World Rabbit Sci. 2020, 28: 169-179
doi:10.4995/wrs.2020.11189
WRSA, UPV, 2003

BENEFICIAL EFFECTS OF *ENTEROCOCCUS FAECIUM* EF9A ADMINISTRATION IN RABBIT DIET

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Abstract: Forty-eight rabbits aged five weeks (Hycole breed, both sexes) were divided into experimental (EG) and control (CG) groups, 24 animals in each, and fed a commercial diet with access to water *ad libitum*. Rabbits in EG had *Enterococcus faecium* EF9a probiotic strain added to their drinking water (1.0×10^9 colony forming units/mL 500 µL/d/animal) for 28 d (between 35 and 63 d). The experiment lasted for 42 d. The animals remained in good health condition throughout the experiment, and no morbidity and mortality was noted. There was a higher live weight at 63 d of age (+34 g; $P<0.0001$), final live weight at 77 d of age (+158 g; $P=0.0483$), and average daily weight gain between 63 and 77 d of age in the EG group rabbits than in CG group rabbits (+8 g/d; $P<0.0001$). No significant changes in caecal lactic acid and total volatile fatty acid concentrations, jejunal morphological parameters and phagocytic activity were noted during the treatment. The tested serum parameters were within the range of the reference values. EF9a strain sufficiently established itself in the rabbit's gastrointestinal tract. At 63 d of age, a significant decrease in coliforms ($P<0.05$), coagulase-positive staphylococci ($P<0.01$), pseudomonads ($P<0.01$) and coagulase-negative staphylococci (CoNS, $P<0.001$) was noted in the faeces of the EG group rabbits compared to the CG rabbits. Antimicrobial effects of EF9a strain in the caecum against coliforms ($P<0.001$), CoNS ($P=0.0002$) and pseudomonads ($P=0.0603$) and in the appendix (coliforms, $P<0.05$) were detected.

Key Words: blood parameters, microbiota, morphometry, phagocytic activity, probiotic, rabbit.

INTRODUCTION

Rabbit husbandry has great potential in many countries because of the animals´ small body size, short generation interval, rapid growth rate, high productive capacity and healthy, easily-digestible meat (Dalle Zotte and Szendrő, 2011; Cullere and Dalle Zotte, 2018). In rabbit breeding, the most critical period is the weaning, when rabbits are most susceptible to various gastrointestinal infections and spoilage agents, and there may be negative effects on feed consumption, growth performance and health status of animals in this period. To reduce economic losses and to stabilise and improve the health status, gastrointestinal tract development and growth performance, antibiotics and growth promoters have been widely used for several decades. Although these synthetic drugs show good results based on production indicators, on the other hand, they also cause the spreading of some specific kinds of resistance to the environment, whereby the quality of meat, food and human health could be threatened (Salyers *et al.,* 2004). For this reason, antimicrobial growth promoters have been banned by the European Union since 2006, and antibiotics have been replaced with new, naturally based supplements: probiotics, prebiotics, synbiotics, bacteriocins, organic acids, herbs and their extracts, which are well-tried tools for disease prevention and therapy

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https://doi.org/10.4995/wrs.2020.11189

in various animal species. There are several studies/reviews dealing with the use and beneficial effects of bioactive compounds and/or their combinations in rabbits (Falcão-e-Cunha *et al.,* 2007; Dalle Zotte *et al.,* 2016; Kalma *et al.,* 2016; Bhatt *et al.,* 2017). The positive effects of these additives on the control of pathogens and parasites, nutrient utilisation, metabolism changes, oxidative stress, immunomodulation and growth performance have also been demonstrated in several studies (Pogány Simonová *et al.,* 2009; Bhatt *et al.,* 2017; Cunha *et al.,* 2017). Probiotics, e.g. lactobacilli, enterococci, bifidobacteria and yeasts, are live microorganisms which create beneficial conditions for nutrient utilisation and intestinal flora balance and show positive effects on the growth performance, health status and immune system of animals and their products (e.g. meat, eggs; Markowiak and Śliżewska, 2018). Based on the fact that lactobacilli are rarely found in the rabbit intestine (Linaje *et al.,* 2004; Yu and Tsen, 1993), enterococci with probiotic properties could represent a new opportunity for prevention and treatment of some rabbit diseases because of their antimicrobial ability due to enterocins (Nes *et al.,* 1996; Pogány Simonová *et al.,* 2013a; Lauková *et al.,* 2014). Effects of probiotics on growth performance, microbial composition and nutrient content in the gastrointestinal tract, blood and immune parameters in rabbits have already been documented (Simonová *et al.,* 2008; Kalma *et al.,* 2016; Cunha *et al.,* 2017). However, further research is necessary to complete the already-known facts concerning the application of new natural substances in rabbits, which will help us better understand the relationships between physiological, biochemical, immune and microbiological changes.

