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Bifidobacterium longum and lactulose suppress azoxymethane-induced colonic aberrant crypt foci in rats

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Bifidobacterium longum **has been shown to afford protection against colon tumorigenesis. Lactulose, a keto analog of lactose, serves as a substrate for preferential growth of *Bifidobacterium*. It is not known whether feeding lactulose along with *B.longum* will have any advantage over feeding of *B.longum* alone. To test this combination effect, 61 male Fisher 344 weanling rats were divided into four groups of 15 rats each (16 in the control group) and assigned to one of the following four diets for 13 weeks: (i) AIN76A (control, C); (ii) C + 0.5% *B.longum* (C+Bl, containing 1×10^8 viable cells/g feed); (iii) C + 2.5% lactulose (C+L); (iv) C + 0.5% *B.longum* + 2.5% lactulose (C+Bl+L). All animals received a s.c. injection of azoxymethane at 16 mg/kg body wt at 7 and 8 weeks of age. Colons of 10 rats from each dietary group were analyzed for aberrant crypt foci (ACF), which are preneoplastic markers. Colonic mucosa and livers from five rats were analyzed for glutathione S-transferase (GST, a Phase II enzyme marker). Results indicate that feeding of lactulose and *B.longum* singly and in combination reduces the number of ACF ($P = 0.0001$) and the total number of aberrant crypts significantly ($P = 0.0005$). The total number of ACF in diets C, C+Bl, C+L and C+Bl+L were 187 ± 9, 143 ± 9, 145 ± 11 and 97 ± 11 respectively. There was no significant difference in weight gain among treatments. Colonic mucosal GST levels were significantly ($P = 0.05$) higher in the Bl and L groups compared with group C. Initially there was a mild diarrhea in lactulose-fed rats. There was a positive correlation between higher cecal pH and number of ACF. Results of the study indicate that *Bifidobacterium* and lactulose exert an additive antitumorigenic effect in rat colon.**

Introduction

Colorectal cancer is the second largest cause of cancer death in western countries. Epidemiological evidence points to the fact that environmental factors far outweigh hereditary factors in the etiology of colon cancer (1,2). Hill *et al.* (3) suggested the involvement of intestinal microflora in the pathogenesis of colon cancer. Individuals at high risk of developing colon cancer have been shown to harbor intestinal microflora with increased ability to metabolize steroids and to hydrolyze glucuronides (3–5). Goldin and Gorbach (6) have shown that the intestinal microflora of rats fed a beef diet are able to hydrolyze glucuronides and to reduce azo compounds and aromatic nitro compounds at a high rate.

The antineoplastic property of fermented milk and cultures used therein has long been recognized (7–9). Goldin and Gorbach (10) demonstrated that ingestion of milk fermented with *Lactobacillus acidophilus* reduces both the quantities of β-glucuronidase, azoreductase and nitroreductase formed by intestinal microflora and the counts of fecal putrefactive bacteria such as coliforms, while increasing the levels of lactobacilli in the gut, thereby suppressing the growth of putrefactive organisms presumably involved in the production of tumor promoters and putative procarcinogens (11). Lactobacilli have also been shown to degrade nitrosamines (12). Feeding of fermented milk has also been shown to increase the survival rate of rats with chemically induced colon cancer (13). Bodana and Rao (14) demonstrated antimutagenic properties of milk fermented by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* using *Salmonella typhimurium* strains TA 100 and TA 98 as test organisms.

The protective effect of *Bifidobacterium* was first discovered in 1899 (15) and its therapeutic effect was made use of by the Japanese, who included it in their diets (16). Bifidobacteria are the predominant bacteria in human gut microflora and have been considered to exert a beneficial effect on human health by maintaining the equilibrium of the colonic microflora. The bacterial production of organic acids, particularly lactic acid, stimulation of the immune system and the accumulation of specific metabolites are the determining factors in its probiotic action (17). The inhibitory effect of *Bifidobacteriun longum* on colon, mammary and liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline was shown by Reddy and Rivenson (18). Their study indicated that *B.longum* significantly inhibited the incidence of colon and liver tumors and the multiplicity of colon, liver and small intestine tumors in male rats, while in female rats it suppressed mammary tumors to a lesser extent. Later Kulkarni and Reddy (19) demonstrated that *B.longum* suppresses azoxymethane (AOM*)-induced aberrant crypt foci in the rat colon. More recently, Gallaher *et al.* (20) also demonstrated that feeding bifidobacteria (species not reported) reduces dimethylhydrazine-induced aberrant crypt foci in the distal colon of rats.

