EFFECTS OF DIETARY INCLUSION OF TOYOCERIN® (*BACILLUS CEREUS* VAR. *TOYOI*) ON PERFORMANCE, HEALTH AND FAECAL NITROGEN EXCRETION IN GROWING RABBITS

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ABSTRACT

The effect of dietary supplementation of *Bacillus cereus* var. *toyoi* on the performance, health, caecal fermentation and microbiota, and faecal N excretion of growing rabbits in an Epizootic Rabbit Enteropathy scenario was evaluated. Two diets were manufactured including 0 (Diet C) or 1000 (Diet T) ppm of Toyocerin® (1×10^9 spores of *Bacillus cereus* var. *toyoi* per g). For the growth trial, 100 weaned rabbits (50 per diet) were randomly housed in collective cages (5 per cage) and had free access to one of the experimental diets from 28 to 58 d of age. Mortality and morbidity were recorded daily and individual live weight and average feed intake of the cage were checked at 28, 42 and 58 d. To evaluate the effect of the product on caecal microbiota and fermentative parameters, 8 animals per diet were slaughtered at 58 d of age. Another group of 19 rabbits of 42 d (8 per diet) was used to determine faecal N excretion in a digestibility trial. The dietary inclusion of the probiotic did not affect rabbit growth rate, feed intake and feed conversion rate during the experimental period. However, the inclusion of Toyocerin® significantly reduced the mortality (–6%) and sanitary risk index (–12%), confirming the general reduction of digestive problems with diets supplemented with this probiotic in previous works. As what concerns caecal fermentation, only a reduction of branched chain fatty acids (isobutyric+isovaleric) frequently related with the presence of *Clostridium* sp was observed. A trend was observed where the presence of *Clostridium* decreased and lactic acid bacteria counts increased in animals fed with diet T. The addition of Toyocerin did not change the total cellulolytic bacteria counts. Finally, the dietary supplementation of this probiotic did not affect DM and crude protein digestibility coefficients. The reduction of faecal excretion of N observed (–14%; $P<0.05$) for the animals with diet T was probably due to the slightly lower DM intake observed with this diet (–8%; $P<0.10$) during the digestibility trial, the faecal excretion of N being corrected by a similar feed intake for both diets. In conclusion, dietary supplementation with 1000 ppm of Toyocerin® did not affect the growth and feed conversion rate, but significantly reduced the mortality and sanitary risk index of rabbits during the fattening period. This improvement in growing rabbits health could be related to positive changes in hindgut microflora.

Key words: Rabbit, *Bacillus toyoi*, Health, Nitrogen excretion, Caecal microbiology.

INTRODUCTION

The use of antibiotics as growth promoters was banned in the European Union at the beginning of 2006 and is now being considered in the USA. After an initial transition period, where this lack was compensated by increasing therapeutic usage of antibiotics on farms, potential alternatives must be found for this new reality (Falçao-E-Cunha *et al.*, 2007). Among these alternatives, probiotics are one of most studied and developed, with only two of them being registered for rabbits in EU.

One of them, *Bacillus cereus* var. *toyoi* (Toyocerin®), has been found to improve the performance and/or health of growing rabbits (Trocino *et al.*, 2005) and reproductive rabbit does (Nicodemus *et al.*, 2004). However, no study has been performed to evaluate the contribution of the dietary inclusion of this product either on caecal fermentation and microbiota of growing rabbits in cases of Epizootic Rabbit Enteropathy (ERE), an illness widely suffered on commercial rabbit farms in the last decade, or on the nutrient digestibility and faecal N excretion. The aim of the present work was to study the effect of dietary supplementation of *Bacillus cereus* var. *toyoi* on the performance, health, caecal fermentation and microbiota and faecal N excretion of growing rabbits in an ERE scenario.

MATERIALS AND METHODS

Diets

Starting from the same mix formula [containing (g/kg): barley grain, 80; sunflower meal, 50; lucerne, 650; beet pulp, 160; soya oil, 30; lysine, 3; DL-methionine, 2; threonine, 2; $NaHPO_4$, 15; NaCl, 3; mineral and vitamin premix, 5], two diets were manufactured including 0 or 1000 ppm of Toyocerin® (concentration: 1×10^9 spores of *Bacillus cereus* var. *toyoi* per g of Toyocerin®, provided by Rubinum SA). Both diets had an identical chemical composition, the CP (144 g/kg) and ADF (284 g/kg) contents being below the recommendations of de Blas and Mateos (1998) for growing rabbits. In order to minimise the effects of ERE in the trial, feeds were medicated providing 308, 200 and 50.4 ppm of neomycin, oxytetracycline and tiamulin, respectively.

