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Bifidobacterium longum, a lactic acid-producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis

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The human colon can be described as a complex microbial ecosystem, comprising several hundred bacterial species. Some of these enteric bacteria are beneficial to the host and have been shown to exert antimutagenic and anti-carcinogenic properties. We have investigated the colon tumor inhibitory activity of *Bifidobacterium longum*, a lactic acid-producing enterobacterium. The modifying effects of this lactic culture on colonic mucosal and/or tumor cell proliferation, ODC activity and ras-p21 oncoprotein expression in colon carcinogenesis were also analyzed. Male F344 rats were fed a modified AIN-76A diet containing 0 or 2% lyophilized cultures of *B.longum* and s.c. administered azoxymethane (AOM) dissolved in normal saline at a dose of 15 mg/kg body wt, once weekly for 2 weeks. Vehicle controls received an equal volume of normal saline s.c. Animals were maintained on control or experimental diets until termination of the study. Animals intended for analysis of cell proliferation were killed 20 weeks after the second AOM injection, whereas animals intended for colon tumor analysis and measurement of ODC activity and ras-p21 expression were killed 40 weeks after the last AOM injection. The data demonstrate that dietary administration of lyophilized cultures of *B.longum* resulted in significant suppression of colon tumor incidence and tumor multiplicity and also reduced tumor volume. Results also revealed that ingestion of *B.longum* significantly inhibited AOM-induced cell proliferation, ODC activity and expression of ras-p21 oncoprotein. Data suggest that oral administration of probiotic *B.longum* exerts strong antitumor activity, as indicated by modulation of the intermediate biomarkers of colon cancer, and consequently reduced tumor outcome.

Introduction

Colorectal cancer remains one of the leading causes of cancer morbidity and mortality among men and women in Western countries, including the USA, where an estimated 133 500 new cases will lead to ~55 000 deaths in 1996 (1). Despite several advances in the treatment of cancer, the therapeutic outcome of this malignancy is posing a significant challenge to modern medicine. Consequently, primary prevention, early detection and secondary prevention are emerging as the most promising approaches for reducing the morbidity and mortality from colorectal cancer (2). Epidemiological and experimental studies have demonstrated that high dietary fat intake and lack of adequate amounts of dietary fiber increase the risk of colon cancer development (3), whereas several micronutrients, trace elements present in fruits and vegetables and their synthetic analogs reduce the risk of colon cancer (4,5). Of special interest in this regard is the beneficial effect of certain lactic acid-producing enterobacterial food supplements, the so-called probiotics, in the prevention of chronic conditions such as cardiovascular disease and cancer (6,7). These lactic cultures, which are primarily used for fermentation of milk and other dairy products, have been shown to possess antimutagenic and anticarcinogenic properties (8–10). In fact, the data from epidemiological and experimental studies indicate that ingestion of certain lactic cultures, such as lactobacilli and bifidobacteria, or their fermented dairy products reduce the risk of certain types of cancer and inhibit tumor growth (11–13). In a study in Japan, Kubota found that colon cancer incidence was lowest when the colonic population of bifidobacteria was highest and that of *Clostridium perfringens* was lowest (14). Goldin and Gorbach have shown that dietary supplements of *Lactobacillus acidophilus* suppressed DMH-induced colon tumor incidence and enhanced tumor latency in rodents (15). Shackelford *et al.* have described an increased survival rate among carcinogen-treated animals fed fermented milk (16). Recent data from our laboratory indicate that dietary intake of *Bifidobacterium longum* cultures significantly inhibits the development of azoxymethane (AOM*)-induced aberrant crypt foci representing putative premalignant lesions (17) and blocks the induction of colon and liver tumors by 2-amino-3-methylimidazo[4,5-*f*]quinolone (IQ), a food mutagen (18). However, the precise mechanism by which these lactic cultures exert their antitumorigenic influence is not clear. The antimutagenic activity of lactic acid-producing bacteria is suspected to reside in the cell wall (19), as lactic acid itself has no reported antimutagenic effects (20). Sekine *et al.* (21) and Okawa *et al.* (22) demonstrated that bifidobacterial as well as lactobacterial cell wall preparations induce immunity against tumor induction both *in vivo* and *in vitro*, with the characteristics of a biological response modifier. Studies by Zhang and Ohta (23,24) and by Orrhage *et al.* (25) have indicated that cells of lactic acid-producing bacteria bind to food-derived mutagens and decrease their absorption in the gut by physically removing them from the intestine via feces.

Polyamines play an essential role in cell proliferation and differentiation and participate in macromolecular synthesis. Ornithine decarboxylase (ODC, EC 4.1.1.17) is the first and rate limiting enzyme of this crucial polyamine biosynthetic pathway (26). Increased ODC activity has been observed in colon adenomas and carcinomas (27,28) as well as in normal appearing colon mucosa adjacent to adenomas (29), reflecting the underlying hyperproliferative state of colonic mucosa. We and others have found that inhibitors of ODC activity such

***Abbreviations:** AOM, azoxymethane; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinolone; ODC, ornithine decarboxylase; BrdU, bromodeoxyuridine; DTT, dithiothreitol; TBST, Tris-buffered saline containing 0.1% Tween-20.

as D,L-α-difluoromethylornithine suppress chemically induced colorectal tumorigenesis (30,31). Increasing interest has been focused on alterations in ODC activity in association with clinically premalignant and malignant lesions. This has led to studies on the potential use of ODC as an intermediate biomarker of cancer risk (32,33). Another putative biological marker that has been extensively analyzed in a variety of tissues is the degree of cell proliferation. Enhanced cellular proliferation resulting in anomalous expansion of epithelial cells within the colonic crypts has been found in patients known to be at high risk for colon cancer (34,35). A similar expansion of the proliferative region of actively renewing epithelium has been noted in humans with precancerous lesions of the stomach (36), esophagus (37) and cervix (38) and in various organs of carcinogen-treated animals (39). It has also been observed that several antitumor agents and nutritional factors that have been shown to inhibit colon carcinogenesis in rodents are also inhibitors of cellular proliferation (40).

