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Pilot Experiment in Chickens Challenged with *Campylobacter jejuni* CCM6191 Administered Enterocin M-producing Probiotic Strain *Enterococcus faecium* CCM8558 To Check Its Protective Effect

Andrea Laukova[1], Monika Pogany Simonova[1], Ivana Kubasova[1], Sona Gancarcikova[2], Iveta Placha[1], Jana Scerbova[1], Viera Revajova[2], Robert Herich[2], Mikulas Levkut Sn.[2], Viola Strompfova[1]*

[1]Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic

[2]University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovak Republic

**Corresponding author: laukova@saske.sk*

ABSTRACT

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Campylobacter jejuni is one of the most common food-borne pathogens and chickens are the main source of these bacteria. *Enterococcus faecium* AL41, enterocin M-producing strain (deponed to the Czech Culture Collection of Microorganisms, Brno, Czech Republic – CCM8558) is our isolate previously applied e.g. in broiler rabbits with beneficial effect. In this study it was used in a 11-day experiment with chickens (1-day-old, breed Cobb 500, n = 40) challenged with *C. jejuni*. Birds had free access to feed and water; they were randomly divided into four groups per 10 chicks each: control (CG), *E. faecium* CCM8558 (EG1), CCM8558 + *C. jejuni* CCM6191 (EG2), CCM6191 (EG3). *E. faecium* CCM8558 (10^9 CFU/ml, 200 µl) in Ringer solution was administered daily *per os* to EG1 and EG2 for 7 days (from day 0–1). EG3 and EG2 were infected individually *per os* (day 4, CCM6191, 10^8 CFU/ml in Ringer solution, 200 µl). For microbiota evaluation, faecal mixtures (n = 5) were sampled on day 0–1 (1st sampling), on day 7 (2nd sampling), and on day 11 (3rd sampling) (day 7 of CCM8558 application, day 4 post-infection; day 4 of CCM8558 cessation, day 7 post-infection). Five birds from each group were sacrificed. CCM8558 sufficiently colonized chickens. In faeces of EG2 a tendency to reduce *Campylobacter* spp. (day 7 of application, day 4 of infection) was noted compared to EG2 (day 11, day 4 of cessation, day 7 post-infection; difference 1.21 log cycles), while in EG3 CCM6191 strain was not reduced. Phagocytic activity (PA) values were significantly higher in infected groups compared to CG and EG1. A significant increase in PA was also noted in EG2 and EG3 at the end of experiment compared to CG or EG1. The strain additive did not evoke oxidative stress. Biochemical parameters were influenced to the reference levels.

Keywords: enterococci; effect; chick; microbiota; physiological parameters; *Campylobacter* challenge

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Campylobacter jejuni is one of the most common food-borne pathogens, and chickens are the main source of these bacteria. Fowls and birds in general are prone to *C. jejuni* colonization more than any other animals (Sahin et al. 2001). Chickens are usually colonized by *C. jejuni* within 5–6 weeks of age and remain as a reservoir throughout their lifespan. Campylobacteriosis is the most commonly reported zoonosis (EFSA 2015). To reduce human infections with *Campylobacters*, it is necessary to reduce the colonization in the chickens (Cisek and Binek 2014). Modern broiler rearing has taken attention to natural substances which can help them maintain healthy status of fowls. Therefore, farmers, breeders have been looking for probiotic microbiota. In poultry breeding probiotic strains of the genus Lactobacillus are commercially mostly used (Cisek and Binek 2014); *Bacillus* spp. are also frequently used (Novak et al. 2011); at recent time representatives of the genus Enterococcus have also been reported especially in relation with immunological parameters (Stasova et al. 2015; Levkut Jr. et al. 2016; Karaffova et al. 2017). The probiotic strains of the last mentioned genus, however, usually show a broad antimicrobial acitivity against both, Gram-positive and Gram-negative causative bacteria and this is associated with the production of bacteriocins – enterocins (Laukova et al. 2004a; Franz et al. 2007; Scerbova and Laukova 2016). Bacteriocins are antimicrobial proteinaceous substances produced by some bacteria to kill other more or less related bacteria (Franz et al. 2007). Their beneficial effect in food-producing animal husbandries was previously shown e.g. in rabbits (Szaboova et al. 2011; Laukova et al. 2012) but beneficial effects due to enterocin-producing strains were also reported (Laukova et al. 2003a, 2015, 2016; Pogany Simonova et al. 2013). *Enterococcus faecium* AL41 is our environmental strain previously more detailedly characterized in our laboratory (Laukova et al. 1998, 2004b); it is known to produce a new type of enterocin named enterocin M, which is very similar to enterocin P (Marekova et al. 2007). It was deponed to the Czech Culture Collection of Microorganisms in Brno, Czech Republic (CCM8558). Its beneficial effect was checked not only in animals but also in animal-derived food such as in sausage (Laukova et al. 2003b). Because of complex information we decided to use it in chickens challenged with *Campylobacter jejuni* to reach results for its recommendation and inclusion in a further probiotic product.

