peptide. The peptide is a seven-transmembrane protein, comprising an α-helix embedded in the plasma membrane, a connected random coil, and a β-fold. The results of qPCR showed that *NPFFR1* gene was expressed in the hypothalamic-pituitary-testicular axis (HPTa), with a significantly higher mRNA expression level observed in the hypothalamus compared to the pituitary and testis ($P<0.05$). After birth, the expression of *NPFFR1* gene in the hypothalamus was observed to be significantly lower at the age of 90 days in comparison to 11, 30, 60, and 150 days ($P<0.05$). Additionally, the pituitary mRNA level of *NPFFR1* gene was found to be lower in rabbits aged 30 or 120 days than in those aged 11 or 90 days ($P<0.05$). Furthermore, the testicular mRNA levels of *NPFFR1* gene were also lower in rabbits aged 30 days than those aged 11 or 90 days ($P<0.05$). Compared with the vehicle, the administration of 5 and 50 μg GnIH significantly increased the mRNA level of *NPFFR1* gene within the hypothalamus ($P<0.05$). However, no alterations were found in the mRNA levels of *NPFFR1* in the pituitary gland and testis, regardless of the administered dose of GnIH polypeptide. In conclusion, this study offers valuable theoretical support and a reference point for further research into the function of the rabbit *NPFFR1* gene.

Keywords: rabbit, gonadotropin-inhibitory hormone, Neuropeptide FF receptor 1, cloning, gene expression.

INTRODUCTION

Gonadotropin-inhibitory hormone (GnIH) is a neuropeptide secreted by the hypothalamus of vertebrates It has the capacity to inhibit the synthesis and release of gonadotropin hormone (GtH) in the pituitary gland by binding to GnIH receptors. Bonini et al. (2000) identified two potential GnIH receptors, namely NPFFR1 and NPFFR2. Among these, NPFFR1 was identified as having a high affinity for GnIH and was found to be involved in the regulation of the HPTa by GnIH. Therefore, it is considered to be the main receptor for GnIH. At present, the full-length cDNA of *NPFFR1* gene has been cloned and sequenced in many mammals, but there is no report on *NPFFR1* gene in rabbits. The aim of this study was to clone the cDNA sequence of rabbit *NPFFR1* gene, analyse its bioinformatics, detect its temporal and spatial expression profile, and test the effect of exogenous GnIH polypeptide on *NPFFR1* gene expression in HPTa. The results will provide a theoretical reference for the related functional study of *NPFFR1* in rabbits.

MATERIALS AND METHODS

The Minxinan black rabbits employed in the experiment were all procured from the Longyan Tongxian Rabbit Rearing Co., Ltd. in Shanghang County, Fujian Province. The animals were allowed ad libitum access to feed and water, and were maintained on a 12L/12D (light/dark) cycle.

Cloning of full-length cDNA of the *NPFFR1* gene and its sequence analysis

Three male Minxinan black rabbits, aged 90 days, were randomly selected and slaughtered. They were healthy and of a similar age. The full-length cDNA of the *NPFFR1* gene was cloned from the hypothalamus using Trizol™ Reagent (Invitrogen, USA) and SMART™ RACE cDNA Amplification Kit (Takara, China). The predicted protein structure was analysed using PSIPRED, Phyre2, and PSORT Ⅱ Prediction.

Analysis of expression profiles of *NPFFR1* gene

The mRNA expression levels of *NPFFR1* gene were tested from a total of 30 male rabbits aged 10, 30, 60, 90, 120, and 150 days (n=5 per age group) using a qPCR method.

Effects of ectogenous GnIH on the expression of NPFFR1 gene in the HPTa

Fourty-eight male rabbits, aged 80 days, were selected and randomly divided into four groups (n=12 per group). Four different doses of qGnIH (0, 0.5, 5, and 50 μg) were selected for the treatment. Rabbits received intraperitoneal injections of qGnIH daily for 10 days and slaughtered on the 11th day. Samples of the hypothalamus, pituitary gland, and testis were collected and stored in liquid nitrogen until RNA extraction. The mRNA expression levels of *NPFFR1* gene were then tested by a qPCR method.

Data statistical analyses

Data were analyzed using a one-way analysis of variance (ANOVA) in SPSS version 22.0.

RESULTS AND DISCUSSION

Cloning and Characterization of *NPFFR1*

The cloned cDNA sequence is also 3,712 base pairs in length and encodes 432 amino acids. It has been submitted to GenBank with the same entry number, OP791886. Protein structure prediction indicates that NPFFR1 is a seven-transmembrane protein, comprising an α-helix embedded in the plasma membrane, a connected random coil, and a β-fold.

