

# Reaction of Immune Cells to *Campylobacter Jejuni* in Chicken PBMC Treated by Different Probiotic Bacteria *In Vitro*

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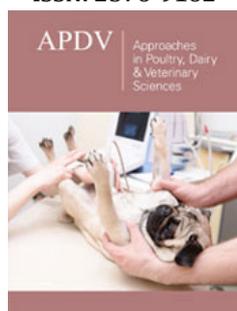
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ISSN: 2576-9162



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Submission:  January 27, 2020

Published:  February 07, 2020

Volume 7 - Issue 3

**How to cite this article:** Viera Revajová, Viera Karaffová, Mária Levkutová, Miroslava Šefcová, Andrea Lauková, et al. Reaction of Immune Cells to *Campylobacter Jejuni* in Chicken PBMC Treated by Different Probiotic Bacteria *In Vitro*. *Appro Poultry Dairy & Vet Sci* 7(3). APDV.000665.2020. DOI: [10.31031/APDV.2020.07.000665](https://doi.org/10.31031/APDV.2020.07.000665)

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## Abstract

The immune response of *Enterococcus faecium* AL41 (EF AL41), *Enterococcus faecium* H31 (EF H31), and *Lactobacillus fermentum* AD1 (LF AD1) against *Campylobacter jejuni* CCM6191 (CJ) were investigated *in vitro* by flow cytometry and immunophenotyping of cultivated peripheral blood mononuclear cells (PBMC). It may be postulated that using of probiotic bacteria strains in single arrangement during 48h *in vitro* cultivation displayed different influence to evaluated T (CD3, CD4, CD8) and B (BU1, IgA, IgM) immune cells. *E. faecium* AL41 acted predominantly 24h pi with *C. jejuni* while *L. fermentum* AD1 effect was detected 48 h pi. Both probiotic strains also displayed distinct manner of T and B cells activation. *E. faecium* H3 showed the lowest effect to both, controls and *C. jejuni*

## Introduction

Chickens are a natural host for *Campylobacter* species and are often colonized by *Campylobacter jejuni* and *Campylobacter coli* [1]. Particularly broiler chickens are commonly regarded as a natural host for this zoonotic pathogen and infected birds carry a very high *C. jejuni* load in their gastrointestinal tract, especially the ceca. This results in contaminated carcasses during processing [2]. Meat contaminated with *C. jejuni* is an important source of foodborne gastroenteritis and poses a serious health burden in industrialized countries. *C. jejuni* isolates can have different potential of colonization and have been divided into three phenotypes. First phenotype fails to colonize 14-day-old chickens, second can colonize but are readily eliminated and are classified as transient, third show efficient and sustained colonization [3]. These three colonization phenotypes were found to be stable and independent of *in vivo* passages and the number of viable bacteria in the inoculum. Enhanced colonization capacity and increased virulence after *in vivo* passage through chicks has been shown [4]. The *C. jejuni* induced adaptive T-cell response in the intestine in *ex vivo* infected explants of human colon tissue. After reached subepithelial compartments, adhesion and invasion of intestinal epithelial cells follows. The direct contact of the invading bacteria with neutrophils, macrophages, and dendritic cells initiated Th1 lymphocytes and responses characterized by the release of IFN- $\gamma$ , IL-1 $\beta$ , IL-12, and IL-6 [5]. An increased IL-12 and IL-23 level indicates the dendritic cell activation and maturation, which is subsequently driving the response of distinct Th17 cell subsets. Elevated numbers of Th17, Th1, and Th17/Th1 double-positive cells were well in line with increased concentrations of IL-22 and IL-17 which orchestrate the eradication of the pathogen by induction of innate responses and defensin production in the epithelium. A possible way to reduce *Campylobacter* contamination in poultry is to develop new actions at the primary production level. Therefore, it has become necessary to develop alternatives such as beneficial microorganisms (probiotics). The use of probiotics, which can help to improve the natural defence of animals against pathogenic bacteria, is an alternative and effective approach to antibiotic administration for livestock to reduce bacterial contamination. *In vitro* experiments showed that *Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* isolated from healthy chicken gut inhibited the growth of *C. jejuni*. *In vivo* administration of *Enterococcus*