MATERIAL AND METHODS

Strains preparation. An enterocin M-producing strain *E. faecium* CCM8558 (AL41) possessing probiotic properties was prepared as previously referred to by Laukova et al. (2015). The strain CCM8558 was grown in De Mann-Rogose-Sharpe broth (MRS; Merck, Germany) for 18 h at 37°C. Broth culture was centrifuged at 10 000 *g* (at 4°C) for 30 min. Supernatant was removed and cells of CCM8558 strain were re-suspended in Ringer solution (Merck) to achieve the concentration of 10^9 colony forming units per ml (CFU/ml). The concentration of CCM8558 cells was checked by dilutions spread on M-Enterococcus agar (Becton and Dickinson, USA) according to the International Organization for Standardization (ISO 7899) and expressed in CFU/ml. Before application but also after CCM8558 isolation from gastrointestinal tract (GIT), its identity was confirmed by polymerase chain reaction (PCR) analysis (Woodford et al. 1997) and by the MALDI-TOF identification system as well (Alatoom et al. 2011). To distinguish *E. faecium* CCM8558 strain from the other enterococci in the samples, CCM8558 strain treated with rifampicin (100 μg/ml) was used (Laukova et al. 2012).

Campylobacter jejuni CCM6191 (supplied by the Czech Culture Collection, Brno, Czech Republic) was grown on *Campylobacter* agar base Karmali CM0935 with supplement SR0167 (Oxoid Ltd., UK) in the jar with microaerophilic atmospheare (CampyGen, Oxoid Ltd.) placed in an incubator at 42°C for 2 days. Broth culture of *Campylobacter jejuni* CCM6191 was prepared in Brian Heart broth (Difco Laboratories Inc., USA) under the former mentioned condition; triplet of tubes was prepared. Final cells concentration of *Campylobacter jejuni* CCM6191 was 10^8 CFU/ml. After its isolation from passaging in the gastrointestinal tract, its identity was checked by the MALDI-TOF identification system again.

Experimental design. Forty chicks (1-day-old, Cobb 500 breed) were used in the experiment. The experiment lasted for 11 days. Chicks were placed in large pens with cellulose cotton (Pehazell, Slovakia) and reared under the regimen 23 h light and 1 h darkness, initial room temperature 32–33°C was reduced weekly by 1°C to a final temperature 28°C. Relative humidity was within a range of 50–60%. Birds had free acces to feed (BR1 – starter diet) and

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water. Application of cleaning and feeding regimens prevented them from cross-contamination effectively throughout the experiment. Chicks were randomly divided into four groups per 10 chicks each (n = 10): control (CG), *E. faecium* CCM8558 (EG1), combined *E. faecium* CCM8558 + *C. jejuni* CCM6191 (EG2), and *C. jejuni* CCM6191 (EG3). *E. faecium* CCM8558 at concentration 10^9 CFU/ml (200 µl) in Ringer solution was administered daily *per os* to the groups EG1 and EG2 for 7 days of the experiment (from day 0–1). Experimental infection of *C. jejuni* CCM6191 (EG3) and CCM8558 + *C. jejuni* CCM6191 (EG2) groups was carried out individually *per os* on day 4 of the experiment (*C. jejuni* CCM6191, concentration 10^8 CFU/ml in Ringer solution) as a single dose 200 µl. For microbiota evaluation, faecal mixtures (n = 5) were sampled on day 0–1 (from each group of birds, 1st sampling), on day 7 (2nd sampling), and on day 11 (3rd sampling) (it means on day 7 of CCM8558 application, day 4 *Campylobacter* post-infection, and on day 4 of CCM8558 cessation, day 7 post-infection). Moreover, at those times, five birds from each group were sacrificed using intraperitoneal injection of xylasine (Rometar 2%; Spofa, Czech Republic) at a dose 0.6 ml/kg and ketamine (Narkamon 5%; Spofa) at a dose 0.7 ml/kg (Levkut et al. 2012); caecal and liver contents were sampled to check microbiota.

