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Regular paper

Preventive use of *Lactobacillus plantarum* LS/07 and inulin to relieve symptoms of acute colitis

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The aim of presented study was to investigate the influence of *Lactobacillus plantarum* LS/07 and inulin on the activity of β-glucuronidase enzyme, and counts of coliform and lactobacilli in fresh caecal digesta, cytokine levels (IL-6, IL-8), and trancription nuclear factor kappa beta (NFκB) activities in colon tissue and blood samples of rats with dextran sulphate sodium (DSS) induced acute colitis. The rats were randomly divided into four groups — CG, AC, AC+PRE and AC+PRO. Colitis was induced using of 5% DSS in drinking water for 7d. DSS application increased activity of β-glucuronidase ($P<0.001$), increased counts of coliforms, and decreased lactobacilli counts ($P<0.05$) in comparison to control group. Serum and tissue levels of IL-6 and IL-8 as well as tissue NFκB activities showed increased expression in acute colitis group. Inulin diet modified counts of microorganims and decreased β-glucuronidase activity, suppressed NFκB activities ($P<0.001$) and down regulate synthesis of IL-6 ($P<0.01$) in serum and colon tissue and tissue IL-8 ($P<0.05$). *Lactobacillus plantarum* LS/07 decreased β-glucuronidase activity ($P<0.05$), levels of IL-6 and IL-8 ($P<0.001$). These results were consistent with the addition of histological findings. Our results indicate that dietary intake of *Lactobacillus plantarum* LS/07 and inulin suppressed expression observed markers, which play an important role in the inflammatory process, which predisposes their use in prevention or treatment of acute colitis.

Key words: colitis, Sprague-Dawley rats, inulin, *Lactobacillus plantarum* LS/07

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INTRODUCTION

Ulcerative colitis (Colitis ulcerosa —- CU) as one of nosological unit inflammatory bowel diseases (IBD) is a chronic, nonspecific relapsing disease characterised by diffuse mucosal inflammation limited to the colon, serious medical and socio-economic problem continuing increase in the incidence and prevalence. The aetiology of ulcerative colitis is not yet understood. Failure of immunoregulatory mechanisms due to external environmental factors in genetically predisposed individuals suggests the current concept of the pathophysiology of inflammatory diseases. The course of CU is characterized by an impact frequency of unpredictable occurrence of relapse (acute inflammation) and remission (inflammation in decline). In genetically susceptible individuals, abnormal communication between the intestinal microbial flora and mucosal immune system form the basis of the defect, which is accompanied by mucosal inflammatory lesions of the gastrointestinal tract. The studies with animal models of colitis indicated that dysregulation of host/microbial interactions included the loss of epithelial and cell barrier function were pre-requisite for the development of IBD (Poritz *et al*., 2007; Liu *et al*., 2009). Risk factors for colorectal carcinoma development in CU include disease duration, earlier age of onset, family history of sporadic colorectal carcinoma, and persistent inflammation of the colon (Jones *et al*., 2014). Patients with longstanding CU have a higher risk of development colitis-associated cancer (CAC) approximately 8–10 years after the initial diagnosis as compared to general population (Eaden *et al*., 2001). 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). A high morbidity, significant early and late complications, and decreased quality of life is the reason for seeking new possibilities for the prevention, rational diagnosis and treatment. It has been suggested that modifying the bacterial flora with probiotics and prebiotics may attenuate the inflammatory process and prevent relapses and maintenance of remission in ulcerative colitis (Hijova & Soltesova, 2013).

The goal of the presented study was to obtain informations about the effect of *Lactobacillus plantarum* LS/07 and inulin on the activity of β-glucuronidase enzyme and counts of coliform and lactobacilli in fresh caecal digesta, cytokine levels (IL-6, IL-8), and transcription nuclear factor kappa beta (NFκB) activities in colon tissue and blood samples of rats with dextran sulphate sodium (DSS) induced acute colitis.

