EFFECT OF POTENTIAL PROBIOTIC ACTIVITY OF ENTEROCOCCUS FAECIUM EE3 STRAIN AGAINST SALMONELLA INFECTION IN JAPANESE QUAILS

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Abstract

Thirty-two one-day-old Japanese quails were divided into 4 equal groups. The first group was the reference control group. The second control group (PT4CG) was infected per os with Salmonella PT4 strain (1.0 x 107 cfu/ml), the third control group (EE3CG) was treated with Enterococcus faecium EE3 strain (1.0 x 108 cfu/ml) and the fourth experimental group (EG) was infected with PT4 strain 8 h before the application of EE3 strain. The birds of the EG and EE3CG received 100 µl of an overnight culture of EE3 strain. The birds in the PT4CG received a placebo. Faecal samples were taken at 0, 16, 72, 118 and 168 h after the first application of EE3 strain. The quails were sacrificed and the survival of EE3 strain as well as PT4 strain was estimated not only in faeces but also in the caecum. The inhibitory effect of EE3 strain against Salmonella PT4 in the EG was found after 16 h; the difference 0.24 log cycles between PT4 and EE3 counting was detected. This effect was prolonged up to the end of the experiment. In the caecum no influence of S. enterica due to EE3 strain was detected. The increase of average daily weight in birds was 7.6% in the EE3CG in comparison to the average daily gain in the reference control group. The average daily gain was higher (10%) in the EG in comparison to the average daily gain in the PT4CG. Comparing the EG and the EE3CG, a 11% lower daily gain in birds was noted in the EG. The lowest value of lactic acid (LA) was in the reference samples. On the other hand, the highest value of LA was observed in the EG of birds (58.12 ± 10.91 mmol/l). Glutathione-peroxidase (Px) activity as well as the evidently good health of Japanese quails indicated that oxidative stress was not evoked by Salmonella infection in this experiment.

Key words: Japanese quails, Enterococcus faecium, Salmonella, glutathione-peroxidase, inhibitory activity.

Salmonellosis has become in developed countries one of the most important zoonoses transmitted by meat (5). Domestic animals, including poultry, are large reservoirs of Salmonella (4) and Salmonella-infected poultry or other animals represent a source of infection for humans (10). It was reported that animals can be infected by contaminated feed, chronic carriers introduced into the herd, rodents or people who visited a contaminated farm before entering the production unit (15, 23). Once ingested, Salmonella attaches to and penetrates the intestinal mucosa and invades the lamina propria where it is phagocytized by macrophages. Salmonella is then spread throughout the body into organs such as tonsils, Peyer’s patches and gastric, hepatic, jejunal; and ileo-caecal lymph nodes (11). Although infections by S. enterica serovar Enteritidis in man has been decreased a little bit during last 5 years, it still is a serious problem. Much of that has been associated with eating undercooked or raw eggs. Transovarian transmission is probably the primary means of its spread to man (7, 9). In many countries, efforts are now being made to reduce the incidence of Salmonella infection at the farm level (15). Nisbet et al. (19) used competitive exclusion to control Salmonella in swine. One form of treatment or protection may be provided by probiotics - live microbial cultures that can be administered to animals and to humans (21).

Enterococci belong to those lactic-acid bacteria that are inhabitants of human and animal intestines (8). But enterococci can be also found in the environment (14). Strains with probiotic character were also detected among enterococci (1).

Living organisms have developed a complex antioxidant network to neutralize excessive and inappropriate reactive oxygen species continuously formed during metabolic processes, mainly during various pathological events. The most commonly examined antioxidant enzyme is a specific selenoprotein glutathione – peroxidase (GSH-Px) as a part of the cellular antioxidant defense which play major role in scavenging of such oxidants (18).

The purpose of the work reported here was to study the microbiological parameters in the ecosystem of Japanese quails after application of Enterococcus.
faecium EE3 strain with probiotic character to demonstrate its antagonistic activity against S. enterica serovar Enteritidis PT4 strain. Moreover, its influence on bird weight as well as GSH-Px blood enzyme was investigated.

Material and Methods

Ent. faecium EE3 strain was isolated from the commercial dog feed. It was genotyped using tDNA-intergenic PCR according to Baele et al. (3). The EE3 strain possess strong adhesive capability to human as well as to canine mucosa (human 7.3%, canine 7.4%). It is resistant to ampicillin, tetracycline, kanamycin, gentamycin and rifampicin and sensitive to vancomycin. In addition, EE3 strain is bile tolerant (growing even in the presence of 5% of oxgall) and lactic acid producing (0.99 ± 0.17 mmol/l) with ureolytic activity (1.05 ± 0.17 nkat/ml). For in vivo test using model animals - Japanese quails, the rifampicin resistant mutant of EE3 strain was used. It was obtained by subsequent cultivation of EE3 strain using Todd-Hewitt agar (Becton and Dickinson, Cockeysville, USA) enriched with rifampicin (100 µg/ml) at 37°C. S. enterica serovar Enteritidis PT4 strain was supplied by Dr. Šišák (Institute of Veterinary Medicine, Brno, Czech Republic). It was grown in Trypticase soy broth (Becton and Dickinson) at 37°C.