Enterococcus faecium EF9a is a bacteriocin-producing strain with probiotic properties isolated from the faeces of the Hungarian Pannon White rabbit breed (Lauková *et al.,* 2019). Because of our previous application of beneficial *E. faecium* strains (Szabóová *et al.,* 2012; Pogány Simonová *et al.,* 2013b; Lauková *et al.,* 2016) using different breeds in Slovakia, we decided to focus on determining the dietary effects of autochthonous *E. faecium* EF9a strain on selected parameters. In this study, the *in vivo* influence of the EF9a strain was tested on the growth performance, bacterial counts in rabbit faeces, caecum and appendix contents, caecal volatile fatty acid content, blood biochemical parameters and phagocytic activity, and the morphometry of the intestinal villi was also documented.

MATERIAL AND METHODS

The experiment was performed in cooperation with our colleagues in Nitra (National Agricultural and Food Centre - NAFC; Slovakia). All care and experimental procedures involving animals followed the guideline stated in the Guide for the Care and Use of Laboratory Animals and the trials were accepted by the Ethical Commission (permission code: SK CH 17016 and SK U18016) at the Institute of Animal Physiology in Košice and by the Slovak Veterinary and Food Administration.

Animals and husbandry

Forty-eight rabbits, aged 5 wk (after weaning, Hycole breed, both sexes) were used. The rabbits were randomly divided into the experimental (EG) and control (CG) groups; 24 rabbits in each group. They were housed in standard cages (0.61×0.34×0.33 m, of the type D-KV-72 supplied by the Kovobel company, Czech Republic), two animals per cage. The cages allowed faeces separation. A cycle of 16 h of light and 8 h of dark was used throughout the experiment. Temperature and humidity were recorded continuously by a digital thermograph positioned at the same level as the cages. Heating and forced ventilation systems allowed air temperature in the building to be maintained within 16±4°C during the experiment. Relative humidity was about 70±5%.

Experiment schedule and diet, sampling

The animals were fed a commercial pellet diet for growing rabbits (Tekro Nitra company, Párovské Háje-Vráble, Slovakia; dry matter 886 g/kg; crude fibre 174 g/kg; crude protein 181 g/kg) with access to feed *ad libitum.* Every day, at the same time in the morning, the rabbits in EG were administered cells of *E. faecium* EF9a strain in Ringer solution (1.0×10^9 colony forming units [CFU]/mL, pH 7.0) at a dose 500 µL/animal d in their drinking water for 28 s (from 35 to 63 d of age). On the basis of our previous experiments, we had information about the volume of water drunk by rabbits, so firstly the rabbits consumed 100 mL of drinking water with EF9a strain and after consuming the total volume, they had access to water *ad libitum*. The experiment lasted for 42 d.

For *in vivo* testing, a rifampicin-marked variant of *E. faecium* EF9a was used to differentiate it from the other enterococci; it was subsequently cultivated at 37°C using Todd-Hewitt agar (TH, Imuna, Šarišské Michaľany, Slovakia) enriched with rifampicin (100 µg/mL). Briefly, an 18-h culture of strain EF9a was plated onto obliqued TH agar enriched with rifampicin (100 µg/mL) and cultivated at 37°C. Colonies with the highest concentration of rifampicin (colonies which were grown on the highest agar) were checked, reinoculated onto rifampicin-TH agar and cultivated; the inoculation and cultivation were repeated two or three times (during 48-72 h).

Performance traits, slaughtering and sampling

The rabbits' weight was checked daily; average daily weight gain (ADWG) were calculated mathematically. Mortality and morbidity were also recorded in groups daily (n=24/group). Freshly-voided faeces were collected using nets mounted under the cages, five nets under 12 cages. As there were two animals housed in each cage (12 cages=12 samples/group) and in some places the faeces were mixed, we decided to collect mixed samples, one mixed sample per net, i.e. per group. Sampling of faeces was done at 35 d of age (day 0; the start of the experiment; n=10 from both, EG and CG – initial microbial background, without EF9a administration), at 63 d of age (day 28; the end of EF9a strain application; n=5/group) and at 77 d of age (day 42; the end of the trial, 14 d after the strain cessation; n=5/group). Blood was sampled from six rabbits, individually from each of them, at days 0, 28 and 42 (n=6/group).

