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# Effect of a Functional Food Containing *Bacillus polyfermenticus* on Dimethylhydrazine-Induced Colon Aberrant Crypt Formation and the Antioxidant System in Fisher 344 Male Rats

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**Abstract** The goal of this study was to investigate the effects of a newly developed functional food containing *Bacillus polyfermenticus* (BP) and other physiologically active materials on the antioxidant system and the process of colon carcinogenesis in male F344 rats. Following a one-week adaptation period, the rats were divided into 3 groups and fed either a high-fat, low-fiber diet (control and DMH groups), or a high-fat, low-fiber diet supplemented with *B. polyfermenticus* ($3.1\times 10^8$ CFU/day) and other physiologically active materials (chitosan, chicory, α-tocopherol, and flavonoids) (DMH+*BP* group). One week after the initiation of the diets, 2 groups of rats were subjected to six weeks of treatment with 1,2-dimethylhydrazine (DMH, 180 mg/kg BW, s.c.). The dietary treatments remained consistent throughout the entire experimental period. Nine weeks after the initial DMH injection, the rats supplemented with *B. polyfermenticus* had significantly lower numbers of aberrant crypt foci than those in the DMH group. Injections with DMH resulted in significantly higher leukocytic DNA damage and plasma lipid peroxidation levels, as well as in a lower plasma total antioxidant potential. These effects were reversed following supplementation with *B. polyfermenticus* and other physiological materials. Our results indicate that a functional food containing *B. polyfermenticus* exerts a protective effect on the antioxidant system and on the process of colon carcinogenesis, thereby suppressing the development of preneoplastic lesions.

**Key words:** functional food, *Bacillus polyfermenticus*, colon carcinogenesis, DNA damage, antioxidant system

## Introduction

Probiotics, which are defined as viable microbial dietary supplements that have beneficial effects on a host organism through their effects on the intestinal tract, are quickly gaining attention as functional foods in this era of self-care and complementary medicine (1). Probiotics, which have historically been used for medicinal purposes, include strains of *Bifidobacterium, Lactobacillus, Enterococcus, Clostridium butyticum, Lactobacillus sporogenes, Bacillus subtilits*, and *Bacillus polyfermenticus* (1, 2). Strains of *B. polyfermenticus*, commonly known as 'Bispan' strains, have been used to treat long-term intestinal disorders, since the live strains can reach the intestine in the form of active endospores. This strain produces a variety of enzymes that lyse a number of pathogenic strains such as typhoid bacillus, paratyphoid bacillus, shigella, and cholera (3). We recently reported on the *in vitro* antioxidative effects of the culture supernatant of *B. polyfermenticus* (4). *B. polyfermenticus* was also found to protect against the DNA damage induced by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), a direct-acting carcinogen with alkylating properties, in CHO-$K_1$ cells and in human lymphocytes. *B. polyfermenticus* was also found to inhibit the growth of Caco-2 colon cancer cells in humans in a dose-dependent manner, as shown by the results of an MTT assay (5).

Colon cancer is one of the leading causes of morbidity and mortality not only in western industrialized countries but also in developed Asian countries. This suggests that a strong linkage exists between colon cancer and changes in the lifestyles of Asian people, especially in regards to their diet (6). Much attention has been focused on reducing the colon cancer risk through dietary alterations, particularly in terms of increasing dietary fiber intake and the consumption of probiotics (7).

During colon carcinogenesis, aberrant crypts are recognized as early neoplastic lesions both in rodent and human colons (8). A number of natural chemopreventive agents or medicinal plants that inhibit aberrant crypt foci (ACF) development have also been proven to prevent colon cancer development in rodents (9, 10), suggesting that the ACF assay in the rodent colon can be used as a good biomarker for colon carcinogenesis. It is also known that colon carcinogenesis is a pathological consequence of persistent oxidative stress, which leads to DNA damage and mutations in cancer-related genes (11-14).

We have recently developed a functional food containing *B. polyfermenticus* as its primary material and that includes other physiologically active materials such as chitosan, chicory, α-tocopherol, and flavonoids. Therefore, the objective of this study was to determine the effects of this newly developed functional food on 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis via the correlation of antioxidant status and DNA damage, and by the concurrent modulation of ACF in F344 rats.

