INFLUENCE OF ORAL APPLICATION OF *ENTEROCOCCUS FAECIUM* AL41 ON TGF-β4 AND IL-17 EXPRESSION AND IMMUNOCOMPETENT CELL DISTRIBUTION IN CHICKENS CHALLENGED WITH *CAMPYLOBACTER JEJUNI*

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Campylobacteriosis is mainly caused by infection with *Campylobacter jejuni* following consumption or handling of *Campylobacter*-contaminated poultry meat. The aim of this study was to investigate the effect of probiotic *Enterococcus faecium* AL41 on TGF-β4 and IL-17 expression and on immunocompetent cell distribution after *C. jejuni* infection in broiler chicken, as a second part of the previous study of Karaffová et al. (2017). Accordingly, day-old chicks were randomly divided into four experimental groups of 10 chicks each (n = 10): control (C), *E. faecium* AL41 (EFAL41), *C. jejuni* CCM6191 (CJ), and combined *E. faecium* AL41 + *C. jejuni* CCM6191 (EFAL41 + CJ). Samples from the caecum were collected on days 4 and 7 post *Campylobacter* infection (dpi), for the isolation of mRNA of TGF-β4, IL-17 and for immunohistochemistry. The relative mRNA expression of TGF-β4 was upregulated in the combined (EFAL41 + CJ) group compared to other groups during both samplings, but the expression of IL-17 was downregulated. Similarly, the highest density of CD3+ was detected in the combined group at 7 dpi, but the number of IgA+ cells was increased in both groups with EFAL41. It was concluded that the EFAL41 probiotic *E. faecium* strain can modulate the expression of selected cytokines (upregulation of TGF-β4 but downregulation of IL-17 relative expression), and activate IgA-producing cells in the caeca of chicks infected with *C. jejuni* CCM6191.

Key words: *Enterococcus faecium, Campylobacter,* chicken, local immunity

Campylobacter jejuni is one of the most common causes of acute enteritis in the developed world and can colonise the intestinal mucosa of most warm-

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blooded animals (Gruntar et al., 2010). Worldwide, the broiler caecum has been demonstrated to be colonised to a high degree by *Campylobacter* species like *C. jejuni* (EFSA, 2016).

The innate responses of the immune system lead to adaptive responses that can be measured as both mucosal and systemic specific antibodies (de Zoete et al., 2007). Studies of T lymphocyte function in the gastrointestinal tract during *C. jejuni* infection are very limited, although T cell responses have been shown in the liver during invasive infections (Jennings et al., 2011). One potential criticism of the past work is that many studies used specific-pathogen-free (SPF) chickens with high challenge doses that have only limited relevance to infection in common broiler breeds (Humphrey et al., 2014). However, previous studies have shown that *C. jejuni* may directly contribute to poor gut health in broilers (Colles et al., 2008; Williams et al., 2013).

Researchers have previously demonstrated that the avian immune response can be modulated with probiotics, which may provide a mechanism for the reported reductions in pathogens (Stringfellow et al., 2011). Probiotics are defined as live microorganisms which, when administered in sufficient amounts, confer a health benefit to the host (FAO/WHO, 2002). Probiotics are likely to have an impact through the gut mucosa by balancing the local microbiota by inhibiting the growth of pathogenic microorganisms (Servin, 2004) and by enhancing local and systemic immune responses (Bodera and Chcialowski, 2009). *Enterococcus faecium* AL41 is an Enterocin M-producing, probiotic strain which has previously shown beneficial effects on the immune response in broiler chicken (Karaffová et al., 2015; Lauková et al., 2015).

Our recent findings indicate that the antibacterial effect of *E. faecium* AL41 on *C. jejuni* in the caecum is mediated by the stimulation of Toll-like receptors (TLRs) and their co-stimulative molecules (IFNβ, MD-2, CD14) (Karaffová et al., 2017), but the question about the local expression of regulatory cytokine (TGF-β4) and pro-inflammatory cytokine (IL-17) has remained open, although it is important because of the possible development of autoimmune reactions after *C. jejuni* infection in broiler breeds. We also investigated the effect of probiotic *E. faecium* AL41 on the distribution of immunocompetent cells in the caecum.