Microbial analyses. The standard microbiological method (ISO, International Organization for Standardization) was used, dilutions in Ringer solution 1 : 9 (Merck) by mixing using Stomacher (Masticator, Spain). Dilutions were spread onto appropriate selective media as follows: *E. faecium* CCM8558 strain was determined on M-Enterococcus agar (Difco Laboratories Inc.) enriched with rifampicin; the total enterococcal count was determined on M-Enterococcus agar (method No. 7889). *Campylobacter* agar base Karmali CM0935 with supplement SR0167 (Oxoid Ltd.) was used to count *Campylobacter* spp. placed in the jar with microaerophilic atmosphere (CampyGen, Oxoid Ltd.) and in the incubator. Coliform bacteria were counted on MacConkey agar (Becton and Dickinson), *Salmonella* sp. were precultivated in Rappaport-Vassiliadis broth (Merck) and then spread onto Brillant Green agar (Becton and Dickinson). Bacteria were cultivated at 37°C for 18–24 h. *Campylobacter* spp. were cultivated at 42°C for 2 days. The bacterial counts were expressed in CFU/g (± SD).

Biochemical analyses, phagocytic activity, gluthatione-peroxidase test. On days 7 and 11 (that is, on day 7 of CCM8558 application, day 4 post-infection with *Campylobacter*, day 4 of CCM8558 cessation, day 7 post-infection) blood was sampled into heparinized and/or unheparinized tubes (following blood analyses) by intracardial punction (n = 5). Phagocytic activity (PA) was measured by a direct counting procedure using microspheric hydrophilic particles (MSHPs). Ingestion of MSHPs by polymorphonuclear cells was described by Vetvicka et al. (1982). Blood smears were stained with May-Gruenwald and Giemsa-Romanowski stains. PA was calculated as the number of white cells containing at least three engulfed particles per 100 white cells (neutrophils) and expressed in percentage. Index of PA (IPA) was calculated as the number of engulfed particles per total number of neutrophils observed. The percentage of phagocytic cells was evaluated using an optical microscope, by counting polymorphonuclear cells up to 100.

The enzyme glutathione-peroxidase (GPx) was determined using a Ransel kit (Randox Laboratories, Ltd., UK), expressed in U/gHb ± SD.

To test the biochemical parameters such as the total proteins (g/l), albumins (g/l), creatinine (µmol/l), triglycerides (mmol/l), total cholesterol (mmol/l), alanine aminotransferase (ALT) (µkat/l), aspartate aminotransferase (AST) (µkat/l), total cholesterol (mmol/l), α-amylase (µkat/l), calcium (mmol/l), phosphorus (mmol/l), and magnesium, a commercial kit Dialab (Czech Republic) and an analyzer ELLIPSE (AMS, Italy) were used; blood was sampled into nonheparinized sterile Eppendorf tubes. Blood serum was received by centrifugation at 3000 *g* for 10 min and stored frozen in plastic vials until analysis.

The experiment was carried out in accordance with the established standards for use of animals (Guide for the Care and Use of Laboratory Animals) and the experimental schedule was approved by the Ethic Commission of the University of Veterinary Medicine and Pharmacy in Košice, Institute of Animal Physiology, Slovak Academy of Sciences and approved by the Slovak Veterinary and Food Administration.

Statistical analysis. Statistical significance was assessed using one-way analysis of variance with the *post hoc* Tukey's multiple comparison test (one-way ANOVA). Probability values of less than 0.05 were considered siginificant.

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RESULTS

On day 7 (2nd sampling, Table 1), *E. faecium* CCM8558 reached in faeces of EG1 (CCM8558) and EG2 chickens (CCM8558 + *C. jejuni* CCM6191) up to 5.0 (log 10) CFU/g; it indicates a sufficient colonization of CCM8558 strain in the digestive tract of broiler chickens. Significantly higher counts of CCM8558 strain in chickens of EG1and EG2 (day 7 of application, day 4 post-infection, 2nd sampling) were found compared to its counts on day 11 (3rd sampling, day 4 of CCM8558 cessation, day 7 post-infection, a : a, $P < 0.05$). Moreover, almost the same counts of CCM8558 strain as in faeces were determined in caecum of EG1 and EG2 (Table 2, 2nd sampling). Higher counts of CCM8558 were noted in EG1 at 3rd sampling compared to its count in EG1 at 2nd sampling (Table 2). PCR analysis using primers and MALDI-TOF identification confirmed its identity (*E. faecium*). In liver, CCM8558 was not detected (Table 3).

