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Inhibitory Effects of Lactic Acid Bacteria (LAB) on the Azoxymethane-induced Colonic Preneoplastic Lesions

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Epidemiological and experimental studies provide evidences that diet and intestinal microflora play an important role in colon carcinogenesis. In recent years, it has been suggested that lactic acid bacteria (LAB) used to ferment dairy products have an inhibitory effect on the colon cancer. This study was designed to determine the effect of *Bifidobacterium longum* HY8001 (Bif) and *Lactobacillus acidophilus* HY2104 (Lac) of Korean origin on azoxymethane (AOM)-induced colonic preneoplastic lesions such as aberrant crypt foci (ACF) formation and cecal pH. At five weeks of age, Spraque-Dawley rats were divided at random into four (AOM alone, Bif, Lac, and Bif+Lac) groups. Animals were weighed weekly and oral administration of LAB cultures were performed daily until the termination of the study. Two weeks later, all animals were given a subcutaneous injection of AOM dissolved in normal saline at a dose of 15 mg/kg of body weight once per week for 2 weeks. All rats were necropsied 7 weeks after the last AOM injection, and the ACF were visualized under light microscopy in the formalin-fixed, unsectioned methylene blue-stained colons. The total number of aberrant crypt in Bif, Lac, and Bif+Lac groups were significantly lower than that of the AOM alone group and the percentage of inhibitions weas 35.0, 45.4 and 45.0%, respectively. Significant inhibition ($p<0.001$) in the total number of ACF was also observed in LAB treated groups (Bif, Lac, and Bif+Lac group by 30.3, 38.6, and 41.2%, respectively). Furthermore, cecal pH appeared to significantly decrease by LAB administration. The results of present study provide some evidences for potential colon tumor-inhibitory properties of lactic cultures and fermented dairy products.

Key words: *Bifidobacterium longum* HY8001, *Lactobacillus acidophilus* HY2104, azoxymethane, colon cancer, aberrant crypt foci (ACF), cecal pH

Colon cancer incidence has been increasing in recent years in Korea. The recent changes in diet of Korean to "Western-type" which is characteristically high-fat, high-protein, low-carbohydrates and low-fiber are thought to be an important factor for the increase in colon cancer incidence (9, 11, 26, 24). Several studies have suggested that interactions between the intestinal microflora and certain dietary factors are involved in the development of colon cancer (13, 14, 21, 26, 24). Reddy *et al.* (25) found that germ-free animals had a 20% incidence of dimethylhydrazine-induced colon tumors compared with a 93% incidence in conventional animals with a normal flora. Hill *et al.* (13) have demonstrated that populations at high risk for colon cancer have an intestinal microflora with an increased ability to metabolize steroids and to hydrolyze glucuronides compared with individuals at low risk. In a study of Japan, Kubota (16) found that colon cancer incidence was lowest when the colonic population of *Bifidobacterium* was highest and that of *Clostridium perfringens* was lowest.

Probiotics can be defined as live microbial food supplements which beneficially affect the host animal by improving its intestinal microbial balance (7). Lactic acid bacteria (LAB) such as bifidobacteria and lactobacilli have been widely used as probiotics in humans and animals. In particular, a possible role of dietary supplement of LAB in the prevention of colon cancer has received wide interest. Supporting results for the protective effects of LAB and fermented milk against cancer have been obtained from *in vitro* experiments and in studies involving animals and human volunteers. Reddy *et al.* (28) found that Ehrich tumor cell proliferation was reduced by 28% in male Swiss mice fed yogurt. Goldin and Gorbach (10) demonstrated that dietary supplements of *L. acidophilus* not only reduced the incidence of dimethylhydrazine (DMH)-induced colon cancer, but also increased its latency period. The inhibitory effect of *Bifidobacterium longum* on colon, mammary and liver carcinogenesis induced by

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2-amino-3-methylimidazo[4,5-f] quinoline was shown by Reddy and Riverson (25).

