Nutrition and Cancer

A Synbiotic Combination of Resistant Starch and *Bifidobacterium lactis* Facilitates Apoptotic Deletion of Carcinogen-Damaged Cells in Rat Colon[1]

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ABSTRACT Recent reports suggest that combinations of prebiotics and probiotics may be protective against colorectal cancer. We examined in rats the effects of probiotic bacteria, resistant starch (RS), and their interaction on luminal and epithelial events of relevance to the development of colorectal cancer. Lyophilized cultures (1×10^{10} cfu/g) of *Lactobacillus acidophilus* and/or *Bifidobacterium lactis* were added at a concentration of 1% by weight to a semipurified diet containing either low-RS (no supplemented RS) or moderate-RS (10% Hi-maize®) and fed to male Sprague-Dawley rats for 4 wk. Experimental end-points included cecal bacterial enumeration, fecal and cecal pH, SCFA levels, cell proliferation, and the acute apoptotic response to a genotoxic carcinogen (AARGC; measured 6 h after a single azoxymethane injection). A significant interaction between dietary RS and supplemental bacteria was observed for the AARGC in the colon and fecal pH ($P < 0.01$). Rats fed the moderate-RS diet in combination with *B. lactis* had a significantly greater AARGC in the colon than those fed that diet without *B. lactis.* Fecal pH was elevated in the moderate-RS fed rats supplemented with bacteria. The moderate-RS diet increased cell proliferation and crypt column height ($P < 0.001$) compared with the low-RS diet. SCFA levels and numbers of bifidobacteria and lactobacilli species were also increased ($P < 0.001$) by the moderate-RS diet, whereas pH levels and total coliforms were lowered ($P < 0.001$). The synbiotic combination of RS and *B. lactis* significantly facilitated the apoptotic response to a genotoxic carcinogen in the distal colon of rats. It appears likely that ingested RS acts as a metabolic substrate, thus creating the right conditions for *B. lactis* to exert its proapoptotic action. Because the synbiotic combination of these agents facilitates the apoptotic response to DNA damage by a cancer initiator in the colon of rats, it warrants further study for its capacity to protect against colorectal cancer. J. Nutr. 135: 996–1001, 2005.

KEY WORDS: • *apoptosis* • *prebiotics* • *probiotics* • *butyrate* • *colon cancer*

Colonic microflora are increasingly being shown to be capable of influencing gastrointestinal diseases and disorders including colorectal cancer (1,2). There exists a potential role for foods that contain probiotics and/or prebiotics, which can change the colonic microflora in a way that might prevent diseases such as colorectal cancer. Probiotics are live microorganisms that are used as dietary supplements with the aim of benefiting health by influencing the intestinal microbial balance (3). Among the major genera of colonic bacteria, bifidobacteria and lactobacilli are thought to have beneficial effects on the human host (4). These probiotic bacteria were shown previously to exert some cancer protective effects in vitro and in vivo (5–7). The precise mechanisms by which certain probiotic bacteria exert their antitumorigenic influence are uncertain, but might involve modifying gut pH and increasing the net production rate of SCFA (mainly acetate, propionate, and butyrate) (8), antagonizing pathogens through production of antimicrobial and antibacterial compounds (such as bacteriocins, cytokines and butyrate), stimulating immunomodulatory cells (9), or competing with pathogens for available nutrients, receptors, and growth factors (10).

Prebiotics are nondigestible dietary components that pass through the digestive tract to the colon. Thus, prebiotics are a potential substrate for fermentation by the microbiota and are capable of stimulating proliferation and/or activity of endogenous desirable bacteria (11). Prebiotics such as resistant starch (RS),[3] in the form of Hi-maize® (20–30% wt:wt) and oligosaccharides (5–10% wt:wt) (12,13) as well as wheat bran (equivalent to 5 or 10% total dietary fiber) (14,15) were

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[3] Abbreviations used: AARGC, acute apoptotic response to a genotoxic carcinogen; ACF, aberrant crypt foci; AOM, azoxymethane; low-RS, no supplemented resistant starch; moderate-RS, supplemented with 10% Hi-maize®; NSP, nonstarch polysaccharide; RS, resistant starch; TNF-α, tumor necrosis factor α.

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shown to stimulate the acute apoptotic response to a genotoxic carcinogen (AARGC) in the rat colon. The 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. Oligosaccharides also suppress azoxymethane (AOM)-induced preneoplastic aberrant crypt foci (ACF) and colon carcinogenesis in the rat colon (2,16,17) as does wheat bran (18–20). Prebiotics may exert their cancer protective effects via modulation of fermentative events, possibly by increasing SCFA production or by altering gut microbiota toward a more beneficial composition.