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## Materials and Methods

**Reagents** Casein, mineral mixture, and vitamin mixture were ICN Pharmaceuticals Inc. (Costa Mesa, CA, USA). Corn starch was obtained from Daesang Co. (Seoul, Korea) and corn oil and lard were from Cheiljedang Co. (Seoul. Korea) and Shinhan Oil Co. (Gosung, Korea), respectively. Tryptic soy broth (TSB) was purchased from Difco Laboratories (Detroit, MI, USA), and cyclohexane was from Merck (Whitehouse Station, NJ, USA). All other chemicals used in this study were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA).

**Preparation of *B. polyfermenticus*** The producer strain of *B. polyfermenticus* was maintained at -70°C in a TSB to which 20%(v/v) glycerol was added. The working cultures were propagated in TSB with shaking at 37°C. *B. polyfermenticus* production was performed as previously described (15). Briefly, *B. polyfermenticus* was inoculated into 60 mL of sterile TSB and the seed culture (2%, v/v) was then transferred to a jar fermenter (3 L working volume; Korea Fermenter Co., Incheon, Korea). The temperature was maintained at 37°C and the pH was maintained at 7.0±0.1. *B. polyfermenticus* was obtained by centrifugation (8,000×g, 30 min) of the resulting stationary-phase cells following 72 hr of incubation. The pellets were then freeze-dried and stored at 4°C.

**Animal and diets** Five week-old male F344 rats (185±10 g) were purchased from Samtako Inc. (Osan, Korea) and were housed individually in hanging wire cages in a room controlled for humidity (55%) and temperature (25°C) were kept under a 12/12-hr light-dark cycle. The animals were cared for in accordance with the Guide for Care and Use of Laboratory Animals (16). The rats were allowed free access to water and were fed a commercially prepared pelleted diet for adjustment for the first week. The F344 rats were divided into 3 groups of 10 animals each, and were fed either a high-fat, low-fiber diet (control and DMH group) or a high-fat, low-fiber diet supplemented with *B. polyfermenticus* ($3\times10^8$ CFU/day) (DMH+*BP* group). The *B. polyfermenticus* products also contained chitosan (0.25 g/day), chicory (0.3 g/day), α-tocopherol (0.007 g/day), and flavonoids (0.02 g/day). The diet consisted of starch (51%), casein (20%), corn oil (10%), lard (12%), cellulose (2%), a vitamin and mineral mixture (3.5 and 1%, AIN-93; American Institute of Nutrition, 1993) (17), choline bitartarate (0.25%), DL-methionine (0.3%) and butylated hydroxy toluene (0.001 %). One week after the initiation of the diets, the rats were treated with 1,2-dimethylhydrazine (DMH, 30 mg/kg, s.c.) for 6 weeks with the exception of the normal control group, which was treated with saline instead. The dietary treat-ments were sustained throughout the entire experiment. The general health of the animals was assessed daily and their body weights were recorded every week for the duration of the study. Following the 10-week experimental period, the rats were anesthetized with ethyl ether and the entire colon was collected from each animal for aberrant crypt analysis and blood was collected from the abdominal artery and placed in a heparinated sterile tube. Whole blood was freshly prepared for use in the comet assay. Plasma was obtained from the blood samples by centrifugation (700×g for 30 min) and stored at -80°C until further analysis.

**Aberrant crypt analysis** Aberrant crypt analysis was performed on a 5 cm segment corresponding to the distal part of the colon. The segment was washed with physiological saline (0.9% NaCl), cut open, and fixed in a 10% buffered formalin solution for at least 24 hr. The colon samples were then stained with 0.2% methylene blue for 5 min, and the mucosal side was placed on a glass slide and examined microscopically under a 10× objective in order to assess the number of aberrant crypts according to the procedure described by Bird (8).

**DNA damage determination by alkaline comet assay** The alkaline comet assay was conducted according to the protocol established by Singh *et al.* (18), with slight modification. Frosted slides (Fisher Scientific, Pittsburgh, PA, USA) were prepared with a basal layer of 0.5% NMA, and 5 μL of whole blood, mixed with 75 μL of 0.7% low melting agarose (LMA) was then added to the slides. The slides were again covered with coverslips and refrigerated for 10 min. The coverslips were then removed and a top layer of 75 μL of 0.7% LMA was added prior to placing the slides (with coverslips) in the refrigerator for another 10 min. After removal of the coverslips, the slides were immersed in a jar containing cold lysing solution (pH 10), consisting of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylsarcosine; 1% Triton X-100 and 10% DMSO were freshly added to the solution, which was then refridgerated for 1 hr. After lysis, the slides were placed in a horizontal electrophoresis tank (Threeshine Co., Ltd., Daejeon, Korea). The slides were then covered with a fresh alkaline buffer (300 mM NaOH, 10 mM $Na_2EDTA$, pH 13.0) and incubated at 4°C for 40 min. Electrophoresis of the DNA was executed by applying an electric current of 25 V/300±3 mA for 20 min at 4°C. The slides were washed 3 times with neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C and were then treated with ethanol for another 5 min. All steps following the lysis treatment were performed in darkness in order to prevent any additional DNA damage. Fifty μL of ethidium bromide (20 μg/mL) were added to each slide and the slides were then analyzed using a fluorescence microscope (LEICA DMLB, Wetzlar, Germany). Images of 100 cells randomly selected from each subject (50 cells from each of two replicate slides) were analyzed, and measurements were made by image analysis (Komet 4.0; Kinetic Imaging, Liverpool, UK), to determine the percentage of DNA in the tail, the tail length and the tail moment (TM, calculated as the percentage of DNA in the tail multiplied by the tail length).

