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Effects of Lactic Acid Bacteria on Intestinal Microbial Enzyme Activity and Composition in Rats Treated with Azoxymethane

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In recent years, colon cancer has been reported to be one of the most important causes of cancer morbidity and mortality in Korea. Epidemiological and experimental studies suggest that lactic acid bacteria (LAB) used to ferment dairy products inhibits colon carcinogenesis. The present study was designed to determine whether the colon cancer inhibitory effect of LAB (*Bifidobacterium longum* HY8001; Bif and *Lactobacillus acidophilus* HY2104; Lac) of Korean origin, is associated with intestinal microflora composition and certain enzyme activity in rats treated with azoxymethane (AOM). At five weeks of age, SD rats were divided at random into four (AOM alone, Bif, Lac, and Bif+Lac) groups. Oral administration of lactic acid bacteria 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 weekly for 2 weeks. Every two weeks for 10 weeks, five of the rats in each group were randomly chosen for fecal specimen collection. The fecal specimens were used for assay of β-glucuronidase and nitroreductase, and analysis of intestinal microflora composition. The activity of β-glucuronidase which plays an important role in the production of the carcinogenic metabolite of azoxymethane was remarkably increased in the AOM alone group after AOM injection and maintained the high level during the experiment. However, LAB inhibited the AOM-induced increase in β-glucuronidase activity. Nitroreductase activity decreased by 30-40% in LAB treated groups in comparison with that of the AOM alone group. The results of the present study suggest that LAB inhibits colon carcinogenesis by modulating the metabolic activity of intestinal microflora and improving the composition of intestinal microflora.

Key words: *Bifidobacterium longum* HY8001, *Lactobacillus acidophilus* HY2104, azoxymethane, colon cancer, β-glucuronidase, nitroreductase, intestinal microflora

The human gastrointestinal track contains an extraordinarily diverse complex of bacterial microflora which includes more than 10^{12} microorganisms and more than 400 different aerobic and anaerobic bacterial species (32). The relationship between intestinal microflora and the host is so specific that alteration in the balance of organisms might result in health problems such as diarrhea, constipation, infections, liver damage, carcinogenesis, hypertension, aging and intestinal putrefaction. Especially, the role of intestinal microflora in colon carcinogenesis has intrigued investigators for many years. Reddy *et al.* (24) found that germ-free animals had a 20% incidence of dimethylhydrazine-induced colon tumors compared with 93% incidence in conventional animals with normal flora. Hill *et al.* (13) have demonstrated that populations at high risk for colon cancer have intestinal microflora with an increased ability to metabolize steroids and to hydrolyze glucuronides when compared with individuals at low risk. Kubota (16) found that colon cancer incidence was lowest when the colonic population of *Bifidobacterium* was highest and that of *Clostridium perfringens* was lowest. The intestinal microflora have a wide range of metabolic capabilities and possess a great number of inducible and repressible enzymes. Bacterial enzymes such as β-glucuronidase, nitroreductase, azoreductase, 7-α-dehydroxylase, and cholesterol dehydrogenase can mediate the formation of mutagens, carcinogens and tumor promoters in the gastrointestinal track (7, 10, 12, 28, 30, 31). For this reason, the activity of these enzymes has been used to monitor colon carcinogenesis.

Probiotics can be defined as "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (5). Lactic acid bacteria such as bifidobacteria and lactobacilli have been widely used as probiotics in humans and animals. In particular, a possible role of a dietary supplement of lactic acid bacteria in the prevention of colon cancer has received special attention. Goldin and Gorbach (8) dem-

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onstrated that dietary supplements of *L. acidophilus* not only reduced the incidence of dimethylhydrazine (DMH)-induced colon cancer, but also increased the latency period. The inhibitory effect of *Bifidobacterium longum* on colon, mammary and liver carcinogenesis induced by 2-amino-3-methylimidazo [4,5-*f*] quinoline was shown by Reddy and Rivenson (27).

