

Symposium: Probiotic Bacteria: Implications for Human Health

The Role of Probiotic Cultures in the Prevention of Colon Cancer^{1,2}

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ABSTRACT Risk factors for colon cancer include both hereditary and environmental factors. Dietary patterns represent controllable risk factors for the development of colon cancer. Much attention has focused on decreasing colon cancer risk through increasing intake of dietary fiber; recently, this has included interest in the consumption of prebiotics and probiotics. Because factors involved in the initiation and promotion of colon cancer might be separated in time from actual tumor development, it is difficult to choose “outcomes” or “end points” that are definitive indicators of efficacy of probiotics or prebiotics. Studies that have explored the cause-effect relationship directly have used animal models. In this review, we have confined our discussion to animal studies from the last 10 years that have examined most directly the relationship between prebiotic and probiotic consumption and colon cancer development. To present the consensus of these studies first, it appears that probiotics with or without prebiotics have an inhibitory effect on the development of aberrant crypts (precancerous lesions) and tumors in animal models. The effect is not completely consistent and is small in some studies, but this may represent a dose or time effect. *J. Nutr.* 130: 410S–414S, 2000.

KEY WORDS: • *probiotic* • *prebiotic* • *colon cancer*

A 1999 report on cancer statistics from the National Cancer Institute (NCI)¹ was released in April 1999; it states that from 1990 to 1996, four cancer sites, i.e., lung, prostate, breast and colon and rectum, accounted for more than half of all new cancer cases; these cancers were also the leading causes of cancer deaths. Tracking trends for those primary sites shows that rates are going down for prostate cancer incidence and mortality. Breast cancer incidence rates have shown little change in the 1990s, whereas breast cancer death rates have been declining ~2%/y since 1990. Colorectal cancer incidence and death rates continued to decline for both men and women. However, even with a decrease, the NCI indicates that colon cancer is the second most frequently diagnosed cancer among both men and women in the United States and the second most common cause of cancer death. Between 133,000 and 160,000 new cases of colorectal cancers are diagnosed each year, with a combined death total of 50,000–60,000 people. Risk factors for developing cancer include both

hereditary and environmental factors. Hereditary factors include familial polyposis, hereditary nonpolyposis colon cancer, Lynch syndromes I and II, and ulcerative colitis. Environmental factors, such as living in an industrialized area, physical inactivity, exposure to certain chemicals and consumption of a high fat, low fiber diet, are of greater interest because they represent controllable risk factors. In particular, much attention has focused on decreasing cancer risk through diet alterations, particularly increasing intake of dietary fiber (including “prebiotics”) and consumption of probiotics. A probiotic is defined as a “a viable microbial dietary supplement which beneficially affects the host through its effects on the intestinal tract” (Gibson and Roberfroid 1995). A prebiotic is defined as a “nondigestible food ingredient which beneficially affects the host by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health promoting bacteria in the intestinal tract, thus improving the host’s intestinal balance” (Gibson and Roberfroid 1995).

Development of colon cancer represents a sequence of events that, although incompletely understood, occurs in definable steps. First is an initiating step, in which a carcinogen produces an alteration in the DNA. This step may be preceded by a metabolic activation of a precursor to produce the carcinogen. At present, it is believed that several mutations must occur for a tumor to develop. The post-initiation steps are much less clear, but usually involve changes in signal transduction pathways. The next clearly observable step is an overgrowth in the colonic crypts, which can be seen morphologically as an aberrant crypt. Aberrant crypts, which are considered preneoplastic structures, are enlarged and elevated relative to normal crypts, and have a serpentine growth pat-

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⁴ Abbreviations used: AC, aberrant crypt; ACF, aberrant crypt foci; AOM, azoxymethane; DMH, 1,2 dimethylhydrazine; FOS, fructooligosaccharide; NCI, National Cancer Institute; SCFA, short-chain fatty acids.

tern. Aberrant crypts may occur singly or as groups of aberrant crypts within a single focus. A certain small but unknown fraction of these aberrant crypts will progress to polyps and eventually to tumors.

