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Original Scientific Article

THE DETECTION OF RELATIVE mRNA EXPRESSION OF CYTOKINE IN CHICKENS AFTER *Enterococcus faecium* EF55 ADMINISTRATION AND *Salmonella enterica* Enteritidis INFECTION

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Summary: Quantitative RT-PCR was used to determine the mRNA expression of pro-inflammatory cytokines IL-15, IL-17, IL-18, LITAF, iNOS, and LyTact chemokine at the caecum and spleen of experimentally infected chicks with *S. enterica* serovar Enteritidis SE147 preventively treated with *Enterococcus faecium* EF55. One-day-old ROSS 308 female chicks (100) were randomly divided into 4 groups (n=25). The chicks of the probiotic (EF) and *Salmonella*+ probiotic (EFSE) groups received per os *E. faecium* EF55 (10^9 CFU/day) from 1 to 7 days of the experiment. The birds of the *Salmonella* (SE) and EFSE groups were perorally infected with *Salmonella* Enteritidis SE147 in a single dose (10^8 CFU) on Day 4 of the experiment.
The preventive administration of *E. faecium* EF55 showed higher, however no significant, mRNA levels of studied pro-inflammatory cytokines and LyTact chemokine except of iNOS in caecum of EFSE experimental group of chicks mainly at Day 1 after *S.* Enteritidis SE147 infection compared to other groups. In contrast, the dynamics of cytokine/chemokine gene specific responses in caecum are not correlated with responses in the spleen.

Key words: cytokines; chemokines; qRT-PCR; chickens; *E. faecium* EF55; *S.* Enteritidis SE147

Introduction

Salmonella enterica is one of the major causes of human food-borne gastroenteritis worldwide. Poultry is considered to be the most significant source of *S. Enteritidis* for humans (6). Therefore, knowledge of the host immune response against *Salmonella* is essential for the understanding of its pathophysiology, prevention and treatment.

Aviansystemic salmonellosis has three distinct phases characterised by significant interaction withthe immune system. In the first phase, the invasion via the gastrointestinal tract occurs (11). *Salmonella* spp.penetrates the intestinal epithelium and enters the Peyer's patches. From the Peyer's patches, *Salmonella* spp. moves to the mesenteric lymph nodes, spreads to the circulatory system, and leads to transient bacteraemia (27). In this phase, there is massive chemotaxis of chemokines (IL-8, CXC, MIP-1β) together with IL-1 and IL-6 into intestinal mucosa. Bacteria are rapidly cleared from the blood by phagocytes in spleen and liver, and a large fraction of bacteria are killed by these cells (4). The second phase includes the establishment of systemic infection, mainly as an intracellular infection of macrophages (11). Activation of macrophages leads to the secretion of pro-inflammatory Th1 cytokines, such as IL-1, TNFα, IFNγ, IL-12, IL-15 and IL-18. Finally, infection may be cleared by the immune response, the birds may succumb to the infection or a subclinical carrier state may develop.

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Manipulationof the gut microbiota of chickens by the administration of probiotic bacteria may help to control enteric bacterial infections, including *Salmonella* invasion (20). The mechanisms of probioticeffects are, however, poorly understood, especially at the molecular level. Several mechanisms might be involved in mediating the effects, including the competitive exclusion, production/presence of antibacterial substances (e.g. bacteriocins or colicins), and the modulation of immune responses (22, 9). These activities include the ability to induce cytokine production leading to the regulation of innate and acquired immune responses. Finally, it has been shown that certain species of lactobacilli strains induce the production of cytokine-promoting Th1 effector functions, such as IL-12 (13, 10), while the other strains of probiotic bacteria induce the production of regulatory inflammatory cytokines, such as IL-10 and TGF-β (23).

Therefore, the current study was undertaken to determine the influence of probiotic bacteria on the pro-inflammatory cytokine and chemokine mRNA profiles during *S.* Enteritidis SE147 infection in chickens to study their potential role in pathogenesis and activation of the avian immune system.

Materials and methods

Experimental animals

Atotal of 100 one-day-old hybrid ROSS 308female chicks were included in the experiment. One day-old chicks were placed in large pens with cellulose cotton (Pehazell, Slovakia) and reared with a lighting regimen of 23^h light and 1^h dark. The initial room temperature of 32–33°C was reduced weekly by 1°C to a final temperature of 28°C. Relative humidity was within a range of 50–6 0%. Birds had free acces to feed (BR1 – starter diet) and water. Application of cleaning and feeding regimens prevented them from cross-contamination effectively throughout the experiment. Chicks were randomly divided into 4 groups (n=25): control (C), *E. faecium* EF55 (EF), combined *E. faecium*EF55+*S.* Enteritidis SE147 (EFSE) and *S.* EnteritidisSE147 (SE).The probiotic strain *Enterococcus faecium* EF55 (provided by Lauková, IAP SAS, Košice, Slovakia) was individually *per os* administered to EF and EFSE groups from 1 to 7 days (1.10^9 CFU/0.2 ml PBS). Experimental infection of SE and EFSE groups was also done individually *per os* using *Salmonella enterica* serovar Enteritidis SE 147 (provided by Rychlík, VRI, Brno, Czech Republic) in a single dose (1.10^8 CFU/0.2 ml PBS) on Day 4. Five chicks from each group were euthanized on Days 1, 2, 3, 4 and 7 post infection (p.i.) with *salmonella*. Samples of spleen and the caudal part of both caeca were taken from each animal during necropsy.