Diverse mechanisms by which lactic acid bacteria inhibit colon cancer have been reported; i) enhancing the host's immune response, ii) binding and degrading potential carcinogens, iii) quantitative and/or qualitative alterations in the intestinal microflora, iv) production of antimutagenic compounds in the colon, v) alteration of metabolic activities of intestinal microflora, and vi) alteration of physicochemical conditions in the colon (21). There have been numerous extensive studies on the anticarcinogenic effect of lactic acid bacteria (2, 3, 6, 8, 21, 27, 30). At present, however, there is no clear understanding of the correlation among intestinal microflora composition, intestinal microflora enzyme activity and colon cancer development in rats administered with lactic acid bacteria because analysis of intestinal microflora is difficult and needs special techniques. Therefore, the present study was designed to determine whether administration of *B. longum* HY8001 and *L. acidophilus* HY2104 of Korean origin, decreases activities of certain bacterial enzymes, and whether this inhibitory effect is associated with the intestinal microflora composition in rats treated with azoxymethane. In addition, we determined whether the combined adminstration of *B. longum* HY8001 and *L. acidophilus* HY2104 would show potential additive or synergistic effects.

Materials and Methods

Animals

Three-week-old male Sprague-Dawley rats were obtained from Yuhan Research Center and housed in polycarbonate cages (4 rats/cage) with wood chip bedding. The temperature in the room ranged from 21°C 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 distributed by body weight into four groups.

The lactic acid bacteria culture

Bifidobacterium longum HY8001 (14) and *Lactobacillus acidophilus* HY2104 (33) were provided from R&D Center of Korea Yakult Co. Ltd. They were cultured in lactobacilli MRS broth at 37°C in an anaerobic 'steel wool' jar filled with O_2-free CO_2 gas (18). The viable bacterial count in the bacterial preparation was 2 to 5×10^9 per ml.

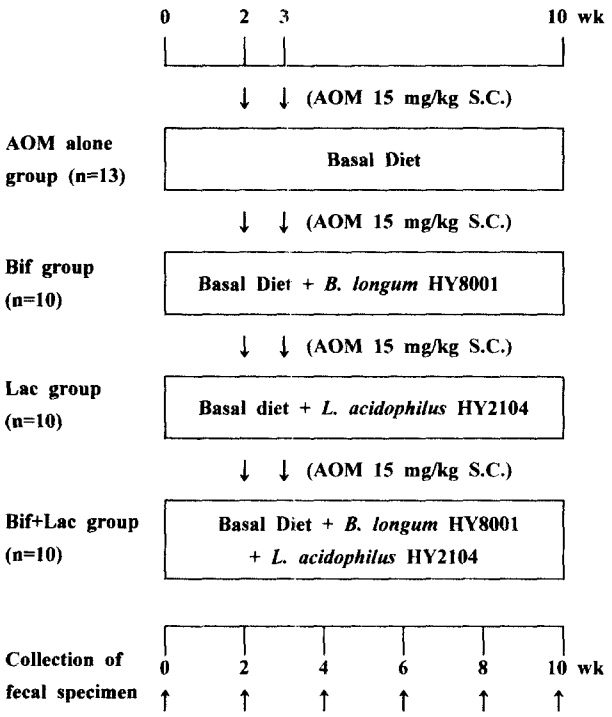

Fig. 1. Schematic diagrams for experimental design.

Carcinogen

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

Experimental design

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 administrated either *B. longum* HY8001 (Bif group), *L. acidophilus* HY2104 (Lac group), *B. longum* HY8001 or *L. acidophilus* HY2104 (Bif+Lac group). 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. Every two weeks for 10 weeks, five of the rats in each group were randomly chosen for fecal specimen collection.

Collection of fecal specimens

Fecal specimens were collected by gently squeezing the rat's rectal region to excise the fecal pellets. This insured fresh material. The fecal specimens were assayed immediately after collection.

Enzyme assay

One gram of fresh fecal specimen was weighed and rapidly transferred under a flow of O_2 free-CO_2 gas into tubes containing 9 ml of cold prereduced 0.1 M PPB (potassium

phosphate buffer, pH 7.0). Then, these tubes were sealed with butyl rubber stoppers. An aliquot of fecal suspension was removed for analysis of intestinal microflora. The remainder of the contents was used for the β-glucuronidase and nitroreductase assays.

