Enterococcus faecium CCM7420, bacteriocin PPB CCM7420 and their effect in the digestive tract of rabbits

M. Pogány Simonová[1], A. Lauková[1], Ľ. Chrastinová[2], V. Strompfová[1], Š. Faix[1], Z. Vasilková[3], Ľ. Ondruška[2], R. Jurčík[2], J. Rafay[2]

[1]Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic
[2]Slovak Centre of Agricultural Research, Institute of Small Animal Production, Nitra, Slovak Republic
[3]Parasitological Institute of Slovak Academy of Sciences, Košice, Slovak Republic

ABSTRACT: The effect of *Enterococcus faecium* CCM7420, bacteriocin-producing strain with probiotic properties and its partially purified bacteriocin PPB CCM7420 on growth performance, microflora, *Eimeria* sp. oocysts, biochemical blood parameters and glutathione-peroxidase activity in rabbits was determined. An increase in the body weight of rabbits ($P < 0.01$) was achieved after *E. faecium* CCM7420 application. A non-significant reduction of faecal *E. coli* (including haemolytic *E. coli*), coagulase-positive staphylococci (CPS) and *Staphylococcus aureus* was found in rabbits administered the *E. faecium* CCM7420 strain and lower (non-significant) counts of *S. aureus* and *Clostridium*-like sp. were detected in PPB CCM7420 group, compared to the control. In the caecum, a significant reduction of CPS was noted in both experimental groups ($P < 0.001$ and $P < 0.05$ for EG1 and EG2, respectively). Biochemical blood parameters increased in both experimental groups ($P < 0.05$ and $P < 0.001$ for EG2 and EG1, respectively). In the CCM7420 group, the lowest activity of glutathione-peroxidase was measured ($P < 0.001$). After the application of PPB CCM7420 ($P < 0.05$; day 21), a reduction of *Eimeria* sp. oocysts was recorded.

Keywords: rabbits; microflora; coccidia; enterocins; blood parameters; probiotic

In rabbits, the majority of the digestive events occur in the caecum, i.e. at a place of a wide variety of microbial population and digestion. Most studies deal with the anaerobic – strictly and facultative – bacteria (Canganella et al., 1992); their presence, counts and activity depend on the age of rabbits. Digestive disorders evoked by harmful bacteria are a predominant cause of mortality in many rabbitries. Young animals are susceptible to the development of gastrointestinal diseases caused by feed-borne pathogens, particularly colibacillosis and clostridiosis (Peeters et al., 1995). Most strains of *Escherichia coli* are a harmless component of the intestinal flora, but some of them (especially enteropathogenic *E. coli*) are primary intestinal pathogens, e.g. serotype O103:K:H2 (Boullier et al., 2003). Furthermore, non-enteropathogenic *E. coli* and *Clostridium perfringens* were repeatedly isolated at high faecal counts from naturally infected animals by Epizootic Rabbit Enteropathy (ERE), first identified in France in 1997 (Lebas and Coudert, 1997; Marlier et al., 2006).

Supported by Slovak Academy of Sciences of the Slovak Republic (Project 2/0008/08).

To replace the antibiotics, new ways are used for prevention and control of these infections which can modulate the gut microflora. These are nonantibiotic compounds with bacteriostatic or bactericidal activity – probiotics, prebiotics, bacteriocins and organic acids (Lauková and Mareková, 2001; Marounek et al., 2003). The natural substances with antimicrobial activity mentioned above represent a well-tried tool for disease prevention and therapy in various animal species. There are some studies dealing with the use and beneficial effects of probiotics, prebiotics and fatty acids in rabbits, but many of them show preliminary and partially results, and do not focus on the specific (specific parameters) and/or diseases; others show zero or even negative effect (Maertens et al., 2006; Falcao-e-Cunha et al., 2007). The most commonly used bacteria as probiotics include the lactic acid bacteria – lactobacilli, enterococci, bifidobacteria and yeasts. Based on the fact that lactobacilli are rarely found in rabbit intestines (Yu and Tsen, 1993; Linaje et al., 2004), enterococci with probiotic properties could represent a new chance in the prevention and treatment of some rabbit diseases because of their antimicrobial ability due to enterocins (Nes and Holo, 2000; Lauková et al., 2006).

In general, probiotics create beneficial conditions for nutrient utilisation. The influence of probiotics on better intestinal digestion and more efficient energy utilisation in rabbits has been documented (Canzi et al., 2000; Amber et al., 2004; Simonová et al., 2008a); this fact provides both economic and health benefits. Blood parameters give the opportunity to detect conditions of stress; manipulation, environmental and diet changes in rabbit husbandries often induce physiological or pathological oxidative stress. To avoid these reactions, an antioxidant defence system has been developed in aerobic organisms for free radical elimination, where the glutathione peroxidase (GSH-Px) enzyme family is a prominent member. There are several factors affecting the GSH-Px activity; among them the nutritional factors are the most essential (Erdelyi et al., 2004). The influence of natural antimicrobials, mainly probiotics and plant extracts, on GSH-Px activity in rabbit blood samples was also recorded (Simonová et al., 2008a,b; Szabóová et al., 2008a,b). However, it is necessary to complete these data concerning the application of new natural substances, e.g. probiotics, enterocins, in rabbit husbandries.

