EFFECT OF DIETARY SUPPLEMENTATION OF ORGANIC ACIDS AND ESSENTIAL OILS ON IMMUNE FUNCTION AND INTESTINAL CHARACTERISTICS OF EXPERIMENTALLY INFECTED RABBITS

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ABSTRACT

To analyze the effect of dietary organic acids and essential oils supplementation 120 young rabbits (28 d old) were divided into three groups and submitted to the following dietary treatments: Control diet; Zinc Bacitracin diet (Control diet+150 ppm Zinc Bacitracine); FormaXol diet (Control diet+0.4% FormaXol - mixture of microincapsulated formic and citric acids and essential oils). At 38 d of age, all rabbits were experimentally infected with *Escherichia coli O103* and *Clostridium perfringens type A* and clinically monitored for 3 weeks. To evaluate the native serum immunity (lysozyme, haemolytic complement assay and serum bactericidal activity) haematic samples were collected by cardiac puncture at regular intervals. Twenty one days after infection, all rabbits were euthanized and intestinal swabs were collected from different tracts of the small intestine, colon and caecum to investigate the aerobic and anaerobic bacteria. A jejune portion was excised to analyze the height of the *villi*. The haemolytic complement values showed a drastic reduction after infection in Control and Zinc Bacitracine diet animals; on the contrary, animals of FormaXol diet showed a steady trend in the first period (9 d) and a drastic reduction in the final period (11 d) like the others group. The better immune response in animals of FormaXol diet could be lied to higher values of serum bactericidal activity and lower values of lysozyme. The Control diet showed higher increase of lysozyme values after infection. All the studied immune parameters are in homeostatic equilibrium being subject to opposite phenomenon as production and consume. A similar percentage of *E. coli O103* and *C. perfringens type A* were found in the Zinc Bacitracin and FormaXol groups. The highest percentage of non-pathogen bacteria was found in the Zinc Bacitracin and FormaXol rabbits. The results obtained measuring the *villi* height confirmed the previous analyses with a better value when FormaXol diet was used (P<0.05). Considered together these results show that 0.4% integration of microincapsulated formic acid and citric acids and essential oils reduces the damage of both Gram^{-} and Gram^{+} pathogen bacteria permitting to obtain a better serum innate response in experimentally infected rabbits.

Key words: Rabbit, Innate immunity, Intestinal characteristics.

INTRODUCTION

Digestive disorders constitute the main health problem in weaned rabbits and antibiotics are widely used for prevention of infections and as a growth promoter, altering the gut flora, suppressing bacterial catabolism and reducing bacterial fermentation (Pinheiro *et al*., 2004). Zinc bacitracin is the antibiotic most used in rabbits to prevent these syndromes. Organic acids are widely used as feed additives for the control of pathogenic bacteria (Hansen *et al*., 2007) and their effects are probably related to factors than their direct antimicrobial action (Skrivanovà and Marounek, 2007). Despite the above mentioned use of organic acids as feed additives to control pathogenic bacteria, the data available to improve gut health, innate immunity and intestinal environment in weaned rabbits with enteric infections are insufficient. The immune system has a significant correlation with the health status of animals (Moscati *et al*., 2003). Anyway, the defence of mucous membrane against pathogen bacteria is ensured by many factors (Forthun-Lamothe *et al*., 2004). The mucosa of the small intestine has a major role in the digestion and absorption of nutrients and represents an important area of defence against antigenic aggressions in young rabbits (Gallois *et al*., 2005). The aim of this trial was to investigate the effect dietary supplementation of organic acids and essential oils on innate immune status and some intestinal characteristics of experimentally infected rabbits.

MATERIALS AND METHODS

Animals and experimental design

The trial was carried out at the experimental station of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche (Italy). One hundred and twenty rabbits, immediately after weaning (28 days old), were divided into three groups and submitted to the three different dietary treatments: Control diet (in Table 1 formulation and chemical composition); Zinc Bacitracin diet (Control diet + 150 ppm zinc bacitracin); FormaXol diet (Control diet + 0.4% FormaXol – mixture of microencapsulated formic and citric acids and essential oil). At 38 days of age all rabbits were experimentally infected with *Escherichia coli O103* and *Clostridium perfringens type A* and were clinically monitored for three weeks. To determine the innate immunity, blood samples from 5 animals/group were collected by cardiac puncture each week. Animals sampled were randomly selected and from the second blood sampling attention was made in order to avoid already sampled rabbits. Twenty one days after infection, all rabbits were euthanized and intestinal swabs were collected from different tracts of the small intestine, colon and *caecum* to determine the aerobic and anaerobic bacteria. Three-cm samples from the middle part of the jejunum were also taken to estimate mucosa morphology and stored in formalin solution 10% until processed in the Animal Production Department of Polytechnic University of Madrid (Spain).