Because factors involved in initiation and postinitiation steps might be separated in time from actual tumor development, it is difficult to choose "outcomes" or "endpoints" that are definitive indicators of efficacy of a given treatment such as probiotics. In many animal and human studies of colon cancer, investigators have measured how diets or treatments affect predisposing factors, such as increases in enzyme activities that activate carcinogens, increase procarcinogenic chemicals within the colon or alter populations of certain bacterial genera or species. A number of studies have now shown that these predisposing factors are altered favorably by consumption of certain probiotics or prebiotics. However, these studies do not demonstrate a causal relationship to development of colon cancer and are at best circumstantial. Studies that do explore the cause-effect relationship directly are, by necessity, animal studies. In this review, we have confined our discussion to animal studies from the last 10 years that have examined most directly the relationship between pre- and probiotic consumption and colon cancer development. We will note human studies that provide support for the conclusions drawn from the animal studies. To present the conclusion first, it appears from these studies that probiotics with or without prebiotics have an inhibitory effect on the development of aberrant crypts (precancerous lesions) and tumors in animal models. The effect is not completely consistent and is small in some studies, but this likely represents a dose effect.

Animal and human studies

Early studies examined the effects of milk fermented with lactobacilli and *Candida* on tumor formation (Takano et al. 1985). The investigators found that colon tumorigenesis induced by 1,2 dimethylhydrazine (DMH) was reduced in rats given the fermented milk. Shackelford et al. (1983) studied the effects of milk fermented by *Streptococcus thermophilus* or *Lactobacillus bulgaricus* on DMH-induced colon tumors. Survival rate was greater in rats fed the fermented milk, but numbers of colon tumors were not different among the control skim milk group, the group given *S. thermophilus*-fermented milk and the group given *L. bulgaricus*-fermented milk. Abdelali et al. (1995) studied aberrant crypt (AC) formation in rats fed skim milk, skim milk fermented with *Bifidobacterium* sp. Bio, and the same bacteria incorporated into the diet. The test diets reduced the incidence of AC by ~50%. There was no difference in cecal pH, but the groups consuming the bifidobacteria had decreased cecal β -glucuronidase activity. Tsuda et al. (1998) actually studied the influence of lactoferrin on azoxymethane-induced aberrant crypts, but used *Bifidobacterium longum* (3% of diet) as a positive control in their studies. Both lactoferrin and *B. longum* reduced aberrant crypt foci (ACF).

Koo and Rao (1991) reported that administration of both bifidobacteria (*B. pseudolongum*) and 5% neosugar [fructooligosaccharide (FOS)] to female mice given DMH resulted in ~50% as many AC as in control animals at 18 and 38 wk. There were also decreased numbers of ACF at 18 and 38 wk after DMH injection. Bifidobacteria in feces were measured at 38 wk only; the numbers of bifidobacteria were slightly but significantly elevated (8.85 ± 0.2 vs. 9.45 ± 0.19) over controls in mice fed the treatment. The decrease in aberrant crypts was a positive effect on the mouse host; however, several key pieces of data would have been useful. The groups

of mice were as follows: controls given the AIN-76 defined diet, mice fed DMH only and the same diet, and those given DMH + bifidobacteria + neosugar and the same diet. The design does not allow the effects of bifidobacteria alone or neosugar alone to be determined. Although the differences in numbers of fecal bifidobacteria at wk 38 were significant, our experience has been that changes in numbers of bifidobacteria of less than ~1 log-fold usually do not reach significance, even with 15–20 animals/group. Another indicator of these small changes in numbers of bifidobacteria in relation to other genera might be changes in the short-chain fatty acid (SCFA) profile. In this study, acetic acid in the cecal contents was not significantly different between the DMH and DMH + bifidobacteria + neosugar groups. Acetic acid and lactic acid are produced by bifidobacteria, whereas butyrate and propionate are not produced; one might expect an increase in acetic acid if numbers of bacteria producing them increase significantly. It is important to remember, however, that concentrations of SCFA represent both production and utilization. The investigators did measure a host outcome (AC) that has been accepted as a predictor of the development of colon tumors. The question that remained at the closure of this study is whether the small changes in bifidobacteria due to the treatment are responsible for the decrease in lesion formation or whether some other factor, such as changes in other SCFA or inhibitory substances or even other groups of bacteria, played a role.

