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Synbiotic intervention of *Bifidobacterium lactis* and resistant starch protects against colorectal cancer development in rats

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This study evaluated the effect of a probiotic bacteria '*Bifidobacterium lactis*', the carbohydrate 'resistant starch' (RS) and their combination (synbiotic), on their ability to protect against colorectal cancer (CRC). *Bifidobacterium lactis* has been shown previously to utilize RS as a substrate and up-regulate the acute apoptotic response to a carcinogen in the colon [Le Leu *et al.* (2005) J. Nutr., 135, 996–1001]. Sprague–Dawley rats were divided into six equal groups and fed semi-purified diets for 30 weeks. Colonic neoplasms were induced by 2 weekly injections of azoxymethane (15 mg/kg body wt). The experimental groups were as follows: control—no added dietary fibre or RS; RS in two forms—Hi-maize 958 or Hi-maize 260; *B.lactis* (lyophilized)—added to control and RS diets (six treatment groups in all). Rats fed RS in combination with *B.lactis* showed significantly lowered incidence and multiplicity of colonic neoplasms ($P < 0.01$) by >50% compared with the control group. There was a trend for protection by RS alone ($P = 0.07$), whereas no protection against cancer was seen in the group supplemented with only *B.lactis*. Fermentation events [short-chain fatty acid (SCFA), pH] were altered by the inclusion of RS into the diet, whereas the inclusion of *B.lactis* into the diet had no significant effect on the fermentation parameters. The synbiotic combination of RS and *B.lactis* significantly protects against the development of CRC in the rat-azoxymethane model. Synbiotic combination of prebiotic and probiotic seems likely to be a superior preventive strategy to prebiotic alone.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in affluent countries and is a leading cause of cancer-related mortality in USA and Australia (1,2). Evidence from epidemiological and experimental studies implies that diet is an important environmental factor in the aetiology of CRC, implying that CRC is potentially preventable (3). The colonic microbiota has been identified as being capable of influencing gastrointestinal diseases and disorders including that of CRC (4–6). There exists a potential role for foods that contain probiotics (live microbial food ingredients that may be beneficial to health, such as lactobacilli or bifidobacteria) and/or prebiotics (non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of resident colonic bacteria) to change the colonic microbiota in a way that might prevent diseases such as CRC.

The precise mechanisms by which probiotics exert their antitumourigenic influence are uncertain but might involve modifying gut pH and increasing the net production rate of short-chain fatty acid (SCFA) (mainly acetate, propionate and butyrate) (7), antagonizing pathogens through production of antimicrobial and antibacterial compounds (such as bacteriocins, cytokines and butyrate) and stimulating immunomodulatory cells (8) or competition with pathogens for available nutrients, receptors and growth factors (9). Prebiotics may exert their cancer-protective effects via modulation of fermentative events possibly by increasing SCFA production or by altering gut microbiota towards a more beneficial composition (10) or by modifying important biological consequences related to cancer development such as apoptosis or cell proliferation (11,12).

It has been suggested that a combination of a probiotic and a prebiotic, termed synbiotics, might be more active than either a probiotic or prebiotic alone (13) in preventing CRC. In a human intervention study, several CRC biomarkers were shown to be altered favourably by a synbiotic intervention (14). There are also several reports in experimental animals whereby a synbiotic combination showed biological and anticancer effects beyond those of the individual components (5,15,16). Although each of these studies suggested several mechanisms of action by which synbiotics might be protective, there is little direct evidence to implicate specific effects on biological events of relevance to oncogenesis.

In a recent study, we have shown that the combination of the prebiotic 'resistant starch' (RS) and the probiotic '*Bifidobacterium lactis*', a probiotic that specifically utilizes RS as a substrate for fermentation (17,18), can significantly stimulate the acute apoptotic response to a genotoxic carcinogen (AARGC) in the rat colon, measured 6 h after carcinogen exposure (19). AARGC might regulate mutational load in the colon and eliminate DNA-damaged cells that might otherwise progress to malignancy, thereby exerting a protective effect at the early stages in the onset of cancer (20,21).

