

## Effect of *Lactobacillus plantarum* LS/07 in combination with flaxseed oil on the microflora, enzymatic activity, and histological changes in the development of chemically induced precancerous growth in the rat colon

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**ABSTRACT:** This experimental study showed the effect of *L. plantarum* LS/07 on the changes in total counts of coliform bacteria, enzymatic activities, cytokine and histological changes in the colonic mucosa, chemically induced by the application of 1.2-dimethylhydrazine dihydrochloride (1.2-DMH) in rats once a week during five weeks in the positive control group, probioticum (PRO) group, and probioticum and oil (PRO-O) group. A total of 32 rats were randomly assigned to four treatment groups: negative control group (diet without any supplements), positive control group (1.2-DMH without any supplements), PRO group (*L. plantarum* LS/07 at the dose (average concentration  $2.88 \times 10^9$  CFU/ml) + 1.2-DMH), PRO-O group (*L. plantarum* LS/07 + flaxseed oil (40 g/kg feed) + 1.2-DMH). All rats were kept on fat diet (35 g/kg). The results showed a significant ( $P < 0.05$ ) decrease in total counts of coliform bacteria in PRO-O group. Results of enzymatic activities of isolated strain showed that *L. plantarum* LS/07 does not produce enzyme  $\beta$ -glucuronidase. Significantly higher activity of  $\beta$ -galactosidase was in the groups PRO ( $P < 0.001$ ) and PRO-O ( $P < 0.01$ ). On the other hand, significantly lower ( $P < 0.001$ ) enzymatic activity of  $\beta$ -glucuronidase and  $\beta$ -glucosidase was found in PRO-O group compared with positive control group. The cytokine activity of tumor necrosis factor (TNF $\alpha$ ) was significantly decreased in groups PRO ( $P < 0.001$ ) and PRO-O ( $P < 0.01$ ) compared to the positive control group. The histological examination showed different changes (aberrant crypts and goblet cells) in experimental groups in comparison to the negative control group. Our study indicates that *L. plantarum* LS/07 in combination with flaxseed oil had a positive influence on colon microflora, enzymatic activities, and cytokine activity. The total results showed that potentiating effects of natural components were markedly achieved in the experimental group PRO-O.

**Keywords:** probiotic; PUFA; 1.2-DMH; coliform bacteria;  $\beta$ -glucuronidase;  $\beta$ -glucosidase;  $\beta$ -galactosidase; TNF $\alpha$ ; histology; rats

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## INTRODUCTION

Gastrointestinal tracts (GIT) in humans and animals are inhabited by a variety of microbiota. The large intestine hosts the largest microbial population of the body ( $10^{10}$ – $10^{12}$  CFU/ml) (DiBaise et al., 2008). Physiological balance of this microbiota is greatly influenced by intestinal environment. The intestinal microbiota plays an important role in the development of the gut immune system, digestion of food, production of short-chain fatty acids and essential vitamins, and resistance to colonization from pathogenic microorganisms (Hooper and Gordon, 2001).

Disrupting this balance in the gastrointestinal tract (stress, infection, antibiotic therapy) can lead to proliferation of undesirable or pathogenic microbes and increased risk of clinical disorders such as inflammatory diseases, infectious illnesses, and others (Brown and Valiere, 2004). The broad variety of bacteria in the gut produces diverse and often physiologically active metabolites that influence the normal development and function of the host (Commane et al., 2005).

A large number of xenobiotic substances taken orally among human and animal may act as mutagenic compounds resulting in a change of native microbiota of intestine and could induce cancer. Epidemiological studies show that colon cancer has especially high incidence in the developed countries of the Western world (Ferlay et al., 2001). The EUROPREVAL project estimates a lifetime risk for colorectal cancer development for 2% of the European population (Capocaccia et al., 2002). While this may be a reason in part related to a genetic susceptibility. The high-fat low-fibre diet is typical of Western culture (Kiss et al., 2000).

Among the numerous intestinal bacteria that beneficially affect the most of intestine, some could be recognized as probiotics (Ishibashi and Yamazaki, 2001). Probiotic is a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). To date, experimental evidence for anticarcinogenic activity of probiotics comes primarily from *in vitro* studies of antigenotoxic effects and *in vivo* work, showing the suppression of pre-neoplastic lesions and chemically induced colon tumors in rodent models (Burns and Rowland, 2000).