Materials and methods

Preparation of strains

Probiotic and enterocin-M producing *E. faecium* AL41 strain and *Campylobacter jejuni* CCM6191 (supplied by the Culture Collection, Brno, Czech Republic) were isolated and inocula prepared at the Institute of Animal Physiology (SAS in Košice, Slovakia) as described in the paper of Karaffová et al. (2017).

Experimental design

The experimental design has been described earlier in detail (Karaffová et al., 2017). In short, 40 *Campylobacter*-free chicks of the Cobb 500 breed were included in 11-day experiments. Day-old chicks were randomly divided into four experimental groups of 10 chicks each (n = 10): control (C), *E. faecium* AL41 (EFAL41), *C. jejuni* CCM6191 (CJ), and combined *E. faecium* AL41 + *C. jejuni* CCM6191 (EFAL41 + CJ). *E. faecium* AL41 at the concentration of 10^9 CFU/ 0.2 ml in Ringer's solution was administered *per os* daily to the EFAL41 and EFAL41 + CJ groups from days 1 to 7 of the experiment. *Campylobacter jejuni* CCM6191 was administered orally on day 4 of the experiment in a single dose of 1×10^8 CFU in 0.2 ml PBS to the CJ and EFAL41 + CJ experimental groups. Samples from the caudal part of the caecum were collected on days 4 and 7 post-*Campylobacter* infection (dpi) for isolation of the mRNA of TGF-β4 and IL-17 and for immunohistochemistry.

Specific experiments were approved by the Ethics Committee of the University of Veterinary Medicine and Pharmacy in Košice followed by the Committee for Animal Welfare of the Ministry of Agriculture of the Slovak Republic (permit number 2636/14-221).

Homogenisation of tissue and isolation of total RNA

Tissues from the caudal part of the caecum were cut into 20 mg sample, placed immediately into RNA Later solution (Qiagen, UK) and stored at –70 °C prior to RNA purification. The isolation of total RNA was described by Karaffová et al. (2017).

Quantitative real-time PCR

The mRNA levels of TGF-β4 and IL-17 were determined. In addition, mRNA relative expression of a reference gene coding GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used for data normalisation. The primer sequences used for qPCR are listed in Table 1. All primer sets allowed DNA amplification efficiencies between 94% and 100%.

Table 1

List of primers used in RT-PCR for cytokines mRNA detection in chickens

Primer	Sequence 5'–3'	References
TGF-β4 For	AGGATCTGCAGTGGAAGTGGAT	Swaggerty et al., 2004
TGF-β4 Rev	CCCCGGGTTGTGTGTTGGT	
IL-17 For	TATCAGCAAACGCTCACTGG	Crhánová et al., 2011
IL-17 Rev	AGTTCACGCACCTGGAATG	
GAPDH For	CCTGCATCTGCCCATTT	De Boever et al., 2008
GAPDH Rev	GGCACGCCATCACTATC	

Amplification and detection of specific products were performed using CFX 96 RT system (Bio-Rad, USA) with the following temperature–time profile: initial denaturation 95 °C for 10 min and 40 cycles; denaturation 94 °C for 15 sec, annealing 59 °C for 30 sec, and final elongation 72 °C for 36 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 real-time PCR in duplicate and the mean values of duplicates were used for subsequent analysis. We also confirmed that the 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 the genes studied were normalised to the 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.

