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Prevention of colon cancer by pre- and probiotics: evidence from laboratory studies

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Oligofructose and inulin, selective fermentable chicory fructans, have been shown to stimulate the growth of bifidobacteria which are regarded as beneficial strains in the colon. Studies were designed to evaluate inulin (Raftiline) and oligofructose (Raftilose), for their potential inhibitory properties against aberrant crypt foci (ACF) formation in the colon of rats. ACF are putative preneoplastic lesions from which adenomas and carcinomas may develop. The results of this study demonstrate that dietary administration of oligofructose and inulin inhibits the formation of preneoplastic lesions in the colon suggesting the potential colon tumour inhibitory properties of chicory fructans. Since these prebiotics selectively stimulate the growth of bifidobacteria, tumour inhibitory activity of lyophilized cultures of *Bifidobacterium longum* (BL) against azoxymethane (AOM)-induced colon carcinogenesis in rats and modulating effect of these cultures on colonic tumour cell proliferation, ornithine decarboxylase (ODC) activity, and *ras*-p21 oncoprotein expression were investigated. Dietary administration of lyophilized cultures of BL strongly suppressed AOM-induced colon tumour development. Inhibition of colon carcinogenesis was associated with a decrease in colonic mucosal cell proliferation and colonic mucosal and tumour ODC and *ras*-p21 activities.

Colon cancer: Oligofructose: Inulin: *Bifidobacterium longum*

Colorectal cancer is one of the leading causes of cancer mortality in both men and women in the Western countries including the USA (Parker *et al.* 1997). Epidemiological studies indicate that increased consumption of fruits and vegetables and high total dietary fibre reduce the risk of development of colon cancer (Howe *et al.* 1992). Human metabolic studies suggest that beneficial effects of dietary fibre in relation to colon cancer development depends on the composition and physical properties of fibre (Reddy *et al.* 1992). Animal model studies also demonstrate that tumour-inhibitory properties of dietary fibre in the colon depends on its composition (Reddy, 1995). Among the types of dietary fibre, inulin and oligofructose are β-(2-1)D fructans which are fermented by colonic microflora and behave as soluble fibres (Gibson & Roberfroid, 1995). Oligofructose and inulin which occur in common foodstuffs such as chicory, leeks, garlic, onion, artichoke and asparagus at high levels selectively stimulate the growth of bifidobacteria at the expense of bacteroides, clostridia or coliforms which are maintained at low levels (Gibson & Roberfroid, 1995; Gibson *et al.* 1995). Bacterial fermentation of chicory fructans and other oligofructoses produces short-chain fatty acids (SCFA) in the colon including a small amount of butyric acid (Gibson & Roberfroid, 1995; Campbell *et al.* 1997) which has been shown to increase apoptosis in the colon (Hague *et al.* 1993). Furthermore, there are studies to demonstrate that cultures of bifidobacteria increase the host's immune response (Sekine *et al.* 1995). These observations raise the possibility that selective fermentable non-digestible oligosaccharides that enhance the growth of bifidobacteria in the gut could potentially inhibit colon carcinogenesis. It was therefore of interest to evaluate the inhibitory properties of dietary oligofructose, inulin and bifidobacteria against colon carcinogenesis.

Oligofructose and inulin

This study was designed to determine the potential inhibitory properties of oligofructose (Raftilose) and inulin (Raftiline) on azoxymethane (AOM)-induced colon carcinogenesis in male F344 rats using colonic aberrant crypt foci (ACF) as the endpoint (Reddy *et al.* 1997). ACF, which are recognized as early preneoplastic lesions in the colon, have consistently been observed in experimentally induced colon carcinogenesis in laboratory animals and in the colonic mucosa of patients with colon cancer (McLellan *et al.* 1991; Pretlow *et al.* 1992). Aberrant crypts are putative precursor lesions from which adenomas and carcinomas may develop in the colon. ACF express mutations in the *apc* gene and *ras* oncogene that appear to be involved in colon cancer development (Vivona *et al.* 1993). Several inhibitors of ACF formation have been shown to reduce the incidence of colon tumours in laboratory animals (Wargovich *et al.* 1996) suggesting that ACF induction can be used to evaluate novel agents for their potential chemopreventive properties against colon cancer.

