

## Expression of cytokines in chicken peripheral mononuclear blood cells (PMBCs) exposed to probiotic strains and *Salmonella* Enteritidis

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### Abstract

The mRNA expression of interleukin (IL)-1 $\beta$ , LITAF, iNOS, macrophage inflammatory protein (MIP1- $\beta$ ), and K60 were examined in peripheral blood mononuclear cells (PMBCs). The PMBCs were isolated from the chicken blood and *in vitro* exposed to the probiotic strains *E. faecium* AL41, *E. faecium* H31, *L. fermentum* AD1, and infected with *Salmonella enterica* serovar Enteritidis (SE147). The PMBCs were evaluated for mRNA expression levels at 24 h and 48 h post infection (p.i.) using the reverse transcriptase polymerase chain reaction (RT-PCR). The level of expression of IL-1 $\beta$  and MIP1- $\beta$  was upregulated ( $P < 0.001$ ) in the EFAL41+SE (*S. Enteritidis* + *E. faecium* AL41) group 48 h p.i. compared to 24 h p.i. Similarly, expression of LITAF was upregulated ( $P < 0.001$ ) in the EFAL41 + SE group compared to the control (C - no infected) and *S. Enteritidis* (SE) group 48 h p.i. In PMBCs treated with *E. faecium* H31 and *S. Enteritidis* expression of IL-1 $\beta$  ( $P < 0.01$ ) and chemokines K60 and MIP1- $\beta$  was upregulated ( $P < 0.001$ ) in the EFH31 + SE group 24 h p.i. The iNOS showed upregulated expression ( $P < 0.001$ ) in the EFAL41 + SE group compared to the control 24 h p.i. and to the C and SE groups 48 h p.i. The results showed that *E. faecium* AL41 demonstrated the highest immunostimulatory effect on expression of selected cytokines by chicken PMBCs after *Salmonella* infection. It is supposed that the differences in cytokine induction within SE groups are related to lymphocytes isolated from different animals.

*mRNA, RT-PCR, immune system, Enterococcus faecium, in vitro*

Over the course of evolution, lactic acid bacteria (LAB) have been abundant colonisers of the small intestinal mucosa, coexisting in relationships with the host. Some members of these groups exert additional probiotic properties that provide health benefits to the host via the regulation of the immune system and other physiological functions (MacDonald and Monteleone 2005; Konstantinov et al. 2008). The introduction of probiotic strains has been found to influence the established microbiota and have also been reported to exert immunomodulatory effects both *in vivo* and *in vitro* conditions. The strains of *Lactobacillus*, *Enterococcus* and others are included among the numerous microorganisms, which have been reported to show beneficial properties involving both the antibacterial effects on possible pathogenic strains and the immunomodulatory effects correlating with reductions in infections as well as improved recovery (Heineman et al. 2012; Hemarajata and Versalovic 2013). The use of probiotics is considered to be a potentially important strategy for modulating infectious and inflammatory responses in the gastrointestinal tract of the host. The effect of these probiotics is diverse and includes the modulation of the gut immune system through the interaction with gut epithelial cells and immune cells (Pamer et al. 2007). The testing of probiotics is performed by studies on cell and animal models. *In vitro* studies may provide valuable information, such as the mechanism through which the probiotic bacteria are effective.

Some of the important cytokines and chemokines involved in the initiation and modulation of the inflammatory immune activities in chickens include IL-1 $\beta$ , LITAF, K60, MIP1- $\beta$ ,

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and iNOS. We aimed at the determination of the levels of cytokines, particularly the pro-inflammatory cytokines IL-1 $\beta$  and LITAF, produced following the invasion of *S. Enteritidis* into chicken cells *in vitro*; and finally, to evaluate whether the strain characteristics of commensal bacteria can be immunologically effective for the prevention and treatment of *Salmonella* infection in chickens.