The primary objective of the current study was to determine whether the synbiotic combination of RS and *B.lactis* can protect against azoxymethane (AOM)-induced CRC in rodents. It was our hypothesis that the synbiotic combination of RS with *B.lactis* would be more protective than either the RS or the *B.lactis* alone because the synbiotic combination of these agents facilitates the apoptotic response to DNA damage by a cancer initiator (AOM) in the colon of rats (19). Two forms of high-amylose maize starches (HAMS) with similar RS content were used in the current study, namely a native HAMS (which is a commercially rich source of dietary fibre and RS, with an amylose content of 85%) and a hydrothermically (heat–moisture)-treated native HAMS. Hydrothermal processing is a means of significantly increasing the dietary fibre content of HAMS (22). We examined two forms of HAMS to determine whether the extra variable of the dietary fibre content played any role in modulating the impact of RS on development of AOM-induced colorectal tumours in the rat model.

Materials and methods

Animals and diets

A total of 180 male Sprague–Dawley rats, 5 weeks of age, were obtained from the Animal Resource Centre, Perth, Western Australia. Animals were divided randomly into six experimental groups and housed two or three per plastic cage in an animal holding room under controlled conditions of 22 ± 2°C (SD), 80 ± 10% humidity and 12 h light/dark cycle. Animals were given free access to water and weighed weekly throughout the study.

The diets were modified forms of the AIN-76a standard for purified diets for rats and mice. Two forms of HAMS were used in the study as the source of RS, namely a native HAMS (Hi-maize® 958, which is a commercially rich source of dietary fibre and RS, with an amylose content of 85%) and a hydrothermically (heat–moisture)-treated native HAMS (Hi-maize® 260). The hydrothermal process was conducted using a native HAMS under conditions where the starch had a moisture content of 25% and it was heated to 125°C for 120 min (22). The total dietary fibre (TDF) and RS values were determined on a dry weight basis for the two different amylose maize starches by Association of Official Analytical Chemists (AOAC) Method 991.43 and AOAC 2002.02,

Abbreviations: AARGC, acute apoptotic response to a genotoxic carcinogen; AOM, azoxymethane; CRC, colorectal cancer; HAMS, high-amylose maize starches; PCNA, proliferating cell nuclear antigen; RS, resistant starch; SCFA, short-chain fatty acid; TDF, total dietary fibre; TNF, tumour necrosis factor.

Table I. Composition of experimental diets (grams/100 g diet)[a,b,c]

Ingredient	Control	Bif	RS(A)	RS(B)	RS(A) + Bif	RS(B) + Bif
Casein	20.00	20.00	20.00	20.00	20.00	20.00
Cornstarch	46.15	46.15	36.15	36.15	36.15	36.15
Hi-maize® 958[c]	—	—	10.00		10.00	—
Hi-maize® 260[c]			—	10.00	—	10.00
Corn oil	18.00	18.00	18.00	18.00	18.00	18.00
Sucrose	10.95	9.95	10.95	10.95	9.95	9.95
Bifidobacterium lactis[b]		1.0			1.0	1.0
DL-methionine	0.3	0.3	0.3	0.3	0.3	0.3
Choline	0.1	0.1	0.1	0.1	0.1	0.1
AIN-76 mineral mix	3.5	3.5	3.5	3.5	3.5	3.5
AIN-76 vitamin mix	1.0	1.0	1.0	1.0	1.0	1.0

[a]High-amylose maize starch used as source of RS.
[b]*Bifidobacterium lactis* in form of lyophilized culture (1×10^{11} c.f.u./g).
[c]The TDF and RS values were determined on a dry weight basis for the two different amylose maize starches by AOAC Method 991.43 and AOAC 2002.02, respectively. The values are as follows: Hi-maize 958 (TDF = 30; RS = 50), Hi-maize 260 (TDF = 60; RS = 46).

