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The Effect of Bifidobacteria and Various Oligosaccharides Consumption on the Risk of Colon Cancer in Rats*

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This study examined the effect of viable bifidobacteria and non-digestible carbohydrates on the cecal pH, colonic neoplastic lesion (aberrant crypt) and proliferating cell nuclear antigen (PCNA) labeling index in carcinogen-treated rats. Animals received s.c. injection of dimethylhydrazine (DMH) (15 mg/kg body weight) twice 3 days apart. Three days after the second carcinogen administration, the treatments were begun. The treatments were basal diet (AIN-76) with skim milk (Basal/skim), or the following diets with daily gavage of 10^8 bifidobacteria: basal (Basal/bifido), 2% fructo-oligosaccharide (FOS/bifido), 2% soybean oligosaccharide (SBO/bifido), 2% wheat bran oligosaccharide (WBO/bifido) and 8.4% wheat bran (WB/bifido). After 4 weeks of treatment, cecal pH was measured using a pH probe. The number of aberrant crypt (AC), aberrant crypt foci (ACF) and crypt multiplicity were enumerated and colonic PCNA labeling index was determined using immunohistochemistry. Cecal pH was significantly reduced in SBO/bifido and FOS/bifido groups compared to control group. However, there were no significant differences in either number of AC or rates of cell proliferation as shown by PCNA labeling index among the groups, although rats fed FOS/bifido reduced the numbers of ACF compared to Basal/skim group. The SBO/bifido group did not reduce the number of ACF or PCNA labeling index. Also, other oligosaccharides did not reduce the risk of colon cancer compared to control group. The concomitant reduction of cecal pH and number of ACF suggest that the combination of bifidobacteria and FOS may reduce the risk of colon cancer.

Key words: Colon cancer, Bifidobacteria, Oligosaccharide, Aberrant crypt, Rats

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INTRODUCTION

Epidemiological and animal studies have demonstrated that there is a strong association between diet, intestinal microflora and the incidence of colon cancer. It has been suggested that the balance between pathogenic and nonpathogenic members of colonic microflora will influence the colonic health[1,2] and diet has a major influence on the modulating capacity of the composition and metabolic activity of intestinal microflora.[3] The ability of microflora to compete for available nutrients and possibility for adhesion sites of microflora on the colonic mucosa is likely to be an important factor that determines the composition of the intestinal flora. Thus, there has been a strong interest in manipulating the composition of microflora in the colons to a more favorable direction, by increasing the populations and activities of beneficial bacterial groups such as bifidobacterium and lactobacillus and decreasing the numbers of potential pathogenic bacteria, *Clostridium perfringens*, bacteroides.

Probiotics, as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance[4], are mostly lactic acid bacteria producing acetic and lactic acid, which tend to lower the colonic pH, therefore presumed to inhibit the proliferation of pathogenic microorganisms. The beneficial effects of bifidobacteria pertaining to the improvement of intestinal flora have been reviewed by Sanders.[5] Of several beneficial effects of bifidobacteria, the protective effect against colon tumorigenesis has been examined in several studies.[6-8]

Colonic bacterial population may also be modulated by consumption of nonabsorbable, but highly fermentable carbohydrates that selectively stimulate the growth of beneficial microflora. A prebiotic, as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, improves host health.[9,10] Gibson *et al.*[11] reported that adults on fructo-oligosaccharides

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(FOS) and inulin feeding significantly increased populations of bifidobacteria whereas numbers of harmful bacteria, bacteriodes, clostridia and fusobacteria were decreased. Since FOS was frequently used as prebiotics in several experiments, we have questioned if other types of oligosaccharides exert similar properties of prebiotics. The type of oligosaccharide appears to influence colonic bacterial populations and bacterial enzyme activity.[12,13] It has been noted that galacto-oligosaccharides and soybean oligosaccharides may also act as prebiotics.[9,14]