By the targeted application of probiotics alone or in combination with natural components we can

influence quantitative and qualitative representation of colon microbiota. By application of new molecular techniques that allow us to explore the mechanisms of action of probiotics, the definition of probiotics can be specified. Probiotics are live microorganisms which modulate the specific function of organism by activation of specific molecular pathways (Bomba et al., 2012).

Flaxseed, or its extracted oil exert anticarcinogenic effects in some *in vitro* and *in vivo* experiments, and related extracts also play an important dietary role in various biological activities in the body (Basch et al., 2007). Flaxseed is the richest plant source of omega 3 fatty acid ( $\alpha$ -linolenic acid) and the phytohormone lignans. The positive effect of some polyunsaturated fatty acids (PUFA) in diet plays an important role in the metabolism of human and animal colonic function (Topping and Clifton, 2001). PUFA affect the attachment sites for the intestinal microbiota, possibly by modifying the fatty acids composition of intestinal wall. PUFA can modify bacterial adhesion, especially of lactic acid bacteria, to the gastrointestinal mucosa and increase counts of B lymphocytes in the peripheral blood (Kankaanpää et al., 2001; Kaštel et al., 2003).

The aim of our study was to determine the effect of orally administered probiotic strain *L. plantarum* LS/07 in pasteurized milk, alone or in combination with flaxseed oil, on selected microbial, enzymatic, immunological, and histological changes in 1.2-dimethylhydrazine dihydrochloride (1.2-DMH) exposed rat colon. We focused on changes in the total counts of coliform bacteria, enzymatic activity of  $\beta$ -glucuronidase,  $\beta$ -glucosidase,  $\beta$ -galactosidase in colon, cytokine TNF $\alpha$  activity in jejunal mucosa, and on the studies of histological changes in colon epithelium of rats.

## MATERIAL AND METHODS

The experiment was conducted in accordance with the principles outlined in Law No. 23/2009 of the Slovak Republic for the Care and Use of Laboratory Animals, and was approved by the Ethical Committee of the Faculty of Medicine of P.J. Šafárik University in Košice. The rats were housed in plastic cages in central vivarium. The clinical status of the rats, consumption of feed and water as well as temperature were monitored daily. In this experiment 32 rats (Sprague Dawley) at the age of 8 weeks were used. They were randomly

Table 1. Experimental design

Group	Diet	Procarcinogen
Negative control 1	CD	–
Positive control 2	CD	1.2-DMH
PRO	CD + <i>L. plantarum</i>	1.2-DMH
PRO-O	CD + <i>L. plantarum</i> + flaxseed oil (40 g/kg)	1.2-DMH

1.2-DMH = 1.2-dimethylhydrazine dihydrochloride, CD = conventional diet

divided into 4 groups per 8 animals. The experiment lasted for 24 weeks. The experimental design is presented in Table 1. During the experiment the rats received conventional diet in the form of pellets each day. Sunflower oil commercially available for rats as a basic diet was supplied in feed at the total concentrations of 35 g/kg.

The composition of the diet is given in Table 2. Flaxseed oil (*Linum usitatissimum* L.) at the dose of 40 g/kg feed, containing a high amount of polyunsaturated fatty acids (omega-3 PUFA) was added into the diet of group PRO-O (Table 3). The oil was purchased from Dr. Kulich Pharma, Hradec Králové, Czech Republic.

**Microbiological assays.** The strain *Lactobacillus plantarum* LS/07 was isolated from rectal swabs of healthy man. The rectal swab was diluted with physiological solution (100 µl each) and was spread-plated on lactobacilli selective LS agar (Imuna-Pharm, a.s., Šarišské Michal'any, Slovak Republic). The isolated lactobacilli strains were randomly selected and Gram stained and visualized under a microscope for morphological characterization. The gram-positive and catalase-negative lactobacilli strains were tested for the ability to

Table 2. Ingredients and chemical composition (g/kg dry matter) of the basal diet

