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Effect of potential probiotic *Enterococcus faecium* strains on selected microflora in turkeys

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ABSTRACT: A group of twenty-six turkeys at the age of seven weeks was divided into four groups (7 birds in three groups 5 in control). The first group of turkeys was used as the control group. The other three groups were inoculated for 7 days with the following bacteria: *Enterococcus faecium* EE3 strain (2.1×10^9 cfu/ml), *E. faecium* EK13 strain (1.76×10^{10} cfu/ml) and *E. faecium* EF55 strain (5×10^8 cfu/ml). Sampling of faeces from each turkey was done at the beginning of experiment (at day 0) and in 7 days from the strain application. The total counts of EE3 strain in faeces of turkeys in EE3 group at day 7 were 4.07 ± 1.04 log cfu/g. At the same day bacteriocin-producing strain EK13 reached the value 5.26 ± 0.2 log cfu/g and the counts of EF55 strain amounted to 4.13 ± 0.64 log cfu/g. When total counts of *E. coli* colonies were checked at day 7 after the application of EE3 and EF55 strains to turkeys, significant differences in cell counts were found out (in EE3 group a difference of 2.43 log, $P < 0.01$; in EF55 group a difference of 1.93 log, $P < 0.001$) compared to the control and EE3 and EF55 groups. The highest reduction of *Pseudomonas* spp. was determined at day 7 after EE3 strain application (difference of 1.85; $P < 0.01$) and after EF55 strain application (difference of 0.78 log, $P < 0.05$) in comparison with the control group. EK13 strain did not influence the counts of *E. coli* and *Pseudomonas* spp. in faeces of turkeys. The average daily gain increased in all probiotic groups compared to the control group (EE3 group – 12.2%, EK13 group – 12.9%, EF55 group – 11.8%). At the beginning of experiment the values of total proteins were below the physiological limit in all groups. Although the intake of probiotic strains was associated with an increase in total proteins, the values were not adjusted to the physiological level. The values of total lipids were above the physiological level at the beginning of experiment. Administration of only EE3 strain significantly decreased the level of total lipids (difference of 1.22; log $P < 0.01$). The values of haematocrit, haemoglobin and activity of the blood enzyme glutathione peroxidase were not influenced.

Keywords: *Enterococcus faecium*; probiotic; intestinal microflora; daily gain; biochemical and zootechnical parameters

Diarrhoeas caused by infectious agents are responsible for large economic losses in animal production, including poultry farms. Antibiotics have traditionally been the most frequently administered drugs to control this type of infections. However, the current trend is to totally eliminate the prophylactic use of antibiotics in animal husbandry. The

use of several antibiotics has already been banned (Jin et al., 2000). Therefore, in an effort to prevent or reduce these disorders, alternatives are searched. Probiotics and bacteriocin-producing strains with probiotic properties have been suggested as alternative possibilities for the reduction or prevention of gastroenteritis (Elmer et al., 1996; Filho-Lima et al.,

2000). *In vitro* antagonistic effects of probiotics were already reported by several authors (Bomba et al., 1996; Jacobsen et al., 1999). Probiotic preparations are added to animal feeds with the intention to improve feed conversion, growth rate and prevention of diarrhoea (Fuller, 1999). Desired effects on weight gain and feed conversion in the range of 1 to 5% can be measured in many cases (Simon et al., 2001).

Enterococci belong to the group of lactic acid bacteria. Moreover, they are known as bacteriocin (antimicrobial) active bacteria (Franz et al., 1999). Although lactate exerts a bacteriostatic effect on bacteria, it is considered predominantly in lactobacilli as one of the probiotic effects in the control of pathogenic intestinal bacteria (Nousiainen and Setälä, 1993). Enterococci are mostly active by the effect of bacteriocin or both, lactic acid and bacteriocin. That is why even less lactic acid producing strains of enterococci (e.g. *Enterococcus faecium* EK13), however bacteriocin-producing ones (EK13), can have an effective probiotic influence in the animals. In addition, lactic acid bacteria are able to grow at lower pH values and thus dominate the proximal digestive tract of poultry (Kovalenko et al., 1989).