Three animals from each group were randomly selected for slaughter at 63 and 77 d of age (days 28 and 42 of the experiment) to test organic acid analyses in the caecum and for morphometry testing in the jejunum; they were stunned with electronarcosis (90 V for 5 s), immediately hung by the hind legs on the processing line and quickly bled by cutting the jugular veins. After bleeding and eviscerating, the caecum and appendix contents were collected in sterile sampling tubes individually from each slaughtered animal.

Morphometry and organic acid analyses

To test morphometry (villus cut surface, villus circumference, villus height – VH, crypt depth – CD, villus height:crypt depth ratio – VH:CD; n=3/group), intestinal tissue (1 cm^2) of the proximal jejunum was sampled and treated as previously described by Žitňan *et al.* (1998).

Lactic acid (g/100 g) and volatile fatty acid (VFA) values - acetic, propionic, butyric, iso-butyric, valeric, iso-valeric and caproic acids (mmoL/L; n=3/group) were determined using gas chromatography (Perkin Elmer gas chromatograph, USA) from the samples of caecal content (15 g) on days 28 and 42. A glass column (average diameter 3 mm, length 180 cm) was filled with N_2 (30 mL), H_2 (20 mL) and air (240 mL) and a sample (1 µL) for diffusion. As the standard column was used, isocaproic acid (SP 1200 H_3PO_4) on Chromosorbe WAW was separated at 130°C and at 125°C on Chromatone N-AW-DMCS. The pH value was measured with a Jenwey 3310 pH-meter (Germany).

Bacterial enumeration

To test microbiota, the samples of faeces, caecal and appendix content (1 g) were treated using the standard microbiological dilution method (ISO, the International Organisation for Standardisation). The appropriate dilutions in Ringer solution (pH 7.0; Oxoid Ltd., Basingstoke, Hampshire, England) were plated onto the following media: M-Enterococcus agar (NF-V04503, Difco Laboratories, Detroit, USA) for enterococci, Todd-Hewitt agar (Imuna) enriched with rifampicin (100 µg/mL) for *E. faecium* EF9a, De Mann-Rogosa-Sharpe agar (ISO 15214, Merck, Germany) for lactic acid bacteria (LAB), Mannitol Salt agar for coagulase-negative staphylococci (CoNS, ISO 6888, Difco), Baird-Parker agar enriched with egg yolk tellurite supplement (ISO 21527-1, Difco) for coagulase-positive staphylococci (CoPS), *Clostridium difficile* agar with the supplement SR0096E 7% (v/v) defibrinated horse blood (SR0050, ISO 15883, Oxoid) for *Clostridium* species (anaerobic cultivation), Mac Conkey agar (ISO 7402, Oxoid) for coliforms. Pseudomonads were isolated on *Pseudomonas* agar (Biomark, India). Cultivation was performed at 30°C and/or 37°C for 24-48 h depending on the bacterial genera. The bacterial counts were expressed in log 10 of colony forming units per gram (log_{10} CFU/g±standard deviation).

Table 1: The effect of *E. faecium* EF9a administration (means±standard deviation) on the growth performance of rabbits.

	EG	CG	*P*-value
Initial live weight[1] (at 35 d of age, weaning, g)	1001±114	1007±105	0.9791
Intermediate live weight[1] (at 63 d of age, g)	2059±192	2025±212	<0.0001
Final weight[2] (at 77 of age, g)	2574±232	2416±178	0.0483
ADWG (35-63 d of age; g/d)	35.0±5.2	34.3±9.1	0.8907
ADWG (63-77 d of age; g/d)	38.5±1.5	30.5±2.5	<0.0001
ADWG (35-77 d of age; g/d)	36.2±4.7	33.0±7.8	0.3012

[1]24 rabbits per group. [2]21 rabbits per group.
EG=experimental group, application of *E. faecium* EF9a into water for 28 d (from 35 to 63 d of age). CG=control group of rabbits. ADWG=average daily weight gain.