Enterococci in faeces of EG1 chicks (CCM8558) (3rd sampling) were significantly higher (c : c, $P < 0.01$) compared to enterococci in EG1 (2nd sampling); enterococci in EG3 (3rd sampling) were significantly higher compared to enterococci in EG1 (2nd sampling, $P < 0.01$). Enterococci in EG2 (3rd sampling) were significantly higher compared to enterococci in EG1, EG3 (2nd sampling, e : e, e : f, $P < 0.01$). Enterococci in caecum were not influenced; they reached almost the same counts as enterococci in faeces (Table 2). In liver, lower counts of enterococci were detected in EG2 at 3rd sampling than in EG2 (2nd sampling, a : a, $P < 0.05$, Table 3).

Coliform bacteria in faeces, caecum or liver were not influenced by CCM8558 strain application (Tables 1–3). *C. jejuni* CCM6191 reached high counts in faeces of EG2 chicks (CCM8558 + *C. jejuni* CCM6191) post-infection as well as in EG3 group (*C. jejuni* CCM6191, Table 1); its initial inoculation was 8.0 CFU/ml; on day 7 (day 4 post-infection) CCM6191 reached up to (log 10) 6.0 CFU/g in EG2 and in EG4 up to 5.0 CFU/g. On day 11 (end of experiment, day 7 post-infection), *C. jejuni* CCM6191 counts in EG2 chicks reached still up to 5.0 (log10) CFU/g (Table 1). However, in faeces of EG2 (2nd sampling) a tendency to reduce *Campylobacters* (day 7 of application, day 4 of infection) was found if compared to EG2 (3rd sampling, day 11 – end of experiment, day 4 of cessation, day 7 post-infection; difference 1.21 log cycles) (Table 2), while in EG3 at this time

Table 1. Microbial counts in faeces of chickens of control (CG) and experimental groups (EG) (log10, CFU/g) ± SD

	CCM8558	Enterococci	Campylobacters	Coliform bacteria
1st sampling, day 0–1 (n = 5)				
	nt	6.68 (0.25)	3.54 (0.88)	6.10 (0.00)
2nd sampling, day 7 (application)				
CG	nt	7.17 (0.67)	< 1.00	6.39 (0.52)
EG1	4.67 (0.16)^{a}	6.76 (0.26)^{cdf}	2.27 (0.66)	7.70 (0.77)
EG2, day 4 of infection	4.32 (0.20)^{a}	7.30 (0.70)^{e}	5.62 (0.37)^{b}	7.89 (0.80)
EG3, day 4 of infection	nt	6.99 (0.04)^{d}	4.17 (2.04)	7.87 (0.80)
3rd sampling, day 11 (end of experiment)				
CG	nt	7.32 (0.70)	2.95 (0.71)	7.75 (0.78)
EG1	1.22 (0.11)^{a}	8.63 (0.93)^{c}	3.70 (0.92)	8.20 (0.83)
EG2	1.62 (0.27)^{a}	8.60 (0.90)^{e}	4.41 (2.10)^{b}	8.56 (0.92)
EG3	nt	8.45 (0.90)^{d}	4.78 (2.18)	8.13 (0.85)

1st sampling, day 0–1 = start of experiment; 2nd sampling, day 7 = *Enterococcus faecium* (CCM8558) application, day 4 of *Campylobacter jejuni* infection; 3rd sampling, day 11 = end of experiment and day 4 of CCM8558 cessation, day 7 post-infection; CG = control group; EG1 = CCM8558; EG2 = CCM8558 + *C. jejuni* CCM6191; EG3 = *C. jejuni* CCM6191; nt = not tested; CCM8558 *Enterococcus faecium*, 2nd sampling : 3rd sampling, a : a, $P < 0.05$; Enterococci in EG1, 3rd sampling : 2nd sampling, $P < 0.01$; in EG3 (3rd sampling) were significantly higher compared to EG1 (2nd sampling, d : c, $P < 0.01$); in EG2 (3rd sampling, d) were significantly higher compared to EG1c, EG3d (2nd sampling, d : c, d : d, $P < 0.01$); in EG2 (3rd sampling, e): 2nd sampling EG2, e : e , d : f, e : f, $P < 0.01$; *Campylobacter* spp., 2nd sampling in EG2 : 3rd sampling in EG2, b : b (difference 1.21 log cycle)

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Table 2. Microbial counts in caecum of chickens of control (CG) and experimental groups (EG) (log10, CFU/g) ± SD