It was suggested that a combination of a probiotic and a prebiotic, termed synbiotics, might be more active than either a probiotic or prebiotic alone (21). This idea was supported by Rowland et al. (2) who showed that administration of the prebiotic inulin with the probiotic *Bifidobacterium longum* to rats resulted in additive effects, with a more potent inhibition of AOM-induced ACF than administration of either inulin or *B. longum* separately.

These findings raise the possibility that probiotics, prebiotics, or synbiotic combinations thereof might be protective against colorectal cancer by regulating the consequences of carcinogen-induced damage to colonic epithelial cells. Thus, we aimed to determine whether supplementation with either the probiotic bacteria *Lactobacillus acidophilus* and *B. lactis* alone or in combination with a moderate amount of RS could influence the acute apoptotic response to a genotoxic carcinogen (AARGC) and fermentative events in the colon rats.

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats ($n = 96$, 5 wk of age) [body weight (mean ± SEM) 155 ± 2 g] were obtained from the Animal Resource Centre, Perth, Western Australia. After arrival, rats were quarantined for 2 wk during which they were fed a modified AIN-76 diet (22) (see low-RS, **Table 1**). Rats were then divided randomly into 8 experimental groups and housed 3/plastic cage with wire bottom grills and wire tops in an animal holding room under controlled conditions of 22 ± 2°C, 80 ± 10% humidity, and a 12-h light:dark cycle and fed experimental diets for 4 wk. Rats had free access to water and were weighed weekly throughout the study. The experimental diets were based on the AIN-76 standard for purified diets for rats and mice (22) and contained either low-RS or moderate-RS (10% Hi-maize 958) (Table 1). The moderate-RS diet was equivalent to the low-RS diet with the exception that 100 g/kg diet of Hi-maize 958 was added at the expense of an equal amount of cornstarch. Lyophilized cultures (1×10^{10} cfu/g) of *L. acidophilus* (LAFTI® L10), *B. lactis* (LAFTI B94) (DSM Food Specialties Australia) and a combination of the 2 cultures were added at a concentration of 1% each by weight to semipurified diets containing low-RS or moderate-RS at the expense of sucrose. Hi-maize 958 is a natural, unmodified, food-grade, high-amylose maize starch that is an important source of total dietary fiber (22% analyzed by AOAC) and RS (61.8%) (23). The Hi-maize 958 was supplied by the National Starch and Chemical Company.

TABLE 1

Composition of the experimental diets

Ingredient	Low-RS	Moderate-RS
	g/100 g diet	
Casein	20.0	20.0
Cornstarch	46.1	36.1
Hi-maize	—	10.0
Corn oil	18.0	18.0
Sucrose	11.0	11.0
DL-Methionine	0.3	0.3
Choline	0.1	0.1
Mineral mix[1]	3.5	3.5
Vitamin mix[1]	1.0	1.0

[1] AIN-76 vitamin and mineral mixtures (22).

Experimental procedure. Fresh fecal samples were collected from each rat on the last 3 d of the experimental period by gently handling the rats until they produced a fecal sample. For fecal pH, fresh feces were homogenized in 3 volumes of saline and the pH recorded (TPB, digital pH meter, model 1852mV). Fresh feces were diluted in 3 volumes of internal standard solution (heptanoic acid, 1.68 mmol/L) and stored at −20°C for later analysis of SCFA concentrations.

On the final day of the experimental period, each rat was administered a single i.p. injection of AOM (10 mg/kg body weight, Sigma Chemical) to induce apoptosis (14); 6 h later, rats were killed by CO_2-induced narcosis. The entire colon was rapidly removed and divided into proximal and distal portions; the limit of the proximal portion was defined by the "herring bone" pattern. These were flushed clean with ice-cold saline, and segments (2 cm) were taken from the cecal end of the proximal portion and the rectal end of the distal portion. These segments were placed in 10% buffered formalin for 24 h, then washed and stored in 70% ethanol. The cecum was excised, weighed, and a known weight of digesta placed in 3 volumes of saline for pH measurement; a known weight of digesta was also diluted in 3 volumes of internal standard solution (heptanoic acid, 1.68 mmol/L) and stored at −20°C for later analysis of SCFA. Cecal digesta was also collected using aseptic techniques and diluted in 9 volumes of storage media and stored at −20°C for microbial population determination. The Flinders University of South Australia Animal Welfare Committee approved all experimental procedures.

Measurement of apoptosis. Colon sections (0.5 cm × 0.5 cm) fixed in 70% ethanol were cut from proximal and distal segments of the colon and embedded in paraffin. Paraffin-embedded sections (5 μm) were stained with hematoxylin and evaluated under a light microscope for apoptotic cells. Apoptotic cells were identified in 20 randomly chosen intact crypts by characteristic morphologic changes of cell shrinkage, the presence of condensed chromatin, and sharply delineated cell borders surrounded with a clear halo (14) as described by Potten et al. (24). In all cases, an independent observer who was unaware of the experimental dietary treatment determined the quantification of apoptotic cells. The percentage of apoptotic nuclei (apoptotic index) was calculated as the mean number of apoptotic cells per crypt column divided by the total number of cells in the column and multiplied by 100. The length of each crypt was determined along with the position of apoptotic cells.