Homogenization of tissue and isolation of total RNA

Tissue samples (spleen and caecum) were cut into 20 mg pieces, immediately placed into RNALater solution (Qiagen, UK) and stored at -70 °C prior to RNA purification. After storage, a single tissue fragment was transferred into 1 ml of TRI Reagent (Molecular Research Center, USA) and homogenized using zirconium silica beads (BioSpec Products, USA) and a vortex mixer (Labnet, USA). To separate the phases, 50 µl of 4-bromanisole (Molecular Research Center, USA) was added. The entire content of the tube was centrifuged and the upper aqueous phase was collected for total RNA purification by using the RNAeasy mini-kit (Qiagen, UK) following the manufacturer's instructions. A TurboDNA-free kit (Ambion, USA) was used for treatment of RNA samples to remove genomic DNA. The purity and concentration of RNA was determined spectrophotometricallywith NanoDrop 200c, (Thermo Scientific, USA) and 1 µg of total RNA was immediately reverse transcribed by using iScriptcDNA Synthesis Kit (Bio-Rad, USA). The resulting cDNA was 10× diluted in UltraPure™ DNase/RNase-Free distilled water (Invitrogen, USA) and used as atemplate in real-time PCR (RT-PCR) or stored at -20 °C until used.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The mRNA levels of IL-15, IL-17, IL-18, lipopoly-saccharide-induced TNF-α factor (LITAF), iNOS and chemokine Lymphotactin (LyTact) were determined. In addition, mRNA expression of two references genes, coding for GAPDH (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitin (UB), was determined and used for data normalization. The primer sequences used for qPCR are listed in Table 1. All primer sets allowed DNA amplification efficiencies between 94% and 100%.

The amplification and detection of specific products were performed using CFX 96 RT system

(Bio-Rad, USA) with the following temperature-time profile: initial denaturation 15 min 95 °C and 45 cycles: denaturation 95 °C for 20 sec, annealing 60 °C for 30 sec, and final elongation 72° C for 30 sec. A melting curve from 50 °C to 95 °C with a reading at every 0.5 °C was performed for each individual RT-PCR plate. Each sample was subjected to RT-PCR in duplicate and the mean values of the duplicates were used for subsequent analysis. We also confirmed that the efficiency of amplification of each target gene (including GAPDH, UB) was essentially 100% in the exponential phase of the reaction, where the cycle quantification (Cq) was calculated. The Cq values of interest genes were normalised to an average Cq value of the reference genes (ΔCq), and the relative expression of each representative value was calculated as $2^{-\Delta Cq}$. These expression levels were then used for comparative data analysis. Relative mRNA expression of cytokine in the spleen and caecum were determined in five independent animals, and we present the results combined from all of these animalsin the figures.

Statistical analysis

Statistical analysis of the results was performed using one-way ANOVA with Tukey post-test by Minitab 16 software (SC&C Partner, Brno, Czech Republic). In the results, the average values are expressed as mean ± SEM. The values of $P < 0.05$ were considered significant.

Results

All studied genes for cytokines (IL-15, IL-17, IL-18, LITAF,iNOS, as well as chemokine LyTact) were detected during whole experiment in allsamplings and groups; however, no statistically significant differences were determined.

Interleukin-15 achieved the maximal levels in the spleen (Fig.1a),and picked up the highest levels of expression in the SE group from 1-4 day p.i. whencomparedto controls, followed with the EFSE group on days 1 and 2 p.i. In three days p.i., the values of EFSE group were higher than SE and the maximal improvement showed EF group. In contrast, the IL-15 levels of EFSE group in the caecum exceeded the level of C and SE groups in all samplings with exception on Day 4 p.i. (Fig.1b).

Interleukin-17 showed in spleen thehighest expression in SE group on Day 2 p.i. butin EF group on Day 1 p.i.(Fig. 2a). In the caecum,the expression of EFSE group at Day 1 p.i. reached the maximum level, similarly to IL-15 (Fig. 2b).

Interleukin-18 in the spleen achieved the highest expression on Day 1p.i. in SE group followed with EFSE group (Fig.3a), butin the caecum this combined group over-exceeded the values of SE infected group at this day (Fig. 3b).

Table 1: List of primers used for chicken cytokine mRNA quantification

Primer	**Sequence 5'–3'**	**References**
GAPDH For	CCTGCATCTGCCCATTT	(De Boever *et al.*,2008)
GAPDH Rev	GGCACGCCATCACTATC	
UB For	GGGATGCAGATCTTCGTGAAA	(De Boever *et al.*,2008))
UB Rev	CTTGCCAGCAAAGATCAACCTT	
IL-15 For	TGGAGCTGATCAAGACATCTG	(Kolesárová *et al.*, 2011)
IL-15 Rev	CATTACAGGTTCCTGGCATTC	
IL-17 For	TATCAGCAAACGCTCACTGG	(Crhánová *et al.*,2011)
IL-17 Rev	AGTTCACGCACCTGGAATG	
IL-18 For	ACGTGGCAGCTTTTGAAGAT	(Rýchlik *et al.*, 2009)
IL-18 Rev	GCGGTGGTTTTGTAACAGTG	
LITAF For	AATTTGCAGGCTGTTTCTGC	(Kolesárová *et al.*, 2011)
LITAF Rev	TATGAAGGTGGTGCAGATGG	
LymphotactinFor	CATAGTCTGGCTTGGCGTCTT	(Withanage *et al.*, 2004)
LymphotactinRev	GCGCATTGACTGACTTGCA	
iNOS For	GAACAGCCAGCTCATCCGATA	(Berndt *et al.*, 2007)
iNOS Rev	CCCAAGCTCAATGCACAACTT	