At the molecular level, recent evidence indicates that activation of *ras* proto-oncogenes, coupled with the loss or inactivation of suppressor genes (anti-oncogenes) induces a malignant phenotype in colonic cells (41). The *ras* proto-oncogenes (c-Ki-*ras*, c-Ha-*ras* and N-*ras*) constitute a family of highly conserved genes encoding a structurally and functionally related 21 kd protein, referred to as ras-p21, which is anchored to the cytoplasmic face of the plasma membrane, binds to the guanine nucleotides GTP and GDP and is believed to function as a molecular switch in transmembrane signaling events of cell growth and differentiation (42). The malignant potential of *ras* genes is attributed to mutational activation by single base substitution, critically in codons 12, 13 or 61, or by enhanced expression of ras-p21, or both (43,44). More than 50% of human colon tumors have been shown to carry mutations in codon 12 of K-*ras* (44). An even higher incidence of similar mutations has been observed in aberrant crypt foci representing putative premalignant lesions (45), in colon adenomas (46) and also in histologically normal appearing mucosa adjacent to regions of colon carcinomas (47). Enhanced levels of oncogenic forms as well as normal cellular ras-p21 have been detected in a variety of human tumors, including colon cancer (48,49). We and others (50,51) have seen predominantly K-*ras* codon 12 mutations and also enhanced expression of normal as well as mutated ras-p21 in colon tumors and in uninvolved colonic mucosa of carcinogen-treated rodents, suggesting an association between expression of activated ras and colon tumorigenesis. We and others (51,52) have also provided evidence for inhibition of carcinogen-induced *ras* mutations and suppression of expression of both normal as well as mutated ras-p21 by inhibitors of ODC activity and other agents known to block colon tumor development. Thus, it is likely that changes in ODC activity and modulation of cell proliferation and ras-p21 expression that are associated with colon tumorigenesis would also be involved in colon tumor inhibition by *B.longum*.

It was therefore of interest to evaluate the colon tumor inhibitory properties of dietary *B.longum* in the established colon cancer model. We have analyzed the effect of dietary *B.longum* on AOM-induced colon tumorigenesis in male F344 rats. We have also examined how this enterobacterial culture influences ODC activity, cell proliferation and the expression of mutated as well as normal cellular ras-p21 during AOM-induced colon carcinogenesis in order to better understand the underlying mechanisms.

Materials and methods

Animals, diets and carcinogen

Weanling male F344 rats were obtained from Charles River Breeding Laboratories (Kingston, NY). Lyophilized cultures of *B.longum* were a generous gift from the Morinaga Milk Industry Co. Ltd (Zama City, Japan). *Bifidobacterium longum* was cultured in a fermentor of 30 l capacity in medium containing 2% glucose, 1% peptone, 1% yeast extract and 0.5% salt. The incubation time was 12–14 h at 37°C until the viable cells reached ~3–4×10^9/ml. The cells were harvested by centrifugation and washed in saline. After mixing with a cryoprotectant solution containing 1% sodium glutamate and 3.5% sucrose, the cells were lyophilized at the Research and Development Center of Morinaga Milk Industry Co. Ltd. The viable cells were enumerated by anerobic plate count methods as described by Rasic (53). Each gram of lyophilized culture contained ~2×10^{10} live bacterial cells. All ingredients of the semipurified AIN-76A diet were obtained from Dyets Inc. (Bethlehem, PA) and stored at 4°C prior to preparation. The composition of the control diet was as follows (54): 20% casein, 0.3% D,L-methionine, 52% corn starch, 13% dextrose, 5% corn oil, 5% Alphacel, 3.5% mineral mix (AIN-76A), 1% vitamin mix (AIN-76A) and 0.2% choline bitartrate. Lyophilized cultures of *B.longum* at the 2% level, equivalent to 4×10^{10} live cells/g diet, were added to the semipurified AIN-76A diet at the expense of dextrose. All the control and experimental diets were prepared weekly in our laboratory and were stored in a cold room. Animals had access to food and water at all times and food cups were replenished with fresh diet three times per week. AOM (CAS 25843-45-2) was obtained from Ash Stevens (Detroit, MI).

Experimental procedure

Male F344 rats received at weaning were quarantined for 1 week. Animals were assigned to either AOM-treated or vehicle-treated groups and were housed in plastic cages with filter tops under controlled environmental conditions of 21°C temperature and 50% humidity on a 12 h light/dark cycle. Beginning at 5 weeks of age, groups of animals were fed the modified AIN 76-A diet containing 0 (for controls) or 2% lyophilized *B.longum* cultures (for experimental groups). Two weeks later, animals intended for carcinogen treatment were given s.c. injections of AOM dissolved in normal saline at a dose of 15 mg/kg body wt once weekly for 2 weeks. Vehicle controls received s.c. injections of a corresponding volume of normal saline. The animals were maintained on control or experimental diets until termination of the experiment. Body weights were recorded weekly until 16 weeks of age and then every 4 weeks until termination of the study. Groups of animals intended for cell proliferation analysis were killed 20 weeks after the second AOM injection, whereas animals intended for colon tumor evaluation, ODC activity and ras-p21 analysis were killed 40 weeks after the second AOM injection.

