Inhibitory Effect of *Bifidobacterium longum* on Colon, Mammary, and Liver Carcinogenesis Induced by 2-Amino-3-methylimidazo[4,5-*f*]quinoline, a Food Mutagen[1]

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ABSTRACT

The inhibitory effect of lyophilized cultures of *Bifidobacterium longum* on 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-induced carcinogenesis was investigated in male and female F344 rats. Beginning at 5 weeks of age, male and female rats were divided into various experimental groups and fed one of the high-fat, semipurified diets containing 0 and 0.5% lyophilized cultures of *B. longum* with or without 125 ppm IQ in the diet. All animals were continued on this regimen until the termination of the study. All animals were necropsied during the 58th week. The results indicated that dietary *B. longum* significantly inhibited the IQ-induced incidence (percentage of animals with tumors) of colon (100% inhibition) and liver (80% inhibition) tumors and multiplicity (tumors/animal) of colon, liver, and small intestinal tumors in male rats. In female rats, dietary supplementation of *Bifidobacterium* cultures also suppressed the IQ-induced mammary carcinogenesis to 50% and liver carcinogenesis to 27% of those observed in animals fed the control diet, but the differences did not reach a statistical significance at $P < 0.05$; however, the mammary tumor multiplicity (tumors/animal) was significantly ($P < 0.05$) inhibited in female rats fed the diet containing *Bifidobacterium* cultures. These findings suggest that *Bifidobacterium* supplements in the diet inhibit IQ-induced colon and liver tumors and to a lesser extent mammary tumors in F344 rats.

INTRODUCTION

There is a growing consensus on the beneficial aspects of fermented dairy products such as fermented milk and yogurt and of bacterial cultures that ferment the dairy products in human and animal nutrition (1–4). Epidemiological and experimental studies provide evidence that fermented milk and bacterial cultures that are routinely used to ferment the milk reduce the risk of certain types of cancer and inhibit the growth of certain tumors and tumor cells (1, 2, 5–9). An inverse relationship has been demonstrated between the frequency of consumption of yogurt and other fermented milk products and breast cancer in women (5, 6). There are also indications that fermentation of milk may result in the production of inhibitors of carcinogenesis (10).

Several investigations revealed that dietary intake of fermented milk containing lactic bacteria altered the intestinal microecology of the host. Consumption of fermented milk containing *Lactobacillus acidophilus* has been shown to reduce significantly the counts of fecal putrefactive bacteria such as coliforms and increased the levels of lactobacilli in the intestine (2, 11) suggesting that supplemental *L. acidophilus* has a beneficial effect on the intestinal microeclogy by suppressing the putrefactive organisms that are presumably involved in the production of tumor promoters and putative precarcinogens. Goldin *et al.* (12) demonstrated that supplemental *L. acidophilus* cultures to healthy subjects consuming a western diet significantly decreased the metabolic activity of certain classes of intestinal microflora as indicated by fecal bacterial β-glucuronidase and nitroreductase activities.

Several recent studies suggest that fermented milk and certain bacterial cultures that are used to ferment the dairy products possess antimutagenic and anticarcinogenic properties (10). Bodana and Rao (13) demonstrated antimutagenic properties of milk fermented with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* using *Salmonella typhimurium* strains TA 100 and TA 98 suggesting the production of antimutagenic compounds during the fermentation of milk. A recent study by Zhang and Ohta (14) indicated that the cells of lactic acid bacteria including *L. acidophilus* and *Bifidobacterium bifidum* bind various fried-food mutagens thereby suppressing the mutagenicity of these compounds by removing them from the intestine. It has also been demonstrated that certain lactobacilli degrade the carcinogens such as dimethylnitrosanine and diphenylnitrosamine (15). With regard to anticancer properties of *Lactobacillus* sp., several studies demonstrate that feeding of fermented milk or cultures containing *L. acidophilus* and *Lactobacillus bulgaricus* and/or *Lactobacillus casei* inhibited Ehrlich ascites tumor cell growth or suppressed the growth of Sarcoma 180 in mice (7, 16, 17). Goldin and Gorbach (9) showed that dietary supplements of *L. acidophilus* not only suppressed the incidence of 1,2-dimethylhydrazine-induced colon carcinogenesis but increased the latency period. Shackelford *et al.* (8) demonstrated that the survival rate of rats fed fermented milk was higher than that of the animals fed the nonfermented milk. There are studies to suggest that cultures of *Bifidobacterium longum* increase the host's immune response (18). These studies indicate that cultured dairy products or cultures of lactic bacteria inhibit tumorigenesis by enhancing the host's immune response, suppressing the growth of intestinal microflora incriminated in generating putative carcinogen(s) and promoters, binding potential carcinogens, and/or producing antitumorigenic or antimutagenic compounds in the colon.

