sciendo

DOI: 10.2478/fv-2020-0006

FOLIA VETERINARIA, 64, 1: 39—47, 2020

IN VITRO STUDY OF IMMUNE PROPERTIES OF NEW LACTOBACILLI ISOLATES FROM PHEASANT GUT

Karaffová, V.[1], Revajová, V.[1], Nemcová, R.[2]
Ševčíková, Z.[1], Levkutová, M.[3], Levkut, M.[1, 4]

[1]Institute of Pathological Anatomy
[2]Department of Microbiology and Immunology
[3]Institute of Epizootology and Preventive Veterinary Medicine
University of Veterinary Medicine and Pharmacy in Košice, Košice
[4]Institute of Neuroimmunology, Slovak Academy of Sciences, Bratislava, Slovakia

viera.karaffova@uvlf.sk

ABSTRACT

The goal of this paper was to study the effect of *Lactobacillus reuteri* B1/1, B2/1 and B6/1 on the relative expression of selected interleukins (IL-1β, IL-15), macrophage inflammatory protein (MIP-1β), and the relative percentage of T lymphocyte subpopulations in peripheral mononuclear blood cells (PMBCs). The mRNA expression levels of interleukins and MIP-1β of PMBCs were evaluated at 24 h and 48 h post inoculation using the quantitative real-time polymerase chain reaction (qRT-PCR). The percentage of T lymphocyte subpopulations in PMBCs was determined by flow cytometry. The group that was administered *L. reuteri* B1/1 had the most significant stimulation of the expression of pro-inflammatory interleukins and MIP-1β, in particular after 24 h. Similarly, we observed a rise in the relative percentage of T cells including CD3+, CD4+ and CD8+ lymphocytes in the groups with *L. reuteri* B1/1 and *L. reuteri* B2/1. Overall, *L. reuteri* B1/1 and *L. reuteri* B2/1 showed a promising stimulatory effect on the relative expression of pro-inflammatory interleukins, MIP-1β and percentage of T cell subpopulations *in vitro*. On the flip side, *L. reuteri* B6/1 did not induce the expression of the IL-1β gene.

Key words: *Campylobacter*; cytokine; lactobacili; peripheral mononuclear blood cells

INTRODUCTION

Poultry meat can become contaminated with *Campylobacter* spp. during slaughter and may be passed on to humans through consumption and handling of contaminated poultry products. Importantly, campylobacteriosis is the most commonly reported zoonosis and chicken meat is considered the main source of this infection [16]. Moreover, the broiler immune system is usually inefficiently activated by *Campylobacter jejuni*. Colonization and the expression of relevant immune proteins is suppressed [2].

Unquestionably, cytokines and chemokines are key regulators of innate immunity via the development of in-

flammatory responses to *Campylobacter* infection. Differentiated T helper cells rapidly secrete particular cytokines upon antigen challenge [18]. The use of new probiotic preparations could be a promising form for the prevention and treatment of *Campylobacter* infections in poultry by the modulation of cytokine production and thus an immune answer as well as by inhibition of the pathogen.

In recent years, great attention has been focused on the use of probiotic lactobacilli derived from natural sources to improve human and animal health. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer health benefits on the host" [11].

Lactobacillus is the largest genus of lactic acid bacteria (LAB), which includes 183 species. They are Gram-positive bacteria that ferment glucose, produce lactic acid and other substances. Moreover, lactobacilli are the dominant bacteria in the animal gastrointestinal system. They play an important role mainly in the maintenance and recovery of gut health [37, 39]. In chickens treated with various *Lactobacillus* species it has been observed that they modulate many aspects of the immune response to pathogens [17].

The development of each new probiotic preparation needs research into the selected strain to be used, how does it affects the host organism, mechanism of action, safety and genetic stability must be documented [14]. Many other factors have to be tested, including the survival in gastric and intestinal fluids, the capability to adhere to the intestinal surface, and the sensitivity to antibiotics during *in vitro* experiments [9]. In terms of protection against pathogenic organisms, it is necessary to test the immunomodulatory effect and antimicrobial activities of newly isolated potential probiotic strains.

Among other things, one of the selection criteria for safety is that the potential probiotic strain should demonstrate ATB (Antibiotic)-sensitive based on the EFSA (European Food Safety Association) requirement [10] and does not contain transferable (acquired) ATB resistance genes located for example on conjugated plasmids or transposons. Probiotic microorganisms should not increase the existing risks of antibiotic resistance. If the lactobacilli live in an environment that is regularly exposed to ATB, such as the intestines of poultry kept in intensive farm conditions, acquired resistance may also develop. Therefore, we decided to isolate potential probiotic strains from wild birds—pheasants, where a low incidence of resistant isolates might be expected.

For the possible use of new, potentially probiotic lactobacilli, isolated from pheasant gut designed for the prevention or treatment of campylobacteriosis in chicken, we focused on the study of the effect of *L. reuteri* B1/1, B2/1 and B6/1. Specifically, we focused on the relative gene- expression of selected pro-inflammatory interleukins, MIP-1β, the relative percentage of T lymphocyte subpopulations in the PMBCs of chickens. Moreover, we tested antimicrobial activities of *L. reuteri* isolates against selected pathogens.

