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(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<sup>a\*</sup>, Tomáš Langerholc<sup>b</sup>, Cristian Botta<sup>c</sup>, Primož Treven<sup>d</sup>, Lidija Gradišnik<sup>b</sup>, Rastislav Salaj<sup>e</sup>, Alena  
4 Šoltésová<sup>a</sup>, Izabela Bertková<sup>a</sup>, Zdenka Hertelyová<sup>a</sup>, Alojz Bomba<sup>a</sup>

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7 <sup>a</sup>Department of Experimental Medicine, Faculty of Medicine, P.J. Šafárik University in Košice, Trieda SNP 1, 04011  
8 Košice, Slovak Republic

9 <sup>b</sup> Department of Microbiology, Biochemistry, Molecular Biology and Biotechnology, Faculty of Agriculture and  
10 Life Sciences, University of Maribor, Pivola 10,  
11 2311 Hoče, Slovenia

12 <sup>c</sup>Department of Forestry, Agriculture and Food Sciences, University of Torino, Largo Paolo Braccini 2, Grugliasco,  
13 10095 Torino, Italy

14 <sup>d</sup>Institute of Dairy Science and Probiotics, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale,  
15 Slovenia  
16 <sup>e</sup> 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- $\alpha$ , IL-1, IL-6, IL-8) and anti-inflammatory  
46 cytokines (IL-10, TGF- $\beta$ ) 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- $\kappa$ 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%CO<sub>2</sub>. 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 10<sup>5</sup> 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% CO<sub>2</sub>  
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  $2 \times 10^5$  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/cm<sup>2</sup>. TLT cells were  
115 seeded in 12 well plates (Corning) at a concentration of  $3 \times 10^5$  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 10<sup>6</sup>  
124 CFU/ml of *L. plantarum* LS/07, 10<sup>6</sup> CFU/ml of LGG and 10 ng/ml LPS (from *Escherichia coli* 0111:B4, Sigma-  
125 Aldrich) respectively. The concentration 10<sup>6</sup> 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% CO<sub>2</sub>, 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 10<sup>9</sup> 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 mm<sup>2</sup>. 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- $\kappa$ B quantification in the intestinal mucosa**

185 The concentration of cytokines IL-6, TNF- $\alpha$ , IL-10 and NF- $\kappa$ 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- $\kappa$ 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- $\kappa$ B p65 ELISA kit (Elabscience Biotechnology, China) according to the  
190 manufacturer's instructions. The plates were read at 450 nm using Eon<sup>TM</sup> 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- $\kappa$ B in intestinal mucosa**

246 Seven days after colitis induction by DSS, tissue concentrations of NF- $\kappa$ B p65, TNF- $\alpha$  and IL-6 in DSS-group  
247 increased significantly compared to the NC group. DSS-induced increase in both NF- $\kappa$ B p65 and pro-inflammatory  
248 cytokines IL-6 and TNF- $\alpha$  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- $\alpha$  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- $\beta$ 1, IL-1 $\beta$  and TNF- $\alpha$  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- $\alpha$  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 $\beta$   
274 upon a direct contact with TLT cells. Conversely, *L. plantarum* LS/07 significantly stimulated IL-10, TGF- $\beta$  but also  
275 TNF- $\alpha$  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- $\kappa$ 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-1b, TNF-a, IL-10 and TGF-b1 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 10<sup>6</sup> CFU/ml of L. rhamnosus GG; LP – cells stimulated with 10<sup>6</sup> 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- $\alpha$ , IL-10 (A) and  
357 transcription factor NF- $\kappa$ 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- $\alpha$ , IFN- $\gamma$  and anti-inflammatory IL-4 and IL-10 are down-regulated  
363 [36]. Considering that activated NF- $\kappa$ B up-regulates many genes associated with pathogenesis of IBD, including  
364 IL-1b, TNF- $\alpha$ , 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- $\kappa$ B activation in vitro and in  
367 vivo [37,38]. In our study, the level of pro- inflammatory mediators NF- $\kappa$ B p65, TNF- $\alpha$  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