A series of studies examining the influence of bifidobacteria and/or FOS and inulin on aberrant crypts or tumors was presented by Reddy and colleagues (Kulkarni and Reddy 1994, Reddy and Rivenson 1993, Reddy et al. 1997, Reddy 1998). The initial study examined the induction of tumors by 2-amino-3-methylimidazo[4,5-f]quinoline, a food mutagen. Both male and female rats were fed a high fat diet (AIN-76), with or without the mutagen and with or without the addition of *B. longum* for 58 wk. The diets were mixed weekly and kept in air-tight plastic containers. The *B. longum* was lyophilized in a cryoprotectant solution containing glutamate and sucrose. The authors state that each gram of lyophilized material contained 2×10^{10} live bacterial cells, but it is not clear whether this measurement was taken at the time of lyophilization or the time of feeding or both. There were differences between male and female rats in incidence of colon tumors. Females did not develop colon tumors on either the diet + mutagen or diet + mutagen + lyophilized culture. Males fed the control diet + the mutagen developed 23 tumors, whereas males fed the same diet + mutagen + the lyophilized culture did not develop any tumors. This study found more tumor development in liver in both sexes than in colon, suggesting that this particular mutagen is not a potent inducer of colon tumors. No measurements of viable bifidobacteria in the feces or intestinal contents were reported for any of the groups. Similarly, the relationship between viable bifidobacteria and feeding of the lyophilized *B. longum* is not clear, nor is the relationship between live bifidobacteria in the colon and the presence or absence of tumors because the bifidobacteria were not measured.

Kulkarni and Reddy (1994) induced colonic aberrant crypts in male F344 rats by azoxymethane (AOM) treatment. At 5 wk of age, groups of rats were fed either the AIN-76 diet or AIN-76 + 1.5 or 3.0% of lyophilized culture of bifidobacteria as described above. At 10 wk of age, the rats were given the AOM injection. Six weeks later, rats were killed and AC in the colons determined. The consumption of the lyophilized cultures inhibited the development of AC in the colon by ~50%; fecal β -glucuronidase was also decreased in feces of rats

fed the cultures. Again, fecal bifidobacteria were not measured in the study, and it is impossible to draw conclusions about the relationship of numbers of viable bifidobacteria and the outcome measured (in this case, AC). In addition, the effect of the addition of lyophilized culture was not linear; 1.5% was equally as effective as 3.0% addition to the diet. This is not surprising, considering that the absolute numbers of bifidobacteria in the two groups differed by a small factor. The measurement of β -glucuronidase represents an indirect indicator of risk because it is not clear which bacteria produce it and whether it has a direct effect on the outcome measured. However, in this case, it correlated with the decrease in AC.

Reddy et al. (1997) fed 10% oligofructose or 10% inulin as part of the AIN-76 diet to male F334 rats that were given AOM in a design similar to the one reported above. The rats were killed 7 wk after the last dose of AOM. Total numbers of AC per colon were significantly less (120 ± 28 for control; 92 ± 28 for oligofructose; 78 ± 37 for inulin) in rats that consumed these prebiotics at 10% of the diet. No bacterial cultures of feces or colon contents were reported, but the authors cited the data of other studies, which reported that these prebiotics increase bifidobacteria and decrease other less desirable organisms. Again, based on our own observations, the effect of these prebiotics in rats (numbers of bifidobacteria and clostridia or AC) is not consistent; thus, measurement of the change in bacteria is critical in drawing conclusions about the relationship between bacteria and aberrant crypt formation. Oligofructose or inulin fed at 10% of the diet is a high amount of dietary fiber intake of a specific type for rats; it would be interesting to determine whether other dietary fibers at this level have similar effects or whether the effect is specific for these two oligosaccharides. The authors refer in detail to other studies that note changes in bacteria numbers and production of SCFA, such as lactic acid and butyric acid. They refer to studies in which the feeding of oligosaccharides increases butyrate levels in the colon as a positive outcome because butyrate has been associated with apoptosis and decreased cellular proliferation. However, increased butyrate concentration is not directly consistent with increasing numbers of bifidobacteria and displacement of less desirable organisms such as clostridia because bifidobacteria do not produce butyrate, whereas clostridia do produce it.