Aberrant crypt foci (ACF) are suggested as the earliest detectable preneoplastic lesions of colon found in animals treated with a colon carcinogen and in humans with an increased risk of colon cancer.[16] Aberrant crypts (AC) are larger than normal crypts, have a thicker epithelial lining, often have elliptically shaped lumina, and have an increased pericryptal zone. Considerable evidence supports the concept that the number of AC is predictive of eventual tumor formation.[17] Enhanced cell proliferation of colonic epithelium has been associated in both humans and animals with a higher risk of colon carcinogenesis[18] and has been proposed as a marker of increased susceptibility of carcinogenesis.[19] Proliferating cell nuclear antigen (PCNA) is the auxiliary protein of DNA polymerase δ. PCNA labeling index has been used as a marker of cell proliferation as its value is correlated with the uptake of DNA precursor labels such as bromodeoxyuridine (BrdU).[20] In fact, a study by Singh *et al.*,[7] reported that *B. longum* supplementation significantly inhibited azoxymethane (AOM)-induced cell proliferation using BrdU labeling index as a marker.

Therefore, the purpose of this study was to examine the effect of the administration of viable bifidobacteria and various kinds of oligosaccharides (fructo-oligosaccharide, soybean oligosaccharide and wheat bran oligosaccharide) in amount that would be achievable in physiological range, on the cecal pH, development of AC and ACF, and the changes of colonic cell proliferation using PCNA labeling index. We added wheat bran group to compare the effect of pure wheat bran oligosaccharide and wheat bran that would contain comparable amount of wheat bran oligosaccharides.

MATERIALS AND METHODS

1. Animals and Diets

Male Wistar rats with initial weights of 75-100 g were housed individually in wire-bottomed stainless steel cages in a temperature-controlled room (25 ℃) with the dark period from 1800 to 0600 hr. One hundred twenty six rats were given the same basal diet, a modified AIN-76,[31] before and during carcinogen treatment. The composition of basal diet was as follows (g/kg): casein, 200; cornstarch, 450; sucrose, 150; corn oil, 100; cellulose, 50; DL-methionine, 3; AIN-76 mineral mix, 35; AIN-76 vitamin mix, 10; choline bitartrate, 2; butylated hydroxy toluene (BHT), 0.02; and menadione sodium bisulfite complex, 0.0013. The BHT and menadione sodium bisulfite were dissolved in the oil. Experimental diets consisted of the basal diet and 2% fructo-oligosaccharide (FOS, GTC, USA), 2% soybean oligosaccharide (SBO, Ajinomoto, Japan), 2% wheat bran oligosaccharide (WBO, Warriwood, Australia), an arabino-xylan and 8.4% wheat bran. In all experimental diets, the oligosaccharides were added to a final concentration of 2% by whole diet dilution. Wheat bran was added to a final concentration of 8.4%, which provided an amount of oligosaccharide equivalent to the 2% of wheat bran oligosaccharide. Rats were given free access to food and water throughout the experimental period. Food intake and body weights were measured weekly.

2. Experimental Design

Rats were treated with the colon carcinogen 1,2-dimethylhydrazine (DMH, Sigma, USA) to induce AC formation. Each rat was injected subcutaneously with two doses of DMH (15 mg/kg body weight) 3 days apart. Three days after the last carcinogen administration, the rats were divided into 6 treatment groups (n=21) and received a daily gavage of 10^8 CFU bifidobacteria delivered in 1 mL of skim milk, except the control group, which was gavaged with only skim milk. Thus, the dietary treatments were as follows: Basal/skim (AIN-76+skim milk), Basal/bifido (AIN-76+bifidobacteria), FOS/bifido (2% FOS+bifidobacteria), SBO/bifido (2% SBO+bifidobacteria), WBO/bifido (2% WBO+bifidobacteria) or WB/bifido (8.4% wheat bran+ bifidobacteria). WB/bifido group was added to compare whether wheat bran containing similar concentration of WBO had comparable influence on the risk of colon cancer. Wheat bran was added to achieve the same concentration of WBO group. After 4 weeks of experimental treatment, rats were killed by CO_2 asphyxiation. The cecum was excised from the rats and pH of the cecal contents was measured using a small pH probe. Bifidobacteria and *C. perfringens* were enumerated from cecal contents. The colons of 15 rats from each group were prepared for aberrant crypt enumeration. The ACF of remaining rats were dissected under dissecting microscope and these ACF sections were embedded in paraffin and processed for the immunohistochemical determination of PCNA.