Immunohistochemistry

The caeca were collected into phosphate-buffered saline (PBS; pH 7.6), and then frozen and cut at –20 °C with Thermo Scientific Cryotome E (Shandon, USA). Frozen 5-µm sections fixed in cold acetone and rinsed in PBS were incubated with 100 µl 0.03% H_2O_2 in PBS for 20 s to block endogenous peroxidase. A streptavidin-biotin amplified peroxidase detection system (VECTASTAIN Elite ABC kit, Mouse IgG, PK 6102, Vector Laboratories, USA) was used to detect IgA-, IgM- and CD3-positive lymphocytes. Unlabelled primary mouse anti-chicken monoclonal antibodies (Abs) IgA (IgA-UNLB, Clone A-1, Cat. No. 8330-01), IgM (IgM-UNLB, Clone M-1, Cat. No. 8300-01) and CD3 (CD3-UNLB, Clone CT-3, Cat. No. 8200-01, Southern Biotech, USA) were used in 1:10 dilution with PBS. Mouse IgG1-UNLB antibody (Clone 15H6, Cat. No. 0102-01, Southern Biotech, USA) was used as negative control. All incubations were done at room temperature according to the manual instructions. The sections were rinsed three times with PBS between the two consecutive incubations. The specific colour reaction was developed for 5 min with 3.5 mmol/L 3,3'-diaminobenzidine (DAB, Sigma, Germany), and 30 ppm hydrogen peroxide in 200 mmol/L Tris-HCl (pH 7.6). Subsequently the sections were counterstained with haematoxylin and mounted into Pertex (Histolab AB, Swedish). Quantification of IgA+, IgM+ and CD3+ lymphocytes was performed under light microscope (NIKON Labophot 2, Germany) at a magnification of × 200. In every section of the caecum six very well-preserved villi were chosen at random and examined. Labelled cells were counted in the epithelium and lamina propria from the base of the villi up to the apex. Calibrated ocular graticule LTD 0.25 mm IdXG rd (Tonbridge, Kent, UK) was used to perform quantitative analysis. Each sample was analysed in three sections.

Statistical analysis

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

Results

The relative mRNA expression of TGF-β4 was upregulated in the EFAL41 + CJ group in the caecum compared to groups EFAL41 ($P < 0.05$) and CJ ($P < 0.001$) at 4 dpi. Similarly, at 7 dpi the relative mRNA expression of TGF-β4 was upregulated in the combined group EFAL41 + CJ compared to the other groups ($P < 0.001$) (Fig. 1).
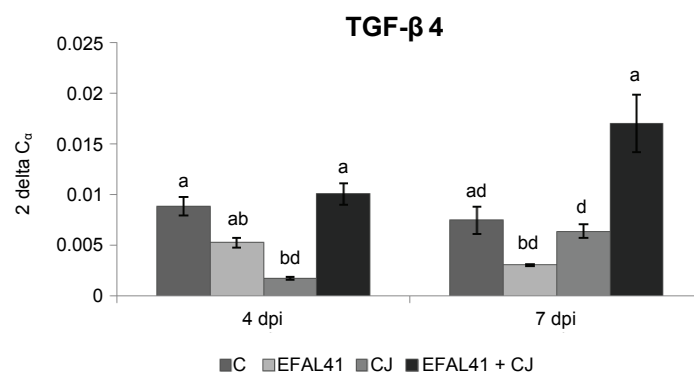

Fig. 1. Relative expression level of TGF-β4 in the caecum of chickens. Real-time PCR results at each time point are the median of $2^{-\Delta Cq}$. Means with different superscripts are significantly different ($^{ab}P < 0.05$, $^{ad}P < 0.001$)

The relative mRNA expression of IL-17 was upregulated in Group CJ compared to Groups EFAL41 and EFAL41 + CJ ($P < 0.05$) at 4 dpi. The same tendency was observed at 7 dpi in the CJ group compared to groups C ($P < 0.05$), EFAL41 ($P < 0.01$) and EFAL41 + CJ ($P < 0.05$) (Fig. 2).

The quantification of IgA+ (IEL + LPL) cells showed a moderate increase in the EFAL41 group compared to the control group and the CJ group ($P < 0.05$) at 4 dpi. Our experiment showed higher density of IgA+ cells in the EFAL41 and EFAL41 + CJ groups than in the control and CJ groups ($P < 0.001$) at 7 dpi (Fig. 3).

