COMBINED EFFECT OF ENTEROCIN CCM4231 AND SAGE IN RABBITS

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ABSTRACT

Enterocin CCM4213 produced by bacteriocinogenic and probiotic *Enterococcus faecium* CCM4231 strain and sage plant extract were applied to rabbits separately and in the combination. A total of 96 rabbits (5-weeks old, male sex, HyPlus breed) were divided into three experimental (EG1, EG2, EG3) groups and one control group (CG). Animals of EG1 received *Salvia officinalis* plant extract (10 µl/animal/day into water), rabbits of EG2 received partially purified bacteriocin (PPB)–enterocin CCM4231 (50 µl/animal/day into water) produced by *E. faecium* CCM4231 strain, animals of EG3 received the combination of PPB CCM4231 (50 µl/animal/day into water) with sage plant extract (10 µl/animal/day into water) for 21 days. The experiment lasted 42 days. The animals were fed by the complete granulated mixture. Faeces were sampled at the start of the experiment, at day 7 (1 week of administration), at day 21 (3 weeks of administration) and 28 (1 week after cessation of application) and at day 42 (3 weeks after cessation of application) to count rabbit intestinal microflora as well as the occurence of *Eimeria* sp. oocysts. Samples of caecal contents (3 slaughtered animals of each group) were collected at days 21 and 42 to determine caecal bacterial counts. To check phagocytic activity, the animals were sampled at the start of the experiment, at days 21 and 42. The reduction of *E.coli* in EG2 (difference 1.3 $\log_{10}$ CFU/g) was noted at day 7 as well as the reduction of *Clostridium*-like sp. (difference 1.57 $\log_{10}$ CFU/g) at day 42 in comparison to CG. Reductive effect against *Pseudomonas*-like sp. ($P<0.001$) was also noted in EG3 at day 42 in comparison to CG. The other bacteria were not influenced by additives. The bacterial counts in the caecum were lower than in the faeces and no significant changes were noted. In the experimental groups prolonged immunostimulative effect ($P<0.001$) was observed in comparison to CG at day 42. The most pronounced anticoccidial effect was recorded in EG3 at day 7 compared to CG (in CG 65 OPG, in EG3 10 OPG). The oocysts shedding were also reduced in EG2 (20 OPG) compared to CG (65 OPG). At day 21, oocysts were also reduced in EG1 (30 OPG to CG - 1184OPG); in EG2 and EG3 were found only rarely. Results of our experiment suggest possible commercial utilization of natural additives tested.

Key words: Bacteriocin, Enterocin, Rabbit, Sage.

INTRODUCTION

Enteric diseases frequently occur in rabbits during weaning period. However, it is widely known, that the use of the antibiotic feed additives in the European Union was canceled in January 2006; consequently, new alternatives are searched, for example phytoadditives, probiotics as well as bacteriocins. In animal nutrition and medicine the species of the genus *Enterococcus* are the most frequently utilized as probiotic microorganisms. Enterococci represent Gram-positive, facultative anaerobic, lactic-acid bacteria (LAB) which belong to the normal intestinal flora in humans and animals, including birds, reptiles or insects (Devriese *et al.,* 1992). LAB are able to produce specific substances termed bacteriocins – ribosomally synthesized antimicrobial peptides with activity directed not only against closely related species (Nes *et al.,* 1996, Lauková *et al.* 1993). Enterococci produce

bacteriocins generally called enterocins. At present, a simplified classification scheme is proposed for enterocins, including four classes (Franz *et al.*, 2007). Class I enterocins (lantibiotic enterocins), Class II enterocins (small, nonlantibiotic peptides), Class III enterocins (cyclic enterocins) and Class IV enterocins (large proteins). Class II can be subdivided into three subclasses: II.1, enterocins of the pediocin family; II.2, enterocins synthesized without a leader peptide; and II.3, other linear, nonpediocin-type enterocins.

Sage (*Salvia officinalis*) is a member of the mint (*Labiatae*) family. From its Latin name, " *Salvia*" meaning to cure and "*officinalis*" meaning medicinal, it is clear that sage has a historical reputation for promotion of health and treatment of ailments. Sage is widely cultivated medicinal plant with a large scale of bioactive compounds. The ethanolic tinctures and decoctions from sage have long been known for their curing effect in various inflammations of oral cavity, digestive and intestinal tract. Several studies have shown sage to be one of the sources of some potent antioxidants, the antioxidant properties were found to be related to the presence of diterpenoids and phenolic acids (Cuvelier *et al.*, 1994; 1996).