In this study, groups of 7-week-old male F344 rats were fed the AIN-76A (control) and the experimental diets

Table 1. Effect of dietary oligofructose and inulin on colonic ACF formation in male F344 rats*

Experimental group	Total ACF/colon	Foci containing number of aberrant crypts			
		1 crypt/focus	2 crypts/focus	3 crypts/focus	4 or more crypts/focus
Control diet	120 ± 28†	19·5 ± 7·3	43·7 ± 7·8	28·2 ± 7·5	28·3 ± 8·2
Oligofructose, 10%	92 ± 28‡ ($P<0.024$)	15·4 ± 7·5	31·2 ± 13‡ ($P<0·01$)	21·3 ± 7·8‡ ($P<0·04$)	23·9 ± 8·2
Inulin, 10%	78 ± 37‡ ($P<0·006$)	15·7 ± 8·2	24 ± 12‡ ($P<0·0001$)	16·6 ± 7·2‡ ($P<0·02$)	21·8 ± 14.2

*Reddy *et al.* (1997)
†Mean ± SD.
‡Significantly different from the control diet. The level of significance is shown in parentheses.

containing 10% oligofructose (Raftilose) or inulin (Raftiline). At 7 weeks of age, all animals received s.c. injection of AOM dissolved in normal saline at a dose rate of 15 mg/kg body weight, once weekly for 2 weeks. The animals were necropsied 7 weeks after the last AOM injection, and the ACF were visualized under light microscopy in the formalin-fixed, unsectioned methylene blue-stained colons. They were distinguished by their increased size, more prominent epithelial cells and pericryptal space. AOM treatment induced on the average ~120 ACF/colon (Table 1) ACF were predominantly observed in the distal colons. Efficacy endpoints used in this study were inhibition of the total number of ACF/colon as well as the reduction of the number of multicrypt clusters (≥2) of aberrant crypts/focus. Administration of oligofructose or inulin in the diet significantly inhibited the total number of ACF/colon as compared to the control diet; the degree of inhibition was more pronounced in the animals fed inulin ($P<0·0006$) than in those fed oligofructose ($P<0·02$). Crypt multiplicity in terms of two or three aberrant crypts/focus were also significantly inhibited in animals fed inulin ($P<0·02$–$0·0001$) and oligofructose ($P<0.04$–0.01). These findings suggest that chicory fructan supplements inhibit ACF formation, an early preneoplastic marker of malignant potential in the process of colon carcinogenesis. The mechanisms by which oligofructose and inulin inhibit preneoplastic lesions of the colon may involve the modulation of microflora (Gibson & Roberfroid, 1995; Gibson *et al.* 1995) in the colon. *In vitro* studies showed that incubation of faecal bacterial cultures with oligofructose and inulin selectively stimulated the growth of bifidobacteria while maintaining the *Escherichia coli* or clostridia at low levels (Wang & Gibson, 1993). Gibson *et al.* (1995) demonstrated that dietary administration of oligofructose or inulin significantly increased faecal bifidobacteria, whereas *Bacteroides*, clostridia and fusobacteria and/or Gram-positive cocci were decreased on total faecal bacterial count. The colonizing cells of bifidobacteria produce lactic acid, thereby lowering the intestinal pH to create a bacteriocidal environment for putative enteropathogens such as *E. coli* and *C. perfringens* thus developing a favourable microenvironment in the gut. This favourable microenvironment may also involve the modulation of bacterial enzymes such as β-glucuronidase that can convert procarcinogens to proximate carcinogens (Kulkarni & Reddy, 1994). In addition to selective modulation of bifidobacteria, oligofructose and inulin increase the production of SCFA especially butyrate in the colon by microbial fermentation (Gibson & Roberfroid, 1995). Although the production of butyrate is around 5% of total SCFA, it is of particular interest because it inhibits proliferation of a number of cell types *in vitro* and induces a more differentiated phenotype including colorectal tumor cells (Gamet *et al.* 1992).

