

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1831405> since 2022-01-11T15:05:57Z

Published version:

DOI:10.1016/j.biopha.2017.07.138

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

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

2

3 Jana Štofilová^{a*}, Tomáš Langerholc^b, Cristian Botta^c, Primož Treven^d, Lidija Gradišnik^b, Rastislav Salaj^e, Alena
4 Šoltéssová^a, Izabela Bertková^a, Zdenka Hertelyová^a, Alojz Bomba^a

5

6

7 ^aDepartment of Experimental Medicine, Faculty of Medicine, P.J. Šafárik University in Košice, Trieda SNP 1, 04011
8 Košice, Slovak Republic

9 ^b Department of Microbiology, Biochemistry, Molecular Biology and Biotechnology, Faculty of Agriculture and
10 Life Sciences, University of Maribor, Pivola 10,
11 2311 Hoce, Slovenia

12 ^cDepartment of Forestry, Agriculture and Food Sciences, University of Torino, Largo Paolo Braccini 2, Grugliasco,
13 10095 Torino, Italy

14 ^dInstitute of Dairy Science and Probiotics, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale,
15 Slovenia
16 ^e Medirex, a.s., Magnezitárska 2/C, 040 13 Košice, Slovak Republic

16

17 **Keywords:** Lactobacillus plantarum Cytokine Functional gut model Inflammation Colitis

18

19

20 **Abstract**

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

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

36

37

38 **1. Introduction**

39 Inflammatory bowel disease (IBD) is a chronic disease of the digestive tract, which includes two distinct clinical
40 forms, Crohn's disease (CD) and ulcerative colitis (UC). Both forms of IBD share almost identical
41 pathophysiological background characterized by chronic and spontaneously relapsing intestinal inflammation
42 occurring in the gut. Although the aetiology remains unclear, IBD is an inappropriate immune response to
43 endogenous bacteria, which occurs in genetically susceptible individuals as the result of complex interactions
44 among the intestinal immune system, gut microbiota and environmental factors [1]. It has been reported
45 that there is a disturbed balance between pro-inflammatory (TNF- α , IL-1, IL-6, IL-8) and anti-inflammatory
46 cytokines (IL-10, TGF- β) in IBD patients [2]. Studies in animal models of IBD and some clinical trials have shown
47 that the specific manipulation of targeted cytokines seems to reduce disease severity and maintains remission,
48 thus in the last decade, several attempts were made to treat patients with cytokine-modulating agents, such as
49 recombinant anti-inflammatory cytokines or antibodies specific against pro-inflammatory cytokines [3].
50 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 activities 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 combination 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, epithelium 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, histopathological signs, cytokine production and activity of
75 transcription factor NF- κ B in the intestine.

76

77 **2. Materials and methods**

78

79 **2.1. Bacteria and growth conditions**

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

87

88 **2.2. Growth and maintenance of in vitro cell models**

89 Macrophages differentiated from human blood monocytes (MDBMs) were prepared according to Menck et al.
90 [12]. Human monocytes were isolated from 6 healthy donors (age 32–45 years) by a double density gradient
91 centrifugation. An informed consent was obtained before the sample collection, and the experiments were
92 approved by the local ethical committee. PBMCs obtained without brake) using HISTOPAQUE1 x1077 (Sigma-
93 Aldrich) were after the first density gradient centrifugation (400 x g, 30 min, brake) using iso-osmotic Percoll1
94 (Sigma-Aldrich) gradient to proceeded to the second centrifugation (550 x g, 30 min, without separate
95 monocytes. The monocytes were then cultured in the presence of 2,5 ng/ml macrophage colony-stimulating
96 factor (M-CSF; Biolegend, San Diego, USA) in RPMI 1640 with stable glutamine (Biosera, Nuaille, France)
97 supplemented with 10% foetal bovine serum (Biosera) and antibiotics (ThermoFisher Scientific, Waltham MA,
98 USA) at 37 °C and 5%CO₂. After six days, differentiated macrophages were harvested and the viability was tested
99 by trypane-blue, which was more that 95%. Macrophages from each participant were seeded into 12 well plates
100 in duplicate at concentration 10⁵ cells/ml and allowed to adhere for 2 h. The intestinal epithelial cell line H4-1