MATERIALS AND METHODS

Cultivation and isolation of peripheral mononuclear blood cells

The State Veterinary and Food Administration of the Slovak Republic approved the experimental protocol number 836/17-221 and the animals were handled in a humane manner.

Peripheral mononuclear blood cells (PMBC) were obtained from the peripheral blood of clinically healthy poultry reared under standard conditions by puncture of vena cutanea ulnaris using Heparin (10—20 U.ml^{-1} PBS, Zentiva, Czech Republic) as an anticoagulant. The blood was diluted in a ratio of 1 : 2 with PBS, transferred and layered into Leucosep tubes (Greiner bio-one, DE) containing Histopaque-1077 (Sigma-Aldrich, UK). The isolation of the PMBC was done by centrifugation (19 000 × g, 40 min, 20 °C; Hettich Rotina 420R Centrifuge, DJB Labcare, UK). The mononuclear cells collected from the gradient interface were washed two times with PBS (16 000 × g, 5 min) [3]. Trypan blue was used to determine cell viability and a Bürker chamber to count the total number of cells diluted in Türk solution. Isolated PMBCs were placed on a 12-well cultivation plate (Orange Scientific, BE) with concentration 1×10^7 cells.ml^{-1} and cultured overnight (39 °C, 5 % CO_2) in RPMI 1640 medium enriched with 10-mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Lonza, BE) and 10 % foetal bovine serum (Lonza, BE). Each *Lactobacillus* isolate at a concentration of 1×10^9 CFU.ml^{-1} in 200 µl PBS was added to the individual PMBCs cultures with cultivation lasting 24 and 48 h. The cultivation (culture medium, growth conditions) of the new lactobacilli isolates were performed as described previously [32].

Bacterial strains

The strains of lactobacilli used in the present study were isolated from the gut contents of healthy pheasants. In a previous study, the strains were identified by matrix-assisted laser desorption/ionization—time of flight mass spectrometry (MALDI-TOF MS) as *Lactobacillus reuteri* B6/1, B2/1 and B1/1. The strains were characterized by the production of exopolysaccharides [31].

Homogenization and isolation of the total RNA from PMBCs

The PMBCs were collected from each well by pipetting and then centrifuged for 1 min at 8000 × g. The cell lysate was homogenised two times for 45 s at 2700 rpm using 1.0 mm zirconia/silica beads (BioSpec Products, USA) and 1 ml of TRI Reagent (Sigma-Aldrich, Germany) in a Mag-Na Lyser instrument (Roche, UK). In the separation phase, 50 µl of 4-bromanisole (Molecular Research Center, USA) was added. For the purification of total RNA from the cell lysate, the RNAeasy mini kit (Qiagen, UK) was used. The purity and concentration of the total RNA was determined on a NanoPhotometer (Implen, Germany), and 1 µg of the total RNA was reverse transcribed by using Maxima first strand cDNA synthesis kit (Thermofisher, USA) and oligo-dT primers. The resulting cDNA was 10× diluted in DNase/RNase-Free distilled water (Invitrogen, USA) and used as a template for qRT-PCR or stored at −20 °C.

Quantitative Real-time PCR

The mRNA levels of interleukins and MIP-1β were determined. The mRNA relative expression of a reference gene, coding for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used for data normalization. The specific primer sequences used for qPCR are listed in Table 1. All primer sets allowed DNA amplification efficiencies between 94—100 %. Amplification and detection of specific products were performed via the CFX 96 RT system (Bio-Rad, USA). The cycling conditions included an initial denaturation: at 95 °C for 15 min and 38 cycles; denaturation 95 °C for 20 s, annealing 60 °C for 30 s and final elongation 72 °C for 30 s. A melting curve ranging from 50—95 °C, with readings taken every 0.5 °C, was obtained for each individual qRT-PCR plate. Each sample was subjected to quantitative real-time PCR in triplicate, and mean values of triplicates were used for subsequent analysis. We confirmed that the efficacy of amplification of each gene (including GAPDH) was essentially 100 % in the exponential phase of the reaction, where the cycle of quantification (Cq) was calculated. The Cq values of the genes studied were normalized to an average Cq value of the reference gene (ΔCq), and the relative expression of each gene was computed mathematically as 2−ΔCq.