*faecium* 55 to chicks challenged with *Salmonella Enteritidis* 147 in combined group (EF+SE) ultimately resulted in increased number of blood heterophiles, caecal IgA+ IEL, and decreased caecal expression of MUC compared to SE group on 7 days post infection. Detection of IL-15 and IL-17 mRNA cytokine level in caecum showed a tendency to their increase on 1-day post salmonella infection in combined EF+SE group [6]. The aim of our *in vitro* study was to determine by flow cytometry the stimulation of main T and B lymphocyte subpopulations in peripheral blood mononuclear cell culture after preventive administration of probiotic bacterial strains *Enterococcus faecium* AL41, *Enterococcus faecium* H31 and *Lactobacillus fermentum* AD1 and *Campylobacter jejuni* infection.

## Material and Methods

### Isolation and cultivation of PBMC

Blood was collected from the vena cutanea ulnaris to 1.5% EDTA from clinically healthy poultry reared under standard condition. 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 40min at 19000×g at 20 °C (Hettich Rotina 420R Centrifuge, DJB Labcare, UK). Mononuclear cells were collected from the gradient interface and washed 2 times for 5min at 16000 × g with PBS. Cell viability and number were determined by trypan blue exclusion. Isolated PBMC (Peripheral Blood Mononuclear Cells) were placed on 12-well cultivation plate (Orange Scientific, BE) at the concentration of 1×10<sup>7</sup> cells/mL and cultured overnight (39 °C, 5% CO<sub>2</sub>) in RPMI 1640 medium enriched with 10mM HEPES (Lonza, BE) and 10% FBS (Lonza, BE). Followed by 24 hours cultivation 200µl of particular probiotic bacterial strains (provided by Dr. Lauková, IAP SAS, Košice, Slovakia) were added to the individual PMBCs cultures. The concentrations of *C. jejuni* CCM6191 was 1×10<sup>8</sup> CFU/ml. *Enterococcus faecium* AL41, *Enterococcus faecium* H31 and *Lactobacillus fermentum* AD1 were added at the concentration of 1×10<sup>9</sup> CFU/ml. After the addition of probiotic bacteria PMBCs were cultured for 24 and 48 hours. The cultivation of bacterial strains, culture medium and growth conditions were performed as described previously [7,8].

### Flow cytometry procedure

Mouse anti-chicken monoclonal antibodies CD3, CD4, CD8 (T-cells), BU1, IgA, IgM (B-cells) labelled with FITC (SouthernBiotech, USA) were used for immunophenotyping of lymphocytes by direct immunofluorescent method. After cultivation the lymphocytes were washed twice with phosphate buffered saline (PBS). Fifty µL of cellular suspension (1.10<sup>7</sup> lymphocytes in PBS) and 2µl of specific or control MoAbs were mixed and incubated in dark at 22 °C for 15min. After being stained, the cells were washed once in 0.5mL PBS and resuspended in 0.2mL of PBS with 0.1% paraformaldehyde. Measurement and analysis of stained cells was performed by FACS system (Becton Dickinson, Germany) provided with a 15mV argon ion laser. The analysis examined a dot plot of the leukocytes obtained by the forward and side scattering of the

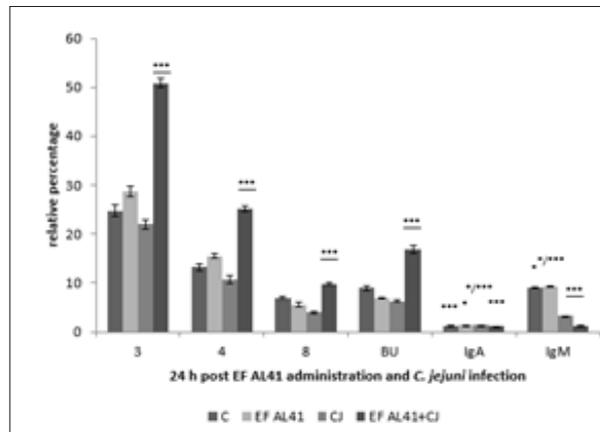
physical character of the lymphocyte population. Gates were drawn around lymphocytes based on 90° and forward-angle light scatter. The fluorescence data were collected on at least 10,000 lymphocytes using the Becton Dickinson Cell Quest programme. The results are therefore expressed as the relative percentage of the lymphocyte subpopulation which was positive for a specific MoAb. Samples were measured in triplets before cultivation, 24h after cultivation, 24h and 48h post infection (pi) in following arrangement: controls, probiotic strain, campylobacter and also co-cultivation probiotic + campylobacter group. Statistical analysis was done using GraphPad Software and one-way analysis of variance (ANOVA) with the post hoc Tukey multiple comparison test. The differences between the mean values for the groups of chickens were considered significant when P<0.05.

