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Prebiotics and bioactive natural substances induce changes of composition and metabolic activities of the colonic microflora in cancerous rats

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Prebiotics are defined as selectively fermented food ingredients that induce specific changes in the composition and/or activity in the gastrointestinal microbiota beneficial to the host well-being and health. The aim of the presented experiment was to investigate the effect of a prebiotic applied alone or in combination with *Hyppocastani extractum* siccum, and *Lini oleum* virginale in rats with dimethylhydrazine induced colon cancer. Wistar albino rats were fed high fat diet supplemented with the prebiotic alone or in combination with Horse chestnut and flaxseed oil. The activity of faecal glycolytic enzymes, lipid parameters, bile acids, short chain fatty acids and counts of coliforms and lactobacilli were determined. Treatment with the prebiotic alone and in combination with selected substances significantly decreased the activity of glycolytic bacterial enzyme β-glucuronidase (*P*<0.001) and increased activities of β-galactosidase and β-glucosidase. Bile acids concentration was significantly decreased (*P*<0.01) except for the combination of the prebiotic with Horse chestnut. The prebiotic alone decreased the lipid parameters (*P*<0.001) and enhanced production of short chain fatty acids. Application of prebiotic and bioactive natural substances significantly reduced number of coliforms (*P*<0.05). Prebiotic alone significantly increased the count of lactobacilli (*P*<0.05). These results show that prebiotics have a protective effect and may be the useful for colon cancer prevention and treatment.

Keywords: colon cancer, prebiotic, flaxseed oil, Horse chestnut, rats

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INTRODUCTION

Colorectal cancer (CRC) is among the most frequent tumors worldwide (World Health Statistics, 2008). Lifestyle factors, especially dietary intake, affect the risk of CRC development. Suitable risk biomarkers are required in order to assess the effect that specific dietary components have on CRC risk. In order to achieve optimal digestion, absorption, and nutritional health, we must harbor appropiate microflora. In this context, dietary ingredients improving intestinal function and ensuring a healthy gastrointestinal tract environment attract a great deal of interest. One of the mechanisms increasing the number and metabolic activities of purportedly beneficial microbes (e.g., lactic acid bacteria, bifidobacteria) in the gut is ingestion of prebiotics. Prebiotics are generally defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth or activity of health-promoting lactobacilli and bifidobacteria, may enhance defence mechanisms of the host, increase resistance to various health challenges and accelerate recovery of gastrointestinal tract disturbances. Prebiotics can complement function of probiotics and as synbiotics might represent novel therapeutic or preventive agents (Geier *et al.*, 2006; Trafalska *et al.*, 2006; Rafter *et al.*, 2007).

The aim of the present study was to obtain information about the efficacy of the prebiotic inulin applied alone or in combination with *Hyppocastani extractum* siccum as nutritional plant extract or *Lini oleum* virginale on the activities of the bacterial glycolytic enzymes, lipid parameters, bile acids, short chain fatty acids (SCFA), and counts of coliforms and lactobacilli in rats with dimethylhydrazine (DMH) induced colon cancer.

MATERIALS AND METHODS

Animals. Wistar albino rats (n=60) (central vivarium, Faculty of Medicine, P. J. Šafárik University), six months old with mean body weight 372±15 g were housed in plastic cages with tops and maintained at 22°C, on 12 h light/dark cycle, according to the principles provided in Law No. 23/2009 of the Slovak Republic for the Care and Use of Laboratory Animals. Animals were fed high fat diet (HF) containing 10 percent fat (Biofer, Slovak Republic), similar to the diet of some western populations at risk for colon cancer, supplied with drinking water *ad* libitum. All rats were additionally treated with N,N-dimethylhydrazine (DMH, Merck, Germany). Food and water consumption were monitored daily. The rats were divided into 5 groups of 12 animals each. Control group (CG) was a group fed HF diet with DMH, without administration of prebiotic or bioactive food components. Animals in all experimental groups (EG) were fed HF diet with DMH and treated with selected bioactive food components. Experimental group 1 (EG1=PRE) was administered the prebiotic inulin (PRE), experimental group 2 (EG2=PRE+HES) was administered a combination of the prebiotic and Hypocastani extractum sic-

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Abbreviations: CRC, colorectal cancer; DMH, dimethylhydrazine; SCFA, short chain fatty acids; HF, high fat diet; CG, control group; EG, experimental group; PRE, prebiotic; HES, hyppocastani extractum siccum; O, lini oleum virginale; PUFA, polyunsaturated fatty acids; β-GAL, β-galactosidase; β-GLUCUR, β-glucuronidase; β-GLU, β-glucosidase.