The immune response elicited by *B.longum* and *B.animalis* was studied by Sekine *et al.* (21), who observed that both *B.longum* and *B.animalis* promote the induction of inflammatory cytokines (IL-6 and TNF-α) in mouse peritoneal cells. In an earlier study, Sekine *et al.* (22) had shown that whole peptidoglycan (WPG), a new cell wall preparation from *B.infantis*, was an active stimulator of the host-mediated response and had a higher efficiency of regression of established tumors in mice. Their study demonstrated that purified cell walls are responsible for the antitumor activity of *B.infantis* and that a cell wall preparation which had the physical integrity of the cell wall structure (WPG) was more effective against progressively growing tumor cells than cell walls that were

***Abbreviations:** WPG, whole peptidoglycan; AOM, azoxymethane; ACF, aberrant crypt foci; GST, glutathione S-transferase.

Table I. Dietary composition

Ingredient	Diet			
	Control (AIN76A)	Bl	L	Bl+L
Casein, vitamin-free	20.0%	20.0%	20.0%	20.0%
Corn starch	52.0%	51.5%	49.5%	49.0%
B.longum[a] (BB-536)	0.0%	0.5%	0.0%	0.5%
Lactulose	0.0%	0.0%	2.5%	2.5%
Common ingredients[b]	28.0%	28.0%	28.0%	28.0%

[a]Lyophilized powder containing 2×10^{10} live cells/g; final concentration in feed 10^8 viable cells/g feed.
[b]Dextrose 13%, corn oil 5%, Alphacel 5%, methionine 0.3%, AIN76 vitamin mix 1%, AIN mineral mix 3.5%, choline bitartarate 0.2%.

disrupted, suggesting the presence of an active principal structure in the crude cell wall portion. The antitumor activity of WPG of *B.infantis* on peritoneal and thoracic tumor-bearing animals was studied by Sekine *et al.* (23) and results of their study revealed that WPG induces and activates non-specific phagocytes (polymorphonuclear cells and macrophages) *in situ* to reject growing tumor cells in peritoneal and thoracic cavities of animals. That WPG has adjuvant activity was shown earlier by Sekine *et al.* (24).

Lactulose (4-*O*-β-D-galactopyranosyl-D-fructofuranose), a disaccharide, is a keto analog of lactose and has been used as a laxative for several decades. Lactulose resists the hydrolytic action of β-galactosidase and is therefore not absorbed in the small intestine. Incorporation of lactulose in an infant feeding formula was shown to enhance the development of intestinal flora containing *B.bifidus* that resembled the flora of breast fed infants (25). In humans, a diet containing lactulose (3 g/day for 3 weeks) was shown to significantly increase the number of bifidobacteria in feces, decrease the mean fecal pH and increase the fecal water content. Fecal β-glucuronidase, nitroreductase and azoreductase were also found to decrease significantly (26).

Though there are some studies demonstrating the beneficial effect of feeding *Bifidobacterium* on colon tumorigenesis and very few on the protective effect of lactulose, there are no reports in the literature on the effect of feeding both bifidobacteria and lactulose on colon carcinogenesis. Since lactulose serves as a substrate for the growth of bifidobacteria, the objective of our study was to examine the effects of feeding lactulose and *B.longum*, alone and in combination, on AOM-induced colon carcinogenesis in rats.

Materials and methods

Animals, housing and diets

Sixty one male Fisher 344 weanling rats were obtained from Charles River Laboratories (Wilmington, MA) and housed in stainless steel wire cages (2 rats/cage). The temperature and relative humidity were maintained at 21°C and 50% respectively. Light and dark cycles were 12 h each. Feed and water were provided *ad libitum*. After a 1 week period of acclimatization, the animals were divided into four groups of 15 rats each (16 in the control group) and fed the experimental diets for 13 weeks (Table I). During this time weekly body weights and daily feed intake were recorded. Animals in group 1 received the semi-purified AIN76A diet (control). Those in group 2 received the control diet containing 0.5% *B.longum* (Bl, 1×10^8 viable cells/g feed; strain BB536, a gift from Morinaga Milk Industry, Japan). Animals in group 3 received control diet containing 2.5% lactulose (L) (a gift from Morinaga Milk Industry, Japan), while those in group 4 received the control diet containing 0.5% *B.longum* and 2.5% lactulose (Bl+L). Initially the level of lactulose was 5% in the diet, however, after 10 days of feeding, two rats in group L and one rat in group Bl+L died due to severe diarrhea and so all the rats in groups L and Bl+L were switched to diets containing 2.5% lactulose). *Bifidobacterium longum* and lactulose were added at the expense of cornstarch. All diets were prepared on a weekly basis and stored at 4°C until use.