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Each group of animals was fed an experimental diet based on the control diet (Table I). Choline, methionine, minerals and vitamins were added as previously (19). The first group 'control' consumed a diet containing no added fibre or RS. The second group 'Bif' consumed the same diet as control with 1% lyophilized culture (1×10^{11} c.f.u./g) of *B.lactis*. The third group 'RS(A)' was fed Hi-maize® 958 at a level of 100 g/kg diet. The fourth group 'RS(B)' was fed Hi-maize 260 at a level of 100 g/kg diet. The fifth group 'RS(A) + Bif' was fed Hi-maize® 958 at a level of 100 g/kg diet + 1% lyophilized culture (1×10^{11} c.f.u./g) of *B.lactis*. The sixth group 'RS(B) + Bif' was fed Hi-maize® 260 at a level of 100 g/kg diet + 1% lyophilized culture (1×10^{11} c.f.u./g) of *B.lactis*.

The different types of RSs were supplied by National Starch and Food Innovation, Bridgewater, NJ, USA HAMS was added to the diets at the expense of an equal amount of digestible cornstarch. Lyophilized cultures of *B.lactis* were purchased from DSM Food Specialties (Sydney, Australia) and added to the diets at the expense of sucrose.

Experimental procedure

After 4 weeks on experimental diets, each rat received subcutaneous injections of azoxymethane (15 mg/kg body wt; Sigma Chemical Co., St Louis, MO) once weekly for 2 weeks and then maintained on their dietary regimen until termination of the study at 26 weeks after the second azoxymethane injection. The rats in each group were weighed once weekly. As scheduled, all rats were killed by CO_2 asphyxiation. After laparotomy, the entire stomach, small intestine and large intestine were resected. They were opened longitudinally and the contents emptied. They were examined for intestinal tumours, and the location and number of tumours were assessed with a dissection microscope and recorded. Distal, proximal and caecal digesta were collected and diluted in 3 vols of internal standard solution (heptanoic acid, 1.68 mmol/l) and stored at −20°C for later analysis of SCFA concentrations.

The Flinders University of South Australia Animals Welfare Committee approved all experimental procedures.

Colonic tumours

Using a dissecting microscope, the small intestine and colon were scored for tumour number and location by an experienced gastroenterologist/pathologist (GY) who was blinded to the treatment groups by random coding of slides (23). Tumours were removed and embedded in paraffin (5 μm) for histopathological analysis. All tumours were classified as either an adenoma or adenocarcinoma based on the criteria of Pozharisski (24). Adenoma was characterized by expansion of the mucosa layer, reduction in goblet cell number, cellular dysplasia and moderate loss of mucosal architecture by glandular growth and lack of invasion through the basement membrane. Adenocarcinoma was identified when there was typical cytological change, prominent cellular atypia, loss of cell polarity, marked distortion of glandular architecture and invasion (24).

Epithelial cellular processes

Colon sections (0.5 × 0.5 cm) stored in 70% ethanol were cut from distal segments of the colon free of neoplasms and embedded in paraffin. Paraffin-embedded sections (5 mm) were stained with haematoxylin and evaluated under a light microscope for apoptotic cells. Apoptotic cells were identified in 20 randomly chosen intact crypts by cell shrinkage, presence of condensed chromatin and sharply delineated cell borders surrounded with a clear halo as reported previously (23).

To assess the proliferative activity and the distribution of proliferating cells in the colonic crypts, the proliferating cell nuclear antigen (PCNA) was performed using standard immunohistochemical procedures (23). Briefly, deparaffinized sections were rehydrated in a graded series of ethanol from 100 to 50% and then to distilled water. The primary mouse monoclonal antibody (PC-10, Santa Cruz, CA, USA) was placed on the slides (1/500 dilution) and incubated overnight at room temperature. A level 2 Ultra Streptavidin Detection System (Signet Laboratories, CA, USA) was used utilizing biotinylated goat anti-mouse as the secondary antibody. The slides were counterstained for 3 min with haematoxylin. PCNA-positive cells were identified in 20 randomly chosen intact crypts. An independent observer (JW) who was blinded to the treatment groups by random coding of the slides determined the quantification of proliferative cells and apoptotic cells.