The formation of mutagens upon broiling fish and meat was first discovered by Sugimura *et al.* (19). IQ, a heterocyclic aromatic amine produced from food pyrolysis, was first isolated from broiled fish (20). Subsequently, it was isolated from a variety of broiled or cooked fish and meat (21, 22). IQ is a strong mutagen in *S. typhimurium* and also induces mutations in Chinese hamster lung cells and hepatocellular carcinomas in rodents and nonhuman primates (23, 24). Other cooked food mutagens, which are heterocyclic aromatic amines, include IQ,[3] 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. They demonstrate a multitarget organospecificity with specific cancer induction in Zymbal gland, skin, colon, oral cavity, and mammary gland of rodents (23). The precursors of IQ-type heterocyclic amines are creatinine, amino acids, and sugars in meat and fish (25). It has been shown that IQ requires metabolic activation by liver microsomes for conversion to its ultimate carcinogen (26) and forms high levels of DNA adducts in a number of organs (27). Although it is not clear whether these heterocyclic amines

may contribute to human cancer development, it is certain that these compounds are present in cooked foods and pose a credible risk to humans.

Because IQ induces colon and mammary tumors in male and female rats, respectively, and bacterial cultures that ferment milk possess anticarcinogenic properties, the possibility exists that these bacterial cultures may prevent IQ-induced carcinogenesis. Accordingly, the present study was designed to investigate the efficacy of cultures of *B. longum*, a lactic bacteria indigenous to human intestine, on IQ-induced carcinogenesis in male and female F344 rats fed the high-fat diet. The rationale for the high-fat content of the experimental diet was to simulate a western-style diet. It is hoped that the results generated from this study provide a rationale for additional studies to elucidate the mechanism(s) of action of *Bifidobacterium* cultures in inhibiting carcinogenesis.

MATERIALS AND METHODS

Animals, Diets, and Carcinogen. A total of 156 weanling male and female F344 rats obtained from Charles River Breeding Laboratories (Kingston, NY) were quarantined for 10 days and then housed in plastic cages with wood chip bedding and filter tops in an animal holding room under controlled environmental conditions of a 12-h light-dark cycle, 50% humidity, and 22°C temperature. This study was conducted within the guidelines of our Institute's Animal Care and Use Committee. They were all randomly assigned by weight into 2 treatment groups (male, IQ-fed, 60; female, IQ-fed, 60; male, without IQ, 18; female, without IQ, 18). Lyophilized *B. longum* (BB-536) cultures were kindly provided by Morinaga Milk Industry Co., Ltd. (Zama City, Japan). *B. longum* was cultured in a medium containing glucose, peptone, yeast extract, and salts. The cells were harvested by centrifugation and washed using a saline solution. After being mixed with a cryoprotectant solution containing sodium glutamate and sucrose, the cells were lyophilized. Each g of lyophilized material contained about 2×10^{10} live bacterial cells. IQ (CAS 76180-96-6) was purchased from Toronto Research Chemicals (Downsview, Ontario, Canada). A high-fat semipurified diet was used throughout the study (28). All ingredients of semipurified diet were obtained from Dyets, Inc. (Bethlehem, PA). A high-fat control (modified AIN-76A) diet with or without IQ and the experimental diets with or without IQ but containing 0.5% lyophilized *B. longum* cultures were prepared in our laboratory once weekly and stored in a cold room in air-tight plastic containers filled with N_2 (Table 1). The amount of IQ added to the diets was 125 ppm.

Experimental Procedure. At 5 weeks of age, male and female animals were divided at random into various experimental groups and fed one of the high-fat diets containing 0 and 0.5% *B. longum* with or without IQ in the diet (Table 1). All animals were fed the control and experimental diets until the termination of the experiment. Animals were weighed weekly until they attained 16 weeks of age and then every 4–6 weeks until the termination of the study. Female animals were palpated for mammary tumors every 2 weeks, beginning 8 weeks on experimental diets. As scheduled, the experiment was terminated 58 weeks after the start of experimental diets.

All animals were sacrificed by CO_2 euthanasia. All organs including the intestine, liver, and mammary glands were examined grossly under the dissec-

tion microscope. They were fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by routine histological methods with the use of hematoxylin and eosin stains. The histological criteria used for tumors of intestine, liver, and mammary gland were as described previously (28, 29).

