The effect of new probiotic strain *Lactobacillus plantarum* on counts of coliforms, lactobacilli and bacterial enzyme activities in rats exposed to N,N-dimethylhydrazine (chemical carcinogen)

Denisa Čokášová[1], Alojz Bomba[1], Ladislav Strojný[1], Beata Pramuková[1], Viktória Szabadosová[1], Rastislav Salaj[1], Jana Žofčáková[1], Andrea Brandeburová[1], Anna Supuková[1], Alena Šoltésová[1], Emília Hijová[1], Júlia Ričanyová[1], Leonard Siegfried[2], Peter Supuka[3]

[1]Institute of Experimental Medicine, Faculty of Medicine, [2]Institute of Medical and Clinical Microbiology, P. J. Šafárik University, Košice, Slovak Republic
[3]Institute of Animal Husbandry, University of Veterinary Medicine and Pharmacy, Košice, Slovak Republic

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Abstract

The aim of the present study was to evaluate the effect of the new probiotic strain *Lactobacillus plantarum* on chemically induced carcinogenesis in rats. *Sprague dowley* rats (n = 33) were divided into control and experimental groups and were fed a conventional laboratory diet. In the experimental group, rats were treated with the probiotic at the dose of 1×10^9 CFU (colony-forming units)/ml. Two weeks after the beginning of the trial, N,N-dimethylhydrazine (chemical carcinogen) injections were applied s.c. at the dose of 21 mg/kg b.w., 5 × weekly. At the end of the 8-month experimental period, faeces samples were taken from the rats and used for laboratory analysis. The counts of lactobacilli and coliforms and bacterial enzyme activity were determined. The probiotic strain *L. plantarum* as single species or in combination with oil *(Lini oleum virginale)* decreased the count of total coliforms and increased lactobacilli in faeces of rats. Application of probiotic microorganisms significantly ($P < 0.05$) decreased the activities of bacterial enzymes (β-galactosidase and β-glucuronidase) compared to the control group rats. The results of this study indicate that probiotic microorganisms could exert a preventive effect on colon carcinogenesis induced by N,N-dimethylhydrazine.

Colon cancer, prevention, faeces, Sprague dowley

Colon cancer is one of the most common forms of malignant tumours in humans, and its incidence is increasing. Cancer mortality will continue to rise with an estimated 9 milion people dying from cancer in 2015, and 11.4 milion dying in 2030 according to World Health Statistics (2008). Diet makes an important contribution to colon cancer risk, which implies the risks of colon cancer are potentially reducible (Rafter 2004). Lactic acid bacteria probiotics are viable microbial food ingredients supposed to be beneficial through their effect on 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). Some studies showed that a high fat diet increased the risk of colon cancer (Meyerhardt et al. 2007). 1,-2-dimethylhydrazine is a common colon carcinogen often used in developing colorectal cancer 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 (1,2-dimethylhydrazine) in the colonic mucosa of rats in a trial of 40 weeks.

Address for correspondence:
MVDr. Denisa Čokášová
P. J. Šafárik University in Košice, Faculty of Medicine
Institute of Experimental Medicine
Trieda SNP 1, 040 01 Košice, Slovak Republic
Phone: +421 556 404 323
E-mail: denisa.cokasova@gmail.com
http://actavet.vfu.cz/

The aim of this study was to evaluate the effects of the new probiotic strain *Lactobacillus plantarum* on counts of coliforms, lactobacilli and bacterial enzyme activities in rats exposed to N,N-dimethylhydrazine.

Materials and Methods

Animals and feeding

Rats (n = 33, mixed sex) of 6 months of age were used in the study. The rats (*Sprague dowley*) were reared in the Central Vivarium, Medical Faculty, P. J. Šafárik University, Košice, Slovak Republik. They were housed in plastic cages at controlled climate (temperature of 22 °C) favourable for their growth and welfare. The experiment was performed complying with the ethical requirements for animal handling pursuant to Acts No. 289/2003 and 489/2003 of the Slovak Republic. The experiment was approved by the Ethics Committee. The animals were fed a conventional diet for 8 months of the experiment, manufactured by Biofer (Slovak Republic). *Lini oleum virginale* (Dr. Kulich Pharma, Czech Republic) at a dose of 4% was obtained from *Linum usitatissimum* flaxseed containing high concentrations of polyunsaturated fatty acids. Feed and water were provided *ad libitum*. At the end of the 8 months the rats were anaesthetized by ketamine 100 mg/kg + xylazine 15 mg/kg b.w., i.p. and the fresh samples of faeces were taken from the intestine part of the caecum in all experimental rats after death. During the study there was a death of three animales (males), one rat in group 3 and two rats in group 4.