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

108 To obtain polarised monolayers of epithelial cell line, H4-1 cells were seeded on Transwell filter inserts (0,4 mm
109 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
110 apical compartment and concentration of 2×10^5 cells/ml media. The filters were main- 1.5 mL in the basolateral
111 compartment. Medium was changed every two days and cells were cultured for 13–14 days to achieve fully
112 differentiated monolayers. Trans-epithelial electrical resistance (TEER) was determined continuously using the
113 Millicell-ERS volt-ohmmeter (Millipore, Bedford, MA). Functional polarity was established when TEER between
114 the apical and basolateral surface of the monolayers reached a minimal value of 600 V/cm². TLT cells were
115 seeded in 12 well plates (Corning) at a concentration of 3×10^5 cells/ml and further incubated until confluence
116 (24–48 h). To create the functional gut model, inserts containing fully differentiated H4-1 monolayers were
117 transferred to 12 well plates with TLT cell monolayer.

118

119 2.3. Stimulation of cell cultures with bacteria and cytokine determination

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

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

130

131 **2.4. Animals**

132 Animal experiment was conducted according to the principles described in the Law No. 377/2012 and No.
133 436/2012 of Slovak Republic for the Care and Use of Laboratory Animals, and were approved by the Ethical
134 Committee of the P.J. Šafárik University, Faculty of Medicine. The specific license to carry out this experiment on
135 animals was obtained from the State Veterinary and Food Administration of the Slovak Republic (Ro-1136/14-
136 221). Male Sprague Dawley rats (seven-weeks old, n = 24) were kept humidity (50–55%) with 12-h light/12-h
137 dark cycle in the under standard conditions of temperature (21 ±2°C) and relative Laboratory of Research Bio-
138 models, Faculty of Medicine, University of P.J. Šafárik in Košice, Slovak Republic. The rats were fed with standard
139 laboratory chow (containing 3.5% fat). Feed and water were provided ad libitum. The state of health, body
140 weights, feed and water usage were monitored daily.

141

142 **2.5. Preparation and administration of probiotic**

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

148

149 **2.6. Experimental induction of colitis and treatment**

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

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

163

164 **2.7. Macroscopic assessment of colonic inflammation**

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

171

172 **2.8. Histological assessment of colonic damage**

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

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

183

184 **2.9. Cytokine and NF- κ B quantification in the intestinal mucosa**

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

192

193 **2.10. Statistical analysis**

194 The experimental data were expressed as means \pm SD or \pm SE and statistically evaluated by one-way ANOVA
195 followed by Tukey's post-hoc test (MINITAB Release 11, 1996). Differences were considered significant at P <
196 0.05.

197

198 **3. Results**

199

200 **3.1. Effects of *L. plantarum* LS/07 on cytokines release in vitro**

201 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
202 after 24 h of culture with either monocyte/macrophages monolayers alone or co-culture
203 monocyte/macrophages with epithelial cell line H4 (Fig. 2A–C). LPS from *E. coli* significantly induced pro-
204 inflammatory cytokines (IL-1b, TNF-a) in both MDBMs and TLT cells compared with non-stimulated control. None
205 of stimulants (LPS, LP, LGG) elicited the production of IL-10 in TLT cells grown in a monolayer, whereas MDBMs
206 responded by massive production of IL-10 after 24 h incubation with LPS and *L. plantarum* LS/07. The level of
207 TGF- b1 was not detected in MDBMs after 24 h stimulation. In the functional gut model, LPS decreased
208 significantly the concentration of IL-10 and stimulated both pro-inflammatory cytokines TNF-a and IL-1b
209 compared to untreated control. Noticeably, the production of TNF-a, IL-10 and TGF-b increased significantly in
210 the functional gut model treated with *L. plantarum* LS/07 compared to the negative control, while the same
211 cytokines were weakly induced in TLT cells grown in a monolayer. The stimulant capacity of *L. plantarum* LS/07
212 was comparable with that of LGG in all cell models. However, *L. plantarum* LS/07 induced a significant higher
213 production of TNF-a and IL-10 in the MDBMs and in the functional gut model compared with the LGG. Moreover,
214 we found out that there were considerable changes in the level of cytokines produced by MDBMs and TLT cells.
215 The production of IL- 10 by TLT cells depended on the source of their activation.

