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ENTEROCIN 4231 PRODUCED BY *ENTEROCOCCUS FAECIUM* CCM 4231 AND ITS USE IN RABBITS

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The aim of this experiment was to study the effect of Enterocin (Ent) 4231 produced by Enterococcus faecium. The group of 48 rabbits (Hy-Plus breed) was divided into experimental (EG) and control (CG) group. In EG Ent 4231 was applied for 21 days in a dose of 50 μL/animal/day into drinking water. The experiment lasted for 42 days. Samples were collected on day 0-1, day 7, day 21 (3 weeks of administration) and on day 42 (3 weeks after cessation of application) to measure the occurence of Eimeria sp. oocysts. Samples of caecal contents were collected on days 21 and 42 to determine bacterial counts. The phagocytic activity was examined on days 0-1, 21 and 42. On days 21 and 42 the reduction of Clostridium-like sp. was detected in EG (difference 1.53 $\log_{10}$ CFU/g; 1.57 $\log_{10}$ CFU/g) in comparison with CG. In EG on day 7 the decrease of E. coli (difference 1.30 $\log_{10}$ CFU/g) was also noted in comparison with CG. In EG prolonged immunostimulative effect ($p<0.001$) was observed on day 42 in comparison with CG. The oocysts of Eimeria sp. were reduced in EG comparing with CG at day 42. Enrichment of the diet for rabbits with Ent 4231 did not influence biochemical, zootechnical parameters and the values of caecal content volatile acids during the whole experiment.

Key words: Enterococcus faecium CCM4231, Enterocin 4231, rabbit

INTRODUCTION

Rabbit production for meat is an important livestock activity in many countries. Advantages of farming rabbits are a high fertility rate with rapid growth rate; high feed efficiency and early marketing age; high muscle-bone ratio. Rabbit meat has high protein content, low level of cholesterol and low total lipids. It is of high nutritive value in human diets (Hernández *et al.,* 2000). However, rabbits after weaning are susceptible to various infectious and diarrhoeal diseases caused by e.g. *Escherichia coli* or *Clostridium* sp. (Gidenne *et al*., 2005, 2008; Kristas *et al.,*

2008). Moreover, eimeriosis represents a permanent problem in rabbit breeding (Suvegová, 2004). Because the use of antibiotics as feed additives has been banned by EU and the use of coccidiostats will be forbiden in EU in the near future, new alternatives are searched.

Nowadays, natural substances such as probiotics, phytoaditives, enzymes, and organic acids have been used as alternatives with increasing frequency in veterinary medicine (Marounek *et al*., 2002; Lauková *et al.,* 2006a). The genus *Enterococcus* from division *Firmicutes* belongs to the group of microbiota known as lactic acid bacteria (LAB). Enterococci are Gram-positive, facultative anaerobic bacteria and some of them have the ability to produce bacteriocins-enterocins, small peptides with antimicrobial activity towards more or less related bacteria (Nes *et al.,* 1996; Franz *et al*., 2007). In animal nutrition and medicine *Enterococcus faecium* is the species most frequently utilized as a probiotic microorganism. *Enterococcus faecium* CCM4231, (isolated in Laboratory of Animal Microbiology of Institute of Animal Physiology, Slovak Academy of Sciences) was found as the first bacteriocin-producing strain of ruminal origin with probiotic character (Lauková *et al.,* 1993). Enterocin 4231 is an antimicrobial termo-stable peptid (3-10kD) with optimum pH for its production between 4.0 and 7.5 in log phase of growth with activity 3200 AU/mL (Lauková *et al*., 1997). Producer strain and its bacteriocin (*Enterocin) Ent* 4231 have already been successfully applied into many ecosystems to reduce spoilage flora (Lauková *et al.,* 1998a; 1998b; 1999a; 1999b; 2001a; 2001b). Even the producer strain *E. faecium* CCM 4231 was tested on rabbits (Szabóová *et al*., 2008). Based on our previous results, the aim of this study was to test the antimicrobial effect of *Ent* 4231 in a model experiment with rabbits and to check its effect on zootechnical, immunological and biochemical blood parameters and occurence of *Eimeria* spp. oocysts.

MATERIAL AND METHODS

Animals and experimental design

A group of 48 rabbits, 5-weeks old (male sex, Hy-Plus breed) were used in this experiment. All care and experimental procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Laboratory Animals which was accepted by Slovak Governmental Veterinary Institution. Rabbits were divided into 2 groups; the experimental group (EG) and control group (CG) of 24 animals in each. Rabbits were kept in standard cages, two animals per cage. All animals were fed a commercial diet for growing rabbits (ANPRO.FEED, VKZ Bučany, Slovakia) with free access to water. Partially purified *Ent* 4231 in dose 50 μL/animal/day (prepared according to Lauková *et al.,* 1997) was applied into drinking water for 21 days. The experiment lasted for 42 days.