**Plasma total radical trapping antioxidant potential (TRAP)** The plasma total radical trapping antioxidant potential (TRAP) was measured using a modification of the photometric method developed by Rice-Evans and Miller (19). The method used to measure the antioxidant activity is predicated on the antioxidant-induced inhibition of the absorbance of the radical cation of 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate) ($ABTS^+$). The $ABTS^+$

radical cation is formed by the interaction of $ABTS^+$ (150 μM) with the ferrylmyoglobin radical species, which is generated by the $H_2O_2$-induced (75 μM) activation of metmyoglobin (2.5 μM). Ten mL of sample/buffer/Trolox-standard were added to tubes containing 400 μL of PBS buffer, 20 μL of metmyoglobin, and 400 μL of ABTS, and the solution was then vortexed. The reaction was initiated by the addition of 170 μL of $H_2O_2$. Following 6 min of incubation, the absorbance was measured at 734 nm using a spectrophotometer (Shimazu, Tokyo, Japan). These values are expressed as the Trolox equivalent antioxidant capacity (TEAC) and are defined as the millimolar concentration of the Trolox antioxidant capacity of a calibration curve.

**Baseline conjugated dienes in LDL** The baseline low density lipoprotein (LDL) conjugated diene levels were determined according to the methods outlined by Ahotupa *et al.* (20), with slight modifications. Plasma (100 μL) was added to 700 μL of heparin citrate buffer (0.064 M trisodium citrate, 50,000 IU/L heparin, pH 5.05), and this suspension was incubated for 10 min at room temperature. The insoluble lipoproteins were then sedimented by centrifugation at 1,000×g for 10 min. The pellet was resuspended in 100 μL of 0.1 M Na-phosphate buffer containing 0.9% NaCl (pH 7.4). Lipids were extracted from 100 mL of the LDL suspension with chloroform-methanol (2:1), dried under nitrogen, redissolved in cyclohexane, and analyzed at 234 nm using a spectrophotometer (Shimazu). Ethylenediamine tetraacetate (EDTA) was added to the sample to prevent oxidation during sample preparation.

**Statistical analysis** The data was analyzed using the SPSS package for Windows (Version 10). The values were expressed as the mean ± standard error (SE). The data was evaluated using one-way ANOVA and the differences between the means were assessed using Duncan's multiple range test. A Student's *t*-test was employed to compare the DMH group with the *B. polyfermenticus* supplement group. The differences were considered significant at $p<0.05$.

## Results and Discussion

**Food intake, weight gain, and organ weight** During this experiment, no treatment-associated signs of adverse effects in the clinical appearance of the animals were observed. Although DMH injection and *B. polyfermenticus* supplementation induced a statistically significant re duction in food consumption and final body weight, no differences in the food efficiency ratio (FER) were observed among the groups (Table 1). DMH injection resulted in a slight reduction in the relative organ weights in comparison to the saline-injected control group, with the exception of the spleen. However, no differences were detected in the relative organ weights between the DMH group and the DMH+*BP* group (Table 1).