Bifidobacterial cultures

Recent study from our laboratory demonstrates that dietary intake of lyophilized cultures of *Bifidobacterium longum* (BL) significantly suppressed the development of AOM-induced ACF in the colon (Kulkarni & Reddy, 1994). It was therefore of interest to evaluate the colon tumour-inhibitory properties of dietary BL in the colon cancer model. The effects of dietary BL on AOM-induced as well as 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-induced colon tumourigenesis were analysed in male F344 rats (Reddy & Rivenson, 1993; Singh *et al.* 1997). We have also examined how this enterobacterial culture influences ornithine decarboxylase (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 (Singh *et al.* 1997).

Effect on AOM-induced colon carcinogenesis

Male F344 rats were fed the modified AIN-76A diet containing 0 or 2% lyophilized cultures of BL and administered s.c. AOM dissolved in normal saline at a dose rate of 15 mg/kg body weight, once weekly, for 2 weeks. Vehicle controls received s.c. equal volume of normal saline. Animals were maintained on control or experimental diets until termination of the study. Animals intended for analysis of cell proliferation were sacrificed 20 weeks after the second AOM injection, whereas animals intended for colon tumour histopathology and measurement of ODC activity and *ras*-p21 expression were sacrificed 40 weeks after last AOM injection. Mucosal cell proliferation, ODC activity and differential expression of total as well as mutant *ras*-21 were measured as described (Singh *et al.* 1997).

Table 2 summarizes the AOM-induced tumours in the colon in terms of tumour incidence (percentage of animals with tumours) and colon tumour multiplicity (number of tumours/animal). Dietary administration of BL cultures

Table 2. Inhibitory effect of lyophilized cultures of *Bifidobacterium longum* on AOM-induced colon carcinogenesis in F344 rats*

	Colon tumourigenesis		Colon mucosa			Colon tumors	
Dietary regimen	% animals with tumours	Tumours/animal	Labelling index (%)	ODC activity	Total *ras*-p21 activity	ODC activity	Total *ras*-p21 activity
Control diet	77	1·8 ± 1·27†	18·9 ± 1·1	66 ± 10	14·2 ± 3·0	456 ± 147	32·7 ± 5·9
2% BL	53‡	0·83 ± 0·98‡	12·8 ± 1·1‡	32 ± 6‡	8·9 ± 2·7§	101 ± 30¶	19·8 ± 4·8§

*Singh *et al.* (1997).
†Mean ± SD.
‡Significantly different from control diet, $P<0·05$.
§Significantly different from control diet, $P<0·01$.
¶Significantly different from control diet, $P<0·001$.

significantly inhibited the incidence of colon adenocarcinomas ($P<0·05$), and colon tumour multiplicity in terms of tumours/animal ($P<0·001$).