Reddy (1998) reviewed the various studies from his group. In addition to the data cited above, he presented data showing that the colonic labeling index, ornithine decarboxylase activity, and ras-p21 oncogene activity were decreased in rats fed the lyophilized cultures of *B. longum*. These measures are thought to reflect cell proliferation, and correlation with AC numbers is not surprising. However, these indices are not necessarily indicative of a cause-effect in terms of AC or tumor formation because relatively few AC progress to tumors and we do not understand completely the factors that influence tumor formation.

Our own studies in male Wistar rats have not been consistent in terms of increases in numbers of bifidobacteria, decreases in clostridia, or AC formation in response to feeding of bifidobacteria or FOS (Gallaher et al. 1996). We used DMH as the carcinogen and measured the ability of probiotics and FOS to inhibit AC formation in the postinitiation phase. In our first experiment of the series, we gavaged 10^9 bifidobacteria per day and fed 2% FOS (Gallaher et al. 1996). Feeding bifidobacteria + FOS inhibited AC formation in this experiment by almost 50%, but there was not an inverse correlation of AC with the numbers of cecal bifidobacteria, nor was there any correlation with numbers of cecal *Clostridium perfringens*. In a second experiment, we used a saline-gavaged control group, a

milk-gavaged control group, a group gavaged with milk + bifidobacteria, a group gavaged with milk + FOS, and a group gavaged with milk + both bifidobacteria and FOS. We found no differences in AC with any treatment and no correlations with cecal bacteria. In a third experiment, we repeated the second study. We found marginal decreases of ACF in the rats gavaged with bifidobacteria + FOS compared with control rats gavaged with skim milk. ACF numbers did not correlate with numbers of fecal bifidobacteria or *C. perfringens*. In another experiment, we changed this design to include the following: control (rats gavaged with skim milk), rats gavaged with skim milk + FOS, rats gavaged with skim milk + bifidobacteria + FOS, rats gavaged with *Lactobacillus acidophilus* + FOS, and a group that was gavaged with *L. acidophilus*, bifidobacteria and FOS. We found no differences in AC numbers, but in this case, did find decreased numbers of fecal clostridia in the rats that received bifidobacteria-FOS, *L. acidophilus*-FOS, or bifidobacteria + *L. acidophilus* + FOS. We found no consistent correlation of bacterial numbers with AC, nor did we find effects of bifidobacteria or FOS on AC formation. Last, we examined the effects of various oligosaccharides consumed in the diet + bifidobacteria. We found that the group of rats fed FOS and bifidobacteria did have significantly decreased AC, but AC did not correlate with changes in bifidobacteria or clostridia. When data from all experiments were plotted as the relationship between bifidobacteria and clostridia, we did see an inverse relationship of bifidobacteria with clostridia. We were careful to provide for the consistent consumption of numbers of viable bacteria that the rats received each day. We gavaged live cultures that were made up fresh daily and assayed for viability randomly during the experiment from the same mix as was given to rats. We also used 2% FOS in the diets; this is less FOS than used in other studies and might be the reason for differences in effects observed. However, we felt that this level was reasonable in terms of amounts consumed. Our conclusion was that bifidobacteria + FOS had some slight effect on AC numbers in rat colon, but this effect was not due directly to numbers of culturable bifidobacteria in the colon.

Challa et al. (1997) examined AOM-induced AC in rats consuming *B. longum* ± lactulose. Both *B. longum* and lactulose singly and together reduced ACF formation. The authors concluded that the effect of *B. longum* and lactulose was additive, but numbers of bifidobacteria in gut contents were not measured. This makes it difficult to ascribe the results directly to changes in colonic bifidobacteria.