Table 1. List of primers used in qRT-PCR for IL-1β, IL-15 and MIP-1β mRNA detection in PMBC culture

Primer	Sequences 5′–3′	References
IL-15 For	TGGAGCTGATCAAGACATCTG	[26]
IL-15 Rev	CATTACAGGTTCCTGGCATTC	
IL-1β For	GAAGTGCTTCGTGCTGGAGT	[5]
IL-1β Rev	ACTGGCATCTGCCCAGTTC	
MIP1-β For	GGCAGACTACTACGAGACCAACAG	[36]
MIP1-β Rev	ACGGCCCTTCCTGGTGAT	
UB For	GGGATGCAGATCTTCGTGAAA	[7]
UB Rev	CTTGCCAGCAAAGATCAACCTT	

Flow cytometry procedure

The direct immunofluorescent method and double immunostaining labelled isolated lymphocytes with mouse anti-chicken monoclonal antibodies (SouthernBiotech, USA) in arrangement CD3PE/CD4FITC, CD3PE/CD8 FITC (T-cells) were used. Polyclonal goat-anti mouse FITC-conjugated immunoglobulin F(ab')2 fragment (Dako, Denmark) was used at a working dilution of 1 : 50 with PBS as the control antibody. Fifty µL of cellular suspension (1.10^7 lymphocytes in PBS) and 2 µl of specific or control MoAbs were mixed and incubated in the dark at 22 °C for 15 min. After washing in 0.5 ml PBS and re-suspended in 0.2 ml of PBS with 0.1 % paraformaldehyde, the cells were measured by FACScan (Becton Dickinson, Germany) with a 15 mV argon ion laser. The fluorescence data was collected and analysed on at least 10,000 lymphocytes using the Becton Dickinson Cell Quest programme (Germany). The results were expressed as the relative percentage of the lymphocyte subpopulation that was positive for a specific MoAb. Samples were measured in triplets before cultivation, 24 h and 48 h post inoculation (p. i.) in the following arrangement: control (C) and lactobacilli strains marked B1/1, B1/2 and B1/6.

Determination of antagonism *in vitro*

The method to test the antagonistic activity of *Lactobacillus reuteri* B1/1, B2/1, B6/1 was performed according to J a c o b s e n et al. [23] with some modifications. Sterile discs with a diameter of 6 mm (BBL, Cockeysville, USA) were placed on the surface of 20 ml of peptone-yeast extract-glucose (PYG) agar in Petri dishes. The composition of PYG agar was as follows: peptone for bacteriology 5 g; enzymatic casein hydrolysate 5 g; yeast extract 10 g; glucose 10 g; and agar 18 g.1000 ml^{-1} distilled water (pH 6.9). The discs were inoculated with 10 µl of the night cultures of lactobacilli (1×10^8 $CFU.ml^{-1}$) in de Man-Rogosa-Sharpe broth (MRS; Carl Roth GmbH + CO. KG, Karlsruhe, Germany) and the plates were then cultivated anaerobically (Gas Pak Plus, BBL Microbiology systems, Cockeysville, Maryland, USA) at 37 °C for 48 hours. After incubation, paper discs were removed and the plates were overlaid with 3 ml of 0.7 % PYG agar, inoculated with 0.3 ml of the night culture of a respective indicator strain and incubated aerobically at 37 °C for 24 hours. The following indicator strains were used: *Escherichia coli* 0149 F4 (Research Institute of Veterinary Medicine in Brno, CR); *Staphylococcus aureus* CCM 4223; *Salmonella Typhimurium* CCM 7205 (Czech Collection of Microorganisms in Brno, CR) and *Bacillus cereus* (isolate obtained from the Laboratory of gnotobiology, UVMP in Košice, SR). Two controls were used: blank disc and discs inoculated with 10 µl of sterile MRS medium. The results are presented as the arithmetic means of three measurements (mm) ± SD.

Statistical analysis

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

RESULTS

Relative expression of cytokines by quantitative real-time PCR

The relative expression for IL-1β gene (Fig. 1a) was upregulated in the B1/1 group compared to the control ($P < 0.001$) and B2/1 ($P < 0.01$) 24 h p.i. After 48 h p.i., the relative expression of IL-1β was upregulated in the same group (B1/1) compared to other groups ($P < 0.001$). Interestingly, group B6/1 did not exhibit gene expression for IL-1β.

Similarly, the relative expression for IL-15 gene (Fig. 1b) was upregulated in both the B1/1 and B2/1 groups compared to the control and B6/1 ($P < 0.001$) group, as well as in the B1/1 group compared to the B2/1 group ($P < 0.01$) 24 h p.i. In the second sampling, the upregulation of the