β-Glucuronidase assay

β-Glucuronidase assay was performed by a slight modification of Goldin and Gorbach's method (10) previously used in our laboratory (19). Briefly, the fecal suspension was disrupted by sonication for 30 minutes at 4°C and then centrifuged at 1000 × g for 10 minutes. The supernatant was used for the enzyme assay and fecal protein concentration. The enzyme reaction was run at 37°C (pH 6.8) in a total volume of 1 ml containing 0.02 M potassium phosphate buffer, 0.1 mM EDTA, 1 mM phenolphthalein-β-D-glucuronide (Sigma Chemical Co., St. Louis, Mo.) as substrate, and 0.1 ml fecal extract. After incubation for 1 hr, the reaction was stopped by addition of 5 ml of 0.2 M glycine buffer (pH 10.4) containing 0.2 M NaCl. The phenolphthalein liberated was measured at 540 nm using a spectrophotometer (DU-650, Beckman). The amount of phenolphthalein released was determined by comparison with a standard phenolphthalein curve. β-Glucuronidase activity was expressed as microgram of released phenolphthalein per hour per milligram of fecal protein.

Nitroreductase assay

Nitroreductase activity in the fecal suspension was assayed anaerobically using *m*-nitrobenzoic acid as a substrate by the method previously used in our laboratory (19). One ml of fecal suspension was transferred into a tube containing 4 ml of 1.5 mM nitrobenzoic acid (pH 7.0) under a flow of O_2 free-CO_2 gas. Then the tube was sealed with a butyl rubber stopper. The enzyme reaction was run anaerobically at 37°C for 1 hr and stopped by the addition of 2.5 ml of 1.2 N HCl. It was then centrifuged at 5000 rpm for 10 min., and the supernatant was used to determine the amount of *m*-aminobenzoic acid formed, as described (19). The fecal protein concentration was also determined. Nitroreductase activity was expressed as a microgram of formed aminobenzoic acid per hour per milligram of fecal protein.

Protein determination

The fecal protein concentrations were determined by the method of Lowry *et al.* (20) with bovine serum albumin as the standard.

Bacteriological analysis

The intestinal microflora was analyzed using the method and media of Mitsuoka *et al.* (22). Three non-selective agar plates: EG agar and BL agar for anaerobes, and TS for aerobes and five selective agar media: BS agar for *Bifidobacterium*, NN agar for lecithinase-positive *Clostridium*, LBS for *Lactobacillus*, TATAC agar for *Enterococcus* and streptococci and DHL agar for Enterobacteriaceae were used. The EG, BL, BS, NN and LBS agar plates were incubated at 37°C for 48 hr in an anaerobic 'steel-wool' jar filled with oxygen-free CO_2 gas (18). Each of TATAC, DHL and TS agar plates was incubated aerobically at 37°C for 24 hr. After incubation, each plate was examined for bacterial colonies. Identification was performed with colonial and cellular morphologies, Gram-reaction, spore formation and aerobic growth. The bacterial counts per gram of feces were calculated and converted into a logarithmic equivalent for each bacterial species.

Statistical analysis

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

Results

β-Glucuronidase activity

Fig. 2. shows the effect of lactic acid bacteria on β-glucuronidase activity in rats treated with AOM. Before administration of lactic acid bacteria culture, this enzyme activity ranged from 99.4± 12.21 to 102.2± 12.19. In the

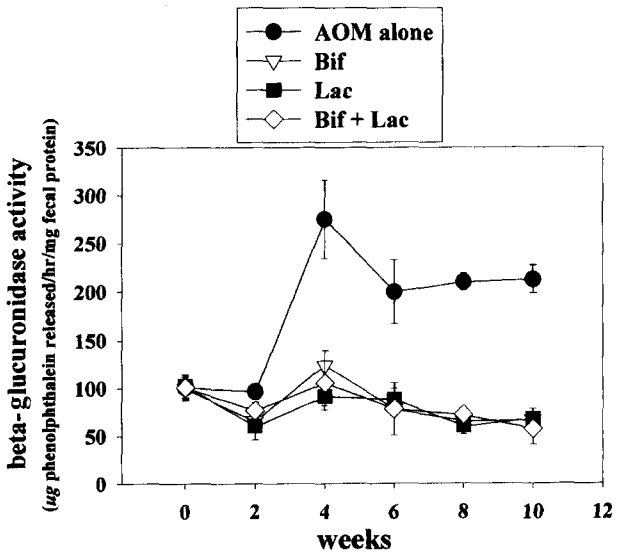

Fig. 2. Effects of *B. longum* HY8001 and *L. acidophilus* HY2104 on the activity of β-glucuronidase in the intestinal microflora of rats treated with AOM. Oral administration of lactic acid bacteria cultures were performed daily until the termination of the study. Two weeks later, all animals were given S.C. injection of AOM dissolved in normal saline at a dose of 15 mg/kg body weight once weekly for 2 weeks. Every two weeks, the β-glucuronidase activity of the fecal suspension from the 5 rats randomly chosen in each group was assayed aerobically using phenolphthalein-β-D-glucuronide as a substrate. Values represent the mean ± SE.

