Cytokine production in vitro and in rat model of colitis in response to Lactobacillus plantarum LS/07

Cytokine production in vitro and in rat model of colitis in response to *Lactobacillus plantarum* LS/07

Jana Štofilová[a]*, Tomaž Langerholc[b], Cristian Botta[c], Primož Treven[d], Lidija Gradišnik[b], Rastislav Salaj[e], Alena Šoltésová[a], Izabela Bertková[a], Zdenka Hertelyová[a], Alojz Bomba[a]

[a] Department of Experimental Medicine, Faculty of Medicine, P.J. Šafárik University in Košice, Trieda SNP 1, 04011 Košice, Slovak Republic

[b] Department of Microbiology, Biochemistry, Molecular Biology and Biotechnology, Faculty of Agriculture and Life Sciences, University of Maribor, Pivola 10,

2311 Hoce, Slovenia

[c] Department of Forestry, Agriculture and Food Sciences, University of Torino, Largo Paolo Braccini 2, Grugliasco, 10095 Torino, Italy

[d] Institute of Dairy Science and Probiotics, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia e Medirex, a.s., Magnezitárska 2/C, 040 13 Košice, Slovak Republic

Keywords: Lactobacillus plantarum Cytokine Functional gut model Inflammation Colitis

Abstract

Over the past decade, it has become clear that specific probiotic lactobacilli are valuable in the prevention and treatment of infectious and inflammatory diseases of gastrointestinal tract but their successful application would benefit greatly from a better understanding of the mechanisms of individual strains. Hence, each probiotic strain should be characterized for their immune activity before being proposed for clinical applications. The aim of the study was to characterize the immunomodulatory activity of the strain Lactobacillus (L.) plantarum LS/07 in vitro

using functional gut model and to study its anti- inflammatory potential in dextran sulphate sodium (DSS)-induced colitis in rats. We showed that L. plantarum LS/07 induced production of IL-10 in macrophages derived from blood monocytes as well as monocyte/macrophages cell line stimulated indirectly via enterocytes in vitro. In rat model of colitis, L. plantarum LS/07 attenuated the DSS-induced signs of inflammatory process in colon such as weight loss, diarrhoea, infiltration of inflammatory cells associated with decreased colon weight/length ratio, inhibited gut mucosa destruction and depletion of goblet cells. Moreover, the strain increased the concentration of anti-inflammatory cytokine IL-10 in mucosal tissue. In conclusion, the protective effects of L. plantarum LS/07 in the DSS-induced colitis model seem to be related to the stimulation of IL-10 and the restoration of goblet cells and indicate it as a good candidate to prevent and treat diseases associated with inflammation.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic disease of the digestive tract, which includes two distinct clinical forms, Crohn's disease (CD) and ulcerative colitis (UC). Both forms of IBD share almost identical pathophysiological background characterized by chronic and spontaneously relapsing intestinal inflammation occurring in the gut. Although the aetiology remains unclear, IBD is an inappropriate immune response to endogenous bacteria, which occurs in genetically susceptible individuals as the result of complex interactions among the intestinal immune system, gut microbiota and environmental factors [1]. It has been reported that there is a disturbed balance between pro-inflammatory (TNF-a, IL- 1, IL-6, IL-8) and anti-inflammatory cytokines (IL-10, TGF-b) in IBD patients [2]. Studies in animal models of IBD and some clinical trials have shown that the specific manipulation of targeted cytokines seems to reduce disease severity and maintains remission, thus in the last decade, several attempts were made to treat patients with cytokine-modulating agents, such as recombinant anti-inflammatory cytokines or antibodies specific against pro-inflammatory cytokines [3]. However, the use of anti- inflammatory cytokines, such as anti-TNF, have side effects connected with an

51 increased risk of bacterial, viral and fungal infections [4] and only some of them are efficient in clinical trials while
52 other are not [3,5]. Considering the above facts, it is not surprising that probiotics, which are well documented
53 to modulate composition of gut microbiota, reinforce the epithelial barrier and affect the immune system, may
54 represent attractive and effective alternative or adjuvant therapeutic approaches in the treatment of gut
55 inflammatory diseases. Moreover, their advantage is their availability in the intestine and commonly the effect
56 of probiotics on mucosal immune system is complex and not restricted to a single cell type, molecular pathway
57 or molecule. Selected immunomodulatory probiotic bacteria can counteract
58 inflammation of the intestine through multiple regulatory activi- ties including cytokine production [6] and may
59 be either complementary or an alternative to conventional treatments toward IBD. However, some animal
60 studies and clinical trials have demonstrated that not all probiotic bacteria or bacterial combina- tion may be
61 effective in the treatment or prevention of IBD [7,8] because of differences in their immunomodulatory
62 properties [9]. The diversity among probiotic strains and the complexity of their interplay with the immune
63 system warrant a careful selection process before using them in clinical trials. A single mechanism of action for
64 all probiotics is unlikely, therefore, it is necessary to perform appropriate in vitro and in vivo studies to
65 characterize and compare the immunomodulatory activity of different probiotic strains. Foligne et al. [10]
66 suggested that the peripheral blood mononuclear cells (PBMCs) from healthy donors can be used as a tool for
67 screening the immunomodulatory activity of probiotic bacteria and this assay appeared to be a good indicator
68 of in vivo anti-inflammatory effects of individual strains. However, the primary site of probiotic action is the gut
69 where bacteria adhere to the intestinal wall and interactions between bacteria, epitheli- um and underlying
70 mucous immune system occur. Accordingly, to evaluate immunomodulatory effect of L. plantarum LS/07, more
71 complex in vitro functional gut model was used in the study. The strain L. plantarum LS/07 was selected from
72 different Lactobacillus strains isolated from human rectal swabs because it was most potent inhibitor of
73 pathogenic E. coli [11]. Subsequently, we examined the potential ability of L. plantarum LS/07 to attenuate DSS-
74 induced colitis concerning disease symptoms, histopatholog- ical signs, cytokine production and activity of
75 transcription factor NF-kB in the intestine.

2. Materials and methods

2.1. Bacteria and growth conditions

The L. plantarum LS/07 was isolated from faeces of healthy human and characterized in our laboratory using molecular methods [11]. The probiotic strain Lactobacillus rhamnosus GG (LGG) ATCC 53103 was used as the reference strain. Strains were cryopreserved at 80 °C in 20% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO) with De Man Rogosa Sharpe broth (MRS, Merck Germany). Prior to testing on cell lines, lactobacilli were revitalised by overnight growth in MRS broth at 37 °C. The initial concentration of bacterial strains was determined by measuring optical density at 595 nm and all the suspensions were set to the same initial count using an internal calibration curve for each strain.

