

Improvement of colitis by *Lactobacillus plantarum* LS/07 and inulin

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Abstract

The objective of this study was to investigate the effect of the probiotic strain *Lactobacillus plantarum* LS/07 and prebiotic inulin on the activity of β -glucuronidase, transcription nuclear factor kappa B (NF κ B), myeloperoxidase (MPO), cytokine levels (IL-6, IL-8, IL-13), and counts of coliforms and lactobacilli in rats with dextran sulphate sodium (DSS) induced acute colitis. Sprague-Dawley rats were divided into groups: control (C), acute colitis (AC), prebiotic (Pre), and probiotic (Pro). Dextran sulphate sodium induced inflammatory process in the colonic tissue, increased the activity of β -glucuronidase ($P < 0.05$), increased counts of coliforms, decreased lactobacilli counts ($P < 0.05$), and activated production of the measured indicators NF κ B, MPO, IL-6, IL-8, except of IL-13. Diet supplemented with *L. plantarum* and inulin alleviated DSS induced inflammatory process by inhibiting production of IL-6, IL-8, activities of NF κ B and MPO, and by stimulation of IL-13. These results indicate that the dietary intake of *Lactobacillus plantarum* LS/07 and inulin suppressed the expression of markers playing an important role in the inflammatory process, which predisposes their use in prevention or treatment of acute colitis.

Inflammatory bowel diseases, rats, inflammation, prebiotics, probiotics

Ulcerative colitis (UC) as a nosological unit within inflammatory bowel diseases (IBD) is a chronic, nonspecific relapsing disease characterised by diffuse mucosal inflammation limited to the colon but it can also affect other areas of the digestive tract in humans. Similar disorders are also present in several animal species (Kalck 2009; Cerquetella et al. 2010).

The course of UC is characterized by an impact frequency of unpredictable occurrence of relapse (acute inflammation) and remission (inflammation in decline), but the aetiology of UC has not yet been understood. In genetically susceptible individuals, an abnormal communication between the intestinal microbiota and the mucosal immune system forms the basis of the defect, which is accompanied by mucosal inflammatory lesions of the gastrointestinal tract. Studies on animal models of colitis has led to the hypothesis that genetic factors and enteric bacteria can play a pivotal role in the pathogenesis of these disorders, owing to the abnormal intestinal response to commensal microflora (Burgener et al. 2008; Liu et al. 2009). Long-standing UC has a higher risk of developing colitis-associated cancer (CAC) approximately 8–10 years after the initial diagnosis when compared to the general population (Jones et al. 2014). In addition to genomic instability that underlies the process of tumorigenesis, continuous inflammation in the intestine seems to be a key factor in CAC development since chronic inflammation is associated with an overexpression of proinflammatory cytokines, chemokines, growth factors as well as their receptors, and reactive oxygen species (Kanneganti et al. 2011; Hijová et al. 2014). Ulcerative colitis is a serious medical and socio-economic problem with an increasing incidence and prevalence. The decreased quality of life associated with it is the reason for seeking new possibilities for its prevention and treatment.

In general, the first approach with UC usually involves dietary modification of bacterial

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flora, the use of probiotics and prebiotics may attenuate the inflammatory process and prevent relapses and maintenance of remission in ulcerative colitis.

The aim of the presented study was to obtain information regarding the effects of the probiotic *Lactobacillus plantarum* LS/07 and of prebiotic inulin on the activity of β -glucuronidase and counts of coliform and lactobacilli in fresh caecal digesta, cytokine levels (IL-6, IL-8, IL-13), transcription nuclear factor kappa B (NF κ B) and myeloperoxidase (MPO) activities in the colon tissue and blood samples of rats with dextran sulphate sodium (DSS) induced acute colitis.

Materials and Methods

Male Sprague-Dawley rats ($n = 32$, 7 weeks old, 220–290 g body weight) were placed in the Laboratory of Research Bio-models of the Faculty of Medicine, University of P. J. Šafárik, Slovak Republic (SK PC 4013). The room was maintained at 21 ± 1 °C with 50–60% humidity and 12-h light/dark cycle. All animal experiments were conducted in accordance with the principles outlined in Law No. 377/2012 and No. 436/2012 of the Slovak Republic for the Care and Use of Laboratory Animals, and were approved by the Ethics Committee of the University of P.J. Šafárik, Faculty of Medicine and State Veterinary and Food Administration of the Slovak Republic (Ro-1136/14-221).