Parameters	Quantity
Dry matter	880 g/kg
Crude protein	180 g/kg
Lysine	6 g/kg
Ca	15 g/kg
P	8 g/kg
Na	2 g/kg
Sunflower oil	35 g/kg
Vitamin A	8 000 m.j.
Vitamin B	4 mg/kg

inhibit the growth of *Escherichia coli* Serotype 06:K2:H1 (ATC1938, CNTC Eck 63/59 Czech collection of microorganisms) using the agar diffuse test. One isolated strain exhibited the best ability to create inhibited zones and was subjected to the molecular DNA detection (unpublished data). *L. plantarum* was identified as the most suitable strain by molecular genetics method of polymerase chain reaction (PCR) using specific primers 16S rRNA (5'-GCTGGATCACCTCCTTTC-3') forward, 23S rRNA (5'-ATGAGGTATTCAACTTATG-3') reverse, according to Berthier and Ehrlich (1998). The enzymatic activities of the isolated strain *L. plantarum* were determined using an API ZYM kit (Biomérieux, Lyon, France) according to the manufacturer's instructions and expressed on the scale of 0 (negative reaction) to 5 (maximum activity). The isolated strain did not produce β-glucuronidase enzyme.

At the beginning of the trial 1 ml MRS broth (Merck, Darmstadt, Germany) of *L. plantarum* (average concentration  $2.88 \times 10^9$  CFU/ml) was added to 39 ml of pasteurized milk (0.5% fat). The total volume (40 ml) of milk was given to four rats. The milk was poured into screw-capped bottles and was administered to PRO and PRO-O groups of rats every day prior to feeding. The last 24 h before finishing the experimental trial the rats were given no milk. Water was available for *ad libitum* intake.

**Application of 1.2-dimethylhydrazine dihydrochloride.** Two weeks after beginning the trial, positive control, PRO, and PRO-O groups of rats were treated with 1.2-DMH (Merck) at the dose of 21 mg/kg subcutaneously once a week during five weeks. The rats were anaesthetized by the solution of Ketamine (Gedeon Richter Plc, Budapest, Hungary) (100 mg/kg) and were sacrificed by the solution of Xylazine (Bioveta, Ivanovice Na Hané, Czech Republic) (15 mg/kg body weight) applied intraperitoneally after 24 weeks of the experiment.

Table 3. Fatty acid profile of flaxseed oil

Analyzed acids	Contents (%)
Palmitic	6.2
Palmitooleic	0.1
Stearic	3.8
Oleic	19.8
Linolenic	16.5
Arachidic	0.1

**Preparation of colon samples.** Fresh samples of the intestinal content from colon were collected from all experimental rats after death. Samples (1 g) were put into sterile plastic polyethylene Stomacher Lab Blender bags (Interscience, Mourjou, France) with sterile diluents (9 ml) of Ringer's solution and mixed in Stomacher 400 Bag mixer (Interscience). The series of 10-fold dilutions (from  $10^{-2}$  to  $10^{-8}$ ) were made in the same sterile diluents. The dilutions (100  $\mu$ l each) were spread-plated onto selective MacConkey agars (Merck) for coliform bacteria. The plates for coliform bacteria were incubated aerobically at 37°C for 24. The viable counts were expressed as the log of colony-forming units per g (CFU/g) of the wet colon content.

**Evaluation of bacterial enzymes activity.** Bacterial enzymes activity of colon content was measured by the rate of release of *p*- or *o*-nitrophenol from their nitrophenyl glucosides according to the modified method of Djouzi and Andrieux (1997) described in details by Juskiwicz et al. (2002). A measurement unit of bacterial activity was  $\mu$ mol of *p*-nitrophenol/min/g of colon content.

**Evaluation of interleukin activity.** Mucous samples were prepared according to the method by Doligalska et al. (2006) by scraping the inner layer of the first 10 cm of the small intestine with a glass microscope slide. The samples were diluted, homogenized in glass in 1 ml of cold 0.1M PBS (pH 7.4), containing inhibitor cocktails tablets (Complete, Mini, and Roche) and centrifuged at 10 000 g for 30 min. The supernatant was stored at  $-80^{\circ}\text{C}$ .