216

217 **3.2. Protective effects of *L. plantarum* LS/07 on manifestation of colonic inflammation**

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

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

230

231 **3.3. Protective effects of *L. plantarum* LS/07 on histopathological changes of the colonic mucosa induced by** 232 **DSS**

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

244

245 **3.4. Effects of *L. plantarum* LS/07 on the level of cytokines and NF- κ B in intestinal mucosa**

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

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

252

253 **4. Discussion**

254 In this study, the capacity of the probiotic strain *L. plantarum* LS/07 to modulate production of selected cytokines
255 in vitro and in vivo was addressed, using human intestinal cells and animal model of IBD respectively. Many
256 recent studies have indicated that several probiotic lactic acid bacteria, such as lactobacilli, can stimulate in vitro
257 and in strain-dependent manner the production of cytokines in PBMCs, macrophages, dendritic cells (DC) and
258 epithelial cells [16,17]. Previous studies have shown that the ability of different lactobacilli to induce a high ratio
259 of IL-10/IL-12 or IL-10/TNF- α production in culture of PBMCs correlates with their capacity to provide significant
260 protection in rodent colitis models [10,18]. However, Mileti et al. [18] have shown that strains of lactobacilli can
261 prevent trinitrobenzenesulfonic acids (TNBS)-induced colitis in mice, despite they elicited a reduction of IL-10/IL-
262 12 ratio in vitro. This could be due to a simplified model used in their study, which was composed of PBMCs and
263 bacteria only. Furthermore, immunomodulatory activity of bacteria can be affected by cellular interactions in
264 the intestinal environment [19]. Nevertheless, IL-10 is the major anti-inflammatory cytokine and probiotic strains
265 capable of inducing its secretion would likely be good candidates to be used in IBD studies. Based on these
266 evidences, we evaluated the ability of *L.*

267 *plantarum* LS/07 to stimulate the production of IL-10, TGF- β 1, IL-1 β and TNF- α in monocyte/macrophages either
268 directly or indirectly via the action of epithelial cells H4. The functional gut model, composed of human
269 enterocytes H4-1 and TLT cell line [13], was chosen following the concept of cellular cross-talk between epithelial
270 and underlying immunocompetent cells in response to probiotic bacteria at the mucosal surface to simulate the
271 in vivo situation. Overall, lactobacilli seem to be strong inducers of pro-inflammatory cytokines IL-12, TNF- α and
272 weak inducers of anti-inflammatory cytokine IL-10 in PBMCs [10,20,21]. In accordance with aforementioned
273 reports, in the study both tested lactobacilli did not induce IL-10 and strongly stimulated production of IL-1 β
274 upon a direct contact with TLT cells. Conversely, *L. plantarum* LS/07 significantly stimulated IL-10, TGF- β but also
275 TNF- α 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- κ B 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

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

310

311

312

313

314 **Conclusion**

315

316 **Acknowledgments**

317 **References**

318

319

320 **Legend of figures:**

321

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

326

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

335

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

342

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

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

347

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

355

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

361 In the pathogenesis of IBD, the anti-inflammatory cytokine balance is altered towards over-expression of pro-
362 inflammatory molecules IL-1, IL-6, IL-8, TNF- α , IFN- γ and anti-inflammatory IL-4 and IL-10 are down-regulated
363 [36]. Considering that activated NF- κ B up-regulates many genes associated with pathogenesis of IBD, including
364 IL-1b, TNF- α , IL-6, IL-2, IL-8, IL-12, nitric oxide synthase, cyclooxygenase 2, intercellular and vascular adhesion
365 molecules and MHC II, this molecule is an attractive target for reducing the inflammatory response. Several
366 studies have suggested that *L. plantarum* or its conditioned medium suppressed NF- κ B activation in vitro and in
367 vivo [37,38]. In our study, the level of pro- inflammatory mediators NF- κ B p65, TNF- α and IL-6 was not affected
368 in *L. plantarum* LS/07 treated group. Notwithstanding, administration of the strain attenuated colon
369 inflammation induced by DSS, which was confirmed by histopathological observations and by the significantly