MATERIALS AND METHODS

Animals. 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 Ethical Committee of the University of

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Abbreviations: CAC, colitis-associated cancer; CU, Colitis ulcerosa; IBD, Inflammatory bowel disease; NFκB, nuclear factor kappa beta

P.J. Šafárik, Faculty of Medicine and State Veterinary and Food Administration of the Slovak Republic (Ro-1136/14-221). Male Sprague-Dawley rats (8 per group, 7 weeks old, 220–290 g body weight) were placed in Laboratory of Research Bio-models of the Faculty of Medicine, P. J. Šafárik University, Slovak Republic with a 12-h light/dark cycle. The room was maintained at 21°C ± 1°C with 50% to 60% humidity. The rats were randomly assigned to the following groups: control group (**CG**) received the conventional feed (Snina, Slovak Republic) at an interval of 14 days, acute colitis group (**AC**) received conventional feed without DSS at an interval of 7 days and followed by 7 days of feed with DSS, prebiotic group (**AC+PRE**) group received 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 the same feed with DSS, and probiotic group (**AC+PRO**) received conventional feed with 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 7 days simultaneously with DSS.

All animals had free access to water and dietary intake. Animal weights and clinically monitoring of health status were recorded daily. After 14 days of consuming the experimental diets, the animals were euthanized under anesthesia (Zoletil (Virbac S.A., France) administered at a dose of 50 mg/kg body weight with Xylazin (Riemser, Germany) at a dose of 15 mg/kg body weight, intramuscular). Blood samples were drawn by cardiac puncture to measure selected parameters. Caecal and tissue samples from colon were recovered for microbial, biochemical, immunological and histological analysis.

Induction of colitis. 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 replemished daily and mean DSS consumption was noted per cage at the end of 7-days treatment.

Disease activity index. Disease activity index (DAI) is the combined score of animal weight loss, stool consistency and bleeding in stool as detailed in Table 1. Stool probes were tested to evaluate rectal bleeding by using HemoFEC test (Roche Diagnostics, Slovak Republic). All parameters were assessed on a scale from 0 to 3, or 4, the maximum score was 10. These parameters 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).

Probiotic strain. The probiotic strain of *Lactobacillus plantarum* LS/07 was isolated from rectal human swabs reported by Strojný and coworkers (2011). The strain was cultured in MRS broth (Merck, Germany) prepared as night cultures at 37°C aerobically and provided in dose 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 every day. Each rat received approximately 1.5×10^9 CFU lactobacilli *via* the oral route.

Bacteriological examination. Microbial analyses (total counts of lactobacilli and coliform) of the fecal samples were carried out after completion of the experiment. Feces (1g) were placed in a sterile polyethylene Stomacher Lab Blenders bag (Seward, France) with 9 mL sterile 0.9% NaCl and mixed in BagMixer 400 (Interscience, France). A series of 10-fold dilution (10^{-2} to 10^{-8}) were made with the same sterile diluent. From each dilution, 0.1 mL aliquots were spread onto selective McConkey agar plates (Merck, Germany) for coliforms and Rogosa agar plates (Biokar Diagnostics, France) for lactobacilli. The plates for lactobacilli culturing were maintained under anaerobic conditions (BD GasPak, Becton, Dickinson and Company, USA) and incubating at 37°C for 48 h. Plate 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 feces.

Measurement of caecal β-glucuronidase activity. The activity of β — glucuronidase (β-GLUCUR) bacterial enzyme was measured in fresh caecal digesta taken after completion of the experiment by determining the rate of *p*- or *o*-nitrophenol as previously described by the Juskiewicz and coworkers (2002). The reaction contained 0.3 mL a substrate solution (5 mM) *p*-nitrophenyl-β-D-glucuronide for β-glucuronidase (Sigma Aldrich, USA) and 0.2 mL of 1:10 (v/v) dilution of the caecal digesta in 100 mM phosphate buffer (pH 7.0) centrifuged at $10\,000 \times g$ for 15 min at 4°C. Incubation was carried out at 37°C for 10 min, and *p*- or *o*-nitrophenol was quantified after addition of 0.25 M cold sodium carbonate and measured absorbation at 400 nm. A measurement unit of enzymatic activity is expressed as μmol of *p*–nitrophenol per min per gram digesta.