Measurement of cell proliferation. 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. 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 Biotechnology) was placed on the slides (1/500 dilution) and incubated overnight at room temperature. A Level 2 Ultra Streptavidin detection system (Signet Laboratories) was used with biotinylated goat anti-mouse IgG as the secondary antibody. The slides were counterstained for 3 min with hematoxylin. In all cases, an independent observer who was unaware of the experimental dietary treatment determined the quantification of proliferative cells. The scoring for cell proliferation was the same as the method used to score apoptosis.

SCFA analysis. Fecal and cecal samples were homogenized in 3 volumes of internal standard solution (heptanoic acid, 1.68 mmol/L) and centrifuged at 3000 × *g* for 10 min. The supernatant was then distilled and 0.3 μL injected into a gas chromatograph (Hewlett

Packard 5890 Series II A) equipped with a flame ionization detector and a capillary column (Zebron ZB-FFAP, 30 m × 0.53 mm i.d, 1-μm film, SGE). Helium was used as the carrier gas; the initial oven temperature was 120°C and was increased at 30°C/min to 190°C; the injector temperature was 210°C and the detector temperature was 210°C. A standard SCFA mixture containing acetate, propionate, and butyrate was used for calculation and the results are expressed as μmol/g of sample.

Microbiology. Samples of digesta (1 g) from the cecum were diluted into 9 mL of storage medium containing 20 g/L buffered peptone (Oxoid CM509), 20 g/L lab-lemco powder (Oxoid L29) and 20% v:v glycerol before storage at −20°C. Suspensions were thawed at 4°C, homogenized, and further diluted in 10-fold dilutions using prereduced buffered peptone (20 g/L buffered peptone Oxoid CM509, 0.5% cysteine HCl and 0.1% Tween 80). Four appropriate dilutions (100 μL each) were inoculated in duplicate directly onto Bifidus blood, (25) Rogosa (Oxoid CM627), Columbia blood (Oxoid CM 331), and Chromogenic *Escherichia coli*/Coliform (Oxoid CM956) medium for the selective enumeration of *Bifidobacteria*, *Lactobacilli*, total anaerobes, *E. coli*, total coliforms, and total aerobes, respectively. Bifidus blood, Columbia Blood, and Rogosa plates were incubated at 37°C under anaerobic conditions using Anaerogen Compact (Oxoid AN010C) for a period of 5 d for Bifidus blood and Columbia Blood plates and 3 d for Rogosa plates. Chromogenic plates were incubated at 37°C under aerobic conditions for 24 h. Colonies characteristic of each bacterial group were counted visually and the concentration calculated as cfu/g wet weight. All colonies were counted on both Rogosa and Columbia agar.

Statistical analysis. Results are presented as means ± SEM. Data were tested by 2-way ANOVA using the SPSS version 12 statistical package. The 2 factors were diet (Low-RS and Moderate-RS) and bacteria (Control, *L. acidophilus*, *B lactis*, *L. acidophilus* + *B lactis*). Differences were considered significant at $P \leq 0.05$. When the diet × bacteria interaction term was not significant, the interaction term was removed from the model. Where there was a significant interaction, each diet was analyzed separately for differences due to bacteria using 1-way ANOVA and the Sidak post hoc test.

RESULTS

Rats. Body weight gains did not differ among the rats fed the different experimental diets. During the 4-wk experiment, weight gains were as follows: Low-RS, 49.0 ± 1.6 g; Low-RS + *L. acidophilus*, 50.0 ± 3.5 g; Low-RS + *B. lactis*, 49.8 ± 2.2 g; Low-RS + *L. acidophilus* + *B. lactis*, 46.8 ± 2.0 g; Moderate-RS, 49.9 ± 1.6 g; Moderate-RS + *L. acidophilus*, 50.1 ± 2.1 g; Moderate-RS + *B. lactis*, 49.4 ± 2.4; Moderate-RS + *L. acidophilus* + *B. lactis*, 48.9 ± 2.7 g.

Fecal fermentation variables. Fecal pH was used as an indicator of distal lumenal pH (26) (**Table 2**). For fecal pH, there was a significant interaction between diet and bacteria. Fecal pH did not differ among the bacteria-treated groups fed the low-RS diets ($P = 0.094$), but in rats fed the moderate-RS diet, it was elevated in those treated with bacteria ($P < 0.05$).

The rats consuming the moderate-RS diet had higher ($P < 0.001$) total SCFA, acetate, propionate, and butyrate concentrations in the feces compared with those fed the low-RS diet (Table 2). Probiotic bacteria supplementation did not affect fecal SCFA concentrations in rats fed either the low or moderate-RS diets.