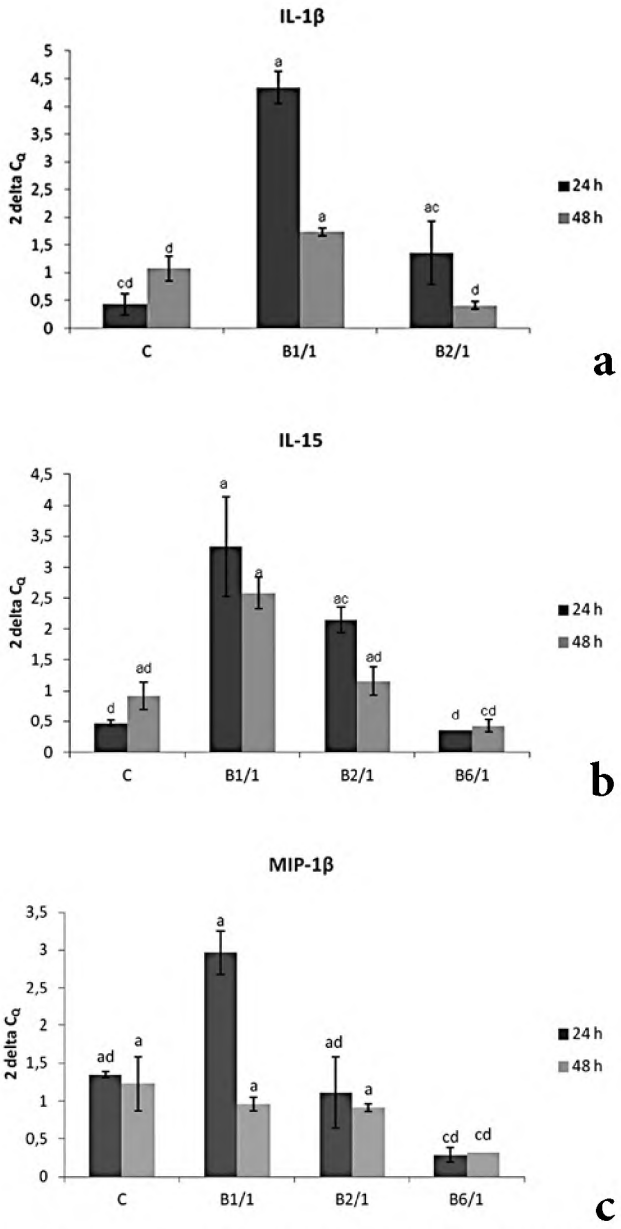

Fig. 1. Relative expression level of a) IL-1β; b) IL-15; and c) MIP-1β in PMBC culture. Results at each time point are the median of 2−ΔCq. Means with different superscripts are significantly different acP < 0.01; adP < 0.001

relative expression for IL-15 was recorded mainly in the B1/1 group compared to the other groups ($P < 0.001$).

A significant increase in the relative expression for MIP-1β (Fig. 1c) was observed in the B1/1 group compared to the other groups ($P < 0.001$) 24 h p.i. In contrast, during the second sampling (48 h p.i.) the relative expression of MIP-1β was downregulated in all treated groups compared to the control ($P < 0.001$).

Immunophenotyping of lymphocytes

The relative percentages of CD3+, CD4+ and CD8+ cells are presented in Fig 2. In the first sampling (24 h p.i.) we noted significantly higher proportions of T cells including CD3+, CD4+ and CD8+ lymphocytes in lactobacilli B1/1 and B2/1 groups in comparison with the control ($P < 0.001$). The highest relative percentage was observed in the B2/1 group, and it was significantly higher compared to the lowest values, which were observed in the B6/1 group. Forty eight hours post inoculation, the T cells showed an increase only in the B2/1 group of lactobacilli compared to the control and all other groups. This increase was observed in CD3+ and CD8+ ($P < 0.001$), as well as CD4+ ($P < 0.01$) subpopulations.

Antimicrobial activities of lactobacilli

The highest inhibitory activity against *S. aureus* CCM4223 was shown by *L. reuteri* B6/1 and B1/1 compared to *L. reuteri* B2/1 ($P < 0.01$). On the other hand, the efficiency of inhibitory activity against *S. Typhimurium* was the highest in *L. reuteri* B2/1 compared to other lactobacilli strains ($P < 0.001$). Antibacterial activity against *E. coli* O149F4 was the highest in *L. reuteri* B1/1 compared to other lactobacilli strains ($P < 0.05$) (Table 2).