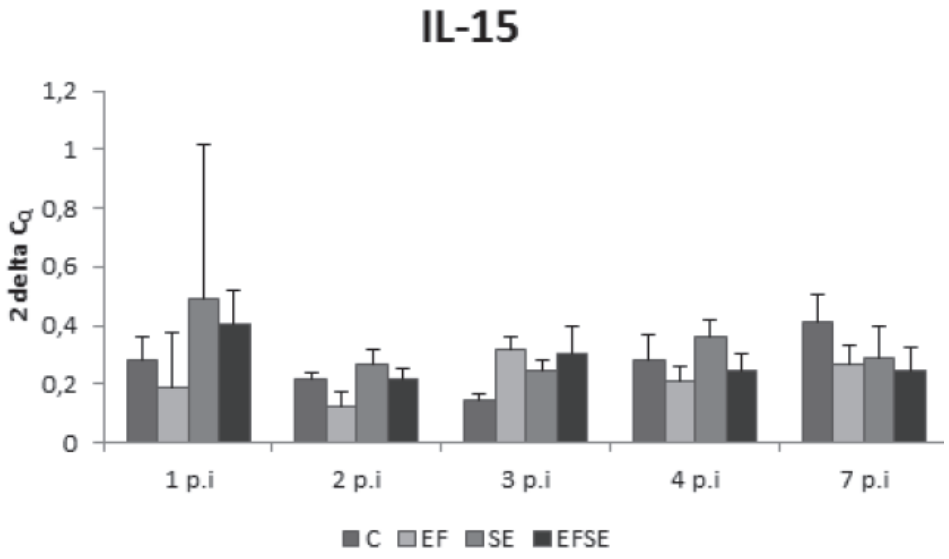

Figure 1a: Relative expression of IL-15 mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147, EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

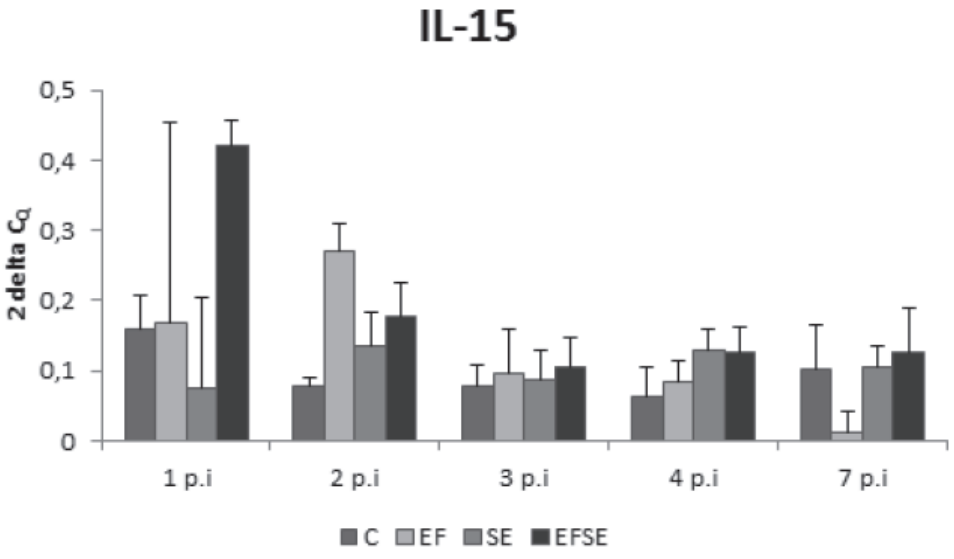

Figure 1b: Relative expression of IL-15 mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