Blood analysis and statistical analysis

Blood from 6 rabbits from each group was sampled from the marginal ear vein (*Vena auricularis*) into dry non-heparinised Eppendorf tube at days 0, 28 and 42 for biochemical analyses. Biochemical parameters in blood serum were determined by colorimetric methods (Spectrophotometer UV-2500 Shimadzu, Japan) using kits (Randox, UK) for the following parameters: total protein (TP245), triglyceride (TR210), cholesterol (CH200), glucose (GL2623), calcium (CA590), alanine aminotransferase (ALT; AL10). To assess phagocytic activity (PA) and index of phagocytic activity (IPA), blood was sampled into Eppendorf tubes containing microspheric hydrophilic particles (MSHP) and heparin. Direct counting was performed; ingestion of MSHP by polymorphonuclear cells (PMN) was determined using a modified test described by Vetvička *et al.* (1982): 50 µL of MSHP suspension (ARTIM, Prague, Czech Republic) was mixed with 100 µL of blood in an Eppendorf-type test tube and incubated at 37°C for 1 h. Blood smears were then prepared and stained in accordance with May-Grünwald and Giemsa-Romanowski. Index of PA (IPA) was calculated

Table 2: The effect of *E. faecium* EF9a administration on the phagocytic activity and jejunal morphometry in rabbits.

	EG	CG	*P*-value
Phagocytic activity (%)			
63 day of age	30.33±2.34	30.00±2.10	0.7709
77 day of age	36.67±2.42	29.83±1.94	<0.0001
Index of phagocytic activity			
63 d of age	1.03±0.12	1.03±0.12	1.0000
77 d of age	1.12±0.08	1.00±0.09	0.0312
Morphometry (63 d of age)			
Villus cut surface (µm^2)	81311.0±4650.0	80152.0±3867.0	0.7566
Villus circumference (µm)	1568.0±82.0	1552.0±49.0	0.7861
Villus height (µm)	677.0±54.0	659.0±43.0	0.6749
Crypt depth (µm)	185.0±21.0	179.0±9.0	0.6728
Villus height:crypt depth	3.73±0.66	3.70±0.42	0.9502
Morphometry (77 d of age)			
Villus cut surface (µm^2)	79625.0±1694.0	78322.0±2359.0	0.4805
Villus circumference (µm)	1556.0±53.0	1545.0±37.0	0.7828
Villus height (µm)	671.0±24.0	653.0±27.0	0.4368
Crypt depth (µm)	183.0±7.0	181.0±10.0	0.7907
Villus height:crypt depth	3.68±0.27	3.61±0.23	0.7497

EG=experimental group, application of *E. faecium* EF9a into water for 28 d (from 35 to 63 d of age). CG=control group.

Table 3: The effect of *E. faecium* EF9a administration on the pH, the lactic acid and volatile fatty acids concentrations in caecum of rabbits.

	EG	CG	*P*-value
At 63 d of age			
Acetic acid (mmoL/100 g)	2.750±0.700	3.900±1.210	0.2273
Propionic acid (mmoL/100 g)	0.170±0.040	0.220±0.020	0.1249
Iso-butyric acid (mmoL/100 g)	0.004±0.001	0.004±0.002	1.0000
Butyric acid (mmoL/100 g)	0.630±0.120	0.923±0.279	0.1700
Iso-valeric acid (mmoL/100 g)	0.040±0.010	0.040±0.009	1.0000
Valeric acid (mmoL/100 g)	0.036±0.001	0.048±0.008	0.0615
Caproic acid (mmoL/100 g)	0.034±0.010	0.049±0.012	0.1716
VFA (mmoL/100 g)	3.666±0.863	5.180±1.501	0.2045
Lactic acid (g/100 g)	0.009±0.003	0.006±0.004	0.3574
pH	6.240±0.040	5.920±0.110	0.0091
At 77 day of age			
Acetic acid (mmoL/100 g)	5.358±0.600	6.518±1.398	0.2571
Propionic acid (mmoL/100 g)	0.446±0.022	0.414±0.139	0.7138
Iso-butyric acid (mmoL/100 g)	0.014±0.009	0.004±0.001	0.1283
Butyric acid (mmoL/100 g)	1.704±0.198	1.880±0.688	0.6922
Iso-valeric acid (mmoL/100 g)	0.043±0.016	0.023±0.008	0.1249
Valeric acid (mmoL/100 g)	0.108±0.002	0.084±0.022	0.1330
Caproic acid (mmoL/100 g)	0.046±0.013	0.063±0.022	0.3134
VFA (mmoL/100 g)	7.719±0.724	8.985±2.201	0.3975
Lactic acid (g/100 g)	0.009±0.004	0.012±0.003	0.3574
pH	6.040±0.180	6.070±0.230	0.8674

EG=experimental group, application of *E. faecium* EF9a into water for 28 d (from 35 to 63 d of age). CG=control group. VFA=volatile fatty acids.

as the number of white cells containing at least three engulfed particles/100 white cells (neutrophils and monocytes). The percentage of phagocytic cells was evaluated using an optical microscope, by counting PMN up to 100.