The objective of this study was to determine the effect of three *Enterococcus faecium* strains (orally administered) on the composition of intestinal microflora and on biochemical parameters of turkey poults. Moreover, their influence on bird weight as well as haematocrit, haemoglobin and activity of the blood enzyme glutathione-peroxidase (GSH-Px) was investigated.

MATERIAL AND METHODS

Enterococcus faecium strains EE3, EK13 and EF55 (isolated in our laboratory from commercial feed, sewage and from the crop content of a chick) were selected according to criteria for potential probiotics. They produce lactic acid and possess sufficient adhesive capability to the mucus (for EE3 strain it is 7.3%–7.4%; for EK13 strain it is 6.3%–6.7%; for EF55 strain it is 4.2%–5.8%). Moreover, *E. faecium* EK13 produces an antimicrobial substance enterocin A (Mareková et al., 2003) and *E. faecium* EF55 produces a bacteriocin-like substance EF55. Enterocin produced by EK13 strain are active against Gram-positive and Gram-negative bacteria, and the bacteriocin-like substance produced by EF55 strain inhibits the growth of Gram-positive bacteria (Mareková et al., 2003; Strompfová et al., 2003).

A group of twenty-six turkeys (BRANKO, Nitra, Slovak republic) at the age of seven weeks was divided into four groups: five birds in the control group and seven birds in the other groups. The experiment lasted for seven days. All birds received the commercial diet HYD 15 (TAJBA, Čaña, Slovak republic) and had an *ad libitum* access to water. The experiment was designed for a 4-week duration. However, according to technological parameters it was necessary to terminate it already after one week.

The first group of turkeys was used as the control group. The other three groups were applied for seven days at the same hour in the morning rifampicin resistant mutants of the following bacteria: the first EE3 group received *E. faecium* EE3 strain (10^9 cfu per ml), the second EK13 group was fed *E. faecium* EK13 strain (10^{10} cfu/ml) and the third EF55 group received *E. faecium* EF55 strain (10^8 cfu per ml). The doses of the strains (100 µl) were given *per os*. Rifampicin resistant mutants were obtained by subsequent cultivation of the strains using Todd-Hewitt agar (Becton and Dickinson, Cockeysville, USA) enriched with rifampicin (100 µg/ml) at 37°C. Sampling of faeces was carried out by the technique of attachment of polyethylene bags according to Grešáková et al. (2003) from each turkey at the beginning of experiment, i.e. before the strain application (at day 0), and then after 7 days to monitor the effect of added strains on the microflora in the faeces of turkeys. The samples of faeces were serially diluted in Ringer solution (pH 7; Oxoid, Basingstoke, England) according to the standard microbiological method and plated on the following media: Todd-Hewitt agar enriched with rifampicin for enumeration of rifampicin resistant mutants of EE3, EK13 and EF55 strains, *M-Enterococcus* agar (Becton and Dickinson, Cockeysville, USA) for enumeration of enterococci, De Man-Rogosa-Sharpe agar (Merck, Darmstadt, Germany) for lactic acid bacteria (LAB), MacConkey agar (Becton and Dickinson) for *E. coli*, Mannitol salt agar (Becton and Dickinson) for coagulase-negative staphylococci and Baird-Parker agar (Becton and Dickinson) for coagulase-positive staphylococci, Pseudocel agar (Becton and Dickinson) for pseudomonads. Enterococci and *E. coli* were cultivated at 37°C for 24 h. LAB and staphylococci were cultivated at 30°C for 48 h and pseudomonads were cultivated at 25°C for 48 h. Numbers of colony forming units (cfu) were expressed in \log_{10} cfu/ml/g \pm SD.

The birds were weighed at the beginning of experiment and after 7 days to record the influence of probiotic strains on daily gain.