The concentration of proinflammatory cytokine TNF $\alpha$  was measured in homogenisate of jejunal mucosa by ELISA kit (RayBiotech Inc., Norcross, USA), according to manufacturer's instructions. The samples of mucosa (50 mg) were diluted with 2 ml of cold PBS (pH 7.4) and centrifuged at 15 000 g at 4°C for 15 min. The supernatant and serum were stored at  $-20^{\circ}\text{C}$  until cytokines measurements. The plates were read at 450 nm

by using the ELX 808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, USA).

**Histological and pathological evaluation of precancerous changes.** At the end of the experiment, the colons were immediately removed from their proximal end to the beginning of the rectum of each animal by ventral incision. The biopsy samples from colon were fixed in neutral 10% formalin and embedded in paraffin blocks. Paraffin blocks were sectioned and stained by hematoxylin-eosin and alcian blue (alcian blue with safranin). After staining and using light microscope OLYMPUS BX 43 (OLYMPUS Corp., Tokyo, Japan) at magnifications 40 $\times$ , 100 $\times$ , 200 $\times$ , histopathological findings on the degree of mononuclear cells infiltration and hyperplasia of crypts were investigated. The sections coloured by alcian blue were stained and total counts of goblet cells were performed.

The total numbers of goblet cells were determined in the proximal and distal colon in crypts at 400 $\times$  magnification, field of view area 0.238 mm $^2$ . Goblet cells in ten fields of view were calculated and average numbers of goblet cells were recalculated on the surface of 1 mm $^2$  of tissue column.

**Statistical analyses.** The results were statistically analyzed using a multifactorial Analysis of Variance (ANOVA). Duncan's multiple-range test was then performed to identify significant differences between the mean values of each treatment group.

## RESULTS AND DISCUSSION

**Weight of experimental rats.** During the experimental trial, no clinical toxicity and subsequent mortality were observed in the rats. The mean body weight of the rats at the beginning of the experiment and at the end of the experiment is documented in Table 4.

The statistical changes in body weight during the experiment ( $P < 0.05$ ) were observed in groups PRO and PRO-O as compared to positive control

Table 4. Mean body weight of rats fed different dietary treatment

	Negative control	Positive control	PRO	PRO-O
Initial BW (g)	289.38 $\pm$ 17.87	286.88 $\pm$ 23.17	329.38 $\pm$ 28.18	294.00 $\pm$ 22.83
Final BW (g)	316.25 $\pm$ 33.52	281.25 $\pm$ 29.06	343.13 $\pm$ 26.41*	327.50 $\pm$ 15.67*

BW = body weight, 1,2-DMH = 1,2-dimethylhydrazine dihydrochloride, negative control = without 1,2-DMH, positive control = with 1,2-DMH, PRO = *L. plantarum* with 1,2-DMH, PRO-O = *L. plantarum* and flaxseed oil with 1,2-DMH

\*data are expressed as Least Squares Means (LSM) with pooled SEM ( $n = 8$ ); LSM values without a common superscript differ ( $P < 0.05$ )

group. The mean body weight was increased by 8.5% in negative control group, by 4.0% in PRO group, and by 10.2% in PRO-O group. In positive control group, the mean body weight was decreased by 1.96%. Food consumption was changed proportionally to the body weight of rats.

**Counts of coliform bacteria.** As far as the total number of coliform bacteria is concerned, we recorded decreased values in PRO group as compared to positive control group. However, significant reduction in the number of coliform bacteria was found in PRO-O group in comparison to positive control group (Table 5).

In our experiment we found statistical decrease ( $P < 0.05$ ) in total count of coliform bacteria in the colon content in PRO-O group of rats. In this group, *L. plantarum* in combination with flaxseed oil was administered. We assume that the counts of coliform bacteria were affected by addition of probioticum and PUFA into diet. Interaction between probiotic microorganisms and PUFA in flaxseed oil clearly enhances antimicrobial properties of probiotic pathogen bacteria. However, similar effect of *L. acidophilus* KFRI 342 (isolated from Korean traditional food kimchi) on reducing the total count of *E. coli* bacteria was observed in the experimental study of 1.2-DMH induced precancerous growths in the colon of rats (Chang et al., 2012).