Chemical and Histological Analyses

Lysozyme was measured according to the Osserman and Lawlor (1966) method in order to reveal a possible inflammatory status; haemolytic complement assay (HCA) was performed according to Barta and Barta (1993) to observe the animals ability to defend themselves against environmental pathogens; serum bactericidal activity (SBA) was determined according to Amadori *et al*. (1997) with the purpose of assessing the serum capacity to inhibit bacterial growth. Jejune specimens were first embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin. Mounted samples were magnified at 40× using an Olympus BX-40 light microscope and photographed for later analysis with a camera attached to a computer for image processing (P: Soft software, version 3.2 C4040Z, Olympus, Soft Imaging System GmbH, Germany). *Villus* heights were determined according to the procedure described by Hampson (1986). To screen aerobic bacteria, the intestinal swab samples were sown on 5% ram blood agar and Mac Conkey agar; the material was incubated for 24 hours at 37°C. To identify anaerobic bacteria, incubation was performed with the same methods, but in anaerobic conditions. Macroscopic and microscopic morphological aspects of the colony were evaluated together with their biochemical characteristics to determine their genus and species. *E. coli* typification was made by agglutination with specific hyperimmune anti-O103 serum (IZSUM). In the case of *C. perfringens*, the characterization of 5 sero-types (A, B, C, D, E) was performed by multiplex PCR with specific primers to identify genes encoding the principal toxins (α, β, ε, ι) and the new β_2 and CPE enterotoxins. The amplified products were examined by electrophoresis in 2% agarose gel and stained with ethidium bromide (0.25 μg/ml).

Statistical Analysis

Statistical analysis was carried out using a linear model (GLM/SAS, 1990) with the fixed effect of dietary treatment which took into account the repeated measures on the same animal.

Table 1: Diet ingredients and chemical composition of Control diet

Diet ingredients	Ingredients (%):
Alfalfa meal	39.0
Wheat bran	35.0
Barley	9.0
Soybean meal S.E. 48%	13.0
Salt	0.5
Limestone	1.0
Ca phosphate	0.5
Vitamin-mineral premix1	1.0
Vegetal fat	1.0
Chemical analysis	(g/kg fresh matter)
Moisture	108
Crude protein	177
Crude fiber	167
Ether extract	39
Ash	103
NDF	294
ADF	177
ADL	37
Starch[2]	153
Soluble sugars[2]	126
Digestible energy (MJ/kg f.m.)3	106

[1]Premix provided per kg of diet: vitamin A, 11,000 IU; vitamin D_3, 2,000 IU; vitamin B_1, 2.5 mg; vitamin B_2, 4 mg; vitamin B_6, 1.25 mg; vitamin B_{12}, 0.01 mg; vitamin E, 25 mg; biotin, 0.06 mg; vitamin K, 2.5 mg; niacin, 15mg; folic acid, 0.30 mg; D-pantothenic ac., 10 mg; choline, 600 mg; Mn, 60 mg; Cu, 3 mg; Fe, 50 mg; Zn, 15 mg; I, 0.5 mg; Co, 0.5 mg; lysine, 0.5 mg and methionine, 0.5 mg. [2]According to Castellini et al. (1997). [3]According to Maertens *et al*. (1988)

RESULTS AND DISCUSSION

The chemical composition of the diets was consistent with the standard requirements for rabbits (De Blas and Wiseman, 1998). Supplementations with zinc-bacitracin and mixture of microencapsulated formic and citric acid and essential oil affected the innate immunity parameters in the experimentally infected rabbits (Table 2). The HCA is a measure which allows us to assess the risk of infectious disease onset or already existing pathologies. After infection HCA showed a different timing of reduction, but at the end of study a drastic reduction of HCA values was reached in all animals. The SBA is a major parameter of innate immunity, modulates concentration of natural antibodies targeted to different environmental bacteria, mainly $Gram^{-}$. After infection this parameter showed a drastic decrease reaching very poor values at the end of the trial in all animals. Lysozyme is a strong antibacterial enzyme (against $Gram^{+}$) which has a synergic action with the immune humoral response and the serum complement system (Carrol and Martínez, 1979). In contrast to the other groups, the Control diet rabbits showed a higher values of lysozyme after one week from infection probably to ascribe to the lack of any supplementation against bacteria. The Zinc Bacitracin and FormaXol diets groups, although with different trends, showed the lowest values of this parameter, probably related to the higher percentage of non pathogen bacteria and to the better health status. In the last experimental period, those immune parameters the drastic values reduction could demonstrate an unspecific serum defence reduction age depending effect. Moreover, it should be considered that all the immune parameters studied are homeostatic being subject to opposite phenomenon as production and consume, consequently their restoration time should to be deeply investigated.

Such immune parameters are mainly affected by management practices and environment as reported in pigs (Moscati *et al*., 2003) and dairy cows (Amadori *et al*., 1997), where reductions of immune defences affect both their health status and performance. The Zinc Bacitracin and FormaXol diets groups harboured similar percentages of *E. coli O103* and *C. perfringens type A* (Figure 1). Rabbits fed Zinc Bacitracin and FormaXol diets harboured the highest percentage of non-pathogen bacteria.