	CCM8558	Enterococci	Campylobacters	Coliform bacteria
1st sampling, day 0–1 (n = 5)				
	nt	nt	nt	nt
2nd sampling, day 7 (application)				
CG	nt	7.26 (2.69)	1.36 (0.16)	8.59 (0.93)
EG1	4.58 (0.14)[a]	7.28 (2.69)	4.73 (0.17)	8.29 (0.85)
EG2, day 4 of infection	4.51 (0.12)[b]	7.81 (0.79)	3.60 (1.89)	8.40 (0.89)
EG3, day 4 of infection	nt	7.75 (0.78)	2.51 (1.59)	8.70 (0.94)
3rd sampling, day 11 (end of experiment)				
CG	nt	7.61 (2.75)	1.30 (0.00)	8.80 (0.96)
EG1	2.60 (0.61)[a]	7.76 (2.78)	4.73 (2.17)	8.63 (0.93)
EG2	1.56 (0.24)[b]	7.57 (0.75)	6.90 (0.62)	8.57 (0.93)
EG3	nt	8.39 (0.89)	6.68 (2.58)	8.85 (0.91)

1st sampling, day 0–1 = start of experiment; 2nd sampling, day 7 = *Enterococcus faecium* (CCM8558) application, day 4 of *Campylobacter jejuni* infection; 3rd sampling, day 11 = end of experiment and day 4 of CCM8558 cessation, day 7 post-infection; CG = control group; EG1 = CCM8558; EG2 = CCM8558 + *C. jejuni* CCM6191; EG3 = *C. jejuni* CCM6191; nt = not tested; CCM8558 *Enterococcus faecium*, 2nd sampling, 3rd sampling in EG1, a : a, difference 1.98 log cycle; in EG2 : EG1, 2nd sampling, 3rd sampling, b : a, difference 1.91 log cycle; in EG2 : EG2, 2nd sampling : 3rd sampling (difference 2.95 log cycle)

CCM6191 strain counts were not reduced, similarly as in caecum (Table 2); in liver mostly less than 1.00 (log 10) CFU/g was detected (Table 3). No *Salmonella* cells were detected in chicken.

At 2nd sampling, PA values were significantly higher in infected groups (EG3, EG2; Table 4) compared to CG and EG1 (a : b, a : c, $P < 0.01$; d : b, d : c, $P < 0.001$). Significant increase in PA was also noted in EG2 and EG3 at 3rd sampling compared to CG or EG1 (a : b, d : b, $P < 0.01$, $P < 0.001$; c : b, $P < 0.01$). Regarding the IPA, siginificant increase was noted in EG3 compared to CG, and in EG2 compared to CG, a : d, d : b, $P < 0.05$) at 3rd sampling; i.e. in infected groups (Table 4).

Table 3. Microbial counts in liver of chickens of control (CG) and experimental groups (EG) (log10, CFU/g) ± SD

CCM8558		Enterococci	Campylobacters	Coliform bacteria
1st sampling, day 0–1 (n = 5)				
	nt	nt	nt	nt
2nd sampling, day 7 (application)				
CG	nt	2.76 (0.66)	1.68 (0.29)	2.72 (1.64)
EG1	–	1.95 (0.39)	< 1.00	2.04 (0.42)
EG2, day 4 of infection	–	2.14 (0.46)[a]	< 1.00	2.22 (0.48)
EG3, day 4 of infection	–	1.61 (0.02)	< 1.00	2.02 (0.42)
3rd sampling, day 11 (end of experiment)**)**				
CG	nt	2.69 (0.63)	< 1.00	2.90 (0.70)
EG1	nt	1.71 (0.30)	< 1.00	1.97 (0.04)
EG2	nt	1.25 (0.11)[a]	< 1.00	1.93 (0.39)
EG3	nt	1.50 (0.02)	1.50 (0.02)	2.29 (0.51)

1st sampling, day 0–1 = start of experiment; 2nd sampling, day 7 = *Enterococcus faecium* (CCM8558) application, day 4 of *Campylobacter jejuni* infection; 3rd sampling, day 11 = end of experiment and day 4 of CCM8558 cessation, day 7 post-infection; CG = control group; EG1 = CCM8558; EG2 = CCM8558 + *C. jejuni* CCM6191; EG3 = *C. jejuni* CCM6191; nt = not tested; Enterococci in EG2, 2nd sampling : 3rd sampling, a : a, $P < 0.05$

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Table 4. Phagocytic activity (PA) values and index of phagocytic activity (IPA) (%)