Thirty-two 1-day-old Japanese quails (Farm Rozhanovec, Slovakia) were divided into four equal groups. The first group was used as the reference control group (without infection and treating with microorganisms). The second control group (PT4 group) was infected with Ent. enterica, the third control group (EE3 group) was inoculated with Ent. faecium and the fourth - experimental group was treated with S. enterica and then with Ent. faecium.

The birds were housed in boxes (each group in the separate box) in one room. The birds were fed a commercial feeding mixture (KZBŽ-1, Rozhanovce, Slovakia) and had free access to water. The experiment lasted for seven days.

At the start of the experiment, the birds of PT4 and experimental groups were infected with 100 µl of PT4 strain of S. enterica serovar Enteritidis (1.0 x 10⁶ c.f.u./ml). Then every day, at the same time in the morning, the birds of EE3 and experimental groups received 100 µl of an overnight culture of EE3 strain of Ent. faecium (1.0 x 10⁶ c.f.u/ml). The first dose was given per os (using syringe) 8 h after the infection with S. enterica. The later doses were applied into the drinking water. The birds of PT4 group received a placebo.

Samples of faeces (1 g) were collected at 0, 16, 72, 118 and 168 h after the first application of EE3 strain and examined for both, S. enterica and Ent. faecium. The quails were then sacrificed according to Ethic Commission of Regional Veterinary Administration and the survival of S. enterica as well as the growth of EE 3 strain in the faeces and caecum were checked. The survival of S. enterica and growth of Ent. faecium were determined by plating of appropriate dilutions of the samples in saline solution (0.85%; pH 7.0) on TH agar with rifampicin for EE3 strain of Ent. faecium and on Brilliant green agar (Becton and Dickinson, Cockeysville, USA) for PT4 strain of S. enterica. The plates were incubated at 37°C and checked for colonies after incubation for 24 – 48 h. The growth of enterococci in the reference control group was examined using M-Enterococcus agar (Becton and Dickinson). The bacterial counts are expressed in log 10 c. f. u./g ± SD.

The birds were weighed at the beginning as well as at the end of experiment and the increase or decrease in average daily gain was recorded.

Lactic acid production from jejunal content was tested using capillary isotachophoresis and expressed in mmol ± SD. The blood samples were taken after sacrificing of the birds (at the end of the experiment). The activity of blood glutathione-peroxidase (GSH-Px) was determined by commercial standard set RANSEL from Randox, UK (22) and expressed in U/ml of blood. Statistical analysis of the GSH-Px activity was done by one-way analysis of variance (ANOVA) with the post hoc Tukey post-test. The results are quoted as means ± SEM.

Results

The total counts of enterococci in faeces of the birds from the reference control group reached 7.26 ± 0.29 log 10 cfu/ml/g at the start of the experiment; at the end of experiment it was 5.17 ± 0.28 log 10 cfu/ml/g (Table 1). After 16 h enterococci reached 5.71 ± 0.38 log 10 cfu/ml/g in the caecum of the reference birds. Faeces and caecum of reference birds were during all the experiment Salmonella free. At the start of the experiment (0 h) the counts of EE3 strain in faeces from EE3 group as well as from experimental group reached 7.34 ± 0.23 log 10 cfu/ml/g. After 16 h EE3 strain reached the count of 6.39 ± 0.32 log 10 cfu/ml/g in experimental group (infected with PT4 strain and simultaneously treated with EE3 strain) and 6.48 ± 0.37 log 10 cfu/ml/g in EE3 group (Table 1). After 16 h the inhibitory effect of EE3 strain against Salmonella PT4 strain was found; in experimental group and the difference 0.24 log cycles between PT4 and EE3 strain counting was detected (Table 1). This effect was prolonged up to the end of the experiment (after 118 h - 1.00 log cycles, after 168 h - 0.88 log cycles). Surprisingly, in the caecum no influence of S. enterica due to EE3 strain was detected.

The average daily weight gain of birds in the control EE3 group was 22.6 g, while in the reference control group it was 19.5 g. That is, the difference (7.6%) between these two groups was recorded. In the experimental group, the average daily weight gain was 18.7 g. Comparing the experimental and the control PT4 groups, a 10% higher average daily weight gain was recorded (18.7 g; 17.0 g) in the experimental group. Comparing the experimental and EE3 groups, a 11% lower daily weight gain was noted in the experimental group (18.7 g; 22.6 g). The lowest value of lactic acid
(LA) was measured in the reference samples (average 32.29 ± 6.18 mmol/l). On the other hand, the highest value of LA was determined in the experimental group (58.12 ± 10.91 mmol/l). In both control EE3 and PT4 groups similar values of LA were measured (40.55 ± 8.576; 41.83 ± 5.98 mmol/l). No influence of Salmonella contamination as a potential inducer of oxidative stress was detected in the activity of blood GSH-Px (Table 2). No significant effect of the probiotic EE3 strain used was determined as well.