## Results

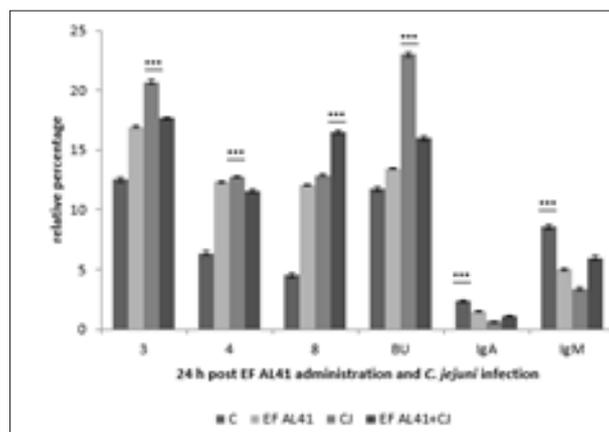
Subpopulations of lymphocytes in controls 24 hours after cultivation showed the increase of values in comparison with samples before cultivation (data not shown). Probiotic strain *Enterococcus faecium* AL41 24h pi (Figure 1) showed increase (\*\*p<0.001) of all examined T-cell subpopulations (CD3+, CD4+, CD8+) and BU1+ in combined EF AL41+CJ group in comparison with other groups. IgA expression was the highest in CJ group (\*\*p<0.001 vs C and EF AL41+CJ, \*p<0.05 vs EF AL41), and IgM+ subpopulation in EF AL41 group (\*p<0.05 vs C, \*\*p<0.001 vs CJ and EF AL41+CJ). 48 h pi (Figure 2) was recorded the increase of examined subpopulations in experimental groups compared to control, except of IgA+ and IgM+ with the peak in C groups (\*\*p<0.001). However, the highest values were found in CJ groups (\*\*p<0.001), overstimulation persisted only in CD8+ of EF AL41+CJ group. Controls showed maximal IgA+ and IgM+ values while probiotic groups overexpressed CJ groups. Probiotic strain *Enterococcus faecium* H31 24 h pi (Figure 3) surprisingly overstimulated almost all determined lymphocyte subpopulations in control groups (\*\*p<0.001) when compared to experimental groups, except BU+ cells showed maximum level (\*\*p<0.001) in EF H31 alone and combined probiotic group EF H31+CJ. Strain *C. jejuni* exceeded the expression of probiotic groups in CD4+, CD8+ and IgA+ subpopulations. 48 hours post infection (Figure 4) on the contrary upregulation showed experimental groups, mainly EF H31 (\*\*p<0.001) in T-cell and IgA+ subpopulations compared to controls, CJ and EF H31+CJ. Infected CJ group reached the higher values in BU expression (\*\*p<0.001) than control and probiotic groups. On the other hand, the values of IgM+ lymphocytes in EF H31 group were downregulated (\*\*p<0.001 vs C, CJ, EF H31+CJ). Probiotic strain *Lactobacillus fermentum* AD1 24h pi (Figure 5) upgraded the subpopulations of experimental groups in comparison with controls except of slightly higher CD3 expression (\*\*p<0.01 vs LF AD1, \*p<0.05 vs CJ, \*\*p<0.001 vs LF AD1+CJ). Strain *C. jejuni* (CJ) overnumbered both probiotic groups only in CD4+ subpopulation (\*p<0.01). Overstimulation of combine group LF AD1+CJ (\*\*p<0.001) was observed in CD8+ T-cells, as well as BU+ and IgM+ B-cells, while IgA+ showed overexpression in alone probiotic LF AD1 group (\*\*p<0.001) compared to others. 48 hours

pi (Figure 6) the higher values of experimental groups persisted (\*\*p<0.001) when compared to the controls. While CD3+, CD8+ and BU+ subpopulations showed overstimulation in LF AD1+CJ