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

381

382 **5. Conclusion**

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

391

392 **Acknowledgements**

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

395 Medical Faculty4/GSD/2012.

396

397 **References**

398 [1] S. Danese, C. Fiocchi, Etiopathogenesis of inflammatory bowel diseases, *World J. Gastroenterol.* 12 (2006)
399 4807–4812.

400 [2] F. Sanchez-Muñoz, A. Dominguez-Lopez, K. Yamamoto-Furusho, Role of cytokines in inflammatory bowel
401 disease, *World J. Gastroenterol.* 14 (2008) 4280–4288.

402 [3] C. Perrier, P. Rutgeers, Cytokine blockade in inflammatory bowel disease, *Immunotherapy* 3 (2011) 1341–
403 1352.

404 [4] T. Ali, S. Kaitha, S. Mahmood, A. Ftesi, J. Stone, et al., Clinical use of anti-TNF therapy and increased risk of
405 infections, *Drug Healthc. Patient Saf.* 5 (2013) 79– 99.

406 [5] G. Fiorino, L. Peyrin-Biroulet, A. Repici, A. Malesci, S. Danese, Adalimumab in ulcerative colitis: hypes and
407 hopes, *Expert Opin Biol. Ther.* 11 (2011) 109–116.

408 [6] S.K. Hegazy, M. El-Bedewy, Effect of probiotics on pro-inflammatory cytokines and NF-kB activation in
409 ulcerative colitis, *World J. Gastroenterol.* 16 (2010) 4145–4151.

410 [7] M.S. Geier, R.N. Butler, P.M. Giffard, G.S. Howarth, *Lactobacillus fermentum* BR11 a potential new probiotic,
411 alleviates symptoms of colitis induced by dextran sulfate sodium (DSS) in rats, *Int. J. Food Microbiol.* 114 (2007)
412 267–274.

413 [8] S.F. Ghasemi-Niri, S. Solki, T. Didari, S. Mozaffari, M. Baeeri, et al., Better efficacy of *Lactobacillus casei* in
414 combination with *bifidobacterium bifidum* or *Saccharomyces boulardii* in recovery of inflammatory markers of
415 colitis in rat, *Asian J. Anim. Vet. Adv.* 7 (2012) 1148–1156.

416 [9] T.S. Kemgang, S. Kapila, V.P. Shanmugam, R. Kapila, Cross-talk between probiotic lactobacilli and host immune
417 system, *J. Appl. Microbiol.* 117 (2014) 303–319.

418 [10] B. Foligne, S. Nutten, C. Grangette, V. Dennin, D. Goudercourt, et al., Correlation between in vitro and in
419 vivo immunomodulatory properties of lactic acid bacteria, *World J. Gastroenterol.* 13 (2007) 236–243.