2.2. Growth and maintenance of in vitro cell models

Macrophages differentiated from human blood monocytes (MDBMs) were prepared according to Menck et al. [12]. Human monocytes were isolated from 6 healthy donors (age 32–45 years) by a double density gradient centrifugation. An informed consent was obtained before the sample collection, and the experiments were approved by the local ethical committee. PBMCs obtained without brake) using HISTOPAQUE1 x1077 (Sigma-Aldrich) were after the first density gradient centrifugation (400 x g, 30 min, brake) using iso-osmotic Percoll1 (Sigma-Aldrich) gradient to proceeded to the second centrifugation (550 x g, 30 min, without separate monocytes. The monocytes were then cultured in the presence of 2,5 ng/ml macrophage colony-stimulating factor (M- CSF; Biolegend, San Diego, USA) in RPMI 1640 with stable glutamine (Biosera, Nuaille, France) supplemented with 10% foetal bovine serum (Biosera) and antibiotics (ThermoFisher Scientific, Waltham MA, USA) at 37 °C and 5%CO2. After six days, differentiated macrophages were harvested and the viability was tested by trypane-blue, which was more that 95%. Macrophages from each participant were seeded into 12 well plates in duplicate at concentration 105 cells/ml and allowed to adhere for 2 h. The intestinal epithelial cell line H4-1

(human foetal small intestinal cell line clone 1) and normal monocyte/macrophage cell line TLT, isolated from human blood, were established at the Department of Microbiology, Biochemistry, Molecular Biology and Biotechnology at the Faculty of Agriculture and Life Sciences, University of Maribor (Maribor, Slovenia) [13]. Cell lines were grown in advanced Dulbecco0s Modified Eagle0s Medium (DMEM; ThermoFisher Scientific), supplemented with 5% foetal calf serum (Lonza, Basel, Switzerland), L-glutamine (2 mM, ThermoFisher Scientific) and antibiotics (ThermoFisher Scientific) and maintained at 37 °C in a humidified atmosphere containing 5% CO2 and 95% air, until confluent monolayers were obtained.

To obtain polarised monolayers of epithelial cell line, H4-1 cells were seeded on Transwell filter inserts (0,4 mm pore size, 12 mm, Corning, NY, USA) placed into 12 well plates (22,1 mm, Corning) with a volume of 0.5 mL in the apical compartment and concentration of 2 x 105 cells/ml media. The filters were main- 1.5 mL in the basolateral compartment. Medium was changed every two days and cells were cultured for 13–14 days to achieve fully differentiated monolayers. Trans-epithelial electrical resistance (TEER) was determined continuously using the Millicell-ERS volt-ohmmeter (Millipore, Bedford, MA). Functional polarity was established when TEER between the apical and basolateral surface of the monolayers reached a minimal value of 600 V/cm2. TLT cells were seeded in 12 well plates (Corning) at a concentration of 3 ? 105 cells/ml and further incubated until confluence (24–48 h). To create the functional gut model, inserts containing fully differentiated H4-1 monolayers were transferred to 12 well plates with TLT cell monolayer.

2.3. Stimulation of cell cultures with bacteria and cytokine determination

To evaluate the interaction among bacteria, differentiated epithelial H4-1 cells and macrophages, two different experimental approaches were conducted: 1) direct stimulation of MDBMs or TLT cells with bacteria (Fig. 1A and B) and 2) indirect stimulation of TLT cells via bacteria-challenged H4-1 cells seeded on Transwell filter inserts (gut model) (Fig. 1C). The apical surface of MDBMs, TLT and H4-1 monolayers was challenged by the addition of 106 CFU/ml of L. plantarum LS/07, 106 CFU/ml of LGG and 10 ng/ml LPS (from Escherichia coli 0111:B4, Sigma-Aldrich) respectively. The concentration 106 CFU/ml of bacteria was chosen based on previously performed

cytotoxicity assay with crystal violet (data not shown). After 24 h co-cultivation at 37 °C in an atmosphere of air with 5% CO2, culture supernatants (from the basolateral side in the case of gut model) were harvested and stored at -20 °C until cytokine analysis. Cytokines were measured by commercially available ELISA kits for human IL-1b, IL- 10, TNF-a and TGF-b1 (USCN Life Science Inc., USA), according to the manufacturer's instructions.

2.4. Animals

Animal experiment was conducted according to the principles described in the Law No. 377/2012 and No. 436/2012 of Slovak Republic for the Care and Use of Laboratory Animals, and were approved by the Ethical Committee of the P.J. Šafárik University, Faculty of Medicine. The specific license to carry out this experiment on animals was obtained from the State Veterinary and Food Administration of the Slovak Republic (Ro-1136/14-221). Male Sprague Dawley rats (seven-weeks old, n = 24) were kept humidity (50–55%) with 12-h light/12-h dark cycle in the under standard conditions of temperature (21 ?2?C) and relative Laboratory of Research Bio-models, Faculty of Medicine, University of P.J. Šafárik in Košice, Slovak Republic. The rats were fed with standard laboratory chow (containing 3.5% fat). Feed and water were provided ad libitum. The state of health, body weights, feed and water usage were monitored daily.

2.5. Preparation and administration of probiotic

The L. plantarum LS/07 was grown in MRS broth (Merck) for 18 h at 37 #C. The aerobically prepared night bacterial culture was mixed with the skim milk (fat content 0.5%, temperature 22–25 °C) and filled into screw-bottles for daily feeding (10 mL per animal). Each animal daily received approximately 109 CFU of L. plantarum LS/07. The concentration of bacteria was determined by measuring the optical density (DENSILAMETER, Erba Lachema, Czech Republic).

2.6. Experimental induction of colitis and treatment

Colitis was induced using a previously described method [14]. DSS (molecular weight 40 kDa; TdB Consultancy

AB, Upsala, Sweden) was added to drinking water at the final concentration of 5% (w/v) for 7 days. The animals in negative control group received normal drinking water only. The DSS solution was replenished daily and mean DSS consumption was noted per animal at the end of 7-days treatment. Rats were divided into the following experimental groups: (1) NC (negative control group, n = 8, body weight 281.25 ? 30.05 g), (2) DSS group (positive control group, DSS, n = 8, body weight NC (negative control group, n = 8, body weight 281.25 ? 30.05 g), body weight 235 ? 26.77 g). The whole experiment lasted 14 days. 222.5 ?18.52 g), and (3) PRO group (DSS + L. plantarum LS/07, n = 8, L. plantarum LS/07 resuspended in milk was administered once a day starting from 7 days before colitis induction and continuing until death to study preventive mode of probiotic action. Both NC and DSS groups were supplemented with milk free of probiotic. After 14 days, experiment was terminated and animals were sacrificed under deep anaesthesia (50 mg/kg of b.w., i.m. Zoletil; Virbac S.A., France). The small and large intestine from each rat were immediately removed, weighed, macroscopically evaluated and processed for cytokine measurements or histological assessment.

2.7. Macroscopic assessment of colonic inflammation

The colon was opened longitudinally, gently cleared of faecal material with PBS and cleaned of fat and mesentery. Each specimen was weighed and its length was measured to calculate the weight/ length ratio as a marker of colonic inflammation. Disease activity index (DAI) was used to evaluate grade and extent of colonic inflammation based on a previously published grading system [14] with slight modifications. DAI is the combined score of weight loss, stool consistency and bleeding, as detailed in Table 1. All parameters were scored every day since the first DSS administration.