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* (1×10^9/ml) was aerobically prepared at 37 °C. Firstly, 4 ml of the cultures were mixed with 36 ml of skimmed milk. The milk was then filled (at a temperature of 22–25 °C) into screw-capped bottles and administered daily to drink rats (groups 3, 4).

Experimental design

The rats were randomly divided into 4 groups of 7–9 rats each:

Group 1 - control group without any supplements,
Group 2 - control group without any supplements and chemically induced carcinogenesis with N,N-dimethylhydrazine,
Group 3 - chemically induced carcinogenesis with N,N-dimethylhydrazine + *L. plantarum* (probiotic) in milk,
Group 4 - chemically induced carcinogenesis with N,N-dimethylhydrazine + *L. plantarum* (probiotic) in milk + 4% oil *(Lini oleum virginale)* in feed.

Application of N,N-dimethylhydrazine

Experimental groups of rats (groups 2, 3 and 4) were treated with N,N-dimethylhydrazine (Merck, Germany) at a dose of 21 mg/kg s.c., twice a week, 2 weeks after the administration of diets. Group 1 of rats was not treated with N,N-dimethylhydrazine. After 8 months of the experiment the rats were anaesthetised i.p. by ketamine 100 mg/kg and xylazine 15 mg/kg b.w.

Preparation of faecal samples

Fresh samples of faeces were taken from the intestinal 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 saline and mixed in a Stomacher 400 Bag mixer (France). 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 put 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 enzymes - galactosidase (α-GAL), β-galactosidase (β-GAL), β-glucuronidase (β-GLUCUR), α-glucosidase (α-GLU) and β-glucosidase (β-GLU) using an (SIGMA-ALDRICH, Bratislava). Bacterial enzymes activity in the caecal digesta is measured by the rate of release of *p*- or *o*-nitrophenol from their nitrophenylglucosides according to the modified method of Djouzi and Andrieux (1997) described in details by Juśkiewicz et al. (2002).

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 parafin blocks. Histological sections were stained by haematoxylin-eosin and determined microscopically.

Statistical analysis

Statistical analysis was performed by Student's *t*-test and analysis of variance (ANOVA) with significance $P < 0.05$; $P < 0.01$ and $P < 0.001$.

Results

Counts of lactobacilli and coliforms

As shown in Table 1, administration of the probiotic caused an increase in the counts of lactobacilli (9.51 ± 0. 34 × 10^8 CFU/g) in group 3 compared to control group 2 (8.86 ± 0.64 × 10^8 CFU/g). However, significant increase ($P < 0.001$) in counts of lactobacilli was observed after the administration of the combination of probiotic-oil in group 4 (10.25 ± 0.39 × 10^8 CFU/g) compared to control group 2. When comparing groups 1 and 2, no significant difference was observed. We recorded an increase in the counts of lactobacilli in probiotic and probiotic-oil groups of rats.

Table 1. Counts of lactobacilli and coliforms (CFU/g) administration of probiotic (*Lactobacillus plantarum*) and probiotic-oil to experimental rats

Groups*	Supplement	*Lactobacilli*	*Coliforms*
1		8.99 ± 0.45	6.16 ± 0.55
2	N,N-dimethylhydrazine	8.86 ± 0.64	6.34 ± 0.24
3	N,N-dimethylhydrazine+probiotic	9.51 ± 0.34***	5.14 ± 0.48***
4	N,N-dimethylhydrazine+probiotic+oil	10.25 ± 0.39***	5.97 ± 0.44***