- 420 [11] L. Stojný, A. Bomba, E. Hijová, A. Chmelárová, G. Mojžišová, et al., Effects of a probiotic in combination with
421 prebiotics on intestinal lactobacilli and coliforms and activities of bacterial enzymes in 1,2-dimethylhydrazine
422 exposed rats, *Czech J. Anim. Sci.* 56 (2011) 99–106.
- 423 [12] K. Menck, D. Behme, M. Pantke, N. Reiling, C. Binder, T. Pukrop, F. Klemm, Isolation of human monocytes
424 by double gradient centrifugation and their differentiation to macrophages in teflon-coated cell culture bags,
425 *JOVE* 91 (2014) e51554.
- 426 [13] A. Cenci9c, T. Langerholc, Functional cell models of the gut and their applications in food microbiology—a
427 review, *Int. J. Food Microbiol.* 141 (2010) S4–S14.
- 428 [14] V. Vasina, M. Broccoli, M.G. Ursino, D. Canistro, L. Valgimigli, et al., Non- peptidyl low molecular weight
429 radical scavenger IAC attenuates DSS-induced colitis in rats, *World J. Gastroenterol.* 16 (2010) 3642–3650.
- 430 [15] M. Doligalska, J. Rzepecka, N. Drela, K. Donskow, M. Gerwel-Wronka, The role of TGF- β in mice infected with
431 *Heligmosomoides polygyrus*, *Parasite Immunol.* 28 (2006) 387–395.
- 432 [16] N. Habil, W. Al-Murrani, J. Beal, A.D. Foey, Probiotic bacterial strains differentially modulate macrophage
433 cytokine production in a strain- dependent and cell subset-specific manner, *Beneficial Microbes* 2 (2011) 283–
434 293.
- 435 [17] R. Ashraf, T. Vasiljevic, S.L. Day, S.C. Smith, O.N. Donkor, Lactic acid bacteria and probiotic organisms induce
436 different cytokine profile and regulatory T cells mechanisms, *J. Funct. Foods* 6 (2014) 395–409.
- 437 [18] E. Mileti, G. Matteoli, I.D. Iliev, M. Rescigno, Comparison of the immunomodulatory properties of three
438 probiotic strains of Lactobacilli using complex culture systems: prediction for in vivo efficacy, *PLoS One* 4 (2009)
439 e7056.
- 440 [19] M. Tiittanen, J. Keto, J. Haiko, J. Mättö, J. Partanen, et al., Interaction with intestinal epithelial cells promotes
441 an immunosuppressive phenotype in *Lactobacillus casei*, *PLoS One* 8 (2013) e78420.
- 442 [20] G. Zompopoulou, E. Tsakalidou, J. Dewulf, B. Pot, C. Grangette, Differential crosstalk between epithelial cells,
443 dendritic cells and bacteria in a co-culture model, *Int. J. Food Microbiol.* 131 (2009) 40–51.
- 444 [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- κ B 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.*

470 Bowel Dis. 20 (2014) 2364–2378.

471 [32] N.H. Javed, M.B. Alsahly, J. Khubchandani, Oral feeding of probiotic bifidobacterium infantis: colonic
472 morphological changes in rat model of TNBS-induced colitis, Scientifica 2016 (2016) 9572596.

473 [33] R. Toumi, K. Abdelouhaba, H. Rafea, I. Souflia, D. Raissi-Kerbouab, et al., Beneficial role of the probiotic
474 mixture Ultrabiotique on maintaining the integrity of intestinal mucosal barrier in DSS-induced experimental
475 colitis, Immunopharmacol. Immunotoxicol. 35 (2013) 403–409.

476 [34] D. Ulluwishewa, R.C. Anderson, W.C. McNabb, P.J. Moughan, J.M. Wells, et al., Regulation of tight junction
477 permeability by intestinal bacteria and dietary components, J. Nutr. 141 (2011) 769–776

478 [35] R. Mariman, B. Kremer, M. van Erk, T. Lagerweij, F. Koning, et al., Gene expression profiling identifies
479 mechanisms of protection to recurrent trinitrobenzene sulfonic acid colitis mediated by probiotics, Inflamm.
480 Bowel Dis. 18 (2012) 1424–1433.

481 [36] G. Roda, M. Marocchi, A. Sartini, E. Roda, Cytokine networks in ulcerative colitis, Ulcers 2011 (2011) 391787.

482 [37] E.O. Petrof, E.C. Claud, J. Sun, T. Abramova, Y. Guo, et al., Bacteria-free solution derived from lactobacillus
483 plantarum inhibits multiple NF-kappaB pathways and inhibits proteasome function, Inflamm. Bowel Dis. 15
484 (2009) 1537–1547.

485 [38] P. Van Baarlen, F.J. Troost, S. van Hemert, C. van der Meer, W.M. de Vos, et al., Differential NF-kB pathways
486 induction by Lactobacillus plantarum in the duodenum of healthy humans correlating with immune tolerance,
487 Proc. Natl. Acad. Sci. 106 (2009) 2371–2376.