All animals were killed by CO_2 asphyxiation at the scheduled times and were carefully necropsied. After laparotomy, the entire stomach and the small and large intestines were resected and opened longitudinally. The intestinal contents were flushed with ice-cold normal saline. Using a dissection microscope, tumors in the colon and small intestine were grossly recorded for their location, number and size. For each tumor, the length (L), width (W) and depth (D) were measured with calipers and estimates of tumor volume (V) were made according to the formula $V = L \times W \times D \times \pi/6$ (53). Colon tumors with a diameter of >0.4 cm were cut into two halves; one portion was used for the analysis of ODC and ras-p21 and the other half was processed for histopathological examination of tumor type. In addition, tumors <0.4 cm in diameter were used for histopathology. Colonic mucosa that was free of tumors from AOM-treated animals and from saline-treated animals was scraped with a microscope slide, snap-frozen in liquid nitrogen and stored at –80°C until used for ODC and ras-p21 analysis. For histopathological evaluation, colonic and small intestinal tumors were fixed in 10% buffered formalin, embedded in paraffin blocks and processed for hematoxylin and eosin staining. Stained sections were examined for tumor types as described (55). Most of the tumors in this study were adenocarcinomas.

Cell proliferation assay

Mucosal cell proliferation was analyzed using the bromodeoxyuridine (BrdU) labeling method as described (56). One hour before being killed by CO_2 asphyxiation, animals intended for cell proliferation assay were i.p. injected with 20 mg/kg body wt BrdU. Their colons were removed, carefully slit open longitudinally from cecal end to rectum, fixed in 80% ethanol and embedded in paraffin. Sections of 4 μm thickness were cut perpendicular to the mucosal surface, deparaffinized, rehydrated and incubated for 30 min with 0.3% H_2O_2 in methanol to quench the endogenous peroxidase activity and were incubated again for 20 min in 4 N HCl to denature the DNA. BrdU incorporation in the nucleus was detected using monoclonal anti-BrdU as primary antibody (Becton-Dickinson, Mountview, CA) and a biotinylated secondary antibody with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Slides

Table I. Body weights of male F344 rats treated with AOM or vehicle and fed the control or experimental diets

Experimental group	Body wt (g) at weeks after last AOM/saline injection[a]					
	0	8	16	24	32	40
AOM-treated						
Control diet (42)[b]	173 ± 10	292 ± 16	350 ± 21	382 ± 24	412 ± 27	439 ± 36
2% *B.longum* diet (42)	175 ± 8.6	297 ± 15	358 ± 20	399 ± 23	420 ± 39	451 ± 38
Saline-treated						
Control diet (12)	181 ± 8	311 ± 16	370 ± 21	413 ± 26	445 ± 31	476 ± 42
2% *B.longum* diet (12)	181 ± 12	305 ± 18	360 ± 24	395 ± 24	435 ± 27	469 ± 27

[a]Animals were injected s.c. with AOM or saline during the second and third week of being on the control or experimental diet.
[b]Number of animals at the beginning of the study. Twenty weeks after the last AOM injection, 12 animals from each control and experimental dietary goup were used for cell proliferation studies.

were then counterstained with hematoxylin, dehydrated, clarified, mounted in Permount and examined under a standard light microscope.

Approximately 35–40 longitudinally oriented crypts were analyzed from each animal. Each crypt was scored to determine the crypt height, which is defined as total number of cells per crypt column, and the number and position of labeled cells within each crypt column. Each crypt column was divided into three compartments of equal size, compartment 1 being in the lower third of the crypt, compartment 2 in the middle third and compartment 3 in the upper third near the luminal surface. Our attention was confined to well-oriented crypts in which the base, lumen and top of the crypt could be observed. The percentage of labeled cells (labeling index) was determined by tabulating the ratio of labeled cells to total number of cells in that region/crypt ×100. The total number of cells per crypt column of each animal was also determined. The distribution in the proliferative zone was measured as a percentage of total labeled cells per crypt column.

ODC assay

The colonic mucosal and tumor samples obtained 40 weeks after the last AOM or saline injection at the termination of the study were assayed for ODC activity using previously described methods (57). Briefly, specimens were homogenized in ice-cold buffer [25 mM Tris–HCl, pH 7.5, 2.5 mM dithiothreitol (DTT), 0.1 mM EDTA] and centrifuged at 40 000 *g* for 30 min at 4°C. Aliquots of clear suparnatants were added to 50 µl of the reaction mixture [15 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 2.5 mM DTT, 0.04 mM pyridoxal phosphate, 0.4 mM L-ornithine and 25 µCi D,L-[^{14}C]ornithine hydrochloride (56.6 mCi/mmol; Amersham International, Arlington Heights, IL)]. The reaction mixture was incubated at 37°C for 1 h in 16×100 mm glass tubes sealed with rubber stoppers supporting a center well (Kontes, Morton Grove, IL). The released $^{14}CO_2$ was trapped on microglass fiber discs (934-AH, GFA; Whatmann) soaked in saturated barium hydroxide. The reaction was stopped by injecting 0.1 ml 2 N sulfuric acid through the rubber septum directly into the reaction mixture. The incubation was continued for an additional 1 h to completely trap the released $^{14}CO_2$. Center wells along with the filter discs were then transferred to glass scintillation vials and the radioactivity was counted in 10 ml scintillation cocktail (Scintisol; ISOLAB Inc.). ODC activity was determined by measuring $^{14}CO_2$ liberated from L-[1-^{14}C]ornithine and expressed as pmol $^{14}CO_2$ released/mg protein/min.

Measurement of ras-p21 expression

Differential expression of total as well as mutant ras-p21 was estimated as described (58). Briefly, colonic mucosa and tumor samples were washed in ice-cold phosphate-buffered saline and suspended in disruption buffer (50 mM Tris–HCl, pH 7.8, 100 mM NaCl, 10 mM sodium deoxycholate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) containing 0.1 mM leupeptin and 0.2 µg/ml aprotinin as protease inhibitors. The samples were homogenized and left on ice for 30 min. The extracts were clarified by centrifugation at 15 000 *g* for 15 min. at 4°C.