Bif group, the activity of β-glucuronidase was significantly decreased ($p>0.05$, 66.56± 8.94) in week 2 in comparison with that of the AOM alone group. In the Lac and Bif + Lac groups, the decrease of β-glucuronidase activity was also observed (60.6 ± 14.86 and 79.7 ± 8.45, respectively) but there were no significant differences from the AOM alone group. After the rats were inoculated with AOM (in week 4), the activity of β-glucuronidase was increased in all groups. We noted that the β-glucuronidase activity had increased 2.6 fold by AOM treatment and maintained a high level of activity up to 10 weeks in the AOM alone group (Fig. 2). However, AOM-induced elevation of β-glucuronidase activity was significantly inhibited by administration of lactic acid bacteria. Furthermore, the enzyme activity was less than that of week 0 in weeks 6, 8 and 10.

Nitroreductase activity

The effect of lactic acid bacteria on nitroreductase activity in rats treated with AOM is shown in Fig. 3. The nitroreductase activity remained relatively constant in the AOM alone group throughout the experiment. However, it was significantly decreased in weeks 4, 6 and 8 (16.6 ± 2.31, 15.1 ± 0.98, 15.0 ± 1.48 respectively) in the Bif group compared with that of the AOM alone group (24.0± 2.11, 26.4± 2.59 and 24.1± 3.01, respectively) and in weeks 6 and 8 (17.4 ± 1.29 and 14.7 ± 1.45, respectively) in the Bif + Lac group. In the Lac group, this enzyme activity showed tendencies to decrease but the difference did not reach a statistical significance ($p>0.05$). However, this enzyme activity was not affected by AOM treatment in this study.

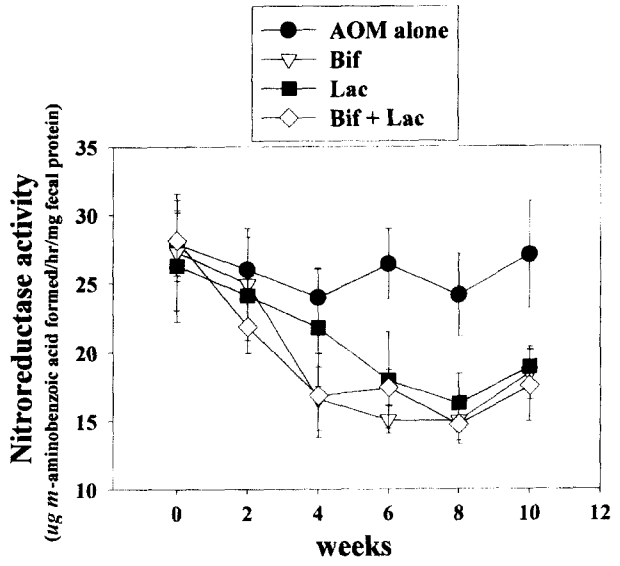

Fig. 3. Effects of *B. longum* HY8001 and *L. acidophilus* HY2104 on the activity of nitroreductase in the intestinal microflora of rats treated with AOM. Oral administration of lactic acid bacteria cultures were performed daily until the termination of the study. Two weeks later, all animals were given S.C. injection of AOM dissolved in normal saline at a dose of 15 mg/kg body weight once weekly for 2 weeks. Every two weeks, nitroreductase activity of fecal suspension from of the 5 rats randomly chosen in each group was assayed aerobically using nitrobenzoic acid as a substrate. Values represent the mean ± SE.

Intestinal microflora analysis

The effect of *B. longum* HY8001 and *L. acidophilus* HY2104 on the intestinal microflora composition of male SD rats treated with AOM are shown in Table 1. The number of *Bifidobacterium* decreased in AOM alone and Lac groups by 10-100 fold after week 4 but remained constant in Bif and Bif+Lac groups throughout the experiment. The significant differences compared with the AOM alone group were observed in the Bif group in weeks 4, 6 and 10 ($p<0.001$, 0.001 and 0.05, respectively) and in the Bif+Lac group in weeks 4, 6 and 8 ($p<0.01$, 0.01 and 0.05, respectively).