3. Aberrant Crypt Enumeration.

AC and ACF were enumerated in the distal section by a modification of the method of Bird.[21] Colons were rinsed with phosphate buffered saline (PBS) to remove the fecal contents, then slid onto a 2 mL glass pipette and fixed for 5 minutes in 10% formalin-PBS. The colons were sliced open and fixed flat between filter paper submerged in 10% formalin-PBS until microscopic examination. A 2×5 cm^2 section of the colon, ~2 cm from the anal end was removed and stained with 0.2% methylene blue chloride-PBS and counted at 100×magnification with the mucosal side up. Aberrant crypts (AC) were defined as easily recognizable mucosal alterations that were characteristically larger and more elongated than normal crypts. The number of ACF was counted by AC appearing together as a cluster forming a single unit. Crypt multiplicity was calculated by the number of AC divided by the number of ACF.

4. Immunohistochemical Detection of PCNA.

Cell proliferation was determined using PCNA labeling index of colonic crypts. The labeling index was determined as the ratio of antibody stained cells per total number of cells in each crypt. Colonic tissue sections were embedded in paraffin and processed for the PCNA immunohistochemistry. Thin sections (4 μm) were placed on polylysine-coated slides. Sections were deparaffinized in xylene and rehydrated with a graded ethanol series. Endogenous peroxidase was quenched by incubation in 0.3% H_2O_2, followed by rinsing with PBS. Detection of PCNA was accomplished with a mouse monoclonal PCNA antibody (Ab-1, Oncogene Science, USA) and a commercial kit (Unitect kit, Oncogene Science, USA) using an avidin-biotin complex (ABC) method. Cells with darkly stained nuclei were assumed to be PCNA positive and were considered to be in proliferative stage.

5. Statistical Analysis.

Data were analyzed by one-way analysis of variance (ANOVA) using Sigmastat for Windows version 1.0 (Jandel Scientific, USA). AC and ACF were analyzed using non-parametric analysis of variance due to lack of equal variance among groups. When the p value was<0.05, differences among groups were analyzed by use of the Student-Newman-Keuls method.

RESULTS

1. Body Weights and Food Intakes

Body weights at the start and final point of experimental diets and food intake in the final week are summarized in Table 1. No statistically significant differences were observed in weekly body weight among the experimental groups throughout the study. Food intake of the first and second week did not differ among groups, but during the third and fourth week, there were significant differences among treatments. Since the trends of weekly food intake were similar for both weeks, only results of final week of food intake are presented here. Food intake was significantly less in the WBO/bifido group compared with the Basal/bifido, SBO/bifido and WB/bifido group.

Table 1. Body Weight and Daily Food Intake[1,2]

Diet Groups	Initial weight at start of treatment(g)	Final body weight(g)	Food intake (g/day[3])
Basal/skim	131.7±2.3	388.1±10.1	26.5±0.9^{ab}
Basal/bifido	131.6±2.5	386.6±10.3	27.9±0.7^{a}
FOS/bifido	131.6±2.2	380.5± 8.6	26.3±0.7^{ab}
SBO/bifido	131.6±2.7	382.6± 8.3	28.2±0.9^{a}
WBO/bifido	131.7±2.2	363.8± 6.6	24.2±0.6^{b}
WB/bifido	131.5±2.1	388.5± 8.2	27.0±0.5^{a}

1) Values are mean±SEM, n=21.
Values in a column not sharing a superscript are significantly different ($p<0.05$) by one way ANOVA followed by Student-Newman-Keuls method.
2) Abbreviations used: Basal/skim = AIN-76 diet and 1 mL skim milk daily; Basal/bifido = AIN-76 diet and 1 mL skim milk containing 10^8 bifidobacteria daily; FOS/bifido = AIN-76 diet + 2% fructooligosaccharides and skim milk containing 10^8 bifidobacteria; SBO/bifido = AIN-76 diet + 2% soybean oligosaccharides and 1 mL skim milk containing 108 bifidobacteria; WBO/bifido = AIN-76 diet + 2% wheat bran oligosaccharides and 1 mL skim milk containing 10^8 bifidobacteria; WB/Bifido = AIN-76 diet + 8.4% wheat bran and 1 mL skim milk containing 10^8 bifidobacteria.
3) Since the first and second week of food intakes were not statistically different and the trends of weekly food intake were similar for third and final week, only final week of food intake are presented.