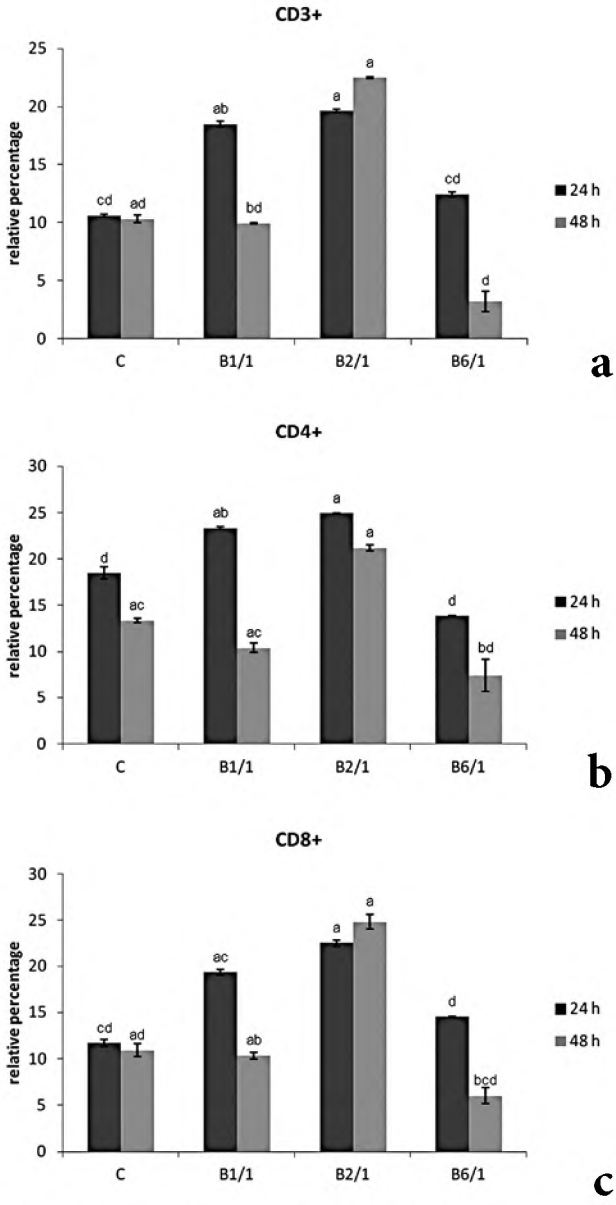

Fig. 2. Relative percentage of the determined lymphocyte' subpopulations a) CD3+; b) CD4+; and c) CD8+. Means with different superscripts are significantly different abP < 0.05; acP < 0.01; adP < 0.001

Table 2. Antimicrobial activities of lactobacilli towards indicator bacteria. The results are presented as the arithmetic means of three measurements (mm) ± SD, Means with different superscripts are significantly different abP < 0.05; acP < 0.01; adP < 0.001

Lactobacilli	Indicator strains				
	S. aureus CCM 4223	*B. cereus*	*S. Typhimurium* CCM 7205	*E. coli* O149 F4	*pH after* 48 h incubation
***L. reuteri* B1/1**	13.33±0.58a	34.00±0.00	25.00±1.00d	40.33±2.08a	3.46±0.05
***L. reuteri* B2/1**	9.00±1.73c	34.67±2.08	54.33±0.58a	32.00±1.00b	3.52±0.06
***L. reuteri* B6/1**	15.33±0.58a	34.67±1.15	31.00±1.00d	28.00±3.61c	3.69±0.02

DISCUSSION

This study was mainly focused on the evaluation of the immune properties and antimicrobial activities of new lactobacilli isolates from pheasant guts.

The most significant stimulating effect on the expression of all pro-inflammatory interleukins and MIP-1β was found in the group that was administered *L. reuteri* B1/1 mainly 24 h p.i. Similarly, H o f f m a n n et al. [19] reported that *L. reuteri* 100-23 significantly induced the expression of IL-1, IL-6 and MIP-2 in intestinal epithelial cells of mice. Interestingly, *L. reuteri* B6/1 did not induce the expression of the IL-1β gene in PMBCs. For the initiation of inflammation IL-1β represents a key cytokine. Moreover, IL-1β plays an important role in the regulation of MIP-1β expression and thereby contributing to the maintenance of inflammation [38]. Likewise IL-15 induces the expression of chemokines and their receptors on T-cells. IL-15 improves the proliferation of cytotoxic and helper T cells, and directs heterophils and NK cells to the sites of inflammation [34].

We observed a rise in the relative percentage of T cells including CD3+, CD4+ and CD8+ lymphocytes in the groups with *L. reuteri* B2/1 and *L. reuteri* B1/1 as compared to the control 24 h p. i., and this indicates the ability of new lactobacilli isolates to stimulate the required cellular immune response. It is known, that the individual strains of *Lactobacillus* spp. are able to induce the production of Th1 proinflammatory cytokines [21], while other strains induce the production of Th2 regulatory anti-inflammatory cytokines [30]. In agreement with a previous statement, in subsequent studies it would be appropriate to confirm this effect in *in vivo* conditions.

L i n et al. [27] demonstrated that *Lactobacillus reuteri* 6475 suppressed the activity of AP-1 transcript factor, which regulates the expression of pro-inflammatory cytokines in response to TLR activation. In another study, *L. reuteri* GMNL-263 reduced serum MCP-1, TNF, and IL-6 levels in mice fed with a high fat diet [20]. Taken together, the studies indicate that several strains of *Lactobacillus* can keep the balance in Th polarization (Th1/Th2), thereby creating better conditions for inflammation control [22].

Conversely, no stimulatory effect of *Lactobacillus reuteri* B6/1 on the relative expression of cytokines, chemokine and percentage of T cell subpopulations were observed. Several authors noticed, that certain isolates of *Lactobacillus reuteri*, affected neither: colonic microbial composition in piglets [28], IgA concentration and cytokine levels in saliva [4, 24] nor the incidence of nosocomial diarrhoea, including rotavirus infection [35]. We assumed that the final effect of the *L. reuteri* strain that was used in this study is also dependent on the real concentration of antimicrobial substances. In any case, the production of antimicrobial substances is essentially involved in the antimicrobial activity of lactobacilli; but their concentration could be different among certain isolates [15].

The antimicrobial activity is one of the most important criteria for selecting a probiotic strain against potentially harmful pathogens. *Lactobacillus* strains are unique in their production of antimicrobials substances against pathogens. Their antimicrobial activity is mainly due to the production of hydrogen peroxide, organic acids, especially lactic acid and acetic acid, and bacteriocins [33, 25].