Statistical analysis of the results was performed using nonpairing T-test with the level of significance set at $P<0.05$. The results are quoted as means±standard deviation.

RESULTS AND DISCUSSION

All animals remained in good health condition throughout the experiment, with no symptoms of disorders; no mortality and morbidity were noted. There was a higher live weight at 63 d of age (+34 g; $P<0.0001$), final live weight at 77 d of age (+158 g; $P=0.0483$) and ADWG between 63 and 77 d of age (+8 g/d; $P<0.0001$) in the EG group rabbits (Table 1). In this respect, other authors also observed the beneficial effects of feed supplementation with probiotics on body weight of rabbits (Matusevičius *et al.,* 2004; Kritas *et al.,* 2008; Kalma *et al.,* 2016; Fathi *et al.,* 2017). Furthermore, improved ADWG was also noted during previous experiments with both autochthonous and non-autochthonous probiotic strains of *E. faecium* administration in rabbits (Pogány Simonová *et al.,* 2009; Szabóová *et al.,* 2012; Lauková *et al.,* 2016), which agrees to some extent with the results of the present study. Surprisingly, in this study, during EF9a administration ADWG increased only slightly (by 2.1%) compared to the control group rabbits, while after the cessation of probiotic administration a significantly higher ADWG was noted ($P<0.0001$; by 20.8%). We suppose that EF9a application had a prolonged effect on the rabbits' growth, which was noted after cessation, in contrast to the CCM7420 strain, when higher ADWG was already noted during the treatment period (Pogány Simonová *et al.,* 2009). There were no significant effects of *E. faecium* EF9a administration on the

Table 4: Counts of bacteria (log_{10} colony forming units/g±standard deviation) in faeces of rabbits after application of *E. faecium* EF9a.

	EG	CG	*P*-value
At 35 d of age			
RMS-EF9a		–	
Enterococcus sp.		3.69±0.76	
LAB		3.54±0.59	
CoNS		3.45±0.81	
CoPS		2.31±0.82	
Coliforms		3.52±0.39	
Pseudomonas-like sp.		4.91±0.88	
Clostridium-like sp.		2.21±0.64	
At 63 d of age			
RMS-EF9a	3.99±0.89	–	
Enterococcus sp.	3.99±0.89	4.58±0.60	0.2540
LAB	4.21±0.61	5.58±0.83	0.0178
CoNS	4.10±0.15	4.88±0.19	<0.0001
CoPS	2.65±1.15	4.70±0.70	0.0093
Coliforms	2.29±1.80	5.03±1.08	0.0193
Pseudomonas-like sp.	4.54±0.99	7.03±1.17	0.0067
Clostridium-like sp.	2.88±0.33	3.89±1.06	0.0763
At 77 d of age			
RMS-EF9a	<1.00	–	
Enterococcus sp.	3.57±1.05	3.81±0.60	0.6690
LAB	2.92±1.24	3.77±0.96	0.2601
CoNS	3.85±0.34	3.68±0.46	0.5250
CoPS	3.23±0.45	3.11±1.10	0.8270
Coliforms	3.23±0.91	2.92±0.80	0.5830
Pseudomonas-like sp.	4.51±0.41	5.34±0.31	0.0069
Clostridium-like sp.	NT	NT	

EG=experimental group, application of *E. faecium* EF9a into water for 28 d (from 35 to 63 d of age). [2]CG=control group. RMS=rifampicine marked strain. LAB=lactic acid bacteria. CoNS=coagulase-negative staphylococci. CoPS=coagulase-positive staphylococci. NT=not tested.

jejunal morphometry in rabbits (Table 2). Similarly, Oso *et al.* (2013) observed no significant effects on morphological parameters in the rabbit ileum after probiotics inclusion. On the contrary, Pogány Simonová *et al.* (2015) and Lauková *et al.* (2014, 2017) observed that administration of rabbit-derived CCM7420 strain as well as bacteriocins enterocin EF55 (produced by *E. faecium* EF55) and nisin to rabbits altered their jejunal morphometry.