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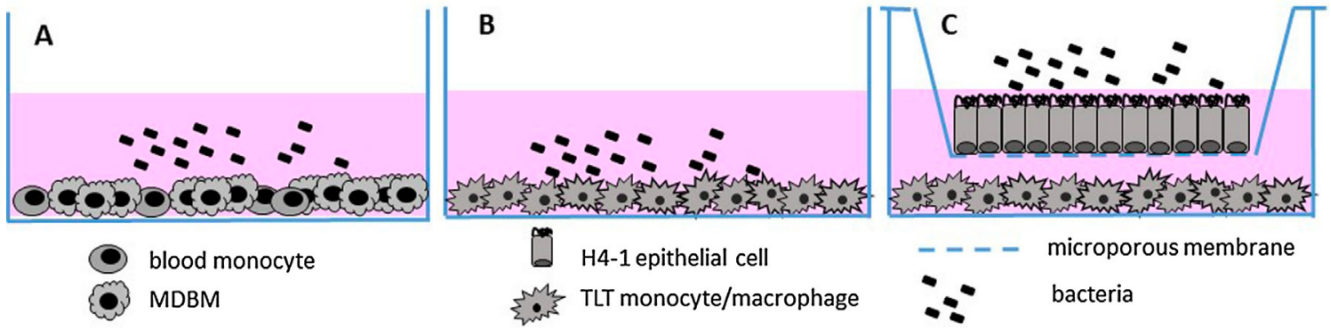
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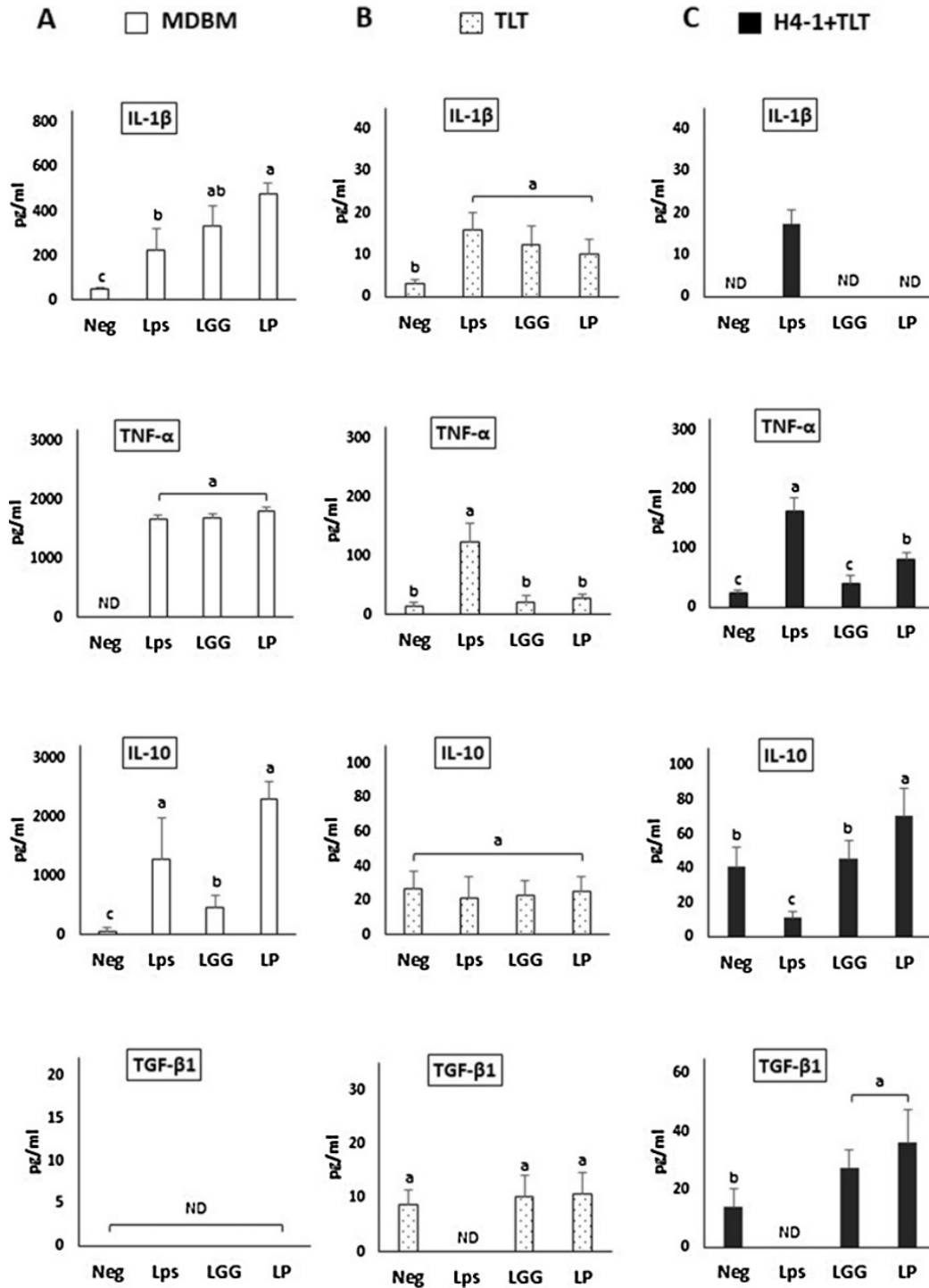