According to our results, the tested lactobacilli strains inhibited the growth of both gram-positive and gram-negative indicator bacteria probably by reducing the pH of the environment as a result of organic acid production. After 48 hours of lactobacilli incubation, the pH ranged from 3.46—3.78 which is unsuitable for most pathogenic bacteria. In addition, the lipophilic and undissociated form of lactic acid and acetic acid allows these molecules to exhibit antibacterial character through the penetration of the bacterial membrane. Undissociated acid fractions diffuse passively across the membrane and subsequently ionize depending on intracellular pH, causing the cytoplasm to acidify and form inhibitors [29]. We noted a difference in the size of the inhibitory zones among the individual strains. Gram-positive Bacillus cereus was more sensitive than *S. aureus* CCM 4223. The sensitivity of *E. coli* O149 F4 and *S. Typhimurium* CCM 7205 to the lactobacilli tested was approximately the same. Likewise, B i l k o v á et al. [1] referred to *S. aureus* as the bacteria that are most resistant to *L. reuteri*, *L. murinus* and *L. mucosae probiotics* compare to *Yersinia enterocolitica* and *Listeria monocytogenes*, which were sensitive to the antimicrobial activity of lactobacilli. G e o r g i e v a et al. [13] tested the inhibitory activity of the 23 lactobacilli strains by the disc-diffusion method, which demonstrated their antimicrobial activity against pathogenic strains of *S. aureus*, *Bacillus cereus* and *E. coli*, with the majority of strains recording a correlation between the acidification intensity (pH) and the inhibitory zone size. D e A n g e l i s et al. [6] confirmed antimicro-

bial activity against *S. aureus* and *E. coli* in *L. plantarum* and *L. reuteri* (isolated from swine feces). The antimicrobial activity of *L. rhamnosus* against *S. Typhimurium* was demonstrated as a result of lactic acid accumulation [8] and pH reduction [12].

CONCLUSIONS

In summary, *L. reuteri* B1/1 and *L. reuteri* B2/1 showed promising stimulatory effects on the relative expression of pro-inflammatory interleukins, MIP-1β and the percentage of T cell subpopulations *in vitro*. On the flip side, *L. reuteri* B6/1 suppressed the relative expression of selected interleukins and MIP-1β and did not affect the percentage of T cells. Overall, selected lactobacilli strains inhibited the growth of both gram-positive and gram-negative indicator bacteria. These conclusions show the importance of individual testing of each probiotic strain for its various effects on the immune status of the host, mainly for their wide uses throughout the fermented dairy, food, and meat processing industries. In addition, it is necessary to confirm their properties also *in vivo* conditions, because they may not be the same. Based on the results, *L. reuteri* B1/1 was selected for a subsequent *in vivo* experiment in broiler chickens infected with *Campylobacter jejuni* CCM6189, which is currently in progress.

ACKNOWLEDGEMENTS

This study was supported by the Grant Agency for Science of the Slovak Republic VEGA 1/0112/18 and the Slovak Research and Developmental Agency APVV-15-0165.

REFERENCES

1. **Bilková, A., Kiňová Sepová, H., Bukovský, M., Bezáková, L., 2011:** Antibacterial potential of lactobacilli isolated from a lamb. *Vet. Med.*, 56, 7, 319—324. DOI: 10.17221/1583-VETMED.
2. **Bouwman, L. I., de Zoete, M. R., Bleumink-Pluym, N. M., Flavell, R. A., van Putten, J. P., 2014:** Inflammasome activation by *Campylobacter jejuni*. *J. Immunol.*, 193, 9, 4548—4557. DOI: 10.4049/jimmunol.1400648.
3. **Boyum, M. A., 1974:** Separation of blood leukocytes, granulocytes and lymphocytes. *Tissue Antigens*, 4, 4, 269—274.
4. **Braathen, G., Ingildsen, V., Twetman, S., Ericson, D., Jorgensen, M. R., 2017:** Presence of *Lactobacillus reuteri* in saliva coincide with higher salivary IgA in young adults after intake of probiotic lozenges. *Benef. Microbes.*, 8, 1, 17—22. DOI: 10.3920/BM2016.0081.
5. **Crhanova, M., Hradecka, H., Faldynova, M., Matulova, M., Havlickova, H., Sisak, F., Rychlík, I., 2011:** Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica* serovar *Enteritidis infection*. *Infect. Immun.*, 79, 7, 2755—2763. DOI: 10.1128/IAI.01375-10.
6. **De Angelis, M., Siragusa, S., Berloco, M., Caputo, L., Settanni, L., Alfonsi, G., et al., 2006:** Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. *Res. Microbiol.*, 157, 8, 792—801. DOI: 10.1016/j.resmic.2006.05.003.
7. **De Boever, S., Vangestel, C., De Backer, P., Croubels, S., Sys, S. U., 2008:** Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. *Vet. Immunol. Immunopathol.*, 122, 3—4, 312—317. DOI: 10.1016/j.vetimm.2007.12.002.
8. **De Keersmaecker, S. C., Verhoeven, T. L., Desair, J., Marchal, K., Vanderleyden, J., Nagy, I., 2006:** Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. *FEMS Microbiol. Lett.*, 259, 1, 89—96. DOI: 10.1111/j.1574-6968.2006.00250.x.
9. **De Vos, W. M., 2011:** Systems solutions by lactic acid bacteria: from paradigms to practice. *Microb. Cell Fact.*, 10, Suppl. S2. DOI: 10.1186/1475-2859-10-S1-S2.
10. **EFSA, 2005:** Opinion of the Scientific Committee on a request from EFSA related to A generic approach to the safety assessment by EFSA of microorganisms used in food/feed and the production of food/feed additives. *The EFSA Journal*, 226, 1—12.
11. **FAO/WHO, 2002:** Report of a Joint FAO/WHO Working Group on Drafting. *Guidelines for the Evaluation of Probiotics in Food*. London, Ontario, Canada, April 30 and May 1, 2002.
12. **Fayol-Messaoudi, D., Berger, C. N., Coconnier-Polter, M. H., Liévin-Le Moal, V., Servin, A. L., 2005:** pH-, lactic acid-, and non-lactic acid-dependent activities of probiotic lactobacilli against *Salmonella enterica* serovar *Typhimurium*. *Appl. Environ. Microbiol.*, 71, 10, 6008—6013. DOI: 10.1128/AEM.71.10.6008-6013.2005.