Long-term carcinogenicity studies do not allow the assessment of the role of dietary factors in the multiple steps progressing toward disease. In addition, carcinogenicity studies to identify carcinogens and modulators of carcinogenesis are time consuming and very expensive and cannot be applied to each suspected agents. Bird (2) has recently proposed that aberrant crypt foci (ACF) are the earliest identifiable preneoplastic lesions of colon in carcinogen-treated rats. ACF appear in the rat colon within 5 days of colon carcinogen administration (35). Aberrant crypts (AC) are observed topographically on the whole mount of colonic mucosal surface stained with methylene blue. They exhibit dilated, irregular luminal openings, thicker epithelial lining and pericryptal zone, and exist as a single crypt or as a cluster of crypts forming foci (2). In addition, ACF have been reported to be associated with alterations in enzyme activity, and express mutations in the *apc* gene and the *ras* oncogene that appear to be biomarkers of colon cancer development (23, 33). Pretlow *et al.* (22) have demonstrated that colonic ACF in human colonic mucosa are putative preneoplastic lesions which develope into adenoma and adenocarcinoma. The ACF induced by DMH or AOM in rodents persist and grow. Therefore the number and growth features of ACF have been used to identify initiators and modulators of colon carcinogenesis (3).

There have been numerous extensive studies concerning the anticarcinogenic effect of LAB. However, there have been no reports on the anticarcinogenic properties of commercial LAB, which are derived from Korean intestinal microflora, in a chemically induced colon cancer model.

This study was designed to determine whether the administration of *B. longum* HY8001 and *L. acidophilus* HY2104, both of Korean origin, decreased colon cancer risk as measured by the number of ACF. In addition, it was aimed to determine whether the combined adminstration of *B. longum* HY8001 and *L. acidophilus* HY2104 would show potential additive or synergistic effect.

Materials and Methods

Animal

Three-week-old male Sprague-Dawley rats were obtained from the Yuhan research center and housed in polycarbonate cages (4 rats/cage) with wood chip bedding. The temperature in the animal room ranged from 21 to 24°C and a 12-h light-dark cycle was maintained. Commercial solid feed (Samyang Co., Korea) and water were provided *ad libitum*. After a 2 week period of acclimatization, animals were randomly assigned by body weight into four groups.

The cultures of lactic acid bacteria

B. longum HY8001 (14) and *L. acidophilus* HY2104 (31) were provided from the R&D center of Korea Yakult Co. Ltd. They were plated onto BL agar and incubated at 37°C for 48 h in an anaerobic steel wool jar filled with O_2-free CO_2 gas (18). Then, several colonies were transferred into tubes containing 10 ml of lactobacilli MRS broth and incubated at 37°C for 16 h (*L. acidophilus* HY2104) or 48 h (*B. longum* HY8001). The cultures of *B. longum* HY8001 and *L. acidophilus* HY2104 were prepared by inoculating 10 ml cultures of these bacteria (48 and 16 h, respectively) into 100 ml of fresh lactobacilli MRS broth. These cultures were prepared three times a week for oral administration and stored at 4°C. All rats of experimental groups were administered daily by 1 ml of cultured LAB throughout the experiment. The viable bacterial count in the LAB preparation was 2 to 5 $\times$ 10^9 per ml. It was confirmed that there was no contamination during the experiments.

Carcinogen

Azoxymethane (AOM), a colon carcinogen, was purchased from Sigma Chemical Co. AOM was dissolved in saline just before injection.

Experimental designs

The experimental protocol is shown schematically in Fig. 1. At five weeks of age, 43 rats were divided at random into four groups of 10 rats each (13 rats in the AOM alone group) and orally administered with one of the following; *B. longum* HY8001 (Bif group), *L. acidophilus* HY2104 (Lac group), *B. longum* HY8001 and *L. acidophilus* HY2104 (Bif+Lac group). Animals were weighed weekly and oral administration of LAB cultures were performed daily until the termination of the study. Two weeks later,