SCFA analysis

SCFA including acetate, propionate and butyrate, as well as branched chain fatty acids isobutyrate, isovaleric and valeric, were determined in the caecal, colon contents and faeces of rats as described previously (25).

Statistical analysis

The effect of the diets on tumour incidence and tumour counts were assessed using binomial logistic and Poisson regression models, respectively. All tumour analysis was carried out using Stata version 10.1 (StataCorp, Texas, USA). The effect of diet on SCFA, apoptosis, crypt height and cell proliferation was analysed using one-way analysis of variance and the Sidak post hoc test (SPSS version 17). The criterion for statistical significance was set at $P < 0.05$ for each endpoint.

Results

No differences between the two RS groups [i.e. RS(A) and RS(B)] were observed in each of the outcomes; therefore, the two RS groups were combined in order to increase the power to test our main study hypothesis—that the synbiotic combination of RS with *B.lactis* would be more protective than either the RS or *B.lactis* alone.

Animals

There were no significant variations in final mean bodyweights of the different treatment groups. Data not shown.

Colonic tumours

The effects of the experimental diets on neoplasm incidence (proportion of rats who develop neoplasia), number of neoplasms per colon and type of neoplasm (adenoma or cancer) in the colon are shown in Table II. The synbiotic diet (RS + Bif) significantly reduced the incidence of neoplasms in the colon (18.3%) compared with the control group (53.3%) ($P < 0.001$). There was also a trend towards reduction of neoplasm incidence with RS (33.3%) ($P = 0.07$). No protection was seen for the probiotic alone on any measure of colonic neoplasms.

When the incidence of adenomas and cancers were analysed separately, there was a significant reduction in the incidence of adenomas in the synbiotic diet group (5.0%) compared with the control group (23.3%) ($P = 0.02$) and a borderline reduction in the incidence of cancers among the synbiotic diet group (13.3%) versus control group (30.0%).

A similar pattern of protection was seen in regard to colonic neoplasm multiplicity. Significant protection against the number of neoplasms per rat colon was observed for the synbiotic group (0.2 ± 0.4) versus the control group (0.7 ± 0.8) ($P = 0.001$). The number of cancers per rat colon was also reduced (0.15 ± 0.40) compared with the control group (0.43 ± 0.8) ($P = 0.01$) and number of adenomas per rat colon was also reduced with the synbiotic (0.03 ± 0.18) in comparison with the control group (0.23 ± 0.19) ($P = 0.02$).

SCFA levels

SCFA concentrations (micromoles/gram) in the caecum and proximal and distal colon are shown in Table III. Overall, SCFA concentrations

Table II. Effect of experimental diets on AOM-induced colonic neoplasms in rats

Experimental group	Control	Bif	RS			RS + Bif			*P* values*		
			RS(A)	RS(B)	Pooled	RS(A) + Bif	RS(B) + Bif	Pooled	Bif	RS	RS + Bif
Neoplasm incidence (%)											
All colonic neoplasms	53	50	33	33	33	23	13	18	0.80*	0.07	0.001
Cancer	33	33	17	30	23	17	10	13	0.78	0.61	0.06
Adenomas	23	27	19	7	13	3	3	3	0.76	0.33	0.02
Number of neoplasms per rat colon (multiplicity)											
All colonic neoplasms	0.7	0.67	0.43	0.37	0.40	0.27	0.13	0.20	0.88	0.08	0.001
Cancer	0.43	0.37	0.17	0.30	0.24	0.23	0.10	0.15	0.68	0.16	0.01
Adenomas	0.23	0.30	0.24	0.10	0.17	0.03	0.03	0.03	0.62	0.52	0.02

RS, HAMS used as source of RS.
*Versus control diet. Binary logistic regression was performed for assessing the incidence of neoplasms and Poisson regression was performed for assessing number of neoplasms. The two RS groups, namely RS(A) and RS(B), and the two RS + Bif groups, namely RS(A) + Bif and RS(B) + Bif, were pooled for analysis since effects were not different for the separate RS groups or for the separate RS + Bif groups.