The highest density of IgM+ cells was detected in the CJ group compared to the control, EFAL41 + CJ ($P < 0.001$) and EFAL41 groups ($P < 0.01$) at 4 dpi. At 7 dpi, the cell numbers significantly increased in all observed groups compared to the control (Fig. 4).
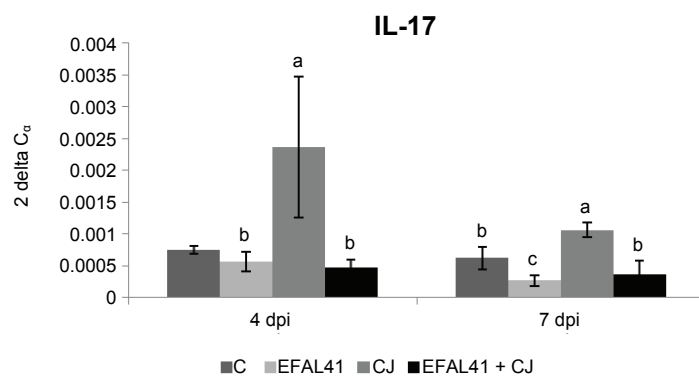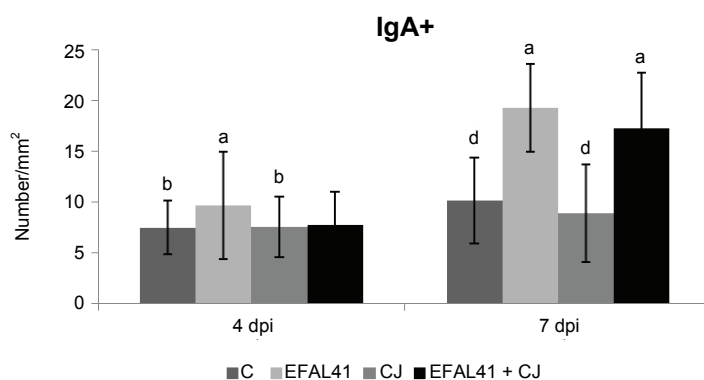
IL-17

IgA+

IgM+

The quantification of CD3+ (IEL + LPL) cells showed higher density in the EFAL41 group when compared to groups CJ (P < 0.01) and EFAL41 + CJ (P < 0.05) at 4 dpi. At 7 dpi the CD3+ cells showed a higher density in the EFAL41 + CJ group than in the other groups (P < 0.001) (Fig. 5).

Fig. 5. Quantification of CD3+ cells in the caecum of chickens. Means with different superscripts are significantly different ([ab]P < 0.05, [ac]P < 0.01, [ad]P < 0.001)
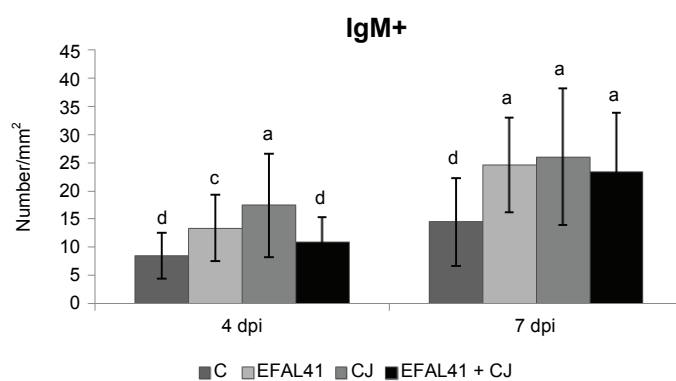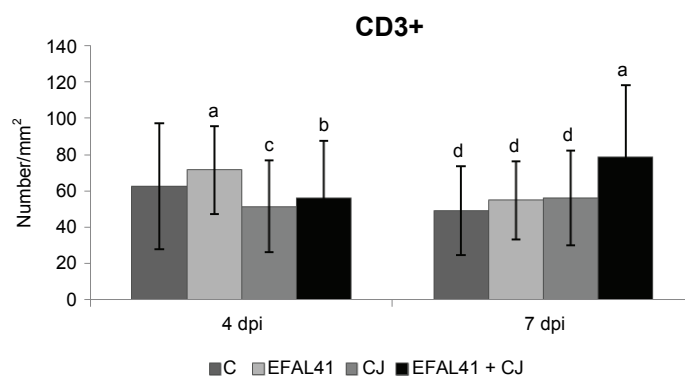
Discussion