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497 Figure 1  
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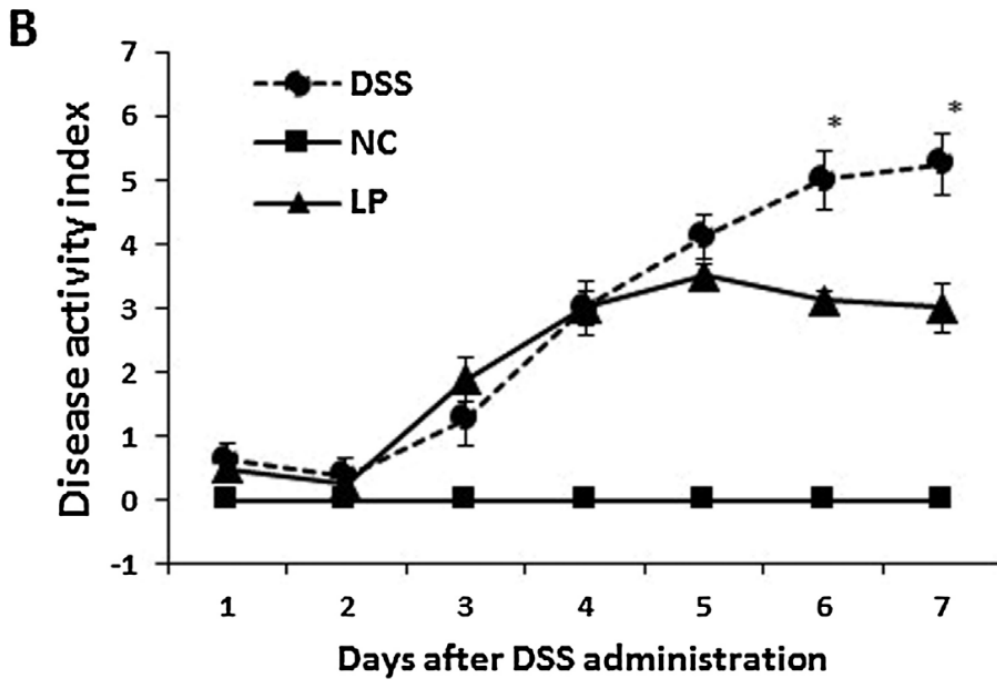
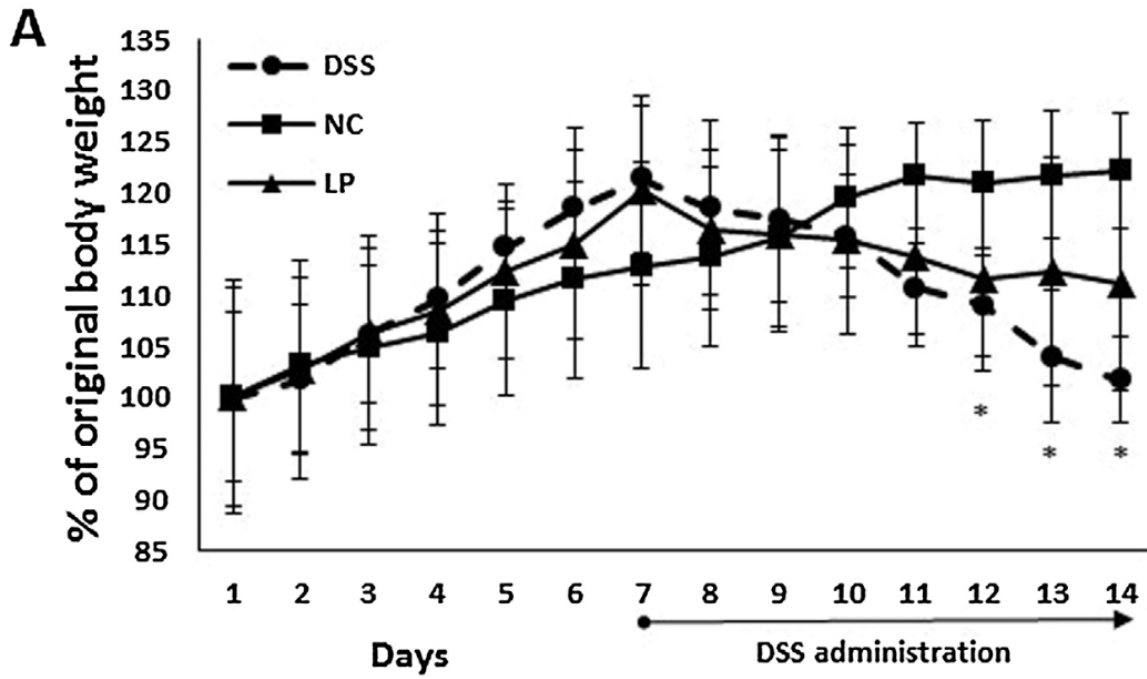