In this study, *in vivo* antimicrobial activity of *Enterococcus faecium* CCM7420 strain (our isolate from rabbit) and its partially purified bacteriocin PPB CCM7420 was tested on bacteria and *Eimeria* sp. oocysts in rabbit faeces and caecum and their influence on blood parameters (total lipids and proteins, cholesterol, glucose, calcium and glutathione-peroxidase) was also studied; moreover, the probiotic influence on an antioxidant condition in the rabbit organism (measured by the activity of GSH-Px enzyme) is a pioneering study.

MATERIAL AND METHODS

Preparation of inoculum and partially purified bacteriocin (PPB) CCM7420

For an *in vivo* test, the rifampicin-resistant variant of *E. faecium* CCM7420 (previously EF2019, our isolate from rabbit faeces; Simonová et al., 2005; Simonová and Lauková, 2007; deposited in the Czech Collection of Microorganisms, number CCM7420) was used to differ it from the other enterococci; it was subsequently cultivated at 37°C using Todd-Hewitt agar (TH, Imuna, Šarišské Michaľany, Slovak Republic) enriched with rifampicin (100 µg/ml). Briefly, an 18 hours culture of strain CCM7420 was plated onto obliqued TH agar enriched with rifampicin (100 µg/ml) and cultivated at 37°C. Colonies with the highest concentration of rifampicin were checked; the inoculation and cultivation were continually repeated.

The PPB of *E. faecium* CCM7420 (a thermostable substance of proteinaceous character, with stable bacteriocin activity alread at pH 4.0; Simonová and Lauková, 2007) was prepared according to Mareková et al. (2003). Briefly, to remove cells, a 15 h culture (300 ml) of CCM7420 in MRS broth (Merck, Germany) was centrifuged for 30 min at 10 000 × g. After the supernatant adjusting to pH 5.5, precipitation with ammonium sulphate was carried out (w/v – 40% saturation); the mixture was stirred at 4°C for 4 h. After centrifugation at 10 000 × g for 30 min, the pellet was resuspended in 10 mmol/l of sodium phosphate buffer (pH 5.0). The activity of PPB was determined by the agar spot test (De Vuyst et al., 1996) on Brain Heart agar and Trypticase Soy agar (Becton and Dickinson, Cockeysville, USA) plates against the indicators; activity tested by the agar spot test was defined as the reciprocal of the highest twofold dilution demonstrating the complete inhibitory activity of the indicator strain and was expressed in arbitrary

units per millilitre of culture medium (AU/ml; Table 1).

Experiment schedule

A total of 96 rabbits (Hy-plus breed, female sex), weaned at 5 weeks of age, were divided into 2 experimental (EG1, EG2) groups and 2 control (CG1, CG2) groups of 24 rabbits each (also 5 weeks old at the beginning of the experiment, day 0). The experiment lasted for 42 days. Rabbits were housed in standard cages (0.61 m × 0.34 m × 0.33 m), 2 animals per cage. The rabbits were fed a commercial diet for growing rabbits (ANPRO.FEED, VKZ Bučany, Slovakia, Table 2; diet for the weaning period was fed until day 56 of life and diet for the fattening period was fed from day 56 to the end of the experiment) and had access to water *ad libitum*. The diet was granulated and supplemented with salinomycin (a coccidiostat) at 24 mg/kg in the group CG2 during 2 weeks, while rabbits in groups EG1, EG2 and CG1 were fed the unsupplemented diet. Every day, at the same time in the morning, the rabbits in EG1 were administered an overnight culture of *E. faecium* CCM7420 strain (1.0×10^9 cfu/ml, 500 µl/animal/day) for 21 days. Rabbits in EG2 consumed PPB CCM7420 (25 600 AU/ml activity; 50 µl/animal/day). Probiotic doses as well as PPB were added into drinking water. All care and experimental procedures involving animals followed the guidelines defined in the Guide to the Care and Use of Laboratory Animals (1996) and the trials were agreed by the Ethical Commission of the Institute of Animal Physiology in Košice, SR.

Body weight and feed consumption of rabbits were measured every week during the experi-

Table 1. Antimicrobial activity of partially purified bacteriocin produced by the *Enterococcus faecium* CCM7420 strain

Indicator organism	TS	Source	IS	Activity (AU/ml)
Ent. avium EA 5[a]	1	faeces of piglets	1	3 200
L. innocua LMG 13568[b]	1		1	3 200
L. monocytogenes CCM 4699[c]	1	from collection	1	3 200
Enterococcus sp.[a]	5	faeces of piglets	3	100
Enterococcus sp.[a]	11	dog's feed	11	100–3 200
Enterococcus sp.[a]	6	faeces of dogs	6	800–6 400
Enterococcus sp.[a]	22	faeces of rabbits	21	100–6 400
Enterococcus sp.[a]	4	rodents	3	200–1 600
Enterococcus sp.[a]	2	silage	2	400–1 600
Staphylococcus sp.[a]	8	dog's feed	2	100–200
Staphylococcus sp.[a]	9	silage	8	100–400
Staphylococcus sp.[a]	5	faeces of rabbits	1	200
Clostridium sp[a]	4	faeces of rabbits	1	200
Pseudomonas sp.[a]	5	faeces of rabbits	1	100
Pseudomonas sp.[a]	27	meat	1	100
Escherichia coli[a]	14	faeces of rabbits	1	100
Enterobacteriaceae[a]	19	meat	2	100–200
Salm. enterica ser. Enteritidis PT4[d]	1	from collection	0	0

TS – the number of tested indicator strains; IS – the number of inhibited indicator strains;
[a]our isolate; [b]supplied by Prof. De Vuyst (University of Brussels, Belgium); [c]Czech Collection of Microorganisms, Brno, Czech Republic; [d]Dr. Šišák, Institute of Veterinary Medicine, Brno, Czech Republic

