Effect of preventive application of *Enterococcus faecium* EF55 on intestinal mucosa during salmonellosis in chickens

R. Herich[1], T. Kokinčáková[2], A. Lauková[3], M. Levkutová[2]

[1]Institute of Pathology, University of Veterinary Medicine, Košice, Slovak Republic
[2]Institute of Epizootology and Infectious Diseases, University of Veterinary Medicine, Košice, Slovak Republic
[3]Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic

ABSTRACT: In the present study the effect of preventive application of *Enterococcus faecium* EF 55 on the intestinal mucosa was evaluated in experimentally infected chickens with *Salmonella enterica* subsp. *Enteritidis*. A total of 120, one-day-old *Salmonella*-free chickens of Isa Brown hybrid were divided into 4 groups. The chickens in groups E and ES were perorally inoculated with *E. faecium* EF55 in a dose of 1×10^9 CFU/ml for 7 consecutive days. Placebo was applied to birds in control group C and group S during the first 7 days of life. At the age of 8 days chickens in groups ES and S were perorally infected with *S. enterica* subsp. *Enteritidis* phage type 4 in a dose of 1×10^8 CFU/ml. In birds treated with *E. faecium* EF 55 (group ES) a decreased number of *Salmonella* spp. positive individuals was recorded from 28.5% 2 days post infection (p.i.) to 10% 14 days p.i. when the difference between group ES and group with the application of *Salmonella* Enteritidis alone (group S) was significant ($P < 0.01$). On the contrary, in birds of group S the percentage of *Salmonella* spp. positive animals showed no constant changes. It increased from 12.5% 2 days p.i. to 37.5% 4 days p.i. The maximum of positive samples 83.3% was found 14 days p.i. The application of *E. faecium* EF55 reduced colonisation of caeca and minimized translocation of salmonellae into the liver and spleen. Two days p.i. the shortest villi in the jejunum were observed in group S – 1 266.2 μm, when compared to group E with the highest jejunal villi – 1 605 μm ($P < 0.05$). The growth of the villi was observed 14 days p.i. in all groups except group S. The early exposition of chickens to *E. faecium* EF55 led to more rapid development of intestinal villi when compared to the untreated control ($P < 0.05$). Reduced colonisation of the intestinal tract by salmonellae in birds treated with *E. faecium* EF 55 also preserved the microenvironment of the intestine from harmful effects of the pathogen.

Keywords: *Enterococcus faecium*; *Salmonella* spp.; PCR; villi; chickens

Salmonellosis is one of the most widespread zoonoses throughout the world. It is difficult to take efficient control measures because there are numerous potential sources of infection and product contamination in an integrated poultry enterprise. In live birds the transmission is usually asymptomatic, and the organisms are shed, some intermittently, in the faeces.

Salmonellas are not native members of the intestinal microbiota, but young chicks are readily colonised, and the organisms may persist in the host for some weeks or during all of the rearing period.

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The oral route of infection of chickens with *Salmonella enterica* subsp. *Enteritidis* results in attachment and entering of bacteria to the epithelial cells of intestinal villi (Asheg et al., 2003). After lysis of the host cells, bacteria are found in the *lamina propria* lying free or inside macrophage-like cells (Desmidt et al., 1998).

One of the control measures to reduce the intestinal colonisation and lower the shedding of salmonellae is the prophylactic treatment of chicks using competitive exclusion. Protective microflora produces antibacterial substances and stimulates the production of specific antibodies and mucus. The early colonization of the gut by living microorganisms is important for the development of the gut protection barrier (Herich and Levkut, 2002). Several experiments demonstrated that probiotic bacteria significantly decreased the caecum colonisation by pathogens (Pascual et al., 1999; La Ragione et al., 2001).

Limited information is available about the penetration of salmonellae into the internal organs in the colonisation of the gut by probiotics. Probiotics could represent an effective alternative to synthetic substances recently used in nutrition and medicine (Strompfová et al., 2004).