SDS–polyacrylamide gel electrophoresis and Western blotting. SDS–PAGE and Western transfers were carried out essentially by the methods of Laemmli (59) and Towbin *et al.* (60) respectively. Clear extracts of colonic mucosa and tumors corresponding to 200 or 100 µg total protein respectively were solubilized in sample buffer (10% SDS, 600 mM Tris–HCl, pH 6.7, 50% glycerol) containing 2-mercaptoethanol and 50 µg/ml bromophenol blue. Samples were boiled for 2 min and resolved on 12.5% reduced polyacrylamide vertical slab gels with an overlay of 5% polyacrylamide along with low range SDS–PAGE pre-stained molecular weight markers (BioRad Laboratories, Richmond, CA) and ras-p21 Western blot standards (Oncogene Science, Manhasset, NY). Electrophoretically resolved proteins were electrotransferred onto nitrocellulose membrane (Hybond ECL; Amersham International) in a Trans-blot Electrophoretic Transfer Cell (BioRad Laboratories).

Immunodetection and quantification of ras-p21 using enhanced chemiluminescence (ECL). After transblotting the electrophoretically resolved proteins, blots were blocked with a 5% solution of non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) and were then incubated with mouse monoclonal antibody pan-ras (Ab-2) or rabbit polyclonal antibody pan-ras^{Asp12} (Ab-1) (Oncogene Science) diluted in TBST containing 0.5% non-fat dried milk. Ab-2 is broadly reactive to p-21 translational products of the H-, K- and N-*ras* genes, whereas Ab-1 specifically reacts only with the mutant ras-p21^{Asp12} and not with ras-p21^{Gly12} or ras-p21^{Val12}. The rationale for determining differential expression of wild-type and Asp12- specific mutant ras-p21 was that we had, in our previous studies, observed predominantly G→A transitions in codon 12 of K-*ras*, substituting Gly with Asp, during AOM-induced colon carcinogenesis (50). Blots were extensively washed in TBST and reincubated with peroxidase-linked secondary antibody (anti-mouse Ig or anti-rabbit Ig; Amersham International) diluted in TBST containing 0.5% non-fat dried milk. The blots were thoroughly washed in excess TBST and probed with an ECL Western blot detection system (Amersham International) using Reflection autoradiography films (Du Pont NEN, Boston, MA). The autoradiography films were scanned using an Image-master sharp laser scanner (PDI, Huntington, NY) and the peak areas representing ras-p21 bands of both the standards and samples were integrated.

Protein determination

Protein contents in colonic mucosal and tumor homogenates were determined by the method of Bradford (61), using bovine serum albumin as standard.

Statistical analysis

Body weights, tumor incidence, tumor multiplicity, tumor volume, ODC activity, cell proliferation and ras-p21 expression were compared between the animals fed control and experimental diets. Tumor incidence, i.e. the percentage of animals with tumors, was analyzed by χ^2 test. Tumor multiplicity, expressed as the mean number of tumors per animal, was analyzed by unpaired *t*-test. The data on ODC activity, cell proliferation, ras-p21 expression and body weights were analyzed using unpaired *t*-test and one-way analysis of variance. Differences were considered statistically significant at $P < 0.05$.

Results

General observations

Table I presents the body weights of AOM- and vehicle-treated animals fed the control or experimental diets. Analysis of data reveals that there were no significant differences in the body weights of vehicle-treated animals fed control or *B.longum* diet nor between vehicle-treated and AOM-treated animals fed the *B.longum* diet. However, body weights of AOM-treated animals maintained on the control diet were slightly, though not significantly, lower in comparison with their counterparts on the *B.longum* diet from 16 weeks after the last AOM injection. This may be due to AOM carcinogenicity and consequent tumor burden.

Tumor data

Table II summarizes the AOM-induced tumors in the colon and small intestine in terms of tumor incidence (% animals

Table II. Effect of dietary lyophilized *B.longum* on intestinal tumor incidence in male F344 rats

	Control diet	2% *B.longum* diet
Colon		
Incidence (% animals with tumors)	77 (23/30)[a,b]	53 (16/30)
Tumors/animal	1.8 ± 1.27[d]	0.83 ± 0.98
Tumors/tumor-bearing animal	2.3 ± 0.9[c]	1.56 ± 0.80
Tumor volume (mm^3)	550 ± 3099	32 ± 49
Small intestine		
Incidence (% animals with tumors)	43 (13/30)	30 (9/30)
Tumors/animal	0.47 ± 0.57	0.30 ± 0.4
Tumors/tumor-bearing animal	1.08 ± 0.28	1.0 ± 0.07

[a]Values in parentheses are number of animals with tumors/effective number of animals in that group.
[b,c,d]Significantly different from *B.longum* group at $P < 0.05$, 0.01 and 0.001 respectively.

with tumors), colon tumor multiplicity (number of tumors/animal and number of tumors/tumor-bearing animal) and colon tumor volume. No tumors were found in vehicle-treated animals fed the control or *B.longum* diets. Dietary administration of *B.longum* cultures significantly inhibited the incidence of colon adenocarcinomas ($P < 0.05$), and colon tumor multiplicity in terms of tumors/animal ($P < 0.001$) and tumors/tumor-bearing animal ($P < 0.01$). Although there was a 91% reduction in colon tumor volume in the animals fed the *B.longum* diet, this difference was not statistically significant due to a large standard deviation. Animals fed the *B.longum* diet had fewer but a statistically insignificant number of small intestinal tumors than those fed the control diet.