Based on our previous experience we postulated that heat stable enterocin CCM4231 produced by conjugated linolenic acid-producing *Enterococcus faecium* CCM4231 strain (Lauková *et al.*, 1993; Marciňáková *et al.*, 2006) offers possibility to prevent bacterial disorders in rabbit breeding (clostridiosis, colibacillosis, staphylococcosis). The aim of this study was to compare the combined effect of enterocin CCM4231 and *Salvia officinalis* plant extract on selected parameters in rabbits (antimicrobial, immunological, biochemical, zootechnical, occurence of *Eimeria spp.* oocysts).

MATERIALS AND METHODS

Animals and experimental design

A total of ninety six rabbits (5-weeks old, male sex, HyPlus breed) were divided into 3 experimental (EG1, EG2, EG3) groups and one control group (CG). Rabbits were kept in standard cages, two animals per cage. The rabbits in the experimental groups as well as control group were fed with a complete granulated mixture (ANPRO.FEED,VKZ Bučany, Slovakia, Table 1). In the EG1 the animals received plant extract of *Salvia officinalis* (contained 24% of thujone, 18% of borneol, 15% of cineole; Calendula company, Nová Ľubovňa, Slovak Republic, dose 10 µl for animal/day into the known value of drinking water) for 21 days. Rabbits in EG2 received every day orally in drinking water partially purified enterocin CCM4231 (dose 50 µl/animal/day; prepared according to Lauková *et al.*, 1997) for 21 days. Sage plant extract (10 µl for animal/day) and enterocin CCM4231 (50 µl/animal/day) were administered in EG3 into drinking water for 21 days. The experiment lasted for 42 days. 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 Office.

Samples of faeces were taken at the beginning of the experiment, at day 7 (1 week of natural substances administration, NSA), 21 (3 weeks of NSA administration), 28 (1 week after cessation of NSA application) and 42 (3 weeks after cessation of NSA) to monitor the effect of enterocin CCM4231 and sage separately and together on the rabbit microflora. 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 Baird-Parker agar supplemented with egg yolk tellurite solution (Becton & Dickinson, Cockeysville, USA), Mannitol Salt Agar (Difco Laboratories, Detroit, USA) and *Clostridium difficile* agar with selective supplement (SR0096E) and 7% (v/v) defibrinated horse blood (SR0050, Oxoid Ltd., Basingstoke, Hampshire, England) were used to enumerate coagulase-positive staphylococci (CPS, including *Staphylococcus aureus*), coagulase-negative staphylococci (CNS) and *Clostridium*-like sp. 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 and were

expressed as $\log_{10}$ of colony forming units (CFU) per gram. Three animals in each group were slaughtered at days 21, 42 and caecal contents were collected to count bacteria. They were serially diluted in Ringer solution and plated on the media mentioned above.

Biochemical and immunological parameters were examined at the start of the experiment, at days 21, 42: serum levels of total proteins and lipids (g/l), cholesterol (mmol/l), glucose (mmol/l), calcium (mmol/l), glutathione peroxidase (U/ml) using commercial kit Randox (England). The phagocytic activity (PA) was monitored and expressed as percentage of the number of bacteria ingested per phagocyte during a limited period of incubation of particules suspension and phagocytes in serum (Hrubiško *et al*., 1981).

The zootechnical parameters (feed consumption, weight gain, feed conversion, mortality) were evaluated daily.

Eimeria sp. oocysts were enumerated in the faecal samples microscopically at days 1, 7, 21 and 42 of the experiment and expressed in 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, Fischeries and Food, UK, 1986).

Table 1: Ingredients and chemical composition and nutritive value of diets

Ingredients (%):		Composition (g/kg)	
Clover (grass) meal	27.00	Crude protein	197.00
Extracted sugar beet pulp	10.00	Crude fibre	166.50
Barley	15.00	Crude fat	39.00
Oats	13.00	Ash	80.00
Wheat bran	6.00	Organic matter	921.00
Soybean meal	7.50	Starch	178.00
Sunflower meal	14.00	Lysine	7.50
Monocalcium phosphate	0.60	Methionine+cysteine	6.50
Dicalcium carbonate	0.90	Cholinchloride	0.80
Salt	0.30	ME (MJ/kg)	10.0
Carob-breadfruit of *Ceratonia siliqua*	2.50		
DL-Methionine+wheat meal	0.10+0.10		
Mineral and vitamin premix[1]	3.00		

[1]Premix (per kg diet): vitamin A, 10,000 IU; vitamin D_3, 2,000 IU; vitamin E acetate, 30 mg; vitamin B_2, 5 mg; vitamin B_6, 2 mg; vitamin B_{12}, 8 mg; Ca, 9.25 g; P, 6.2 g; Na, 1.6 g; Mg, 1.0 g; k, 10.8 g; Fe, 327.5 mg; Mn, 80 mg; Zn, 0.7 mg

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. Microbial concentrations were formed in $\log_{10}$ CFU/g ± SD.