The rats were randomly assigned to four groups: control group (C) received the conventional MP-OŠ-06 feed (Snina, Slovak Republic) at an interval of 14 days; the acute colitis group (AC) received the conventional feed without DSS at an interval of 7 days followed by 7 days of feed with DSS; the prebiotic group (Pre) received the conventional feed supplemented with prebiotic inulin (BeneoSynergy 1, ORAFTI, Tienen, Belgium) at a dose of 80 g/kg feed without DSS at an interval of 7 days, followed by 7 days of the same feed with DSS; and the probiotic group (Pro) received the conventional feed with the probiotic *Lactobacillus plantarum* LS/07 given in pasteurized milk containing 0.5% fat at a daily dose 1.5×10^9 CFU/1 ml, without DSS at an interval of 7 days, and then for 7 days with DSS. Colitis was induced using DSS (molecular weight 40 000, TdB Consulting AB, Uppsala, Sweden) added to drinking water at a final concentration of 5% (wt/vol) for 7 days. Controls were all time-matched and consisted of rats receiving normal drinking water only. The DSS solution was replenished daily and mean DSS consumption was noted per cage at the end of the 7-day treatment. All animals had free access to water and feed. Animal weights and clinical monitoring of the health status were recorded daily. After 14 days of consuming the experimental diets, the animals were euthanized under anaesthesia Zoletil (Virbac S.A., France) administered at a dose of 50 mg/kg body weight with xylazine (Riemser, Germany) at a dose of 15 mg/kg body weight, intramuscularly. Blood samples were drawn by cardiac puncture to measure selected indices. Caecal and tissue samples from the colon were recovered for biochemical and immunological analyses.

The disease activity index (DAI) is the combined score of animal weight loss, stool consistency and bleeding in stool. Stool probes were tested to evaluate rectal bleeding using HemoFEC test (Roche Diagnostics, Slovak Republic). All indices were assessed on a scale from 0 to 3 or 4, the maximum score was 10. These indices were each assigned a score and utilized to calculate an average daily disease activity index score for each rat as previously described (Vasina et al. 2010).

The probiotic strain of *Lactobacillus plantarum* LS/07 was isolated from rectal human swabs reported by Strojný et al. (2011). The strain was cultured in MRS broth (Merck, Germany) prepared as night cultures at 37 °C aerobically and provided at a dose of 3×10^9 CFU of strain/1 ml. Then 0.5 ml of lactobacilli strains mixed with 9 ml of pasteurized milk (20–22 °C) was filled into bottles and administered individually every day. Each rat received approximately 1.5×10^9 CFU lactobacilli via the oral route. The Rogosa agar plates for lactobacilli culturing were maintained under anaerobic conditions (BD GasPak, Becton, Dickinson and Company, USA) and incubated at 37 °C for 48 h. McConkey agar plates used for coliform culturing were incubated aerobically at 37 °C for 16–18 h. The numbers of colony forming units (CFU) are expressed as \log_{10} CFU per gram of faeces.

The activity of β -glucuronidase (β -GLUCUR) enzyme was measured in fresh caecal digesta with a method previously described by Juskiewicz et al. (2002). A measurement unit of enzymatic activity is expressed as μmol of p -nitrophenol per min per gram digesta.

Samples of blood were to clot for 2 h at room temperature before centrifugation for 15 min at $1\,000 \times g$, removed serum samples were stored at -20 °C. Tissue samples from the entire colon were rinsed in ice-cold PBS (pH 7.0–7.2) to thoroughly remove excess blood, then they were cut longitudinally and homogenized in PBS with a homogenizer on ice (Dispenser T10 Basic Ultra Turrax, Germany), and stored overnight at -20 °C. Two freeze-thaw cycles were performed to break the cell membranes. Next, the homogenates were centrifuged for 5 min at $5\,000 \times g$ at 4 °C; supernatants were removed and stored at -20 °C. All endpoints were measured by ELISA method as follows: NF κ B in tissue by USCN Life Science, Inc., USA; IL-6 in blood and tissue by eBioscience, IL-8 and IL-13 in tissue by Cusabio (Biotech Co., China), and MPO in tissue and blood by BlueGene (Biotech Co., China). The final values of each indicator were measured on the Synergy H4 multiplate reader (BioTek Instruments, Inc. USA).