*Group 1 – control group without any supplements,
Group 2 – control group without any supplements, (chemically induced carcinogenesis) with N,N-dimethylhydrazine,
Group 3 – chemically induced carcinogenesis with N,N-dimethylhydrazine + *L. plantarum* (probiotic) in milk,
Group 4 – chemically induced carcinogenesis with N,N-dimethylhydrazine + *L. plantarum* (probiotic) in milk + 4% of oil *(Lini oleum virginale)* in feed
Data are expressed as mean ± SD
Significant differences calculated from group 2 are designated as *** ($P < 0.001$)

When comparing the coliforms in group 1 and group 2, no significant differences were observed. A significant ($P < 0.001$) decrease of coliforms (5.97 ± 0.44 × 10^4 CFU/g) was found after administration of probiotic-oil in group 4 (Table 1) compared to control group 2 (4.67 ± 1.03 × 10^4 CFU/g). Administration of probiotic in group 3 (5.14 ± 0.48 × 10^4 CFU/g) decreased the counts of coliforms compared to control group 2 (6.34 ± 0.24 × 10^4 CFU/g).

Bacterial enzyme activity

The results of the present study showed a decrease in the activities of bacterial enzymes after the addition of probiotic or probiotic-oil to the rats' diet (Table 2). Significant decreases in the activities of β-GAL and β-GLUCUR were observed after the addition of probiotic and probiotic-oil in groups 3 and 4.

Our results confirmed 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-galactosidase.

Histopathological changes

Rats from control group 1 did not reveal any histopathological changes. The epithelium showed significant histological changes; epithelial cells produced sufficient deep crypts.

Table 2. Changes in activity of bacterial enzymes in rats.

Groups*	α-GAL	β-GAL	β-GLUCUR	α-GLU	β-GLU
1	0.21 ± 0.06	0.06 ± 0.02	0.28 ± 0.10	0.08 ± 0.04	0.08 ± 0.02***
2	0.11 ± 0.06**	0.07 ± 0.04	0.37 ± 0.15	0.15 ± 0.05**	0.04 ± 0.01***
3	0.32 ± 0.15**	0.03 ± 0.01*	0.17 ± 0.05**	0.18 ± 0.04	0.15 ± 0.02***
4	0.29 ± 0.13**	0.06 ± 0.02	0.3 ± 0.14	0.12 ± 0.08	0.06 ± 0.01*

*Group 1 – control group without any supplements,
Group 2 – control group without any supplements, (chemically induced carcinogenesis) with N, N-dimethylhydrazine,
Group 3 – chemically induced carcinogenesis with N,N-dimethylhydrazine + *L. plantarum* (probiotic) in milk,
Group 4 – chemically induced carcinogenesis with N,N-dimethylhydrazine + *L. plantarum* (probiotic) in milk + 4% of oil (*Lini oleum virginale)*, in feed
α-GAL (α-galactosidase), β-GAL (β-galactosidase), β-GLUCUR (β-glucuronidase), α-GLU (α-glucosidase), β-GLU (β-glucosidase)
Data are expressed as means ± SD, significant differences calculated from group 1 are designated as: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

In control group 2 histological changes showed enlarged lymph follicles that were brought to the surface of damaged epithelial lining of the *tunica mucosa* layer. A cluster of morphologically altered cells showing neoplastic changes was found in the layer of *tunica mucosa*. Histopathological changes showed an ongoing long-term chronic inflammatory process of the lining of the colon induced by experimental application of the chemical N,N dimethylhydrazine.

Discussion

The effect of probiotics on prevention of colorectal cancer confirms the importance of bacterial flora in the aetiology of colorectal cancer and at the same time represents an appropriate way of decreasing its incidence.

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

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 dimethylhydrazine and fed yoghurt were used in their 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 compared to healthy controls suggesting a role of this enzyme in carcinogenesis (Kim and Jin 2001). Studies in humans have demonstrated that the capacity of probiotics to decrease the activity of bacterial enzymes is strain specific (Goossens et al. 2003).

Based on an experimental study, Bertková et al. (2010) suggested that *Hippocastani extractum siccum* may be a useful candidate agent for colon cancer chemoprevention and treatment. They investigated the effect of probiotic microorganisms and selected bioactive compounds in different combinations on decreasing the concentration of bile acids in blood serum activity of bacterial enzymes in colon contents, short-chain fatty acids, IL-6 levels and increased TNF-α- level in comparison with their individual application. The results of their experiment indicate that probiotic microorganisms and bioactive compounds could exert a preventive effect on colon carcinogenesis induced by dimethylhydrazine.