Table 2: Effects of diets on serum immunity parameters

	Groups			
Days	Control	Zinc bacitracin	FormaXol	SED
HCA				
37	84.8	84.9	82.0	4.7
41	46.8a	40.4a	67.2b	3.4
44	77.0b	69.7a	66.2a	3.0
47	50.7a	56.6a	70.2b	6.9
53	25.3	19.1	25.9	2.4
58	21.7	16.7	20.4	2.0
SBA				
37	16.1	14.3	15.1	1.9
41	19.8a	19.3a	38.7b	4.8
44	17.0a	30.6b	30.3b	2.9
47	4.6a	7.6a	15.3b	2.4
53	0.0a	0.6b	0.0a	0.8
58	0.0a	1.0b	0.0a	0.2
Lysozyme				
37	30.0	28.4	30.4	2.6
41	31.3	29.2	36.9	3.3
44	44.5b	30.7a	25.5a	2.9
47	27.5	24.1	20.0	1.9
53	26.0	18.7	18.1	2.3
58	26.3	26.0	26.1	1.7

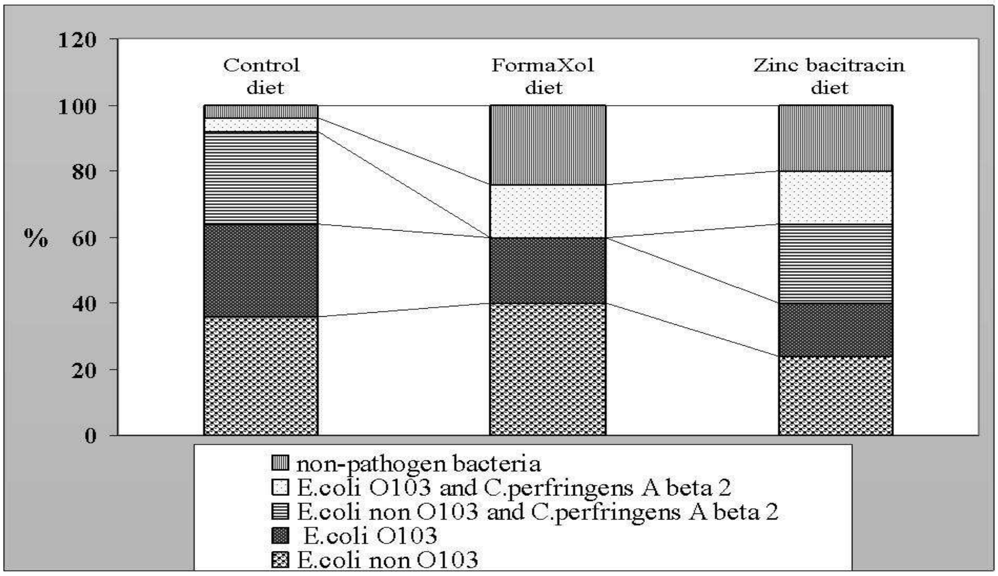

Figure 1: Enteric bacteria of the 3 groups of rabbits 20 days after experimental infection

Also mortality confirmed this trend (15 and 20 vs. 30%, in Zinc bacitracin, FormaXol and Control diet. Data not shown). Height of *villi* (Table 3) was greater in FormaXol diet than in the Control diet group. In all groups, the apical ridge of the *villi* was damaged, with many lymphocytes concentrated in their tip and in *lamina propria* showing that the effect of infections was not completely overcame by dietetic treatment. Indeed, in healthy animals of the same age, Rebollar *et al*. (2004; 2006) and Gutiérrez *et al.* (2002) showed lower lymphocyte concentration and less histological damages. Our histological results are consistent with others where similar pathologic agents of diarrheic processes were studied (Heczo *et al*. 2000, Cialet *et al*. 1998; Hara-Kudo *et al*. 1997).

Table 3: Height of *villi* of rabbits 21 days after experimetal infection (Means ± s.d.)

Diets	Control	Zinc bacitracin	FormaXol	P
Height of *villus* (µm)	269.43^{a} ± 9.91	304.51^{ab} ± 14.39	360.08^{b} ± 12.7	0.05

N Control = 28; N Zinc bacitracin = 34; N FormaXol = 32

CONCLUSIONS

This trial allowed us to analyze the effect of a mixture of microencapsulated formic and citric acids and essential oil source on experimentally infected young rabbits. The innate immunity parameter of this animals helped us to understand the trend of the humoral response during infection and intestinal swab samples and height of *villi* confirmed these results. Also the results about enteric bacteria demonstrates and confirms the efficacy of microencapsulated formic and citric acid and essential oils to reduce the damage caused by both Gram^{-} and Gram^{+} pathogen bacteria in experimentally infected rabbits.

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