Probiotics are cultures of potentially beneficial bacteria that positively affect the host by regulating the microbial balance and by restoring the normal intestinal permeability and gut micro-ecology (Herich and Levkut, 2002). They also improve the intestine immunological barrier function (Vazquez-Tores et al., 1999). The small intestine undergoes age-dependent important changes and the preservation of the well developed mucosal surface ensures optimal digestion and absorbent abilities. The presence of normal and probiotic microflora influences the structure of the host intestinal mucous membrane (Gugolek et al., 2004).

Most of probiotic bacteria are of intestinal origin and belong to the genera *Bifidobacterium* and *Lactobacillus.* Strains from other genera used as probiotics include also *Enterococcus faecium* (Holzapfel et al., 1998). *E. faecium* EF55 is a bacteriocin-producing strain isolated and characterised in the Laboratory of Animal Microbiology, Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovakia (Strompfová et al., 2003), which was successfully used in many experiments.

The present study evaluates the effect of preventive application of *E. faecium* EF55 in chickens experimentally infected with *S. enterica* subsp. *Enteritidis* strain.

MATERIAL AND METHODS

Experiment design

A total of 120, one-day-old *Salmonella*-free chickens of Isa Brown hybrid were divided into 4 groups. The pen (1 000 × 950 × 500 mm) was lit continuously and the optimal temperature was maintained according to the age of birds. Water and commercial feed mixture for chickens were available *ad libitum*. Chickens received a diet containing 11.9 MJ, 195 g crude protein/kg, 8 g ash per kg, 5 g fibre/kg, 10.5 g lysine/kg, 7.5 g methionine and cysteine/kg, 10 g linolenic acid/kg, 8 g Ca/kg, 5 g P/kg, 2 g NaCl/kg, 0.07 g Mn/kg, 0.06 g Fe/kg, 0.006 g Cu/kg, 0.05 g Zn/kg, 10 000 IU vitamin A per kg, 2 000 IU vitamin D3/kg, 0.015 g vitamin E/kg, 0.004 g vitamin B2/kg, 10 µg vitamin B12/kg and 0.5 g choline/kg. Diciazuril 1 mg/kg was used as a coccidiostatic drug.

The chickens in groups E and ES were perorally inoculated with *E. faecium* EF55 in a dose of 1×10^9 CFU/ml in 0.2 ml of LB for 7 consecutive days. Placebo was applied to birds in control group C and group S during the first 7 days of life. At the age of 8 days chickens in groups ES and S were perorally infected with *S. enterica* subsp. *Enteritidis* phage type 4 (kindly provided by Dr. Šišák – Institute of Veterinary Medicine, Brno, Czech Republic) in a dose of 1×10^8 CFU/ml in 0.2 ml of LB.

Six birds from each group were euthanized 2, 4, 6, 8, and 14 days after infection (p.i.) with the pathogen. For statistical analysis of results the Chi-square test and one-way ANOVA were used.

Ethical Commission of the University of Veterinary Medicine approved the experimental protocol. Animal care was provided according to the acceptance of the State Veterinary and Food Administration of the Slovak Republic.

DNA isolation and PCR amplification

0.2 g of the fresh content of caeca and 0.2 g of samples from liver and spleen were collected and pre-cultivated at 37°C for 8 h in 2 ml of LB medium (Oxoid, UK). The samples were then centrifuged at 16 000 g for 3 min. The pelleted bacteria were washed twice with PBS and finally resuspended in 50 µl of PBS.

The isolation of bacterial genomic DNA was performed with Wizard Genomic DNA purification kit (Promega, USA).

The sequences of the primer pairs used for DNA amplification of the *invA* gene region of *Salmonella* spp. were as follows: INVA-1 5'-ACAGTGCTCGTTTACGACCTGAAT-3' and INVA-2 5'-GACGACTGGTACTGATCGATAAT-3' (Invitrogen, USA), prepared according to Chiu and Ou (1996).