Table 2. Ingredients and determined chemical composition of the commercial diet

Ingredients	Weaning period (%)	Fattening period (%)		(g/kg)[a]/(mg/kg)[b]
Extracted clover (grass) meal	27 + 14	23 + 3	dry matter[a]	884
Extracted sugar beet	10	14	crude protein[a]	173
Oats	10	13	crude fibre[a]	147
Wheat bran	5	16	fat[a]	34
Extracted sunflower meal	15	14	ash[a]	71
Extracted soybean meal	8	7.5	organic compounds[a]	813
Monocalcium phosphate	–	0.6	starch[a]	139
Dicalcium carbonate	–	0.9	calcium[a]	8
Salt	–	0.3	phosphorus[a]	5
Apple pomace	5	–	magnesium[a]	0.9
Carob-breadfruit of *Ceratonia siliqua*	3	2.5	sodium[a]	1.4
Methionine + wheat meal	–	0.1	potassium[a]	9.6
			iron[b]	289.6
			zinc[b]	0.6

weaning period – from weaning (day 35 of life) until day 56
fattening period – from day 56 until day 84 of life

ment. Mortality and morbidity were also recorded in groups daily, over the whole period of experiment. Faecal samples were collected on days 0 (at the start of the experiment; 12 composite samples from all rabbits – initial microbial background), 7 (1 week after probiotic and PPB application; 6 composite samples from each group), 21 (at the end of the probiotic and PPB application; 6 composite samples from each group) as well as on day 42 (at the end of the trial, 28 days after the strain cessation; 6 composite samples from each group) to monitor the counts of *E. faecium* CCM7420 and the effect of *E. faecium* CCM7420 and its bacteriocin PPB CCM7420 on the rabbit microflora as well as on *Eimeria* sp. oocysts. Blood was sampled on days 0, 21 and 42.

Three animals from each group were slaughtered on days 21 and 42 and samples from the caecum of each rabbit were collected to count caecal bacteria.

Bacterial enumeration

Bacteria from faecal and caecal samples were isolated by the standard microbiological method using the appropriate dilutions in Ringer solution (pH 7.0; Oxoid Ltd., Basingstoke, Hampshire, England). Dilutions were plated onto the following media: M-Enterococcus agar for enterococci, Todd-Hewitt agar (Imuna) enriched with rifampicin (100 µg/ml) for *E. faecium* CCM7420, Mannitol Salt agar to detect coagulase-negative staphylococci, Baird-Parker agar enriched with Egg Yolk Tellurite supplement (Becton and Dickinson, Cockeysville, USA) for coagulase-positive staphylococci and *S. aureus*, DeMann-Rogosa-Sharpe agar (Merck, Darmstadt, Germany) for lactic acid bacteria, *Clostridium difficile* agar (Oxoid Ltd.) for *Clostridium*-like species, Mac Conkey agar for *E. coli* and incubated at 37°C for 24–48 h. The bacterial counts were expressed in log 10 of colony forming units per gram ($\log_{10}$ CFU/g ± SEM). Concerning the caecal samples, the results shown are expressed as the average of three parallel replications of each sample.

Blood parameters

Blood samples (12 samples from all rabbits on day 0; 8 samples from each group on days 21, 42) for biochemical and haematological analyses were

collected from the marginal ear vein (*vena auricularis*) into dry non-heparinized glass tubes and blood serum was separated by centrifugation at 3 000 × g for 10 min. Serum was stored frozen in plastic vials until analyzed for total lipids and proteins (g/l ± SEM), glucose and calcium (mmol/l ± SEM). Biochemical parameters were determined by an enzymatic colorimetric procedure using commercial kits of Randox (United Kingdom).

The activity of blood glutathione-peroxidase (GSH-Px; U/ml) was determined by a RANSEL standard set from Randox (United Kingdom).

Eimeria sp. oocysts detection

Eimeria oocysts were microscopically examined in the faecal samples on days 0, 7, 21, and 42 of the experiment. 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). The intensity of the infections was estimated from counts of oocysts per 1 g of faeces – OPG.

Statistical analysis

Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test with the level of significance set at $P < 0.05$. The results are quoted as means ± SEM.

RESULTS

All animals were found in good health conditions throughout the experiment. On day 21, a non-significant increase in rabbit body weight was detect-

Table 3. Weight gain performances of growing rabbits (g)

Number of rabbits	EG1 *E. faecium* CCM7420 (n = 24)	EG2 PPB CCM7420 (n = 24)	CG1 control (n = 24)	CG2 diet with salinomycin (n = 24)
Day 0	977 ± 97	963 ± 101	964 ± 109	976 ± 89
Day 7	1 263 ± 138	1 242 ± 142	1 254 ± 166	1 313 ± 127
Day 14	1 584 ± 131	1 513 ± 170	1 523 ± 165	1 585 ± 153
Day 21	1 850 ± 152	1 788 ± 199	1 810 ± 208	1 864 ± 194
Day 28	2 177 ± 163^{a}	2 011 ± 185^{b}	2 071 ± 247^{ab}	2 186 ± 216^{ac}
Day 35	2 452 ± 148^{a}	2 255 ± 178^{b}	2 291 ± 213^{b}	2 420 ± 169^{c}
Day 42	2 622 ± 104^{a}	2 431 ± 142^{b}	2 465 ± 180^{bc}	2 549 ± 104^{ac}
Feed consumption (g)				
(35–56 days of age)	55 820	56 470	56 230	55 400
(56–84 days of age)	60 500	66 870	60 150	49 310
Feed conversion (kg/kg)				
(35–56 days of age)	2.71	2.68	2.81	2.67
(56–84 days of age)	4.67	3.87	4.02	5.03
During the experiment	3.47	3.22	3.32	3.44
Mortality (n)	3	1	1	5

the values are means SEM; different letters in columns = significant differences ($P < 0.05$)
35–56 days of age – the application of CCM7420 strain and PPB CCM7420
56–84 days of age – after the CCM7420 strain and PPB CCM7420 cessation