Carcinogen injection

All animals received a s.c. injection of AOM in saline at 16 mg/kg body wt, one dose at 7 weeks and another dose at 8 weeks of age.

Sample collection

At 17 weeks of age, all animals were killed using CO_2 asphyxiation. The livers were removed and stored at –80°C. The colons of all rats from each group were removed and flushed with 0.1 M potassium phosphate buffer, pH 7.2. Ten colons of rats from each dietary group (11 from the control) were prepared for counting aberrant crypt foci (ACF), which are preneoplastic lesions and excellent intermediate markers of colon tumorigenesis (27). Colons of the remaining five rats from each dietary group were split open longitudinally and the colonic mucosa scraped using a microscope slide. The colonic mucosal scrapings were stored in a vial at –80°C for glutathione S-transferase (GST) assay.

Measurement of cecal weight, cecal pH and diarrheal index

The cecum from each rat was excised, weighed and then split open and the pH of the cecal contents was recorded. The diarrheal index was measured by assigning the following numbers based on the appearance of the pellets in the colon: 0, normal; 1, mild diarrhea (gas in the colon); 2, moderate diarrhea (gas and soft pellets); 3, overt (semi-solid pellets); 4, severe (watery diarrhea).

Counting the ACF

Colons were prepared and ACF were counted essentially as described by Bird (27). Briefly, the colons were split open longitudinally and placed on a strip of filter paper with their luminal surface open and exposed. Another strip of filter paper was placed on top of the colons. The colons were then placed in a tray containing 10% buffered formalin and fixed overnight. The colons were cut into two halves (proximal and distal). Each half was then cut into 2 cm long segments and placed in a Petri dish containing 0.05% methylene blue solution and stained for 5 min. The segments were transferred to another Petri dish containing buffer (0.1 M potassium phosphate, pH 7.2). The stained segments were placed on a slide and the ACF were counted under low magnification using a light microscope. The total number of ACF as well as the number of crypts per focus were recorded.

GST assay

GST in the liver and colonic mucosal samples was assayed by the procedure of Habig *et al.* (28). Colonic mucosa or liver samples were homogenized in 10 vol. 0.1 M potassium phosphate buffer, pH 7.0, in a Potter–Elvejem homogenizer (10 strokes) at 4°C. The homogenate was centrifuged at 10 000 *g* for 30 min. In the case of colonic mucosa, the supernatant was centrifuged for a second time at 10 000 *g* for 5 min to obtain a clear supernatant, which was used for the assay. In the case of liver, the first supernatant was filtered through Whatman No. 1 filter paper to remove the fat and the filtrate was used for the assay. The assay mixture (1 ml) contained 0.1 M potassium phosphate buffer, pH 6.5, 1 mM 1-chloro-2,4-dinitrobenzene (Sigma Chemical Co., St Louis, MO), 1 mM EDTA (Sigma), 1 mM glutathione (9.6 E/mM/cm; Sigma). The reaction was started by addition of 50–100 µl of sample and change in absorbance at 340 nm as a function of time was monitored in a Cary 1/3 UV/visible dual beam spectrophotometer. The initial velocity of GST was calculated and the concentration determined using the above mentioned extinction coefficient. A unit of activity is defined as µmol product formed/min under the conditions of the assay. GST activity is reported both as U/g tissue as well as U/mg protein.

Statistical analysis

Analysis of variance was done using the Statview™ 512+ statistical program (Abacus Concepts Inc.).