Microbiota enumeration

Faecal samples were taken at the beginning of the experiment (at day 0-1), on day 7 (1 week of *Ent* 4231 administration), and day 21 (3 weeks of *Ent* addition) and 42 (3 weeks after cessation of *Ent*) to monitor the surviving, stability and effect

of *Ent* 4231 in rabbits. The samples were treated by a standard microbiological method using appropriate dilutions in Ringer solution (pH 7.0; Oxoid Ltd., Basingstoke, Hampshire, England). The appropriate dilutions were plated onto M-Enterococcus agar (Becton & Dickinson, Cockeysvile, USA) to determine the counts of enterococci, on Baird-Parker agar supplemented with egg yolk tellurite solution (Becton & Dickinson) to enumerate coagulase-positive staphylococci (CPS) including *Staphylococcus aureus*, on Mannitol Salt Agar (Difco Laboratories, Detroit, USA) for coagulase-negative staphylococci (CNS) and on *Clostridium difficile* agar enriched with selective supplement (SR0096E) and 7% (v/v) defibrinated horse blood (SR0050, Oxoid Ltd., Basingstoke, Hampshire, England) to detect *Clostridium*-like bacteria. MacConkey agar and Cetrimide agar (Becton & Dickinson) were used to count *E. coli* and *Pseudomonas* sp. The plates were incubated at 30°C and/or 37°C for 24-48 h depending on the bacterial species. Bacterial counts were expressed in colony forming units (log_{10} CFU) per gram. Three animals of each group were slaughtered on days 21, 42 and the caecal contents were sampled to count microbiota. They were also treated as described above.

Biochemical, zootechnical parameters, volatile fatty acids measurement in the caecum content and phagocytic activity testing

Biochemical parameters examined on days 0-1, 21, 42 were: serum levels of proteins and lipids (g/L), cholesterol (mmol/L), glucose (mmol/L), calcium (mmol/L), glutathione peroxidase (U/mL) using commercial Randox kits (England). Moreover, the phagocytic activity (PA) was monitored and expressed as percentage of bacteria ingested per phagocyte (100 neutrophils) during a limited period of incubation of particules suspension and phagocytes in serum (Hrubiško *et al.,* 1981). Volatile fatty acid values (lactic acid - g/100 g; acetic, propionic and butyric acids- mmol/l) were determined using gas chromatography from the samples of caecal content on days 21 and 42. The zootechnical parameters (weight gain and feed conversion) were evaluated daily.

Eimeria oocysts evaluation

Eimeria sp. oocysts were enumerated in the faecal samples microscopically on days 0-1, 7, 21 and 42 of the experiment and expressed as counts of oocysts per 1 g of faeces (OPG). The samples were stored at 4°C and then evaluated by the quantitative flotation technique - McMaster method (Ministry of Agriculture, Fisheries and Food, UK, 1986).

Statistical Analysis

The results were quoted as mean ± standard deviation (SD), statistical evaluation of the results was performed by the one-way ANOVA and the Tukey test.

RESULTS AND DISCUSSION

On day 21 as well as on day 42 the reduction of *Clostridium*-like sp. was detected in EG (day 21: 3.47 ± 0.72 CFU/g; difference 1.53 $\log_{10}$ CFU/g; day 42: 3.40 ± 1.53 CFU/g; difference 1.57 $\log_{10}$ CFU/g) comparing with CG (day 21: 5.00 ± 0.74 CFU/g; day 42: 4.97 ± 0.91 CFU/g). In EG at day 7 (1 week of *Ent* 4231 application) the decrease of *E. coli* (1.00 ± 0.00 CFU/g; difference 1.30 $\log_{10}$ CFU/g) was also noted in comparison with CG (2.30 ± 0.63 CFU/g). Lauková *et al.* (2006b) observed in rabbits significant difference of *E. coli* ($p<0.001$) even 2 weeks after cessation of application of probiotic and *Ent* A, P-producing strain *E. faecium* EK13 (CCM7419) comparing control and experimental groups. Previously, *Ent* 4231 has been experimentally added to the rumen fluid, cattle dung water and food products and showed the inhibition of listeriae and staphylococci (Lauková *et al.,* 1998a; 1998b; 1999a; 1999b; 2001a; 2001b). Moreover, the reduction of *Staphylococcus aureus* and *Clostridium*-like bacteria in the rabbits receiving *Ent* 2019 (produced by the rabbit isolate *E. faecium* EF2019-CCM7420) was reported by Simonová *et al.* (2006). In our study the counts of other faecal microbiota were not influenced by the additive used. In general, the bacterial counts in the caecum were lower (from 1 to 2 log cycle) than in the faeces. Moreover, no significant changes of bacterial counts in the caecum were noted.