Escherichia coli DNA isolated by the same protocol was used as negative control template DNA. DNA isolated from the pure culture of *S. enterica* subsp. *Enteritidis* served as positive control.

The PCR mixture contained 0.5 µM of each primer, 0.2mM of each deoxynucleoside (dATP, dTTP, dCTP, dGTP) (Fermentas, Lithuania), 2.5mM $MgCl_2$ (Fermentas, Lithuania), 1× PCR buffer (Fermentas, Lithuania), 1.25 U Taq polymerase (Fermentas, Lithuania), 5 µl of isolated DNA and H_2O to the total volume of 50 µl. The amplification conditions were as follows: initial denaturation at 94°C for 1 min, 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, final elongation at 72° for 10 min. Techne PTC termocycler (Techne, UK) was used for amplification of samples. PCR products (5 µl of each) were separated by electrophoresis in 1% agarose gels buffered with 1X TAE (Merck, Germany) containing 0.5 µg/ml ethidium bromide (Promega, USA). The gels were photographed and analysed with Doc-Print II (Vilber Lourmat, France). The molecular mass standard GeneRuler 100 bp DNA Ladder Plus (Fermentas, Lithuania) was used according to the manufacturer's instructions.

Histological examination

Medial parts of the jejunum were taken immediately after death from 6 birds of each group. The samples were fixed in 10% formalin for 48 hours. After fixation the samples were drained and embedded into paraffin. Three micrometres thick sections were then stained with haematoxylin-eosin and mounted into Canadian balm. The slides were analysed using an optical microscope (Nikon, Japan) at 100× magnification. The height of the villi: 20 villi from each section were measured by Ellipse software for image analysis (Vidito, Slovak Republic).

RESULTS

The amplification of samples with isolated genomic DNA using INVA-1 and INVA-2 primers brought a PCR product of 243 bp size (Figure 1). The Gen Bank program BLAST was used to ensure that the proposed primers would be complementary with the target species.

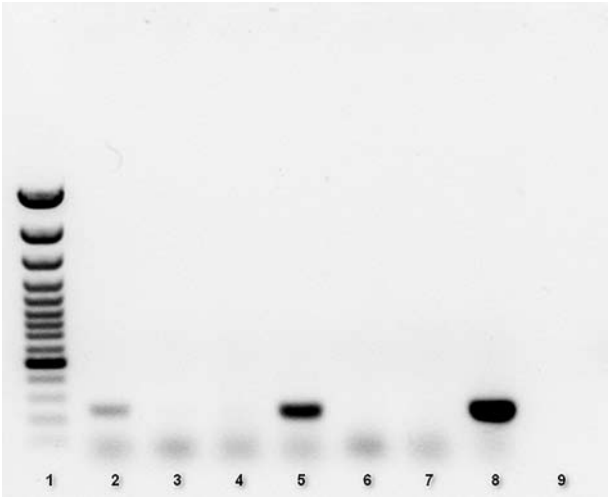

Figure 1. PCR of samples obtained from the content of caeca (lane 1 – Gene Ruler 100 bp DNA Ladder Plus, the lowest size 100 bp, lane 2–4 samples from group ES, lane 5–7 samples from group S, lane 8 – positive control, lane 9 – negative control)

In group S amplicons of this size were detected in more samples during the experiment except 2 days p.i. A significant increase ($P < 0.01$) was recorded 14 days p.i. when compared to the group treated with *E. faecium* EF 55 (Figure 2).

In accordance with overall results the evaluation of detection of salmonellae in particular organs showed less positivity in birds preventively treated with *E. faecium* EF55. The evaluation of *Salmonella* spp. positivity in caeca showed 2.5 times higher numbers of *Salmonella* spp. positive samples detected from day 6 to day 14 p.i. in birds from group S. In liver a similar difference was recorded and salmonellae were detected in twice more samples in group S when compared to the group treated with *E. faecium* EF55 from day 4 to day 14 p.i. In spleen three times more *Salmonella* spp. positive samples were found on day 6 p.i. in group S when compared to group ES. During the followed samplings a smaller difference was found between the infected groups but the ratio of infected samples still reached 60% in group ES compared to 90% in group S.