### Materials and Methods

#### Isolation and cultivation of PMBCs from peripheral blood of poultry

Blood was collected from the vena cutanea ulnaris to 1.5% EDTA from clinically healthy poultry reared under standard conditions. Collected blood was diluted with PBS in a ratio of 1:2 and transferred to Leucoseps tube (Greiner bio-one, DE) containing histopaque-1077 (Sigma-Aldrich, UK) and centrifuged for 40 min at 19 000  $\times$  g at 20 °C (Hettich Rotina 420R Centrifuge, DJB Labcare, UK). Mononuclear cells were collected from the gradient interface and washed twice for 5 min at 16 000  $\times$  g with PBS. Cell viability and number were determined by trypan blue exclusion. Isolated PMBCs (Peripheral Mononuclear Blood Cells) were plated in to 12-well cultivation plate (Orange Scientific, BE) in the number of  $1 \times 10^7$  cells/ml, and cultured overnight (39 °C, 5% CO<sub>2</sub>) in RPMI 1640 medium enriched with 10 mM HEPES (Lonza, BE) and 10% FBS (Lonza, BE). After 24 h cultivation 200  $\mu$ l of *S. Enteritidis* SE147 (provided by Dr. Rychlík, VRI, Brno, Czech Republic) in the number of  $1 \times 10^8$  CFU/ml, and 200  $\mu$ l of *Enterococcus faecium* AL41, *Enterococcus faecium* H31 and *Lactobacillus fermentum* AD1 (provided by Dr. Lauková, IAP SAS, Košice, Slovakia) in a number of  $1 \times 10^9$  CFU/ml were added to PMBCs in particular wells. After the addition of bacteria PMBCs were cultured for 24 and 48 h. The cultivation of bacterial strains, culture medium and growth conditions was performed as described previously (Herich et al. 2005; Stropňová et al. 2005).

#### Isolation of mRNA from PMBCs

The PMBCs were harvested from each well by pipetting and centrifuged for 1 min at 8 000  $\times$  g. Cell lysing buffer containing  $\beta$ -mercaptoethanol (Qiagen, USA) was added to the resulting pellet. Then, 70% ethanol was added to the whole cell lysate at the ratio of 1:1, and the whole mixture was homogenized using a vortex mixer (Labnet, USA) for 1 min. This resulting cell lysate was used for isolation of RNA using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions. The purity and concentration of total RNA was determined spectrophotometrically on NanoDrop 200c (Thermo Scientific, UK) and 1  $\mu$ g of total RNA was immediately reverse transcribed by using iScript cDNA Synthesis Kit (Bio-Rad, USA). The resulting cDNA was diluted  $\times 10$  in UltraPure™ DNase/RNase-Free distilled water (Invitrogen, USA) and used as a template for qRT-PCR or stored at -20 °C until used.

#### RT-PCR

The relative expression of cytokines (IL-1 $\beta$ , LITAF), chemokines (K60, MIP1- $\beta$ ), and iNOS were determined. The expression levels of two house-keeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ubiquitin (UB) were used for data normalisation. Amplification and detection of specific products were performed using the 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 s, annealing 60 °C for 30 s and final elongation 72 °C for 30 s. 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 real-time PCR in duplicate and the mean values of the duplicates were used for subsequent analysis. We also confirmed that efficiency of amplification of each target genes (including GAPDH and UB) were essentially 100% in the exponential phase of the reaction, where the quantification cycle (Cq) is calculated. The Cq values of genes of interest were normalised to a mean Cq value of the reference genes ( $\Delta$ Cq) and the relative expression of each representative was calculated as  $2^{-\Delta$ Cq. These expression levels were then used for comparative data analysis.

#### Statistical analysis

Statistical analysis of obtained data was done by one-way analysis of variance (ANOVA) with *post hoc* Tukey multiple comparison test using GraphPad Software, statistical version 5.0 (USA). Differences between the mean values for different treatment groups were considered significant at  $P < 0.01$ ;  $P < 0.001$ .

## Results

The effect of *E. faecium* AL41 revealed upregulation ( $P < 0.001$ ) on expression of IL-1 $\beta$  in the EFAL41 + SE group compared to the control 48 h p.i. Similarly, in PMBCs treated with *E. faecium* H31 the relative expression of IL-1 $\beta$  was upregulated ( $P < 0.01$ ) in the EFH31 + SE group compared to the control and SE group 24 h p.i. (Fig. 1).