Cell proliferation

The data on colonic epithelial cell proliferation were analyzed as the number of cells/crypt column and as the rate of cell proliferation (labeling index) in the lower, middle and upper third compartments of the crypt column and the total crypt column (Figure 1 and Table III). There was no significant difference in the total number of epithelial cells counted between the control and experimental groups (data not shown). However, as shown in Figure 1, the number and distribution of BrdU-labeled cells per crypt column as well as per crypt column compartment significantly differed in the two groups. As summarized in Table III, dietary *B.longum* significantly suppressed AOM-induced proliferative indices in the lower, middle and upper compartment as well as in the total crypt column ($P < 0.01$–0.001). This inhibitory effect of *B.longum* on AOM-induced cell proliferation was strongly correlated with tumor outcome.

ODC activity

Table IV summarizes ODC activity in the colonic mucosa and in the colon tumors. AOM administration significantly enhanced ODC activity in the colonic mucosa of animals fed the control as well as *B.longum* diets as compared with their saline-treated counterparts. Dietary intake of *B.longum* resulted in a significant inhibition of AOM-induced ODC activity in the colonic mucosa. Ingestion of cultures of *B.longum*, however, did not cause a significant decrease in the steady-state levels of colonic mucosal ODC activity as shown in saline-treated animals. Colon tumors of AOM-treated animals fed the *B.longum* diet exhibited significantly lower levels of ODC activity as compared with the tumors from animals fed the control diet. This inhibitory effect of cultures of *B.longum* on AOM-induced ODC activity is very well correlated with colon tumor outcome.

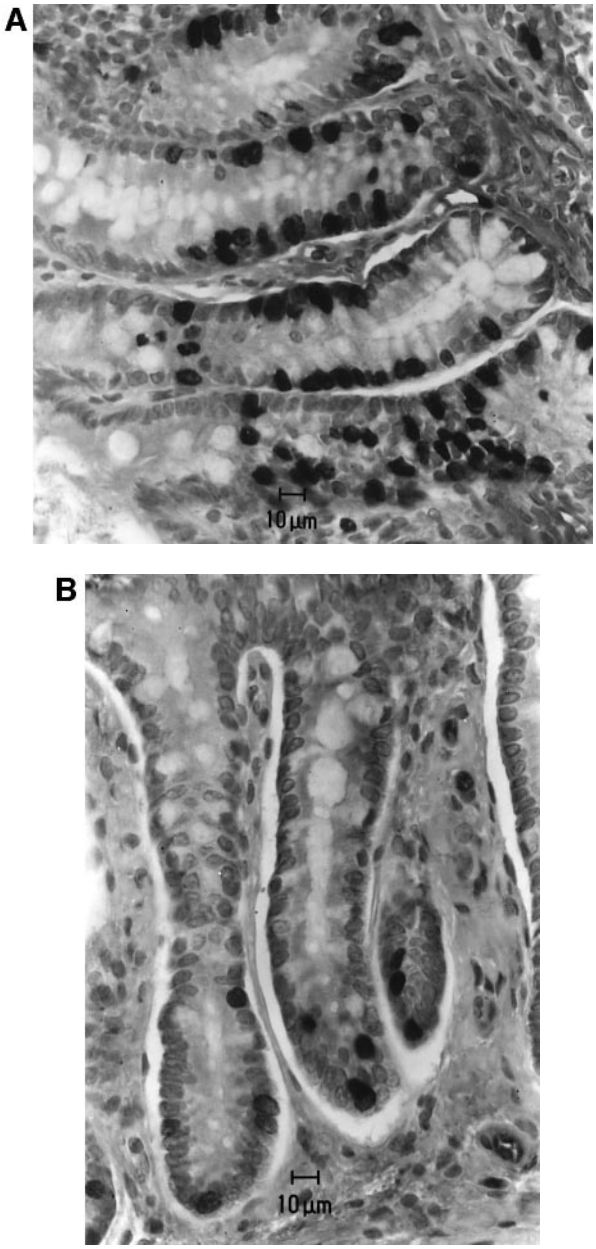

Fig. 1. Photomicrographs of colonic crypts showing BrdU-labeled cells stained immunohistochemically with anti-BrdU monoclonal antibody in male F344 rats as described under Materials and methods. (**A**) AOM-treated animals fed the control diet showing expansion of the proliferative compartment. (**B**) AOM-treated animals fed the *B.longum* diet. (Bar represents 10 µm.)

Western blot analysis and differential expression of ras-p21

Figure 2A and B demonstrates representative examples of Western blot analyses of ras-p21 and ras-$p21^{Asp12}$. The majority of the samples had detectable levels of p21 with pan-reactive anti-ras (Ab-1) mouse monoclonal antibody. Ab-1, which does not distinguish between wild-type and mutated forms of ras-p21, identified a duplet in samples expressing increased levels of p21 species that exhibit different electrophoretic mobilities (Figure 2A). These samples exhibited a single band when probed with anti-pan ras^{Asp12} (Ab-2) rabbit polyclonal antibody representing mutated ras-$p21^{Asp12}$ (Figure 2B), thereby con-

Table III. Effect of dietary lyophilized *B.longum* on rate of colonic mucosal cell proliferation[a] during colon carcinogenesis in male F344 rats

Experimental group	Percent labeled cells/total cells				Percent labeled cells/total cells in compartment		
	Total labeling index	Lower third	Middle third	Upper third	Lower third	Middle third	Upper third
Control diet	18.9 ± 1.1[b]	6.9 ± 0.5	8.8 ± 0.7	3.2 ± 0.5	21.5 ± 1.9	26.7 ± 2.0	9.9 ± 1.4
2% *B.longum* diet	12.8 ± 1.1[d]	5.2 ± 0.5[d]	5.2 ± 0.8[d]	2.2 ± 0.5[d]	15.7 ± 1.7[d]	15.5 ± 2.2[d]	7.1 ± 1.4[c]

[a]Cell proliferation is expressed as colonic crypt labeling index (LI): LI = (no. labeled cells/total number of cells)×100.
[b]Mean ± SD (n = 12).
[c]Significantly different from the control group, $P < 0.01$.
[d]Significantly different from the control group, $P < 0.001$.