**Plasma antioxidant potential and lipid peroxidation** DMH administration resulted in a reduction in the plasma TRAP value, an indicator of the total antioxidant defense, coupled with an increase in plasma conjugated dienes,

**Table 1. Effects of a functional food containing *Bacillus polyfermenticus* on the weight gain, food efficiency ratio, and relative organ weights in rats administrated DMH[1)]**

| | Control[2)] | DMH | DMH+*BP* |
|---|---|---|---|
| Initial body weight (g) | 115.0±1.6[ns4)] | 115.0±7.8 | 115.1±6.8 |
| Final body weight (g) | 308.4±7.5[b5)] | 290.8±5.7[a] | 289.8±3.4[a] |
| Weight gain (g/day) | 2.8±0.1[ns] | 2.5±0.1 | 2.5±0.1 |
| Food intake (g/day) | 19.0±0.1[c] | 18.7±0.1[b] | 18.3±0.1[a] |
| FER[3)] (%) | 14.6±0.5[ns] | 13.5±0.4 | 13.6±0.5 |
| **Organ weight (g/100 g BW)** | | | |
| Liver | 2.43±0.08[b] | 2.18±0.08[a] | 2.14±0.04[a] |
| Heart | 0.31±0.01[b] | 0.28±0.01[ab] | 0.27±0.01[a] |
| Kidney | 0.61±0.01[b] | 0.54±0.01[a] | 0.55±0.01[a] |
| Spleen | 0.19±0.00[ns] | 0.18±0.00 | 0.19±0.00 |

[1)]Values are the mean±SE for 10 animals in each group.
[2)]Control, high-fat and low-fiber diet-fed group; DMH, high-fat and low-fiber diet + DMH injected group; DMH+*BP*, high-fat and low-fiber diet + functional food containing *B. polyfermenticus* ($3.1\times10^8$ CFU/day) + DMH injected group.
[3)]FER, food efficiency ratio. [4)]ns, not significant.
[5)]Values in the same row that do not share a common superscript are significantly different at $p<0.05$.

which function as markers for lipid peroxidation (Table 2). The DMH+*BP* group showed a 32% decrease in plasma conjugated dienes in comparison to DMH group levels ($p<0.05$), whereas the slight increase in the plasma TRAP value was not significant. DMH has been reported to induce carcino-genesis in rodents by producing high levels of reactive free radicals and by decreasing the activity of some antioxidant enzymes (21). DMH is converted into an indirect electro-philic carcinogen via a series of oxidative steps (22). A great deal of the evidence for this finding emanates from the finding that the antioxidants that directly scavenge free radicals, or those that interfere with the generation of free radical-mediated events, inhibit the neoplastic process and that the activities of antioxidant enzymes are altered during tumor formation (23). Our results indicated that oxidant-antioxidant homeostasis was disrupted in DMH-treated rats. The plasma lipid peroxidation level significantly increased, and the plasma total antioxidant potential was significantly reduced in the DMH-treated rats in comparison to the control rats. DMH, a procarcinogen, undergoes oxidative metabolism in the liver, which results in the production of an active carcinogenic electrophile (the diazonium ion), which is subsequently released into the circulation, eventually culminating in lipid peroxidation in the plasma (24). Additionally, Szatrowski and Nathan (25) have suggested that tumor cells generate a substantial amount of $H_2O_2$, which is released into the circulation. Thus, the increased susceptibility of the plasma in DMH-treated rats may be attributed to the production of $H_2O_2$ by tumor cells.

In our previous study, the administration of *B. polyfermenticus* colonized the human intestine, thereby reducing the number of fecal coliforms in healthy adults (26). Therefore, *B. polyfermenticus* has been suggested to function as a non-absorbable antioxidant in the colon or at

the surface of the colonic mucosa, similarly to other lactic acid bacteria (27). It may also be hypothesized that, although the majority of *B. polyfermenticus* taken orally has been determined to reach the intestines in the form of an endospore (3), some of the bacteria may be expected to lyse during transit through the gastrointestinal tract and to release their intracellular antioxidative constituents into the GI tract. The absorbed antioxidants from the small intestine then subsequently flow into the circulation, and may function as antioxidants in blood, thereby resulting in decreased lipid peroxidation levels. It has been reported that bacteria, or their extracts, exerted antioxidative effects *in vitro* (28-30), and they were also found to have protective effects against hemolysis in vitamin E-deficient rats (31). Ito *et al.* (27) reported that treatment for 2 weeks with a diet containing 0.4% *Streptococcus thermophilus* YIT 2001 ($2\times10^8$ CFU/day) resulted in a significant reduction in lipid peroxide (thiobarbituric acid reactive substances) levels in the colonic mucosa of iron-overloaded mice.

The functional food used in our study contained other well-known antioxidant sources, such as α-tocopherol and flavonoids (32, 33). In addition, the antioxidant activity of chitosan has attracted much attention. Xie *et al.* (34) and Xue *et al.* (35) have shown that the scavenging effect of chitosan on hydroxy radicals inhibits the lipid peroxidation of phosphatidylcholine and linoleate liposomes *in vitro*. A recent study has demonstrated that chicory is a good source of health-promoting antioxidant polyphenols, such as caffeic acid, flavones, and flavonoids (36).