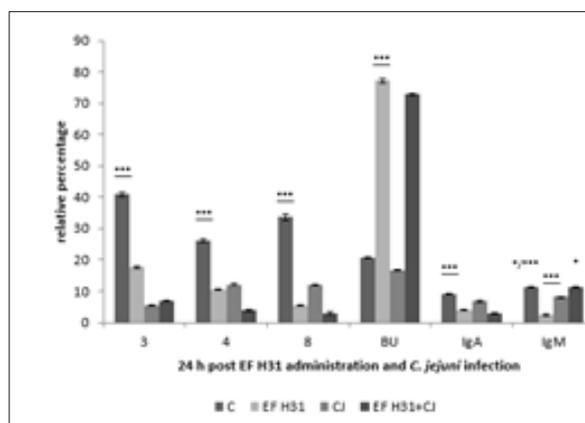
group (\*\*p<0.001) followed by LF AD1, CD4+, IgA+ and IgM+ were overexpressed in CJ groups (\*\*p<0.001).



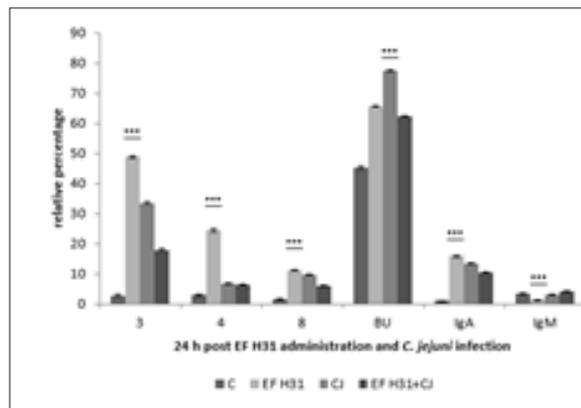
**Figure 1:** Relative percentage of lymphocyte subpopulations 24h post *Enterococcus faecium* AL41 administration and *C. jejuni* infection (\*\*p<0.001, \*p<0.05).



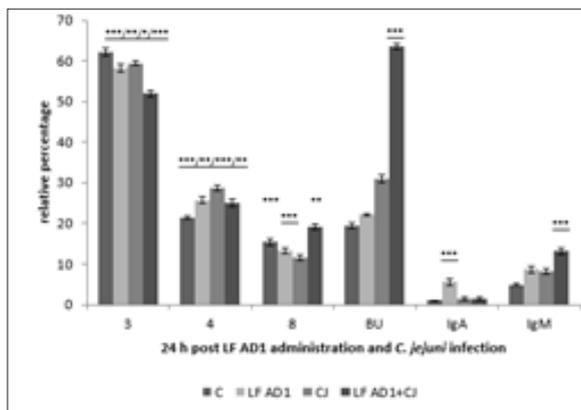
**Figure 2:** Relative percentage of lymphocyte subpopulations 48h post *Enterococcus faecium* AL41 administration and *C. jejuni* infection (\*\*p<0.001).



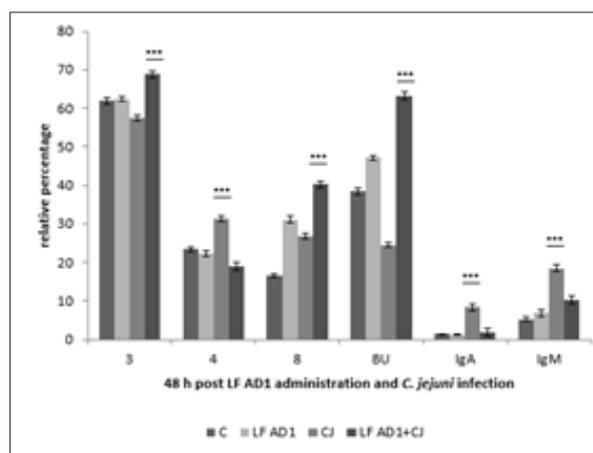
**Figure 3:** Relative percentage of lymphocyte subpopulations 24h pi with *Enterococcus faecium* H31 administration and *C. jejuni* infection (\*\*p<0.001).



**Figure 4:** Relative percentage of lymphocyte subpopulations 48h pi with *Enterococcus faecium* H31 administration and *C. jejuni* infection (\*\* $p < 0.001$ ).