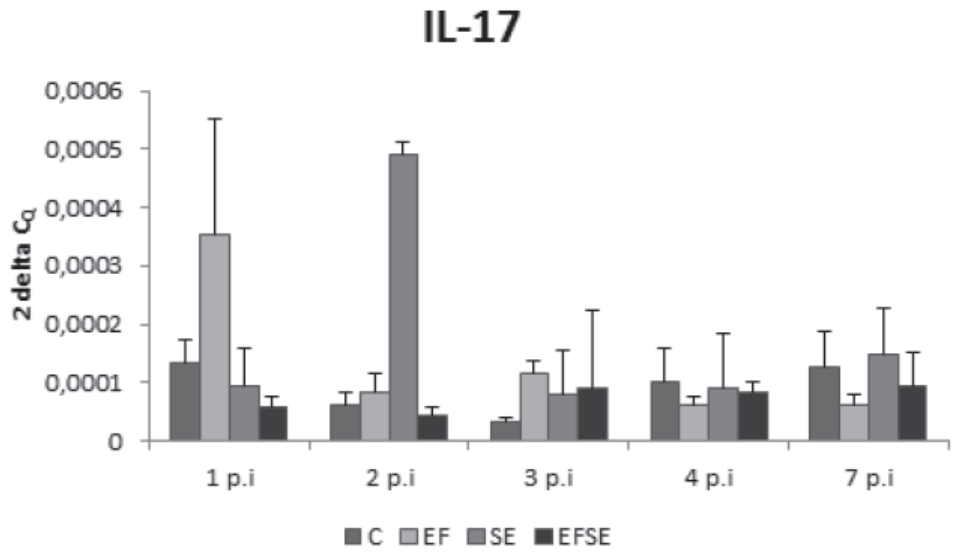

Figure 2a: Relative expression of IL-17 mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.*Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

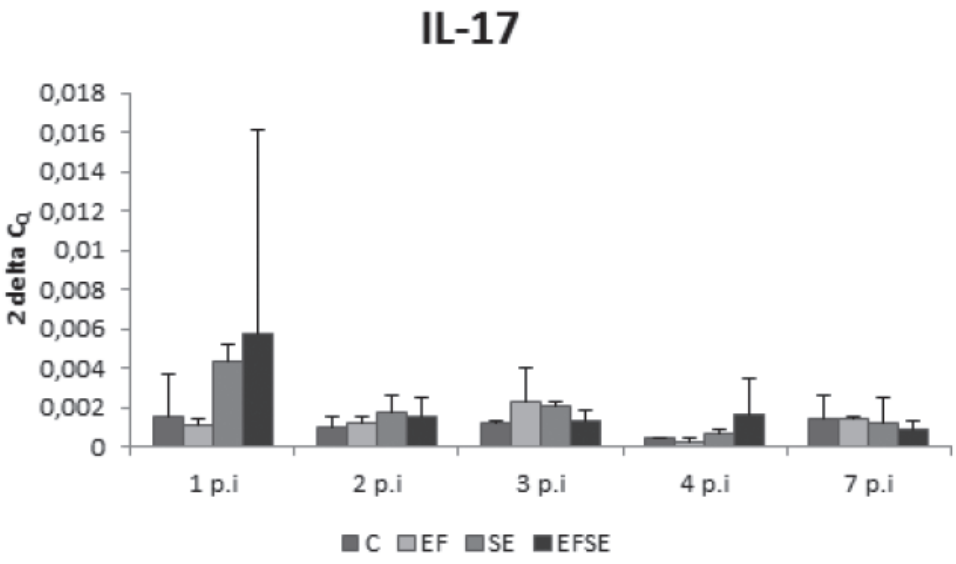

Figure 2b: Relative expression of IL-17 mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.*Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

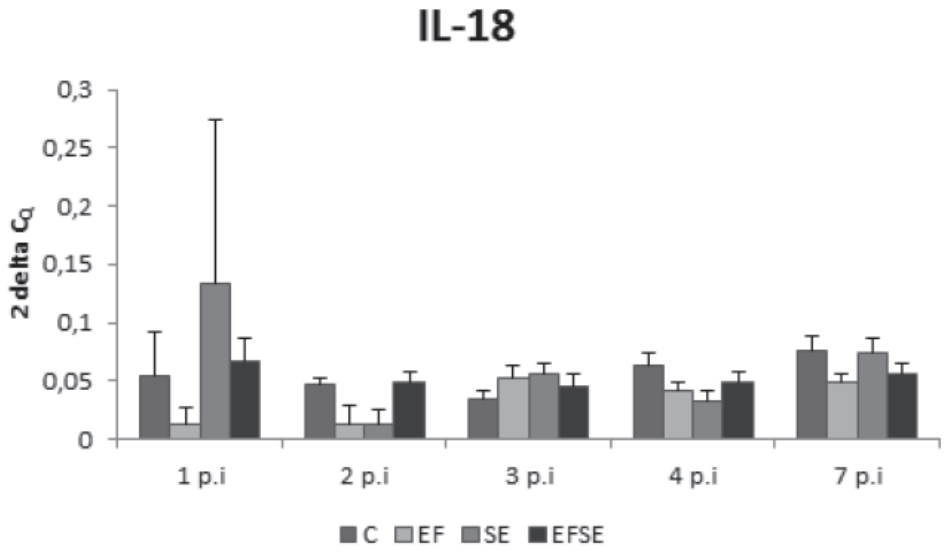

Figure 3a: Relative expression of IL-18 mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.*Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