The data were expressed as mean \pm SD. One-way analysis of variance and Tukey test (MINITAB for Windows 11.21) was used to evaluate significance of differences between the control and experimental groups. Differences were considered significant at $P < 0.05$.

Results

The final mean body weight of the animals compared to the initial weight was increased in group C by 30.92%, in group AC by 9.90%, in group Pre by 14.28%, and in group Pro by 21.74%. The average value of all daily disease activity index score showed that the maximum DAI score = 6.50 was in the AC group. A decrease of the DAI score was observed after application of prebiotic inulin = 4.13 and probiotic *Lactobacillus plantarum* LS/07 = 3.50. Changes in the monitored indices correspond to the changes in weight as well as the results of the DAI score. Within a short experimental period, preventive dietary supplementation with prebiotic inulin and *Lactobacillus plantarum* LS/07 positively decreased the activity of β -glucuronidase which is believed to be a biomarker of increased risk of neoplasm. The activity of β -glucuronidase was significantly increased ($P < 0.05$) in group AC. *Lactobacillus plantarum* LS/07 and inulin positively reduced the activity of β -glucuronidase ($P < 0.05$), (Table 1). Acute colitis elevated (non-significantly) the number of coliforms and significantly ($P < 0.05$) decreased the number of lactobacilli in group AC compared to the control group (Table 1). Table 2 shows changes in NF κ B and MPO activities in serum (s) and colon tissue (t) in the control group, the acute colitis group, and in the groups treated with inulin and probiotic. Serum and tissue cytokine levels of IL-6, IL-8 and IL-13 in C, AC, Pre and Pro groups are shown in Table 3.

Table 1. The effect of administration of inulin and *L. plantarum* LS/07 on the β -glucuronidase activity and total counts of lactobacilli and coliform caecal content in rats.

Indicator	C (n=8)	AC (n=8)	Pre (n=8)	Pro (n=8)
β -GLUCUR μ mol/min/g	0.14 \pm 0.02 ^b	0.54 \pm 0.05 ^a	0.49 \pm 0.18 ^a	0.39 \pm 0.18 ^a
Lactobacilli log ₁₀ CFU/g	7.78 \pm 0.17 ^a	7.15 \pm 0.90 ^b	7.31 \pm 0.45 ^a	7.40 \pm 0.44 ^a
Coliforms log ₁₀ CFU/g	5.18 \pm 0.56	5.74 \pm 1.03	5.51 \pm 0.77	5.19 \pm 0.57

C - control group, AC - acute colitis group, Pre - prebiotic group, Pro - probiotic group. The data are presented as mean \pm standard deviation (S.D.), ^{a,b} – values in a row sharing different superscripts are significantly different ($P < 0.05$)

Table 2. The effect of administration of inulin and *L. plantarum* LS/07 on NF κ B and MPO activities in serum (s) and tissue (t) in rats.

Indicator	C (n=8)	AC (n=8)	Pre (n=8)	Pro (n=8)
NF κ B (t) ng/ml	41.73 \pm 7.41 ^b	60.21 \pm 10.31 ^a	11.65 \pm 1.32 ^c	50.52 \pm 17.56 ^{a,b}
MPO (s) pg/ml	53.38 \pm 30.53 ^b	98.78 \pm 18.26 ^a	88.35 \pm 10.49 ^a	69.94 \pm 32.95 ^{a,b}
MPO (t) pg/ml	362.53 \pm 71.55 ^{ab}	424.12 \pm 73.46 ^a	296.96 \pm 63.78 ^b	346.04 \pm 92.13 ^{a,b}

C - control group, AC - acute colitis group, Pre - prebiotic group, Pro - probiotic group. The data are presented as mean \pm standard deviation (S.D.), ^{a,b,c} – values in a row sharing different superscripts are significantly different ($P < 0.05$)

Table 3. The effect of administration of inulin and *L. plantarum* LS/07 on cytokine levels of IL-6, IL-8 and IL-13 in serum (s) and tissue (t) in rats.