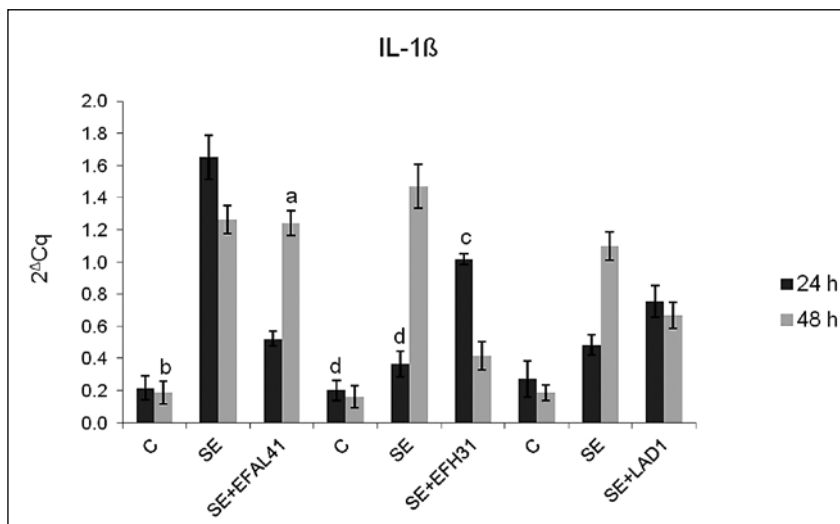


Fig. 1. Relative quantitation - mRNA of IL-1 $\beta$  in PMBCs

C - control; SE - *S. Enteritidis* SE147, SE + EFAL41 - *S. Enteritidis* SE147 + *E. faecium* AL41; SE + EFH31 - *S. Enteritidis* SE147 + *E. faecium* H31; SE + LAD1 - *S. Enteritidis* SE147 + *L. fermentum* AD1; 24 and 48 h - post infection; All values are expressed as mean  $\pm$  SD. Means with different superscripts are significantly different <sup>ab</sup> $P < 0.001$ ; <sup>cd</sup> $P < 0.01$

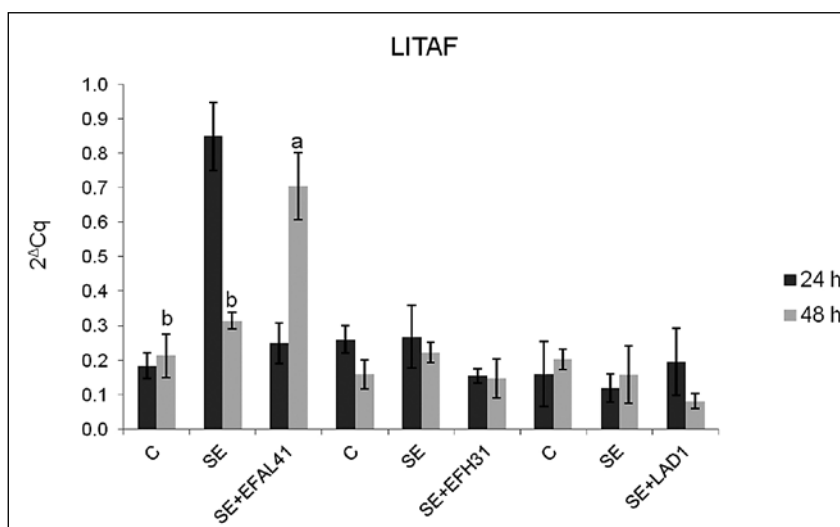


Fig. 2. Relative quantitation - mRNA of LITAF in PMBCs.