Assessment of cytokine levels (IL-6, IL-8), and NFκB in serum and colon homogenates. Samples of blood was to clot for two hours at room temperature before centrifugation for 15 mimutes at $1\,000 \times g$, remove serum samples were store –20°C. Colon tissue samples were rinsed in ice cold PBS (pH 7.0–7.2) to remove excess blood thoroughly, cut longitudinally and homogenized in PBS with a homogenizer on ice (Disperser T10 Basic Ultra Turrax, Germany) and storage overnight at –20°C. After 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 2–8°C, supernatant was removed and store at –20°C. All enpoints were measure by ELISA method as follows: NFkB in tissue by USCN Life Science, Inc., USA; IL-6 in blood and tissue by eBioscience, USA; IL-8 in tissue by Cusabio Biotech Co., Ltd. China. The final values of each parameter were measured on the Synergy H4 multiplate reader (BioTek Instruments, Inc. USA).

Histopathological examinations. The distal portion of the colon was removed, fixed in 10% buffered formalin and embedded in paraffin. Tissue sections (about 5 μm) were prepared, deparaffinized, dehydrated, stained with haematoxylin and eosin (H&E, ×400), and

Table 1. Disease activity index of acute ulcerative colitis

Score	Stool consistency	Bleeding	Weight loss	Maximum score
0	Formed	Normal color stool	No weight loss	10
1	Mild soft	Brown color stool	5–10% weight loss	
2	Very soft	Reddish color stool	11–15% weight loss	
3	Watery stool	Bloody stool	16–20% weight loss	
4			>20% weight loss	

Table 2. Activity of β-glucuronidase and total counts of lactobacilli and coliform

Parameters	CG	AC	AC+PRE	AC+PRO
β-GLUCUR μmol/min/g	0.14±0.02	0.54±0.05 ***	0.49±0.18	0.39±0.18 +
Lactobacilli $\log_{10}$CFU/g	7.78±0.17	7.15±0.90 *	7.31±0.45	7.40±0.44
Coliforms $\log_{10}$CFU/g	5.18±0.56	5.74±1.03	5.51±0.77	5.19±0.57

Data represent mean ±standard deviation. Statistical significance is between *CG/AC and + AC/AC+PRE or AC/AC+PRO: */+P<0.05; ***P<0.001

Table 3. Serum and tissue changes in cytokine levels and NFkB activities

Parameters	CG	AC	AC+PRE	AC+PRO
NFκB t ng/mL	41.73±7.41	60.21±10.31***	11.65±1.32+++	50.52±17.56
IL-6 s pg/mL	49.31±15.83	61.89±15.33	45.83±9.99++	43.00±6.09+++
IL-6 t pg/mL	47.00±8.53	62.65±12.19**	48.77±5.31++	41.27±14.88+++
IL-8 t pg/mL	37.78±7.42	50.12±7.32***	47.67±8.49+	39.34±5.39+++

Data represent mean ±standard deviation. Statistical significance is between *CG/AC and +AC/AC+PRE or AC/AC+PRO: +P<0.05; **/++ P<0.01; ***/+++P<0.001

examined under light microscopy (Olympus, Japan). The histopathological study was performed to evaluate the progression of mucosal injury. The severity of mucosal injury was graded according to (Cooper *et al.*, 1993) as follows: grade 0, intact crypt; grade 1, loss of the one–third of the crypt; grade 2, loss of the basal two thirds of the crypt; grade 3, loss of the entire crypt with the surface epithelial cells remaining intact; grade 4, loss of both-the entire crypt and surface epithelial cells (erosion). The histological index should be calculated as the mean of the scores of each section of the entire colon. Apart from the scoring system, histopathological findings/throughout description was evaluated.

Statistical analysis. The data are presented as mean ± standard deviation (S.D.). Statistical analysis was performed by Student's *t*-test and analysis of variance (ANOVA) to determine the significance. A values P<0.05 were considered to be statistically significant.