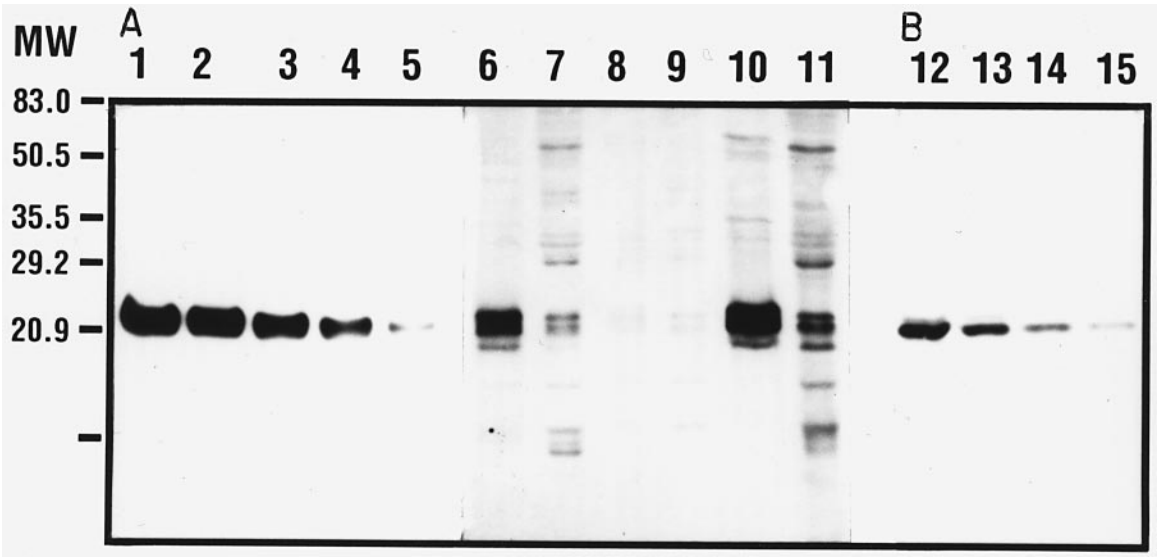

Fig. 2. Western blot analysis of total ras-p21 (**A**) and mutated ras-p21^{Asp12} (**B**) expression. Extracts of colonic mucosa or tumors were resolved by SDS–PAGE, electroblotted onto ECL-Hybond followed by immunodetection using pan-reactive anti-ras-p21 monoclonal antibody (A) or antipan ras-p21^{Asp12} polyclonal antibody (B) as described under Materials and methods. (A) Lanes 1–6, ras-p21 Western blotting standards; lanes 6 and 7, colon mucosa of AOM-treated animals fed the control and experimental diet respectively; lanes 8 and 9, colon mucosa from saline-treated animals fed the control and experimental diets respectively; lanes 10 and 11, colon tumors from AOM-treated animals fed the control and experimental diets respectively. (B) Lanes 12 and 13, colon tumors; lanes 14 and 15, colon mucosa from AOM-treated animals fed the control and experimental diets respectively.

Table IV. Effect of dietary lyophilized *B.longum* on colonic mucosal and tumor ODC activity in male F344 rats[a]

Experimental group	Control diet	2% *B.longum* diet
AOM-treated		
Mucosa	66 ± 10[b,c]	32 ± 6
Tumor	456 ± 147[c]	101 ± 30
Saline-treated		
Mucosa	11.5 ± 4.3	10.3 ± 3.3

[a]ODC activity is defined as pmol $^{14}CO_2$ released/mg protein/min.
[b]Mean ± SD (n = 12).
[c]Significantly different from *B.longum* diet group at $P < 0.001$.

firming the simultaneous occurrence of both wild-type and mutated ras-p21 phenotypes.

A standard curve of integrated optical density from laser densitometric scans representing ras-p21 Western blot standards was plotted to quantify immunoreactive ras-p21 protein (Figure 3). Table V summarizes the results of Western blot analysis for total as well as mutant ras-p21 expression in both colonic mucosa and tumors representing AOM-treated animals fed the control and *B.longum* diets. Dietary *B.longum* significantly suppressed the expression of total and mutated ras-p21 in colonic mucosa and tumors as compared with the control diet ($P < 0.01$). This inhibitory effect of *B.longum* cultures on AOM-induced ras-p21 expression was again strongly correlated with colon tumor outcome.

Discussion

The main purpose of this study was to evaluate the colon tumor inhibitory properties of cultures of *B.longum*. A previous study from our laboratory (17), demonstrating inhibitory effects of dietary *B.longum* on cecal β-glucuronidase activity and the development of AOM-induced aberrant crypt foci, provided the impetus for studying the influence of this lactic culture on colon tumorigenesis in a well-established experimental model. Our experiments demonstrate that whereas AOM administration induces multiple colon tumors in ~77% of treated animals, dietary intake of *B.longum* significantly suppresses the number as well as the size of these tumors. To our knowledge, this is the first study providing evidence that ingestion of lyophilized cultures of *B.longum*, a lactic acid-producing bacterium present in the human colon, inhibits tumor incidence and multiplicity in addition to reducing the overall volume of AOM-induced colon tumors.