13. **Georgieva, R., Yocheva, L., Tserovska, L., Zhelezova, G., Stefanova, N., 2015:** Antimicrobial activity and antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* spp. intended for use as starter and probiotic cultures. *Biotechnol. Biotechnol. Equip.*, 29, 1, 84—91. DOI: 10.1080/13102818.2014.987450.

14. **Gilliland, S. E., Reilly, S. S., Kim, G. B., Kim, H. S., 2002:** Viability during storage of selected probiotic lactobacilli and bifidobacteria in a yogurt-like product. *J. Food Sci.*, 67, 8, 3091—3095. DOI: 10.1111/j.1365-2621.2002.tb08864.x.

15. **Greifová, G., Májeková, H., Greif, G., Body, P., Greifová, M., Dubničková, M., 2017:** Analysis of antimicrobial and immunomodulatory substances produced by heterofermentative *Lactobacillus reuteri*. *Folia Microbiol.*, 62, 6, 515—524. DOI: 10.1007/s12223-017-0524-9.

16. **Gruntar, I., Ocepek, M., Avbersek, J., Mićunović, J., Pate, M., 2010:** A pulsed-field gel electrophoresis study of the genetic diversity of *Campylobacter jejuni* and *Campylobacter coli* in poultry flocks in Slovenia. *Acta Vet. Hung.*, 58, 1, 19—28. DOI: 10.1556/AVet.58.2010.1.2.

17. **Haghighi, H. R., Abdul-Careem, M. F., Dara, R. A., Chambers, J. R., Sharif, S., 2008:** Cytokine gene expression in chicken caecal tonsils following treatment with probiotics and *Salmonella* infection. *Vet. Microbiol.*, 126, 1—3, 225—233. DOI: 10.1016/j.vetmic.2007.06.026.

18. **Helmstetter, C., Flossdorf, M., Peine, M., Kupz, A., Zhu, J., Hegazy, A. N., et al., 2015:** Individual T helper cells have a quantitative cytokine memory. *Immunity*, 42, 1, 108—122. DOI: 10.1016/j.immuni.2014.12.018.

19. **Hoffmann, M., Rath, E., Hölzlwimmer, G., Quintanilla-Martinez, L., Loach, D., Tannock, G., Haller, D., 2008:** *Lactobacillus reuteri* 100-23 transiently activates intestinal epithelial cells of mice that have a complex microbiota during early stages of colonization. *J. Nutr.*, 138, 9, 1684—1691. DOI: 10.1093/jn/138.9.1684.

20. **Hsieh, F. C., Lan, C. C., Huang, T. Y., Chen, K.W., Chai, C. Y., Chen, W.T., Wu, C. S., 2016:** Heat-killed and live *Lactobacillus reuteri* GMNL-263 exhibit similar effects on improving metabolic functions in high-fat diet-induced obese rats. *Food Funct.*, 7, 5, 2374—2388. DOI: 10.1039/c5fo01396h.

21. **Christensen, H. R., Frokiar, H., Pestka, J., 2002:** Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J. Immunol.*, 168, 1, 171—178. DOI: 10.4049/jimmunol.168.1.171.

22. **Isolauri, E., 2003:** Therapy update: Probiotics for infectious diarrhoea. *Gut*, 52, 3, 436—437. DOI:10.1136/gut.52.3.436.

23. **Jacobsen, C. N., Rosenfeldt, N., Hayford, A. E., Møller, P. L., Michaelsen, K. F., Pærregaard, A., et al., 1999:** Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Appl. Environ. Microbiol.*, 65, 11, 4949—4956.

24. **Jorgensen, M. R., Keller, M. K., Kragelund, C., Hamberg, K., Ericson, D., Nielsen, CH., Twetman, S., 2016:** *Lactobacillus reuteri* supplements do not affect salivary IgA or cytokine levels in healthy subjects: a randomized, double-blind, placebo-controlled, cross-over trial. *Acta Odontol. Scand.*, 74, 5, 399—404. DOI: 10.3109/00016357.2016.1169439.