The data were analyzed statistically by the χ^2 test and Fisher's exact test (tumor incidence) and by Student's t test (body weights and tumor multiplicity). Analyses were carried out on a VAX 11/750 computer using the SAS package.

RESULTS

The body weights of male and female animals fed the control and *Bifidobacterium* diets containing IQ were comparable throughout the study (Table 2). In groups that were not given IQ, body weights of male and female animals fed the control diet were similar to those fed the *Bifidobacterium* diet in their respective gender. As expected, IQ-fed animals weighed less than those that were not fed IQ in all dietary groups during the course of the study.

Table 3 summarizes the incidences of IQ-induced colon, small intestine, liver, and mammary gland tumors. There was no evidence of tumors in animals that were not fed IQ. In the present investigation, IQ-induced tumors of colon, small intestine, and mammary gland were all adenocarcinomas. The tumors of the small intestine and colon were well-differentiated adenocarcinomas that invaded the submucosal and muscular layers. Liver tumors were hepatocellular carcinomas. Dietary supplementation of *B. longum* resulted in a significant inhibition of colon, small intestine, and liver tumor incidences in male rats ($P < 0.05$). In female rats, dietary supplementation of *B. longum* also decreased the mammary carcinogenesis to 50% and liver carcinogenesis to 27% of those observed in animals fed the control diet, but the differences did not reach a statistical significance ($P > 0.05$). It is noteworthy that the incidence of liver tumors was lower in female rats as compared to their male counterparts irrespective of dietary treatment. Also, none of the female rats developed IQ-induced colon and small intestinal tumors.

The data summarized in Table 4 show that colon tumor multiplicity (tumors/animal and tumors/tumor-bearing animal) followed the same pattern as tumor incidence summarized in Table 3. Although the effects of dietary *Bifidobacterium* on small intestinal tumor incidence in male rats and on mammary tumor incidence in female rats did not reach a statistical significance (Table 3), it significantly suppressed the multiplicity (tumors/animal) of small intestinal and mammary gland tumors in their respective gender (Table 4); dietary *Bifidobacterium*, however, had no significant effect when small intestinal and mammary tumor data were expressed as tumors/tumor-bearing animal.

DISCUSSION

It is interesting that the results of the present study indicated sex differences in the susceptibility of liver and intestines to IQ-induced carcinogenesis in F344 rats. IQ-induced liver tumors were lower in female rats compared to male animals in both dietary groups; interestingly, none of the female animals developed colon and small intestinal tumors. Our previous studies also demonstrated lower incidence of 3,2'-dimethyl-4-aminobiphenyl-induced colon and small intestinal tumors in female F344 rats compared to their male counterparts (30). Although the precise mechanism of sex differences in the susceptibility of liver and intestine to IQ and other heterocyclic amines remains to be elucidated, it is possible that male and female rats metabolize IQ differently that might explain the organospecificity of IQ in male and female animals.

The purpose of the current study was to investigate the efficacy of dietary *B. longum* cultures on IQ-induced tumorigenesis in male and

Table 1 *Percentage composition of experimental diets*

Diet ingredients	High-fat control diet [a]	High-fat experimental diet [b]
Casein	23.50	23.50
DL-Methionine	0.35	0.35
Corn oil	23.52	23.52
Corn starch	32.90	32.40
Dextrose	8.30	8.30
Alphacel	5.90	5.90
Mineral Mix, AIN	4.11	4.11
Vitamin Mix, AIN revised	1.18	1.18
Choline bitartrate	0.24	0.24
B. longum (lyophilized)	0	0.5

[a] This high-fat diet was formulated on the basis of the American Institute of Nutrition (AIN) standard reference diet with the modification of varying sources of carbohydrate (28).

Table 2 *Body weights of male and female F344 rats*

Dietary regimen	Initial body wt (wk 0)	4	16	32	48	56
	Body wt (g) on experimental diets at wk[a]					
Male rats						
Control diet + IQ (30)[b]	113 ± 6[c, d]	252 ± 14[d]	380 ± 28[d]	461 ± 37[d]	464 ± 44[d]	468 ± 33[d]
0.5% BL diet + IQ (30)	114 ± 6	253 ± 12	380 ± 21	459 ± 30	460 ± 50	466 ± 40
Control diet (9)	115 ± 7[e]	261 ± 12[e]	396 ± 21[e]	482 ± 29[e]	495 ± 39[e]	519 ± 44[e]
0.5% BL diet (9)	116 ± 12	256 ± 15	394 ± 27	482 ± 36	520 ± 48	524 ± 38
Female rats						
Control diet + IQ (30)	91 ± 5[d]	161 ± 8[d]	203 ± 13[d]	234 ± 16[d]	250 ± 22[d]	272 ± 23[d]
0.5% BL diet + IQ (30)	91 ± 5	159 ± 9	197 ± 12	228 ± 16	254 ± 24	278 ± 25
Control diet (9)	90 ± 9[e]	161 ± 9[e]	206 ± 10[e]	242 ± 17[e]	261 ± 19[e]	286 ± 22[e]
0.5%BL diet (9)	94 ± 8	164 ± 9	210 ± 17	237 ± 20	278 ± 24	292 ± 24