The number of *Lactobacillus* was significantly increased in the Lac group in weeks 4 and 8 (9.56 ± 0.05 and 9.62 ± 0.02, respectively) in comparison with those of the AOM alone group (9.46 ± 0.07 and 9.52 ± 0.02, respectively). However, administration of cultures of Bif or Bif and Lac combined did not cause a significant increase in the level of *Lactobacillus*.

The number of Enterobacteriaceae was remarkably decreased by 3-12 fold in LAB treated groups compared to that of the AOM alone group. The most potent inhibitory effect on Enterobacteriaceae was seen in rats treated with *L. acidophilus* HY2104 cultures (Lac group) throughout the experiment. The number of *Clostridium* in LAB treated groups was not significantly different from that of the AOM alone group throughout the experiment, whereas the frequency of occurrence was lower than that of the AOM alone group.

The number of total bacteria of all groups remained relatively constant throughout the experiment. No detectable changes were observed in the number and frequencies of *Bacteroides* and *Streptococcus* throughout the experiment. In the case of *Eubacterium*, the number of bacteria showed a tendency to decrease in all groups and decreased significantly in the Bif group in weeks 8 and 10.

Discussion

Colon cancer is one of the leading causes of cancer morbidity and mortality in western countries. In Korea, the incidence of stomach cancer has traditionally been high. However, stomach cancer incidence has been decreasing and colon cancer incidence has been increasing in recent years. Based on epidemiological and experimental data, the increase of colon cancer incidence in Korea is thought to be associated with the recent changes

Table 1. Effect of LAB on the intestinal microflora composition

	groups	0 wk	2 wk	4 wk
Enterobacteriaceae	AOM alone	7.42 ± 0.18[a] (100)[b]	7.34 ± 0.25 (100)	6.85 ± 0.13 (100)
	Bif	7.00 ± 0.16 (100)	6.22 ± 0.17 (100)**	6.2 ± 0.19 (100)
	Lac	7.14 ± 0.10 (100)	6.12 ± 0.26 (100)*	5.84 ± 0.14 (100)**
	Bif+Lac	7.52 ± 0.26 (100)	6.1 ± 0.21 (100)	6.02 ± 0.24 (100)
Streptococcus	AOM alone	8.34 ± 0.13 (100)	8.20 ± 0.41 (100)	7.88 ± 0.39 (100)
	Bif	7.78 ± 0.41 (100)	8.06 ± 0.36 (100)	7.90 ± 0.31 (100)
	Lac	8.3 ± 0.21 (100)	8.12 ± 0.25 (100)	7.74 ± 0.44 (100)
	Bif+Lac	8.42 ± 0.18 (100)	7.78 ± 0.38 (100)	7.82 ± 0.30 (100)
Lactobacillus	AOM alone	9.56 ± 0.01 (100)	9.42 ± 0.08 (100)	9.46 ± 0.07 (100)
	Bif	9.48 ± 0.07 (100)	9.52 ± 0.07 (100)	9.42 ± 0.09 (100)
	Lac	9.5 ± 0.18 (100)	9.64 ± 0.07 (100)	9.56 ± 0.05 (100)**
	Bif+Lac	9.44 ± 0.17 (100)	9.6 ± 0.08 (100)	9.54 ± 0.11 (100)
Bifidobacterium	AOM alone	8.86 ± 0.30 (100)	8.44 ± 0.34 (100)	7.08 ± 0.15 (100)
	Bif	8.66 ± 0.12 (100)	8.90 ± 0.09 (100)	8.52 ± 0.10 (100)***
	Lac	8.86 ± 0.07 (100)	8.74 ± 0.14 (100)	7.14 ± 0.44 (100)
	Bif+Lac	8.86 ± 0.15 (100)	8.88 ± 0.10 (100)	8.30 ± 0.28 (100)**
Eubacterium	AOM alone	9.04 ± 0.12 (100)	9.10 ± 0.13 (100)	9.04 ± 0.12 (100)
	Bif	9.06 ± 0.14 (100)	9.04 ± 0.13 (100)	8.94 ± 0.11 (100)
	Lac	8.98 ± 0.11 (100)	8.94 ± 0.09 (100)	8.8 ± 0.21 (100)
	Bif+Lac	9.16 ± 0.07 (100)	9.12 ± 0.14 (100)	9.20 ± 0.10 (100)
Bacteroidaceae	AOM alone	9.32 ± 0.06 (100)	9.34 ± 0.09 (100)	9.44 ± 0.07 (100)
	Bif	9.26 ± 0.14 (100)	9.24 ± 0.12 (100)	9.22 ± 0.05 (100)
	Lac	9.4 ± 0.08 (100)	9.14 ± 0.10 (100)	9.32 ± 0.09 (100)
	Bif+Lac	9.28 ± 0.09 (100)	9.38 ± 0.07 (100)	9.40 ± 0.06 (100)
Clostridium	AOM alone	8.13 ± 0.18 (80)	8.18 ± 0.08 (100)	8.02 ± 0.29 (100)
	Bif	7.10 ± 0.36 (80)	7.20 ± 0.17 (80)	7.30 ± 0.50 (60)
	Lac	7.83 ± 0.34 (60)	7.83 ± 0.23 (60)	7.53 ± 0.14 (80)
	Bif+Lac	7.83 ± 0.18 (60)	7.55 ± 0.75 (40)	6.80 ± 0.50 (80)
Total counts	AOM alone	9.94 ± 0.04 (100)	9.88 ± 0.02 (100)	9.88 ± 0.02 (100)
	Bif	9.90 ± 0.08 (100)	9.92 ± 0.04 (100)	9.78 ± 0.05 (100)
	Lac	9.96 ± 0.07 (100)	9.9 ± 0.04 (100)	9.82 ± 0.05 (100)
	Bif+Lac	9.94 ± 0.04 (100)	9.98 ± 0.04 (100)	10.00 ± 0.08 (100)