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551 **Figure 4**

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553 **Tables**  
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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%           |               |

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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 <sup>a</sup> | 23.938 $\pm$ 2.397 <sup>c</sup> | 0.097 $\pm$ 0.009 <sup>c</sup> |
| DSS           | 2.684 $\pm$ 0.352 <sup>a</sup> | 17.625 $\pm$ 1.767 <sup>a</sup> | 0.149 $\pm$ 0.008 <sup>a</sup> |
| LP            | 2.674 $\pm$ 0.308 <sup>a</sup> | 20.438 $\pm$ 0.728 <sup>b</sup> | 0.131 $\pm$ 0.011 <sup>b</sup> |

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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.

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| Groups of rats | Number of goblet cells/mm <sup>2</sup> | Number of CTMC/mm <sup>2</sup> |
|----------------|--|--------------------------------|
| NC             | 1991.67 $\pm$ 159.71 <sup>a</sup>      | 23.33 $\pm$ 10.08 <sup>b</sup> |
| DSS            | 705.83 $\pm$ 199.19 <sup>c</sup>       | 60.0 $\pm$ 19.19 <sup>a</sup>  |
| LP             | 1465.83 $\pm$ 258.80 <sup>b</sup>      | 37.50 $\pm$ 7.07 <sup>b</sup>  |

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