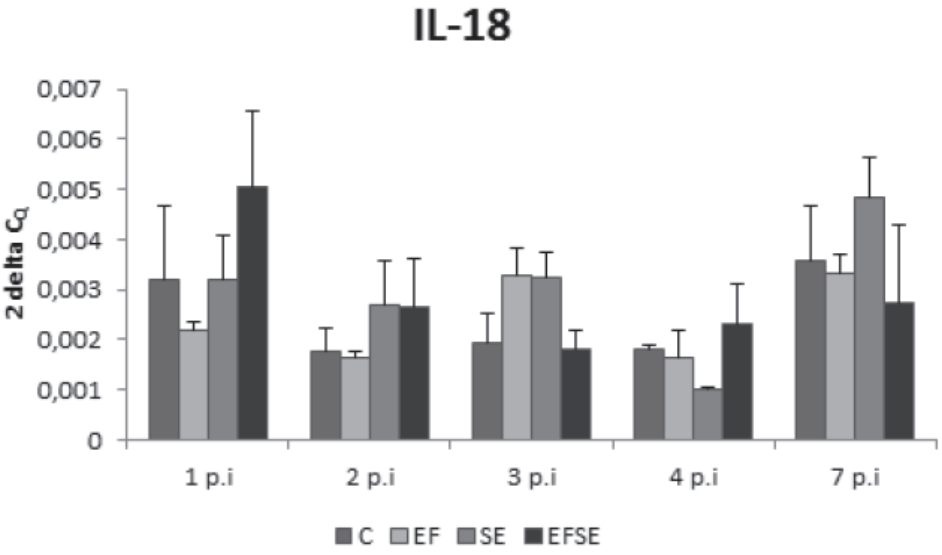

Figure 3b: Relative expression of IL-18 mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

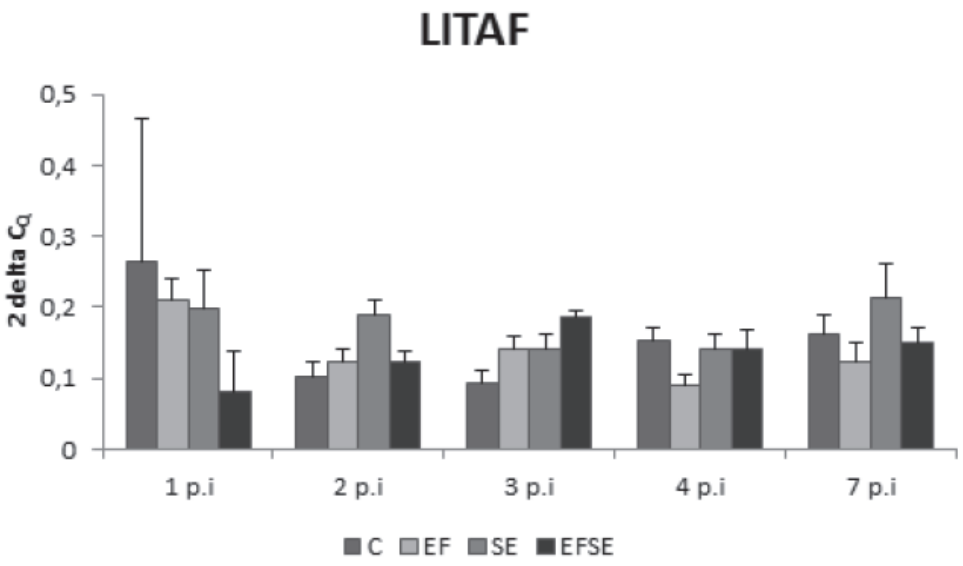

Figure 4a: Relative expression of LITAF mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

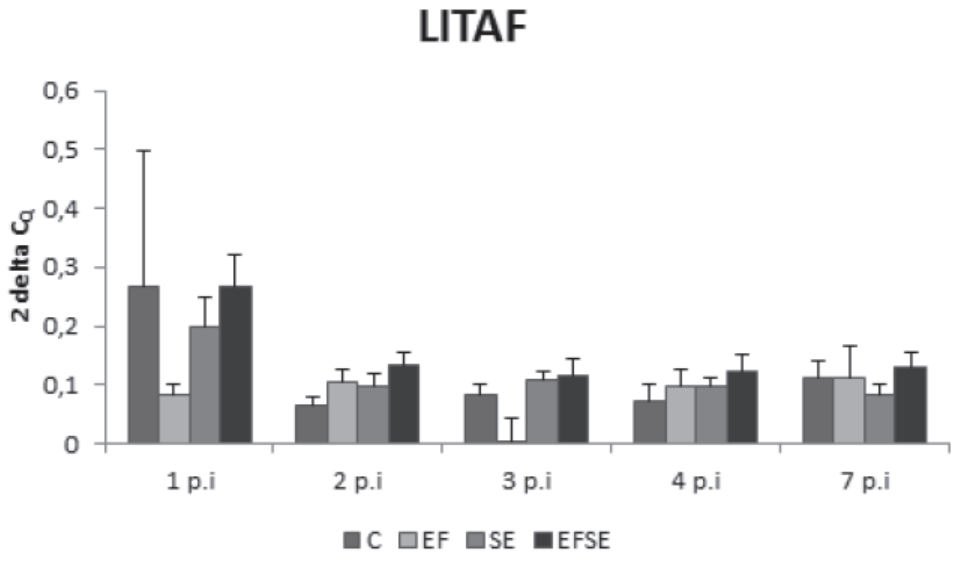

Figure 4b: Relative expression of LITAF mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