Table 1. Activity of bacterial enzymes in faeces in control and experimental groups.
Data represent mean ± standard deviation, *$P<0.05$; **$P<0.001$; ***$P<0.001$

Groups	β-GAL	β-GLUCUR	β-GLU
CG	0.88±0.20	4.25±0.52	0.75±0.28
EG1 (PRE)	1.92±0.37*	0.83±0.52***	1.83±0.26*
EG2 (PRE+HES)	2.00±0.32*	0.72±0.25***	1.52±0.16
EG3 (PRE+O)	1.70±0.23*	2.18±0.33***	1.35±0.39
EG4 (PRE+HES+O)	2.11±0.55**	2.22±0.56***	1.92±0.46*

Statistical significance is comparison between CG *versus* EG1, EG2, EG3, EG4

cum, experimental group 3 (EG3=PRE+O) was given a combination of the prebiotic and Lini oleum virginale. Experimental group 4 (EG4=PRE+HES+O) was administered a mixture of all selected substances.

Treatments. Treatment consisted of oligofructose-enriched inulin (PRE, BeneoSynergy 1, ORAFTI, Tienen, Belgium) at a dose of 2% of HF diet. It is a commercialized food ingredient composed of a mixture of long-chain inulin and short-chain oligofructose. Extract of *Aesculus hippocastanum* L. (HES, Calendula, Slovak Republic) was administered at a dose of 1% of HF diet. Most of the beneficial effects of the extract of *Aesculus hippocastanum* L., (Hippocastanaceae) commonly known as Horse chestnut are attributed to its principal component beta-escin or aescin. *Lini oleum* virginale (Dr. Kulich Pharma, Czech Republic) is obtained from flaxseed *Linum usitatissimum* L. containing a high amount of ω-3 polyunsatured fatty acids (ω-3 PUFA), and was administered at a dose of 2% of HF diet.

Two weeks after begining of the experiments, rats were treated with DMH at a dose of 20 mg/kg body weight s.c., two times a week, and the dietary treatments were continued for an additional six weeks. Then the rats were anaesthetized (Ketamine 100 mg/kg + Xylazine 15 mg/kg body weight i.p.), blood samples were taken from the heart by puncture and feaces samples from the colon.

Biochemical Analysis. Blood samples were centrifuged at $2500\times g$ for 15 min and serum used for determination of bile acids with a commercial kit (Trinity Biotech, Ireland) and lipid parameters with commercial kits (Biolatest, Czech Republic). The measurement was carried out on an automatic spectrophotometric analyser Cobas Mira S (Roche, Schwitzerland). Freshly collected faeces samples were examined for activities of the bacterial glycolytic enzymes — β-galactosidase (β-GAL), β-glucuronidase (β-GLUCUR), and β-glucosidase (β-GLU) using an API-ZYM kit (Biomérieux, France). Activities were determined according to the manufacturer's instructions and expressed on the scale of 0 (negative reaction) to 5 (maximum activity). The SCFA were analyzed in the colon contents using gas chromatography (Hewlett Packard 6890 Plus, USA). Total SCFA (mmol/100 ml of wet cecal digesta) = acetic + propionic + butyric + isobutyric + isovaleric + valeric + caproic.

Bacteriological Examination. Microbial analyses (total lactobacilli and coliforms) of the faecal samples were carried out after the completion of the experiment. Faeces (1 g) was placed in a sterile polyethylene Stomacher Lab Blender bag with 9 ml of sterile 0.9% NaCl. Series of 10-fold dilutions (10^{-2} to 10^{-8}) were made in the same sterile diluent. From appropriate dilution, 0.1 ml aliquots were spread onto two selective Mc Conkey agar (Merck, Germany) for coliforms and Rogosa agar (Biocar diagnostic, France) for lactobacilli. The plates for lactobacilli were made anaerobic (Gas PaK, USA) and incubated at 37°C for 48 hours. The plates for coliforms were incubated aerobically at 37°C for 16–18 hours. Colonies were counted and bacteria were Gram$^-$ stained and viewed under a microscope for morphological characterization. The viable counts are expressed as the log 10 of colony forming units (CFU) per gram of faeces. The colonic pH was measured using a pH meter kit with pH electrode SP 1DT (Merck). 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. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

Weight gain of the rats

The mean body weight of the rats at the begining of the experiment was 372.57±15.01 g and at the end of the experiment it rose to 398.16±33.19 g ($P<0.05$). Respectively, at the end of experiment in the control group the mean body weight increased by 2.1%, by 2.8% in EG1, by 0.8% in EG2, by 10.9% in EG3, and by 17.2% EG4. Food consumption was changed proportional to body weight of rats.

The effect of treatment on activities of glycolytic enzymes

The changes in activity of glycolytic enzymes in control group and experimental groups are summarized in Table 1. Inulin treatment alone (EG1) and in combination with bioactive natural substances significantly decreased activity of β-glucuronidase ($P<0.001$), and increased activities of β-galactosidase and β-glucosidase as compared to the control group. The tendency of changes in activity of glycolytic enzymes was similar in all experimental groups.

