Effects of a probiotic in combination with prebiotics on intestinal lactobacilli and coliforms and activities of bacterial enzymes in 1,2-dimethylhydrazine exposed rats

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ABSTRACT: Effects of the probiotic (PRO) *Lactobacillus plantarum* and of the combination of PRO and the prebiotic (PRE) inulin enriched with oligofructose (2%), and PRO with *Lini oleum virginale* (O) on counts of lactobacilli and coliforms and enzymatic activities in faeces of rats were studied. The rats ($n = 60$) were divided into 5 groups of 12 subjects. The animals were fed on a high fat diet (10%) for 8 weeks of experiment. Colon cancer was induced by the application of 1,2-dimethylhydrazine (DMH) twice a week in a dose of 20 mg/kg *s.c.* in groups G2–G5. The rats in group 1 (control 1) received a diet without any supplements. The rats in group 2 (control 2) received 1,2 DMH without any supplements. The rats in group 3 received PRO, group 4 PRO and PRE, and group 5 received PRO and O. A significant decrease ($P < 0.05$) of coliforms was found out after the application of PRO, PRO-O, and PRO-PRE in comparison with control group G2. Significantly higher ($P < 0.05$) counts of lactobacilli were determined after the application of PRO-O and PRO-PRE. Significantly lower ($P < 0.001$) activities of β-galactosidase, β-glucuronidase and α-glucosidase were observed in PRO, PRO-PRE and PRO-O, while in the case of the enzyme β-glucosidase the activity was lower only after the addition of PRO-O. The protective effect of lactobacilli was observed in the order PRO-O, PRO-PRE, and PRO. It was shown that combinations of PRO-O and PRO-PRE had a synergistic effect which was higher than the effect of administering only PRO.

Keywords: *L. plantarum*; inulin; 1,2-dimethylhydrazine; lactobacilli; coliforms; faeces; rats

The intestinal microflora plays a crucial role in the health of humans and animals. All functions of the absorption of nutrients are connected with the valid digestive tract. Approximately 70% of the immune function is derived from the gut, being composed of the mucosal barrier, submucosal glands and the mucosa-associated lymphoid tissue (Bengmark, 2002). Lactic acid bacteria – probiotics

are viable microbial food ingredients supposed to be beneficial through their effect in the intestinal tract. The majority of the probiotics used today belong to the *Lactobacillus* group (Jenkins et al., 2005). New probiotic strains with antimicrobial effects can be isolated from plants and animals (Marciňáková et al., 2008; Herich et al., 2010).

The bacteria (e.g. coliforms, bacteroides, clostridia) play an important role in the process of development of colorectal carcinoma (Brady and Gallaher, 2000). Disturbances in the microflora of the colon (the reasons are often wrong food habits) are the overgrowth of microorganisms with an increase in the prevalence of coliform bacteria, and the production of bacterial enzymes increases that are able to change procarcinogens to carcinogens, mutagens and produce a variety of tumour promoters.

Colorectal cancer (CRC) is the third most prevalent form of cancer in men with a 5-year survival rate of 63%, decreasing to 10% in patients with metastatic disease (Goldberg, 2005). The diet makes an important contribution to CRC risk implying that risks are potentially reducible (Rafter, 2004). Some studies showed that a high fat diet increased the risk of colon cancer (Meyerhardt et al., 2007). 1,2-DMH is a common colon carcinogen often used in developing CRC in various experimental animals (Fukui et al., 2001; Lunz et al., 2008).

Lactic acid bacteria or a soluble compound produced by the bacteria may interact directly with tumour cells in culture and inhibit their growth. Kumar et al. (2009) observed that the oral administration of lactic acid bacteria *Lactobacillus acidophilus* and *Lactobacillus casei* and curd culture of *Lactococcus lactis* biovar *diacetylactis* DRC-1 in milk effectively reduced DNA damage, induced by chemical carcinogens (DMH), in the colonic mucosa in rats in a trial of 40 weeks.

Prebiotic (PRE) inulin-type fructans are classified as negative modulators of the carcinogenic process. The mechanisms proposed to explain these beneficial effects include changes in the composition and/or activity of colonic microflora (prebiotic effect) and in the composition of the pool of short-chain fatty acids and especially an increased relative proportion of butyrate as a result of their anaerobic fermentation (Roberfroid, 2000). The prebiotics inulin and oligofructose have been demonstrated to reduce the severity of 1,2-dimethylhydrazine induced colon cancers in rats (Hughes and Rowland, 2001).