In uninfected groups (E and C) all samples were negative for the *Salmonella* spp. presence during the whole experiment.

At the age of 10 days (2 days p.i.) histomorphological changes in the jejunum showed significant

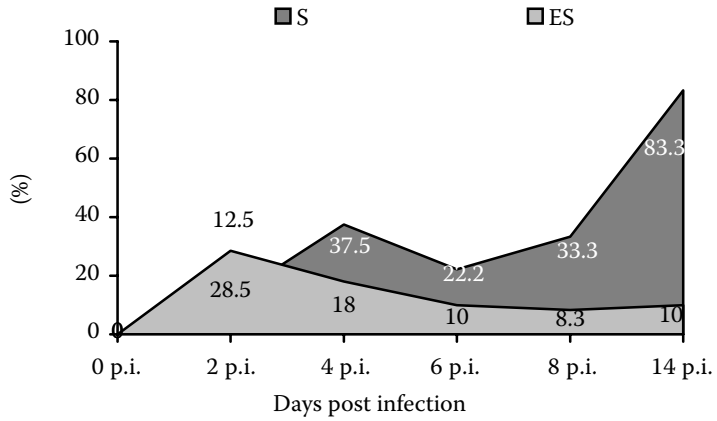

Figure 2. Percentage of *Salmonella* spp. positive samples found in group ES and S during the experiment

differences ($P < 0.01$, $P < 0.05$) among groups treated with *E. faecium* EF55 and untreated groups. The shortest villi were observed in the group of birds infected only by salmonellae – 1 266.2 μm (Table 1). The growth of villi was recorded in all experimental groups 14 days p.i. with the exception of group S. The recorded differences were significant ($P < 0.05$). The highest jejunal villi were measured in the group preventively treated with *E. faecium* EF 55 – 1 611.8 μm (Table 1).

DISCUSSION

The intestinal mucosa represents the most active defence barrier against the continuous challenge of food antigens and pathogenic microorganisms present in the intestinal lumen. The establishment of beneficial bacterial communities and metabolites from these complex ecosystems has varying consequences for the host health (Nava et al., 2005).

The critical stages of the gut colonisation appear on the days after hatching. In young chicks, only a few bacterial species are present initially, and it can take more than 4 weeks for the microbiota to reach maturity (Revolledo et al., 2006).

Mechanisms by which probiotic bacteria affect the micro-ecology of the gastrointestinal tract are not well understood, but at least three mechanisms of action have been proposed: production/presence of antibacterial substances (e.g. bacteriocins or colicins), modulation of immune responses and specific competition for adhesion receptors to the intestinal epithelium (Nava et al., 2005).

The principal site of *Salmonella* spp. multiplication in poultry is the digestive tract, particularly caecum and caecal tonsils, and they can occur also in the upper part of the small intestine, which may result in widespread contamination of the environment (Popier and Turnbull, 1995). Pathogenic *Salmonella* species possess an array of invasion genes that produce proteins, which are used by the bacteria to penetrate the intestinal mucosa by invading and destroying specialised epithelial M cells of the Peyer's patches (Jones and Falkow, 1996). After crossing the intestinal mucosa through M cells salmonellae first encounter dendritic cells concentrated in the Peyer's patches (Hopkins et al., 2000).

Table 1. The average length of jejunal villi (μm) in all groups two and 14 days post infection with *Salmonella enterica* subsp. *enteritidis*

	Group			
	control	*E. faecium* EF55	*E. faecium* EF55 + *Salmonella enterica*	*Salmonella enterica*
2 days p.i.	1 313.4[a]	1 605.0[a,b]	1 475.5[a]	1 266.2[a,c]
SD	229.7	182.8	294.9	325.6
14 days p.i.	1 565.3[a]	1 611.8[a,b]	1 562.0[a,b]	1 206.3[a,c]
SD	152.1	72.8	139.2	163.4

[a,b,c]means with different superscripts within a row are significantly different at $P < 0.05$

Salmonellae colonise Peyer's patches and trigger the recruitment of macrophages in response to the chemokines released by infected enterocytes.

