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Interaction of TGF-β4 and IL-17 with IgA secretion in the intestine of chickens fed with *E. faecium* AL41 and challenged with *S.* Enteritidis

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The relative mRNA expression of IgA, TGF-β4, IL-17, and concentration of secretory IgA (sIgA) in small intestine of chickens pretreated with *Enterococcus faecium* AL41 and challenged with *Salmonella* Enteritidis PT4 were studied. *Salmonella*-free day-old chicks (40) Cobb 500 breed, were divided into four groups of 10 chicks each (n = 10): control (C), treated with *E. faecium* AL41 strain (EFAL41), challenged with *Salmonella* Enteritidis PT4 (SE), and combined (EFAL41+SE). Expression of IgA and sIgA concentration was upregulated in EFAL41 group in jejunum and ileum on 4 days post-*Salmonella* infection (dpi). Chicks in combined group demonstrated upregulation of cytokines and IgA expression, and increased sIgA concentration in the intestine flush on 7 dpi. The experiment demonstrated beneficial effect of *E. faecium* AL41 on IgA production and secretion in intestine. Findings also indicated that IgA played important role in decrease of *S.* Enteritidis in the intestine, and cytokines TGF-β4 and IL-17 contributed to the increased IgA secretion.

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1. Introduction

Salmonella enterica serovar Enteritidis carried by chicks and poultry products is the major source of human intestinal infections. The outbreaks have been found to be associated with consumption of contaminated and undercooked poultry products, such as eggs and egg-containing foods, and have become a serious economic and public health problem (Sheela et al., 2003).

Salmonella can multiply in gastrointestinal tract of birds and contaminate the environment due to excretion of bacteria through feces. These bacteria can also invade the intestinal mucosa, cecal tonsils, and Peyer's patches, survive and multiply in macrophages to spread to other organ systems (Dunkley et al., 2009).

Dietary fed microbes reduce clinical signs of bacterial enteric diseases such as clostridial disease (Geier et al., 2010) and *Salmonella* spp. (Zhang et al., 2002). *Enterococci*, a group of lactic acid bacteria, are ubiquitous microbiota, which constitute a large proportion of autochthonous microflora found in the gastrointestinal tract of human and animals (Strompfová and Lauková, 2007). Species *E. faecium* is the best studied concerning enterocin production and its characteristics (Giraffa, 2002). *E. faecium* EF55 strain has shown inhibitory activity against many bacteria including *S. enterica* (Levkut et al., 2009). Influence of *E. faecium* EF55 on the dynamics of intestinal mucin production in *S. enterica* infected birds has also been reported (Levkut et al., 2012).

A key intestinal strategy to generate immune protection in non-inflammatory manner is the production of immunoglobulin A (IgA), the most abundant antibody isotype produced in the body (Macpherson et al., 2008). Follicular inductive sites in the intestine are rich in cytokines with IgA-inducing functions, including transforming growth factor β (TGF-β) and interleukin 17 (IL-17) (Cerutti and Rescigno, 2008).

The goal of this paper was to study the effect of enterocin M producing probiotic strain *E. faecium* AL41 on IgA mRNA expression and secretion into intestinal lumen after challenge of chicks with *Salmonella enterica* serovar Enteritidis phage type 167/3 4b (PT 167/3 4b). Moreover, the cytokines inducing IgA functions (TGF-β4, IL-17) were followed-up.

2. Materials and methods

2.1. Experimental design

Salmonella-free day-old chicks (40), Cobb 500 breed, were included in an 11-day experiment. Day-old chicks were placed in large pens with cellulose cotton (Pehazell, Slovakia) and reared with lighting regimen 23 h light and 1 h dark. 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 50–60%. Birds had free acces

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to feed (BR1 – starter diet) and water. Application of cleaning and feeding regimens prevented them from cross-contamination effectively throughout the experiment. Day-old chicks were randomly divided into 4 groups of 10 chicks each (n = 10): control (C), *E. faecium* AL41 (EFAL41), *S.* Enteritidis PT4 (SE), and combined *E. faecium* AL41+*S.* Enteritidis PT4 (EFAL41+SE). *E. faecium* AL41 (provided by Andrea Lauková, Institute of Animal Physiology, Slovak Academy of Science, Košice, Slovakia) at 10^9 CFU/0.2 mL Ringer's solution was administered daily *per os* to EFAL41 and EFAL41+SE groups during 1–7 days of experiment. Experimental infection of SE and EFAL41+SE groups was carried out individually *per os* on day 4 of the experiment using *Salmonella enterica* serovar Enteritidis PT phage type 167/3 4b (provided by František Šišák, VRI, Brno, Czech Republic) at 1×10^8 CFU/0.2 mL PBS as a single dose. Samples from intestine (caudal part of the jejunum and ileum) for determination of sIgA, mRNA IgA, TGF-β4, and IL-17 were taken at 4 and 7 days post-*Salmonella* infection (dpi) during necropsy.

Specific experiments were approved by the Ethics Committee of the Veterinary Medicine and Pharmacy followed by the Committee for Animal Welfare of Ministry of Agriculture of the Slovak Republic (permit number 2730/13-221).