RESULTS

During the experimental trial, clinical changes observed in rats did not lead to death. The mean body weight of the rats at the beginning of the experiment and at the end of the experiment in CG was increased by 30.92% (242.50 g *vs.* 317.50 g), in AC by 9.90% (252.50 g *vs* 277.5 g), in AC+PRE by 14.28% (253.75 g *vs* 290.0 g), and in AC+PRO by 21.74% (258.75 g *vs* 315.0 g). The average value of all daily disease activity index score showed that the maximum DAI score (6.5) was in the AC group. The decline DAI score was observed after application of prebiotic inulin (4.13) and probiotic *Lactobacillus plantarum* LS/07 (3.5). Applicated DSS significantly increased activity of β-glucuronidase (P<0.001) in comparison to control group, applicated probiotic and prebiotic alleviate activity of this enzyme probiotic significantly (P<0.05) and prebiotic non-significantly as shown Table 2. Changes of total counts of coliform and lactobacilli in different experimental group are summarized in Table 2. In Table 3 are shown changes in cytokines levels and NFκB activities in serum (s) and tissue (t) in control group, acute colitis group and in treated groups with inulin and probiotic. Mucosal damage was evaluated by the histological index and histological grading method. The highest histological index was found in the AC group (3.38), in AC+PRO group with applicated *Lactobacillus plantarum* LS/07 was 2.42 and in AC+PRE group with inulin was 3.12. Histological indexes in experimental groups corresponded histopathological changes shown in (Fig. 1).

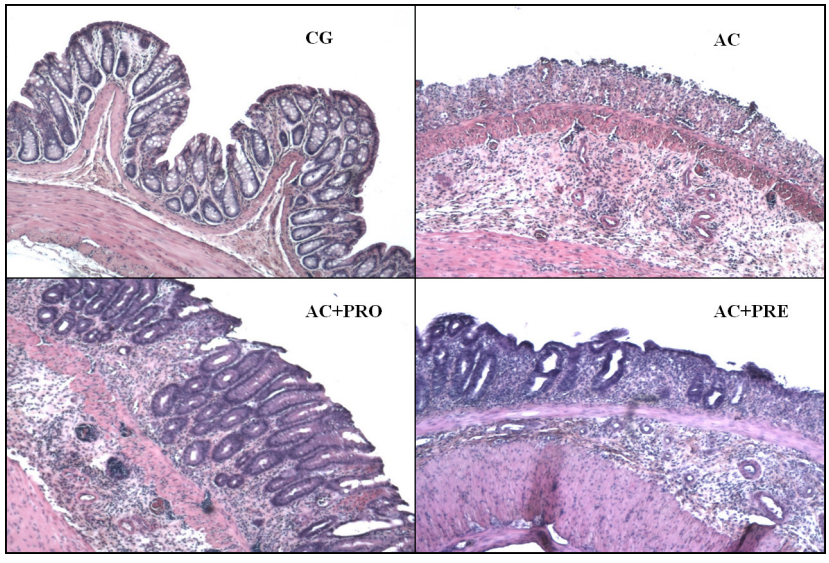

Figure 1. Histopathological changes.
(CG) intact crypts. (**AC**) no epithelial lining, no crypts visible. Tunica mucosa is completely disintegrated, *lamina propria mucosae* is infiltrated by lymphoid tissue (neutrophils, plasma cells, macrophages), distinguishable necrosis of the mucosa is present. (**AC+PRO**) epithelial lining is discontinuous, partly disintegrated. In *lamina propria mucosae* inflammatory infiltration is visible. Visible cell erosion in the crypts is present. (**AC+PRE**) complete disintegration of the epithelial lining. Crypts are present; however epithelial lining of the crypt is disturbed.

DISCUSSION

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders that affect individuals throughout life. The role of interactions between genetic, immunologic, microbial and environmental factors is expected in UC, but exact etiology and pathogenesis still remains unclear. Long-standing UC has an increased risk to devel-

op of CAC/CRC (colon carcinogenesis), (Danese *et al.,* 2011). It was also widely accepted that chronic inflammation promotes carcinogenesis by inducing production of a variety of cytokines and chemokines that propagate a localized inflammatory response by activating transcription factors such as NF-ϰB which is accompanied by increased COX-2, iNOS and pro-inflammatory cytokines. These finding have important implications for the possible development of anticancer therapies and offer an opportunity to devise strategies which support opinion that probiotics and prebiotics can provide an alternative or adjuvant approach to conventional therapy by altering the intestinal microflora and modulating the host immune system. The goal of the this study was to obtain informations about the effect of prebiotic inulin and probiotic *Lactobacillus plantarum* LS/07 administered in DSS-induced acute colitis rat model.