2.8. Histological assessment of colonic damage

Colon samples were fixed in 4% neutral formaldehyde solution and embedded in paraffin blocks following the classical procedure. A part of full-thickness sections of 5 mm were stained with Mayer's haematoxylin and eosin to determine the intensity of the inflammation in the colon. Toluidine Blue staining protocol was used to visualize

the enzymes tryptase and chymase localised in granules of connective tissue mast cells (CTMC), seen in pink. Staining with Alcian Blue/Safranin solutions enabled the visualisation of goblet cells in the blue color. Both cell types were and then their numbers were calculated for an area of 1 mm2. counted on at least 30 images captured using 400 x magnifications Histological evaluation and subsequently quantifications were performed on the sections from each animal and finally the mean cell number (± SD) was calculated for each group. Morphometric analysis was performed by Olympus Microscope BX51 equipped with Digital Analysis Imaging system "Analysis Docu" (Czech Republic).

2.9. Cytokine and NF-kB quantification in the intestinal mucosa

The concentration of cytokines IL-6, TNF-a, IL-10 and NF-kB, the marker of inflammation, was measured in the homogenate of ileal mucosa by ELISA. Intestinal mucosa was prepared as previously described by Doligalska et al. [15]. The concentration of cytokines and NF-kB were determined by commercially available kits Rat IL-6 Platinum ELISA (eBioscience, USA), Rat IL- 10 Platinum ELISA (eBioscience, USA), Rat TNF-alpha ELISA kit (RayBiotech, Inc., USA) a Rat NF-kB p65 ELISA kit (Elabscience Biotechnology, China) according to the manufacturer0s instruc- tions. The plates were read at 450 nm using EonTM Microplate Spectrophotometer (Bio-Tek Instruments, USA). The final concentration was expressed as ng/g of wet mucosa

2.10. Statistical analysis

The experimental data were expressed as means ± SD or ± SE and statistically evaluated by one-way ANOVA followed by Tukey's post-hoc test (MINITAB Release 11, 1996). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of L. plantarum LS/07 on cytokines release in vitro

The strain L. plantarum LS/07 was tested for its capacity to induce the secretion of IL-1b, TNF-a, IL-10 and TGF-b after 24 h of culture with either monocyte/macrophages monolayers alone or co-culture monocyte/macrophages with epithelial cell line H4 (Fig. 2A–C). LPS from E. coli significantly induced pro-inflammatory cytokines (IL-1b, TNF-a) in both MDBMs and TLT cells compared with non-stimulated control. None of stimulants (LPS, LP, LGG) elicited the production of IL-10 in TLT cells grown in a monolayer, whereas MDBMs responded by massive production of IL-10 after 24 h incubation with LPS and L. plantarum LS/07. The level of TGF- b1 was not detected in MDBMs after 24 h stimulation. In the functional gut model, LPS decreased significantly the concentration of IL-10 and stimulated both pro-inflammatory cytokines TNF-a and IL-1b compared to untreated control. Noticeably, the production of TNF-a, IL-10 and TGF-b increased significantly in the functional gut model treated with L. plantarum LS/07 compared to the negative control, while the same cytokines were weakly induced in TLT cells grown in a monolayer. The stimulant capacity of *L. plantarum* LS/07 was comparable with that of LGG in all cell models. However, *L. plantarum* LS/07 induced a significant higher production of TNF-a and IL-10 in the MDBMs and in the functional gut model compared with the LGG. Moreover, we found out that there were considerable changes in the level of cytokines produced by MDBMs and TLT cells. The production of IL- 10 by TLT cells depended on the source of their activation.

3.2. Protective effects of *L. plantarum* LS/07 on manifestation of colonic inflammation

Weight changes of experimental animals over the entire study period are shown in Fig. 3A. The mean body weight of rats in DSS group significantly decreased on the fifth day after DSS administration in comparison with the weight before DSS treatment. Animals in the DSS group exhibited watery and bloody diarrhoea on the sixth day after DSS administration, which was associated with significantly increased DAI (Fig. 3B). Compared to the NC group, the colon weight/length ratio, the index of colonic inflammation, was significantly elevated in the DSS treated group (Table 2). Macroscopic evaluation of the colon in DSS group revealed that the colonic mucosa was edematous and erythematous with occasional areas of mucosal erosions. Diet supplementation with L. plantarum LS/07 attenuated the DSS-induced loss of weight until the end of experiment (Fig. 3A). Fourteen-day

feed supplementation with L. plantarum LS/07 alleviated clinical signs and symptoms of colitis induced by DSS, such as weight loss and diarrhoea. The DAI of probiotic-supplemented animals was significantly lower on day 6 after DSS administration in comparison with the DSS group (Fig. 3B). Treatment with L. plantarum LS/ 07 suppressed the effect of DSS on colon weight gain and length shortening (Table 2).

3.3. Protective effects of L. plantarum LS/07 on histopathological changes of the colonic mucosa induced by DSS

No histological abnormalities were detected in the colonic mucosa of rats from the NC group (Figs. 4 A and 5 A and D). Histological assessments of colonic samples revealed that seven- days administration of 5% DSS in drinking water led to disruption of crypt architecture, depletion of goblet cells and diffuse leukocyte infiltration (Figs. 4 B and C and 5 B). Inflammatory cells originated from lymphoid follicles infiltrated tunica mucosa and submucosa and erosion of the epithelial layer was observed (Fig. 4C). In the DSS group, the mean number of goblet cells was significantly lower in comparison with the NC group (Table 3). On the contrary, administration of DSS resulted in the elevation of CTCM localised mainly in the tela submucosa (Fig. 5E). The diet supplementation. The diet supplementation with L. plantarum LS/07 alleviated harmful effect of DSS on the colon mucosa, including the reduction of epithelial lesions and inflammatory infiltrates (Fig. 4D) accompanied with significantly decreased colon weight/length ratio. Probiotic treatment resulted in the restoration of goblet cells number (Fig. 5C) and decreased number of the CTMC in tela submucosa (Table 3; Fig. 5F).

3.4. Effects of L. plantarum LS/07 on the level of cytokines and NF-kB in intestinal mucosa

Seven days after colitis induction by DSS, tissue concentrations of NF-kB p65, TNF-a and IL-6 in DSS-group increased significantly compared to the NC group. DSS-induced increase in both NF-kB p65 and pro-inflammatory cytokines IL-6 and TNF-a concentration were suppressed by the administration of L. plantarum LS/07, but not significantly (Fig. 6). However, DSS treatment significantly decreased the mucosal IL-10 concentration in comparison with negative control. Daily intake of probiotics in the preventive mode, significantly increased IL-10

concentration in mucosal tissue in contrast to the DSS-group (Fig. 6A).