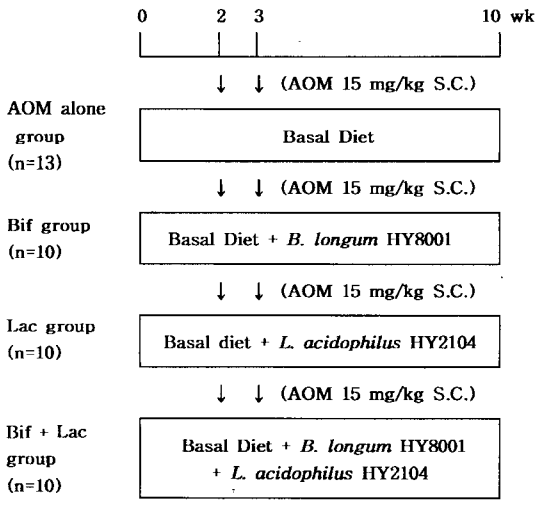

Fig. 1. Schematic diagrams for experimental design. AOM was injected sub-cutraneously (S.C.) at the weeks indicated by arrows.

all animals were given a sub-cutaneous (S.C.) injection of AOM dissolved in normal saline at a dose of 15 mg/kg of body weight once weekly for 2 weeks. All rats were sacrificed at 10 weeks after the start of the experiment. The liver and kidney were removed and weighed.

Assesment of aberrant crypt foci

For the aberrant crypt foci (ACF) assay, the colons were removed from rats after sacrifice. They were gently inflated with 10% neutral phosphate-buffered formalin and fixed for 10 min. Then they were slit open along the longitudinal median axis from the anus to cecum and fixed flat between filter papers. They were stored in 10% neutral phosphate-buffered formalin for further fixation. Each colon then was cut into 4 segments and stained with 0.25% methylene blue solution for a few minutes. They were then placed on microscope slides with the mucosal side up and aberrant crypts were scored under a light microscope at a magnification of 40 or 100 according to standard procedures (2, 3). The ACF was identified from the normal crypts by their increased size, increased pericryptal zone, elliptic or circular luminal opening, and greater thickness of the epithelial cell lining. Crypts overlying lymphoid follicles were excluded from the score since the normal crypt in this area can occasionally be confused with the aberrant crypt (AC). The number of ACF observed per colon and the number of aberrant crypts observed in each focus were recorded. All colons were scored by one observer. Thus, the repeated scores yield very similar values.

Measurement of cecal pH

Cecal pH was measured with a pH meter (920A, Orion), by inserting the electrode directly into the cecal content after sacrifice.

Statistical analysis

All laboratory data were analyzed statistically using an unpaired Student's *t* test.

Results

Body and organ weights

Body weights for each of the four groups are shown Fig. 2. After three weeks of experiment, lactic acid bacteria (LAB) treated groups were heavier than that of the AOM alone group but there were no significant differences throughout the experiment. Organ weights and relative organ weights (g/100 g body weight) for four groups are summarized in Table 1. The relative liver weights and total kidney weights of experimental groups were lighter than those of the AOM alone group but there were no significant differences.

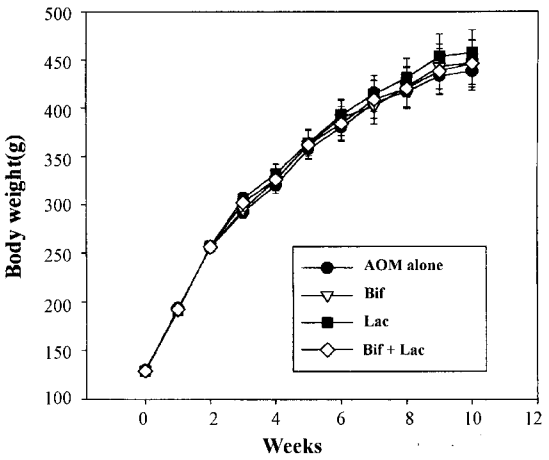

Fig. 2. Average body weights of male SD rats treated with AOM. At five weeks of age, 43 rats were divided at random into four groups of 10 rats each (13 rats in the AOM alone group). Animals were weighed weekly and oral administration of lactic acid bacteria cultures were performed daily until the termination of the study. Two weeks later, all animals were given S.C. injection of AOM dissolved in normal saline at a dose of 15 mg/kg body weight once weekly for 2 weeks. Values represent mean ± SE.