[a]Expressed as mean ± SE of $\log_{10}$ number per gram of feces. [b]Frequency of occurrence (%)
*p<0.05, **p<0.01, ***p<0.001; compared to AOM alone group.

in diet to the "Western-type", which is characteristically high-fat, high-protein, low-carbohydrate and low-fiber (7, 25, 28, 32). Epidemiological and experimental evidence points to the fact that environmental factors, especially diet and intestinal microflora play an important role in colon carcinogenesis. It has also been suggested that fermented milk and lactic acid bacteria possess anticarcinogenic properties. The purpose of this study was to determine whether the anticarcinogenic effect of LAB is associated with changing the intestinal microbial composition and enzyme activity.

Experimental evidence suggests that lactic acid bacteria can reduce the activity of certain microbial enzymes related to formation of carcinogen or tumor promoters (3, 9, 11, 26, 30). Azoxymethane, used in this study, is a potent, organ-specific colon carcinogen and metabolizes to an ultimate carcinogen, methylazoxymethanol (MAM) by microbial β-glucuronidase in the colon (4). β-Glucuronidase is an inducible enzyme so that continued exposure of intestinal microflora to procarcinogens leads to increases in enzyme levels. In the present study, the remarkable increase of microbial β-glucuronidase activity was observed after AOM treatment in the AOM alone group. It probably reflects higher production of MAM in the colon. Goldin and Gorbach (9) observed a very similar result in that DMH increased the fecal β-glucuronidase activity in male F344 rats. However, in LAB treated groups, β-glucuronidase activity increased slightly and then decreased again. This result indicates that AOM-induced elevation of β-glucuronidase activity is inhibited