4. Discussion

In this study, the capacity of the probiotic strain L. plantarum LS/07 to modulate production of selected cytokines in vitro and in vivo was addressed, using human intestinal cells and animal model of IBD respectively. Many recent studies have indicated that several probiotic lactic acid bacteria, such as lactobacilli, can stimulate in vitro and in strain-dependent manner the production of cytokines in PBMCs, macrophages, dendritic cells (DC) and epithelial cells [16,17]. Previous studies have shown that the ability of different lactobacilli to induce a high ratio of IL-10/IL-12 or IL-10/TNF-a production in culture of PBMCs correlates with their capacity to provide significant protection in rodent colitis models [10,18]. However, Mileti et al. [18] have shown that strains of lactobacilli can prevent trinitrobenzenesulfonic acids (TNBS)-induced colitis in mice, despite they elicited a reduction of IL-10/IL-12 ratio in vitro. This could be due to a simplified model used in their study, which was composed of PBMCs and bacteria only. Furthermore, immunomodulatory activity of bacteria can be affected by cellular interactions in the intestinal environment [19]. Nevertheless, IL-10 is the major anti-inflammatory cytokine and probiotic strains capable of inducing its secretion would likely be good candidates to be used in IBD studies. Based on these evidences, we evaluated the ability of L.

plantarum LS/07 to stimulate the production of IL-10, TGF-b1, IL- 1b and TNF-a in monocyte/macrophages either directly or indirectly via the action of epithelial cells H4. The functional gut model, composed of human enterocytes H4-1 and TLT cell line [13], was chosen following the concept of cellular cross-talk between epithelial and underlying immunocompetent cells in response to probiotic bacteria at the mucosal surface to simulate the in vivo situation. Overall, lactobacilli seem to be strong inducers of pro- inflammatory cytokines IL-12, TNF-a and weak inducers of anti- inflammatory cytokine IL-10 in PBMCs [10,20,21]. In accordance with aforementioned reports, in the study both tested lactobacilli did not induce IL-10 and strongly stimulated production of IL-1b upon a direct contact with TLT cells. Conversely, L. plantarum LS/07 significantly stimulated IL-10, TGF-b but also TNF-a production in TLT cells through trans-epithelial activation. Activation of Toll-like receptors (TLR) on

276 epithelial cell leads to influencing activities of antigen presenting cells, such as phagocytic activity, expression of
277 co-stimulatory molecules and cytokine production [22]. Specific strains of Lactobacillus are able to trigger TLR-
278 dependent translocation of NF-kB p65 and STAT1 (signal transducer and activator of transcription 1) in epithelial
279 cells and that this signal translates further into macrophages resulting in expression of cytokines [23]. In the
280 functional gut model, the direct contact between epithelial and immune cells was restricted by the presence of
281 membrane. Accordingly, activity of immune cells was likely affected by the soluble factors secreted by the
282 epithelial cells in response to the presence of bacteria. These results are in agreement with previous studies in
283 which bacteria-induced production of cytokines in immune cells varies depending on the way of their activation,
284 i.e. – direct activation or through the epithelial monolayer [24,25]. With respect to the IL-10 induction profile in
285 the functional gut model, the strain L. plantarum LS/07 was subsequently tested as a preventive agent in DSS-
286 induced colitis in rats. Experimental DSS- induced colitis in rats appeared to be highly reproducible and share
287 most features with human UC [26]. Important pathological alterations induced by DSS involve elevated
288 production of pro- inflammatory cytokines and other inflammatory mediators, leucocyte infiltration in intestine
289 and dysregulation of the gut microflora composition [27].
290 There are few studies demonstrating the protective role of
291 different strains of L. plantarum on symptoms of IBD, nevertheless, the mechanisms involved in anti-colitis effect
292 of the strains differ between studies [28,29]. In this study, preventive treatment of animals with L. plantarum
293 LS/07 in DSS-treated rats attenuated clinical symptoms of colitis, with favourable effects on body weight loss,
294 colon weight/length ratio, disease activity index and modest improvement of colon morphology. UC has been
295 associated with a defective mucus layer, reduced number of goblet cells and increased number and activity of
296 mast cells [30,31]. Although there is a limited number of studies reporting the influence of probiotics on the
297 number of goblet cells in inflammation-impaired mucosa [32],
298 lactobacilli are documented to play an active role in enhancing the intestinal barrier at the mucosal surface in
299 IBD by affecting the mucin expression and epithelial barrier integrity, including tight junction formation [33,34].
300 Mast cells contribute to the inflammation of the gastrointestinal tract induced by DSS by regulating the

permeability of the epithelium, releasing the pro- inflammatory molecules (histamine, chymase, tryptase, prostaglandins, leukotrienes, variety of cytokines, such as IL-1b, IL-6, IL- 8, TNF-a), followed by recruitment of inflammatory cells [31,35]. Mariman et al. [35] confirmed a lower expression of mast cell- associated enzymes connected with a decreased number of these cells in the lamina propria and the submucosa in TNBS-treated animals after long-term administration of L. plantarum NCIMB8826 or probiotic mixture VSL#3. In the present study, we also confirmed the inhibitory effect of L. plantarum LS/07 on depletion of goblet cells and mast cell infiltration in tunica submucosa of DSS-treated rats, which finally could lead to enhancing and strengthening the epithelial barrier integrity and to confer protection against invading the mucosa by pathogen and consequently attenuate colitis development

Conclusion

Acknowledgments

References

Legend of figures:

Fig. 1 . A–C Cell model settings used in the experiment. Direct bacterial stimulation of monolayers consisted of neither (A) macrophages derived from human blood monocytes (MDBM) or (B) TLT cells; (C) indirect stimulation of TLT monocyte/macrophages trough bacteria-activated epithelial H4-1 cells grown on microporous membrane (functional gut model).

Fig. 2 A–C Effect of the lactobacilli on the production of cytokines IL-1b, TNF-a, IL-10 and TGF-b1 by MDBMs (A) and TLT cells (B) stimulated by direct bacterial contact or indirect stimulation by bacteria challenged epithelial cells H4-1 (C). Data are mean ? SD values derived from three independent experiments and tested in duplicate wells. Values within each graph followed by the same letter are not significantly different according to Tukey's post-hoc test ($p < 0.05$). Neg – non-stimulated control; Lps – cells stimulated with 10 ng/ml LPS from E. coli, LGG – cells stimulated with 106 CFU/ml of L. rhamnosus GG; LP – cells stimulated with 106 CFU/ml of L. plantarum LS/07; MDBMs – macrophages derived from human blood monocytes; TLT – monocyte/macrophage human cell line; H4-1–human intestinal epithelial cell line; ND – not detectable

Fig. 3. A–B The effect of L. plantarum LS/07 on body weight change (A) and disease activity index (B) in rats treated with DSS. Data represent means ± SDs/SEs (n = 8). Asterisk indicate significant differences in body weight compared with weight of animals before DSS administration (A) and DAI (B) compared with DSS group tested by one-way ANOVA and Tukey's post-hoc test ($p < 0,05$). Average body weight in individual groups was as follows: NC – 281.25 x 30.05 g; DSS – 222.5 ?18.52 g; Pro – 235 x 26.77 g. NC – negative control group; DSS – rats orally administered with 5% DSS; LP – L. plantarum LS/07 treated group

Fig. 4 A–D Representative histological sections of rat colon stained with H&E (A–D). Magnification 100?. No histological modification was present in the control animals (A). Administration of DSS resulted in the disruption

of epithelium and crypt architecture (B) and extensive inflammatory infiltrates in the mucosa and submucosa (C). Lesser extend of histological damages (D) was observed after L. plantarum LS/07 treatment in DSS rats.