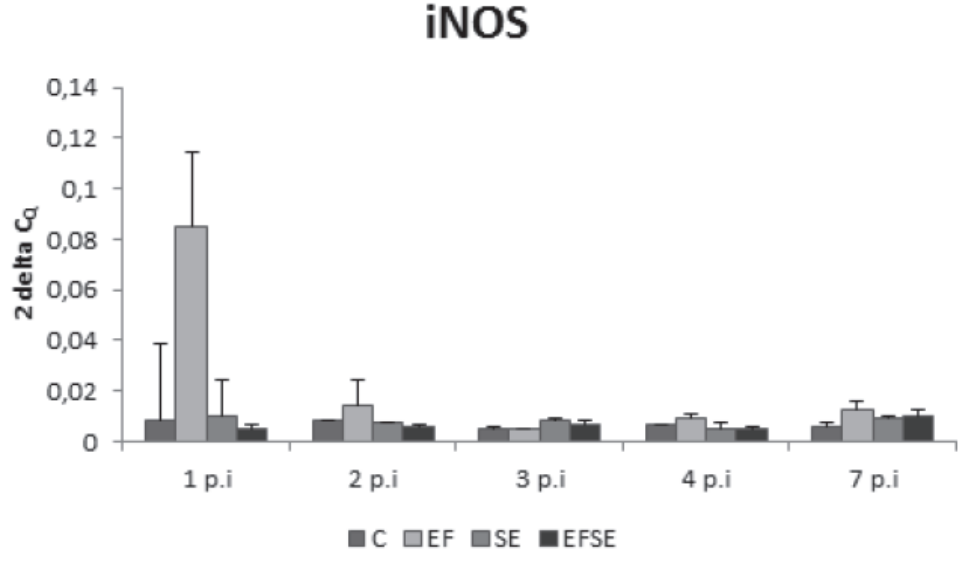

Figure 5a: Relative expression ofiNOS mRNA in the spleen.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

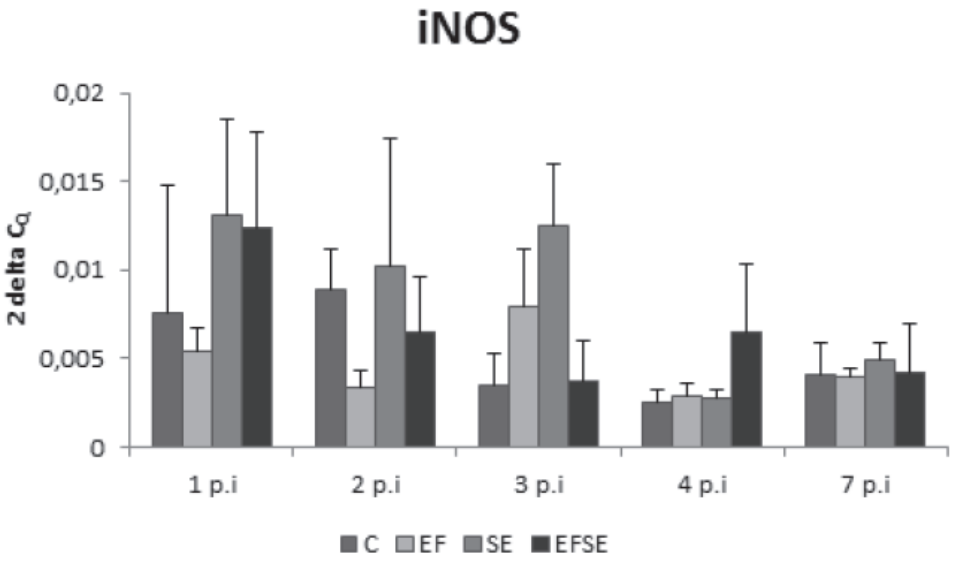

Figure 5b: Relative expression of iNOS mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

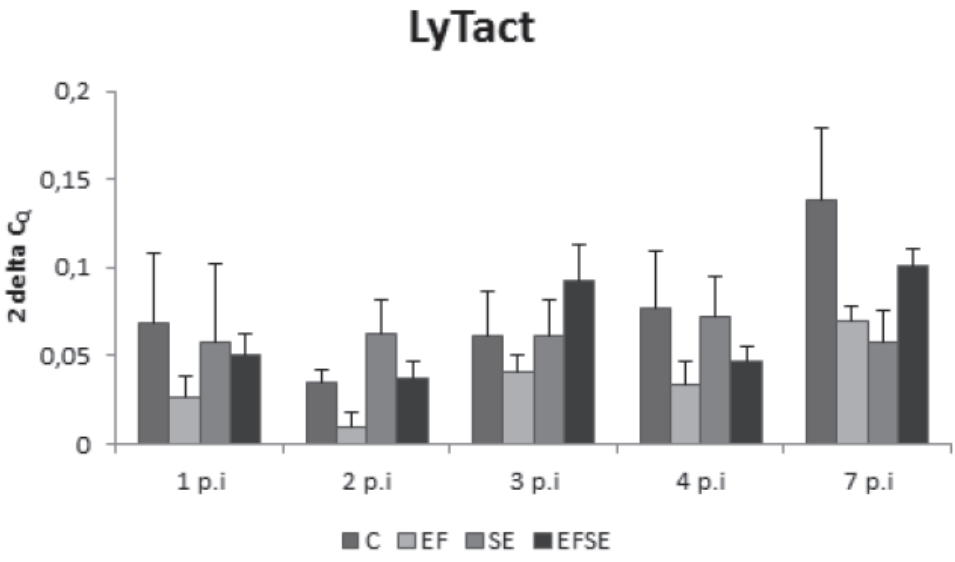

Figure 6a: Relative expression of LyTact mRNA in the spleen.
Legend**:** C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