Table III. Concentrations of SCFA levels in the caecum and colon of rats fed different experimental diets

	Control	Bif	RS			RS + Bif			(*P* values)*			
			RS(A)	RS(B)	Pooled	RS(A) + Bif	RS(B) + Bif	Pooled	Analysis of variance *F*-test	Bif	RS	RS + Bif
Caecum												
Total SCFA	61.6 (20.5)	72.2 (23.3)	82.3 (6.1)	82.2 (4.5)	82.3 (28.8)	82.3 (7.1)	77.9 (5.5)	80.1 (34.6)	0.01	0.64	0.01	0.03
Acetate	38.7 (13.8)	44.5 (15.9)	50.6 (4.2)	47.6 (2.6)	49.1 (19.0)	52.0 (5.0)	46.3 (3.8)	49.1 (24.0)	0.08	0.83	0.11	0.11
Propionate	14.6 (5.0)	18.1 (5.0)	19.4 (1.5)	23.7 (1.7)	21.5 (8.7)	19.3 (1.6)	21.7 (1.6)	20.5 (8.8)	0.001	0.41	0.001	0.005
Butyrate	8.3 (3.8)	9.7 (5.1)	12.3 (1.3)	11.0 (0.9)	11.7 (6.3)	11.0 (1.1)	10.0 (0.8)	10.5 (5.1)	0.05	0.91	0.04	0.38
Proximal colon												
Total SCFA	46.0 (18.4)	50.8 (16.5)	74.6 (5.5)	56.5 (3.9)	64.9 (23.7)	75.8 (4.9)	56.6 (4.2)	66.4 (23.5)	0.001	0.98	0.02	0.007
Acetate	28.6 (13.2)	32.0 (12.3)	46.4 (3.7)	32.9 (2.6)	39.2 (16.4)	50.6 (3.5)	34.9 (3.1)	42.9 (17.6)	0.004	0.98	0.10	0.009
Propionate	11.5 (3.9)	13.0 (0.7)	16.8 (1.4)	16.0 (1.2)	16.4 (6.2)	13.9 (0.9)	14.9 (1.2)	14.4 (5.1)	0.004	0.93	0.005	0.23
Butyrate	6.0 (2.9)	5.8 (2.6)	11.3 (1.1)	7.6 (0.6)	9.3 (4.5)	11.2 (1.1)	6.8 (0.6)	9.1 (4.9)	0.001	1.0	0.01	0.02
Distal colon												
Total SCFA	29.2 (19.5)	23.4 (9.9)	59.8 (5.2)	62.3 (5.7)	60.8 (24.0)	50.2 (5.7)	62.5 (4.4)	56.8 (23.2)	0.001	0.98	0.001	0.001
Acetate	17.4 (12.9)	14.2 (5.9)	34.8 (3.0)	35.1 (3.5)	34.9 (14.2)	31.0 (3.6)	36.5 (3.1)	34.0 (15.1)	0.001	0.99	0.001	0.001
Propionate	7.7 (5.1)	5.7 (2.8)	14.9 (1.5)	16.7 (2.0)	15.7 (7.6)	9.2 (1.1)	17.9 (1.7)	13.9 (8.0)	0.001	0.96	0.001	0.002
Butyrate	4.1 (2.0)	3.5 (1.6)	10.1 (1.3)	9.3 (1.4)	9.8 (5.9)	10.0 (1.6)	8.1 (0.6)	9.0 (5.2)	0.001	1.0	0.001	0.003

Mean (SD).
*One-way analysis of variance and Sidak post hoc comparisons were performed among the four groups: Bif, RS and RS + Bif versus control.

were highest in the caecum followed by the proximal colon and then the distal colon. RS supplementation had the greatest effect on SCFA causing significant increases in SCFA concentrations at all sites. The probiotic alone did not significantly influence SCFA concentrations.