Colonic preneoplastic lesions (ACF)

Table 2 summarizes the effects of *B. longum* HY8001 and *L. acidophilus* HY2104 on ACF formation and its characteristics. The rats treated with AOM showed 100% ACF incidence. Methyleneblue stained ACF in the unsectioned colon of SD rats were shown in Fig. 3. The number of aberrant crypts per focus ranged from one to thirteen, although the majority of the ACF comprised one to three aberrant crypts. The crypt multiplicity was analyzed by categorizing according to the number of crypts/focus. Among the crypt multiplicity (number of crypts/focus), two crypts/focus were highest in all groups compared with one, three, four, five, or more than 6 crypts/focus (Fig. 4). Crypt multiplicity was remarkably suppressed in

Table 1. Organ weights of AOM-treated male Sprague-Dawely rats on week 10

		AOM alone	Bif	Lac	Bif+Lac
Organ weight (g)	Liver	10.45 ± 0.22	10.23 ± 0.29[a]	10.66 ± 0.35	10.34 ± 0.32
	Kidney	2.78 ± 0.09	2.69 ± 0.05	2.75 ± 0.08	2.72 ± 0.12
Relative organ weight (g)[b]	Liver	2.39 ± 0.05	2.29 ± 0.03	2.32 ± 0.05	2.32 ± 0.04
	Kidney	0.64 ± 0.018	0.61 ± 0.03	0.60 ± 0.010	0.61 ± 0.019

[a] Expressed as mean ± SE
[b] Expressed as g/100 g body weight

Table 2. Effects of *B. longum* and *L. acidophilus* on the formation of aberrant crypt foci in the colons of Sprague-Dawley rats treated with AOM[a]

	AOM alone	Bif	Lac	Bif+Lac
1-3AC/foci	238.4 ± 8.96	176.7 ± 8.57* (25.9%)[b]	164.1 ± 11.60* (31.2%)	152.8 ± 10.42* (35.9%)
≥ 4AC/foci	61.2 ± 4.90	32.1 ± 5.10* (47.6%)	19.9 ± 3.51* (67.5%)	24.5 ± 3.82* (60.0%)
ACF/colon	299.6 ± 8.95	208.8 ± 9.56* (30.3%)	184.0 ± 14.46* (38.6%)	177.3 ± 12.20* (41.2%)
AC/colon	769.4 ± 31.91	499.9 ± 28.66* (35.0%)	419.7 ± 38.32* (45.5)	422.3 ± 34.69 (45.1%)

[a] Expressed as mean ± SE
[b] Values in parenthesis represent percentage of inhibition compared with the AOM alone group.
*p<0.001; compared with the AOM alone group.

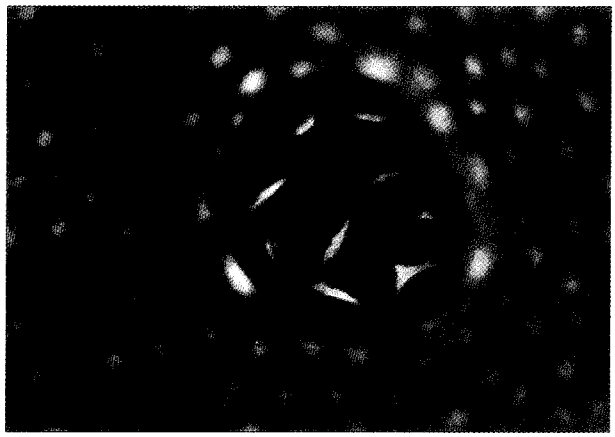

Fig. 3. Topographic view of AOM-induced ACF with nine AC. Rats were injected with AOM (15 mg/kg) sub-cutaneously once weekly for 2 weeks. All rats were necropsied 7 weeks after the last AOM injection, and the ACF were visualized under light microscopy in formalin-fixed, unsectioned methylene blue-stained colons.