Fig. 5. A–F Representative histological sections of rat colon stained with Alcian Blue/Safranin (A–C) and Toluidine Blue (D–F). Magnification 100?. Abundant goblet cells in tunica mucosa of the colon from healthy rats (A). Administration of DSS caused depletion of goblet cells (B). Restoration of goblet cells was observed after L. plantarum LS/07 treatment in DSS rats (C). Representative colon sections of CTMC visualisation in tunica mucosa (arrowheads) (D–F). Sporadic occurrence of mast cells in the colon from the negative control rats (D). Increased numbers of mast cells were seen in tunica submucosa in the DSS-challenged rats (E) and in L. plantarum LS/07 treated rats (F). CTMC – connective tissue mast cells.

Fig 6. A-B The effect of L. plantarum LS/07 on the concentration of cytokines IL-6, TNF-a, IL-10 (A) and transcription factor NF-kB p65 (B) in the ileal mucosa of rats. The concentration of cytokines was expressed as ng/g of wet mucosa. Data represent means ± SDs (n = 8). Asterisks indicate significant differences in comparison with the DSS group tested by one-way ANOVA ($p < 0.05$). NC-negative control group; DSS-rats orally administered with 5% DSS; LP – *Lactobacillus plantarum* 07 treated group.

In the pathogenesis of IBD, the anti-inflammatory cytokine balance is altered towards over-expression of pro-inflammatory molecules IL-1, IL-6, IL-8, TNF-a, IFN-g and anti-inflammatory IL-4 and IL-10 are down-regulated [36]. Considering that activated NF- kB up-regulates many genes associated with pathogenesis of IBD, including IL-1b, TNF-a, IL-6, IL-2, IL-8, IL-12, nitric oxide synthase, cyclooxygenase 2, intercellular and vascular adhesion molecules and MHC II, this molecule is an attractive target for reducing the inflammatory response. Several studies have suggested that L. plantarum or its conditioned medium suppressed NF-kB activation in vitro and in vivo [37,38]. In our study, the level of pro- inflammatory mediators NF-kB p65, TNF-a and IL-6 was not affected in L. plantarum LS/07 treated group. Notwithstanding, administration of the strain attenuated colon inflammation induced by DSS, which was confirmed by histopathological observations and by the significantly

decreased DAI in comparison with control group. Concurrently, L. plantarum LS/07 significantly increased the intestinal level of IL-10, which was diminished by DSS application. The stimulation of IL-10 in the gut of DSS treated rats seems to be crucial for the anti-inflammatory effect of L. plantarum LS/07. Cytokine IL-10 produced by T cells, B cells and monocytes counteracts the effects of pro-inflammatory cytokines such as IL-1b, TNF-a and IL-6 by decreasing the antigen-presenting capacity of monocytes via downregulation of MHC II [36]. Our previous work confirmed anti-inflammatory effect of L. plantarum LS/07 on chronic inflammation in dimethylhydrazine-challenged rats, connected with up-regulation of IL-10 in the intestine [39]. We speculate that L. plantarum LS/07 could influence gut inflammation indirectly by affecting the formation of Treg cells secreting IL-10 and TGF-b, or it could favour polarization of M1 to M2 macrophages associated with high level of IL-10 production. These mechanisms were previously confirmed for other strains of Lactobacillus plantarum [40,41], and should be carefully examined in the future.

5. Conclusion

The anti-inflammatory effects of *L. plantarum* LS/07 in the environment of inflamed gut seem to be related to the stimulation of IL-10 and the recovery of epithelial barrier. Moreover, the study revealed that despite limitations, the functional gut model could represent an effective alternative to monitor probiotic immuno-modulatory properties of uncharacterized probiotic strains, by simulating more closely the in vivo conditions than single monolayers of immune cells. Our future research will be aimed to characterize molecular pathways involved in anti-inflammatory effects of the strain *L. plantarum* LS/07 as well as the bacterial metabolites responsible for this activity. Overall, our data show that *L. plantarum* LS/07 exhibits an anti-inflammatory activity and could be a good candidate to prevent and treat diseases associated with inflammation.

Acknowledgements

This work was partially supported by the Scientific Grant Agency of the Ministry of Education of Slovak Republic and Academy of Sciences VEGA 1/0896/15, the grant of SPP Foundation Hlavi9cka 2012/2013, and the grant of

Medical Faculty4/GSD/2012.

References

[1] S. Danese, C. Fiocchi, Etiopathogenesis of inflammatory bowel diseases, World J. Gastroenterol. 12 (2006) 4807–4812.

[2] F. Sanchez-Muñoz, A. Dominguez-Lopez, K. Yamamoto-Furusho, Role of cytokines in inflammatory bowel disease, World J. Gastroenterol. 14 (2008) 4280–4288.

[3] C. Perrier, P. Rutgeers, Cytokine blockade in inflammatory bowel disease, Immunotherapy 3 (2011) 1341–1352.

[4] T. Ali, S. Kaitha, S. Mahmood, A. Ftesi, J. Stone, et al., Clinical use of anti-TNF therapy and increased risk of infections, Drug Healthc. Patient Saf. 5 (2013) 79– 99.

[5] G. Fiorino, L. Peyrin-Biroulet, A. Repici, A. Malesci, S. Danese, Adalimumab in ulcerative colitis: hypes and hopes, Expert Opin Biol. Ther. 11 (2011) 109–116.

[6] S.K. Hegazy, M. El-Bedewy, Effect of probiotics on pro-inflammatory cytokines and NF-kB activation in ulcerative colitis, World J. Gastroenterol. 16 (2010) 4145–4151.

[7] M.S. Geier, R.N. Butler, P.M. Giffard, G.S. Howarth, Lactobacillus fermentum BR11 a potential new probiotic, alleviates symptoms of colitis induced by dextran sulfate sodium (DSS) in rats, Int. J. Food Microbiol.114 (2007) 267–274.

[8] S.F. Ghasemi-Niri, S. Solki, T. Didari, S. Mozaffari, M. Baeeri, et al., Better efficacy of Lactobacillus casei in combination with bifidobacterium bifidum or Saccharomyces boulardii in recovery of inflammatory markers of colitis in rat, Asian J. Anim. Vet. Adv. 7 (2012) 1148–1156.