25. **Kiňová Sepová, H., Bilková, A., 2013:** Isolation and identification of new lactobacilli from goatling stomach and investigation of reuterin production in *Lactobacillus reuteri* strains. *Folia Microbiol.*, 58, 1, 33—38. DOI: 10.1007/s12223-012-0166-x.

26. **Kolesarova, M., Spisakova, V., Matulova, M., Crhanova, M., Sisak, F., Rychlik, I., 2011:** Characterisation of basal expression of selected cytokines in the liver, spleen, and respiratory, reproductive and intestinal tract of hens. *Vet. Med.*, 56, 7, 325—332. DOI: 10.17221/1586-VETMED.

27. **Lin, Y. P., Thibodeaux, CH., Peña, J. A., Ferry, G. D., Versalovic, J., 2008:** Probiotic *Lactobacillus reuteri* suppress proinflammatory cytokines via c-Jun. *Inflamm. Bowel Dis.*, 14, 8, 1068—1083. DOI: 10.1002/ibd.20448.

28. **Liu, H., Hou, C., Wang, G., Jia, H., Yu, H., Zeng, X., et al., 2017:** *Lactobacillus reuteri* I5007 modulates intestinal host defense peptide expression in the model of IPEC-J2 cells and neonatal piglets. *Nutrients*, 9, 6, E559. DOI: 10.3390/nu 9060559.

29. **Ogueke, C. C., 2007:** The effect of metabolites of *lactobacillus* in fermented milk on the growth of hospital isolates of *E. coli*. *Life Sci. J.*, 5, 1, 46—50.

30. **O'Mahony, L., O'Callaghan, L., McCarthy, J., Shilling, D., Scully, P., Sibartie, S., et al., 2006:** Differential cytokine response from dendritic cells to symbiotic and pathogenic bacteria in different lymphoid compartments in humans. *Am. J. Physiol. Gastrointest. Liver Physiol.*, 290, 4, G839–G845. DOI: 10. 1152/ajpgi.00112.2005.

31. **Ryznerova, D., 2013:** *The Study of the Properties of the Probiotic Bacteria in Terms of their Biological Effects and Applications*. Dissertation thesis, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia, 148 pp.

32. **Strompfová, V., Marciňaková, M., Gancarčíková, S., Jonecová, Z., Sciranková, L., Guba, P., et al., 2005:** New probiotic strain *Lactobacillus fermentum* AD1 and its effect in

Japanese quail. *Vet. Med.-Czech*, 50, 9, 415—420. DOI: 10.17221/5642-VETMED.

33. **Tan, P., Peh, K., Gan, C., Liong, M. T., 2014:** Bioactive dairy ingredients for food and non-food applications. *Acta Aliment. Hung.*, 43, 1, 113—123. DOI: 10.1556/AAlim.43.2014.1.12.
34. **Van Belle, T. L., Dooms, H., Boonefaes, T., Wei, X. Q., Leclercq, G., Grooten, J., 2012:** IL-15 augments TCR-induced CD4+ T cell expansion *in vitro* by inhibiting the suppressive function of CD25 High CD4+ T cells. *PLoS One*, 7, 9, e45299. DOI: 10.1371/journal.pone.0045299.
35. **Wanke, M., Szajewska, H., 2012:** Lack of an effect of *Lactobacillus reuteri* DSM 17938 in preventing nosocomial diarrhoea in children: a randomized, double-blind, placebo-controlled trial. *J. Pediatr.*, 161, 1, 40—43. DOI: 10.1016/j.jpeds.2011.12.049.
36. **Withanage, G. S. K., Wigley, P., Kaiser, P., Mastroeni, P., Brooks, H., Powers, C., et al., 2005:** Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar *Typhimurium* infection in the chicken and in protective immunity to rechallenge. *Infect. Immun.*, 73, 8, 5173—5182. DOI: 10.1128/IAI.73.8.5173-5182.2005.
37. **Zhang, B., Wang, Y., Tan, Z., Li, Z., Jiao, Z., Huang, Q., 2016:** Screening of probiotic activities of lactobacilli strains isolated from traditional tibetan qula, a raw yak milk cheese. *Asian-Australas. J. Anim. Sci.*, 29, 10, 1490—1499. DOI: 10.5713/ajas.15.0849.
38. **Zhang, T., Guo, C. J., Li, Y., Douglas, S. D., Qi, X. X., Song, L., Ho, W. Z., 2003:** Interleukin-1beta induces macrophage inflammatory protein-1beta expression in human hepatocytes. *Cell. Immunol.*, 226, 1, 45—53. DOI: 10.1016/j.cellimm.2003.10.005.
39. **Xu, Y., Zong, X., Han, B., Li, Y., Tang, L., 2016:** *Lactobacillus pentosus* expressing porcine lactoferrin elevates antibacterial activity and improves the efficacy of vaccination against Aujeszky's disease. *Acta Vet. Hung.*, 64, 3, 289—300. DOI: 10.1556/004.2016.028.

Received December 13, 2019

Accepted February 5, 2020