The blood samples were taken at the beginning of experiments as well as after 7 days and the values of total lipids and proteins (g/l \pm SD) were determined. The activity of blood glutathione-peroxidase (GSH-Px) as well as the values of haematocrit (%) and haemoglobin (g/100 ml) were determined by commercial standard kit RANSEL from Randox (The United Kingdom) according to Paglia and Valentine (1967) and activity of GSH-Px was expressed in U/ml of blood.

Statistical analyses

Means and standard deviations were calculated using *t*-test. Statistical analysis of the GSH-Px activity was done by one-way analysis of variance (ANOVA) with the *post hoc* Tukey's post-test.

RESULTS

The total counts of selected bacterial groups in the faeces of turkeys are summarized in Table 1. The intake of probiotic strains of *E. faecium* was not associated with any clinical evidently adverse effect. After 7 days of application the count of EE3 strain in faeces of turkeys in EE3 group was 4.07 ± 1.04 log cfu/g. At the same time, enterocin A producing strain EK13 reached the value 5.26 ± 0.2 log cfu/g and finally EF55 strain amounted to 4.13 ± 0.64 log cfu/g (Table 1). The same counts of total enterococci (8.05 ± 0.02 log cfu/g) were estimated in control as well as in EK13 group after 7 days of its application. Although EE3 and EF55 strains colonized the gastrointestinal tract of turkeys sufficiently, the total counts of enterococci in those groups did not achieve the counts of enterococci in the control group. A similar situation was found out in lactic acid bacteria (LAB, Table 1). However, a slight decrease of staphylococci was detected after the application of EE3 and EF55 strains to turkeys (difference of 0.5 log in EE3 group, difference of 0.43 log in EF55 group). EK13 strain did not influence the presence of staphylococci in faeces of turkeys in comparison with the control group. When total counts of *E. coli* and *Pseudomonas* spp. colonies were compared in control, EE3 and EF55 groups in 7 days from the application of both strains (EE3 and EF55 strains) to turkeys, significant differences in cell counts were found out (difference of 2.43 log for *E. coli*, $P < 0.01$; difference of 1.93 log for *E. coli*,

Table 1. *In vivo* effect of probiotic strains of *E. faecium* on bacteria in the gastrointestinal tract of turkeys

Group	Control group (n = 5)		EE3 group (n = 7)		EK13 group (n = 7)		EF55 group (n = 7)	
	before appl.	after appl.	before appl.	after appl.	before appl.	after appl.	before appl.	after appl.
RIF mutant of strain	–	–	–	4.07 ± 1.04	–	5.26 ± 0.2	–	4.13 ± 0.64
<i>Enterococcus</i> spp.	4.29 ± 0.38	8.04 ± 0.03	5.47 ± 0.95	5.06 ± 2.19	5.91 ± 0.66	8.05 ± 0.02	5.67 ± 0.61	6.02 ± 0.68
LAB	5.99 ± 0.67	7.76 ± 0.23	6.69 ± 0.99	5.72 ± 1.42	6.89 ± 0.4	7.97 ± 0.09	5.46 ± 0.21	6.39 ± 0.76
<i>Staphylococcus</i> spp.	4.78 ± 0.17	5.06 ± 0.68	5.26 ± 0.19	4.56 ± 1.18	5.28 ± 0.17	5.64 ± 0.21	5.11 ± 0.29	4.63 ± 1.08
<i>E. coli</i>	5.48 ± 0.97	6.59 ± 0.41	6.28 ± 0.84	$4.16 \pm 2.01^{**}$	5.74 ± 0.96	6.92 ± 0.14	6.14 ± 0.49	$4.66 \pm 0.62^{***}$
<i>Pseudomonas</i> -like	3.21 ± 1.31	3.37 ± 0.7	3.56 ± 0.51	$1.52 \pm 1.38^{**}$	3.32 ± 0.41	3.96 ± 0.75	3.57 ± 0.46	$2.59 \pm 0.61^*$