Results and discussion

There were no significant ($P > 0.05$) differences among dietary groups in either weight gain or feed intake (Table II). There was a significant ($P = 0.0001$) reduction in the total number of ACF in the colons of the rats consuming the Bl, L or Bl+L diets compared with the control (Table III). However, there was no significant difference between the Bl and L groups in total number of ACF. The total number of ACF in the Bl+L group was significantly ($P < 0.05$) lower than the number of

ACF in the Bl and L dietary groups. Similarly, it was observed that feeding Bl and Bl+L significantly ($P < 0.05$) decreased the total number of aberrant crypts in the colons of rats treated with AOM when compared with the control (Table III; $P < 0.0001$). Rats fed the Bl+L diet had significantly ($P < 0.05$) lower numbers of aberrant crypts than rats consuming the Bl or L diets alone. The effect of feeding the experimental diet on caloric intake and whether any change in the caloric intake was responsible for the lowered number of ACF in this experiment was not considered because it is very difficult to assess the caloric value of lactulose. It is known that lactulose is further metabolized in the cecum of rats (and in the colon of humans) to volatile fatty acids that are further metabolized to release energy.

Total number of foci containing 1–5 crypts/focus were counted in the proximal and distal regions of the colons for all the treatments. Student's *t*-test revealed that the distal segment of the colon had a significantly ($P < 0.05$) higher number of crypts than the proximal segment only when foci contained 2 and/or 3 crypts/focus. Our study also revealed that the total number of foci containing 2 crypts was significantly higher in number than foci containing 1, 3, 4 or 5 crypts for all dietary treatments. Feeding of Bl, L and Bl+L significantly reduced the total number of foci containing 2 crypts (Figure 1). In other studies (29) which report 4 crypts/focus being related to higher tumor yield, the animals were killed 12 weeks after AOM injection, as compared to 9 weeks after AOM injection in our study. Thus the time period after AOM injection seems to influence the preponderance of foci containing 2 or 4 crypts and it should be considered as an important parameter when using ACF as intermediate biomarkers in predicting tumor incidence.

Since lactulose is used therapeutically to induce diarrhea, we measured the diarrheal index in the rats consuming different diets. Groups receiving the L and Bl+L diets had a significantly ($P < 0.05$) higher diarrheal index than the animals in the control group. Lactulose seemed to be responsible for the observed diarrheal symptoms (Table IV). Since lactulose is known to cause a reduction in intestinal and cecal pH (30), the pH of the cecal contents as well as the cecal weight was recorded for the different dietary treatments (Table IV). As expected, cecal pH of the animals on the L and Bl+L diets were significantly ($P < 0.05$) lower than that of the animals in the control group. There was a significant ($P < 0.05$) difference between animals consuming the Bl diet and those on the L as well as the Bl+L diets. Since there was no significant difference between the L and Bl+L groups in cecal pH, it can be safely concluded that lactulose was responsible for the drop in pH. The cecal weights of the animals on the L and Bl+L diets were ($P < 0.05$) significantly higher than those in the control or Bl groups. As anticipated, the cecal weights inclusive of contents increased in both groups consuming lactulose.

The effects of dietary treatment on liver and colonic mucosal total GST levels are shown in Table V. There was no difference in liver GST levels (U/g tissue) among the different dietary treatments. However, the Bl+L group showed significantly lower ($P < 0.05$) GST activity than the Bl and L groups when the activity was represented as U/mg protein. In the colonic mucosa, the levels of GST (U/g tissue) were higher in the animals receiving the Bl, L and Bl+L diets than the levels in the control group, however, only those in the Bl+L group were significantly different from the values in the control group ($P < 0.05$). When the enzyme activity was reported as

Table II. Effect of dietary treatments on weight gain and feed intake

Diet[a]	*n*	Weight gain (g)	Feed intake (g/rat/day)
Control	16	271.7 ± 3.93	14.4 ± 0.27
Bl	15	269.3 ± 4.30	14.3 ± 0.28
L	13	275.1 ± 6.42	15.4 ± 0.30
Bl+L	14	256.4 ± 8.47	14.3 ± 0.29

[a]See Table I for dietary composition and abbreviations.
Data are given as means ± SEM.
There were no significant differences among treatments.