Cecal fermentation variables. Cecal pH was lower in rats fed the moderate-RS diets ($P < 0.001$) compared with rats fed the low-RS diets. Supplementation of probiotics to the RS diet did not affect pH (**Table 3**).

SCFA concentrations in the cecum were greater in those fed moderate-RS diets ($P < 0.001$) (Table 3). Probiotic supplementation did not affect cecal SCFA concentrations.

Microbiological studies. The level of dietary RS affected total anaerobes ($P = 0.021$), total aerobes ($P < 0.001$), lactobacilli ($P < 0.001$), bifidobacteria ($P < 0.001$), and total coliforms ($P < 0.001$) in the cecal digesta (**Table 4**). Furthermore, the type of bacteria supplemented to the diet had a significant effect on lactobacillus species numbers ($P = 0.004$). *B. lactis* supplementation increased Lactobacilli numbers.

Acute apoptotic response to genotoxic carcinogen (AARGC) in the distal colon. There was a significant interaction between the level of dietary RS and bacteria on the AARGC ($P = 0.002$). The AARGC was significantly elevated in the distal colon by *B. lactis* in rats fed RS, with or without *L. acidophilus* (**Fig. 1**). *L. acidophilus* did not affect the AARGC nor did *B. lactis* in rats fed low-RS. The level of dietary RS did not affect the AARGC.

Cell proliferation in distal colon. The moderate-RS diet significantly increased the labeling index and crypt column height ($P < 0.001$) (**Table 5**). Probiotic bacteria supplementation did not affect either variable.

TABLE 2

Effect of low- and moderate-RS diets supplemented with L. acidophilus (LA), B. lactis (BL), or both (LA + BL) on fecal pH and fecal SCFA concentrations in rats[1]

	Low-RS diet				Moderate-RS diet				2-Way ANOVA *P*-values[2]		
	Control	LA	BL	LA + BL	Control	LA	BL	LA + BL	D	B	D × B
Fecal pH	7.5 ± 0.1	7.4 ± 0.1	7.6 ± 0.1	7.6 ± 0.1	6.3 ± 0.1[b]	6.5 ± 0.1[a]	6.5 ± 0.1[a]	6.7 ± 0.1[a]	—	—	0.010
	μmol/g wet feces										
Total SCFA	30.7 ± 4.8	33.9 ± 2.9	31.9 ± 3.7	33.6 ± 3.8	71.9 ± 10.0	76.2 ± 6.7	62.1 ± 5.2	53.5 ± 6.3	<0.001	—	—
Acetate	17.4 ± 3.0	18.8 ± 1.5	18.8 ± 2.3	19.4 ± 2.2	48.1 ± 6.9	53.5 ± 4.6	40.8 ± 3.7	34.4 ± 5.1	<0.001	—	—
Propionate	9.6 ± 1.4	10.7 ± 1.0	9.4 ± 1.0	9.5 ± 1.0	15.8 ± 2.0	14.2 ± 1.7	11.4 ± 0.9	12.5 ± 1.0	<0.001	—	—
Butyrate	3.8 ± 0.8	4.3 ± 0.6	3.7 ± 0.5	4.8 ± 0.8	7.9 ± 1.5	8.4 ± 1.2	9.2 ± 1.1	7.0 ± 1.1	<0.001	—	—

[1] Values are means ± SEM, $n = 12$.

[2] Significant effects ($P < 0.05$) of diet (D), bacteria (B), or their interaction (D × B) are shown. When the interaction was significant, each diet was analyzed separately by 1-way ANOVA and Sidak's test for differences due to bacteria. Means for a diet with superscripts without a common letter differ, $P < 0.05$.

TABLE 3

Effect of low- and moderate-RS diets supplemented with L. acidophilus (LA), B. lactis (BL), or both (LA + BL) on cecal pH and cecal SCFA concentrations in rats[1]

	Low-RS diet				Moderate-RS diet				2-Way ANOVA *P*-values[2]		
	Control	LA	BL	LA + BL	Control	LA	BL	LA + BL	D	B	D × B
Cecal pH	7.4 ± 0.1	7.5 ± 0.1	7.6 ± 0.1	7.7 ± 0.1	6.5 ± 0.1	6.4 ± 0.5	6.4 ± 0.1	6.5 ± 0.1	<0.001	—	—
					μmol/g wet cecal content						
Total SCFA	51.9 ± 4.1	60.2 ± 3.0	49.4 ± 4.8	46.5 ± 4.4	82.9 ± 8.7	75.3 ± 6.0	100.5 ± 11.5	82.2 ± 6.7	<0.001	—	—
Acetate	32.1 ± 3.0	34.5 ± 2.0	29.3 ± 3.5	26.5 ± 3.3	49.0 ± 5.3	45.6 ± 3.5	64.7 ± 9.2	45.0 ± 3.4	<0.001	—	—
Propionate	14.6 ± 0.9	18.3 ± 0.8	14.5 ± 1.1	14.1 ± 1.1	22.1 ± 2.4	19.7 ± 2.3	22.2 ± 2.2	24.1 ± 2.6	<0.001	—	—
Butyrate	5.2 ± 0.5	7.2 ± 0.6	5.5 ± 0.5	5.9 ± 0.4	11.8 ± 1.3	10.0 ± 1.0	13.5 ± 1.1	13.3 ± 1.7	<0.001	—	—