2. Cecal pH

The cecal pH from rats fed the various oligosaccharides and bifidobacteria are shown in Figure 1. FOS and SBO fed groups had significantly reduced cecal pH compared

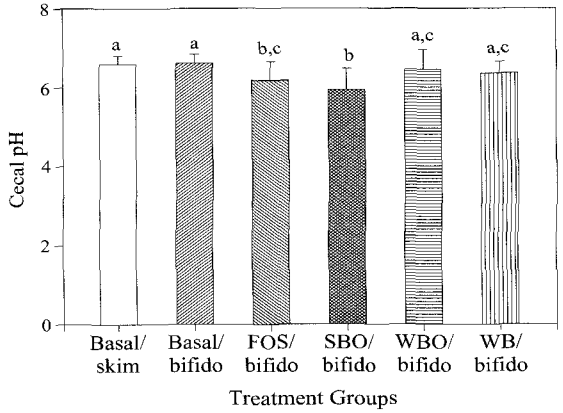

Fig. 1 Effect of various oligosaccharides and bifidobacteria administration on pH in rat cecal contents.

Bars with different letters are significantly different ($P<0.05$) by one way ANOVA followed by Student-Newman-Keuls method.
Values are means±SEM. N=21 rats per group. See Table 1 for dietary group abbreviations.

with control groups. SBO fed rats had significantly lower cecal pH than all groups except FOS fed rats. No significant differences were found among the control, WBO, and WB fed rats.

3. Aberrant Crypt and Foci Enumeration

The effect of different oligosaccharides and bifidobacteria administration on the number of AC and ACF is shown in Table 2. There were no significant differences among the treatment groups in the total number of AC or number of ACF. However, the FOS/bifido group showed a trend toward a lower number of ACF compared to control group (Basal/skim), and this difference was significant by Student t-test (P=0.04).

Table 2. Effect of Oligosaccharides and Bifidobacteria on Aberrant Crypt Formation[1,2] (Per cm^2)

Diet	Aberrant crypt	Aberrant crypt foci	Crypt multiplicity
Basal/skim	1.82±(1.36-3.38)	0.99±(0.91-1.55)	1.61±(1.54-1.83)
Basal/bifido	1.56±(1.24-2.45)	0.95±(0.72-1.32)	1.74±(1.48-2.44)
FOS/bifido	1.16±(0.74-1.55)	0.61±(0.40-1.06)*	1.90±(1.35-2.49)
SBO/bifido	1.44±(0.56-2.85)	0.93±(0.41-1.65)	1.56±(1.30-1.73)
WBO/bifido	1.90±(1.47-2.74)	1.49±(0.85-2.31)	1.76±(1.56-1.88)
WB/bifido	2.31±(1.51-4.11)	1.13±(0.78-1.50)	1.75±(1.59-1.86)

1) Since data are not normally distributed, values were presented as median (10^{th}-90^{th} percentile), n=15 rats per group. No statistical significant differences were found among the groups by one way ANOVA.
2) See Table 1 for dietary group abbreviations.
* Aberrant crypt foci of FOS/bifido group is significantly different from basal/skim group by t-test (p=0.0401).

4. PCNA Labeling Index

The rates of distal colonic mucosal cell proliferation were determined using PCNA labeling index and shown in Figure 2. No statistically significant differences among the experimental groups were detected in the PCNA labeling index by ANOVA. There was no significant correlation between the median number of aberrant crypts and mean labeling index for each group (r = 0.20, P>0.05).