In the caecum, total SCFA concentrations were significantly higher in the RS group (82.3 ± 28.8) ($P = 0.01$) and RS + Bif group (80.1 ± 34.6) ($P = 0.03$) compared with the control group (61.6 ± 20.5). Caecal acetate concentrations were not significantly altered by the different dietary groups. Propionate concentration was significantly higher in the RS group (21.5 ± 8.7) ($P = 0.001$) and RS + Bif group (20.5 ± 8.8) ($P = 0.005$) compared with the control group (14.6 ± 5.0). Butyrate concentration was significantly increased only in the RS group (11.7 ± 6.3) ($P = 0.04$) compared with the control group (8.3 ± 3.8).

In the proximal colon, total SCFA concentration was significantly higher in the RS group (64.9 ± 23.7) ($P = 0.02$) and RS + Bif group (66.4 ± 23.5) ($P = 0.007$) compared with the control group (46.0 ± 18.4). Acetate concentration was increased in the RS + Bif group (42.9 ± 17.6) ($P = 0.009$) compared with that of the control group (28.6 ± 13.2) ($P < 0.001$). Propionate concentration was increased in the RS group (16.4 ± 6.2) ($P = 0.005$) versus control (11.5 ± 3.9). There was a significant increase in butyrate concentration in the RS group (9.3 ± 4.5) ($P = 0.01$) and RS + Bif group (9.1 ± 4.9 ($P = 0.02$) compared with the control group.

In the distal colon, the total SCFA concentration was significantly higher in the RS group (60.8 ± 24.0) ($P = 0.001$) and RS + Bif group (56.8 ± 23.2) ($P = 0.001$) compared with the control group (29.2 ± 19.5). Acetate concentration was also increased in the RS group (34.9 ± 14.2) ($P = 0.001$) and RS + Bif group (34.0 ± 15.1) ($P = 0.001$) compared with the control group (17.4 ± 13.0). Propionate concentration was highest in the RS group (15.7 ± 7.6) ($P = 0.001$) and RS + Bif group (13.9 ± 8.0) ($P = 0.002$) compared with the control group (7.7 ± 5.1). Butyrate concentration was highest in the RS group (9.8 ± 5.9) ($P = 0.001$) and RS + Bif group (9.0 ± 5.2) ($P = 0.003$) compared with the control group (4.1 ± 2.1).

Effects of diet on crypt height, cell proliferation and spontaneous apoptosis in distal colon

A significant increase in crypt column height (cells per crypt column height) was observed in the RS group (33.5 ± 1.2) ($P = 0.003$) and RS + Bif group (33.3 ± 1.4) ($P = 0.02$) in comparison with the control group (31.9 ± 1.3) (Figure 1A).

Cell proliferation was evaluated by assessing the PCNA staining in normal appearing distal colonic crypts measured 26 weeks after the