*E. coli* is predominant microorganism in the initiation of early and chronic ileal lesions of ulcerative colitis, Crohn's disease, and colorectal carcinoma. These strains belong to a potentially pathogenic group of invasive *E. coli*, which was designated as a group of adherent-invasive *E. coli* (Havrišová et al., 2006). PUFA have a specific role

in the elimination of pathogenic bacteria. It is based on potentiating the effects of probiotics. They act on the size of bacterial colonies and adhesive properties of probiotic bacteria. It is manifested by an increase in their number, as well as improved adhesion to epithelial cells.

Many similar experiments were carried out in other animals, e.g. pigs. The effect of *L. plantarum* (isolated from pig) in combination with flaxseed oil was manifested in the adhesion of *L. plantarum* LP-96 Biocenol™ to the intestinal mucosa and in the inhibitory effect on *E. coli* 08:K88ab:H9 in gnotobiotic pigs. The adhesion of *L. plantarum* to jejunal and ileal mucosa as well as the decrease of total count of *E. coli* K88 in the jejunal intestinal content were markedly promoted by flaxseed oil (Nemcová et al., 2012).

The results of this study confirmed the influence of PUFA on lactobacilli adhesion to the jejunal mucosa and on the immunity of gnotobiotic piglets (Bomba et al., 2003).

Kastel' et al. (2007) observed the effect of diets with high content of omega-3 fatty acids (omega-6 : omega-3 – 1 : 10) in combination with *L. casei* subsp. *casei* on adherence properties of probiotics, intestinal metabolisms, and plasma lipid metabolisms in pigs. Differences in colonization of lactobacilli were most prominent at the jejunal mucosa, where their numbers have greatly been increased by PUFA administration compared to the control group.

**Enzymatic study.** The microbial enzymes in gastrointestinal tract play a significant role in the etiology of colon cancer (George et al., 2004). The concentration of ammonia, a putative tumor promoter produced by bacterial degradation of protein and urea, and the activities of certain bacterial enzymes are thought to play a role in colon carcinogenesis,  $\beta$ -glucuronidase and  $\beta$ -glucosidase were also assayed (Rowland et al., 1998).  $\beta$ -glucuronidase activity is perceived as harmful for health as it is able to release carcinogens from hepatically derived glucuronic acid conjugates and it is a critical factor in the enterohepatic circulation of drugs and other foreign compounds (Salminen et al., 1998). The activity of  $\beta$ -glucosidase contributes to the hydrolysis of glucose monomers from nonstarch polysaccharides (e.g. cellulose,  $\beta$ -glucans), but it is also possible for  $\beta$ -glucosidase to be involved in the formation of toxic aglycons from plant glucosides (Pool-Zobel et al., 2002).

In our experimental study, enzymes  $\beta$ -glucuronidase and  $\beta$ -glucosidase were significantly increased in positive control group in comparison to

Table 5. Counts of coliform bacteria in the colon of rats fed different dietary treatment

Experimental groups	Coliform bacteria ( $\log_{10}$ CFU/g)
Negative control	5.42 $\pm$ 0.21
Positive control	5.45 $\pm$ 0.11
PRO	4.96 $\pm$ 0.32
PRO-O	4.81 $\pm$ 0.22*

1.2-DMH = 1.2-dimethylhydrazine dihydrochloride, negative control = without 1.2-DMH, positive control = with 1.2-DMH, PRO = *L. plantarum* with 1.2-DMH, PRO-O = *L. plantarum* and flaxseed oil with 1.2-DMH

\*data are expressed as Least Squares Means (LSM) with pooled SEM ( $n = 8$ ); LSM values without a common superscript differ ( $P < 0.05$ )

Table 6. Activity of selected bacterial enzymes ( $\mu\text{mol/g/min}$ ) in the colon of rats fed different dietary treatment

Experimental groups	$\beta$ -glucuronidase	$\beta$ -glucosidase	$\beta$ -galactosidase
Negative control	0.10 $\pm$ 0.01	0.03 $\pm$ 0.01	0.08 $\pm$ 0.01
Positive control	0.28 $\pm$ 0.02**	0.015 $\pm$ 0.02**	0.07 $\pm$ 0.01
PRO	0.20 $\pm$ 0.02	0.12 $\pm$ 0.01	0.62 $\pm$ 0.12**
PRO-O	0.08 $\pm$ 0.01**	0.06 $\pm$ 0.00*	0.37 $\pm$ 0.02*