LAB – lactic acid bacteria

Faecal samples were tested: before appl. – before application of strains probiotic (at day 0); after appl. – after application of strains probiotic (at day 7)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 2. Selected blood biochemistry in turkeys before and after application of probiotic strains of *E. faecium*

Group	TP (45.0–62.0 g/l)		TL (5.0–7.0 g/l)	
	before appl.	after appl.	before appl.	after appl.
Reference group	33.49 ± 4.89	37.04 ± 6.36	8.85 ± 0.7	8.89 ± 0.77
EE3	33.49 ± 4.89	37.04 ± 3.51	8.85 ± 0.7	7.67 ± 0.37**
EK13	33.49 ± 4.89	40.93 ± 4.62	8.85 ± 0.7	8.38 ± 0.51
EF55	33.49 ± 4.89	43.69 ± 10.71	8.85 ± 0.7	8.72 ± 1.23

TP – total proteins; TL – total lipids

before appl. – before application of strains probiotic (at day 0); after appl. – after application of strains probiotic (at day 7)

** $P < 0.01$

Table 3. Selected blood parameters in turkeys before and after application of probiotic strains of *E. faecium*

Group	Ht (%)		Hb (11.45–12.22 g/dl blood)		GPx (U/g Hb)	
	before appl.	after appl.	before appl.	after appl.	before appl.	after appl.
Reference group	33.48 ± 0.99	36.76 ± 1.61	17.37 ± 1.45	17.91 ± 1.10	44.76 ± 4.38	40.74 ± 8.12
EE3	33.48 ± 0.99	32.42 ± 1.47	17.37 ± 1.45	14.49 ± 3.52	44.76 ± 4.38	41.32 ± 9.80
EK13	33.48 ± 0.99	34.72 ± 2.82	17.37 ± 1.45	17.60 ± 1.42	44.76 ± 4.38	39.39 ± 6.21
EF55	33.48 ± 0.99	32.50 ± 1.77	17.37 ± 1.45	16.05 ± 3.60	44.76 ± 4.38	42.86 ± 11.16

Ht – haematokrit; Hb – haemoglobin; GPx – glutathione peroxidase

before appl. – before application of strains probiotic (at day 0); after appl. – after application of strains probiotic (at day 7)

$P < 0.001$). The highest decrease of *Pseudomonas* spp. was determined at day 7 after EE3 strain application (difference of 1.85; $P < 0.01$) as well as after EF55 strain application (difference of 0.78 log, $P < 0.05$) in comparison with the control group. EK13 strain did not influence the counts of *E. coli* and *Pseudomonas* spp. in faeces of turkeys.

At the beginning of experiment the values of total proteins were below the physiological limit in all groups. Although the intake of probiotic strains was associated with an increase in total proteins, the values were not adjusted to the physiological level. The values of total lipids were above the physiological level at the start of experiment. The administration of only EE3 strain significantly decreased the level of total lipids (difference of 1.22; log $P < 0.01$) compared with the control group. The values of total lipids were not influenced in the other groups (Table 2). The values of haematocrit, haemoglobin and of the blood enzyme glutathione peroxidase were not influenced either (Table 3).

The average daily gain of birds in EE3 group was 5.9 g while it was 4.8 g in the control group. The daily gain in EE3 group expressed as percentage

was 12.2%. In EK13 group, the average daily gain was 6.2 g. Comparing EK13 and control groups, a 12.9% difference in average daily gain was recorded (6.2 g and 4.8 g, respectively; expressed in %) in EK13 group. And finally, in EF55 group an 11.8% increase in daily gain of birds was found out compared to EF55 group, in which the average daily gain was 5.7 g, and to control group.