[1] Values are means ± SEM, $n = 12$.
[2] Significant effects ($P < 0.05$) of diet (D), bacteria (B), or their interaction (D × B) are shown.

DISCUSSION

This study showed that the addition of RS to the diet (10 g Hi-maize 958/100 g diet) markedly changed colonic markers of fermentation, i.e., SCFA levels were higher and pH was lower. Furthermore, a significant prebiotic effect of selective stimulation of growth of lactobacilli and bifidobacteria species was also seen when the diet was supplemented with RS. The acute apoptotic response (i.e., AARGC) to the carcinogen AOM was not affected by either probiotic or the prebiotic alone but it was significantly increased by *B. lactis* in synbiotic combination with RS. *L. acidophilus* did not affect the AARGC under any circumstances. Therefore, the synbiotic combination of *B. lactis* with the fermentable substrate, Hi-maize 958, exerted a specific proapoptotic effect not shared by the individual components.

There are several reports in which a synbiotic combination showed biological effects beyond those of the individual components. Rowland et al. (2) demonstrated a reduction of preneoplastic ACF using the synbiotic combination of inulin and *B. longum*. Gallaher and Khil (27) also showed a reduction in ACF using oligofructose and bifidobacteria. In another study, Femia et al. (17) found that the combined administration of *B. lactis* and *L. rhamnosus* with inulin enriched with oligofructose in rats was able to further reduce the occurrence of malignant tumors induced by the carcinogen AOM compared with the individual components alone.

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. Apoptosis is an important regulatory process in the protection against the development of cancer. Apoptosis provides an innate cellular defense against oncogenesis by processes that include removal of cells with genomic instability that have developed during oncogenesis (28) and by deletion of cells suffering DNA insult from genotoxic agents such as carcinogens (29). Upregulation or facilitation of apoptosis during initiation events might increase the elimination of mutated cells that might otherwise

TABLE 4

Effect of low- and moderate-RS diets supplemented with L. acidophilus (LA), B. lactis (BL), or both (LA + BL) on cecal microbial populations[1]

	Low-RS diet				Moderate-RS diet				2-Way ANOVA *P*-values[2]		
	Control	LA	BL	LA + BL	Control	LA	BL	LA + BL	D	B	D × B
					log_{10}/g wet cecal content						
Total anaerobes	9.26 ± 0.16	9.10 ± 0.10	9.29 ± 0.12	9.32 ± 0.10	8.92 ± 0.11	9.00 ± 0.10	9.31 ± 0.11	9.04 ± 0.05	0.021	—	—
Total aerobes	8.15 ± 0.11	7.63 ± 0.31	7.87 ± 0.16	7.75 ± 0.21	8.31 ± 0.05	8.27 ± 0.08	8.27 ± 0.09	8.31 ± 0.08	<0.001	—	—
Lactobacilli	8.27 ± 0.21	8.42 ± 0.08	8.68 ± 0.17	8.43 ± 0.12	8.79 ± 0.11	9.04 ± 0.09	9.29 ± 0.05	8.83 ± 0.11	<0.001	0.004	
Bifidobacteria	8.33 ± 0.42	8.00 ± 0.50	8.91 ± 0.29	7.80 ± 0.54	9.65 ± 0.24	9.38 ± 0.08	9.83 ± 0.08	9.60 ± 0.17	<0.001	—	—
E. coli	6.68 ± 0.26	7.01 ± 0.13	6.54 ± 0.25	6.28 ± 0.17	6.50 ± 0.13	6.67 ± 0.22	6.16 ± 0.22	6.85 ± 0.20	—	—	—
Total coliforms	8.06 ± 0.18	7.82 ± 0.18	7.55 ± 0.34	7.80 ± 0.21	6.60 ± 0.16	6.76 ± 0.27	6.16 ± 0.22	7.24 ± 0.21	<0.001	—	—

[1] Values are means ± SEM, $n = 12$.
[2] Significant effects ($P < 0.05$) of diet (D), bacteria (B), or their interaction (D × B) are shown. The diets were combined and analyzed by 1-way ANOVA and Sidak's test for differences due to bacteria. BL significantly different from Control ($P < 0.05$).