	Phagocytic activity		Index of phagocytic activity	
	2nd sampling	3rd sampling	2nd sampling	3rd sampling
CG	33.00 (4.30)[b]	32.40 (2.30)[b]	1.48 (0.24)	1.38 (0.19)[b]
EG1	33.80 (3.90)[c]	35.80 (3.70)[c]	1.52 (0.26)	1.44 (0.21)
EG2	36.80 (4.20)[a]	38.60 (2.30)[a]	1.40 (0.16)	1.34 (0.21)[a]
EG3	39.20 (2.49)[d]	41.00 (2.70)[d]	1.76 (0.21)	1.82 (0.26)[d]

CG = control group, EG = experimental group, 2nd sampling, day 7 = *Enterococcus faecium* (CCM8558) application, day 4 of *Campylobacter jejuni* infection; 3rd sampling, day 11 = end of experiment and day 4 of CCM8558 cessation, day 7 post-infection; EG1 = CCM8558; EG2 = CCM8558 + *C. jejuni* CCM6191; EG3 = *C. jejuni* CCM6191; 1st sampling not tested; PA, 2nd sampling, a : b, a : c, $P < 0.01$; d : b, d : c, $P < 0.001$; 3rd sampling, d : b, d : c, $P < 0.001$; a : b, a : c, $P < 0.01$; IPA, 3rd sampling, a : d, d : b, $P < 0.05$

Based on the results achieved (Table 5) comparing values of nitrogen profile (total proteins, albumin, creatinine), it can be concluded that initial hypoproteinaemia in all groups was noted comparing groups EG1, EG2, EG3 to CG ($P < 0.001$, $P < 0.05$, $P < 0.05$), hypoalbuminaemia, hypocreatinaemia (day 7); at the end of experiment, increase of these parameters in the experimental groups (EG1, EG2, EG3) was noted. In opposite, in blood serum of chickens of CG even at the end of experiment hypoproteinaemia and hypoalbuminaemia were noted.

Measured values of AST and ALT were in the framework of reference values. However, α-amylase values were low (Table 6). Regarding the triglycerides on day 4 of infection, slight lipidemia was measured. In all chickens hypocalcaemia was noted; in general, the values of minerals varied (Table 7). At the end of experiment Mg values were increased in EG1 compared to EG2 ($P < 0.01$) and in EG1 compared to EG3 ($P < 0.05$, Table 7).

The values of GPx were lower during the whole experiment in the experimental goups EG1 and EG2 compared to CG; which means a beneficial effect. In EG1 (CCM8558) and EG2 (CCM8558 + *C. jejuni* CCM6191) even at the end of experiment lower counts were noted compared to CG but also compared to EG3 (*C. jejuni* CCM6191) (Table 6). There in EG3 even higher counts of GPx were reached compared to all other groups. On the other hand, the lowest, stable GPx value was noted in EG1 (CCM8558); it means that CCM8558 strain did not evoke oxidative stress; however, in EG3 (*C. jejuni*) it was probably evoked.

Table 5. Nitrogen profile values in blood serum of chickens of control (CG) and experimental groups (EG)

	CG	EG1	EG2	EG3
Day 7 (day 4 of infection)				
Total proteins (g/l)	20.56 (3.47)[a]	10.90 (2.97)[b]	9.35 (0.69)[b]	4.23 (1.71)[b]
Albumin (g/l)	11.50 (0.23)	7.55 (1.11)	6.88 (0.42)	4.61 (0.31)
Creatinine (μmol/l)	12.67 (0.88)	8.00 (1.24)	4.45 (1.03)	9.50 (3.79)
Day 11 (end of experiment)				
Total proteins (g/l)	11.68 (3.47)	16.72 (1.49)	11.06 (4.15)	11.26 (2.57)
Albumin (g/l)	8.80 (0.40)	9.80 (0.45)	6.40 (1.15)	7.66 (0.77)
Creatinine (μmol/l)	10.20 (1.63)	12.20 (1.02)	8.20 (1.56)	15.20 (3.79)

day 7 – 2nd sampling, day 11 – 3rd sampling, 1st sampling was not checked; 1st sampling, day 0–1 = start of experiment; 2nd sampling, day 7 = *Enterococcus faecium* CCM8558 application, day 4 of *Campylobacter jejuni* infection; 3rd sampling, day 11 = end of experiment, day 4 of CCM8558 cessation, day 7 post-infection; CG = control group, EG1 = CCM8558, EG2 = CCM8558 + *C. jejuni* CCM6191; EG3 = *C. jejuni* CCM6191; a : b ($P < 0.001$, $P < 0.05$, $P < 0.05$); reference values: total proteins 27–32 g/l; albumin 11.7–16.0 g/l; creatinine 11.1–30.6 μmol/l