PA as well as IPA values were not increased during EF9a administration. However, PA and IPA values significantly increased after cessation of EF9a administration (at 77 d of age; $P<0.001$; Table 2), in contrast to our previous experiments with other bacteriocinogenic and probiotic *E. faecium* strains AL41/CCM8558, EF2019/CCM7420 and their enterocins EntM, Ent2019 as well as nisin in rabbits, during the application of which significant PA stimulation was noted (Pogány Simonová *et al.,* 2013a; Lauková *et al.,* 2014, 2016). Fathi *et al.* (2017) noted improved cell-mediated immunity after adding *Bacillus subtilis* to rabbits' feed. Moreover, it is well known that probiotics are able to stimulate immunity by improving the intestinal barrier and mucosal immune system *via* modulation of intestinal microbiota and through antibacterial compound production (Fortun-Lamothe and Boullier, 2007). It is noteworthy that our study is one of the few dealing with non-specific immunity stimulation by natural substances (probiotics and bacteriocins).

Table 5: Counts of bacteria (log_{10} colony forming units/g±standard deviation) in caecum of rabbits after application of *E. faecium* EF9a.

	EG	CG	*P*-value
At 63 d of age			
Coliforms	1.71±0.19	4.05±0.05	<0.0001
Enterococcus sp.	0.90±0.00	1.10±0.20	0.1587
CoNS	2.15±0.08	3.46±0.15	0.0002
CoPS	1.85±0.57	1.98±0.60	0.7990
LAB	2.90±0.53	3.25±1.17	0.6615
Clostridium-like sp.	2.40±0.13	2.92±1.21	0.5003
Pseudomonas-like sp.	2.70±0.40	3.66±0.50	0.0603
RMS	0.90±0.00	—	
At 77 d of age			
Coliforms	1.23±0.51	2.46±1.80	0.3184
Enterococcus sp.	1.61±1.23	1.23±0.42	0.6392
CoNS	2.94±0.08	2.97±0.01	0.5543
CoPS	1.60±0.20	1.70±0.24	0.6088
LAB	1.54±0.93	1.20±0.36	0.5866
Clostridium-like sp.	NT	NT	
Pseudomonas-like sp.	2.38±0.12	3.52±0.94	0.1056
RMS	1.96±0.40	—	

EG=experimental group, application of *E. faecium* EF9a into water for 28 d (from 35 to 63 d of age). CG=control group. CoNS=coagulase-negative staphylococci. CoPS=coagulase-positive staphylococci. LAB=lactic acid bacteria. RMS=rifampicine marked strain. NT= not tested.

The lactic acid and volatile fatty acids concentrations in caecum of the rabbits were not significantly influenced by the treatment, and only the caecal pH value was higher in the EG group rabbits at 63 d of age (*P*=0.0091; Table 3) Similarly, no significant shifts in caecal pH or total VFA concentration were noted during treatment of rabbits with several probiotics by Phuoc and Jamikorn (2017). On the other hand, Bovera *et al.* (2012) presented higher total VFA production during *Lactobacillus plantarum* use on pre-weaning rabbits, but the molar proportion of the main VFAs was unaffected by their treatment. Lauková *et al.* (2016) also detected no influence on the acetic, butyric, propionic, valeric and lactic acid concentrations in the chymus of rabbits during AL41 probiotic strain administration, while increased presence of acetic and butyric acids in the rabbits' faeces was noted in our previous study after *E. faecium* EF2019/CCM7420 strain application (Simonová *et al.,* 2008).