488 [39] J. Štofilová, V. Szabadosová, G. Hrčková, R. Salaj, I. Bertková, et al., Co- administration of a probiotic strain
489 Lactobacillus plantarum LS/07 CCM7766 with prebiotic inulin alleviates the intestinal inflammation in rats
490 exposed to N,N-dimethylhydrazine, Int. Immunopharmacol. 24 (2015) 361–368.

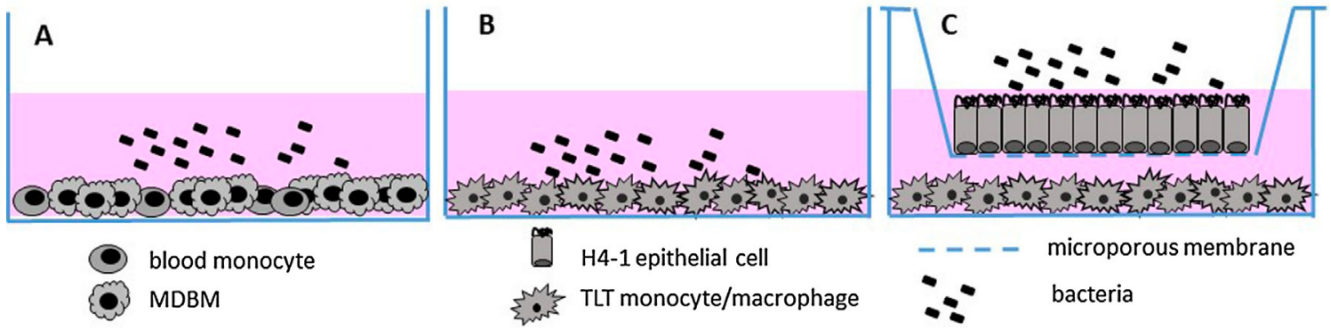
491 [40] M. Chytilová, D. Mudro nová, R. Nemcová, S. Gancarčíková, V. Buleca, et al., Anti-inflammatory and
492 immunoregulatory effects of flax-seed oil and Lactobacillus plantarum–Biocenolá LP96 in gnotobiotic pigs
493 challenged with enterotoxigenic Escherichia coli, Res. Vet. Sci. 95 (2013) 103–109.

494 [41] S.E. Jang, M.J. Han, S.Y. Kim, D.H. Kim, Lactobacillus plantarum CLP-0611 ameliorates colitis in mice by