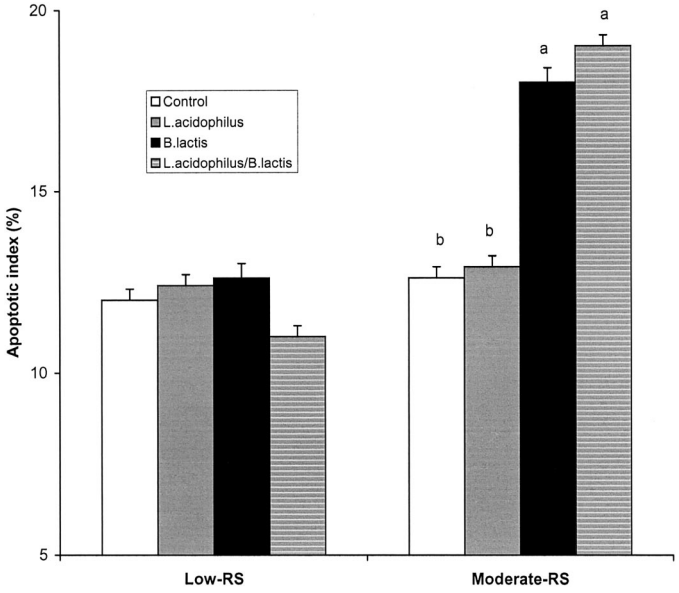

FIGURE 1 Apoptotic index in the distal colon of rats fed a low-RS or moderate-RS diet supplemented with *L. acidophilus, B. lactis*, or both. Data are expressed as means ± SEM, $n = 12$. There was a significant interaction ($P = 0.002$) between diet and bacteria (2-way ANOVA). Each diet was analyzed separately by 1-way ANOVA and Sidak's test for differences due to bacteria. Means for the moderate-RS diet without a common letter differ, $P < 0.05$.

progress to malignancy (14,15). Such an effect might be the mechanism by which prebiotics and/or probiotics act to protect against colorectal cancer.

We showed previously that the moderate amount of RS used in the present study did not affect the AARGC, whereas higher amounts (i.e., 20 or 30 g Hi-maize 958/100 g diet) do have an effect (12). Thus we used this dose of RS to test for a synbiotic effect. Because it was suggested that a mixture of probiotics might be more effective than individual strains, (21) we combined the RS with 2 probiotic strains. The synbiotic combination of RS with *B. lactis* clearly enhanced the apoptotic response, i.e., by ~33%. This is likely to be biologically relevant because it was estimated that only a small change (<1%) in the proportion of apoptotic cells in the crypt is required to influence development of colorectal tumors (24,30,31). This might have important implications for humans because the proportion of starch consumed as RS (10 g Hi-maize 958 per 100 g diet) is feasible in the context of the human diet and not likely to create a serious problem of side effects such as flatulence and bloating (32).

A possible mechanism by which the *B. lactis* in combination with RS facilitated AARGC may be through the immunomodulating properties of probiotic bacteria (33). Cytokines such as tumor necrosis factor α (TNF-α) are capable of inducing apoptosis (34). Although cytokine levels were not measured in the present study, other studies have shown that the levels of cytokines such as TNF-α, interferon-γ and interleukin-10 may be increased with probiotic supplementation (35).

RS influences fermentation variables such as pH and SCFA concentrations, including butyrate (14,36,37). The RS concentration used in the present study consistently altered all fermentation variables measured, including significant increases in butyrate concentrations in the cecum and feces. Butyrate is associated with induction of differentiation, suppression of proliferation, and enhanced apoptosis in vitro (38–41) and may be associated with enhanced apoptosis in vivo (12,14). Increased luminal concentrations have been directly linked to protection in the rodent model (19). Although the probiotic strains used did not influence SCFA production, and with only minor alterations in pH, it seems likely that RS-induced changes in the luminal microenvironment (such as acidification of digesta, SCFA production, or changed balance of microfloral species) might create a situation that enables *B. lactis* to exert a proapoptotic affect. RS in the form of high-amylose maize starch was reported to increase survival of bifidobacteria at low pH in the intestinal tract of mice through adhesion and utilization of the starch granules as an energy source to sustain their growth (42). This might increase amounts of metabolically active *B. lactis* in the colon.

Lactobacilli are not apparently sustained by RS in the rat. Wang et al. (42) reported that none of the lactobacillus stains that they tested showed any starch-degrading activity. This may well explain why *L. acidophilus* in combination with RS had no effect on the AARGC.

In conclusion, the synbiotic combination of RS and *B. lactis* significantly facilitated the apoptotic response to a genotoxic carcinogen in the distal colon of rats. It appears likely that ingested RS acts as a metabolic substrate, thus creating the right conditions for *B. lactis* to exert its proapoptotic action. Because the synbiotic combination of these agents facilitates the apoptotic response to DNA damage by a cancer initiator in the colon of rats, it warrants further study for its capacity to protect against colorectal cancer.