Animals and experimental procedure

For the growth trial, a total of 100 weaned rabbits (50 per experimental diet), aged 28 d with average live weight of 0.56 kg (S.E.: 0.009 kg), were randomly housed in collective cages (5 animals per cage), sharing the kits from the same litter in both diets. All the rabbits were individually identified by tattoo and had free access to one of the experimental diets from 28 to 58 d of age. The experimental design followed the recommendations described by the European Group on Rabbit Nutrition (Fernández-Carmona *et al*., 2005). Mortality and morbidity of the animals were recorded daily. Individual live weight and average feed intake of the cages were controlled at 28, 42 and 58 d. A correction of feed intake data was done when one or more animals died during the week, registering the dates of deaths in order to determine the number of rations offered. For dead animals, it was considered that they did not eat during the day previous to death and the previous days of this week were considered as morbid days. In addition, feed intake and conversion rate values were also corrected considering the number of total morbid days in a cage by a weight procedure (Pascual *et al.*, 2007).

To evaluate the effect of the product on caecal microbiota and fermentative parameters, 8 animals per diet were slaughtered at 58 d of age and an initial group of 8 animals at weaning age (28 d). Animals were electrically stunned, then slaughtered. The caecum was separated and its content was collected into a flask to measure the pH (pH-meter GLP 21, CRISON, Alella, Spain). An aliquot of approximately 5 g was immediately cool-stored in sterile tubes and sent to the lab for microbiological analyses. Aliquots of about 1 g of caecal content were weighted into 5 ml centrifuge tubes and added with 3 ml of 2% sulphuric acid solution or 2 ml of 2% orto-phosphoric acid for analysing NH_3 and short chain fatty acids (SCFA), respectively. Samples for SCFA analysis were centrifuged at 10000×g for 10 min and the liquid phase was collected into Eppendorf vials of 0.5 ml. Finally, all samples were stored at –80 ºC until analysis. The remaining caecal content was stored at –20 ºC until dry matter (DM) analysis. Another group of 19 rabbits (8 per diet), aged 42 d with average live weight of 1.13 kg (S.E.: 0.02 kg), was used to determine faecal N excretion. The rabbits were housed in metabolic cages, and feed and water were offered *ad libitum* during the trial. After an adaptation period of 7 d, the faeces collection lasted 4 d (Perez *et al.*, 1995). Faeces were individually analysed for DM and crude protein (CP).

Chemical and microbiological analyses

Chemical analysis of diets and faeces were performed following the methods of the Association of Official Analytical Chemists (AOAC, 1991) for DM and CP and of Van Soest *et al.* (1991) for acid detergent fibre (ADF). DM and NH_3 concentration in caecal content was determined according to the AOAC (1991) procedures. For short chain fatty acids (SCFA) analysis, samples were previously filtered through a cellulose filter (0.45), and 250 µl of them were transferred to the injection vials. Two µl from each sample were injected into the gas chromatograph (FISONS 8000 series, Milan, Italy) equipped with an automatic injector AS800. The column used was a BD-FFAP 30m×0.25mm×0.25µm. The temperature of the injector and the detector were maintained at 220 and 225ºC, respectively. The concentration of each SCFA was determined using the 4-methyl valeric as the internal standard.

Microbiological samples were weighted and 10-fold serial dilutions in sterile Ringer ¼ were carried out in order to analyse the following counts: total aerobic mesophile bacteria, total Enterobacteriaceae, *Escherichia coli*, total anaerobic bacteria, *Clostridium perfringens*, *Bacteroides* spp., *Prevotella* spp., Lactic acid bacteria and total cellulolytic bacteria.