Table 4. Counts of bacteria (log_{10} CFU/g ± SD) in faeces of rabbits after the application of *E. faecium* CCM7420 and PPB CCM7420

	CG1 control	CG2 diet with salinomycin	EG1 *E. faecium* CCM7420	EG2 PPB CCM7420
Day 0				
E. coli	7.06 ± 0.62	7.06 ± 0.62	7.06 ± 0.62	7.06 ± 0.62
Enterococcus sp.	5.97 ± 0.91	5.97 ± 0.91	5.97 ± 0.91	5.97 ± 0.91
CNS	5.07 ± 1.40	5.07 ± 1.40	5.07 ± 1.40	5.07 ± 1.40
CPS	3.04 ± 0.60	3.04 ± 0.60	3.04 ± 0.60	3.04 ± 0.60
S. aureus	1.46 ± 0.46	1.46 ± 0.46	1.46 ± 0.46	1.46 ± 0.46
LAB	5.05 ± 0.83	5.05 ± 0.83	5.05 ± 0.83	5.05 ± 0.83
Clostridium-like	4.17 ± 1.32	4.17 ± 1.32	4.17 ± 1.32	4.17 ± 1.32
Day 7				
RMS	–	–	4.32 ± 0.34	–
Day 21				
E. coli	5.72 ± 0.87^{ab}	6.17 ± 0.63^{a}	5.63 ± 0.84^{ab}	4.31 ± 1.23^{b}
Enterococcus sp.	4.11 ± 0.08^{a}	4.01 ± 0.02^{a}	5.63 ± 1.21^{b}	6.16 ± 0.59^{b}
CNS	3.06 ± 0.39^{a}	3.00 ± 0.00^{a}	3.33 ± 0.38^{ab}	3.65 ± 0.28^{b}
CPS	3.08 ± 0.47^{a}	1.97 ± 0.22^{a}	3.45 ± 0.76^{ab}	2.38 ± 0.62^{ab}
S. aureus	1.53 ± 0.92	< 1.00	< 1.00	< 1.00
LAB	5.56 ± 0.97^{ab}	6.22 ± 0.26^{a}	5.62 ± 0.30^{ab}	4.60 ± 0.92^{b}
Clostridium-like	4.19 ± 0.41	4.00 ± 0.00	3.86 ± 0.79	4.49 ± 0.88
RMS	–	–	4.34 ± 0.75	–
Day 42				
E. coli	2.47 ± 0.76^{a}	3.89 ± 0.41^{b}	3.01 ± 0.79^{ab}	3.27 ± 0.63^{ab}
Enterococcus sp.	3.20 ± 0.31	4.33 ± 1.40	3.17 ± 0.38	2.90 ± 0.80
CNS	4.01 ± 0.54^{a}	5.18 ± 0.56^{b}	3.82 ± 0.80^{a}	3.76 ± 0.58^{a}
CPS	2.64 ± 0.81	2.96 ± 0.39	2.53 ± 0.75	3.33 ± 0.32
S. aureus	2.36 ± 1.10	1.77 ± 1.08	1.26 ± 0.24	2.02 ± 0.40
LAB	3.45 ± 0.19^{a}	4.96 ± 0.58^{b}	4.84 ± 0.74^{a}	4.45 ± 1.03^{ab}
Clostridium-like	2.80 ± 1.53	3.64 ± 1.09	3.06 ± 1.29	2.79 ± 1.64
RMS	–	–	3.30 ± 0.30	–

CNS – coagulase-negative staphylococci; CPS – coagulase-positive staphylococci; LAB – lactic acid bacteria; RMS – rifampicin marked strain;
the values are means SEM; different letters in columns = significant differences ($P < 0.05$)

ed in groups EG1 (*E. faecium* CCM7420) and CG2 (salinomycin supplemented diet) in comparison with CG1 (Table 3). A significant increase in body weight in EG1 ($P < 0.01$; $P < 0.001$) compared to CG1 and EG2 was found at the end of the experiment. Average daily gain was 9.5% higher in EG1 ($P < 0.001$) and by 4.8% in CG2 ($P < 0.01$) compared to the control group (CG1). The application

of PPB CCM7420 did not influence the growth of rabbits.

After the application of CCM7420 and its bacteriocin to rabbits (EG1, EG2; day 21), a non-significant reduction of faecal *E. coli*, coagulase-positive staphylococci (CPS), *Clostridium*-like sp. and *S. aureus* was observed compared to CG1 (Table 4). In both groups EG1 and EG2, the counts of enterococci significantly increased ($P < 0.05$; $P < 0.01$ vs. CG1; $P < 0.01$; $P < 0.001$ vs. CG2). The counts of CCM7420 were stable during the experiment (day 7, 21, 42; 4.32 ± 0.34, 4.34 ± 0.75, 3.30 ± 0.30 $\log_{10}$ CFU/g). In the group of rabbits receiving the diet with salinomycin (CG2), a decrease in CPS ($P < 0.001$) and *S. aureus* was detected compared to CG1.