Table 1. Continued

	groups	6 wk	8 wk	10 wk
Enterobacteriaceae	AOM alone	7.34 ± 0.16 (100)	7.42 ± 0.20 (100)	7.04 ± 0.19 (100)
	Bif	6.28 ± 0.08 (100)***	6.66 ± 0.10 (100)**	6.10 ± 0.20 (100)**
	Lac	6.02 ± 0.12 (100)***	6.06 ± 0.14 (100)***	6.06 ± 0.18 (100)**
	Bif+Lac	6.72 ± 0.05 (100)**	6.12 ± 0.17 (100)**	6.14 ± 0.14 (100)**
Streptococcus	AOM alone	7.64 ± 0.21 (100)	7.72 ± 0.23 (100)	7.92 ± 0.47 (100)
	Bif	7.40 ± 0.16 (100)	7.8 ± 0.21 (100)	7.76 ± 0.24 (100)
	Lac	7.68 ± 0.34 (100)	7.96 ± 0.34 (100)	8.18 ± 0.27 (100)
	Bif+Lac	7.70 ± 0.20 (100)	7.86 ± 0.40 (100)	7.92 ± 0.40 (100)
Lactobacillus	AOM alone	9.40 ± 0.09 (100)	9.52 ± 0.02 (100)	9.42 ± 0.05 (100)
	Bif	9.44 ± 0.11 (100)	9.50 ± 0.05 (100)	9.36 ± 0.06 (100)
	Lac	9.54 ± 0.07 (100)	9.62 ± 0.02 (100)	9.52 ± 0.06 (100)
	Bif+Lac	9.44 ± 0.19 (100)	9.58 ± 0.06 (100)	9.46 ± 0.05 (100)
Bifidobacterium	AOM alone	6.70 ± 0.21 (100)	7.68 ± 0.34 (100)	7.20 ± 0.50 (100)
	Bif	8.78 ± 0.15 (100)	8.42 ± 0.20 (100)	8.34 ± 0.07 (100)
	Lac	7.10 ± 0.17 (100)	7.52 ± 0.29 (100)	7.42 ± 0.39 (100)
	Bif+Lac	8.24 ± 0.22 (100)**	8.62 ± 0.15 (100)*	7.74 ± 0.33 (100)
Eubacterium	AOM alone	9.18 ± 0.18 (100)	9.18 ± 0.05 (100)	9.08 ± 0.15 (100)
	Bif	9.08 ± 0.08 (100)	8.76 ± 0.14 (100)*	8.48 ± 0.12 (100)*
	Lac	8.74 ± 0.13 (100)	8.92 ± 0.11 (100)	8.66 ± 0.14 (100)
	Bif+Lac	9.06 ± 0.14 (100)	9.04 ± 0.11 (100)	8.90 ± 0.08 (100)
Bacteroidaceae	AOM alone	9.42 ± 0.04 (100)	9.34 ± 0.03 (100)	9.46 ± 0.05 (100)
	Bif	9.26 ± 0.05 (100)	9.38 ± 0.07 (100)	9.24 ± 0.04 (100)
	Lac	9.2 ± 0.10 (100)	9.24 ± 0.02 (100)	9.12 ± 0.07 (100)
	Bif+Lac	9.42 ± 0.07 (100)	9.18 ± 0.24 (100)	9.26 ± 0.08 (100)
Clostridium	AOM alone	8.40 ± 0.22 (80)	7.83 ± 0.08 (80)	8.25 ± 0.05 (80)
	Bif	7.47 ± 0.60 (60)	7.63 ± 0.33 (60)	7.60 ± 0.00 (20)
	Lac	7.50 ± 0.1 (60)	7.63 ± 0.17 (60)	7.10 ± 0.20 (40)
	Bif+Lac	7.63 ± 0.33 (60)	7.86 ± 0.16 (100)	7.26 ± 0.33 (60)
Total counts	AOM alone	9.84 ± 0.04	9.88 ± 0.02	9.96 ± 0.08
	Bif	9.82 ± 0.02	9.82 ± 0.04	9.68 ± 0.05
	Lac	9.78 ± 0.02	9.86 ± 0.04	9.72 ± 0.06
	Bif+Lac	9.84 ± 0.07	9.90 ± 0.04	9.76 ± 0.05

by orally administrated lactic acid bacteria. From the lower activity of β-glucuronidase, it is clear that there is a decreased production of MAM, active carcinogenic metabolites, being transported to the colon and formation of aberrant crypt foci (ACF).

The activity of nitroreductase produced by intestinal microflora was also investigated. Nitroreductase reduces heterocyclic and aromatic nitro compounds, which are extensively used in industry and medicine, to carcinogenic derivatives (31). This enzyme activity was not affected by AOM treatment in this study. In the Bif and Bif+Lac groups, however, significant decreases of nitroreductase activity were observed. The percentage of reduction ranged from 30 to 40%. Administration of *L. acidophilus* HY2104 also inhibited the nitroreductase activity but there were no significant differences compared with that of the AOM alone group. Goldin and Gorbach (11) have demonstrated that rats fed *L. acidophilus* and naphthylamine-glucuronide, 2-nitrofluorene, or 4-phenylazo-2-naphthol substrate for glucuronidase, nitroreductase and azoreductase respectively, excrete lower amounts of free amines and bacterial enzyme products in their feces than rats not receiving *L. acidophilus*. They also have observed that *Lactobacillus* supplements decreased the level of β-glucuronidase, nitroreductase and azoreductase. Furthermore, our previous study demonstrated that β-glucuronidase and nitroreductase activities were significantly decreased in human volunteers during intake of *B. longum* HY8001 (19).