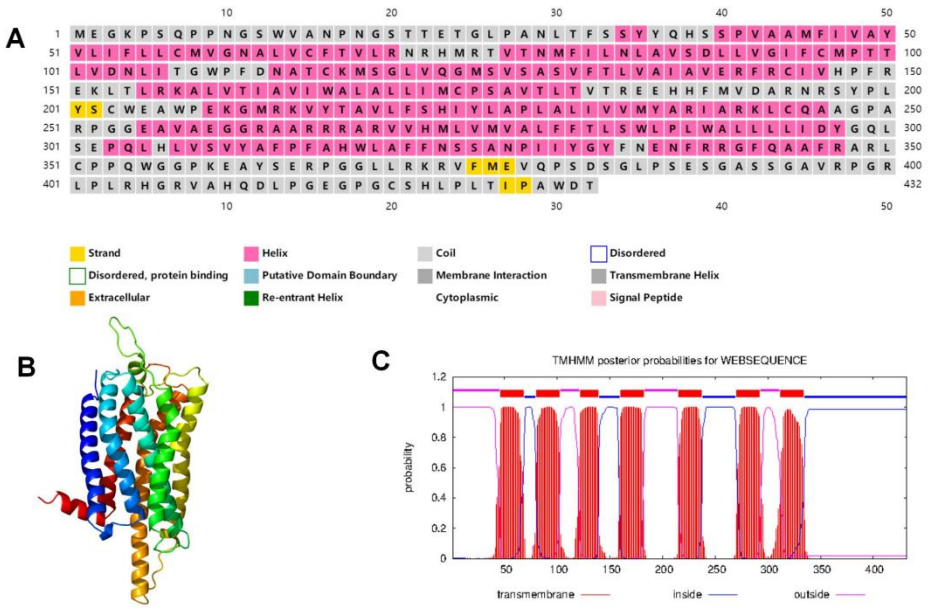

Figure 1: Structure analysis of rabbit NPFFR1 protein. A. Secondary structure prediction; B. Tertiary structure prediction; C. Transmembrane protein structure prediction.

Analysis of expression profiles of *NPFFR1* gene

As illustarted in Figure 2, the qPCR analysis revealed that *NPFFR1* was expressed in all tissues of the HPT axis, with the highest level of mRNA detected in the hypothalamus ($P<0.05$). From birth to 150 days of age, the hypothalamic mRNA level of the *NPFFR1* gene in 90-day-old rabbits was found to be significantly lower in comparison to other age groups ($P<0.05$). Additionally, the pituitary mRNA level of *NPFFR1* gene in rabbits aged 30 or 120 days were found to be lower than in rabbits aged 11 or 90 days ($P<0.05$). The testicular mRNA levels of *NPFFR1* gene in rabbits aged 30 days were also lower than in rabbits aged 11 or 90 days ($P<0.05$).

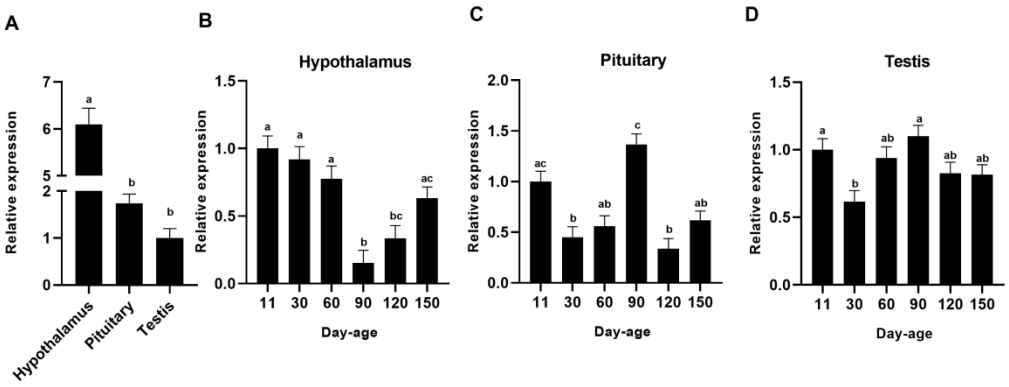

Figure 2: mRNA expression of *NPFFR1* gene in the rabbit hypothalamus-pituitary-testisaxis (HPTa). A. *NPFFR1* expression in the HPTa of 90-day-old male rabbits; B~D. *NPFFR1* expression in the HPTa of male rabbits with different ages. Reference gene: GAPDH. Different lowercase letters indicate significant difference ($P<0.05$).

Effects of chronic intraperitoneal infusion of qGnIH on the *NPFFR1* genes expressed in the HPTa

As shown in Figure 3, the mRNA levels of *NPFFR1* exhibited a significant increase ($P<0.05$) in the hypothalamus at doses of 5 and 50 μg of qGnIH. Furthermore, the mRNA level was higher at a dose of 50 μg of qGnIH in comparison to that at a dose of 5 μg. However, no significant difference was observed in *NPFFR1* mRNA expression levels in the four groups at the pituitary and in three groups of the testis ($P>0.05$).

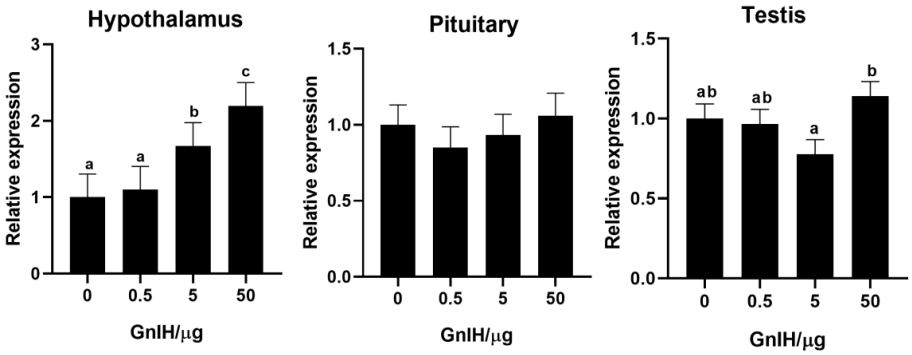

Figure 3: Effect of intraperitoneal injection of different doses of qGnIH on the expression of *NPFFR1* in the HPTa of 90-day-old male rabbits

CONCLUSIONS

In this study, it was discovered that the protein encoded by the *NPFFR1* gene is a 7-transmembrane protein embedded in the cytomembrane. The highest levels of mRNA expression of this gene was identified in the hypothalamus. Furthermore, the injection of 5 and 50 μg exogenous GnIH peptide in 80-day-old male rabbits resulted in a significant increase in the expression of *NPFFR1* in the hypothalamus. This finding provides indirect confirmation that rabbit NPFFR1 functions as the primary GnIH receptor and may play a pivotal role in regulating downstream reproductive hormones in rabbits.

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