S. aureus was absent in the caecal microflora. Enterococci occurred in low numbers at the end of the experiment (< 2.00 $\log_{10}$ CFU/g), they were even absent on day 21. Different ranges of caecal *E. coli* (from 1.49 ± 1.07 to 6.24 ± 2.07 $\log_{10}$ CFU/g) and *Clostridium*-like (from 1.67 ± 0.47 to 5.00 ± 0.00 $\log_{10}$ CFU/g) counts were observed in all groups. CNS, CPS and LAB in the caecum occurred in lower numbers compared to faecal bacteria, except CNS in groups EG1 and EG2 at the end of the treatment; the counts of those bacteria were higher in caecal samples. However, the counts of CPS were significantly reduced (EG1: $P < 0.01$, EG2: $P < 0.05$; day 21) compared to CG1.

A higher level of serum total proteins was observed in groups EG1, EG2, and CG2 ($P < 0.001$) in comparison with CG1 (Table 5); higher total lipid, glucose and calcium concentrations were also observed in these groups on day 21, compared to CG1. In group EG2, the highest lipid values were detected ($P < 0.05$ vs. CG1; $P < 0.001$ vs. CG2) at the end of the experiment. Increased values of serum glucose were measured in animals from groups EG1 ($P < 0.01$ vs. CG1) and CG2 ($P < 0.01$ vs. CG1) at the end of the experiment. After the probiotic

Table 5. Biochemical parameters in the blood serum of rabbits before and after *E. faecium* CCM7420 and PPB CCM7420 application

		EG1 *E. faecium* CCM7420	EG2 PPB CCM7420	CG1 control	CG2 diet with salinomycin
Total proteins – PL (g/l)		40–85 g/l			
	day 0	52.88 ± 5.13*			
	day 21	62.96 ± 6.78	61.64 ± 5.36	60.12 ± 7.84	58.74 ± 4.33
	day 42	66.79 ± 4.93^{a}	60.59 ± 4.51^{a}	49.15 ± 2.39^{b}	61.63 ± 2.22^{a}
Total lipids – PL (g/l)		1.5–9.5 g/l			
	day 0	7.25 ± 2.86*			
	day 21	8.73 ± 2.97	8.22 ± 3.88	6.61 ± 2.96	6.76 ± 1.43
	day 42	2.23 ± 0.51^{a}	5.87 ± 2.07^{b}	3.17 ± 0.74^{ac}	1.80 ± 1.18^{ac}
Glucose – PL (mmol/l)		3–8 mmol/l			
	day 0	4.91 ± 1.43*			
	day 21	6.65 ± 1.45	7.01 ± 1.53	5.95 ± 1.18	6.26 ± 1.13
	day 42	6.87 ± 0.74^{a}	5.32 ± 0.45^{b}	5.07 ± 1.01^{b}	6.97 ± 0.76^{a}
Calcium – PL (mmol/l)		2.4–3.4 mmol/l			
	day 0	2.62 ± 0.21*			
	day 21	2.06 ± 0.63	1.84 ± 0.53	1.45 ± 0.19	2.06 ± 0.43
	day 42	3.10 ± 0.38^{ab}	2.62 ± 0.28^{a}	3.33 ± 0.42^{b}	3.10 ± 0.29^{ab}
GSH-Px (U/ml)	day 0	20.27 ± 3.97*			
	day 21	23.24 ± 5.15^{a}	28.10 ± 4.21^{a}	38.93 ± 5.13^{b}	25.30 ± 4.34^{a}
	day 42	23.40 ± 2.90^{a}	23.40 ± 2.90^{a}	32.47 ± 6.97^{b}	32.96 ± 5.90^{b}

PL – physiological level; *12 samples from all rabbits on day 0
the values are means SEM; different letters in columns = significant differences ($P < 0.05$)

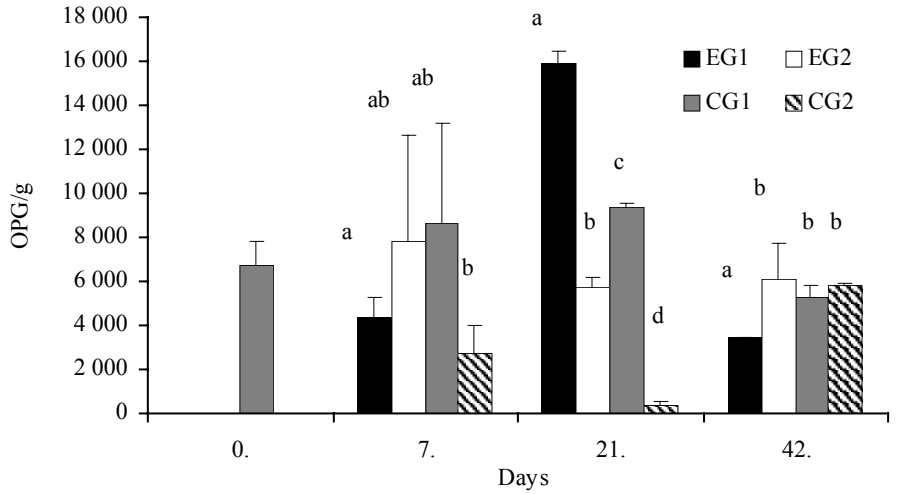

Figure 1. Occurrence of *Eimeria* sp. oocysts in rabbit faeces after the application of *E. faecium* CCM7420 and bacteriocin PPB CCM7420
EG1 – experimental group 1 (*E. faecium* CCM7420), EG2 – experimental group 2 (PPB CCM7420), CG1 – control group 1, CG2 – control group 2 (diet with salinomycin)
The values are means SEM; different letters in columns = significant differences ($P < 0.05$)

application, the lowest level of glutathione-peroxidase (GSH-Px) was detected in group EG1 animals ($P < 0.001$); similarly, in samples of groups EG2 and CG2, a decrease in levels of GSH-Px ($P < 0.05$) was found compared to control values (CG1). At the end of the experiment (day 42), in EG1 and EG2 ($P < 0.05$) the same effect was observed, except group CG2, where the highest GSH-Px concentration was measured.