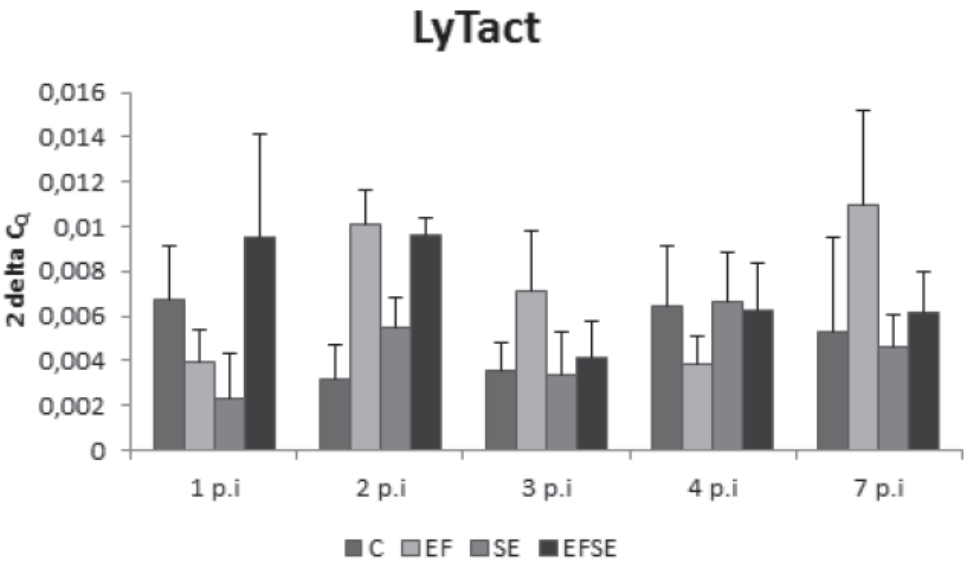

Figure 6b: Relative expression of LyTact mRNA in the caecum.
Legend: C – control; SE – *S.* Enteritidis SE147,EFSE – *E. faecium* EF55+*S.* Enteritidis SE147; EF – *E. faecium*EF55; p.i – days post infection

LITAF expression in higher levels was found in the spleen on Days 2 and 7 p.i. in SE group, as well as in EFSE group on Day 3 p.i. (Fig 4a).In contrast, in the caecum, the expression of LITAF was the highest in the EFSE group compared with another groups from Days 1 to 7 p.i. (Fig.4b).

iNOS detection in the spleen showed the highestlevel of expression in EF group during experiment on Day 1 p.i. in comparison with other groups(Fig.5a). However, in the caecum the maximum levels reached SE groups except for Day 4 p.i. when values of EFSE group were the highest (Fig. 5b).

LyTactchemokine expression in the spleen increased on Day 2 p.i. in SE, and on day 3 p.i. inEFSE groups. (Fig. 6a).Caecum of chickens treated with *E. faecium* EF55 showed the highest levels of expressionon Days 2, 3 and 7 p.i. together with SE+EF group on Days 1, 2, 3, and 7 p.i. (Fig. 6b).

Discussion

Cytokines are essential effector molecules, which initiate and coordinate cellular and humoral immune response against pathogens.Specifically, the increased mRNA expression and protein secretion of chemokines, proinflammatory and Th1 cytokines such as IFN-γ, IL-1, IL-6, IL-8, IL-12, and MIP-1β are observed following infection with various *Salmonella* species (12).

The current results demonstrate the modulation of other kinds ofcytokine mRNA expression in chickens after administration of *E. faecium* EF55 and challenged with *S.* Enteritidis SE147. The early increased expression of IL-17 mRNA in the intestinal mucosa of chickens in EFSE group suggests that enhanced expression of cytokine can be consistent with the moving of phagocytic cells into infected intestine. Earlier *in vivo* study indicated that IL-17 is a potent activator of neutrophils, both through expansion of the lineage via the regulation of G-CSF receptor as well as recruitment through regulation of chemokine expression. Ectopic expression of IL-17 stimulated a strong neutrophilic response (8). Furthermore, our earlier work with *S.* EnteritidisPT4 (17) demonstrated the increased density ofheterophilsin the blood of EFSE group on Day 3 p.i. In the current experiment the increased of IL-17as well as chemokine lymphotactin in the caecum of combined treatment EFSE group on the Day 1p.i confirms the assumption of mutual cytokine interactions at the local level. This result demonstrates the early activation of the natural immunity components in the caecum after preventive *E. faecium* EF55 administration. Moreover, increased mRNA expression of IL-17 only in the caecum may suggestthat cytokine is involved mainly in mucosal immunity.Coccia (2012) showed that IL-17 enhances production of antimicrobial peptides by intestinal epithelial cells. This finding is consistent with our previous report about decreased numbers of *S.* Enteritidis in the caecum of chickens after administration of *E. faecium* EF55 and challenged with *S.* Enteritidis (16). Similarly, our laboratory recently demonstrated the increased number of IgA+ caecal intraepithelial lymphocytes in the EFSE group (18), which suggests an important role of IL-17 in local immune response.