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Table 6. Enzymatic-hepatal, energetic values, and GPx values in blood serum of chickens of control (CG) and experimental groups (EG)

	CG	EG1	EG2	EG3
Day 7 (day 4 of infection)				
AST (μkat/l)	2.35 (0.19)	2.65 (0.18)	0.28 (0.08)	2.46 (0.13)
ALT (μkat/l)	0.09 (0.02)	0.05 (0.02)	0.04 (0.02)	0.04 (0.01)
α-Amylase (μkat/l)	18.14 (1.60)	10.33 (1.35)	18.16 (3.09)	8.98 (1.65)
GPx (μkat/l)	157.53 (72.30)	156.38 (34.57)	139.71 (22.00)	132.81 (54.28)
Total cholesterol (mmol/l)	4.87 (0.12)	4.54 (0.27)	3.78 (0.13)	3.24 (0.16)
Triglycerides (mmol/l)	2.97 (0.14)	2.00 (0.08)	1.85 (0.03)	1.93 (0.29)
Day 11 (end of experiment)				
AST (μkat/l)	2.17 (0.23)	2.88 (0.08)	2.39 (0.22)	2.16 (0.19)
ALT (μkat/l)	0.10 (0.04)	0.02 (0.01)	0.06 (0.02)	0.04 (0.02)
α-Amylase (μkat/l)	10.29 (1.36)	13.79 (1.56)	12.19 (1.18)	11.49 (0.92)
GPx (μkat/l)	193.18 (71.80)	158.68 (47.60)	175.93 (43.61)	216.00 (80.00)
Total cholesterol (mmol/l)	4.27 (0.18)	4.48 (0.45)	3.80 (0.44)	4.22 (0.31)
Triglycerides (mmol/l)	1.58 (0.13)	1.64 (0.06)	1.52 (0.04)	1.50 (0.04)

day 7 = 2nd sampling, day 11 = 3rd sampling, 1st sampling was not checked; CG = control group, EG1= CCM8558, EG2 = CCM8558 + *C. jejuni* CCM6191; EG3 = *C. jejuni* CCM6191; reference values: AST = aspartate aminotransferase 0.30–2.63 μkat/l; ALT = alanine aminotransferase 0.11–0.76 μkat/l; α-amylase 82.47 μkat/l; GPX = glutathione peroxidase; total cholesterol 2.79–5.38 mmol/l; triglycerides 0.96–1.70 mmol/l

DISCUSSION

As in our previous experiments using CCM8558 strain = AL41 (Laukova et al. 2012, 2015), here also sufficient colonization in the GIT of chickens was approved including those chickens infected with *C. jejuni* CCM6191 strain. Followed our previous studies (Herich et al. 2004; Laukova et al. 2015) anti-*Campylobacter* effect or anti-*Salmonella* or anti-coliforms effect was noted. In our study a tendency to reduce *Campylobacter* was also noted. Moreover, in our previous studies, antimicrobial effect of CCM8558 strain was achieved both *in vitro* and *in vivo,* but with a longer application period. It means, to observe the tendency to reduce *Campylobacter* could be more clearly documented by a longer application of CCM8558 strain in chickens. In spite of this, CCM8558 strain probably acts as a protective element; it seems that it does not damage the intestinal barrier (mucosa)

Table 7. Mineral profile values in blood serum of chickens of control (CG) and experimental groups (EG)

	CG	EG1	EG2	EG3
Day 7 (day 4 of infection)				
Calcium (mmol/l)	0.00	0.006 (0.006)	0.00	0.00
Phosphorus (mmol/l)	4.12 (0.24)	0.48 (0.11)	0.33 (0.13)	0.46 (0.12)
Magnesium (mmol/l)	0.61 (0.07)	0.49 (0.03)	0.60 (0.14)	0.57 (0.18)
Day 11 (end of experiment)				
Calcium (mmol/l)	0.00	0.00	0.00	0.39 (0.39)
Phosphorus (mmol/l)	0.93 (0.49)	0.70 (0.36)	0.83 (0.45)	0.86 (0.32)
Magnesium (mmol/l)	0.68 (0.14)	0.95 (0.24)[a]	0.18 (0.04)[b]	0.20 (0.04)[b1]