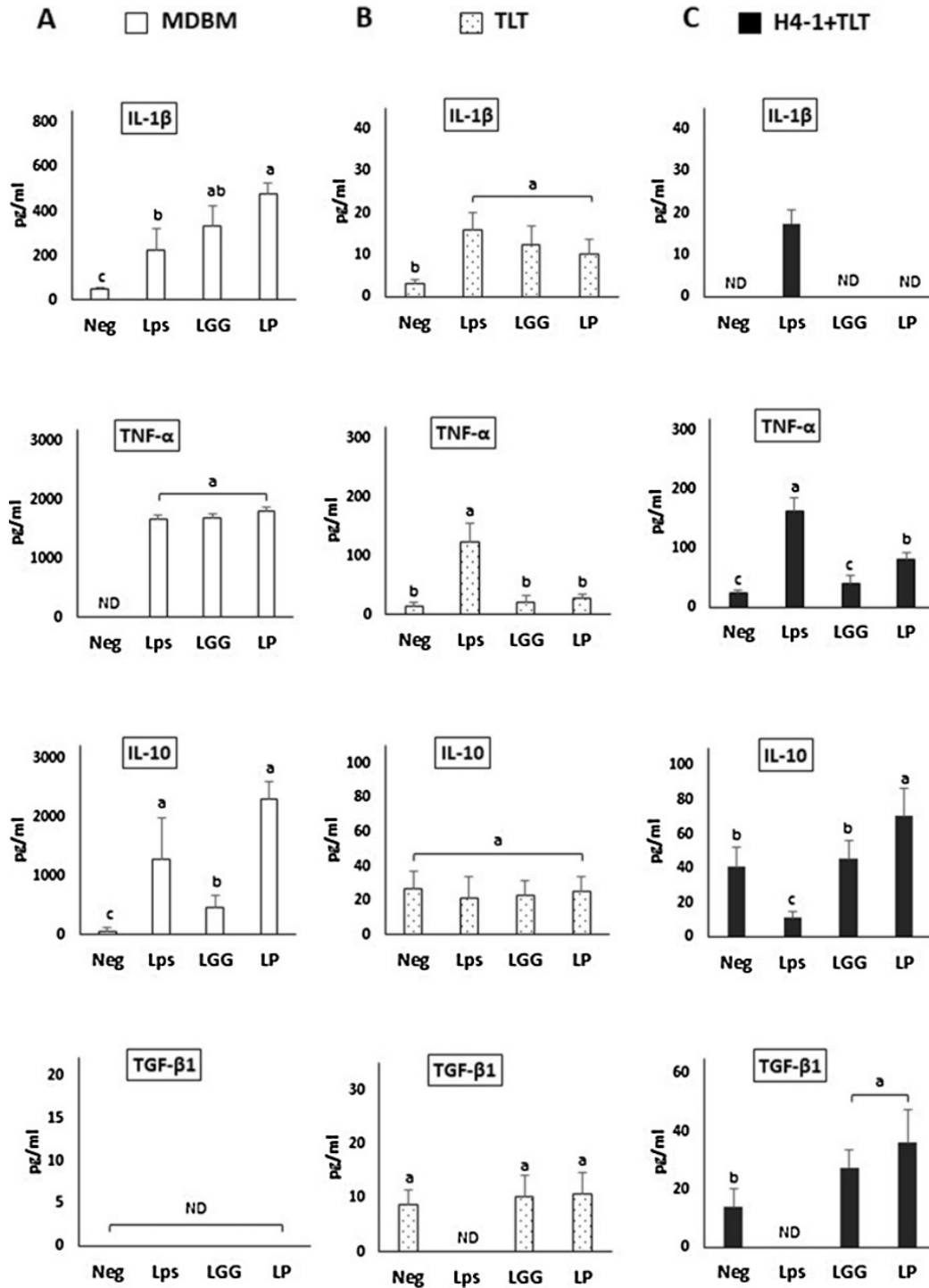
495 polarizing M1 to M2-like macrophages, *Int. Immunopharmacol.* 21 (2014) 186–192.