Campylobacter jejuni is often considered a harmless commensal inhabitant of the chicken gut, and the immune response to it in the intestinal tract is thought to be tolerogenic (Hermans et al., 2014). In our previous study the counts of *Campylobacter*-like spp. in the caecum were high in the infected groups but simultaneously we found that *E. faecium* AL41 sufficiently colonised the caecum of broiler chickens in the groups where it was applied (Karaffová et al., 2017). Recent reports mention that the chicken–*Campylobacter* relationship cannot be regarded as commensal. Mucosal damage can occur as a result of a prolonged pro-inflammatory response (Humphrey et al., 2014). Infection with *C. jejuni* led to the initiation of innate immune responses in the gut that caused an influx of inflammatory cells, including macrophages and heterophils (de Zoete et al., 2010). The results of these studies indicate the strong activation of innate and humoral immunity through increasing the relative expression of proinflammatory cytokine IL-17 and the number of IgM-positive lymphocytes in the *Campylobacter* group (CJ), in addition to modulation of the expression of TLRs and other inflammatory mediators as reported recently (Karaffová et al., 2017).

Probiotics and the gut microbiota have a significant impact on gut homeostasis in the host. Moreover, various mechanisms of activity have been suggested to explain the protective anti-inflammatory effects of probiotics in intestinal inflammation, including the immunoregulation and suppression of Th17 cells and

IL-17 production (Tanabe, 2013). In agreement with the previous statement, we observed a suppressive effect of *E. faecium* AL41 on the relative expression of IL-17 in the EFAL41 + CJ group. The stimulatory effect of *E. faecium* AL41 on the relative expression of TGF-β4, which was recorded in the combined group during both samplings, correlates with the observed presence of a higher IgA cellular response in this group. Transforming growth factor-beta (TGF-beta) has been reported to play an important role in IgA isotype expression when B cells are stimulated with LPS (Ehrhardt et al., 1992). In the LPS system TGF-beta caused a small but significant absolute increase in surface IgA (sIgA) expression and a very definite increase in IgA secretion.

The importance of inhibitory cytokines such as TGF-β in immune homeostasis is reflected in the phenotype of mice unable to produce these molecules. TGF-β1-deficient mice develop a multifocal inflammatory disease with autoimmune manifestations including the production of autoantibodies (Han et al., 2012). The results suggest an anti-inflammatory activity of *E. faecium* AL41 on the local immunity of the caecum. In addition, TGF-β can also stimulate MHC class II expression and induces the differentiation of IgA-secreting plasma cells (Klein et al., 2006).

The microbiota also has an essential role in promoting the development of IgA-producing plasma cells, and there is evidence that this enhancing effect is driven by specific bacterial species (Macpherson and Harris, 2004). Previous studies have shown that probiotic strains of *E. faecium* (EF55 and AL41) increased the number of IgA+ cells in the intestine of chickens at a late phase of infection with *Salmonella* Enteritidis (Bobíková et al., 2015; Karaffová et al., 2015). Similarly, our experiment demonstrated that the highest density of IgA+ cells was found in the groups treated with *E. faecium* AL41 (EFAL41, EFAL41 + CJ) at 7 dpi also in the case of infection with *C. jejuni*. These results confirm that *E. faecium* AL41 is able to influence the mucosal immunity of the host. The results demonstrate the ability of *E. faecium* AL41 to influence infiltration of the mucosal layer in the caecum with IgA+ cells.

Intestinal lamina propria lymphocytes (LPLs) and intraepithelial lymphocytes (IELs) are the effector components of the gut mucosal immune system and may play a critical role in immune surveillance of the epithelium. In addition to their effector function, LPLs also have a role in immunoregulation (Burkey et al., 2009) and IELs with potent cytolytic and immunoregulatory capacities can be recruited quickly to maintain epithelial integrity and protect host tissues from infectious agents (Van Wijk and Cheroutre, 2009). In our study, administration of *E. faecium* AL41 significantly increased the numbers of (LPLs + IELs) CD3+ T cells in the EFAL41 + CJ group at 7 dpi.

In conclusion, the results indicate that the probiotic strain *E. faecium* AL41 can have an immunomodulatory effect mediated by the local regulation of cytokine expression (upregulation of TGF-β4 but downregulation of IL-17 rela-

tive expression) and activation of IgA-producing cells in the caeca of chicks infected with *C. jejuni* CCM6191. Moreover, downregulation of IL-17 may participate in preventing the development of autoimmune reactions, which can occur in rapidly growing broiler breeds.

Acknowledgements

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