The bacterial activity of some fatty acids in *in vitro* conditions has been known for a long time and may be of importance in *in vivo* preventing, eliminating and in some cases treating infections. It is suggested that dietary fatty acids affect the attachment sites for the intestinal microbiota, possibly by modifying the fatty acid composition of the intestinal wall (Ringo et al., 1998). The short-chain fatty acids play an important role in metabolisms in the human colonic function (Topping and Clifton, 2001).

The aim of this study was to compare the effects of a probiotic (PRO) and of the combination of PRO and prebiotics (PRE) and the combination of PRO and oil (O) on counts of coliforms and lactobacilli and activities of enzymes in 1,2-DMH exposed rats.

MATERIAL AND METHODS

Animals and feeding

In this study, rats (n = 60, mixed sex) at 6 months of age were used. The rats (Wistar) were reared in the Central Vivarium, Medical Faculty, P. J. Šafárik University, Košice, SR. The rats were housed in

Table 1. Composition of high fat diet supplied administered to rats

Composition	Value (g/kg)	Minerals	Value (mg/kg)	Vitamins	Value (mg/kg)
Crude protein	234.51	Calcium	15.0	A	8.000 IU/kg
Fat	65.87	Phosphorus	8.0	E	65
Ash	107.07	Sodium	2.0	B 1	4.0
Fiber	46.87			B2	4.0
Lysine	6.0				

*Statistically significant at a level $P < 0.05$

plastic cages at controlled climate (temperature of 22°C) favourable for their growth and welfare. The experiment was performed complying with ethical requirements for animal handling pursuant to Acts No. 289/2003 and 489/2003 of the Slovak Republic. The experiment was approved by the Ethical Commission.

The animals were fed a high fat (HF) diet (10% of fat) for 8 weeks of the experiment that was manufactured in Biofer (SR). The prebiotic Beneo Synergy 1, Orafti, Tienen, (Belgium) was applied in a dose 2% of the HF diet and was prepared with oligofructose-enriched inulin. It is a commercialised food ingredient composed of a mixture of long-chain inulin and short-chain oligofructose. *Lini oleum virginale* (Dr. Kulich Pharma, Czech Republic) in a dose 2% of the HF diet is obtained from *Linum usitatissimum* flaxseed containing high levels of polyunsaturated fatty acids. Feed and water were provided *ad libitum.* The composition of the diet is given in Table 1.

Microbiological assays

Various strains of lactobacilli were isolated from rectal human swabs. The obtained solution (100 μl of each) was spread-plated onto Lactobacillus selective agar (LS agar) (IMUNA-Pharm, Slovak Republic). The isolated lactobacilli strains were randomly selected and Gram stained and visualised under microscope for morphological characterisation.

The gram-positive and catalase-negative strains were tested for the ability to prove inhibited zones against the pathogen *E. coli* (CNCTC Eck 63/59, Czechoslovak Collection of Microorganisms) by the agar diffusion test. The most suitable strain was characterised as *Lactobacillus plantarum* using the molecular genetic method of polymerase chain reaction (PCR) according to Berthier and Ehrlich (1998). The characterised *L. plantarum* (3×10^9/ml) was aerobically prepared at 37°C. Firstly, 4 ml of the cultures were mixed with 36 ml of skim milk. After that, the milk (at a temperature of 22–25°C) was filled into screw-capped bottles and administered daily to drink to G3, G4, and G5 experimental rats.

Experimental design

The rats were randomly divided into 5 groups of 12 rats each.

Group 1 – control group 1 was without any supplements

Group 2 – control group 2 was without any supplements

Group 3 – received *L. plantarum* (PRO) in milk

Group 4 – received *L. plantarum* (PRO) in milk and inulin (PRE) in feed

Group 5 – received *L. plantarum* (PRO) in milk and oil* (O) in feed

*Lini oleum virginale

Application of DMH

Experimental groups of rats (G2, G3, G4, G5) were treated with 1,2-dimethylhydrazine (Merck, Germany) (in a dose of 20 mg/kg *s.c.*, twice a week) 2 weeks after the application of diets. The group G1 of rats was not treated with 1,2-dimethylhydrazine. Rats were killed after 8 weeks of the experiment. Animals were anaesthetised *i.p.* by ketamine 100 mg/kg and xylazine 15 mg/kg b.w.