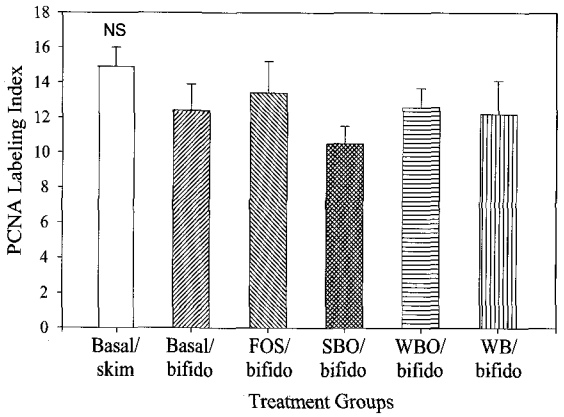

Fig. 2 Effect of various oligosaccharides and bifidobacteria administration on PCNA Labeling Index.
NS, no statistical significant differences were found among the groups by one way ANOVA.
Values are means±SEM.
N=15 rats per group.
See Table 1 for dietary group abbreviations.

DISCUSSION

In the present study, supplementation of a basal diet with 2% (20 g/Kg diet) various oligosaccharides and administration of bifidobacteria for 4 weeks did not affect the body weight of animals treated with the carcinogen DMH, although the last two weeks of weekly food intake were significantly less in a WBO/bifido feeding group. However, the reason for this remains uncertain.

Several studies reported that the administration of bifidobacteria decreases tumor incidence and ACF counts in the colon of animals. Also, *in vivo* studies demonstrated that diets providing oligosaccharides that resist digestion and reach the colon so as to stimulate the growth and metabolism of beneficial bacteria could decrease the risk of colon cancer in animals.[6,16,19]

Koo and Rao[15] reported a protective effect of administration of 10^9 bifidobacteria twice per week and 5% oligofructose by decreasing the number of AC and ACF in DMH-induced colon carcinogenesis in mice. This study did not separate the effect of the bifidobacteria from that of the oligofructose-containing diet. Therefore, we designed the experiment so that the effect of probiotics (bifidobacteria) and prebiotics (oligosaccharides) could be separated and to examine whether an additive effect of the two agents occurred. In addition, it was of interest that other types of oligosaccharides could exert the comparable effect as prebiotics using formation of AC and rates of cell proliferation. Also, whether wheat bran, which in a number of studies has been found to reduce colon tumors, would affect the same way as WBO diet was examined.

Reddy and Rivenson[6] reported that addition of 0.5% Bifidobacterium longum (*B. longum*) in the diet reduced tumor incidence effectively. Kulkarni and Reddy[22] found that rats given carcinogen azoxymethane (AOM) and fed diets containing 1.5% lyophilized cultures of *B. longum* had a 43% reduction in the total number of AC relative to a control diet containing no bifidobacteria. In addition, Singh *et al.*[7] reported the greater reduction of colon tumor incidence, multiplicity and volume by 2% lyophilized cultures of *B. longum* on AOM-induced F344 rats in a 40-week feeding study. These results clearly indicate that the consumption of bifidobacteria reduces colon cancer risk

in carcinogen-treated animals, indicating that bifidobacteria are acting as probiotics. However, administration of bifidobacteria 10^8 CFU by daily gavage in 1 mL of skim milk did not show any inhibitory effect of colon carcinogenesis in the present study. Previously reported studies supplemented 0.5%~2% bifidobacteria in the diet which would be equivalent to 4×10^{10} live bacteria/g diet per rat. This might be the reason of discrepancy which we did not find the effect of bifidobacteria administration on the reduction of ACF number.

Reddy *et al.*[23] examined the effect of consumption of 10% FOS and inulin on AOM-induced AC formation in male F344 rats for 7 weeks. Addition of FOS and inulin in the diet significantly inhibited the total number of AC formation and crypt multiplicity. Also, the administration of 6% of FOS and xylooligosaccharides inhibited the development of ACF and decreased cecal pH in DMH-treated rats.[24]