In the present study, the count of bacteriocin-producing strain *E. faecium* EF9a in the faeces of rabbits (at 63 d of age) was 3.99±0.89 (log10) CFU/g (Table 4). This is a similar count to that detected in our previous *in vivo* experiments using 21-d application of *E. faecium* EF2019/CCM7420 (4.34±0.75 CFU/g; Pogány Simonová *et al.,* 2009), but higher than in the case of *E. faecium* AL41 (1.47±0.16 CFU/g; Lauková *et al.,* 2016). While the counts of CCM7420 strain were still sufficient also at day 42 (21 d after cessation of administration, 3.30±0.30 CFU/g), and in the case of the AL41 strain it was lower (1.47±0.73 CFU/g), the counts of EF9a decreased to less than 1.00 CFU/g in the present study. In spite of the lower counts of the tested EF9a strain in this study, we can conclude its sufficient establishment in the rabbits' gastrointestinal tract, but with lower survival ability compared to CCM7420 or AL41 strains. Although Cunha *et al.* (2017) noted higher counts of autochthonous *E. avium* and *E. faecalis* probiotic strains in rabbit faeces after 25 d of experiment (mean 6.45-6.92 CFU/g), the probiotic bacterial strains did not remain in the gastrointestinal tract for longer than one week after the administration ended. From these findings we can conclude that the survival ability of the tested probiotic strain does not always positively correlate with its high counts and/or its origin in the gastrointestinal tract of animals. The digestive physiology of rabbits is significantly influenced by microbial diversity in their caecum. Moreover, this bacterial population differs from the microbiota of other studied animals because of the

Table 6: Counts of bacteria (log_{10} colony forming units/g±standard deviation) in appendix of rabbits after application of *E. faecium* EF9a.

	EG	CG	*P*-value
At 63 d of age			
Coliforms	2.55±1.33	4.98±0.10	0.0343
Enterococcus sp.	0.90±0.00	0.90±0.00	1.0000
CoNS	2.18±0.10	2.64±0.61	0.2669
CoPS	3.19±1.04	1.96±0.18	0.1137
LAB	5.04±0.00	4.60±1.00	0.4885
Clostridium-like sp.	4.34±0.78	3.34±1.77	0.4212
Pseudomonas-like sp.	4.33±2.35	5.67±2.37	0.5251
RMS	0.90±0.00	—	
At 77 d of age			
Coliforms	1.98±0.35	3.57±0.68	0.0227
Enterococcus sp.	0.90±0.00	1.74±0.00	<0.0001
CoNS	2.76±0.19	2.73±0.08	0.8134
CoPS	1.84±0.62	2.42±0.19	0.1963
LAB	1.96±0.17	1.62±0.73	0.4760
Clostridium-like sp.	NT	NT	
Pseudomonas-like sp.	3.51±1.02	5.06±0.03	0.0581
RMS	0.90±0.00	—	

EG=experimental group, application of *E. faecium* EF9a into water for 28 d (from 35 to 63 d of age). CG=control group. CoNS=coagulase-negative staphylococci. CoPS=coagulase-positive staphylococci. LAB=lactic acid bacteria. RMS=rifampicine marked strain. NT=not tested.

caecotrophy or recycling of microbial proteins in rabbits. The rabbit caecal microflora is diverse, and although most of the bacterial species have already been described, there are still some microorganisms present which have not previously been described. Moreover, it is unknown whether the microbial population of caecotrophic species differs from those of other herbivorous species (Abecia *et al.,* 2005). It is known that probiotic administration modulates the intestinal microbiota by reducing pathogens and spoilage microbes in favour of beneficial bacteria. In the present study (Table 4), a significant decrease in LAB, coliforms ($P<0.05$), CoPS, pseudomonads ($P<0.01$) and CoNS ($P<0.001$) was noted in the faeces of EG (at 63 d of age) compared to CG group. In the case of LAB, the explanation may corroborate the competitive exclusion of the tested probiotic strain together with the beneficial intestinal microbiota, *e.g.* LAB. The previous administration of CCM7420 and AL41 strains in rabbits individually and in combination with *Eleutherococus senticosus* led to a decrease in CoNS, CoPS, coliforms, clostridiae and pseudomonads (Pogány Simonová *et al.,* 2013b; Lauková *et al.,* 2016). The lower occurrence of *E. coli* and *Clostridium perfringens* in probiotic-treated rabbits was also noted by Kritas *et al.* (2008). The antimicrobial effect of EF9a strain against all of the tested bacterial genera was noted in the caecal content, where the counts of coliforms ($P<0.001$), CoNS ($P=0.0002$) and pseudomonads ($P=0.0603$) decreased (Table 5). Reduction in coliforms ($P<0.05$) in the appendix was repeatedly detected during the EF9a administration (Table 6). Oso *et al.* (2013) and Phuoc and Jamikorn (2017) also showed a reduction in caecal coliform populations in rabbits receiving several probiotic supplements on the basis of *Bacillus cereus, Pediococcus acidolactici, Bacillus subtilis* and *Lactobacillus acidophilus* strains. A slight decrease in bacteria in the caecum and appendix contents was found by Lauková *et al.* (2016).