Statistical analysis

Data on feed intake, growth and conversion rate from the fattening trial were analysed using the MIXED procedure of SAS (1996), including the live weight at 28 d of age as a covariate. Percentage of non-morbid days in a cage was included as weight variable for the feed intake and conversion rate data analysis. Data concerning health status of the animals during the fattening period [mortality, morbidity and sanitary risk index (SRI), the latter calculated as the sum of both] and microbiological counts were analysed according to a chi-square test. Statistical analyses of the data from the digestibility trial were carried out according to a GLM procedure by SAS. Data were analysed as a completely randomised design with a model accounting for the fixed effect of the experimental diet.

RESULTS AND DISCUSSION

The dietary inclusion of this probiotic did not affect the growth rate, feed intake and feed conversion rate during the growing period (Table 1). Mean animal performance was lower than expected because of the summer season. These results are in disagreement with those obtained by Trocino *et al.* (2005), where a slight improvement in final live weight (+3%) and feed conversion (–4%) were observed when 200 ppm Toyocerin® was included in the diet of growing rabbits from 35 to 70 or 79 d, without any additional improvement for higher inclusion (1000 ppm). In this sense, Hattori *et al.* (1984) showed a linear increase of final body weight as dietary *Bacillus toyoi* concentration increased from 0 to 5×10^6 spores/g.

Table 1: Effect of dietary supplementation with 1000 ppm of Toyocerin® (Diet T) on rabbit growth performance and health (mean±SE.; n=100)

		Diet C	Diet T	P-value
Live weight at 28 d (g)		557±9	557±9	0.999
Growth rate (g/d):	28-42 d	41.1±0.9	40.8±0.9	0.840
	42-58 d	40.6±0.9	41.0±0.9	0.755
Feed intake (g DM[1]/d):	28-42 d	68.9±3.0	72.1±3.0	0.457
	42-58 d	115.6±3.7	117.7±3.6	0.697
Feed conversion:	28-42 d	1.66±0.07	1.71±0.07	0.589
	42-48 d	2.80±0.10	2.90±0.09	0.478
Mortality[2] (%):		8.0	2.0	<0.05
Morbidity[2] (%):		12.0	6.0	>0.05
Sanitary risk index[23] (%):		20.0	8.0	<0.05

[1]DM: dry matter. [2]Health parameters were analysed using a χ^2 test at P<0.05. [3]Sanitary Risk Index: mortality + morbidity

However, the inclusion of Toyocerin® significantly reduced the mortality (−6%) and sanitary risk index (−12%) of growing rabbits in the present experiment, confirming the general reduction of digestive problems with diets supplemented with this probiotic, whether in growing rabbits (Hattori *et al.*, 1984; Trocino *et al.*, 2005) or in lactating kits (Nicodemus *et al.*, 2004; Pinheiro *et al.*, 2007). Some works have revealed that some *Bacillus sp.* could reduce the colonisation possibilities of the pathogenic flora at gastrointestinal level (Cristofalo *et al.*, 1980; Hattori *et al.*, 1984). In fact, when the effect of dietary Toyocerin® supplementation on caecal fermentation is evaluated (Table 2), the only change observed in the caecal fermentation parameters controlled was a reduction of branched chain fatty acids (BCFA: isobutyric+isovaleric). When the protein is fermented in the hind gut of different species (pigs, rabbits, humans) large amounts of BCFA are produced, and some works have related their production with the presence of *Clostridium* sp (Barker, 1981).

Table 2: Effect of dietary supplementation with 1000 ppm of Toyocerin® (Diet T) on caecal fermentation parameters at 58 days of age (mean±SE.)

				P-value	
	weaning (28 d)	Diet C	Diet T	28 d vs.58 d	C vs. T
No. of animals	11	8	8		
pH	5.87±0.07[a]	6.06±0.08[ab]	6.11±0.08[ab]	0.045	0.649
DM (%)	21.06±0.62b	19.28±0.78[ab]	18.00±0.78[ab]	0.009	0.782
NH_3 (mmol/l)	7.34±0.73[b]	2.89±0.92[a]	3.02±0.86[a]	<0.001	0.916
Short chain fatty acids (%):					
Acetic	89.1±0.7	89.1±0.7	89.2±0.7	0.350	0.929
Propionic	5.25±0.38[b]	3.54±0.40[a]	3.21±0.40[a]	<0.001	0.561
Butyric	5.32±0.56[a]	6.87±0.59[ab]	7.24±0.59[b]	<0.001	0.655
Isobutyric	0.00±0.02[a]	0.07±0.02[b]	0.00±0.02[a]	0.322	0.032
Isovaleric	0.00±0.02	0.09±0.03	0.03±0.03	0.196	0.173
BCFA[1]	0.00±0.05[a]	0.16±0.05[b]	0.03±0.05[a]	0.236	0.036
n-Valeric	0.05±0.02[a]	0.18±0.02[b]	0.12±0.02[b]	0.006	0.077
n-Caproic	0.14±0.03	0.14±0.03	0.17±0.03	0.859	0.613
Heptanoic	0.00±0.02	0.01±0.02	0.04±0.02	0.248	0.220