Rowland et al. (1998) found that consumption of bifidobacteria or inulin or both together inhibited AOM-induced small ACF. These treatments were also associated with decreased β -glucuronidase activity and ammonia concentration in cecal contents of rats. β -Glucosidase and cecal weight were increased with these treatments. There was no measure of numbers of bifidobacteria in the colon or the feces in this study. Again, the enzyme measurements suggest that some alteration in bacterial metabolism that is related to the decreases in ACF has occurred, but do not implicate directly a particular bacteria or suggest changes in numbers of any group or changes in metabolite levels.

Arimochi et al. (1997) studied the effect of numbers of intestinal bacteria on AC formation with AOM as the administered carcinogen. They presented very different conclusions than those of other investigators about the genera of bacteria that decrease AC formation. Bifidobacteria had no effect, whereas both *L. acidophilus* and *C. perfringens* decreased AC formation significantly. The culture supernatants were found to mediate the effect, suggesting a metabolite product (they suggested butyrate produced by *C. perfringens*). The drinks

containing bacterial cultures were prepared freshly each day, but there is no indication of how much the rats drank of each solution, even though similar numbers of each bacteria were added to the drinks. It is possible that bacteria exhibited differential survival in the bottles before actual consumption. The authors did not find that β -glucuronidase activity was affected by *L. acidophilus*, even though AC were decreased by *L. acidophilus*. *C. perfringens* treatment also did not increase β -glucuronidase activity, as would be expected if this enzyme and *C. perfringens* were positively correlated with AC development; in fact, *C. perfringens* was correlated with decreased AC development.

Onoue et al. (1997) studied the effects of inoculating germ-free rats with various combinations of microbiota. Germ-free rats were given *Escherichia coli*, *Enterococcus faecium*, and several strains of *Bacteroides* and *Clostridium* sp. (gnotobiotic) or feces from conventional rats. They were then given DMH injections 3 and 4 wk later and then killed 11 or 34 wk after that. Addition of bacteria to germ-free rats increased both the ACF with four or more AC and the mean number of AC per focus. When *Bifidobacterium breve* was added to the defined inoculation (gnotobiotic) noted above, ACF with four or more AC per focus and crypt multiplicity were significantly lower at 11 wk, but not at 34 wk. *B. breve* addition did not affect the fecal microflora, again making it difficult to attribute the differences to changes in numbers of flora.

Goldin et al. (1996) studied the effect of dietary fat (20 and 5%) and administration of *Lactobacillus casei* on development of tumors in DMH-treated Fischer 344 rats. A lyophilized powder of 10^{11} viable cells/g was added at 1% of the diet. The rats consumed $\sim 2-4 \times 10^{10}$ organisms/d. At 24 wk, in the high fat group fed the lactobacillus before, during and after DMH injections, colon tumors numbered 24 vs. the DMH control number of 74 (not significantly different, but colon tumors per tumor-bearing animal were 3.7 vs. 1.7, which was significantly different. There was also a significant decrease in percentage of rats with tumors, from 100% in the control to 71% in the rats fed lactobacilli. This study highlights problems with expression of data, when various measures that are used to indicate development of precancerous lesions do not correlate. The question of the most meaningful expression of data remains to be answered.

The outcomes measured in human studies are more indirect and provide more circumstantial evidence than is offered by animal studies, but may support or refute data from animal studies in the host of most interest. Advantages of human studies are as follows: 1) this is the real population target of colon cancer prevention and the information gained from studies can be applied more directly (such as efficacy of various strains or degree of colonization); 2) the variability of the population plays an important role, in contrast to studies using homogeneous populations of animals. A few recent studies illustrate the nature of human studies that have addressed various aspects of the relationships among diet, fecal bacteria and colon cancer risk.

Meijer-Severs et al. (1993) compared SCFA concentration and selected bacteria in controls and patients with familial polyposis before and after colectomy. Preoperative patients had bacterial counts similar to those of controls (*B. fragilis* in control: 10^9 ; preop, 10^9 ; postop, 10^7 ; bifidobacteria: control, $10^{9.5}$; preop, $10^{9.75}$; postop, $10^{8.1}$). After colectomy, numbers of Bacteroides and bifidobacteria were decreased compared with preop and controls. The ratio of acetic acid to other SCFA increased, in proportion to decreases in other SCFA.