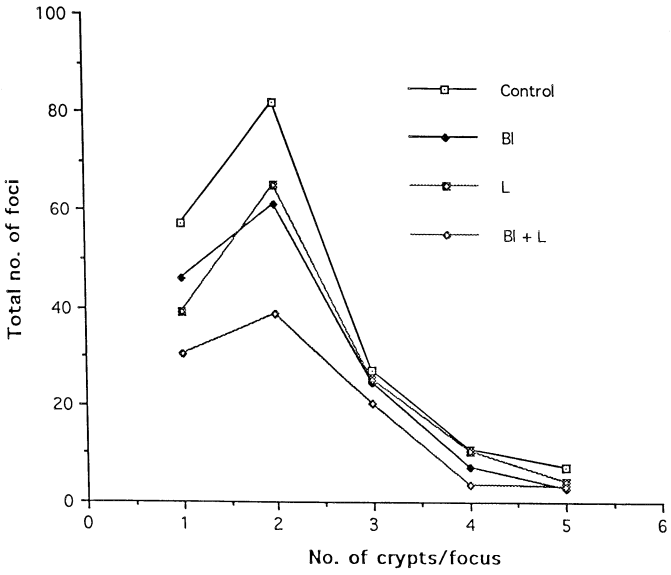

Fig. 1. Effect of dietary treatments on total number of foci containing 1, 2, 3, 4 or 5+ crypts/focus.

Table III. Effect of dietary treatments on total number of ACF and aberrrant crypts (AC) in colon

Diet[a]	*n*	Total no. ACF			Total no. AC		
		Proximal	Distal	Total	Proximal	Distal	Total
Control	11	85.7 ± 4.9[b]	100.9 ± 6.6[b]	186.6 ± 9.1[b]	163.9 ± 11.4[b]	224.5 ± 5.0[b]	393.4 ± 35.1[b]
Bl	10	64.5 ± 6.3[c]	78.6 ± 4.5[c]	143.1 ± 9.4[c]	120.2 ± 11.8[c]	171.3 ± 12.5[bc]	291.5 ± 21.0[c]
L	10	61.1 ± 5.7[c]	84.2 ± 9.6[bc]	145.3 ± 11.2[c]	118.5 ± 14.9[c]	195.5 ± 26.3[b]	315.0 ± 32.2[bc]
Bl+L	10	42.2 ± 5.9[d]	55.0 ± 6.2[d]	97.2 ± 11.0[d]	82.0 ± 10.9[d]	120.0 ± 15.3[cd]	202.0 ± 25.0[d]

[a]See Table I for dietary composition and abbreviations.
[b–d]Means ± SEM in the same column bearing different superscripts are significantly different ($P < 0.05$) by Fisher PLSD mean separation.

Table IV. Effect of diet on diarrheal index (DI), cecal pH and cecal weight

Diet[a]	*n*	DI	*n*	Cecal pH	*n*	Cecal wt (g)
Control	16	0.0^{b}	11	6.5 ± 0.25^{b}	11	3.5 ± 0.11^{b}
Bl	13	0.3 ± 0.13^{bd}	11	6.6 ± 0.17^{b}	11	3.4 ± 0.11^{b}
L	12	0.8 ± 0.18^{cd}	10	6.3 ± 0.10^{c}	10	6.1 ± 0.28^{c}
Bl+L	12	1.1 ± 0.38^{ce}	11	6.0 ± 0.11^{c}	11	6.1 ± 0.33^{c}

[a]See Table I for dietary composition and abbreviations.
[b–e]Means ± SEM in the same column bearing different superscripts are significantly different ($P < 0.05$) by Fisher PLSD mean separation.
DI range: 0, normal; 1, mild diarrhea (gas in the colon); 2, moderate diarrhea (gas and soft pellets); 3, overt (semisolid pellets); 4, severe (watery diarrhea).

Table V. Effect of diet on liver and colonic mucosal GST

Diet[a]	Liver			Colonic mucosa		
	n	U/g tissue	U/mg protein	*n*	U/g tissue	U/mg protein
Control	5	68.5 ± 5.30^{bc}	0.7 ± 0.05^{b}	5	16.4 ± 1.02^{b}	0.6 ± 0.04^{b}
Bl	5	68.9 ± 5.07^{bc}	0.7 ± 0.06^{b}	5	19.9 ± 1.41^{bc}	0.7 ± 0.05^{b}
L	5	71.0 ± 1.55^{b}	0.7 ± 0.03^{b}	3	17.7 ± 1.63^{bc}	0.7 ± 0.09^{b}
Bl+L	5	58.1 ± 1.10^{c}	0.6 ± 0.01^{c}	4	22.7 ± 1.53^{c}	0.7 ± 0.06^{b}

[a]See Table I for dietary composition and abbreviations.
[bc]Means ± SEM in the same column bearing different superscripts are significantly different ($P < 0.05$) by Fisher PLSD mean separation.