### Table 1
Counts of *S. enterica* serovar Enteritidis PT4 and *Ent. faecium* EE3 strains in faeces

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Reference control group</th>
<th>PT4 control group</th>
<th>EE3 control group</th>
<th>Experimental group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PT4</td>
<td>EE3</td>
<td>PT4</td>
</tr>
<tr>
<td>0 h</td>
<td>7.26 ± 0.29</td>
<td>-</td>
<td>7.34 ± 0.23</td>
<td>-</td>
</tr>
<tr>
<td>16 h</td>
<td>-</td>
<td>6.48 ± 0.27</td>
<td>6.48 ± 0.37</td>
<td>-</td>
</tr>
<tr>
<td>72 h</td>
<td>-</td>
<td>6.65 ± 0.32</td>
<td>7.17 ± 0.24</td>
<td>6.82 ± 0.23</td>
</tr>
<tr>
<td>118 h</td>
<td>-</td>
<td>5.68 ± 0.35</td>
<td>7.79 ± 0.29</td>
<td>6.73 ± 0.21</td>
</tr>
<tr>
<td>168 h</td>
<td>5.17 ± 0.28</td>
<td>5.13 ± 0.42</td>
<td>8.07 ± 0.36</td>
<td>6.89 ± 0.37</td>
</tr>
</tbody>
</table>

All counts are expressed in log10 CFU/ml

a The birds with *S. enterica* serovar Enteritidis PT4

b The birds with *Ent. faecium* EE3
c The birds with *Ent. faecium* EE3 and *S. enterica* PT4

d The birds with *Ent. faecium* EE3 and *S. enterica* PT4

### Table 2
The activities of blood glutathione peroxidase (GSH-Px)

<table>
<thead>
<tr>
<th>Reference control group</th>
<th>EE3 control group</th>
<th>PT4 control group</th>
<th>Experimental group</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH-Px (U.ml⁻¹ blood)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>43.24 ± 0.75</td>
<td>48.63 ± 2.88</td>
<td>50.18 ± 5.18</td>
<td>50.70 ± 0.35</td>
</tr>
</tbody>
</table>

All values are expressed in log10 cfu/ml

a The birds with *Ent. faecium* EE3

b The birds with *S. enterica* serovar Enteritidis PT4
c The birds with *Ent. faecium* EE3 and *S. enterica* PT4

d The birds with *Ent. faecium* EE3 and *S. enterica* PT4

### Discussion

In naturally infected, especially young chickens, high mortality has been primarily associated with *S. enteritis* serovar Enteritidis PT4 (20). In this study the reducing effect of *Ent. faecium* EE3 strain against *S. enterica* serovar Enteritidis PT4 was found. Detection of almost the same counts of EE3 strain in the caecum and faeces at the end of the experiment indicates that EE3 strain was probably not strongly adhered to the epithelial tissues; in spite of the fact that EE3 strain possesses good adhesive capability. Lauková et al. (13) reported an antagonistic effect due to ent-A producing *Ent. faecium* EK13 strain against *S. enterica* serovar Düsseldorf SA31 in the model of gnotobiotic Japanese quails. Evenly protective effect of EK13 strain on the duodenal epithelium was found before and after *Salmonella* infection (6). Audisio et al. (1) presented an antagonistic effect of *Ent. faecium* J96 strain against human and poultry isolates of *Salmonella* sp., and suggested its possible use as avian probiotic. The same authors confirmed preventive effect of J96 strain against *Salmonella pullorum* in chickens (2). The administration of *Ent. faecium* provided protection against the experimental challenge with *S. enterica* serovar Typhimurium in gnotobiotic mice (17). However, this protective effect was not due to the reduction of intestinal population of the pathogenic bacteria. In any case, *Ent. faecium* EE3 strain have favorable effect on the daily weight gain of Japanese quails; the average daily weight gain was higher in the control EE3 group to compare with the reference group (7.6%). Lauková et al. (unpublished data) reported benefit influence of bacteriocinogenic *Ent. faecium* EK13 strain with
probiotic character on daily weight gain in rabbits and 1.0% increase in daily weight gain in rabbits was found after administration of probiotic *Ent. faecium* M74 strain. The high value of LA in the jejunum indicates that EE3 strain colonizes the digestive tract of birds sufficiently; however, in spite of its good adhesive capability, it was probably quickly passed. The measurement of the activity of GSH-Px as well as the evidently good health of Japanese quails indicated that oxidative stress was presumably not evoked by *Salmonella* infection in this experiment. However, the GSH-Px is only one indicator of oxidative stress from a complex antioxidative network in the organism. It would be interesting to confirm this explanation by the determination of other antioxidant enzymes and/or using antioxidant strains with desirable properties based on increased resistance of some strains to toxic oxidative compounds (12).

*Ent. faecium* EE3 strain might be potentially used for the protection of the hosts against *Salmonella* infections. Of course, additional experiments are required to better understand this protection, especially in accordance with immunological parameters.

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