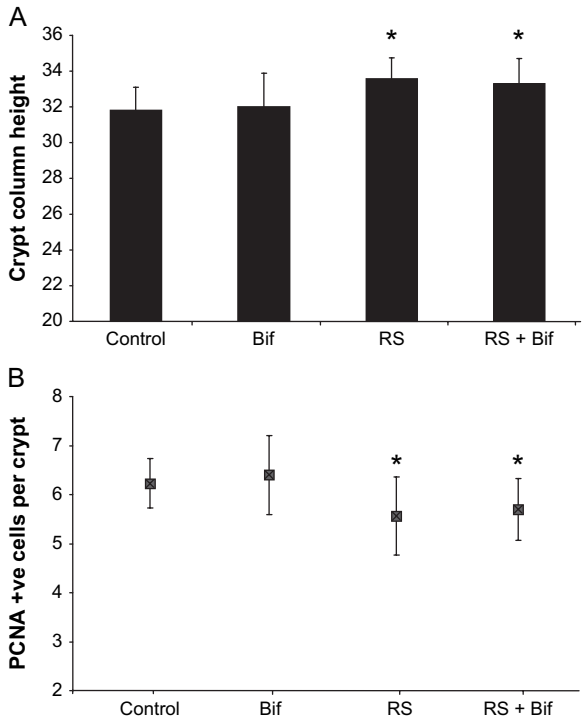

Fig. 1. Effects of diet on cell proliferation and crypt height in distal colon 26 weeks after administration of the last dose of azoxymethane. (**A**) Crypt column height, (**B**) PCNA-positive cells per crypt height. Values are means ± SDs, $n = 15$. The two RS groups, namely RS(A) and RS(B), and the two RS + Bif groups, namely RS(A) + Bif and RS(B) + Bif, were pooled for analysis since effects were not different for the separate RS groups or for the separate RS + Bif groups. Individual mean values ± SDs for crypt column height were as follows: RS(A) 33.6 ± 1.4; RS(B) 33.5 ± 1.0; RS(A) + Bif 33.2 ± 1.4; RS(B) + Bif 33.4 ± 1.5. For PCNA-positive cells per crypt height, they were as follows: RS(A) 5.4 ± 0.8; RS(B) 5.7 ± 0.6; RS(A) + Bif 5.8 ± 0.6; RS(B) + Bif 5.6 ± 0.6. $^{*}P < 0.05$ significantly different from control. One-way analysis of variance and Sidak post hoc comparisons were performed among the four groups; Bif, RS and RS + Bif versus control.

final AOM injection. Figure 1B shows that the number of PCNA-labelled cells per crypt was significantly reduced in the RS group (5.6 ± 0.8) ($P = 0.02$) and RS + Bif group (5.7 ± 0.8) ($P = 0.04$) in relation to the control group (6.3 ± 0.5).

The frequency of spontaneously occurring apoptotic cells in normal appearing mucosa was detected by haematoxylin staining and measured 26 weeks after the final AOM injection. There were no significant differences observed between treatment groups with spontaneous apoptotic cells. The levels were all low and below 0.04 apoptotic cells per crypt (data not shown).

Discussion

The present study is a follow-on from our previous rodent study that demonstrated that the synbiotic combination of RS and *B.lactis* exerted a pro-apototic action in response to the carcinogen, AOM (19). In our previous study, the acute apoptotic response to the carcinogen AOM (AARGC), measured in the colon 6 h after exposure to the carcinogen, was not affected by either probiotic or the prebiotic alone but it was significantly increased (i.e. by 33%) by the synbiotic combination of RS and *B.lactis* (19). The unanswered question from that study was does this up-regulation of apoptosis at the time of cancer initiation translate to increased protection against CRC? The results of the present study show that the synbiotic combination of RS and *B.lactis* protects against CRC in rats treated with the carcinogen AOM, hence supporting the value of a synbiotic combination.

A plausible explanation of why the synbiotic combination of RS and *B.lactis* showed the greatest protection against CRC is through up-regulation of the apoptosis at the time of cancer initiation by the carcinogen AOM (19). This supports the importance of regulating apoptosis for cancer prevention. However, why apoptosis was up-regulated with the synbiotic remains unclear. A possible explanation may be through an interaction of the immunomodulating properties of probiotic bacteria and butyrate produced via fermentation of RS (26). Cytokines such as tumour necrosis factor (TNF)-α are capable of inducing apoptosis (27). Although cytokine levels were not measured in the present study, other studies have shown that cytokine levels such as TNF-α, interferon-γ and interleukin-10 may be increased with probiotic supplementation (28). Studies *in vitro* using CRC and breast cancer cell lines have shown that TNF-α and TNF-related apoptosis-inducing ligand can act synergistically with the SCFA product butyrate, so as to target anti-apoptotic proteins and promote apoptosis (29–32). Interestingly, treatment of cells with TNF-α alone did not induce apoptosis (33).