CG = control group, EG1 = CCM8558, EG2 = CCM8558 + *C. jejuni* CCM6191, EG3 = *C. jejuni* CCM6191; reference values: calcium 2.05–3.0 mmol/l; phosphorus 1.45–2.63 mmol/l; magnesium 0.83–1.30 mmol/l; day 7 = 2nd sampling, day 11 = 3rd sampling, 1st sampling was not checked; EG1 compared to EG2 (a : b, $P < 0.01$) and EG1 compared to EG3 (a : b1, $P < 0.05$)

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and mucus which play an important role as a capture area to catch pathogenic strains (Ohland and MacNaughton 2010). Glyconjugates, which are basic components of mucus, were probably beneficially influenced by *E. faecium* CCM8558. The strain with protective effect should not degrade mucin; this property seems to be associated with CCM8558 strain because it did not translocate in the liver; however, count of total enterococci in liver reached up to 10^2 or 10^3 CFU/g . Similarly, e.g. Levkut et al. (2016) reported beneficial effect of *E. faecium* EF55 strain (producing enterocin 55) on the expression of *MUC* (intestinal mucin gene) and production of IgA + IEL (intraperitoneal lymphocytes) in caecum after injection with *Salmonella.*

Phagocytic activity is an essential component of the cellular innate immunity response and plays an important role in host defense against microbial infection. In the experimental groups, PA activity was significantly increased. In EG1 and EG2 this could be explained through the ability of CCM8558 strain to modulate the Toll-like receptors (TLRs) expression and in EG3 it could be explained via the ability of CCM8558 strain to modify the activation of *MIF*, *IFN-β*, *MD-2*, and *CD14* molecules in chickens challenged with CCM6191 strain as reported by Karaffova et al. (2017). Toll-like receptors are pattern recognition receptors that function as microbial infection sensors and are critical for the initiation of the innate inflammatory and adaptive immune response (Shang et al. 2008). Based on the results of Kavulova et al. (unpublished), *E. faecium* AL41 has probably an antibacterial effect on *C. jejuni* CCM6191 in the caecum mediated by stimulation of local gut immune response. It seems that *E. faecium* AL41 (CCM8558) can regulate inflammatory response in the intestine through the local regulation of *IL-17* and *TGF-β4* expression.

The effect of CCM8558 strain on biochemical blood parameters (e.g. cholesterol or triglycerides) could be explained possibly by the fact that *E. faecium* CCM8558 is a lactic acid producing microorganism, producing enzymes degrading bile, deconjugates it, and it can lead to lower pH. These changes could function by lowering of cholesterol or triglycerides (Bovdisova and Capcarova 2015). Hypoproteinaemia, for example, could be explained by a lower administration of exogenous proteins than loss or reduction of proteins synthesis. Creatinine is a parameter of the condition of glomeral system. From the clinical point of view high levels of creatinine are dangerous; in our study, creatinine values were low or normal. A nitrogenous profile provides information related to possible changes in apetite of the animals. In growing chickens, α-amylase plays an important role and lack of endogenous amylase limits the utilization of starch and reduces nutrition potential of feed. In our study, differences were not significant. Regarding the mineral metabolism, it was insignificantly influenced, too.

GPx belongs to the most abundant antioxidant enzymes protecting cells from oxidative damage (Mangiapane et al. 2014). In our conditions, lower values were assessed in experimental groups with CCM8558 strain; it indicates that this additive did not evoke oxidative stress. Similarly, also e.g. after *Enterococcus durans* ED26E/7 application in broiler rabbits, this enzyme was not influenced negatively (Laukova et al. 2017).

CONCLUSION

E. faecium CCM8558 sufficiently colonized the gastrointestinal tract of chickens. In faeces of EG2 (2^{nd} sampling, CCM8558 + *C. jejuni* CCM6191) a tendency to reduce *Campylobacter* spp. (day 7 of application, day 4 of infection) was noted as compared to EG2 (3^{rd} sampling, day 11, end of experiment, day 4 of cessation, day 7 post-infection; difference 1.21 log cycles); while in EG3 at this time CCM6191 strain counts were reduced neither in faeces, nor in caceum. PA values were significantly higher in infected groups compared to CG and EG1 (CCM8558 group). A significant increase in PA was also noted in EG2 and EG3 at 3^{rd} sampling compared to CG or EG1. Probiotic additive did not evoke oxidative stress, in spite of *C. jejuni* CCM6191 strain; but values of GPx were lower than in CG. Biochemical parameters were influenced to the reference levels.

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