C - control; SE - *S. Enteritidis* SE147, SE + EFAL41 - *S. Enteritidis* SE147 + *E. faecium* AL41; SE + EFH31 - *S. Enteritidis* SE147 + *E. faecium* H31; SE + LAD1 - *S. Enteritidis* SE147 + *L. fermentum* AD1; 24 and 48 h - post infection; All values are expressed as mean  $\pm$  SD. Means with different superscripts are significantly different <sup>ab</sup> $P < 0.001$

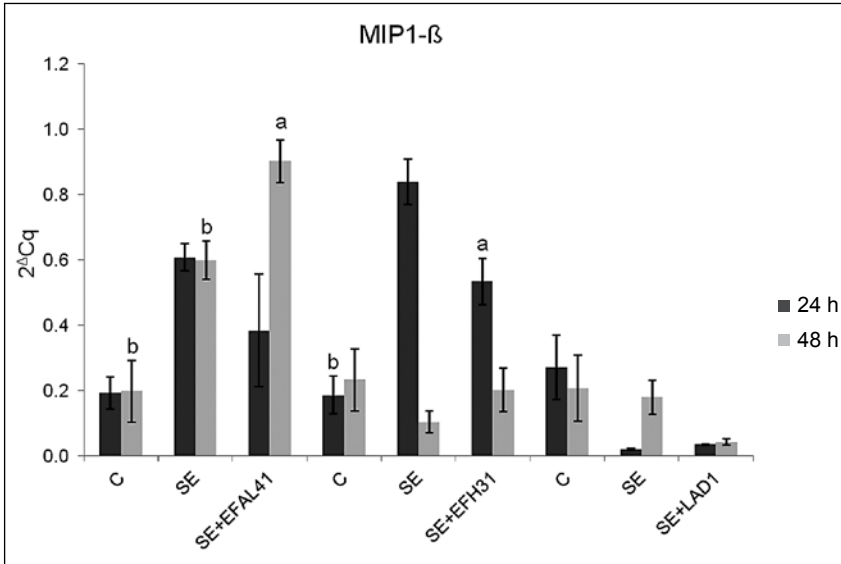


Fig. 3. Relative quantitation - mRNA of MIP1- $\beta$  in PMBCs.

C - control; SE - *S. Enteritidis* SE147, SE+EFAL41 - *S. Enteritidis* SE147 + *E. faecium* AL41; SE+EFH31 - *S. Enteritidis* SE147 + *E. faecium* H31; SE+LAD1 - *S. Enteritidis* SE147 + *L. fermentum* AD1; 24 and 48 h - post infection; All values are expressed as mean  $\pm$  SD. Means with different superscripts are significantly different <sup>ab</sup> $P < 0.001$

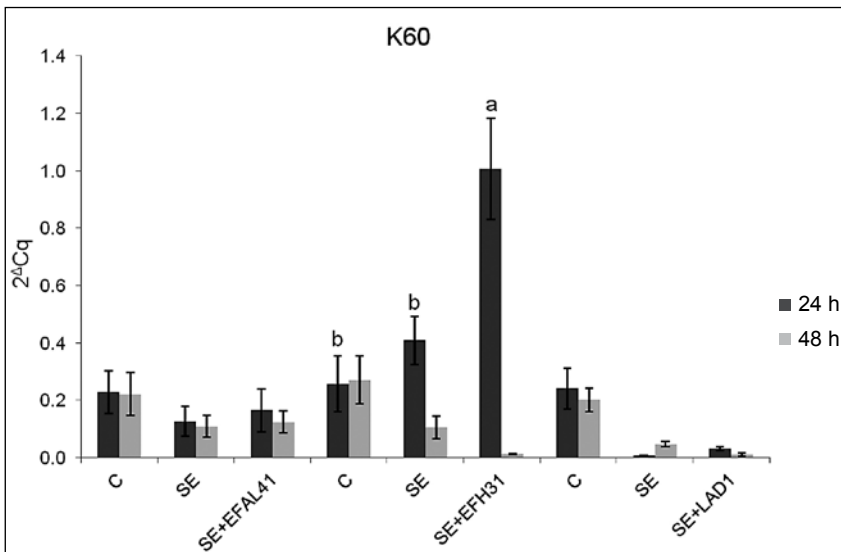


Fig. 4. Relative quantitation - mRNA of K60 in PMBCs.