2.2. Homogenization of tissue and isolation of total RNA

Tissue samples (jejunum and ileum) were cut into 20 mg pieces, immediately placed into RNA Later solution (Qiagen, UK) and stored at −70 °C prior to RNA purification. Single tissue fragment was transferred into 1 mL of TRI Reagent (Molecular Research Center, USA), and homogenized using zirconium silica beads (BioSpec Products, USA) in vortex mixer (Labnet, USA). To separate the phases, 50 μL of 4-bromanisole (Molecular Research Center, USA) was added. Whole content of the tube was centrifuged and upper aqueous phase was collected for total RNA purification using the RNAeasy mini kit (Qiagen, UK) following the manufacturer's instructions. Turbo DNA-free kit (Ambion, USA) was used for treatment of RNA samples to remove genomic DNA. Purity and concentration of RNA was determined spectrophotometrically on NanoDrop 200c (Thermo Scientific, USA) and 1 μg of total RNA was immediately reverse transcribed by using iScript cDNA Synthesis Kit (Bio-Rad, USA). Resulting cDNA was 10× diluted in UltraPure™ DNase/RNase-Free distilled water (Invitrogen, USA) and used as a template in real-time PCR or stored at −20 °C until used.

2.3. Real-time PCR

The mRNA level of IgA, TGF-β4, and IL-17 were determined. In addition, mRNA relative expression of reference gene, coding GAPDH (glyceraldehyde-3- phosphate dehydrogenase), 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%.

Amplification and detection of specific products were performed using CFX 96 RT system (Bio-Rad, USA) with temperature–time profile as initial denaturation 95 °C for 10 min and 40 cycles; denaturation 94 °C for 15 s, annealing 59 °C for 30 s, and final elongation 72 °C for 36 s. A melting curve from 50 °C to 95 °C with 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 mean values of duplicates were used for subsequent analysis. We also confirmed that efficiency of amplification of each target gene (including GAPDH) was essentially 100% in the exponential phase of the reaction, where the quantification cycle (Cq) was calculated. The Cq values of studied genes were normalised to an average Cq value of the reference gene (ΔCq), and the relative expression of each gene was calculated as $2^{-\Delta Cq}$. These expression levels were then used for comparative data analysis. Relative expression of sIgA, TGF-β4, and IL-17 in jejunum and ileum were determined in forty independent animals, and in the figures, we present results combined from all of these animals.

Table 1
List of primers used in RT-PCR for IgA and cytokines mRNA detection in chicks.

Primer	Sequence 5′–3′	References
IgA For	GTCACCGTCACCTGGACTACA	Lammers et al., 2010
IgA Rev	ACCGATGGTCTCCTTCACATC	
TGFβ4 For	AGGATCTGCAGTGGAAGTGGAT	Swaggerty et al., 2004
TGFβ4 Rev	CCCCGGGTTGTGTGTTGGT	
IL-17 For	TATCAGCAAACGCTCACTGG	Crhánova et al., 2011
IL-17 Rev	AGTTCACGCACCTGGAATG	
GAPDH For	CCTGCATCTGCCCATTT	De Boever et al., 2008
GAPDH Rev	GGCACGCCATCACTATC	

2.4. Intestine samples and flush protocol

The flush protocol was assessed as described by Holt et al. (1999) with some modifications recently published (Husáková et al., 2014). Briefly, five randomly chosen chicks from each group were killed by cervical dislocation on days 4 (early phase of infection) and 7 (late phase of infection) post infection with *Salmonella* (dpi). The ileum and terminal section of jejunum were excised, and 10 mL of warm flush solution (1 M tris/glycine buffer with 0.25% Tween 20, pH 7, Sigma Aldrich, USA) was injected into the intestine lumen. The intestine flushes were centrifuged for 5 min at 12,000 *g* (Hettich Rotina 420R Centrifuge, DJB Labcare, UK), and the supernatants from each sample were frozen at −20 °C until the ELISA assay procedure.

2.5. Enzyme-linked immunosorbent assay of IgA (ELISA)

Total IgA was detected by using of chicken IgA ELISA kit (Kamiya Biomedical Company, USA). Briefly, test samples, diluted 1:5 in 1× diluent solution, were added at 100 μL per well in duplicate wells. Undiluted positive and negative controls were also applied at 100 μL per well in duplicate wells. The plate was incubated at room temperature for 20 min, followed by three washings with wash solution (horseradisch peroxidase). Anti-chicken HRPO conjugate was added at 100 μL per well, followed by incubation at room temperature for 20 mts, and 3 washings in wash buffer. One hundred microliters of 3,3,5,5′-tetramethylbenzidine (TMB) substrate solution was added into each well, and incubated at room temperature for 15 mts, followed by addition of stop solution at 100 μL per well. Absorbance at 450 nm [A(450)] was measured in a Microplate reader (Revelation Quicklink, Opsys MR, Dynex Technologies, USA). Interpretation was made using calibration curve prepared according to the manufacturer's protocol.

2.6. Statistical analysis

One way ANOVA with Tukey post-test by Minitab 16 software was used (SC&C Partner, Brno, Czech Republic). Differences between mean values for various treatment groups were considered statistically significant at $P < 0.05$, $P < 0.01$, $P < 0.001$. Values were expressed as means ± standard deviation mean ±SD).

3. Results

Relative mRNA expression of IgA was upregulated in the EFAL41 group in jejunum compared to groups: SE and EFAL41+SE ($P < 0.01$), C ($P < 0.001$); and in ileum compared to groups: SE and EFAL41+SE ($P < 0.05$), C ($P < 0.01$) at 4 dpi. However, at 7 dpi relative mRNA expression of IgA was upregulated in the combined group *E. faecium*

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