**Figure 5:** Relative percentage of lymphocyte subpopulations 24h pi with *Lactobacillus fermentum* AD1 administration and *C. jejuni* infection (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Figure 6:** Relative percentage of lymphocyte subpopulations 48h pi with *Lactobacillus fermentum* AD1 administration and *C. jejuni* infection (\*\* $p < 0.001$ ).

**Discussion**

Campylobacteriosis is a collective description for infectious diseases caused by members of the bacterial genus *Campylobacter*. The only form of campylobacteriosis of major public health

importance is *Campylobacter* enteritis due to *C. jejuni* and *C. coli*. The rate of *Campylobacter* infections worldwide has been increasing, with the number of cases often exceeding those of salmonellosis and shigellosis. This increase, as well as the

expanding spectrum of diseases caused by the organisms, necessitates a clearer understanding of the epidemiology and control of campylobacteriosis [9]. *Campylobacter jejuni* are gram-negative, spiral, microaerophilic, motile bacteria and one of the most common enteric bacterial pathogens globally. The Campylobacter multidrug efflux pump (CME) plays an important role in multidrug resistance in *C. jejuni*, mediating resistance to heavy metals and a broad range of antibiotics and other antimicrobial agents [10]. It is also responsible for resistance to bile salts in the chicken intestinal tract and is therefore essential for successful intestinal colonization in chickens [11]. In mice, ferrets, nonhuman primates, and humans, immunity to sickness is suggested to be controlled by an immune mechanism distinct from that controlling immunity to infection. In ferrets *C. jejuni* CG8421 stimulated both mucosal and systemic antigen-specific IgA and IgG [12]. Hermans et al. [13] mentioned that if antibodies against *C. jejuni* are already present in the chick, the ability of *C. jejuni* to colonize is dramatically reduced, be it due to transfer of maternal antibodies to or through immunization of such birds. Our *in vitro* study showed the gradual increase of IgA+ lymphocytes in *C. jejuni* groups during cultivation that reached the peaks at 24 or 48h in all three experiments with different probiotic bacterial strains. In spite of probiotic strains overstimulation, we missed any prevalence of combined probiotic and *C. jejuni* groups. *C. jejuni* group also presented improvement of IgM, and BU1 subpopulations including into production of antibodies. LF AD1 strain upregulated BU1 subpopulation 24 and 48h post infection. Because genetically polymorphic cell surface antigen, BU-1, is expressed on B cells as well as on a subset of macrophages and no correlation with IgM was observed, we supposed also activation of macrophages. Overstimulation of macrophages proved Karaffová et al. [14] by demonstration that *E. faecium* AL41 can modulate the TLRs expression and modify the activation of MIF, IFN- $\gamma$ , MD-2 and CD-14 molecules in the chicken caecum challenged with *C. jejuni* CCM 6191. A little information connected with *C. jejuni* we found in literature about cell mediated immune response, mainly subpopulation of lymphocytes in peripheral blood and immune organs in chickens. By some authors there is some similarity of immunity in *C. jejuni* to *Salmonella* infection when changes in numbers, distribution and proliferation of T cells and delayed type of hypersensitivity were observed [15].

We found stimulation of T cells in *C. jejuni* groups, mainly CD3, CD4, CD8, CD44, as well as higher expression of CD45 and MHC II antigens as the proof of specific cell immunity including in prevention against intracellular bacteria (unpublished data). Increase of MHC II antigens is important for their presentation of bacterial antigens to T<sub>H</sub> (CD4+) and T<sub>C</sub> (CD8+) lymphocytes. Activated T<sub>C</sub> then are able to kill infected macrophages tend to loss of protective atmosphere for bacteria that results to inhibition of infection or its dissemination.

Probiotics can be defined as live microbial feed supplements that beneficially affect the host animal by improving its intestinal health [16]. Different strains of the same species of probiotics