Preparation of faecal samples

The fresh samples of faeces were taken from the intestine part of the caecum in all experimental rats after death. Faeces of rats (1 g) were placed in sterile polyethylene Stomacher Lab Blender bags with sterile diluents (9 ml) of Ringer's solution and mixed in a Stomacher 400 Bag mixer (France). The series of 10-fold dilutions (from 10^{-2} to 10^{-8}) were made in the same sterile diluents. The dilutions (100 μl of each) were spread-plated onto two selective agars – MacConkey agar (Merck, Germany) for coliforms and Rogosa agar (Biocar Diagnostics, France) for lactobacilli.

The plates for lactobacilli were made into box (Gas PaK, USA) and incubated at 37°C for 48 h. The plates for coliforms were incubated aerobically at 37°C for 24 h. The colonies were counted and bacteria were Gram-stained in a light microscope. The viable counts were expressed as the log 10 of colony-forming units (CFU/g) of faeces.

Evaluation of enzymatic activity of bacterial enzymes

Freshly collected samples of faeces were also used to evaluate the enzymatic activity of bacterial en-

zymes – galactosidase (α-GAL), β-galactosidase (β-GAL), β-glucuronidase (β-GLUCUR), α-glucosidase (α-GLU), β-glucosidase (β-GLU) using an API-ZYM strip (Biomereux, France). Faecal samples (0.1 g) were dissolved in 2 ml of saline. This solution was incubated (90 μl to each cup) on an API ZYM strip. The activities of enzymes were determined according to the manufacturer's instructions and expressed on a scale of 0 (negative reaction) to 5 (maximum activity).

Histopathological evaluation of tumours

Biopsy samples of the caecum were fixed in 10% neutral formalin solution during 48–72 h. The samples were drained and embedded in paraffin blocks. Histological sections were stained by haematoxylin-eosin and determined microscopically.

Statistical analyses

The results were statistically analysed using a multifactor analysis of variance (ANOVA). Statistically significant effects were further analysed and the means were compared using Duncan's multiple range test.

RESULTS AND DISCUSSION

Counts of lactobacilli

As shown in Table 2, the administration of the probiotic PRO caused an increase in the counts of lactobacilli ($9.09 \pm 0.62 \times 10^8$ CFU/g) in group G3 in comparison with control group G2 ($8.87 \pm 0.64 \times 10^8$ CFU/g).

However, the statistically significant increase ($P < 0.05$) in counts of lactobacilli was observed after the application of the combination of PRO-O in group G5 ($9.41 \pm 0.37 \times 10^8$ CFU/g) and PRO-PRE in group G4 ($9.33 \pm 0.32 \times 10^8$ CFU/g) in comparison with control group G2. When comparing group 1 and group 2, no statistically significant difference was observed.

The research into the influence of high fat diet on colon cancer is necessary for the healthy population of humans. Femia et al. (2002) studied the effects of the application of high fat diet (of the same composition as the diet of some western populations at risk of colon cancer) after the ap-

Table 2. Counts of lactobacilli and coliforms (CFU/g) after administration of PRO, PRO-PRE, PRO-O to experimental rats

Groups	Supplement	Lactobacilli	Coliforms
G 1	Control 1	9.00 ± 0.81	3.08 ± 2.39
G 2	Control 2	8.87 ± 0.64	4.67 ± 1.03
G 3	PRO	9.09 ± 0.62	3.34 ± 2.40
G 4	PRO-PRE	9.33 ± 0.32*	2.76 ± 2.51*
G 5	PRO-O	9.40 ± 0.366*	3.31 ± 1.83

plication of azoxymethane to induce colon cancer in rats. In this experiment, the PRO group rats were fed the same HF diet as controls but supplemented with *Lactobacillus rhamnosus* (LGG) and *Bifidobacterium lactis* (Bb 12) to provide 5×10^8 CFU/g of each strain in the diet. No involved bacteria were present in the faeces prior to feeding. After 7 days of feeding the respective counts of LGG and Bb 12 were present in PRO and PRO-PRE groups (inulin enriched with oligofructose). The counts of LGG ($21.1 \pm 18 \times 10^5$ CFU/g of faeces) and Bb 12 ($8.4 \pm 12 \times 10^5$ CFU/g of faeces) were higher in PRO-PRE groups than in PRO groups (LGG $4.8 \pm 3.4 \times 10^5$ CFU/g of faeces and Bb 12 $6.1 \pm 8.1 \times 10^5$ CFU/g of faeces). Our results are in agreement with this study. We recorded an increase in the counts of lactobacilli in PRO and PRO-PRE groups of rats. The combinations of probiotics (LGG and Bb 12) and prebiotics (inulin enriched with oligofructose) used in previous study were also applied as dietary synbiotics in experiments in colon cancer patients (Rafter et al., 2007).