**Leukocytic DNA damage** The data shown in Table 2 shows the effects of *B. polyfermenticus* supplementation on DMH-induced DNA damage, expressed as the tail moment and tail length in rat peripheral blood cells. The DMH treatment resulted in significant increases in leukocytic DNA damage, in comparison to the saline-injected control group. This strong genotoxic effect of DMH was reduced significantly by 54% in the tail moment and 48% in the tail length after 10 weeks of supplementation with the functional food containing *B. polyfermenticus*. DNA damage has been implicated as the initial step in chemical carcinogenesis; therefore, blocking DNA damage should be the first line of defense against cancer induced by carcinogens (37). Furthermore, DNA alkylation is also believed to be important in human cancer (38). Thus, the antigenotoxic effects observed in DMH-induced chemical carcinogenesis in a rodent model may be representative of the human system (37). The comet assay, or single cell gel electrophoresis assay, is a rapid and sensitive procedure used to quantify DNA lesions in individualized cells, both *in vitro* and *in vivo* (39, 40). Since the comet assay has been recommended as a supplementary assay for the *in vivo* genotoxicity induced by carcinogen exposure, we investigated the effects of *B. polyfermenticus* on DMH-induced DNA damage in leukocytes. Our results indicated that *B. polyfermenticus* supplementation effectively attenuated the genotoxic effects of DMH in leukocytes. Circulating leukocytes can be viewed as scouts, continuously surveilling the body for signs of toxic and antitoxic exposures, which are ultimately relevant to many disease processes, including cancer (41). The results of previous studies have revealed a strong correlation between genetic damage in rectal cells and leukocytes (42).

**Table 2. Effect of a functional food containing *Bacillus polyfermenticus* on plasma antioxidant potential, lipid peroxidation and leukocyte DNA damage in rats administrated DMH[1)]**

| | Control[2)] | DMH | DMH+*BP* |
|---|---|---|---|
| **Plasma** | | | |
| TRAP (mM) | 1.20±0.01[b3)] | 1.11±0.03[a] | 1.14±0.01[a] |
| CD (μM)[4)] | 9.16±0.46[a] | 13.2±1.61[b] | 8.99±1.00[a] |
| **Leukocytes** | | | |
| Tail moment | 0.69±0.13[a] | 1.80±0.34[b] | 0.83±0.16[a] |
| Tail length (μm) | 7.53±0.66[a] | 20.23±2.99[b] | 10.54±1.67[a] |

[1)]Values are the mean ± SE for 10 animals in each group.
[2)]Control, high-fat and low-fiber diet-fed group; DMH, high-fat and low-fiber diet + DMH injected group; DMH+*BP*, high-fat and low-fiber diet + functional food containing *B. polyfermenticus* ($3.1\times10^8$ CFU/day) + DMH injected group.
[3)]Values in the same row that do not share a common superscript are significantly different at $p<0.05$.
[4)]CD, conjugated dienes.

**Colon aberrant crypt foci formation** A significant reduction of 44% (182.0±19.3 vs. 101.3±3.2, $p<0.05$) was observed in the average total number of ACF per colon, as well as in the number of foci harboring one and two crypts in the 1,2-DMH-treated rats supplemented with *B. polyfermenticus,* in comparison to the rats treated with the carcinogen alone (Fig. 1A). The total numbers of AC in the colons of the *B. polyfermenticus* supplemented group were significantly ($p<0.05$) decreased by 32% compared to the DMH group (Fig. 1B). Our results clearly indicate that dietary supplementation of *B. polyfermenticus* ($3.1\times10^8$ CFU/day) effectively suppresses the occurrence of colonic ACF induced by DMH when administered one week prior to treatment with the carcinogen. The colonic carcinogen, DMH, induces a high incidence of precancerous lesions, which are referred to as ACF in rats. ACF, which are enlarged and elevated compared to normal crypts, have also been detected in the human colon, and are generally associated with carcinoma (43, 44). Due to the potential progression from early changes to malignancy, the study of premalignant hyperproliferative lesions and aberrant crypts is crucial to our understanding of colon cancer pathogenesis (45). Similar results have been observed in the colons of rats or mice treated with other probiotics administered during the post-initiation phase of DMH-induced carcinogenesis (46-48). Abdelali *et al.* (46) determined that rats administered the colon carcinogen DMH and fed diets containing $6\times10^9$ CFU/day of *Bifidobacterium longum* showed a 63% reduction in the total number of aberrant crypts per colon, relative to a control diet containing no bifidobacteria.