U/mg protein, there were no significant differences between the dietary treatments in GST activity in the colonic mucosa.

Experimental evidence suggests that lactic cultures, especially lactobacilli and bifidobacteria, can reduce the levels of microbial enzymes. It is also well known that carcinogens such as AOM are metabolized to the ultimate carcinogen methylazoxymethanol by bacterial β-glucuronidases in the colon (31). Thus, by reducing the activity of this microbial enzyme, *Bifidobacterium* may well reduce the conversion of AOM to its ultimate carcinogen. Studies by Sekine *et al.* (21) also showed that a cell wall preparation of bifidobacteria has antitumor properties. WPG was shown to induce polymorphonuclear cells followed by macrophages in ICR male mice inoculated i.p. with Ehrlich tumor cells. The preventive effect of WPG of *B.infantis* on the occurrence of mammary tumors induced by 7,12-dimethylbenz[*a*]anthracene in Sd strain female mice has also been demonstrated by Kitamura *et al.* (32). In fact, a recent animal model paper by Reddy and Rivenson (18) demonstrated that dietary *Bifidobacterium* reduces colon tumor incidence dramatically.

Lactulose, in addition to being bifidogenic in nature, also causes a reduction in colonic and cecal pH. There is concern that the fermentation byproducts of lactulose, such as short chain fatty acids, may cause cell proliferation in the intestinal epithelium. However, experimental studies on the effect of osmotic laxatives such as lactulose on intestinal and colonic epithelial cell proliferation by Geboes *et al.* (33) showed that lactulose had no effect on ileal and colonic epithelial cell proliferation and, therefore, is safe and should not be regarded as a tumor promoting substance. However, according to Lupton *et al.* (34), there is a significant inverse correlation between luminal pH and percent cells in S phase in the cecum. Thus, their results show that acidification of the colonic contents by dietary modification leads to increased epithelial cell proliferation. However, in our study, lactulose-fed animals showed a drop in cecal pH and also exihibited significantly lowered numbers of ACF. We also observed a weak yet positive correlation between increased pH values and increased number of ACF. It is known that bacterial enzymes such as 7α-hydoxylase convert primary bile acids to secondary bile acids, which are tumor promoters. This conversion is higher when the colonic pH is high. Hennigan *et al.* (30) studied the role of lactulose in intestinal carcinogenesis and found that lactulose causes a steep decrease in colonic and small intestinal pH and that lactulose is also associated with a sharp rise in the ratio of secondary to primary bile acids. They reported a decrease in tumor yield in the small intestine of rats fed lactulose. However, they did not find a significant reduction in tumor yield in the colon.

GST is one of the Phase II enzymes involved in the detoxification of toxic metabolites and carcinogens. The enzyme catalyzes the conjugation of arene oxides as well as numerous other substrates possessing electrophilic carbon atoms with glutathione to yield products containing exceptionally stable thioether linkages (35), which are either secreted in the bile as GSH conjugates or are further metabolized to mercapturic acid (36). There are several studies showing elevation of hepatic GST by dietary antioxidants (37). However, in this study feeding of the Bl and L diets did not cause an increase in hepatic GST levels, thereby suggesting that perhaps hepatic detoxification of the carcinogen by GST is not the route of anticarcinogenic action by *Bifidobacterium* and lactulose. On the other hand, colonic GST activity (U/g tissue) was significantly higher in the group receiving the Bl+L diet than the control group. Thus, local Phase II enzyme activity may still be responsible for reduced tumorigenesis in the Bl and L groups.

In conclusion, feeding *B.longum* and lactulose significantly reduced the total number of ACF in male Fisher 344 rats. The anticarcinogenic effects of *Bifidobacterium* and lactulose seem to be additive. Altering the metabolic activity of the colonic microflora with bifidobacteria and reduction in cecal pH as well as the bifidogenic effect of lactulose may be the mechanisms by which the anticarcinogenic effect is exerted. Gallaher *et al.* (20) reported an increase in fecal bifidobacterial count in rats fed a skimmed milk/fructooligosaccharide diet compared with

the control. Preliminary results from our experiment indicated the presence of higher bifidobacterial counts in the feces of rats fed the Bl + L diet. Our investigation supports the concept of a synbiosis between a prebiotic (lactulose) and a probiotic (*Bifidobacterium*) (38). Thus, lactulose and *B.longum* might be synbiotic in reducing colon tumorigenesis.

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