At the beginning of the experiment, the counts of *Eimeria* sp. oocysts were 6.7×10^3 OPG in a composite sample from all groups. After 1 week of the application of *E. faecium* CCM7420 strain and its bacteriocin PPB CCM7420, oocysts showed a trend towards a numerical reduction in both experimental groups (EG1: 4.4×10^3 OPG; EG2: 7.9×10^3 OPG; Figure 1), compared to CG1(8.6×10^3 OPG), mainly in the group receiving the diet with coccidiostat (CG2: 2.8×10^3 OPG). A significant reduction of oocysts in EG1 was recorded on day 42 ($P < 0.01$ vs. EG2, CG2; $P < 0.05$ vs. CG1). In group EG2 with PPB CCM7420 addition, a decreasing (non-significant) tendency of oocyst occurrence was observed up to the end of the PPB application.

DISCUSSION

There is some information about the beneficial effect of different probiotics on the body weight of rabbits. E.g. Amber et al. (2004) reported an 9.6% increase in average daily gain in rabbits after their 6 week probiotic (Lact-A-Bac) treatment, Matusevičiuš et al. (2004) reported even an 18% increase in the body weight of rabbits fed the diet supplemented with probiotic YEASTURE for 60 days; under our conditions, the application of bacteriocinogenic *E. faecium* CCM7420 strain as well as the presence of salinomycin in feed (CG2) increased the final body weight of fattened rabbits by similar values (9.5%; 4.8%). The effect of CCM7420 strain on growth and body weight was also confirmed in our previous model experiment (Simonová et al., 2008a). However, the performance results with probiotic addition are significant only in a limited number of experiments (Falcao-e-Cunha et al., 2007).

The studies dealing with the isolation, characterisation of the enterococci from rabbit digestive tract and/or faeces and their further use as probiotics or bio-additives in rabbits are limited and/or they involve mainly basic information (Linaje et al., 2004). However, we focused on complete and spread the data concerning the rabbit probiotic research; that is, by testing the influence of the bacteriocinogenic *E. faecium* CCM7420 strain and its enterocin against undesirable flora in the caecum and faeces, *Eimeria* sp. oocysts and blood parameters *in vivo*. Several reports have described the positive and/or probiotic effect of lactobacilli on pathogenic flora in rabbits (Ogawa et al., 2001), contradictory to observations that lactobacilli are rarely found in the rabbit intestine or they are not found there at all (Yu and Tsen, 1993; Kovács et al., 2004; Linaje et al. 2004). Other reports presented the influence of Toyocerin (*Bacillus cereus* var. *toyoi*; Trocino et al., 2004) or compared the effect of lactobacilli and enterococci on growth performance, microbial and blood parameters (Simonová et al., 2008a). Linaje et al. (2004) concluded that coliforms were rarely isolated from both faeces and the intestinal content of rabbits, or their counts fell below 100 colonies

per g during growth (Kovács et al., 2004); oppositely, we determined a wide range of *E. coli* (even haemolytic *E. coli*; 0.67–7.06 $\log_{10}$ CFU/g) in faecal and caecal contents. On the other hand, no or minimum occurrence of enterococci and *S. aureus* in the caecum was detected, similarly like in Linaje et al. (2004). A decreased frequency of *E. coli* translocation and prevention of *E. coli* O157:H7 growth were studied after the administration of probiotics, prebiotics and C_2–C_{18} fatty acids (Tachikawa et al., 1998; Marounek et al., 2003; Pinherio et al., 2004; Lauková et al., 2006). Similarly to results previously reported by several authors, lower counts of *E. coli* were also detected due to the probiotic strain and PPB CCM7420 during our experiment compared to the control; even faecal *E. coli* occurred in higher amounts than in the caecum. Moreover, the reductive (although insignificant) influence of PPB CCM7420 addition on *E. coli* was more effective. The statistics are not often included in association with microbiological reduction. *Clostridium*-like bacteria in faeces and caecum of rabbits reached lower counts as was described by Canzi et al. (2000). However, these authors observed a significant increase in caecal enterococci in the group receiving *Lactobacillus delbruecki* subsp. *bulgaricus* and *Streptococcus bulgaricus*; in our experiment, enterococci in the caecum showed only a minimal tendency to increase.