Probiotics are defined as living food supplements or components of bacteria that have been shown beneficial effects on human health, and in recent years there has been observed an increased interest for their use in inflammatory bowel disease due to the microbiome role in IBD pathogenesis (Miele *et al.,* 2009; Mack, 2011). The categories of probiotics used today include: bacteria such as lactic acid bacteria (LAB) and *Escherichia coli* strains (such as *E. coli* Nissle 1917, which is one of the few examples of non-LAB probiotic), as well as yeast species including most predominantly *Saccharomyces boulardii* among others. Prebiotics are a family of molecules which meet the three classification criteria for being considered a prebiotic, as defined Gibson and Roberfroid (1995); i.e. resistance to hydrolysis or absorption in the upper gastrointestinal tract, fermentation by the intestinal microbiota, and selective stimulation of the growth and/or activity of beneficial intestinal bacteria, such as *Lactobacillus* species and *Bifidobacterium* species which have positive impact on the health of the host was shown by authors (Gibson *et al.,* 2004; Guarner, 2007; Looijer-van Langen & Dieleman, 2009).

In our study DSS-induced acute colitis elevated (non significantly) the number of coliforms and significantly (P<0.05) decreased the number of lactobacilli in AC group in compare to control group. Within a short experimental period preventive dietary supplementation with inulin and probiotic positively modified values observed microorganisms and activity of β-glucuronidase. Unlike the lactobacilli and coliforms, the activity of β-GLUCUR is believed to be a biomarker of neoplasm and is also perceived as harmful due to the associated release of carcinogens from hepatically derived glucuronic acid conjugates was significantly increased (P<0.001) in AC group. *Lactobacillus plantarum* LS/07 positively reduced activity of β-GLUCUR (P<0.05).

Chronic inflammation is known to lead to derangement in signaling 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 incompletely understood (Nathan & Ding, 2010). Of the signaling pathways involved in colonic inflammation that triggered by NFϰB plays a key role. NFϰB regulates the expression of various cytokines and modulates the inflammatory processes characteristic of IBD (Schottelius & Dinter, 2006; Toumi *et al.,* 2014). Further, NFϰB controls apoptosis, cell-cycle progression, cell proliferation, and differentiation. Although NFϰB activation has been shown to be involved in CRC development, normally functioning NFϰB is essential for maintenance of epithelial cell homeostasis in gut. Translocation of activated NFϰB into the nucleus induces the expression of cytokines such as TNFα and IL-6, and chemokines, all of which contribute to development of inflammation-related tissue damage. The activity of NFϰB in colon tissue samples was markedly increased in AC groups (P<0.001) and may provide a sensitive means of assessing the state of activation of the mucosal immune response. Inulin treatment suppresed activity of critical transcription factor in mucosa cells (P<0.001). Activated NFϰB in acute colitis group significantly activated serum a colon tissue levels of pro-inflammatory cytokine (IL-6 and IL-8) compared with control group. Inulin diet intervention in AC+PRE group statistically significantly down regulate of synthesis of proinflammatory cytokines IL-6 (P<0.01) in serum and colon tissue and IL-8 (P<0.05) in tissue compared to acute colitis group. *Lactobacillus plantarum* LS/07 nonsignificantly suppresed activity of NFϰB, but down regulate of synthesis of IL-6 and IL-8 (P<0.001) compared to AC group.

The results of the present experiment demonstrate the ability of elected possible food supplements, prebiotic inulin and probiotic *Lactobacillus plantarum* LS/07 to intervene and affect the pathophysiological process development of acute colitis. The exact etiology and pathogenesis of ulcerative colitis is not yet known, and in that regard from the use of prebiotics and probiotics suitable form of prevention of acute colitis.

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