can have unique biological activities, such as different sites of adhesion, specific immunological effects, and fermentation characteristics [17] Willis and Reid [18] showed that *C. jejuni* was present at a lower level in broiler chickens fed with a standard diet supplemented with a probiotic formulation containing *L. acidophilus*, *L. casei*, *B. thermophilus*, and *E. faecium* ( $10^8$  cfu/g) with respect to the control. We have done *in vitro* co-culture of *C. jejuni* and three individual probiotic bacterial strains to determine and recognize their immune influence for using of the most effective strain in the future for *in vivo* arrangement experiments in chicks. The aim will be finding out the possible decrease of *C. jejuni* after oral administration these newest probiotic bacterial strains. Immunofenotyping of our studied probiotic bacterial strains *E. faecium* AL41, *E. faecium* EF H3, and *Lactobacillus fermentum* AD1 manifested different immunostimulatory effect when compared to *C. jejuni* and also controls. Many papers reported beneficial effect of probiotics, mainly in connection with reducing of *C. jejuni* shedding. Morishita et al. [19] reported that Avian PAC Soluble, which contains the bacteria *L. acidophilus* and *S. faecium*, was successful in reducing the shedding (70%) and jejunal colonization of *C. jejuni* in market-aged broilers. Morishita et al. [20] also published a 70% reduction in the frequency of *C. jejuni* in chicks with the use of a commercial probiotic containing *L. acidophilus* and *E. faecium*. In our previous *in vivo* experiment, we tested the effectivity of probiotic strain *E. faecium* AL41 (EF AL41) to *S. Enteritidis* PT4 (SE) infection in the peripheral blood of chickens. The probiotic groups (EF AL41 alone and combine EF AL41+SE) on 4 dpi showed tendency to improve expression of CD3, CD4, CD8, IgM, MHCII, as well as IgA subpopulation in EF group [21]. *In vitro* study with *C. jejuni* showed 24h pi the increase of all determined subpopulations -CD3, CD4, CD8, BU1, IgM, and also CD44, CD45 (data do not show) in probiotic groups excepting IgA that was higher in *C. jejuni*. Countertendency was observed 48 h pi when values *C. jejuni* group slightly outnumbered expression of determined subpopulations in probiotic groups except of IgA, IgM and CD44. Immunofenotyping of lymphocyte subpopulation of probiotic strain *E. faecium* H3 showed lower effectivity then EF AL41. Whereas the expression at 24h pi in probiotic groups was similar to detection of EF AL41 in CD3, Bu1, IgM, CD44 and CD45 and overnumbered values in *C. jejuni*, 48 pi higher expression showed only EF AL41 group in T lymphocytes (CD3, CD4, CD8), and IgA subpopulation. The testing of *L. fermentum* AD1 probiotic bacterial strain demonstrated better immunostimulation of LF AD1 and LF AD+CJ at 48h pi presented with increase of expression CD3, CD8, Bu1, CD44, CD45, and MHC I, whereas at 24h pi the improving was detected only in CD8, IgM and MHC I subpopulations. *C. jejuni* group on the contrary showed increase in CD4 and MHC II at 24h pi followed with rising of IgA and IgM at 48h. It may be supposed preference of humoral immunity in *C. jejuni* infection. We did the monitoring of mucosal immunity by determination of cecal intraepithelial (IEL) and lamina propria lymphocytes (LPL) in chickens administered with *Lactobacillus fermentum* CCM 7514 and *Campylobacter coli* challenge that indicated the improve of CD8+ IEL at 4 and 7 dpi in combined

LBCB groups. Intraepithelial CD3+, CD4+, and IgA+ lymphocytes raised also in combined LBCB groups. Cecal LPL showed the highest values of IgA+ at 4 and 7 dpi together with IgM+ cells. Splenic CD4+ and IgA+ lymphocytes were increased at 7 dpi. The preliminary results suggested beneficial effect of *L. fermentum* CCM 7514 mainly by improving of humoral response [22]. In conclusion may be postulated that using of probiotic bacterial strains in single arrangement during 48h *in vitro* cultivation displayed different influence to immunocompetent cells. While *E. faecium* AL41 increased all T cells and BU1 populations 24 h pi, production of IgA and IgM was noticed 48h pi. *E. faecium* H3 showed minimum interference in comparison with controls that overexpressed the values of both EF H3 and EF H3+CJ probiotic groups except of Bu1, CD44 and CD45 at 24h, although at 48h alone EF H3 showed the highest expression of T cells and IgA subpopulations. On the contrary LF AD1 manifested 48h pi mainly activation across CD8 T lymphocytes with increase of BU1. Finally, *E. faecium* AL41 acts earlier than *L. fermentum* AD1 with distinct manner of activation of T and B cells.

### Acknowledgement

This work was supported by Slovak Research and Developmental Agency APVV-15-0165, Grant Agency for Science of Slovak Republic VEGA 1/0112/1 and 1/0355/19.

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