TABLE 5

Effect of low- and moderate-RS diets supplemented with L. acidophilus (LA), B. lactis (BL), or both (LA + BL) on cell proliferation and crypt height in distal colon[1]

	Low-RS diet				Moderate-RS diet				2-Way ANOVA *P*-values[2]		
	Control	LA	BL	LA + BL	Control	LA	BL	LA + BL	D	B	D × B
Labeling index, %	24.1 ± 0.6	24.6 ± 0.7	23.2 ± 0.5	23.7 ± 0.9	28.0 ± 1.2	27.2 ± 1.5	26.8 ± 1.5	26.1 ± 1.7	<0.001	—	—
Crypt height, *cells/column*	30.9 ± 0.5	31.2 ± 0.6	31.4 ± 0.6	31.2 ± 0.5	32.7 ± 0.1	32.1 ± 0.5	33.1 ± 0.9	32.8 ± 0.5	<0.001	—	—

[1] Values are means ± SEM, $n = 12$.
[2] Significant effects ($P < 0.05$) of diet (D), bacteria (B), or their interaction (D × B) are shown.

LITERATURE CITED

1. Guarner, F. & Malagelada, J. R. (2003) Gut flora in health and disease. Lancet 361: 512–519.
2. Rowland, I. R., Rumney, C. J., Coutts, J. T. & Lievense, L. C. (1998) Effect of *Bifidobacterium longum* and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. Carcinogenesis 19: 281–285.
3. Fuller, R. (1989) Probiotics in man and animals. J. Appl. Bacteriol. 66: 365–378.
4. Orrhage, K. & Nord, C. E. (2000) Bifidobacteria and lactobacilli in human health. Drugs Exp. Clin. Res. 26: 95–111.
5. Singh, J., Rivenson, A., Tomita, M., Shimamura, S., Ishibashi, N. & Reddy, B. S. (1997) *Bifidobacterium longum*, a lactic acid-producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. Carcinogenesis 18: 833–841.
6. McIntosh, G. H., Royle, P. J. & Playne, M. J. (1999) A probiotic strain of *L. acidophilus* reduces DMH-induced large intestinal tumors in male Sprague-Dawley rats. Nutr. Cancer 35: 153–159.
7. Wollowski, I., Rechkemmer, G. & Pool-Zobel, B. L. (2001) Protective role of probiotics and prebiotics in colon cancer. Am. J. Clin. Nutr. 73: 451S–455S.
8. Sakata, T., Kojima, T., Fujieda, M., Miyakozawa, M., Takahashi, M. & Ushida, K. (1999) Probiotic preparations dose-dependently increase net production rates of organic acids and decrease that of ammonia by pig cecal bacteria in batch culture. Dig. Dis. Sci. 44: 1485–1493.
9. Rolfe, R. D. (2000) The role of probiotic cultures in the control of gastrointestinal health. J. Nutr. 130: 396S–402S.
10. Fuller, R. & Gibson, G. R. (1997) Modification of the intestinal microflora using probiotics and prebiotics. Scan. J. Gastroenterol. 222 (suppl.): 28–31.
11. Gibson, G. R. & Roberfroid, M. B. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125: 1401–1412.
12. Le Leu, R. K., Brown, I. L., Hu, Y. & Young, G. P. (2003) Effect of resistant starch on genotoxin-induced apoptosis, colonic epithelium, and lumenal contents in rats. Carcinogenesis 24: 1347–1352.
13. Hughes, R. & Rowland, I. R. (2001) Stimulation of apoptosis by two prebiotic chicory fructans in the rat colon. Carcinogenesis 22: 43–47.
14. Le Leu, R. K., Hu, Y. & Young, G. P. (2002) Effects of resistant starch and nonstarch polysaccharides on colonic luminal environment and genotoxin-induced apoptosis in the rat. Carcinogenesis 23: 713–719.
15. Hu, Y., Martin, J., Le Leu, R. & Young, G. P. (2002) The colonic response to genotoxic carcinogens in the rat: regulation by dietary fibre. Carcinogenesis 23: 1131–1137.
16. Verghese, M., Rao, D. R., Chawan, C. B. & Shackelford, L. (2002) Dietary inulin suppresses azoxymethane-induced preneoplastic aberrant crypt foci in mature Fisher 344 rats. J. Nutr. 132: 2804–2808.
17. Femia, A. P., Luceri, C., Dolara, P., Giannini, A., Biggeri, A., Salvadori, M., Clune, Y., Collins, K. J., Paglierani, M. & Caderni, G. (2002) Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* on azoxymethane-induced colon carcinogenesis in rats. Carcinogenesis 23: 1953–1960.
18. McIntosh, G. H., Le Leu, R. K., Royle, P. J. & Young, G. P. (1996) A comparative study of the influence of differing barley brans on DMH-induced intestinal tumours in male Sprague-Dawley rats. J. Gastroenterol. Hepatol. 11: 113–119.
19. McIntyre, A., Gibson, P. R. & Young, G. P. (1993) Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. Gut. 34: 386–391.