[9] T.S. Kemgang, S. Kapila, V.P. Shanmugam, R. Kapila, Cross-talk between probiotic lactobacilli and host immune system, J. Appl. Microbiol. 117 (2014) 303–319.

[10] B. Foligne, S. Nutten, C. Grangette, V. Dennin, D. Goudercourt, et al., Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria, World J. Gastroenterol. 13 (2007) 236–243.

[11] L. Strojný, A. Bomba, E. Hijová, A. Chmelárová, G. Mojžišová, et al., Effects of a probiotic in combination with prebiotics on intestinal lactobacilli and coliforms and activities of bacterial enzymes in 1,2-dimethylhydrazine exposed rats, Czech J. Anim. Sci. 56 (2011) 99–106.

[12] K. Menck, D. Behme, M. Pantke, N. Reiling, C. Binder, T. Pukrop, F. Klemm, Isolation of human monocytes by double gradient centrifugation and their differentiation to macrophages in teflon-coated cell culture bags, JOVE 91 (2014) e51554.

[13] A. Cenci9c, T. Langerholc, Functional cell models of the gut and their applications in food microbiology–a review, Int. J. Food Microbiol. 141 (2010) S4–S14.

[14] V. Vasina, M. Broccoli, M.G. Ursino, D. Canistro, L. Valgimigli, et al., Non- peptidyl low molecular weight radical scavenger IAC attenuates DSS-induced colitis in rats, World J. Gastroenterol. 16 (2010) 3642–3650.

[15] M. Doligalska, J. Rzepecka, N. Drela, K. Donskow, M. Gerwel-Wronka, The role of TGF-b in mice infected with Heligmosomoides polygyrus, Parasite Immunol. 28 (2006) 387–395.

[16] N. Habil, W. Al-Murrani, J. Beal, A.D. Foey, Probiotic bacterial strains differentially modulate macrophage cytokine production in a strain- dependent and cell subset-specific manner, Beneficial Microbes 2 (2011) 283–293.

[17] R. Ashraf, T. Vasiljevic, S.L. Day, S.C. Smith, O.N. Donkor, Lactic acid bacteria and probiotic organisms induce different cytokine profile and regulatory T cells mechanisms, J. Funct. Foods 6 (2014) 395–409.

[18] E. Mileti, G. Matteoli, I.D. Iliev, M. Rescigno, Comparison of the immunomodulatory properties of three probiotic strains of Lactobacilli using complex culture systems: prediction for in vivo efficacy, PLoS One 4 (2009) e7056.

[19] M. Tiittanen, J. Keto, J. Haiko, J. Mättö, J. Partanen, et al., Interaction with intestinal epithelial cells promotes an immunosuppressive phenotype in Lactobacillus casei, PLoS One 8 (2013) e78420.

[20] G. Zompopoulou, E. Tsakalidou, J. Dewulf, B. Pot, C. Grangette, Differential crosstalk between epithelial cells, dendritic cells and bacteria in a co-culture model, Int. J. Food Microbiol. 131 (2009) 40–51.

[21] H. Dong, I. Rowland, P. Yaqoob, Comparative effects of six probiotic strains on immune function in vitro, Br.

445 J. Nutr. 108 (2012) 459–470.
446 [22] R. Medzhitov, TLR-mediated innate immune recognition, Semin. Immunol. 19 (2007) 1–2.
447 [23] M. Trapecar, A. Goropevsek, M. Gorenjak, L. Gradisnik, M.S. Rupnik, A co- culture model of developing small
448 intestine offers new insight in the early immunomodulation of enterocytes and macrophages by Lactobacillus
449 spp. through STAT1 and NF-kB p65 translocation, PLoS One 9 (2014) e86297.
450 [24] Y.M. Vissers, J. Snel, P.F. Zuurendonk, B.A. Smit, H.J. Wichers, et al., Differential effects of Lactobacillus
451 acidophilus and Lactobacillus plantarum strains on cytokine induction in human peripheral blood mononuclear
452 cells, FEMS Immunol. Med. Microbiol. 59 (2010) 60–70.
453 [25] A. Parlesak, D. Haller, S. Brinz, A. Baeuerlein, C. Bode, Modulation of cytokine release by differentiated CACO-
454 2Cells in compartmentalized coculture model with mononuclear leucocytes and nonpathogenic bacteria, Scand.
455 J. Immunol. 60 (2004) 477–485.
456 [26] F. Kullmann, H. Messmann, M. Alt, V. Gross, T. Bocker, et al., Clinical and histopathological features of
457 dextran sulfate sodium induced acute and chronic colitis associated with dysplasia in rats, Int. J. Colorectal Dis.
458 16 (2001) 238–246.
459 [27] Å. Håkansson, N. Tormo-Badia, A. Baridi, J. Xu, G. Molin, et al., Immunological alteration and changes of gut
460 microbiota after dextran sodium (DSS) administration in mice, Clin. Exp. Med. 15 (2015) 107–120.
461 [28] J. Karczewski, F.J. Troost, I. Konings, J. Dekker, M. Kleerebezem, et al., Regulation of human epithelial tight
462 junction proteins by Lactobacillus plantarum in vivo and protective effects on the epithelial barrier, Am. J.
463 Physiol.–Gastrointest. Liver Physiol. 298 (2010) 851–859.
464 [29] Y.W. Liu, Y.W. Su, W.K. Ong, T.H. Cheng, Y.C. Tsai, Oral administration of Lactobacillus plantarum K68
465 ameliorates DSS-induced ulcerative colitis in BALB/c mice via the anti-inflammatory and immunomodulatory
466 activities, Int. Immunopharmacol. 11 (2011) 2159–2166.
467 [30] Y.S. Kim, S.B. Ho, Intestinal goblet cells and mucins in health and disease: recent insights and progress, Curr.
468 Gastroenterol. Rep. 12 (2010) 319–330.
469 [31] M.J. Hamilton, S.M. Frei, R.L. Stevens, The multifaceted mast cell in inflammatory bowel disease, Inflamm.

Bowel Dis. 20 (2014) 2364–2378.

[32] N.H. Javed, M.B. Alsahly, J. Khubchandani, Oral feeding of probiotic bifidobacterium infantis: colonic morphological changes in rat model of TNBS-induced colitis, Scientifica 2016 (2016) 9572596.

[33] R. Toumi, K. Abdelouhaba, H. Rafaa, I. Souflia, D. Raissi-Kerbouab, et al., Beneficial role of the probiotic mixture Ultrabiotique on maintaining the integrity of intestinal mucosal barrier in DSS-induced experimental colitis, Immunopharmacol. Immunotoxicol. 35 (2013) 403–409.

[34] D. Ulluwishewa, R.C. Anderson, W.C. McNabb, P.J. Moughan, J.M. Wells, et al., Regulation of tight junction permeability by intestinal bacteria and dietary components, J. Nutr. 141 (2011) 769–776

[35] R. Mariman, B. Kremer, M. van Erk, T. Lagerweij, F. Koning, et al., Gene expression profiling identifies mechanisms of protection to recurrent trinitrobenzene sulfonic acid colitis mediated by probiotics, Inflamm. Bowel Dis. 18 (2012) 1424–1433.