1.2-DMH = 1.2-dimethylhydrazine dihydrochloride, negative control = without 1.2-DMH, positive control = with 1.2-DMH, PRO = *L. plantarum* with 1.2-DMH, PRO-O = *L. plantarum* and flaxseed oil with 1.2-DMH data are expressed as Least Squares Means (LSM) with pooled SEM ( $n = 8$ ); LSM values without a common superscript differ (\* $P < 0.01$ , \*\* $P < 0.001$ )

negative control group after application of 1.2-DMH. After administration of *L. plantarum* and flaxseed oil, significantly low values of intestinal enzymes ( $\beta$ -glucuronidase,  $\beta$ -glucosidase) were recorded in PRO-O group as compared to positive control group. These changes may have been due to the reduction in coliform bacteria in the colonic content. The obtained results are documented in Table 6.

Similarly significant decrease of enzyme activities of  $\beta$ -glucuronidase and  $\beta$ -glucosidase of colon content at 1.2-DMH rats after administration of *L. acidophilus* KFRI342 was recorded by Chang et al. (2012).

Our results documented that activities of  $\beta$ -galactosidase were significantly increased in PRO and PRO-O groups, in comparison to positive control group, but the highest enzyme activities were observed in PRO group. We believe that high levels of this enzyme were induced by the high dose of *L. plantarum* that we applied every day throughout the experiment. Our *in vitro* test showed that *L. plantarum* LS/07 also produced enzyme  $\beta$ -galactosidase. This enzyme is mainly produced by bifidobacteria and lactobacilli and its increase in large intestine substantiates the stimulatory effect of inulin on lactic acid bacteria (Lay et al., 2004).

**Immunological parameters.** The potential mechanisms of probiotic-induced immune suppression of carcinogenesis are a complex involving many factors: development of immune system cells, regulatory mechanisms of proliferation of tumor cells population, affecting tumor growth, angiogenesis, and formation of metastases. TNF $\alpha$  is a key cytokine that is involved in the regulation of cytokines during inflammatory responses (de Visser et al., 2006). Increasing the concentration of cytokine TNF $\alpha$  creates the conditions for chronic inflammation, which is one of the predisposing factors of colorectal cancer (CRC). The primary effect of the administered substances was reflected

in the small intestine, where these kinds of substances come into contact with the immune cells of lymphoid tissue of the small intestine.

Our results documented that 1.2-DMH applications caused significant ( $P < 0.001$ ) increase in the levels of TNF $\alpha$  in positive control in jejunal mucosa as compared to negative control. After applications of *L. plantarum* in PRO group statistically lower levels of TNF $\alpha$  ( $P < 0.001$ ) than in positive control were found. Statistically lower ( $P < 0.01$ ) levels of TNF $\alpha$  were recorded in PRO-O group as compared to positive control with 1.2-DMH. The achieved results showed a positive effect of reducing the level of TNF $\alpha$  in the jejunal mucosa after application of *L. plantarum* alone or in combination with flaxseed oil.

Results of our study documented that 1.2-DMH applications significantly affected the concentration of the measured cytokine. TNF $\alpha$  in jejunal mucosa in positive control group was compared to PRO and PRO-O groups. The best effect was observed in PRO group where TNF $\alpha$  level was markedly low in mucosa in comparison to positive control group.

Table 7. Concentration of cytokine TNF $\alpha$  in jejunal mucosa of rats fed different dietary treatment

Experimental groups	TNF $\alpha$ (pg/ml)
Negative control	10 780.79 $\pm$ 695.73
Positive control	15 727.43 $\pm$ 366.00**
PRO	8 379.83 $\pm$ 504.06**
PRO-O	9 921.47 $\pm$ 689.85*

TNF $\alpha$  = tumor necrosis factor, 1.2-DMH = 1.2-dimethylhydrazine dihydrochloride, negative control = without 1.2-DMH, positive control = with 1.2-DMH, PRO = *L. plantarum* with 1.2-DMH, PRO-O = *L. plantarum* and flaxseed oil with 1.2-DMH data are expressed as Least Squares Means (LSM) with pooled SEM ( $n = 8$ ); LSM values without a common superscript differ (\* $P < 0.01$ , \*\* $P < 0.001$ )

Changes of cytokine TNF $\alpha$  during the experiment are presented in Table 7. The results show that long-term consumption of *L. plantarum* administered alone and in combination with flaxseed oil has the effect on production of TNF $\alpha$  at local levels.