In this study, we failed to show a significant inhibition of AC or ACF formation by either bifidobacteria supplement alone or a combination of bifidobacteria and various oligosaccharides treatment, although FOS/bifido group showed a trend toward to decrease the number of AC compared to control group. However, in a paired comparison between the control group (Basal/skim) and FOS/bifido group, there was a significant reduction of ACF in FOS/bifido fed rats. This indicates an additive, or synbiotic effect of fructo-oligosaccharide and bifidobacteria for the reduction of colon cancer risk in carcinogen-treated rats. In agreement with our results, a study by Rowland *et al.*[25] reported that *B. longum* (4×10^8 viable cells /g diet or 5.9×10^9 c.f.u./rat daily) and inulin (5%) resulted in the inhibition of ACF development in rats treated with AOM, and there was a synergistic effect of the synbiotic combination. In comparison with the present study, investigators in previous experiments which showed an inhibition of colon carcinogenesis have used greater levels of fructo-oligosaccharide, e.g. 5~10% FOS, but we used 2% of oligosaccharides (including fructo-oligosaccharide) which we believe that it might be achievable in the human diet in the present study.

Gallaher *et al.*[26] conducted a series of studies involved in feeding of fructo-oligosaccharide and administration of viable bifidobacteria, lactobacillus by gavage. They found that either FOS or bifidobacteria alone did not reduce the number of AC or ACF. However, the combination of FOS and bifidobacteria induced significantly fewer number of AC and ACF in one study, however, this effect was not consistent in a series of following studies. In addition, they did not find any association of populations of cecal bifidobacteria and *C. perfringens* with the AC or ACF counting or with the administration of microorganisms. To date, all of the studies performed have characterized the gut flora at the genus level. Distinguishing individual species of bifidobaterium or lactobacillus in the mixed culture fermentation is extremely difficult through standard phenotypic criteria. Therefore, it is necessary to develop convenient and reliable molecular methods of identification of bifidobacterium species, since it is possible that preferred prebiotics would target individual species.

Proposed mechanisms to explain the inhibition of pathogenic bacterial growth include decreased colonic pH by the production of acetate and lactate, competition for substrate with other harmful bacteria species and decreased levels of enzymes that produce carcinogens. Of the proposed mechanisms associated with colon tumor inhibition, decreased pH may reduce the conversion of primary to secondary bile acids, which are thought as tumor promoters.[27] Lower pH may also stimulate mucus production, providing protection against carcinogens.[24,28] The SBO/bifido group significantly reduced the cecal pH, even though, no concurrent reduction of the number of AC occurred. However, the FOS/bifido group lowered the cecal pH compared to the Basal/Skim and decreased AC formation in comparison with Basal/skim group. The protective effect of bifidobacteria through the lowering of pH was inconsistent and depended on the oligosaccharide supplementation.

An abnormal cell proliferation is thought to be an early feature within the carcinogenic process. PCNA labeling index has been reported to be a reliable predictor of the prognosis of patients with malignant diseases such as gastric cancer and correlated well with other cell proliferation marker (i.e. BrdU).[20] We tried to quantify proliferating cells and apoptotic cells in aberrant crypts. However, we could only quantify PCNA labeled cells and were unable to get apoptotic index. It was because due to the difficulty of identifying and determining the ACF sections in longitudinal sections, obtaining longitudinal sections in which the entire crypt and lumen are visible. Also, the tortuous nature of the crypts in ACF was problematic in counting the epithelial cells along the crypt. In addition, due to the time consumed to separate aberrant crypts, there were too many necrosis occurred in colonic epithelium. This effect resulted in high false apoptotic labeling indices. The present study did not find any significant difference in cell proliferation among the treatment groups. In fact, several recent studies have not found any significant difference in rates of cell proliferation in spite of having different tumor incidence among dietary groups.[29,30] Thus,

this inability to identify the risk of colon cancer has been casting doubt on the usefulness of cell proliferation as a marker of cancer susceptibility.

In summary, our study suggests that the combination of probiotics (as bifidobacteria) and prebiotics (fructo-oligosaccharide) reduces the risk of colon cancer using the AC formation as a marker, which can be called an effect of synbiotics. However, the chemopreventive property of bifidobacteria alone and other oligosaccharides is uncertain based on this study. Also, if we were able to quantify the apoptotic index in conjunction with cell proliferation index, that would give us a more defined effect of bifidobacteria and various oligosaccharide consumption on the risk of colon cancer. Therefore, the effect and mechanism of synbiotics in relation with colon carcinogenesis deserves further investigation.

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