Several lines of evidence support the tumor inhibitory

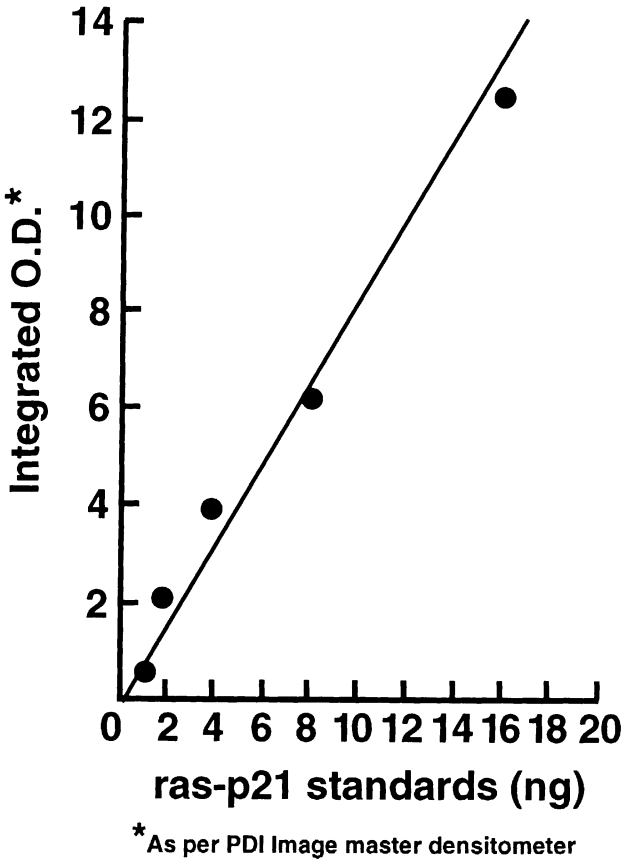

Fig. 3. *ras*-p21 standard curve.

properties of lactic acid bacteria. Epidemiological studies involving cancer patients and populations at increased risk have indicated that consumption of cultured dairy products has an inverse correlation with the risk of colon and breast cancer (11,62–64). In rodents, Shackelford *et al.* (16) demonstrated a protective effect of orally administered dairy products fermented by *Streptococcus thermophilus* or *Lactobacillus bulgaricus* against chemically induced colon tumors. In another experiment, the induction of colon carcinomas by 1,2-dimethylhydrazine in rats was significantly suppressed by feeding animals with viable *L.acidophilus* cells and it was speculated that a similar effect could occur in humans (14). Injections of live or dead *Bifidobacterium* cells into sarcomas has been shown to cause tumor regression in mice (65). Earlier studies from our laboratory have also demonstrated that long-term ingestion of lyophilized cultures of *B.longum* inhibits induction of tumors of the colon, small intestine and liver by IQ, a food-derived carcinogen (18).

While the mechanism of inhibition of colon cancer by cultures of *B.longum* is not clear, we believe that this effect may proceed through diverse mechanisms, including alteration of physiological conditions such as the pH of the colonic microenvironment affecting metabolic activity of the resident intestinal microflora and their associated enzymes, as well as the host's immune response (66). Seki *et al.* (67) reported that administration of *Bifidobacterium* cultured milk to humans significantly enhanced the colonization of bifidobacteria in the colon, as indicated by an increased count of bifidobacterial cells in their stool specimens. These colonizing cells of *B.longum* produce lactic acid, thereby lowering the intestinal pH to create a bacteriocidal environment for putative enteropathogens such as *Escherichia coli* and *C.perfringens*, thus developing a favorable microenvironment in the gut. In a previous study from our laboratory, only 20% of germ-free animals were shown to develop chemically induced colon tumors, as compared with 93% of their counterparts containing normal flora (68). Biasco *et al.* (69), in a study on patients with colonic adenomas, observed a significant reduction in their fecal pH after administering *L.acidophilus* together with *Bifidobacterium bifidus*. Another mechanism may involve the modulation of bacterial fecal enzymes that transform procarcinogens to carcinogens. Goldin *et al.* (14,70) showed that diets supplemented with viable lactic bacilli reduced the fecal β-glucuronidase activity in humans and rodents. A third mechanism could possibly involve cellular uptake of carcinogen metabolites by lactic culture cells, as demonstrated in a number of studies (23–25). AOM, a potent, organ-specific colon carcinogen has been postulated to undergo a series of chemical transformations *in vivo*, including to the proximate carcinogen, methylazoxymethanol, and the ultimate carcinogen, methyldiazonium ion, a highly reactive species that forms methylcarbonium ions and is presumably responsible for methylation of macromolecules both *in vivo* and *in vitro* (71). This alkylation of critical macromolecules is believed to be an essential step in AOM-induced colon carcinogenesis (71). In this study, where animals were administered *B.longum* cultures beginning 1 week prior to AOM treatment and throughout the experiment, it is possible that these lactic cultures, which were colonizing the colon at the expense of the resident microflora (72), may bind to methylazoxymethanol which is released into the intestinal lumen, thereby minimizing its re-absorption into the circulation by physically removing it via feces. It is also possible that the metabolites produced by lactic cultures may affect the mixed function oxygenases, especially the cytochromes P450, which are believed to be involved in the conversion of AOM from proximate to ultimate carcinogen (73). An additional mechanism of tumor suppression may involve a role of *B.longum* as an immunomodulator and biological response modifier (21,22). For example, the administration of viable or non-viable intestinal bacteria to germ-free mice has been shown to enhance intestinal production of IgA plasmacytes (74) and Kohwi *et al.* (65) have demonstrated that repeated intralesional injections of viable or killed *Bifidobacterium* inhibited the growth of Meth-A tumor cells transplanted s.c. into syngeneic BALB/c mice. Furthermore, Sekine *et al.* and others (21,22) have shown *in vivo* and *in vitro* that a water-soluble cell wall fraction, WPG, of bifidobacteria induces an antitumor effect and plays an important role as an immunomodulator in the intestines of humans and animals.