Mortality and incidence of colorectal carcinoma rank as the third after those of prostate and lung cancer in men, breast and lung cancer in women and have shown a low downward trend in the last 20–30 years (Fodiatis, 2008). This has led to an intense interest in factors that can modulate the gut microflora and their metabolism, such as probiotics and prebiotics.

Counts of Coliforms

When comparing the coliforms in group 1 and group 2, no statistically significant difference was observed. A significant decrease ($P < 0.05$) of coliforms ($2.76 \pm 2.51 \times 10^4$ CFU/g) was found out after

the application of PRO-PRE in group G4 (Table 2) in comparison with control group G2 (4.67 ± 1.03 × 10^4 CFU/g). The administration of PRO in group G3 (3.34 ± 2.40 × 10^4 CFU/g) and PRO-O in group G5 (3.31 ± 1.83 × 10^4 CFU/g) decreased the counts of coliforms in comparison with control group G2. Similar results were obtained by Hsu et al. (2004) after the application of prebiotics – xylooligosaccharides (XOS) and fructooligosaccharides (FOS) to rats' diet. They investigated their effect on the intestinal microbiota and precancerous colonic lesion development in rats. The caecal coliform count was negatively associated with the caecal Bifidobacteria count. These results indicated that XOS and FOS supplementation markedly increased the Bifidobacteria population. It can influence the health of the intestinal tract of animals.

In their experimental study Richard et al. (2005) documented the protective effect of probiotics and prebiotics against colorectal cancers after a single azoxymethane injection to rats. Azoxymethane was used for developing CRC in rats. In this study a decrease in total counts of coliforms in the caecum in comparison with the control group was observed.

The influence of probiotics and prebiotics was also described in other animals. Nemcová et al. (1999) found out after the application of the strain *Lactobacillus paracasei* as single species or *L. paracasei* in combination with fructooligosaccharides (Raftilose P95) to experimental piglets that in comparison with control groups the counts of coliforms were significantly decreased by 1 log in group PRO and PRO-PRE.

Activity of bacterial enzymes

Bacterial flora may influence colorectal carcinogenesis and production of enzymes that convert carcinogens into active procarcinogens.

Intestinal microflora is involved in the production of carcinogens from procarcinogenic substances by bacterial enzymes. These include β-glucuronidase, β-glucosidase, azoreductase, nitroreductase, 7 α-steroid dehydrogenase, 7 α-hydroxy steroid dehydrogenase (Goldin, 1986). Their activities may be regarded as a suitable biomarker for cancer risk assessment. Coliform bacteria were investigated mainly due to β-glucuronidase, β-glucosidase activities, i.e. they can assist in the formation of carcinogens from procarcinogenic substances.

The results of the present study show a decrease in the activities of bacterial enzymes after the addition of PRO, PRO-PRE and PRO-O to rat's diet (Table 3). A statistically significant decrease ($P \leq 0.001$) in the activities of β-GAL, β-GLUCUR and α-GLU was observed after the addition of PRO, PRE-PRO, and PRO-O in group G3, G4 and G5. On the other hand, the activity of the enzyme β-GLU was significantly ($P < 0.001$) decreased only after the addition of PRO-O in group G5.

Le Blanc (2005) evaluated the role of yoghurt starter bacteria and their cell-free fermentation products in the reduction of procarcinogenic enzyme activities (β-glucuronidase and nitroreductase). Mice injected with 1,2 DMH and fed yoghurt were used in this study. Feeding yoghurt decreased procarcinogenic enzyme levels in the large intestine contents of mice bearing colon tumour.

β-Glucuronidase is an enzyme responsible for the hydrolysis of glucuronides in the lumen of the gut. This reaction generates toxic and carcinogenic substances which are detoxified by glucuronidase formation in the liver and then enter the bowel via bile. In this way, toxic aglycones can be regenerated *in situ* in the bowel by bacterial β-glucuronidase.