Interleukin-18 is the most important growth factor for avian CD4+ cells and enhances the cytotoxic activity of NK cells (14). IL-15 is indispensable for long-term survival of memory CD8+ T cells and up-regulates the proliferation of cytotoxic and helper T-cells for the direction of heterophils and NK cells to the site of inflammation (19). Chickens infected with *S.*Enteritidis SE147 in the current trial presented the highest increase of IL-18 and IL-15 mRNA expression on the first day p.i. to confirm the activation of specific immune components. However, further sampling showed declined level of IL-18 mRNA expression.

In the EFSE group, the IL-15 and IL-18 expression was maintained approximately at the same levels for all the samplings. The infection of chickens with *S.*Enteritidis showed a significant increase of CD4+ and CD8+ lymphocytes. However, chickens treated with EF55 and infected with *S.* Enteritidis revealed increases of CD4+ and CD8+ cells (25), whichsuggests enhancing the role of IL-15 and IL-18 also in our current trial. Modulation of these cytokines suggests the stimulatory action of *E. faecium* EF55 in the direction of Th1 immune responses.

Several authors demonstrated suppressive effect of some probiotic strains on TNF-α production by host immune cells (13, 24, 29). We observed similar effect of *E. faecium* EF55 on LITAF cytokine production in caecum at the first day p.i. in EF group.

Macrophages and other effector cells express inducible nitric oxide synthase (iNOS) that is an integral part of the host defence mechanisms. The synthases activate the nitric oxide (NO), which is

typical of their strong bacteriostatic effect against intracellular bacteria (28). Similarly, *in vitro* cultivated chickens' macrophages infected with *Salmonella* accounted for the important role of the iNOS enzyme in the defence against *Salmonella* (30). In the current experiment, the highest although not significant iNOSexpression observed in the spleen of EF group at first day p.i.suggests the action of *E. faecium* EF55 in the direction of Th1 polarization. The highest iNOS expression found in the spleen of the SE and EFSE groups at the first day p.i. suggests that *E. faecium* EF55 could also stimulate splenic macrophages.

Chemokines can stimulate the chemotaxis of leukocytes adhering to the activated endothelium via adhesion molecules (selectins, integrins, and members of the large family of immunoglobulin) (3). Our current results showed that *S.* Enteritidis SE147 could affect the expression of immune system components, including chemokines. Lymphotactin acts as a potential chemoatractant for T and NK cells (2). However, Zhang et al. (32) found a decreased level of lymphotactin expression in chickens infected with *S.* Enteritidis. Similarly, we also observed a decreased level of LyTactexpression in the spleen of SE group in comparison with the control group at first day p.i. In contrast, Meyer et al. (21) showed that *in vitro* some probiotic enhanced strains stimulated production of chemokines. In the current study, we found the increased of LyTactexpression in ceaca of EFSE group at Days 1, 2, and 7 p.i. This finding suggests that an increased expression of lymphotactin in the caeca during intestinal infection may play a key role in the migration of cells into site of inflammation.

In conclusion, the preventive administrationof *E. faecium* EF55 to *S.* Enteritidis SE147 infected chicks showed some immunomodulatory effect in the caeca, mainly at the first day p.i. presented by no significant higher levels of pro-inflammatory gene expression cytokines IL-15, IL-17, IL-18, LITAF, and LyTact chemokine. However, the dynamics of cytokine/chemokine gene specific responses in caecum are not correlated with responses in the spleen.

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RAZLIKE V IZRAŽENOSTI GENOV ZA CITOKINE PRI PIŠČANCIH PO DODAJANJU *Enterococcus faecium* EF55 IN OKUŽBI S *Salmonella enterica* enteritidis

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Povzetek: Kvantitativna metoda RT-PCR je bila uporabljena za določanje izražanja mRNK vnetnih citokinov IL-15, IL-17, IL-18, LITAF, iNOS in kemokinov LyTact v slepem črevesu in vranici pri piščancih, poskusno okuženih s *S. enterica* serovar enteritidis SE147 ter nato preventivno zdravljenih z *Enterococcus faecium* EF55. En dan stari piščanci ROSS 308 ženskega spola (100) so bili naključno razdeljeni v 4 skupine (n=25). Piščanci iz probiotične (EF) in salmonele+ probiotične (EFSE) skupine so prejeli per os *E. faecium* EF55 (109 CFU/dan) od 1. do 7. dneva poskusa. Piščanci iz skupin salmonele (SE) in skupin EFSE so bili peroralno okuženi s *Salmonella* enteritidis SE147 v enkratnem odmerku (108 CFU) 4. dan poskusa.
Preventivno dodajanje *E. faecium* EF55 je povzročilo statistično neznačilno povišanje, izraženosti m RNK raziskanih vnetnih citokinov in LyTact kemokina, razen pri izraženosti mRNK za iNOS v slepem črevesu poskusne skupine piščancev EFSE 1. dan po okužbi s *S.* enteritidis SE147. Prav tako niso bili ugotovljeni specifični odzivi v izraženosti citokinov/kemokinskih genov v vranici.

Ključne besede: citokini; kemokini; QRT-PCR; kokoši; *E. faecium* EF55; *S.* enteritidis SE147