Apoptosis provides an innate cellular defence against oncogenesis by processes that include removal of cells with genomic instability that have developed during oncogenesis (34) and by deletion of cells suffering DNA insult from genotoxic agents such as carcinogens(35). Up-regulation or facilitation of apoptosis during initiation events appears likely to increase the elimination of mutated cells that might otherwise progress to malignancy (25,36). Such an effect might be the mechanism by which the synbiotic combination of RS and *B.lactis* acted to protect against CRC. The current study also measured apoptosis levels 26 weeks after carcinogen treatment in the normal appearing colonic mucosa but at this late stage no significant differences between the treatment groups were seen. This form of apoptosis is not the exogenously induced AARGC, which we have shown previously to be regulated by fermentable substrates (25). This spontaneous apoptosis might be a 'normal' phenomenon to maintain the cell mass in intestinal crypt, a response to damage from endogenous products formed during cell metabolism (e.g. free radicals), endogenous events (e.g. replication errors) characteristic of progressive genomic instability during oncogenesis or a response to damage from low-level environmentally induced DNA damage (37). The AARGC occurs rapidly at a much higher rate and in concert with activation of DNA repair mechanisms (38). It also appears to be an important homeostatic control mechanism for removing carcinogen-induced genomic instability (20,25,39).

A trend towards protection against CRC was seen with the RS alone in the present study; however, it was when RS was combined with *B.lactis* that the strongest protection was observed. Interestingly, no significant differences were observed between the two RS forms in terms of AOM-induced colonic neoplasms or SCFA concentrations although the two HAMS differed markedly in their dietary fibre content. The lack of difference in the physiological results from the two different types of HAMS that had similar RS contents but markedly different TDF contents indicates that in experiments focusing on RS it is important to ensure that results are interpreted in relation to the RS content. The RS types we used in the present study were a native HAMS and a hydrothermically treated HAMS, which are both classified as type-2 RSs (40). Previous studies have also demonstrated mild and moderate protection against CRC with similar HAMS as the source of RS (23,41,42) and also a type-3 RS (retrograded starches, formed when starchy foods are cooked and cooled) (43). A mode of action of RS is thought to be through its effects on fermentation parameters particularly butyrate production (10). Butyrate is associated with induction of differentiation, suppression of proliferation and enhanced apoptosis *in vitro* (44–47) and may be associated with enhanced apoptosis *in vivo* (12,25,43). Increased luminal concentrations have been directly linked to protection in the rodent model (48). The present data show that consumption of RS increases SCFA production including butyrate throughout the colon, which is independent of the dietary fibre content of the RS source. No effect on butyrate production or CRC protection was observed with *B.lactis* alone (i.e. not in combination with RS).

Our results showed that colonic cell proliferation (as measured by PCNA-positive cells) was reduced in the rats fed the RS-containing diets. Increased cell proliferation may enhance the risk of mutations, which can lead to an increased risk of developing CRC (49). Similar reductions in cell proliferation have also been observed in rats fed fermentable substrates like RS (23,50) and the carbohydrate oligofructose (51). It is likely that the increased SCFA resulting from fermentation of starch in the colon contributed to the observed effects on colonic epithelial proliferation. Furthermore, colonic crypt column height appears to be dependent on the presence of a fermentative substrate, as crypt height was significantly lower in the rats fed a diet deprived of RS. This effect is consistent with our previous studies (52) and that of others (53,54). Fermentative production of SCFA is considered to have a trophic effect on the colonic epithelium (10).

In conclusion, this study has shown that the synbiotic combination of RS and *B.lactis* significantly protected against the development of CRC in the rat-AOM model beyond the benefit of either agent alone. These results indicate the need to further explore the potential role for the combination of RS and *B.lactis* as a chemopreventive agent for CRC.

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