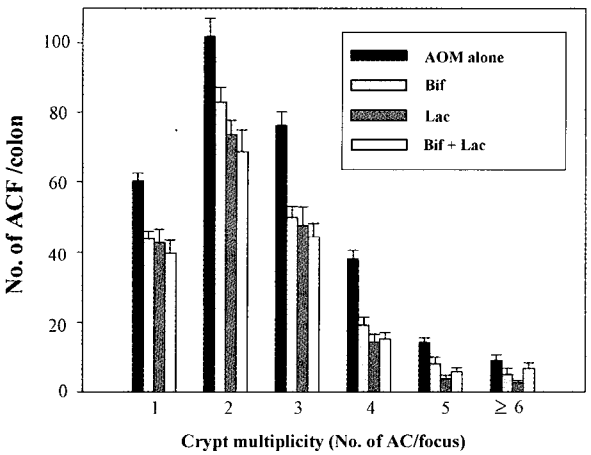

Fig. 4. Effects of *B. longum* HY8001 and *L. acidophilus* HY2104 on the aberrant crypt multiplicity in the colons of rats treated with AOM. After 2 weeks, all rats were treated with AOM once per week for 2 weeks. All rats were necropsied 7 weeks after the last AOM injection, and the ACF were visualized under light microscopy in the formalinfixed, unsectioned, methyleneblue-stained colons. The number of AC per ACF per colon was recorded under × 40 or 100 magnifications. Values represent mean ± SE.

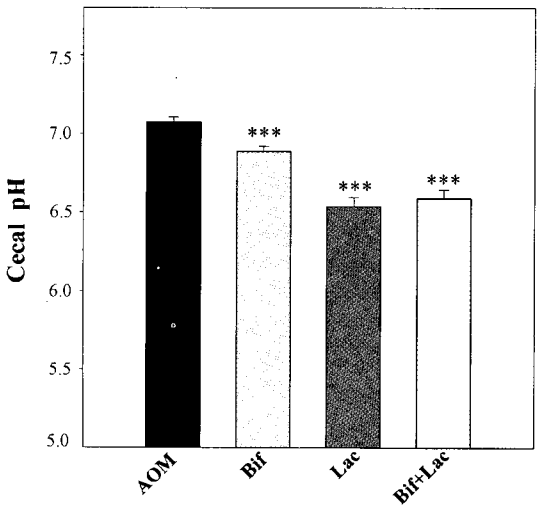

Fig. 5. Effects of *B. longum* HY8001 and *L. acidophilus* HY2104 on AOM-induced cecal pH in rats. Oral administration of lactic acid bacteria cultures were performed daily until the termination of the study. Two weeks later, all animals were given S.C injection of AOM dissolved in normal saline at a dose of 15 mg/kg body weight once weekly for 2 weeks. All rats were necropsied 7 weeks after the last AOM. Cecal pH was measured with a pH meter (920A, Orion), by inserting the electrode directly into the cecal content. Values represent mean ± SE. ***p<0.001; compared with the AOM alone group.

LAB treated groups. The total number of aberrant crypts in the AOM alone group was 769.4 ± 31.9 per colon. The total number of aberrant crypt in the Bif, Lac and Bif+Lac groups were significantly lower than that of the AOM alone group and the percentage inhibitions were 35.0%, 45.5 and 45.1, respectively (Table 2).

As shown in Table 2, the number of small ACF (those comprising 1-3 aberrant crypts per focus) was 238.4 ± 9.0 in the AOM alone group and lowest in the Bif+Lac group (decreased by 35.9%). The large ACF (those comprising more than 4 aberrant crypts per focus) were more strongly inhibited in the LAB-treated groups than the small ACF. The most potent inhibitory effect on the large ACF related to higher tumor yield was seen in the Lac group, in which the number of large ACF was decreased by 67.5%.

Significant inhibition ($p<0.001$) in the development of total number of ACF was observed also in the LAB treated groups. The percentage inhibitions of total ACF in the Bif, Lac, and Bif+Lac group were 30.3, 38.6 and 41.2%, respectively.

pH of cecal contents

The pH of cecal contents was 7.07 ± 0.03 in the AOM alone group. However, the pH was significantly decreased ($p<0.001$) by LAB treatment (Fig. 5). The greatest decrease was seen in the Lac group (6.54 ± 0.06). There were, however, no significant differences between the LAB-treated groups.

Discussion

Epidemiological and experimental studies show results which indicate that environmental factors, especially diet, and intestinal microflora play an important role in colon carcinogenesis. It has been also suggested that fermented milk and lactic acid bacteria possess anticarcinogenic properties. The purpose of this study was to evaluate the inhibitory effects of lactic acid bacteria on the AOM-induced colonic preneoplastic lesions.