[36] G. Roda, M. Marocchi, A. Sartini, E. Roda, Cytokine networks in ulcerative colitis, Ulcers 2011 (2011) 391787.

[37] E.O. Petrof, E.C. Claud, J. Sun, T. Abramova, Y. Guo, et al., Bacteria-free solution derived from lactobacillus plantarum inhibits multiple NF-kappaB pathways and inhibits proteasome function, Inflamm. Bowel Dis. 15 (2009) 1537–1547.

[38] P. Van Baarlen, F.J. Troost, S. van Hemert, C. van der Meer, W.M. de Vos, et al., Differential NF-kB pathways induction by Lactobacillus plantarum in the duodenum of healthy humans correlating with immune tolerance, Proc. Natl. Acad. Sci. 106 (2009) 2371–2376.

[39] J. Štofilová, V. Szabadosová, G. Hr9cková, R. Salaj, I. Bertková, et al., Co- administration of a probiotic strain Lactobacillus plantarum LS/07 CCM7766 with prebiotic inulin alleviates the intestinal inflammation in rats exposed to N,N-dimethylhydrazine, Int. Immunopharmacol. 24 (2015) 361–368.

[40] M. Chytilová, D. Mudro nová, R. Nemcová, S. Gancar9cíková, V. Buleca, et al., Anti-inflammatory and immunoregulatory effects of flax-seed oil and Lactobacillus plantarum–Biocenolá LP96 in gnotobiotic pigs challenged with enterotoxigenic Escherichia coli, Res. Vet. Sci. 95 (2013) 103–109.

[41] S.E. Jang, M.J. Han, S.Y. Kim, D.H. Kim, Lactobacillus plantarum CLP-0611 ameliorates colitis in mice by

polarizing M1 to M2-like macrophages, Int. Immunopharmacol. 21 (2014) 186–192.

Figure 1

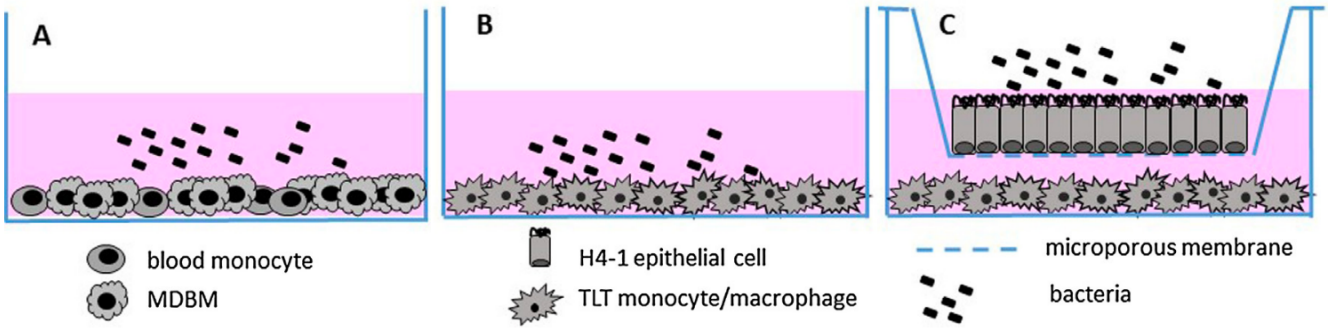
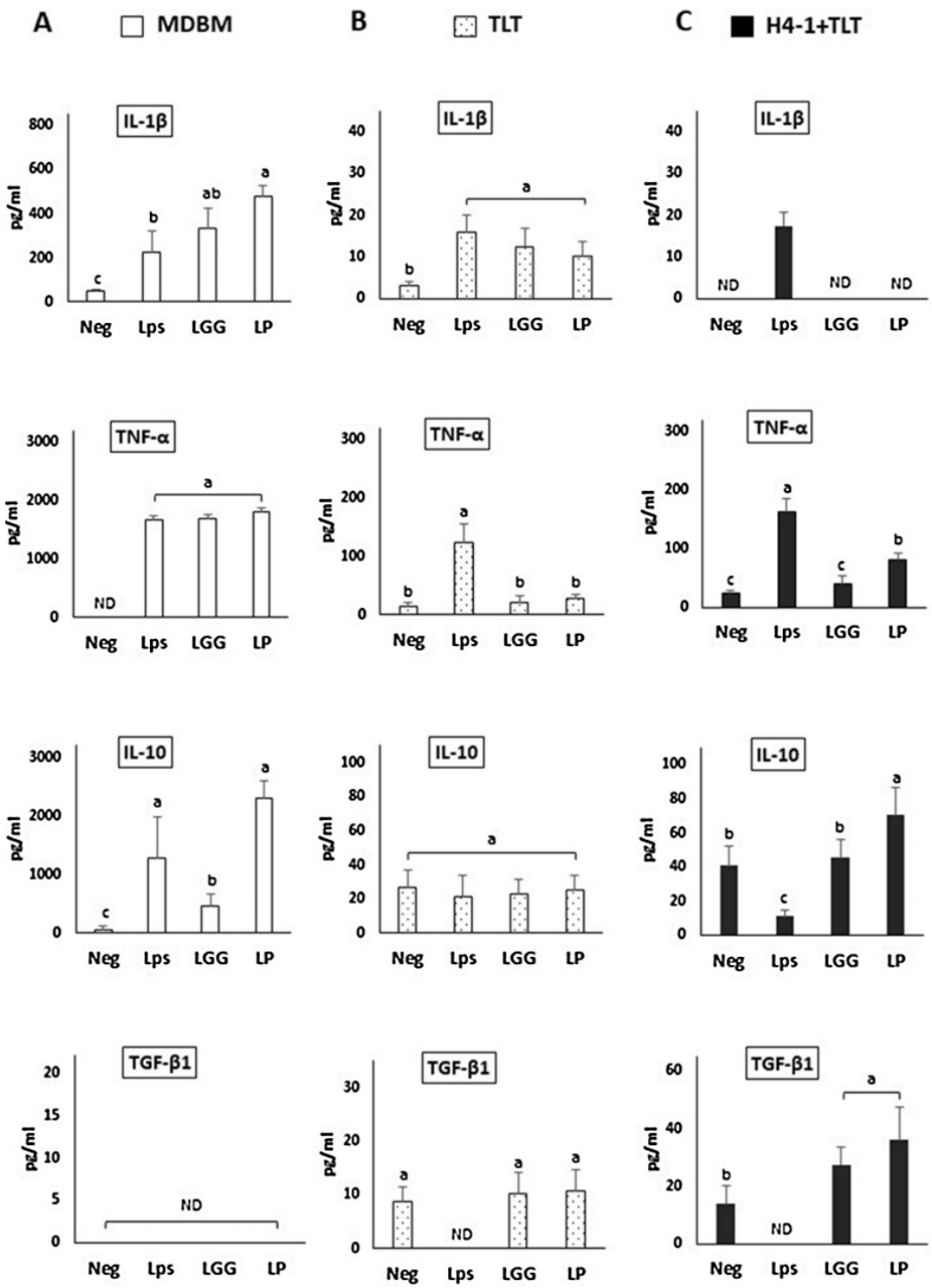
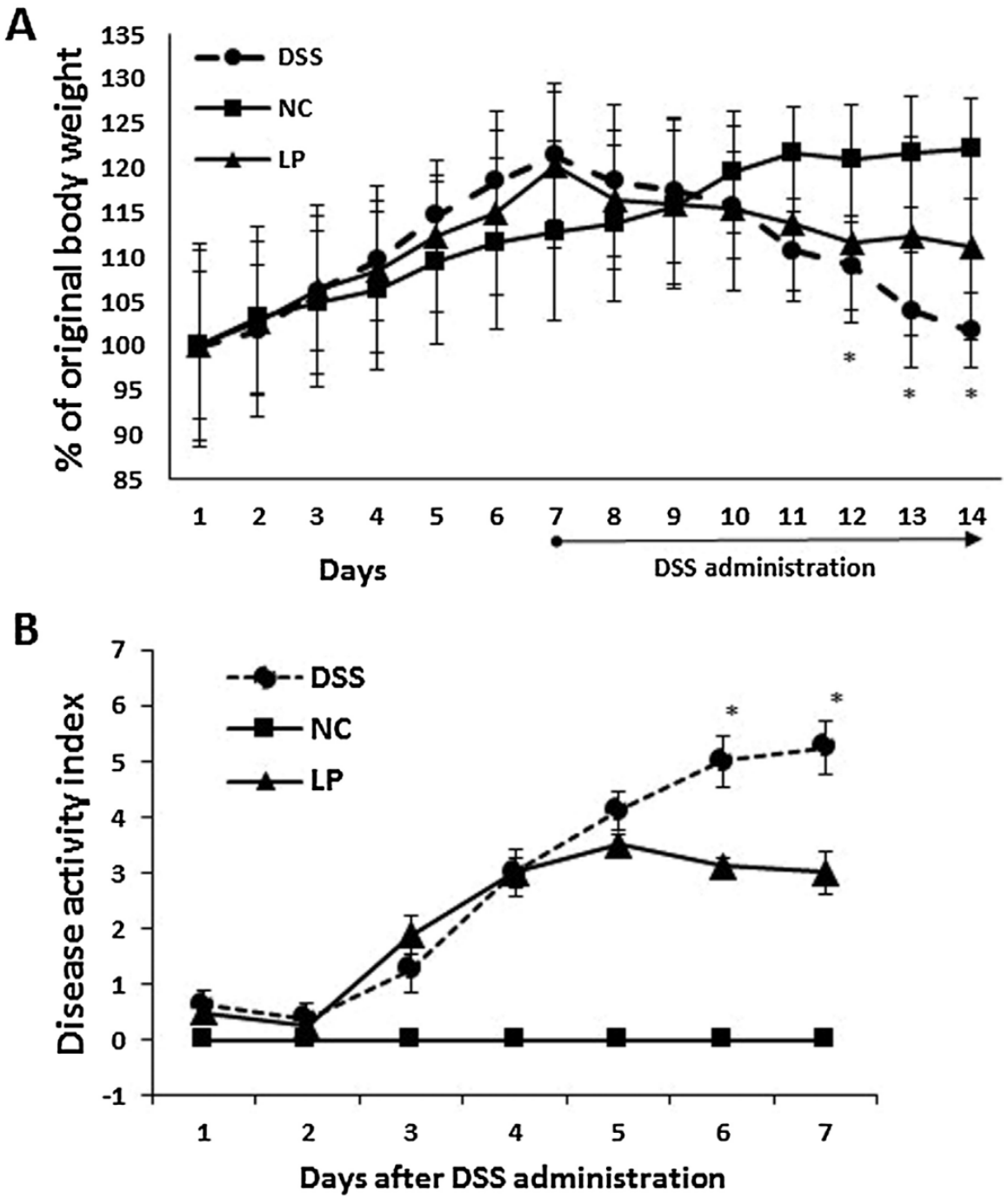