In the present study, we also analyzed the modifying effects of *B.longum* cultures on colonic mucosal and/or tumor cell proliferation, ODC activity and ras-p21 expression in order to determine if the modulation of these cellular, biochemical and molecular events relevant to colon carcinogenesis could be effectively used to monitor inhibition of colon cancer. We found that the administration of *B.longum* cultures significantly suppressed the rate of cellular proliferation, ODC activity and the expression of total as well as mutated ras-p21 in a manner strongly correlating with inhibition of tumor induction by AOM. These results thus indicate that the modulation of intermediate biomarkers such as cell proliferation, ODC activity and ras-p21 expression by lactic cultures can be effectively

Table V. Effect of lyophilized *B.longum* on the expression of total as well as mutated ras-p21 proteins in AOM-induced colon carcinogenesis[a]

Experimental group	Colon mucosa		Colon tumors	
	Total ras-p21	ras-p21^{Asp12}	Total ras-p21	ras-p21^{Asp12}
AOM-treated				
Control diet	14.2 ± 3.0	4.2 ± 1.5	32.7 ± 5.9	8.2 ± 3.1
2% *B.longum* diet	8.9 ± 2.7[b]	1.6 ± 0.9[b]	19.8 ± 4.8[b]	4.0 ± 1.2[b]
Saline-treated				
Control diet	1.5 ± 1.2			
2% *B.longum* diet	1.8 ± 1.4			

[a]Results are expressed as pg ras-p21/mg total protein.
[b]Significantly less than that in control group at $P < 0.01$.

used to monitor the progress of colon cancer inhibition by *B.longum* and to predict colon tumor outcome.

Lipkin (75) and Terpstra *et al.* (35) have suggested that the patterns and rates of mucosal cell proliferation may be acceptable measures of colon cancer risk and that modulation of cellular proliferation by agents known to prevent cancer formation might therefore serve as an intermediate end-point in cancer prevention trials. Hyperproliferation of colonic epithelial cells is induced by administration of bile acids (76), fatty acids (77), partial enteric resection (78) and also by certain carcinogens (39). Enhanced labeling indices in patients with neoplastic lesions and also in carcinogen-treated experimental animals have been observed in all sites of the colon and the uninvolved colonic mucosa. The results of the present study, showing a strong inhibition of AOM-induced cell proliferation that correlates with suppression of AOM-induced colon tumor incidence, multiplicity and tumor volume by dietary *B.longum*, are in line with these observations. Biasco *et al.* (69) observed a significant decrease in mucosal cell proliferative activity in upper colonic crypts of patients with colon adenomas after the administration of *L.acidophilus* and *B.bifidus* cultures. Elevated levels of ODC activity have been reported in neoplastic human colon versus normal appearing colonic mucosa (27–30), in dysplastic polyps versus non-dysplastic polyps (79) and also in non-involved mucosa from polyposis patients versus non-involved mucosa from normal individuals (31). Similarly, ODC activity has been found to be consistently higher in non-familial colon adenocarcinomas compared with adjacent mucosa (27). Evidence that enhanced ODC activity may play an important role in colon tumor development is provided by the observation that DFMO, a highly specific and irreversible inhibitor of ODC, suppresses colon tumor development in a time-dependent manner in carcinogen-treated rodents (30,31). Lans *et al.* (80) showed that supplemental calcium suppressed ODC activity in elderly patients with adenomas, probably by attenuating free fatty acids. In the current study, we observed elevated levels of ODC activity in both colon tumors and in uninvolved colonic mucosa of AOM-treated animals. In addition, ODC activity was significantly decreased in the colonic mucosa as well as in colon tumors of AOM-treated animals fed the *B.longum* diet. Although, the precise mechanism of inhibition of ODC activity by dietary *B.longum* is not clear, it is likely that these effects may proceed through diverse physiological and metabolic alterations.

ras activation represents one of the earliest and most frequently occurring genetic alterations associated with human cancers, especially cancer of the colon (42,43). Elevated levels of ras-p21 have been correlated with increased cell proliferation, histological grade, nuclear anaplasia and degree of undifferentiation (81). In experiments where mutated *ras* genes are selectively inactivated, the pre-existing tumor phenotype reverts to a more normal form, indicating that activated *ras* may be necessary for the maintenance of malignant behavior (82). Recent work from our laboratory and elsewhere has demonstrated a strong correlation between dietary modulation of carcinogen-induced *ras* activation and consequent tumor outcome (50–52). In this study, where dietary intake of *B.longum* cultures began 1 week prior to AOM treatment and continued throughout the experiment, the AOM-induced expression of total and activated ras-p21 was significantly suppressed both in tumors and in uninvolved colonic mucosa. As regards the mechanism of inhibition of *ras* activation afforded by *B.longum*, it is hypothesized that bifidobacterial cells, as a biological response modifier, modulate induction of the methylguanine repair protein O^6-methylguanine DNA methyltransferase, which acts as a suicide enzyme that stoichiometrically accepts a methyl group onto itself, restoring the original guanine in DNA by *in situ* demethylation (83). To our knowledge, there are no other data pertaining to the modulation of *ras* function by lactic cultures. It is, however, clear from our results that *B.longum*-augmented suppression of AOM-induced *ras* activity may interfere with the progression of events leading to colon tumor development. Further, the significant differences in expression levels of immunologically detectable ras-p21 between AOM-treated and vehicle-treated animals fed the control or experimental diets attest to the fact that determination of ras-p21 may be a useful marker to evaluate the effectiveness of tumor inhibitory properties in colon carcinogenesis. Regardless of the mechanism involved, data demonstrate that dietary *B.longum* modulates the process of selective amplification of initiated cells containing activated ras-p21 in AOM-induced colon carcinogenesis.

In conclusion, the results of this study demonstrate that dietary intake of lyophilized cultures of *B.longum*, a lactic acid-producing bacteria present in the human colon, strongly suppresses AOM-induced colon tumor development. Inhibition of colon carcinogenesis is associated with the modulation of colonic cell proliferation and colonic mucosal and tumor ODC and ras-p21.

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