The possible mechanism underlying the reduction of ACF and DNA damage by *B. polyfermenticus* may involve the putative antioxidant activity of *B. polyfermenticus*. In the present study, *B. polyfermenticus* supplementation resulted in a reduction in plasma lipid peroxidation in comparison to the DMH-injected rat group. Other chemopreventive agents with antioxidant properties have also been found to

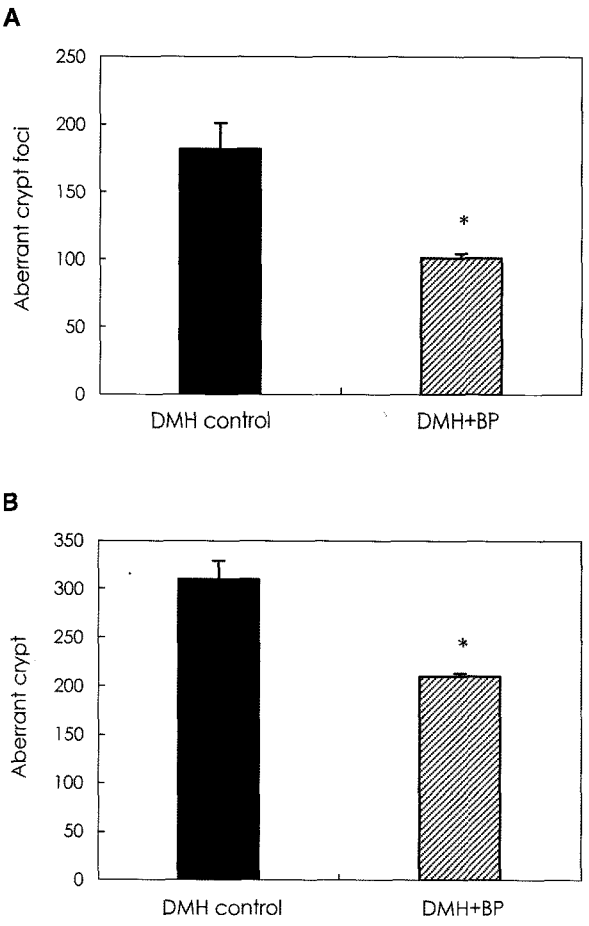


**Fig. 1. Effect of a functional food containing *Bacillus polyfermenticus* on the total number of aberrant crypt foci (ACF) (A) and the total number of aberrant crypts (AC) (B) induced by DMH in the distal colon.** DMH, high-fat and low-fiber diet + DMH injected group; DMH + BP, high-fat and low-fiber diet + functional food containing *B. polyfermenticus* ($3.1\times10^8$ CFU/day) + DMH injected group. Bars represent mean±SE. Significantly different from the control group: * $p<0.05$ (Student's *t*-test)

inhibit DMH-induced colon carcinogenesis and DNA damage in animal models (49, 50).

The beneficial effects of this functional food on colon carcinogenesis may be ascribed not only to *B. polyfermenticus* but also to other components of the functional food, such as chicory, α-tocopherol, and flavonoids. Chicory is a known prebiotic and is primarily composed of inulin, which is a polymer of fructose with β-(2-1) glycosidic linkages (51). Pool-Zobel (52) demonstrated that inulin-type fructans extracted from chicory roots have anti-carcinogenic effects on chemically-induced pre-neoplastic lesions (ACF) or in tumors of the colon in rats and mice. Exon *et al.* (53) found that *d*-α-tocopheryl succinate supplementation in 20 month-old female rats significantly reduced the number of larger ACF induced by azoxymethane. It has also been reported that various types of flavonoids such as quercetin, curcumin, rutin, silymarin, chafuroside, and isoliquiritigenin exert significant and potentially beneficial effects on the large intestine by reducing the number of precancerous lesions (54-56).

Our results suggest that the consumption of a functional food containing *B. polyfermenticus* as its primary material has significant health benefits, which occur *via* the reduction of colonic precarcinogenic events in rats in which colon carcinogenesis had been induced with DMH. Our results also suggest that this effect may be attributed to an increased antioxidant status. The results of this study provide us with new insight into the mechanisms underlying the anticancer properties of *B. polyfermenticus*.

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