In the previous study (Žofčáková et al., 2012) the effect of *L. plantarum* and inulin on immunological parameters was evaluated. Application of 1.2-DMH had strong stimulating effect on the production of interleukins IL-6 and IL-17 and inhibited production of IL-10. Administration of *L. plantarum* and inulin reduced inflammatory process in the jejunal mucosa by inhibiting the production of IL-6 and IL-17 and stimulation of IL-17.

**Histological results.** It appears that probiotics with or without prebiotics have an inhibitory effect on development of aberrant crypts (precancerous lesions) and tumors in animal models (Brady et al., 2000).

A potent procarcinogen administered in the present study is 1.2-DMH, which is a widely accepted standard in *in vivo* rodent models (Commane et al., 2005). DMH and its metabolite azoxymethan (AOM) are widely used agents for the induction of colorectal carcinogenesis in rodents. DMH is metabolically activated in the liver by a series of reactions through intermediates AOM and methylazoxymethanol (MAM) to the ultimate carcinogenic metabolite highly reactive methyl-diazonium ion. MAM can be secreted into the bile and transported to the colon (the development of small intestinal tumors distal to the entrance of the bile duct into the intestine is ascribed to this

path) or can enter directly the epithelial cells of the colon from blood circulation. It is widely accepted today that the adenoma to carcinoma sequence is characterized by recognizable histological changes that start with dysplastic aberrant crypts or intraepithelial neoplasia (Mori et al., 2005).

No histopathological changes were observed in the negative control group of rats (without 1.2-DMH) after administration of conventional laboratory diets. Epithelium showed no histological differences. Epithelial cells produced sufficient deep crypts with a lot of goblet cells (Figure 1). On the other hand, we found inflammation in the column submucosa of a varying intensity, in the positive control group with 1.2-DMH, PRO group, and PRO-O group, after applications of procarcinogen. Application of these substances in the positive control group caused the presence of aberrant crypts in the colon of rats (Figure 3).

In the distal part of the colon in positive control, we observed a strong non-specific chronic inflammation with marked lymphostasis (Figure 2). The structure of crypts was disrupted. In the tissue, solitary lymphatic follicle surrounding fragments crypts with their immersion into lymphoid tissue (cavitations of crypts) were found. These crypts did not contain goblet cells.

Goblet cells play an important role in the development of tumors in the colon, in the form of aberrant crypts in the early preneoplastic lesions in mice with inherited mutation of the *APC* gene, as well as in people with an increased risk of tumors. In all cases, representations of goblet cells



Figure 1. Colonic tissue without histopathological changes in the control negative 1

tissues: *tunica mucosa* (1), *tunica submucosa* (2), *tunica muscularis* (3), lymphoid follicle (4)  
staining: hematoxylin/eosine. 40 $\times$

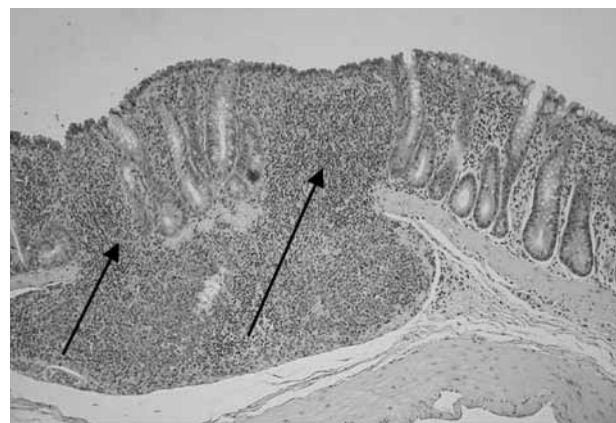


Figure 2. Infiltration of mononuclear cells into the *tunica mucosa* (arrows) and hyperplasia of crypts in the control positive 2