DISCUSSION

Hatching, determination of sex and transport of animals are accompanied by bacterial colonization when enterobacteria and cocci are undoubtedly the first bacterial community in the turkey poult intestine. High numbers of facultative anaerobic bacteria were reported in chickens in the first week of life, which gradually decreased with the chicken age (Vahjen et al., 1998). Bacterial colonization means species and size diversity (Case, 1990) and thus massive doses of probiotics cells can repress potentially harmful bacteria (Vahjen et al., 2002). The probiotic strains used in this study colonized

the gastrointestinal tract of turkeys sufficiently. Moreover, some inhibitory effects against spoilage bacteria (*E. coli*, *Pseudomonas* spp.) were observed after the application of EE3 and EF55 strains. The fact that the total counts of enterococci and LAB in EE3 and EF55 groups did not achieve their counts in the control and EK13 groups could probably be explained by competitive relations within the intestinal microflora of turkeys. Staphylococci were reduced after the application of EE3 and EF55 strains. Stropfová et al. (2003) also reported the greatest cell count reduction of staphylococci (reduction of 22.9%) at the beginning of the administration of bacteriocin-like substance produced by *E. faecium* EF55 in Japanese quails. Jin et al. (1996) reported that chickens fed a diet supplemented with commercial lactobacilli showed reduced numbers of coliforms in the intestine. Francis et al. (1978) found out a significant decrease of coliform bacteria in the intestine and caeca of turkeys after the feeding of probiotic *Lactobacillus* product. A reduction of pathogenic *E. coli* was also observed in the gastrointestinal tract of gnotobiotic chickens dosed with *Lactobacillus acidophilus* (Watkins et al., 1983). A bacteriocin-producing strain can successfully influence microbial community in the ecosystem by its bacteriocin; it seems that in our study *E. faecium* EK13 did not have any influence on the intestinal microflora of turkeys. Although EK13 strain reached 5.26 ± 0.2 log cfu/g in the digestive tract of turkeys at day 7 after its application, probably its growth was so fast that there was a misbalance between growth and bacteriocin production, i.e. bacteriocin production was insufficient. It seems that the beneficial effect of live *E. faecium* strains (EE3 and EF55) was not due only to the reduction of the intestinal population of pathogenic bacteria. On the other hand, *E. faecium* EK13 strain showed a favourable effect on the daily gain in turkeys. Beneficial effects of EE3 strain on daily gain in Japanese quails (7.6%; Marciňáková et al., 2004) as well as the influence of EK13 strain on daily gain in rabbits were recently reported by Lauková et al. (2004).

Although the intake of probiotic strains was associated with an increase in total proteins but with a decrease in total lipids, the values were not adjusted to the physiological level. Similar results were obtained after EE3 strain application to healthy dogs; a decrease in total lipids in the blood of dogs was found out (Marciňáková et al., 2005). On the other hand, Stropfová (2004) and Simonová (personal communication) reported an increase in blood

lipids in dogs and rabbits after the intake of probiotics *Lactobacillus casei* AD1 and *L. rhamnosus* GG strains. So, there are some controversial effects of probiotic bacteria on the lipid metabolism. The discrepancies in the results could be associated with the use of different species and strains (Stropfová, 2004; Marciňáková et al., 2005). The values of haematocrit, haemoglobin and activity of the blood enzyme glutathione peroxidase were not influenced. Comparable results (GSH-Px not influenced) were obtained after the administration of EE3 strain under *in vivo* conditions using animal model – Japanese quails (Marciňáková et al., 2004).

The results indicate that the administration of *E. faecium* EE3 and EF55 strains reduced the levels of spoilage microorganisms such as *E. coli* and pseudomonads in the digestive tract of turkeys. The feeding of EK13 strain increased the body weight of turkeys. This strain also showed sufficient colonization in the digestive tract of turkeys. However, its antimicrobial effect was not shown here.

Although the efficacy of different probiotics was already discussed in literature (Ouweland et al., 2002), under our conditions two mechanisms of the potential influence of probiotics could be speculated; probably they affect by competition to adhere the mostly part of intestinal tissue (EE3, EK13) and/or to produce an inhibitory substance (EK13, EF55).

All strains could be used as potential probiotics; EK13 and EE3 strains improved zootechnical parameters. Moreover, EE3 and EF55 strains showed antimicrobial effects. Of course, a longer period of experiments is requested.

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