The values of blood parameters were changed in the framework of the physiological level (Post Graduate Committee in Veterinary Science). Some authors presented a wide range of blood parameters, mainly of cholesterol (Canzi et al., 2000). Increased levels of the biochemical parameters in blood serum could be explained as a result of better resorption and utilization of these nutrients from the gastrointestinal tract by probiotic and PPB addition; a similar effect of probiotic bacteria was also described in our previous study (Simonová et al., 2008a). The higher level of total lipids in the group administered PPB CCM7420 at the end of the experiment shows a long-term effect of bacteriocin on lipid resorption from the intestine and/or their utilization. The concentration of GSH-Px is often monitored in meat and other organs – liver, kidney in rabbits (Dokoupilová et al., 2007); studies concerning the serum level of GSH-Px enzyme and health status of rabbits are scarce. The measurement of the low activity of GSH-Px in experimental groups as well as the evidently good health of rabbits indicated that no oxidative stress was evoked during the experiment. It is our conclusion because we did not find any studies dealing with probiotic influence and antioxidant condition in rabbits to compare it. From this point of view, our study is a pioneering search.

Coccidiosis in the rabbit may be responsible for important economic losses. Eimerian infections can cause a severe disease depending on the *Eimeria* species, especially in young animals and the oocysts are always present in rabbits and they cannot be completely eliminated even by the use of a coccidiostat; the highest incidence of oocysts was usually found around the weaning period (Zita et al., 2007). In this study, oocyst counts showed a trend towards a numerical reduction during the probiotic and mainly bacteriocin application, similarly to the results from our previous studies (Simonová et al., 2008a). The increased oocyst counts in EG1 on day 21 (at the end of probiotic application) are surprising despite of the fact that their excretion is irregular. The antimicrobial effect of bacteriocins is well-known, however, preliminary results concerning the anticoccidial influence of bacteriocins are new, presented in this study and in our previous studies (Simonová et al., 2008a; Strompfová et al., 2008).

The bacteriocinogenic *E. faecium* CCM7420 strain isolated from the intestinal ecosystem of rabbits could be selected for further utilization as a probiotic feed additive in rabbit husbandry because of its origin. Effectiveness of the bacteriocin-like substance produced by the *E. faecium* CCM7420 strain is also important, particularly as a new alternative way of prevention of diseases of bacterial and/or protozoic origin in rabbit breeding. Further experiments are processed.

Acknowledgements

We are grateful to Dr. Michaela Haviarová and Mrs. Margita Bodnárová for their skilful technical assistance. The part of the results concerning the antimicrobial spectrum of bacteriocin CCM7420 was already reported in Veterinary Research Communications (2007, Simonová and Lauková).

REFERENCES

Amber K.H., Yakout H.M., Hamed Rawya S. (2004): Effect of feedings diets containing Yucca extract or probiotic

on growth, digestibility, nitrogen balance and caecal microbial activity of growing New Zealand White rabbits. In: Proceedings 8th World Rabbit Congress. Puebla, Mexico, 737–745.

Boullier S., Nougayrčde J.P., Marchčs O., Tasca Ch., Boury M., Oswald E., De Rycke J., Milon A. (2003): Genetically engineered enteropathogenic *Escherichia coli* strain elicits a specific immune response and protects against a virulent challenge. Microbes and Infections, 5, 857–867.

Canganella F., Zirletta G., Gualterio L., Massa S., Trovatelli L.D. (1992): Anaerobic facultative bacteria isolated from the gut of rabbits fed different diets. Zentralblatt für Mikrobiologie, 147, 537–540.

Canzi E., Zanchi R., Camaschella P., Cresci A., Greppi G.F., Orpianesi C., Serrantoni M., Ferrari A. (2000): Modulation by lactic-acid bacteria of the intestinal ecosystem and plasma cholesterol in rabbits fed a casein diet. Nutrition Research, 20, 1329–1340.

De Vuyst L., Callevart R., Crabbe K. (1996): Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology, 142, 817–827.

Dokoupilová A., Marounek M., Skřivanová V., Březina P. (2007): Selenium content in tissues and meat quality in rabbits fed selenium yeast. Czech Journal of Animal Science, 52, 165–169.

Falcăo-e-Cunha L., Castro-Solla L., Maertens L., Marounek M., Pinheiro V., Freire J., Mourão J.L. (2007): Alternatives to antibiotic growth promoters in rabbit feeding: a review. World Rabbit Science, 15, 127–140.

Guide For The Care And Use Of Laboratory Animals (1996): Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. National Academy Press, Washington, USA.

Kovács M., Szendrő Zs., Csutorás I., Bóta B., Bencsné K.Z., Orova Z., Radnai I., Biróné N.E., Horn P. (2004): Development of the caecal microflora of newborn rabbits during the first ten days after birth. In: Proc. 8th World Rabbit Congress. Puebla, Mexico, 1091–1096.

Lauková A., Mareková M. (2001): Production of bacteriocins by different enterococcal isolates. Folia Microbiologica., 46, 49–50.

Lauková A., Strompfová V., Skřivanová V., Volek Z., Jindřichová E., Marounek M. (2006): Bacteriocin producing strain of *Enterococcus faecium* EK13 with probiotic character and its application in the digestive tract of rabbits. Biologia, Bratislava, SR, 61, 779–782.

Lebas F., Coudert P. (1997): Entérocolite épizootique et alimentation du lapin. In: Proceedings 7émes Journées de la Recherche Cunicole. Lyon, France, 9–12.

Linaje R., Coloma M.D., Pérez-Martínez G., Zúñiga M. (2004): Characterization of faecal enterococci from rabbits for the selection of probiotic strains. Journal of Applied Microbiology, 96, 761–771.

Maertens L., Falcao-e-Cuńha L., Marounek M. (2006): Feed additives to reduce the use of antibiotics. In: Maertens L., Coudert P. (eds.): Recent Advances in Rabbit Science. ILVO, Melle, Belgium (supported by COST), 259–265.