staining: hematoxylin/eosine. 40 $\times$

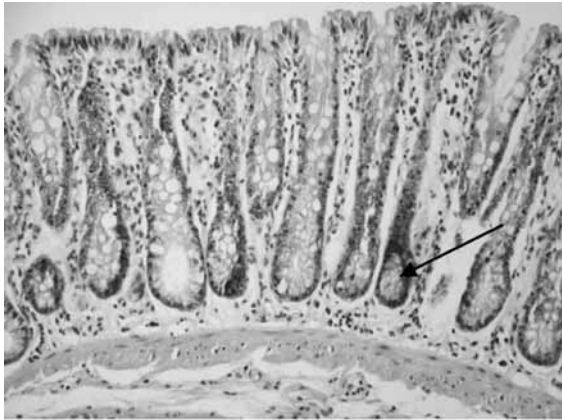


Figure 3. Formation in the aberrant crypt (arrow) in the control positive 2 staining: hematoxylin/eosine. 100×

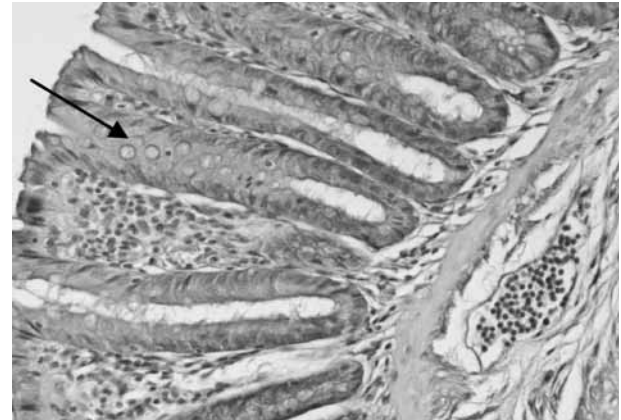


Figure 4. Significantly reduced number of goblet cells in the crypts (arrow) in the control positive 2 staining: alcian blue. 200×

which are major producers of mucus were reduced (Augenlicht et al., 2003).

Importantly, in PRO group, we found chronic inflammation with suspicion of aberrant crypts. The intensity of inflammation was lower than in the positive control group with 1.2-DMH. Certain probiotics have the ability to restore intestinal mucin levels (*Lactobacillus reuterii*, *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici*, and *Lactobacillus salivarius*); they were able to modulate intestinal mucin composition in broilers (Tsirtsikos et al., 2012). Interestingly, the lowest intensity of inflammation in the distal and proximal colon was in PRO-O group, as compared to positive control group with 1.2-DMH. After the evaluation of the total number of goblet cells in the distal proximal colon, we found substantially reduced number of cells in the crypts in the positive control group with addition of 1.2-DMH, as compared to negative control group (Figure 4). Treatment of these natural compounds (probiotics and oil) in our experiment substantially increased the total number of goblet cells.

However, when we administered *L. plantarum* in the PRO group, and mainly in combination with flaxseed oil in the PRO-O group, we found total increase in the number of goblet cells as compared to the positive control group with 1.2-DMH. In the present study the highest number of goblet cells was observed in the PRO-O group, as compared to the positive control group.

Salim et al. (2011) showed in non-treated control rats that the alcian blue staining was strong and intense in all colonic areas. On the other hand, mucosa of DMH treated rats was faint and weak. The mucosa of DMH treated rats which had dietary

flaxseeds oil (5 and 20%) increased in the mucus cells staining ability as compared to that found in the mucosa of only DMH treated rats.

## CONCLUSION

The interactions between the commensal microflora and the host in relation to carcinogenesis are not fully understood. The effects of probiotics and combination with flaxseed oil seem to be beneficial at least in the rodent model. Addition of the probiotic alone and its combination with flaxseed oil had positive effects on the decrease of coliform bacteria, enzymes  $\beta$ -glucuronidase and  $\beta$ -glucosidase, cytokine TNF $\alpha$ , and on the differentiation of goblet cells. Although the 1.2-DMH rat model does not replace studies with patient material, it is a valuable tool for studying the molecular events of CRC and for developing and evaluating a variety of novel cancer chemo-preventive agents or emerging therapeutic strategies that are difficult to address in humans. The results of this experimental study could be beneficial in reducing the risk of inflammation in the intestine and in following preneoplastic changes in the colon of rats, which can be extrapolated to the human system.

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