496

497 Figure 1
498



499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536



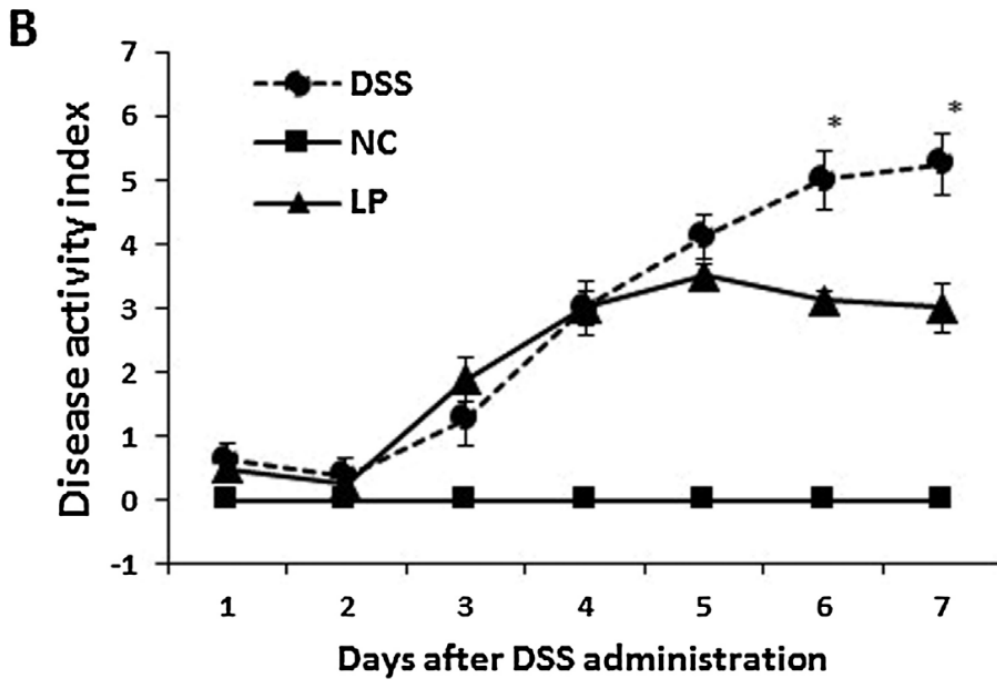
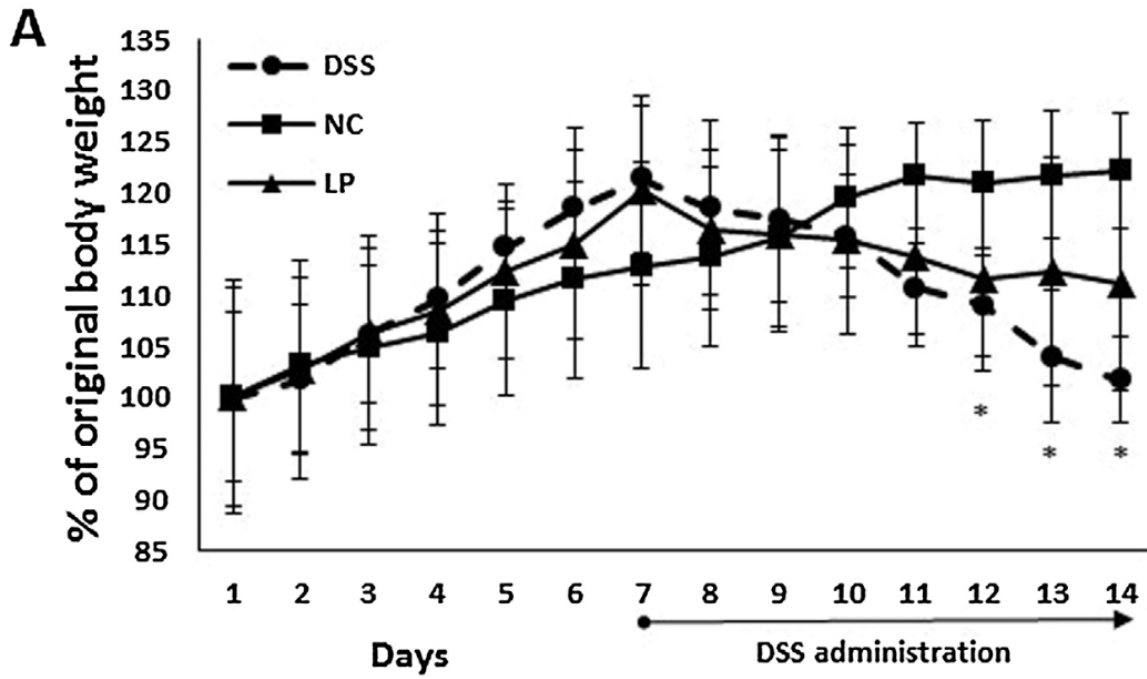
539

540

541

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 \pm 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 \pm 0.439 ^a	23.938 \pm 2.397 ^c	0.097 \pm 0.009 ^c
DSS	2.684 \pm 0.352 ^a	17.625 \pm 1.767 ^a	0.149 \pm 0.008 ^a
LP	2.674 \pm 0.308 ^a	20.438 \pm 0.728 ^b	0.131 \pm 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 \pm 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 \pm 159.71 ^a	23.33 \pm 10.08 ^b
DSS	705.83 \pm 199.19 ^c	60.0 \pm 19.19 ^a
LP	1465.83 \pm 258.80 ^b	37.50 \pm 7.07 ^b

581

582

583

584

585

586

587

588