RESULTS AND DISCUSSION

The reduction of *E.coli* (difference 1.3 $\log_{10}$ CFU/g) was noted at day 7 in EG2 (1 week of the PPB CCM4231 application) as well as the reduction of *Clostridium*-like sp. (difference 1.57 $\log_{10}$ CFU/g) at day 42 (3 weeks of PPB CCM4231 cessation) in comparison to CG. Reductive effect against *Pseudomonas*-like sp. ($P<0.001$) was also noted in EG3 (the combination PPB CCM4231 with sage) at day 42 in comparison to the control group. Lauková *et al.* (2006) reported the antimicrobial activity of probiotic and enterocin A-producing strain *E. faecium* EK13 in rabbits by the significant reduction of *E.coli* counts 2 weeks after EK13 strain application ($P<0.001$). Enterocin CCM4231 has already been experimentally added to the rumen fluid, cattle dung water and food products and showed the inhibitory activity (Lauková *et al.,* 1998a; 1998b; 1999a; 1999b; 2001a; 2001b). The antimicrobial activities of *Salvia officinalis* can be attributed to the presence of high concentrations of thujone, cineole and camphor, three monoterpenes with well documented antibacterial and antifungal potential (Sur *et al.,* 1991; Sivropoulou *et al.,* 1997). Simonová *et al.* (2006) reported the reduction of

Staphylococcus aureus and *Clostridium*-like sp. in the experimental group where enterocin produced by a rabbit isolate *E. faecium* EF2019 strain was applied. The other bacteria were not influenced by additives. In general, the bacterial counts in caecum were lower than in faeces and no significant changes in bacterial counts were noted. In addition, Szabóová *et al.* (2006) showed the antimicrobial activity of probiotic and bacteriocin-producing *E. faecium* CCM4231 strain in rabbit ecosystem by the reduction of *E.coli* strains 7 days after CCM4231 administration (P<0.01) and *Clostridium*-like sp. (P<0.01) at 35 day of the experiment compared to the control group. The phagocytic activity (PA) was significantly increased in EG1 (sage; P<0.001; PA 27.7% ± 0.42) at day 21 in comparison to CG (22.5% ± 0.85). In the experimental groups (EG1; EG2; EG3) prolonged immunostimulative effect (P<0.001; Table 2) was observed in comparison to CG at day 42. In EG1 (sage) PA (30.5% ± 0.96) was monitored; in EG2 (PPB CCM4231; 25.2% ± 0.80); in EG3 (sage with PPB CCM4231; 28.6% ± 0.51) compared to CG (PA 20.4% ± 0.51). Similar immunomodulatory activities have been reported in the case of glucuronoxylan-related polymers as well as polysaccharides isolated from various herbal plants (Capek *et al.*, 2003).

In this experiment, the reduction of *Eimeria* sp. oocysts during bacteriocin CCM4231 and sage administration (in the combination or without combined application) was noted. The most expressive anticoccidial effect was recorded in EG3 at day 7 (PPB CCM4231 with sage) comparing to CG (in CG 65 OPG, in EG3 10 OPG). The oocysts were also reduced in EG2 (enterocin, 20 OPG) comparing to CG (65 OPG); enterocin was probably responsible for oocysts reduction. Moreover, Simonová *et al.* (2007) also reported reduction of *Eimeria* oocysts after application of *Enterococcus faecium* CCM7420 and its PPB EF2019 in rabbits. At day 21, oocysts were also reduced in EG1 (30 OPG to CG - 1184OPG); in EG2 and EG3 were found only rarely. The feeding of natural substances in rabbits did not influence biochemical and zootechnical parameters, as well as it has not negative effect on health status and growth performance of rabbits.

Table 2: Phagocytic activity during experiment in rabbits

	Phagocytic activity (%)		
	Start of experiment	21 days of experiment	42 days of experiment
EG1 – sage		27.7 ± 0.42*	30.5 ± 0.96*
EG2 – PPB CCM4231	21.6 ± 4.22	18.7 ± 0.42	25.2 ± 0.80*
EG3 – sage with PPB CCM4231		20.5 ± 0.43	28.6 ± 0.51*
CG – control group		22.5 ± 0.85*	20.4 ± 0.51*

Phagocytic activity expressed in % as mean ± standard deviation (n=5); *P<0.001; day 21- 3 weeks after application, day 42- the end of experiment - 3 weeks after cessation of application

CONCLUSIONS

It can be concluded from our results that enterocin CCM4231 and sage plant extract were able to reduce bacterial intestinal counts as well as the counts of *Eimeria* sp. oocysts in rabbits. The natural substances showed prolonged immunomodulatory activity.

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