[1]Branched Chain Fatty Acids (isobutyric+isovaleric)

Although there are no statistically significant differences among the animal batches evaluated with regard to the microbiological parameters investigated (Table 3), a clear trend is observed where the presence of *Clostridium* drops and the lactic acid bacteria counts increases in those animals fed with T feed, when comparing the obtained results with the control batch and the C batch. This fact determines a best health status of the animals, as reported by Chamorro *et al.* (2007) and Romero *et al.* (2007), when different diets were assayed that modified *Clostridium* counts and pointed out an improvement of the general status of the animals. The addition of Toyocerin® did not determine a variation in the counts of total cellulolytic bacteria which maintained constant levels in all batches, unlike the results obtained by Amber *et al.* (2004), which emphasised an increase of the counts of this group of micro-organisms after the supplementation of probiotics in the diet.

Table 3: Effect of dietary supplementation with 1000 ppm of Toyocerin® (Diet T) on caecal microbiology at 58 days of age (log CFU/g of caecal content)

	TAMBC	TE	EC	SA	TAN	CL	BC	PR	LAB	TCB
Weaning (28 d)	7.172	4.400	3,126	A	5,995	5,188	1,983	2,000	5,853	6,000
Diet C	5.672	4.774	4,460	A	5,531	4,882	2,000	2,000	5,740	6,000
Diet T	5.829	4.601	4,277	A	5,602	<1	2,000	2,000	6,176	6,000

No significant differences for any of the microbiological parameters studied were found (χ^2 at P<0.05). TAMBC: Total Aerobic Mesophile Bacteria Count; TE: Total Enterobacteriaceae; EC: Escherichia coli; TAN: Total anaerobic bacteria count; CL: Clostridium spp.; BC: Bacteroides spp.; Prevotella spp.; LAB: Lactic Acid Bacteria; TCB: Total cellulolytic bacteria; A: Absence

Finally, Table 4 shows the main results obtained during the digestibility trial, performed to determine the effect of Toyocerin® on faecal excretion of N. As has been previously observed in pigs receiving corn-soya meal based diets (Kornegay and Risley, 1996), the dietary supplementation with *Bacillus* probiotics did not affect DM and CP digestibility coefficients. In the present work, a reduction in faecal excretion of N was observed (−14%; P<0.05) for the animals with diet T, but this was probably due to the slightly lower DM intake observed with this diet (−8%; P<0.10), the faecal excretion of N being corrected by feed intake similar for both diets.

Table 4: Effect of dietary supplementation with 1000 ppm of Toyocerin® (Diet T) on the apparent digestibility of dry matter (DM) and crude protein (CP), and faecal N excretion (mean±SE.; n=18)

	Diet C	Diet T	P-value
Feed intake (g DM/d)	104.4±3.6	95.7±3.6	0.097
Growth rate (g/d):	45.0±2.2	43.3±2.2	0.601
cdDM (%)	54.7±0.7	54.9±0.7	0.860
cdCP (%)	69.1±1.0	68.8±1.0	0.809
Faecal excretion of N: mg N/d	818.5±41.8	704.1±36.8	0.049
mg N/g DM intake	7.51±0.27	7.36±0.23	0.981

CONCLUSIONS

In this study, dietary supplementation with 1000 ppm of Toyocerin® did not affect the growth and feed conversion rate, but significantly reduced the mortality and sanitary risk index of rabbits during the fattening period. This improvement in growing rabbits' health could be related to the changes observed in hindgut microflora − possible lactic bacteria promotion and absence of *Clostridium* sp.− when the animals received this probiotic, which must be confirmed in further works in the future.

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