In humans, the faecal β-glucuronidase activity was shown to be higher in colorectal cancer patients in comparison with healthy controls suggest-

Table 3. Changes of the activity of bacterial enzymes

Groups	α-GAL	β-GAL	β-GLUCUR	α-GLU	β-GLU
G1–Control 1	1.83 ± 0.26	2.60 ± 0.25	2.92 ± 0.20	2.38 ± 0.31	1.67 ± 0.26
G2–Control 2	2.00 ± 0.26	3.92 ± 0.80	4.25 ± 0.52	3.83 ± 0.68	2.08 ± 0.80
G3–PRO	1.96 ± 0.17	2.25 ± 0.27***	2.67 ± 0.28***	2.44 ± 0.27***	1.96 ± 0.17
G4–PRO-PRE	1.86 ± 0.43	2.03 ± 0.44***	1.88 ± 0.22***	2.06 ± 0.39***	1.85 ± 0.44
G5–PRO-O	1.20 ± 0.42	1.15 ± 0.53***	1.66 ± 0.50***	1.41 ± 0.18***	1.31 ± 0.43***

***Statistically significant at a level $P < 0.001$

ing a role of this enzyme in carcinogenesis (Kim and Jin, 2001). Human studies have demonstrated that the capacity of probiotics to decrease the activity of bacterial enzymes is strain specific (Goossens et al., 2003).

Our results confirm the positive impact of lactobacilli on the presence of microorganisms in the intestinal ecosystem (lactobacilli and coliforms) resulting in decreased activity of β-glucuronidase and β-glucosidase.

Histopathological changes

The histopathological tumour changes were not detected in experimental groups in comparison with control groups G1 of rats by reason of the short time of the experiment.

Probiotics and prebiotics affect the prevention of mutations and antigenotoxic activity, determination of active lactic acid bacteria principles, binding of mutagens and inactivation of carcinogens by modification of toxifying and detoxifying and enzymes (Wolowski et al., 2001).

The mechanisms by which probiotic bacteria may inhibit colon cancer have not been fully characterised yet. However, there is evidence for the following: alteration of metabolic activities of the intestinal microflora, alteration of physicochemical conditions in the colon, binding of potential carcinogens, short-chain fatty acid production and production of antitumorigenic or anti-mutagenic compounds, elevating the host's immune response and altering the host's physiology (Fodiatis, 2008).

Obtained results also indicated the positive effect of administering the combination of the strain *L. plantarum* as probiotic (PRO) with oil (O). Although the application of probiotic with oil, specifically with *Lini oleum virginale*, to rats was not documented, the effect of fish oil ingestion on colonic carcinogenesis in rats was studied by Moreira et al. (2009).

In this experiment, male Wistar rats received 4 subcutaneous injections (40 mg/kg body weight) of 1,2-DMH in 3-day intervals and were fed a diet containing 18% fish oil for 36 weeks. Their findings indicated that the chronic FO ingestion protected against DMH-induced preneoplastic colon lesions and adenoma development, but not against carcinoma in rats. The polyunsaturated fatty acid (PUFA) profile of the colon was affected by the diet.

Other studies with pigs confirmed that a high fat diet containing polyunsaturated fatty acids (PUFA) is the main factor affecting the *Lactobacillus* counts in the intestine. Bomba et al. (2003) used seal oil containing essential ω-3 PUFA in gnotobiotic piglets at 22–28 days of age. The piglets were inoculated with *L. paracasei* (1×10^8 bacteria/ml). The results documented that the counts of *L. paracasei* adhering to the jejunum mucosa were 12% higher in the piglets of experimental groups in comparison with the control group. Similarly, Kašteľ et al. (2003) documented the effect of PUFA on some metabolic and immunological parameters in gnotobiotic and conventional piglets.

Our results documented that changes in counts of coliforms and lactobacilli reflected the activities of bacterial enzymes. It was confirmed after the application of the strain *L. plantarum* as single species or of the combination of synbiotics. These studies suggest that synbiotics PRO-PRE may play a role in colorectal cancer treatment.

CONCLUSION

The data presented in our study can be used in modern experimental medicine. This study shows the possibility of using either a probiotic strain (*L. plantarum*) as single species or in combination with inulin or oil (*Lini oleum virginale*) for decreasing the count of total coliforms and increasing lactobacilli. It can be used not only in the treatment of animals, but also mainly in the prevention of colorectal carcinoma in humans. The optimal dietary manipulation could be a good alternative in preventing many, mainly carcinoma diseases. One of the ways of preventing such diseases is the use of probiotics, prebiotics, and natural components. Low fat diet can be recommended as a prevention of colon cancer. This diet is still the best choice for optimal health.

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