In the present study, tested serum parameters (Table 7) remained within the range of normal values defined for these parameters by previous studies using rabbits (Jenkins, 2006; Martinec *et al.,* 2012; Özkan *et al.,* 2012), although there are differences in the physiological or reference ranges in the rabbits' serum found by the present researchers. It should be noted that our probiotic treatment showed insignificant effects in all traits except ALT; at 63 d of age, a higher ALT concentration was measured in the EG group rabbits than in those of the CG group ($P<0.05$), similarly to Lauková *et al.* (2016), who applied the *E. faecium* AL41 probiotic to rabbits. In contrast to our findings, Bovera

Table 7: Biochemical parameters in blood serum of rabbits before and after *E. faecium* EF9a application.

		EG	CG	*P*-value
Total proteins (g/L)	at 35 d of age	51.17±1.48		
	at 63 d of age	55.96±2.63	55.02±1.43	0.4596
	at 77 d of age	53.70±3.22	56.09±1.91	0.1490
Triglycerids (g/L)	at 35 d of age	1.65±0.17		
	at 63 d of age	1.55±0.17	1.49±0.16	0.5431
	at 77 d of age	1.52±0.15	1.69±0.15	0.0780
Cholesterol (mmol/L)	at 35 d of age	3.08±0.33		
	at 63 d of age	3.05±0.40	2.58±0.37	0.0608
	at 77 d of age	2.71±0.23	2.75±0.19	0.7494
Glucose (mmol/L)	at 35 d of age	7.47±2.13		
	at 63 d of age	6.24±0.13	6.09±0.25	0.2215
	at 77 d of age	6.72±0.34	6.72±0.75	1.0000
Calcium (mmol/L)	at 35 d of age	2.80±0.08		
	at 63 d of age	2.77±0.07	2.69±0.11	0.1628
	at 77 d of age	2.77±0.08	2.80±0.07	0.5051
ALT (IU/L)	at 35 d of age	10.3±2.9		
	at 63 d of age	8.3±2.9	5.2±1.1	0.0462
	at 77 d of age	6.3±0.2	7.0±1.0	0.2990

EG=experimental group, application of *E. faecium* EF9a into water for 28 d (from 35 to 63 d of age); CG=control group; ALT=alanine aminotransferase.

et al. (2012) showed a decrease in ALT after *Lactobacillus plantarum* spray application in suckling rabbits. Despite the increase in ALT values, we can assume that EF9a strain application had no adverse effect on the liver, as the measured data were still within the physiological range (Özkan *et al.,* 2012).

CONCLUSIONS

Dietary supplementation with *E. faecium* EF9a enterocin-producing and probiotic strain applied to rabbits could improve their growth and enhance non-specific immunity by increasing phagocytic activity and reducing potential pathogens in the gastrointestinal tract. The results associated with microbiota in the appendix and jejunal morphometry during probiotic strain(s) application are interesting, and they were previously presented only by our laboratory (application of nisin, *E. faecium* CCM7420 and AL41 strains in rabbits; Pogány Simonová *et al.,* 2009; Lauková *et al.,* 2014, 2016); Results of the present study provide a better and deeper explanation of the probiotic challenge in the rabbit organism. However, more research is still required to confirm and extend knowledge about the EF9a effect on rabbits' productivity, metabolism, immunity and microbial diversity, and to achieve more stable and/or improved health status and higher meat production of rabbits.

Acknowledgements: This work was financially supported by the bilateral Slovak – Hungarian project APVV:SK-HU-0006-08 and the national VEGA project 2/0006/17. Part of the preliminary results was presented in the Proceedings from the Conference in Kaposvár, Hungary, 30.05.2012, pp. 89-92. We are grateful to Mrs. M. Bodnárová and Mr. P. Jerga for their skilful technical assistance. We are also grateful to Dr. V. Párkányi and Dr. R. Jurčík, from the National Agricultural and Food Centre in Nitra for blood sampling and Mr. J. Pecho for slaughtering. All care and experimental procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Laboratory Animals and the trials were accepted by the Ethic Commission of the Institute of Animal Physiology in Košice and by the Slovak Veterinary and Food Administration. We would like to thank to Mr. A. Billingham for English language correction.

Conflict of interest: The authors declare that they have no conflict of interest. None of the authors of this paper have a financial or personal relationship with other persons or organisations that could inappropriately influence or bias the content of the paper.

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