Several investigators showed that administration of lactic acid bacteria altered the intestinal microecology of the host. Recently, the intake of fermented milk has been demonstrated to have a beneficial impact on intestinal microflora, changing their composition by substantially

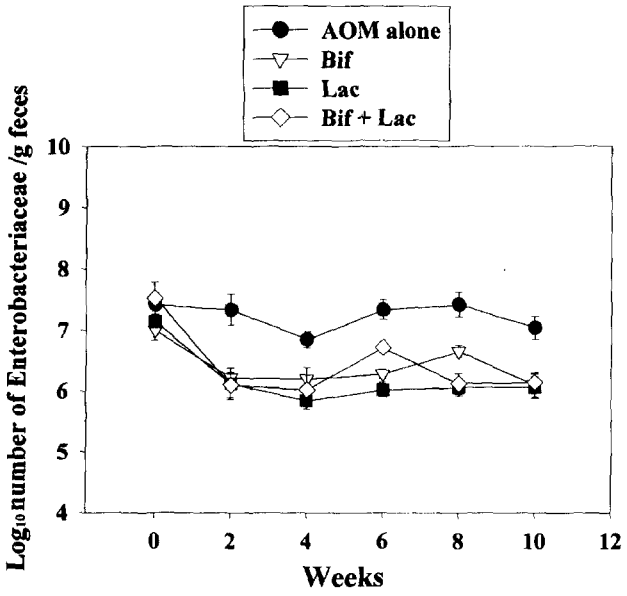

Fig. 4. Effects of *B. longum* HY8001 and *L. acidophilus* HY2104 on Enterobacteriaceae of rats treated with AOM. Fecal suspensions were serially diluted and 0.05ml of aliquots were spread onto EG, BL, TS, BS, LBS, NN, DHL and TATAC agar plates. Then agar plates were incubated at 37 for 24 hr (aerobic culture) or 48 hr (anaerobic culture). Identification was performed with colonial and cellular morphologies, Gram-reaction, spore formation and aerobic growth. The bacterial counts per gram of feces were calculated and converted into a logarithmic equivalent for each bacterial species. Values represent the mean ± SE.

increasing *Bifidobacterium* spp. and decreasing *Clostridium* and *E. coli* (23). In the study of Benno *et al.* (1), a reduction of lecithinase-negative clostridia was caused by *B. longum* feeding. Additionally, the percentages of Bacteroidaceae and lecithinase-negative *Clostridium* to the total microflora were markedly decreased. In this study, we demonstrated that lactic acid bacteria significantly reduced the number of Enterobacteriaceae including *E. coli* and the frequency of occurrence of lecithinase-negative *Clostridium*. *E. coli* and *Clostridium* have the highest level of β-glucuronidase, whereas low levels are found in *Bifidobacterium* and *Lactobacillus* (12). Therefore, the results of the present study may relate to the lower β-glucuronidase activities in LAB treated groups. The number of *Bifidobacterium* remained constant in the Bif and Bif+Lac groups. Unexpectedly, the number of *Bifidobacterium* was reduced in the Lac group and the AOM alone group after AOM inoculation. The reason for this result is not clear but it could be a consequence of the AOM treatment. Intestinal contents of rats and humans have high nitroreductase activity which is proportional to the number of anaerobic bacteria in the gut, especially *Bacteroides, Fusobacterium, Bifidobacterium, Peptostreptococcus, and Eubacterium* (15). Several *Clostridium* and *Bacteroides* spp. are capable of reducing nitroaromatic compounds (15, 29). Although there was no significant correlation between these intestinal bacteria and nitroreductase activity, the decrease in nitroreductase activity in the LAB treated groups could be related to the change in intestinal microflora and their metabolic activities. These findings demonstrate that administration of lactic acid bacteria has a beneficial influence on the distribution of intestinal microflora and the metabolic activities of the fecal enzymes. However, the mechanisms regulating the new microbial balance seem to be complex and so need further extensive investigation.

The results of the present study are closely related to our previous study which showed the inhibitory effect of LAB on the aberrant crypt foci (ACF) in AOM treated rats (17). The reduced level of β-glucuronidase activity and the number of *E. coli* may lead to a lower release of carcinogenic aglycanes and, then, to fewer ACFs. These results are in agreement with other studies showing protection by lactic acid bacteria feeding against ACF (6, 34). Our results are of considerable interest because there is no previous report examining the changes of intestinal microflora and enzyme activity in the carcinogen treated rats. Therefore, further studies are required to corroborate these findings.

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