In EG prolonged immuno-stimulative effect (25.2 ± 0.80%; $p<0.001$) was observed on day 42 in comparison with CG (20.4 ± 0.51%, Table 1). According to Lauková *et al.* (2010), administration of *Ent* M (produced by *E. faecium* AL41 strain) showed stimulating effect on PA in rabbits. Similar immuno-modulatory activities have been reported in the case of glucuronoxylan-related polymers, as well as polysaccharides isolated from various herbal plants (Capek *et al.,* 2003). Neutrophil polymorphonuclear leucocytes (granulocytes) are responsible for the non-specific immune response and in the first line share of phagocytosis intro-defence of the host to infectious and inflammatory actions (Escribano *et al*., 2005).

Table 1. Phagocytic activity during the experiment in rabbits

	Start of the experiment (Day 0-1)	Day 21	Day 42
EG - *Ent* 4231	21.60 ± 4.22	18.67 ± 0.42	25.20 ± 0.80[a]
CG	21.60 ± 4.22	22.50 ± 0.85	20.40 ± 0.51[b]

phagocytic activity expressed in % as mean ± standard deviation (n=5); [a,b]$p<0.001$; at day 21- 3 weeks after application *Ent* 4231, at day 42- the end of experiment - 3 weeks after cessation of application *Ent* 4231

The oocysts of *Eimeria* sp. were reduced in EG (*Ent*, 2250 OPG) comparing with CG (5640 OPG) on day 42. Up to now we cannot explain the exact mechanism of that reduction. However, similarly, Pogány *et al.* (2009) reported a reduction of *Eimeria* oocysts after application of *Ent* - PPB 2019 in rabbits. Enrichment of the diet for rabbits with *Ent* 4231 did not influence biochemical and

zootechnical parameters (weight gain – in EG 33.69 kg; in CG 36.39 kg and feed conversion – in EG 2.81; in CG 3.02) during the whole experiment. The values of caecal content volatile fatty acids were not significantly changed. *Ent* 4231 addition did not evoke oxidative stress (did not influence values of GPx). It has no negative effect on health status and growth performance of rabbits. By contrast to the enterococcal strains, enterococcal bacteriocins produced by heterologous hosts or added as cell-free partially purified preparations has been attractive for applications in feed as was already experimentally proved (Giraffa *et al*., 1994; Lauková *et al*., 1999a, 1999b, 2001; Foulquié-Moreno *et al*., 2003).

CONCLUSION

Ent 4231 showed the anti clostridial and anti-*E. coli* activity, immunomodulatory and anticoccidial effect in rabbits' intestinal ecosystem. The results have been promised for further use of *Enterocin* 4231- antimicrobial peptid in rabbits husbandry. Additional experiments are in processing.

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ENTEROCIN 4231 PROIZVEDEN OD *ENTEROCOCCUS FAECIUM* CCM 4231 I NJEGOVA PRIMENA KOD KUNIĆA

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SADRŽAJ

Cilj ovog eksperimenta je bio ispitivanje efekta enterocina (Ent) 4231 proizvedenog od *Enterococcus faecium*. Grupa od 48 kunića (Hy-Plus rase) je podeljena na eksperimentalnu (E) i kontrolnu (K) grupu. Eksperimentalnoj grupi je Ent 4231 aplikovan u vodi 21 dan u dozi od 50 μL/životinji/dan. Eksperiment je trajao 42 dana. Uzorci su sakupljani 1., 7. i 21. dana (tri nedelje primene leka) i 42. dana (tri nedelje posle prekida primene leka) da bi se određivalo prisustvo oocisti *Eimeria sp.* u crevnoj mikroflori. Uzorci sadržaja cekuma su sakupljeni 21. i 42. dana da bi se utvrdio i broj bakterija. Aktivnost fagocita je ispitivana 1., 21. i 42. dana. U toku ogleda, 21. i 42. dana, je zabeleženo smanjenje broja bakterija *Clostridium sp.* u oglednoj grupi (razlike 1,53 $\log_{10}$ CFU/g; 1,57 $\log_{10}$ CFU/g) u poređenju sa kontrolnom grupom. U E grupi je sedmog dana zabeležen pad broja *E. coli* (razlika 1,30 $\log_{10}$ CFU/g) u poređenju sa grupom K. U eksperimentalnoj grupi je 42. dana zapažen prolongirani imuno-stimulativni efekat ($p<0.001$) u poređenju sa kontrolnom grupom. Broj oocisti *Eimeria sp.* je 42. dana bio smanjen u oglednoj grupi u poređenju sa kontrolnom grupom. Dodavanje Ent 4231 u hranu nije uticalo na biohemijske, zootehničke parametre i koncentraciju isparljivih masnih kiselina u sadržaju cekuma tokom celog eksperimanta.