From Peyer's patches, bacteria disseminate to the reticulo-endothelial system and colonise the liver and spleen (Vazquez-Torres et al., 1999). These organs represent the permissive sites for intracellular proliferation of salmonellae in cases of a systemic disease. Probiotics can protect the intestine by competing with pathogens for attachment, strengthening tight junctions between enterocytes and enhancing the mucosal immune response to pathogens (Lu and Walker, 2001).

Enteroccoci are used as probiotic bacteria mainly because of their abilities to produce anti-bacterial substances. Bacteriocins differ from traditional antibiotics in one critical way: they have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain (Riley and Wertz, 2002).

Audisio et al. (2000) found out that *E. faecium* J96, isolated from a healthy free-range chicken, inhibited *S. pullorum in vitro*, due to its lactic acid and bacteriocin production. In birds preventively treated with our strain of *E. faecium* EF 55 reduced presence of the pathogen was observed in caecum, liver and spleen. It is probably due to competitive exclusion of salmonellae in the gut microenvironment by the applied enterococcus strain. Another mechanism responsible for the lower multiplication of salmonellae in birds treated with *E. faecium* EF55 is production of bacteriocins by this selected strain as shown in the study of Strompfová and Lauková (2007).

Microscopic changes in the mucous membrane are a hidden background for the aggravated absorption abilities in the gut, which is manifested through the weight loss. The application of *E. faecium* EF55 not only reduced the spreading of salmonellae in the gastrointestinal tract but also supported the development of the villi in the jejunum as shown by the results obtained in group E.

Poppe et al. (1993) demonstrated that the course of infection with *S. enterica* depends on bacterial strain, host (age, breed, immune status), environment and experimental design (inoculum dose, housing). The clinical signs of salmonellosis were not manifested during the experiment because of older age of infected chickens. On the other hand, further multiplication of the pathogen in caeca in birds not treated with *E. faecium* EF55 14 days p.i. showed the potential risk for later horizontal or vertical transmission of pathogenic bacteria during clinically inapparent salmonellosis.

The obtained results showed the efficacy of the applied strain in a reduction of colonisation of the gut and internal organs by *S. enterica* subsp. *enteritidis in vivo*. These results confirm inhibition abilities of the selected strain, which were found during *in vitro* studies by Strompfová and Lauková (2007). This information has a great importance especially because of well known phenomena, which is documented in probiotic bacteria. It is a difference of action often observed by the application of probiotic strains, which concerns the inhibition abilities of probiotic bacteria against pathogenic bacteria in *in vitro* studies and in *in vivo* experiments.

It was possible to use PCR as the main examination method in this experiment because of PCR standardisation for detection of pathogens. There exist a lot of different primers and approaches for detection of *Salmonella* spp. in food and in faeces of poultry (Stone et al., 1994; Jitrapakdee et al., 1995; Kwang et al., 1996). *InvA* gene specific primers were used in the present study because the recently evaluated PCR method amplifying *invA* gene fragment for detection of *Salmonella* spp. was validated by Malorny et al. (2003).

It can be concluded that the application of *E. faecium* EF55 reduced colonisation of caeca and minimized translocation of salmonellae into the liver and spleen, which plays an important role in a reduction of the pathogen spread in the flock. Simultaneously, the preventive treatment of the birds supported the development of the intestinal microenvironment which favours the growth of villi and later absorption and digestive abilities.

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Corresponding Author

Robert Herich D.V.M., PhD., Institute of Pathology, University of Veterinary Medicine, Komenského 73, 040 01 Košice, Slovak Republic

Tel. +421 915 984 709, e-mail: herich@uvm.sk