C - control; SE - *S. Enteritidis* SE147, SE + EFAL41 - *S. Enteritidis* SE147 + *E. faecium* AL41; SE + EFH31 - *S. Enteritidis* SE147 + *E. faecium* H31; SE+LAD1 - *S. Enteritidis* SE147 + *L. fermentum* AD1; 24 and 48 h - post infection; All values are expressed as mean  $\pm$  SD. Means with different superscripts are significantly different <sup>ab</sup> $P < 0.001$

The 24 h exposure of PMBCs to *E. faecium* AL41 did not have a stimulatory effect on relative expression of LITAF, but upregulation ( $P < 0.001$ ) was found in the EFAL41 + SE group compared to the C and SE groups 48 h p.i. (Fig. 2).

The relative expression of chemokine MIP1- $\beta$  was upregulated ( $P < 0.001$ ) 48 h p.i., treated with *E. faecium* EFAL41 in the EFAL41 + SE group compared to the control. Similarly, exposure of PMBCs to *E. faecium* EFH31 showed upregulation ( $P < 0.001$ ) of selected chemokines MIP1- $\beta$  and K60 in the EFH31 + SE group compared to the control (Fig. 3) and the C and SE groups 24 h p.i. (Fig. 4).

The level of expression of iNOS was upregulated ( $P < 0.001$ ) in the EFAL41 + SE group compared to the control group 24 h p.i. and to the C and SE groups 48 h p.i. in response to exposure of PMBCs to probiotic strain *E. faecium* AL41 tested in this study (Fig. 5).

The comparison of all three tested probiotic strains in chicken PMBCs challenged with *S. Enteritidis* showed that probiotic strain *E. faecium* AL41 induced upregulation ( $P < 0.001$ ) of expression of proinflammatory cytokines and chemokines compared to the probiotic strains *L. fermentum* AD1 and *E. faecium* H31.

The differences in cytokine induction within SE groups are related to lymphocytes isolated from different animals. Precisely because of different immune response of every animal, all *in vitro* tests included SE group, which represented particular “infectious background”.

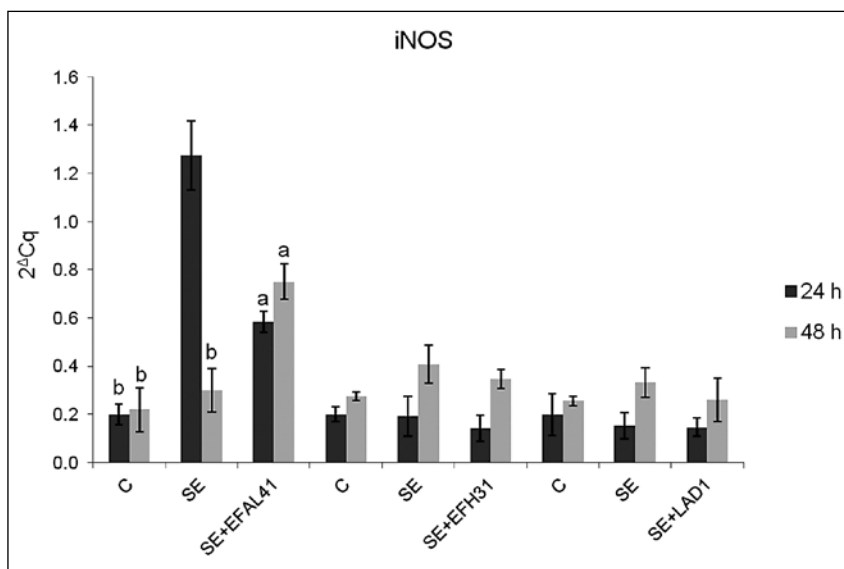


Fig. 5. Relative quantitation - mRNA of iNOS in PMBCs.

C - control; SE - *S. Enteritidis* SE147, SE + EFAL41 - *S. Enteritidis* SE147 + *E. faecium* AL41; SE + EFH31 - *S. Enteritidis* SE147 + *E. faecium* H31; SE+LAD1 - *S. Enteritidis* SE147 + *L. fermentum* AD1; 24 and 48 h – post infection; All values are expressed as mean  $\pm$  SD. Means with different superscripts are significantly different <sup>ab</sup> $P < 0.001$