[a] At 5 weeks of age, groups of male and female rats were fed the control diets with or without IQ and with or without lyophilized *B. longum* cultures (BL). This period is denoted as week 0.
[b] Numbers in parentheses, number of animals.
[c] Mean ± SD.
[d, e] Differences among the dietary subgroups in IQ-fed and in non-IQ-fed animals are not significant, $P > 0.05$.

Table 3 *Effect of dietary B. longum on IQ-induced intestinal, liver, and mammary carcinogenesis in F344 rats*

Dietary regimen	Liver	Colon	Small intestine	Intestine[a]	Mammary gland
	Tumor incidence (% of animals with tumors)				
Male rats (30)[b]					
Control diet	80 (24)[b]	23 (7)	20 (6)	40 (12)	
0.5% BL diet[c]	50 (15)[d]	0[d]	3 (1)	3 (1)[d]	0
Female rats (30)					
Control diet	37 (11)	0	0	0	27 (8)
0.5% BL diet	27 (8)	0	0	0	13 (4)

[a] Intestine represents colon and small intestine.
[b] Numbers in parentheses, number of animals.
[c] BL diet, control diet containing 0.5% lyophilized *B. longum* cultures.
[d] Significantly different from its respective control diet group in the same gender, $P < 0.05$.

because to our knowledge, this is the first report showing that long term administration of cultures of *B. longum*, a human lactic bacterium, can effectively reduce the tumorigenesis induced by IQ, a heterocyclic amine produced from broiling or frying of meat or fish. There are no previous reports on the tumor inhibitory properties of dietary *B. longum* in laboratory animal models. Our results demonstrated that lyophilized cultures of *B. longum* administered in the diet not only inhibited completely the colon (100%) and small intestinal tumors (80%) but also suppressed the liver tumorigenesis (38%) in male F344 rats. Although the inhibitory effect of *Bifidobacterium* cultures on the incidence of IQ-induced mammary carcinogenesis in female rats was not found to be significant at $P < 0.05$, the tumor multiplicity, expressed as tumors/animal, was significantly suppressed in female rats fed the *Bifidobacterium* diet suggesting an inhibitory effect of these bacterial cultures in mammary carcinogenesis. The reason for the insignificant effect of *Bifidobacterium* cultures on small intestinal tumor incidence in males and mammary tumor incidence in females may, in part, be due to low overall incidence of IQ-induced tumors in these organs.

A number of animal model studies have already demonstrated that dietary *L. acidophilus*, a lactic acid-producing bacterium; cultured dairy products; and milk fermented with *L. acidophilus* inhibit 1,2-dimethylhydrazine-induced colon carcinogenesis in rats and proliferation of Ehrlich ascites tumor cells in mice (1, 9, 16, 17). The results of present study, which indicate that the lyophilized cultures of *B. longum*, a lactic acid-producing bacterium indigenous to human colon, administered in the diet inhibit liver, colon, and mammary carcinogenesis, provide further evidence for tumor-inhibitory properties of lactic cultures and fermented dairy products.

While the mechanism of inhibition of colon and mammary carcinogenesis by dietary *B. longum* has not been elucidated, it is likely that the effect of lactic bacteria can proceed through diverse mechanism including the alteration of physiological conditions in the colon affecting the metabolic activity of intestinal microflora, the action of bile acids, and to quantitative and/or qualitative alterations in the bile acid-degrading bacteria. The species of lactobacilli and bifidobacterium, most often suggested as beneficial dietary supplements, have all been reported to exert antagonistic actions toward several entero-

Table 4 *Effect of dietary B. longum on IQ-induced intestinal and mammary tumor multiplicity in F344 rats*