Kanazawa et al. (1996) studied control and high risk patients after treatments for large bowel cancer were completed

and the colon appeared normal again. Feces were collected under CO_2 and packed on ice for shipment, but were not actually cultured until 30 h. later. It is unclear whether dietary intake was determined from only one sample taken on the day before the fecal sample, but analysis indicated that patients consumed more carbohydrate, soluble fiber and calcium than controls. Bacterial cultures revealed that the feces of patients contained more lecithinase-negative clostridia ($10^{9.4}$ vs. $10^{8.8}$), more lactobacilli ($10^{8.37}$ vs. $10^{6.88}$) and less yeast ($10^{3.32}$ vs. $10^{3.96}$). pH was significantly higher in the patient group, as was H_2S and cresol concentration. One question not addressed by this study concerns the cause-effect timeline; did the differences reflect the cause of the high risk or were they the result of the cancer?

Bouhnik et al. (1996) fed 12.5 g FOS/d to 20 healthy human volunteers. A recent paper by the same group found that ≥ 5 g FOS is necessary to increase numbers of bifidobacteria in humans (Bouhnik et al. 1999). Saccharose was used as the placebo control. Consuming 12.5 g of FOS led to increased numbers of fecal bifidobacteria within the 12-d feeding period of FOS (from $10^{7.9}$ to $10^{9.1}$), but the regimen did not significantly affect any measures used to indicate risk of colon cancer development, i.e., total fecal anaerobes, pH, activities of nitroreductase, azoreductase, and β -glucuronidase, bile acids and neutral sterols. In this case, fecal samples were stored at 4°C for up to 12 h before analysis. The authors pointed out the possibility that changes in metabolic parameters might take longer to occur after the occurrence of increases in fecal bifidobacteria than the 12-d measurements that were determined; alternatively, a much longer sustained feeding period might be necessary to see effects. Obviously, these effects would have to be correlated directly with changes in the colon to be meaningful, whatever the length of the study.

Watne et al. (1976) studied fecal neutral and acid steroids and bacterial flora in patients with polyposis coli and controls. Bacterial flora of patients showed an anaerobe/aerobe ratio of 2.7:1 with a relative increase in clostridia and bifidobacteria and decrease in eubacteria and bacteroides. After ileorectostomy, clostridia disappeared, along with ruminococcus, peptostreptococcus and fusobacteria; eubacteria and lactobacilli decreased and bifidobacteria and bacteroides increased. Again, these measures do not imply cause-effect of microbial changes with tumor risk.

Moore et al. (1995) did an epidemiologic study of intestinal floras in population with high risks of colon cancer. The results were not supportive of data linking high numbers of bifidobacteria with low risk for colon cancer. Fecal bacteria were compared in populations of polyp patients, Japanese-Hawaiians, North American Caucasians, rural native Japanese and rural native Africans. The polyp patients and Japanese-Hawaiians were initially considered the high risk groups. Fifteen bacterial groups were associated significantly with high risk of colon cancer (among these Bacteroides and bifidobacteria) and five were associated significantly with low risk (certain lactobacilli species and *Eubacterium aerofaciens*). This study does not indicate cause-effect, but rather associations between bacteria and risk of disease.

SUMMARY

Although research exists that links the consumption of probiotics and prebiotics with decreased risk of colon cancer, the studies can be sorted into those that most directly link consumption inversely with aberrant crypts or tumor development (animal studies) and those that tend to provide more circumstantial evidence. We have made the attempt in this paper to review

critically those animal studies that we feel provide some direct measure of cause and effect. In the case of human studies, we have reviewed those that link bacteria and colon cancer. The obvious assumption in the human data is that humans would react similarly to the animals in all respects.

The major conclusion from the animal data is that there appears to be a synergistic effect of consumption of probiotic bacteria and prebiotics such as fructooligosaccharides on the attenuation of the development of colon cancer. The effect is often not large, but it is possible that it could be beneficial, in combination with other ways to reduce risk. The data also point the way to the opportunities for further investigation, particularly in defining and measuring outcomes/end points in humans that are meaningful and that correlate the consumption of pro- and prebiotics with decreased risk of colon cancer development.

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