The effect of treatment on biochemical parameters and microbial analysis

In all treated groups a noticably decreased bile acid concentration was detected significantly in EG1, EG3 ($P<0.01$), and EG4 ($P<0.001$), and in EG2 nonsignificantly, as shown in Table 2. The total concentration of cholesterol and triacylglycerols was decreased significantly in EG1 ($P<0.001$), and nonsignificantly in the other experimental groups (Table 2). Changes in composition of short chain fatty acids — acetic, propionic, butyric in control and experimental groups are shown in Table 3. The counts of coliforms and lactobacilli are presented in Table 4. In the control group the count of coliforms was 4.67±1.03 $\log_{10}$ CFU/g and of lactobacilli 8.87±0.65

Table 2. Total cholesterol, triacylgylcerols and bile acids in serum in control and experimental groups.
Data represent mean ± standard deviation, ***P*<0.001; *** *P*<0.001

Groups	Total cholesterol (μmol/l)	Triacylglycerols (μmol/l)	Bile acids (μmol/l)
CG	1.39±0.22	1.05±0.32	16.84±6.33
EG1 (PRE)	0.83±0.19***	0.57±0.23***	11.72±4.22**
EG2 (PRE+HES)	0.94±0.16***	0.89±0.68	13.56±6.07
EG3 (PRE+O)	1.05±0.16	1.13±0.46	10.84±3.19**
EG4(PRE+HES+O)	1.14±0.16	0.89±0.54	5.76±2.66***

Statistical significance is comparison between CG *versus* EG1, EG2, EG3, EG4

Table 3. Composition of short chain fatty acids in faeces in control and experimental groups.
Total SCFA (mmol/100 ml of wet cecal digesta) = acetic + propionic + butyric + isobutyric + isovaleric + valeric + caproic. ***P*<0.01

Groups	Total SCFA	Acetic	Propionic	Butyric (% total SCFA)
CG	17.67	64.79	15.67	13.64
EG1 (PRE)	18.21	64.52	15.16	15.98**
EG2 (PRE+HES)	15.30	67.91	13.79	13.07
EG3 (PRE+O)	15.10	63.37	16.88	13.44
EG4(PRE+HES+O)	17.00	64.70	15.88	13.52

Statistical significance is comparison between CG *versus* EG1, EG2, EG3, EG4

Table 4. Counts of lactobacilli, coliforms and pH in faeces in control and experimental groups.
Data represent mean ± standard deviation, **P*<0.05

Groups	pH	Coliforms	Lactobacilli
CG	6.20	4.67±1.03	8.87±0.65
EG1	6.03	3.02±0.80*	9.56±0.75*
EG2	6.46	3.25±1.40*	9.12±0.93
EG3	5.66	2.89±0.25*	9.32±0.24

Statistical significance is between CG *versus* EG1, EG2, EG3, EG4

$\log_{10}$ CFU/g. The application of the prebiotic alone or in combinations decreased the counts of coliforms and increased counts of lactobacilli in all experimental groups.

DISCUSSION

Prebiotics are generally defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of bacteria in the colon that can improve the host health (Gibson *et al.*, 1995). This definition was updated in 2004 (Gibson *et al.,* 2004) and prebiotics are now defined as selectively fermented ingredients that allow specific changes in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host well-being and health. In order to be effective, a prebiotic must escape digestion in the upper gastrointestinal tract so that it can be released in the lower tract and used by the beneficial microorganisms in the colon, mainly bifidobacteria and lactobacilli.