## Discussion

The response of the immune system to probiotic bacteria remains controversial. Some strains modulate the cytokine production *in vitro* and induce a regulatory response, whereas others induce a pro-inflammatory response (Evrard et al. 2011). However,

several studies have provided unequivocal evidence that the specific strains of probiotics are able to stimulate multiple aspects of innate immunity (Weiss et al. 2010) as well as to increase the humoral immunity (Roberfroid 2002). In the current experiment probiotic strains *E. faecium* AL41, *E. faecium* H31 and *L. fermentum* AD1 demonstrated individual response on expression of selected cytokines and chemokines *in vitro*. Kaiser et al. (2000) described down-regulation of IL-1 $\beta$  expression after *Salmonella* infection leading to a reduced rapid inflammatory response in the intestine allowing the initial entry of bacteria. Similarly, our experiment demonstrated down-regulation of IL-1 $\beta$  mRNA expression 24 h p.i., however, 48 h p.i. the level of IL-1 $\beta$  mRNA was upregulated after stimulation of PMBCs with *E. faecium* AL41 and *S. Enteritidis*. It is interesting that regulation of IL-1 $\beta$  mRNA after application of *E. faecium* H31 and *S. Enteritidis* infected cells was upregulated 24 h p.i. Although little is known about the biological function of LITAF in poultry, it has been reported that the expression of this co-stimulatory molecule was upregulated in *S. Typhimurium* LPS-stimulated macrophages. It is also suggested that LITAF plays an important role in driving inflammatory responses and bacterial clearance, though its role in avian salmonellosis remains unclear and requires further investigations. Hong et al. (2006) showed that expression of LITAF to be upregulated following the *in vitro* stimulation of macrophages with *S. Typhimurium* LPS. The data from our experiment showed upregulation in the gene expression level of LITAF in the EFAL41 + SE group 48 h p.i.

The mechanisms of innate immunity are the first line of defence against pathogenic microorganisms. An integral part of this reaction is the production of nitric oxide synthase (iNOS), which has a strong bacteriostatic activity against intracellular bacteria (Richardson et al. 2009). Our results also indicate upregulation of expression of iNOS in the combined group EFAL41 + SE 48 h p.i., which suggests the bacteriostatic effect of *E. faecium* AL41. Moreover, its bacteriostatic activity demonstrated Lauková et al. (2004) after infection of gnotobiotic quails with *Salmonella enterica*. Meyer et al. (2007) in their *in vitro* study showed that some probiotic strains stimulated production of chemokines. Similar results were found by Brisbin et al. (2010) and Withanage et al. (2004) who showed that probiotic bacteria were extensively used as probiotics for the control of intestinal colonization with enteric pathogens. Chemokines are known to be produced by cells during infection or following a pro-inflammatory stimulus and prompt the migration of leukocytes to an injured, or infected site. Such inflammatory chemokines can also activate cells to raise an immune response and commence the wound healing process. K60 might be also chemoattractive for heterophils (Murphy et al. 2000). In our study the upregulation of relative expression of K60 and MIP1- $\beta$  mRNAs 24 h p.i. was demonstrated after treatment with *E. faecium* H31 and challenge with *S. Enteritidis*. On the other hand, upregulation of MIP1- $\beta$  mRNA was determined in the group EFAL41 + SE 48 h p.i.

It follows that increased expression of the studied cytokines and chemokines was demonstrated especially during combined treatments through elevated production of selected cytokines and chemokines, in response of PMBCs to application of probiotic strains *E. faecium* H31 and *E. faecium* AL41. Recently, in our laboratory we have determined early upregulation expression of pro-inflammatory cytokines in the caecum of chickens treated with *Enterococcus faecium* and challenged with *S. Enteritidis* 147 (Levkut et al. 2014). Early reaction of pro-inflammatory cytokines in chickens PMBC exposed to *E. faecium* AL41 was seen also in the current trial.

In conclusion, our results demonstrated that *E. faecium* AL41 showed the highest immunostimulatory effect from all of the tested probiotic strains on the level of relative expression of selected cytokines and chemokines after infection with *S. Enteritidis*. Furthermore, the results showed that the highest cytokine response to probiotic bacteria in our experiment was found in IL-1 $\beta$  and MIP1- $\beta$ . Finally, the *in vitro* method used for

selection of probiotic bacteria on the basis of cytokine activity was suitable for an *in vivo* trial.

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