A
B
C
blood monocyte
MDBM
H4-1 epithelial cell
TLT monocyte/macrophage
microporous membrane
bacteria

Figure 2


A
MDBM
B
TLT
C
H4-1+TLT
IL-1β
TNF-α
IL-10
TGF-β1
pg/ml
Neg
Lps
LGG
LP
ND

542 **Figure 3**

543



544

545

546

547

548

549

550
551 **Figure 4**

552

553 **Tables**

554

555 **Table 1** Disease activity index (DAI) score parameters.

Stool consistency	Bleeding	Weight loss	Maximum score
0 = formed	0 = normal color stool	0 = no weight loss	10
1 = mild-soft	1 = brown color stool	1 = 5–10%	
2 = very soft	2 = reddish color stool	2 = 11–15%	
3 = watery stool	3 = bloody stool	3 = 16–20%	
		4 = >20%	

556

557

558

559 **Table 2** Effect of *L. plantarum* LS/07 on colon weight, length and weight/length ratio in DSS- challenged rats.

560 Data represent means ± SDs (n = 8). Values within rows, followed by the same letter are not significantly different

561 in comparison with DSS group according to one-way ANOVA test and Tukey's post-hoc test ($p < 0,05$). NC-

562 negative control group; DSS – rats orally administered with 5% DSS; LP – *Lactobacillus plantarum* LS/07 treated

563 group.

Group of rats	Colon weight (g)	Colon length (cm)	Weight/length ratio
NC	2.349 ± 0.439^{a}	23.938 ± 2.397^{c}	$0.097 \pm 0{,}009^{c}$
DSS	2.684 ± 0.352^{a}	17.625 ± 1.767^{a}	0.149 ± 0.008^{a}
LP	2.674 ± 0.308^{a}	20.438 ± 0.728^{b}	0.131 ± 0.011^{b}

564

565

566

567

568

569

570

571

572

573

574 **Table 3**. The effect of L. plantarum LS/07 on the number of goblet cells and connective tissue mast cells in the
575 rat colon.
576 Data represent means ± SDs (n = 8). Values within rows, followed by the same letter (s) are not significantly
577 different in comparison with DSS group according to one- way ANOVA test and Tukey's post-hoc test ($p < 0.05$).
578 NC-negative control group; DSS-rats orally administered with 5% DSS; LP – *Lactobacillus plantarum* LS/07 treated
579 group; CTMC – connective tissue mast cells.
580

Groups of rats	Number of goblet cells/mm^2	Number of CTMC/mm^2
NC	1991.67 ± 159.71^{a}	23.33 ± 10.08^{b}
DSS	705.83 ± 199.19^{c}	60.0 ± 19.19^{a}
LP	1465.83 ± 258.80^{b}	37.50 ± 7.07^{b}

581
582
583
584
585
586
587
588