Inulins as prebiotic are a group of non-digestible oligosaccharides, fructans. Inulin-type fructans exctracted from chicory roots are prebiotic food ingredients which in the gut lumen are fermented to lactic acid and short chain fatty acids (Hijova *et al.*, 2007; Alvaro *et al.*, 2008). Of these, butyrate and propionate inhibit growth of colon tumour cells and histone deacetylases. Butyrate also causes apoptosis, reduces metastasis in colon cell lines, and protects from genotoxic carcinogens. The elevated butyric acid concentration, and decreased concentrations of total cholesterol and triacylglycerol during experimental period are in accordance with experimental animal models which revealed that inulin-type fructans have anticarcinogenic properties (Pool-Zobel *et al.*, 2007), hypolipidaemic effect (Beylot, 2005), and anti-atherogenic effects (Rault-Nania *et al.,* 2006). A human intervention study (SYNCAN project) provided experimental evidence that inulin modulates parameters of colon cancer risk in human colon cells (Loo *et al.,* 2005). Activities of the bacterial glycolytic enzymes β-galactosidase (β-GAL), β-glucuronidase (β-GLUCUR), and β-glucosidase (β-GLU) have been determined in this study. Bacterial glycolytic enzyme activities are of relevance for fermentation of nutrients in the large intestine and health of the animal. Diet with a higher load of non-digestible oligosaccharides in the large intestine stimulated higher β-galactosidase activity. Bacterial β-galactosidase could also be involved in the hydrolysis of any undigested lactose reaching the large intestine. This enzyme is mainly produced by bifidobacteria and lactobacilli and its increase in large intestine substantiates a stimulatory effect of inulin on lactic acid bacteria (Lay *et al.*, 2004). β-glucuronidase activity is perceived as harmful for health as it is able to release carcinogens from hepatically derived glucuronic acid conjugates and is a critical factor in the enterohepatic circulation of drugs and other foreign compounds (Salminen *et al.*, 1998). The activity of β-glucosidase contributes to the hydrolysis of glucose monomers from nonstarch polysaccharides (e.g., cellulose, β-glucans), but it is also possible for β-glucosidase to be involved in the formation of toxic aglycons from plant glucosides (Pool-Zobel *et al.*, 2002). An increase in β-glucosidase activity could potentially be regarded as beneficial due to hydrolysis products of plant glucosides, some of which have more antimutagenic, antioxidative, anticarcinogenic and immune stimulatory properties than the respective glucosides. Therefore, its relevance for animal and human health will also depend upon the nature of dietary plant glucosides. The activity of these enzymes with toxicological importance could be altered by the diet, ultimately potentially decreasing the risk of carcinogenesis (Nalini *et al.*, 2004; Manju *et al.*, 2006).

In our study the number of lactobacilli was the highest in the group treated with the prebiotic alone, but a similar tendency was seen in other experimental groups. Coliforms were significantly reduced in the same groups of rats. Current knowledge shows that the colonic microflora is involved in the etiology of colorectal cancer. Intestinal bacteria can produce substances from dietary components that have genotoxic, carcinogenic, and tumour-promoting activities, and human feces have been shown to be genotoxic and cytotoxic to colon cells. The bacteria (e.g., coliforms, clostridia) play an important role in the process of development of colorectal carcinoma. The precise bacterial types associated with colorectal cancer risk have not been elucidated but it is clear that some bacterial groups (lactobacilli and bifidobacteria) have much lower activities of enzymes that can generate carcinogens than do other gut microflora components such as coliforms, clostridia and Bacteroides. This suggests that the balance of microbial types in the gut is important in terms of colorectal cancer risk and beneficial modulation of the intestinal microflora could decrease the colorectal cancer risk.

Most of the beneficial effects of *A. hippocastanum* (Horse chestnut) seed are attributed to its component beta-aescin or aescin; it also contains flavonoids, namely glycosides of quercetin and kaempferol. Beta-aescin is known to generate a wide variety of biochemical and pharmacological effects used in nutraceutical, cosmetic, and food supplement industries. Recent studies suggest that beta-aescin may be used in the treatment of chronic venous insufficiency, edema, hemorrhoids, and may possess anti-inflammatory, chemopreventive, anti-proliferative, apoptotic and anti-obesity efficacy (Patlolla *et al.*, 2006; Niu *et al.*, 2008; Hu *et al.*, 2008). A novel feature found in our study – the positive changes in bacterial enzymes, concentration of bile acids and lipids - confirmed the opinion that Hyppocastani extractum siccum may be a useful candidate agent for colon cancer chemoprevention and treatment. Fatty acid composition of dietary fat plays a vital role in colon tumour development in animal models. Fats containing omega-6 fatty acids (e.g., corn oil) enhanced and omega-3 fatty acids (e.g., flaxseed oil) reduced chemically induced colon tumour development in rats. Dietary flaxseed is high in lignan content. Lignans are phytoestrogens, good sources of dietary fibre, protein, antioxidant, and other nutritional elements and show preventive role in development of colon cancer tumour in experimental animals and humans (Bommareddy *et al.*, 2006; Theodoratou *et al.*, 2007). Although epidemiological and experimental studies indicate an association of elevated faecal levels of secondary bile acids as well as total bile acids with a high risk of colon cancer development, the cellular mechanism for the actions of bile acids is not clear (Cheng *et al.*, 2005; Hagiwara, 2006). Elevated concentration of bile acids in the control group was significantly reduced by administration of selected nutritional products.

In conclusion, dietary habits have been associated with aetiology and prevention of civilization diseases including colon cancer that represent the most serious health, economic, and social problem. For these reasons, the interest in prevention and therapy using substances of biotechnological and natural origin has been increasing worldwide. Among potentially protective foods, growing attention should be devoted to prebiotics which have health benefits. Results of our experiment show that the prebiotic inulin may be a useful candidate for colon cancer prevention and treatment.

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