Although the administration 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). The results obtained from our study indicate a positive effect of administering the combination of the strain *L. plantarum* as probiotic with oil. Changes in counts of coliforms and lactobacilli reflected the activities of bacterial enzymes. Our study shows the possibility of using either a probiotic strain (*L. plantarum*) as single species or in combination with 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. Optimal dietary manipulation could be a good alternative in preventing many, mainly carcinoma diseases, not only in animals but also in humans.

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References

Berthier F, Ehrlich SD: Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rNA spacer region. Microb Letters **161**: 97-106

Bertková I, Hijová E, Chmelárová A, Mojžišová G, Petrášová D, Strojný L, Bomba A, Žitňan R 2010: The effect of probiotic microorganisms and bioactive compounds on chemically induced carcinogenesis in rats. Neoplasma **57**: 422-428

Djouzi Z, Andrieux C 1997: Compared effect of the three oligosaccharides on metabolism of intestinal microflora on rats inoculated with a human faecal flora. Br J Nutr **78**: 313-324

Fukui M, Fujino T, Tsutsui K, Maruyama T, Yoshimura H, Shinohara T, Fukui M, Nada O 2001: The tumour-preventing effect of a mixture of several lactic acid bacteria on 1,2-dimethylhydrazine-induced colon carcinogenesis in mice. Onc Report **8**: 1073-1078

Herich R, Kokinčáková T, Lauková A, Levkutová M 2010: Effect of preventive application of *Enterococcus faecium* EF55 on intestinal mucosa during salmonellosis in chickens. Cz J An Sci **55**: 42-47

Jenkins B, Holsten S, Bengmark S, Martindale R 2005: Probiotics: A practical review of their role in specific clinical scenarios. Nut Clin Prac **20**: 262-270

Juśkiewcz J, Zduńczyk Z, Wróblewska M, Oszmiański J, Hernandez T 2002: The response of rats to feeding with diets containing grapefruit flavonoid extract. Food Res Int **35**: 201-205

Kumar A, Kumar NS, Phushpalata RS 2009: Inhibition of 1,2-dimethylhydrazine induced colon genotoxicity in rats by the administration of probiotics curd. Mol Bio Rep **10**: 1-4

Le Blanc AM, Perdigón G 2005: Reduction of beta-glucuronidase and nitro-reductase activity by yoghurt in a murine colon cancer model. Biocell Mendoza **29**: 1-17

Lunz W, Peluzio MCG, Dias CMGC, Moreira APB, Natali AJ 2008: Long-term aerobic swimming training by rats reduces the number of aberrant crypt foci in 1,2-dimethylhydrazine-induced colon cancer. Braz J Med Bio Res **41**: 1000-1004

Marciňáková M, Lauková A, Simonová M, Strompfová V, Koréneková B, Naď P 2008: A new probiotic and bacteriocin-producing strain of *Enterococcus faecium* EF 9296 and its use in grass enciling. Cz J An Sci **53**: 336-345

Meyerhardt JA, Niedzwiecki D, Hollis D, Saltz LB, Hu FB, Mayer RJ, Nelson H, Whittom R, Hantel A, Thomas J, Fuchs CS 2007: Association of dietary patterns with cancer recurrence and survival in patients with stage III colon cancer. J Am Med Ass **298**: 754-764

Moreira APB, Sabarense CM, Dias CMGC, Lunz W, Natali AJ, Glória MBA, Peluzio MCG 2009: Fish oil ingestion reduces the number of aberrant crypt foci and adenoma in 1,2-dimethylhydrazine-induced colon cancer in rats. Braz J Med Bio Res **12**: 1167-1172

Nemcová R, Bomba A, Gancarčíková S, Herich R, Guba P 1999: Study of the effect of *Lactobacillus paracasei* and fructooligosacharides on the faecal microflora in weanling piglets. Ber Mun Tier Woch **7**: 225-228
Rafter J 2004: The effects of probiotics on colon cancer development. Nutr Res Rev **17**: 277-284
World Health Statistics 2008: WHO Press, May 110 p.