Dietary regimen	Intestine						Mammary gland		
	Colon			Small intestine					
	Total tumors	Tumors/animal	Tumors/TBA[a]	Total tumors	Tumors/animal	Tumors/TBA	Total tumors	Tumors/animal	Tumors/TBA
Male rats									
Control diet	13	0.43 ± 0.89[b]	1.86 ± 0.89	7	0.23 ± 0.5	1.16 ± 0.4	0	0	0
0.5% BL diet	0	0[c]	0[c]	1	0.03 ± 0.18[c]	1.0 ± 0	0	0	0
Female rats									
Control	0	0	0	0	0	0	14	0.46 ± 0.80	1.75 ± 0.46
0.5% BL diet	0	0	0	0	0	0	5	0.16 ± 0.46[c]	1.25 ± 0.5

[a] TBA, tumor-bearing animals; BL diet, control diet containing 0.5% lyophilized *B. longum* cultures.
[b] Mean ± SD.

pathogenic organisms in the intestine such as *Escherichia coli* and *Clostridium perfringens*, to cite a few (31–34). *C. perfringens* and other enteropathogenic anaerobic bacteria contain high levels of 7α-dehydroxylase which is an important enzyme in the formation of the secondary bile acids from the primary bile acids in the colon (35, 36). These secondary bile acids have been shown to play a role as tumor promoters in the colon (36). Hill *et al.* (35) showed a correlation between the incidence of colon cancer and the number of bacteria per g of feces possessing 7α-dehydroxylase enzyme activity in the humans. In view of above results, it is possible that dietary lactic cultures modulate the metabolic activity of intestinal microflora and the activity of 7α-dehydroxylase thereby producing lower levels of secondary bile acids in the colon. Goldin and Gorbach (37) observed that supplementation of normal diet of rats with *L. acidophilus* lowered the activity of fecal bacterial β-glucuronidase, nitroreductase, and azoreductase. The significance of these bacterial enzymes including the 7α-dehydroxylase activity should be considered in the light of their importance in the etiology of certain types of cancer including cancer of the colon (38). It was also demonstrated that lactic acid, a major metabolite produced by lactic bacteria in yogurt and other fermented milk products, and fresh unfermented milk products had no inhibitory effect on Ehrlich ascites tumor cells in mice whereas the lactic fermented milk had an effect (39). In this connection, Ayebo *et al.* (10) reported that this antitumorgenic activity of fermented milk is located in the cell wall fraction of lactic bacteria. Thus, dietary *B. longum* cultures and associated physiological alterations in the intestine could act at one or more of these loci and cause inhibition of IQ-induced carcinogenesis.

Another possible mechanism that should to be considered is the metabolic activation of IQ in the intestine. Heterocyclic aromatic amines such as IQ, like many carcinogens, must be metabolized in order to exert their carcinogenicity. The predominant pathway for the metabolic activation of most carcinogenic heterocyclic amines is through the initial activation step involving *N*-oxidation and is catalyzed predominantly by cytochrome P4501A2 in the liver (40). IQ is also converted to the *N*-glucuronide, a minor metabolite, but to a considerable extent to the 5-hydroxy derivative, excreted via bile into the intestinal tract as glucuronide conjugate (41, 42). The bacterial enzyme, β-glucuronidase, has the ability to hydrolyze many glucuronides due to its wide substrate specificity and thus may liberate aglycones in the colon. It is possible that glucuronide conjugates of IQ metabolites are hydrolyzed in the intestine by bacterial enzyme, β-glucuronidase, to active metabolites and that these active compounds are absorbed and distributed to various target organs including the colon and mammary gland. In this connection, Goldin *et al.* (9, 12) have demonstrated that the addition of viable lactic bacilli supplements to the diets of humans and rats decreased the fecal bacterial β-glucuronidase activity. It is therefore possible that a similar decrease in the β-glucuronidase activity in the colon due to *B. longum*, a lactic bacillus, may result in the decreased production of active metabolites of IQ in the colon and delivery of these metabolites to the colon and to the mammary gland via the blood stream. Another possible mechanism of tumor inhibition by *B. longum* may be explained on the basis that the bacterial cultures bind IQ and other food mutagens in the intestine and eliminate them in the feces (14), thereby reducing the amount available for reabsorption.

In conclusion, the results of this study demonstrate that dietary lyophilized cultures of *B. longum*, a lactic bacillus present in human colon, inhibit IQ-induced intestinal, liver, and mammary carcinogenesis in F344 rats. Although the exact mechanisms by which the cultures of *B. longum* inhibit IQ-induced carcinogenesis in the target organs are not understood at present, the results of earlier studies on

microflora should provide a stimulus to design additional studies to investigate the mechanism of colon, liver, and mammary tumor inhibition by the cultures of *B. longum*.

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