Mareková M., Lauková A., DeVuyst L., Skaugen M., Nes I.F. (2003): Partial characterization of bacteriocins produced by environmental strain *Enterococcus faecium* EK13. Journal of Applied Microbiology, 94, 523–530.

Marlier D., Dewrée R., Lassence C., Licois D., Mainil J., Coudert P., Meulemans L., Ducatelle R., Vindevogel H. (2006): Infectious agents associated with epizootic rabbit enteropathy: Isolation and attempts to reproduce the syndrome. Veterinary Journal, 172, 493–500.

Marounek M., Skřivanová E., Rada V. (2003): Susceptibility of *Escherichia coli* to C_2–C_{18} fatty acids. Folia Microbiologica, 48, 731–735.

Matusevičiuš P., Šliaudaryte R., Antoszkiewicz Z., Bednarska A. (2004): A natural way to improve productivity of rabbits using probiotic *YEASTURE*. Veterinarija Ir Zootechnica, 26, 61–64.

Ministry of Agriculture, Fisheries and Food (1986): Manual of Veterinary Parasitological Techniques, Reference Book. Her Majesty's Stationary Office, London. UK.

Nes I.F., Holo H. (2000): Class II antimicrobial peptides from lactic acid bacteria. Biopolymers, 55, 50–61.

Ogawa M., Shimizu K., Nomoto K., Takahashi M., Watanuki M., Tanaka R., Tanaka T., Hamabata T., Yamasaki S., Takeda Y. (2001): Protective effect of *Lactobacillus casei* strain on shiga toxin-producing *Escherichia coli* O157:H7 infection in infant rabbits. Infection and Immunity, 69, 1101–1108.

Peeters J.E., Maertens L., Orsenigo R., Colin M. (1995): Influence of dietary beet pulp on caecal VFA, experimental colibacillosis and iota-enterotoxemia in rabbits. Animal Feed Science and Technology, 51, 123–139.

Pinheiro V., Alves A., Mourão J.L., Guedes C.M., Pinto L., Spring P., Kocher A. (2004): Effect of mannan oligosaccharides on the ileal morphometry and caecal fermentation of growing rabbits. In: Proceedings 8th World Rabbit Congress, Puebla, Mexico, 936–941.

Post Graduate Committee In Veterinary Science (1990): Rabbits and rodents – laboratory animal science. In: Proceedings Post Grad. Comm. Vet. Sci.. University of Sydney, Australia, No. 142.

Simonová M., Lauková A. (2007): Bacteriocin activity of enterococci from rabbits. Veterinary Research Communications, 31, 143–152.

Simonová M., Lauková A., Štyriak I. (2005): Enterococci from rabbits – potential feed additives. Czech Journal of Animal Sviences, 50, 416–421.

Simonová M., Marciňáková M., Strompfová V., Čobanová K., Gancarčíková S., Vasilková Z., Lauková A. (2008a): Effect of probiotics *Lactobacillus rhamnosus* GG and new isolate *Enterococcus faecium* EF2019 (CCM 7420) on growth, blood parameters, microbiota and coccidia oocysts excretion in rabbits. International Journal Probiotics and Prebiotics, 3, 7–14.

Simonová M., Szabóová R., Chrastinová Ľ., Lauková A., Haviarová M., Strompfová V., Plachá I., Faix Š., Vasilková Z., Mojto J., Rafay J. (2008b): The use of a ginseng extract in rabbits. In: Proceedings 9th World Rabbit Congress. Verona, Italy, 809–814.

Strompfová V., Simonová M., Marciňáková M., Vasilková Z., Lauková A. (2009): Effect of probiotic and bacteriocin-producing lactic acid bacteria towards *Eimeria* spp. Bilógia Bratislava (submitted).

Szabóová R., Chrastinová Ľ., Strompfová V., Simonová M., Vasilková Z., Lauková A., Plachá I., Čobanová K., Chrenková M., Mojto J., Jurčík R. (2008a): Combined effect of bacteriocin-producing *Enterococcus faecium* CCM4231 strain and sage in rabbits. In: Proceedings 9th World Rabbit Congress. Verona, Italy, 821–826.

Szabóová R., Chrastinová Ľ., Strompfová V., Simonová M., Vasilková Z., Lauková A., Čobanová K., Plachá I., Chrenková M., Mojto J., Ondruška Ľ. (2008b): Combined effect of enterocin CCM4231 and sage in rabbits. In: Proceedings 9th World Rabbit Congress. Verona, Italy, 815–820.

Tachikawa T., Seo G., Nakazawa M., Sueyoshi M., OhishiT., Joh K. (1998): Estimation of probiotics by infection model of infant rabbit with enterohemorrhagic *Escherichia coli* O157: H7. Journal of Japanese Association Infectious Diseases, 72, 1300–1305.

Yu B., Tsen H. (1993): *Lactobacillus* cells in the rabbit digestive-tract and the factors affecting their distribution. Journal of Applied Bacteriology, 75, 269–275.

Zita L., Tůmová E., Skřivanová V., Ledvinka Z. (2007): The effect of weaning age on performance and nutrient digestibility of broiler rabbits. Czech Journal of Animal Science, 52, 341–347.

Received: 2008–11–26
Accepted after corrections: 2009–03–24

Corresponding Author

MVDr. Monika Pogány Simonová, PhD., Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4–6, 040 01 Košice, Slovak Republic
Tel. +421 55 6336 251, fax: +421 55 7287 842, e-mail: simonova@saske.sk