Indicator	C (n=8)	AC (n=8)	Pre (n=8)	Pro (n=8)
IL-6 s pg/ml	49.31 ± 15.83 ^{a,b}	61.89 ± 15.33 ^a	45.83 ± 9.99 ^{a,b}	43.00 ± 6.09 ^b
IL-6 t pg/ml	47.00 ± 8.53 ^b	62.65 ± 12.19 ^a	48.77 ± 5.31 ^{a,b}	41.27 ± 14.88 ^b
IL-8 t pg/ml	37.78 ± 7.42 ^c	50.12 ± 7.32 ^a	47.67 ± 8.49 ^{a,b}	39.34 ± 5.39 ^{b,c}
IL-13 t pg/ml	62.81 ± 5.99 ^a	47.87 ± 6.91 ^b	57.93 ± 11.26 ^{a,b}	53.20 ± 14.52 ^{a,b}

C - control group, AC - acute colitis group, Pre - prebiotic group, Pro - probiotic group. The data are presented as mean ± standard deviation (S.D.), ^{a,b,c} – values in a row sharing different superscripts are significantly different ($P < 0.05$)

Discussion

Experimental and clinical data so far support the hypothesis that prebiotics such as inulin and oligofructose and probiotic strains offer the opportunity to prevent or mitigate intestinal inflammatory lesions, modulate the intestinal microbiota and the host immune system in gastrointestinal disorders, and provide an alternative or adjuvant approach to conventional therapy (Looijer van Langen and Dieleman 2009; Verna and Lucak 2010; Mack 2011). For UC, interactions are expected between genetic, immunologic, microbial and environmental factors, while the exact aetiology and pathogenesis are still unclear. Chronic inflammation is known to lead to derangement in the signalling processes and to a local microenvironment described as lying somewhere between pre-cancerous stromal cells and cancer cells, even as the details of the steps in the transformation to a cancer cell are not completely understood (Nathan and Ding 2011). Signalling pathways triggered by NFκB play a key role in the colonic inflammation development. The NFκB regulates the expression of various cytokines and modulates the inflammatory processes characteristic of IBD (Atreya et al. 2008; Luckschander et al. 2010). The NFκB controls apoptosis, cell-cycle progression, cell proliferation, and differentiation. Normally functioning NFκB is essential for the maintenance of epithelial cell homeostasis in the gut. Translocation of activated NFκB into the nucleus induces the expression of genes for cytokines such as TNFα and IL-6, and chemokines, all of which contribute to the development of inflammation-related tissue damage.

In our study, the activity of NFκB in colon tissue samples was markedly increased in group AC ($P < 0.05$) which may provide a sensitive means of assessing the state of activation of the mucosal immune response. Inulin treatment suppressed the activity of critical transcription factor in mucosa cells ($P < 0.05$). The application of *Lactobacillus plantarum* LS/07 decreased colon tissue NFκB slightly. Activated NFκB in the acute colitis group significantly activated serum and colon tissue levels of pro-inflammatory cytokine (IL-6 and IL-8) compared to the control group. Inulin diet intervention in group Pre significantly downregulated the synthesis of proinflammatory cytokines IL-6 ($P < 0.05$) in serum and colon tissue and IL-8 ($P < 0.05$) in tissue compared to group AC. *Lactobacillus plantarum* LS/07 significantly downregulated the synthesis of IL-6 and IL-8 ($P < 0.05$) compared to group AC. Inulin and *Lactobacillus plantarum* LS/07 stimulated IL-13 production thus demonstrating their anti-inflammatory activity and immune enhancing effect.

Evaluating the MPO activity is crucial for understanding its effects in inflammation, since it is the most abundant proinflammatory enzyme stored in the azurophilic granules of neutrophilic granulocytes. Neutrophil accumulation in the inflamed intestinal mucosa

is a prominent feature in ulcerative colitis. Extracellular MPO activity gives an estimate of oxidative stress in inflammatory diseases, whereas intracellular MPO activity correlates well with the tissue neutrophil content (Pulli et al. 2013). The determination of MPO can be used as one of the non-invasive markers of disease and prediction of relapse, and is also considered as an inflammatory damage marker. Increased activities of MPO were found in serum and colon tissue homogenate of rats. Its activity was significantly suppressed by administration of prebiotics and probiotics. The release of MPO correlated with an enhanced local release of the neutrophil activating interleukine-8 (Massodi et al. 2011).

Within the short experimental period, the preventive dietary supplementation with inulin and probiotic positively modified the values of observed microorganisms and activity of β -glucuronidase. Presented experimental data demonstrate the ability of the prebiotic inulin and probiotic *Lactobacillus plantarum* LS/07 to intervene and affect the pathophysiological process of development of acute colitis. The exact aetiology and pathogenesis of ulcerative colitis is not yet known, but the use of prebiotics and probiotics appear to be a suitable form of preventing acute colitis in human and veterinary medicine.

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