Aberrant crypt foci (ACF) may represent the earliest detectable lesions in the development of colon cancer. Considerable evidences support the concept that ACF are indeed preneoplastic lesions and that the number of ACF is predictive of eventual tumor formation (22). Colonic preneoplastic lesions (ACF) were significantly inhibited by lactic acid bacteria supplements in this study. Among the aberrant crypt foci characteristics, crypt multiplicity (number of crypt per focus) has been considered as one of the consistent predictors of tumor outcome. Several investigators have suggested that the number of large ACF with more than four aberrant crypts per focus, is more predictive of eventual tumor incidence than smaller ACF (1-3) aberrant crypts per focus (2, 3, 22). In the present study, large ACF was more strongly inhibited than small ACF by lactic acid bacteria. The induction of small ACF was inhibited most strongly in the Bif+Lac group. However, the most potent inhibition of large ACF was observed in the Lac group. There were, however, no significant differences among the LAB treated groups. These results are in agreement with other studies showing protection by lactic acid bacteria feeding against ACF (8, 32). Kulkarni *et al.* (17) showed that the feeding of lyophilized culture of *B. longum* significantly inhibited ACF formation (53%) and crypt multiplicity in colon. In addition, several studies demonstrated that the combined treatment with lactic acid bacteria and oligosaccharides such as inulin and lactulose exerted additive or synergistic anticarcinogenic effect (5, 29). Rowland *et al.* (29) observed that the combined treatment of rats with *B. longum* and inulin resulted in additive effect was inhibition level reaching 80%. However, any significant synergistic or additive effects were not observed in this study. Azoxymethane used in this study is a potent, organ-specific colon carcinogen and metabolized to the ultimate carcinogen, methylazoxymethanol (MAM) by microbial β-glucuronidase in the colon (6). *E. coli* and *Clostridium* have the highest level of β-glucuronidase, whereas low level are found in *Bifidobacterium* and *Lactobacillus* (12). The dietary intake of fermented milk and LAB cultures have been shown to suppress the number of enteropathogenic organism such as *E. coli* and *C. perfringens*, and decrease the metabolic activity of certain class of intestinal microflora as indicated by fecal bacterial enzymes (1, 11). Our previous study (19) demonstrated that β-glucuronidase and nitroreductase activities significantly decreased in human volunteers during the intake of *B. longum* HY8001. Therefore, the results of the present study may relate to lower β-glucuronidase activities in the LAB treated groups.

Some investigators postulated that a high colonic pH promotes colorectal cancer and a low pH have a protective effect toward colon carcinogenesis (30, 34). Low pH level in the colonic lumen caused by increased production of short chain fatty acids (SCFAs) inhibits the bacterial degradation of primary to secondary bile acids (deoxycholic and lithocholic acids), which have been shown to promote colon cancer in carcinogen treated rats (26, 24, 30). Low pH may also stimulate mucus production, providing protection against carcinogens (4). However, Lupton *et al.* (20) suggested that acidification of the colonic contents by dietary modification led to increased epithelial cell proliferation. However, in this study lactic acid bacteria administrated rats showed a drop in cecal pH and also exhibited a significantly lower number of ACF. Especially, Lac group, in which the most potent inhibition of larger ACF was observed, showed the lowest cecal pH. A decrease in pH of the cecal contents is considered to be the consequence of increased bacterial production of short chain fatty acids and lactic acid in the gut, which have tropic effects on the gut mucosa. Similar results were obtained in other experiments (5. 29). This finding also demonstrates that a lower level of cecal pH for the lactic acid bacteria feedings is related to the inhibitory effects on the development of AOM-induced preneoplastic lesions.

The results of this study are of considerable interest because this is the first report showing that adminstration of lactic acid bacteria, which are indigenous in the intestine of the Korean population and used in commercial yogurt, can effectively reduce azoxymethane-induced colon cancer. In addition, the results of the present study, which indicate that cultures of *B. longum* HY8001 and *L. acidophilus* HY2104 inhibit the formation and multiplicity of ACF in colon, provide some evidence for potential colon tumor-inhibitory properties of lactic cultures and fer-

mented dairy products.

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