20. Reddy, B. S., Hirose, Y., Cohen, L. A., Simi, B., Cooma, I. & Rao, C. V. (2000) Preventive potential of wheat bran fractions against experimental colon carcinogenesis: implications for human colon cancer prevention. Cancer Res. 60: 4792–4797.
21. Roberfroid, M. B. (1998) Prebiotics and synbiotics: concepts and nutritional properties. Br. J. Nutr. 80: S197–S202.
22. American Institute of Nutrition (1977) Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. J. Nutr. 107: 1340–1348.
23. Ferguson, L. R., Tasman-Jones, C., Englyst, H. & Harris, P. J. (2000) Comparative effects of three resistant starch preparations on transit time and short-chain fatty acid production in rats. Nutr. Cancer 36: 230–237.
24. Potten, C. S., Li, Y. Q., O'Connor, P. J. & Winton, D. J. (1992) A possible explanation for the differential cancer incidence in the intestine, based on distribution of the cytotoxic effects of carcinogens in the murine large bowel. Carcinogenesis 13: 2305–2312.
25. Pachenari, A., Conway, P. L. and Playne, M. J. (2001) Bifidus-blood agar—a differentiating medium for the isolation and enumeration of bifidobacteria from faecal samples. Biosci. Microflora 20: 85–88.
26. McIntyre, A., Young, G. P., Taranto, T., Gibson, P. R. & Ward, P. B. (1991) Different fibers have different regional effects on luminal contents of rat colon. Gastroenterology 101: 1274–1281.
27. Gallaher, D. D. & Khil, J. (1999) The effect of synbiotics on colon carcinogenesis in rats. J. Nutr. 129: 1483S–1487S.
28. Thompson, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. Science (Washington, DC) 267: 1456–1462.
29. Potten, C. S. & Grant, H. K. (1998) The relationship between ionizing radiation-induced apoptosis and stem cells in the small and large intestine. Br. J. Cancer 78: 993–1003.
30. Hall, P. A., Coates, P. J., Ansari, B. & Hopwood, D. (1994) Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. J. Cell Sci. 107: 3569–3577.
31. Chang, W. C., Chapkin, R. S. & Lupton, J. R. (1997) Predictive value of proliferation, differentiation and apoptosis as intermediate markers for colon tumorigenesis. Carcinogenesis 18: 721–730.
32. Topping, D. L. & Clifton, P. M. (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol. Rev. 81: 1031–1064.
33. Perdigon, G., Fuller, R. & Raya, R. (2001) Lactic acid bacteria and their effect on the immune system. Curr. Issues Intest. Microbiol. 2: 27–42.
34. Sellers, W. R. & Fisher, D. E. (1999) Apoptosis and cancer drug targeting. J. Clin. Investig. 104: 1655–1661.
35. Perdigon, G., de Moreno de LeBlanc, A., Valdez, J. & Rachid, M. (2002) Role of yoghurt in the prevention of colon cancer. Eur. J. Clin. Nutr. 56 (suppl. 3): S65–S68.
36. Silvi, S., Rumney, C. J., Cresci, A. & Rowland, I. R. (1999) Resistant starch modifies gut microflora and microbial metabolism in human flora-associated rats inoculated with faeces from Italian and UK donors. J. Appl. Microbiol. 86: 521–530.
37. Le Blay, G., Michel, C., Blottiere, H. M. & Cherbut, C. (1999) Enhancement of butyrate production in the rat caecocolonic tract by long-term ingestion of resistant potato starch. Br. J. Nutr. 82: 419–426.
38. Whitehead, R. H., Young, G. P. & Bhathal, P. S. (1986) Effects of short chain fatty acids on a new human colon carcinoma cell line (LIM1215). Gut 27: 1457–1463.
39. Young, G. P. & Gibson, P. R., eds. (1995) Butyrate and the Human Cancer Cell. Cambridge University Press, Cambridge, UK.
40. Heerdt, B. G., Houston, M. A. & Augenlicht, L. H. (1997) Short-chain fatty acid-initiated cell cycle arrest and apoptosis of colonic epithelial cells is linked to mitochondrial function. Cell Growth Differ. 8: 523–532.
41. Medina, V., Edmonds, B., Young, G. P., James, R., Appleton, S. & Zalewski, P. D. (1997) Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. Cancer Res. 57: 3697–3707.
42. Wang, X., Conway, P. L., Brown, I. L. & Evans, A. J. (1999) In vitro utilization of amylopectin and high-amylose maize (Amylomaize) starch granules by human colonic bacteria. Appl. Environ. Microbiol. 65: 4848–4854.