In addition, colon tumour inhibitory property of lyophilized cultures of BL was associated with the inhibition of colonic mucosal cell proliferation, and suppression of ODC activity and expression of total and mutated *ras*-p21, in the colonic mucosa and tumours as compared to that in control diet (Table 2). Biasco *et al.* (1991) observed a significant decrease in mucosal cell proliferation 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 (Porter *et al.* 1987; Singh *et al.* 1992), in dysplastic polyps versus non-dysplastic polyps (Luk & Baylin, 1984) and also in non-involved mucosa from polyposis patients v. non-involved mucosa from normal individuals (Luk *et al.* 1989). Similarly, ODC activity has been found to be consistently higher in non-familial colon adenocarcinomas compared to adjacent mucosa. Evidence that enhanced ODC activity may play an important role in colon tumour-development is provided by the observation that difluoromethylornithine, a high specific and irreversible inhibitor of ODC, suppressed colon tumour development in a time-dependent manner in carcinogen-treated rodent (Singh *et al.* 1992). In the current study, we observed elevated levels of ODC activity both in colon tumours and in uninvolved colonic mucosa of AOM-treated animals (Singh *et al.* 1997). In addition, ODC activity was significantly decreased in colonic mucosa as well as in colon tumours of AOM-treated animals administered lyophilized cultures of BL. Although, the precise mechanism of inhibition of ODC activity by dietary BL cultures 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 (Barbacid, 1990). Elevated levels of *ras*-p21 have been correlated with increased cell proliferation, histological grade, nuclear anaplasia and degree of undifferentiation (Kotsinas *et al.* 1993). In experiments where mutated *ras* genes are selectively inactivated, the pre-existing tumour phenotype reverts to a more normal form, indicating that activated *ras* may be necessary for the maintenance of malignant behaviour (Mukhopadhya *et al.* 1991). As regards the mechanism of inhibition of *ras* activation afforded by BL cultures, it is hypothesized that bifidobacterial cells, as a biological response modifier, modulate the induction of the methylguanine repair protein, O^6-methylguanine DNA methyltransferase, which acts as a suicide enzyme that stochiometrically accepts a methyl group onto itself, restoring the original guanine in DNA by *in situ* demethylation (Pegg & Dolan, 1989). 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 BL-augmented suppression of AOM-induced *ras* activity may interfere with the progression of events leading to colon tumour development.

Table 3. Inhibitory effects of lyophilized cultures of *Bifidobacterium longum* on 2-amino-3-methylimidazo[4,5-*f*]quinoline-induced tumourigenesis in F344 rats*

	Colon		Mammary gland	
Dietary regimen	Incidence†	Multiplicity‡	Incidence†	Multiplicity‡
Male rats				
Control diet	27	0·43 ± 0·89d	0	0
0·5% BL	0¶	0¶	0	0
Female rats				
Control diet	0	0	27	0·46 ± 0·80
0·5% BL	0	0	13	0·19 ± 0·46¶

*Reddy and Rivenson (1993).
†Percentage of animals with tumours.
‡Tumours/animal.
§Mean ± SD.
¶Significantly different from its respective control diet in the same gender, $P<0.05$.

Effect of *Bifidobacterium longum* on 2-amino-3-methylimidazo[4,5-*f*] quinolin-induced colon carcinogenesis

The formation of mutagens upon broiling fish and meat was first discovered by Sugimura *et al.* (1977). IQ, a heterocyclic aromatic amine produced from food pyrolysis, was first isolated from broiled fish. Subsequently, it was isolated from a variety of broiled or cooked fish and meat (Kasai *et al.* 1980). IQ has a multitarget organospecificity with specific cancer induction in Zymbal gland, skin, colon, oral cavity, and mammary gland of rodents (Sugimura *et al.* 1991). 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 tumours in male and female rats, and bacterial cultures that ferment milk possess anti-carcinogenic properties, the possibility exists that these bacterial cultures may prevent IQ-induced carcinogenesis. Accordingly the inhibitory effect of lyophilized cultures of BL on IQ-induced carcinogenesis was investigated in male and female F344 rats (Reddy & Rivenson, 1993). 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 BL with or without 125 p.p.m. IQ in the diet. All animals were continued on this regimen until the termination of the study at week 58.

The results indicated that lyophilized cultures of BL significantly inhibited the IQ-induced incidence (percentage of animals with tumours) of colon (100 % inhibition) tumours and multiplicity (tumours/animal) of colon in male rats (Table 3). In female rats, dietary supplement of BL cultures also suppressed the IQ-induced mammary carcinogenesis to 50 % of those observed in animals fed the control diet. The mammary tumour multiplicity (tumours/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 tumours and to a lesser extent mammary tumours in F344 rats.

In summary, the results thus far generated demonstrate that dietary administration of prebiotics such as oligofructose and inulin and lyophilized cultures of BL inhibits the formation of preneoplastic lesions in the colon and colon and/or mammary carcinogenesis in laboratory animal models.

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