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Research Paper

Suppression of Azoxymethane-Induced Colon Cancer Development in Rats by Dietary Resistant Starch

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KEY WORDS

colorectal cancer, fermentation, butyrate, resistant starch, apoptosis

ABBREVIATIONS

AOM	azoxymethane
SCFA	short chain fatty acid
RS	resistant starch
HAS	high amylose maize starch
PCNA	proliferating cell nuclear antigen
CRC	colorectal cancer

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ABSTRACT

Resistant starch is a complex carbohydrate that reaches the colon where it can be fermented by the colonic microflora resulting in production of short chain fatty acids (SCFA), in particular butyrate. RS effects on colorectal tumorigenesis are contrasting and protection remains controversial. Butyrate has an important role as the preferred metabolic fuel and regulator of colonocyte proliferation, differentiation and apoptosis and may play a role in cancer prevention. Thus variation in butyrate production from different substrates might explain the variation in effect of RS. This study evaluated the hypothesis that feeding dietary resistant starch (as high amylose maize starch) would protect against azoxymethane (AOM)-colon carcinogenesis and favorably influence the colonic luminal environment. Male Sprague-Dawley rats (n = 90) were provided one of three diets: Control (without added dietary fibre or RS), 10% HAS (contained 100 g/kg raw high amylose maize starch) or 20% HAS (contained 200 g/kg high amylose maize starch). Rats were fed their experimental diets for four weeks after which they were injected with AOM (15 mg/kg) during the fifth and six week. Colons were resected (25 weeks post second injection) for evaluation of tumor formation, apoptosis, proliferating cell nuclear antigen (PCNA) labelling index and short chain fatty acid levels. Feeding resistant starch significantly reduced the incidence ($p < 0.01$) and multiplicity ($p < 0.05$) of adenocarcinomas in the colon compared to the Control diet. Both doses of HAS resulted in similar protection against colon tumorigenesis. Feeding RS significantly increased total SCFA concentrations, including butyrate in the distal colon. Apoptosis ($p < 0.01$) was also enhanced while PCNA labelling index was reduced ($p < 0.01$) in the distal colon with resistant starch feeding. The protective effect of consumption of RS as dietary high-amylose cornstarch against colon cancer development appears to be related to active fermentation in the colon, particularly through production of butyrate.

INTRODUCTION

Diet has long been recognised as an important environmental factor in the aetiology of colorectal cancer (CRC).[1] Evidence from numerous studies suggests a protective role of dietary fibre on colorectal cancer.[2,3] While less research has been conducted on a role for dietary starch on colorectal cancer risk, there is evidence that starch may protect.[4] In an international correlative study (across 12 populations) Cassidy et al.[5] found a strong inverse associations between starch intake and both colon and rectal cancer. The effect remained statistically significant when adjusting for fat and protein intake. The authors assumed in this study that around 5% of all starch consumed were resistant to digestion and this component termed 'resistant starch' may have contributed to the protective effect on CRC.

Resistant starch is defined as a component of dietary starch that is not absorbed in the small intestine of healthy individuals and thus reaches the colon undigested, similar to dietary fibre where it also is subjected to fermentation by the colonic anaerobic bacteria.[6] Resistant starch can be classified into four main types based upon structural considerations and bacterial degradation.[6] RS_1 includes physically entrapped starch within whole plant cells and food matrices (e.g., coarsely milled grain). RS_2 consists of native starch granules that are highly resistant to digestion by α-amylases (e.g., green banana, high amylose maize starch). RS_3 comprises retrograded starches, formed when starchy foods are cooked and cooled. RS_4 comprises chemically modified starches (e.g., esterified starches) where the modification interferes with the amylolytic activity of digestive enzymes. Each RS type has different characteristics and different fermentation patterns[7] which may lead to different

effects on luminal environment. As a consequence, different types of resistant starch should not be considered equivalent and may have different effects on CRC development.

A number of studies have examined the effect of resistant starch on experimentally induced colorectal carcinogenesis. These are summarised in Young et al.[4] and the results are conflicting. More recently hydrothermally treated RS_3 protected against dimethylhydrazine (DMH)-induced colon carcinogenesis[8] and high amylose maize starch protected against AOM-induced colon cancer.[9] There are several explanations for the varying results between the reported studies: the different carcinogen protocols (dose and duration), differences in starch type, feeding regimens, lack of effect on fermentation parameters and the comparative control diet. Some of these will alter luminal conditions known to have a major influence on colonic oncogenesis.[10,11]

RS may protect through mechanisms associated with its fermentation in the colon. RS like that of dietary fibre is fermented by the colonic microflora resulting in the production of short chain fatty acids (SCFA) and gases (CO_2, CH_4, H_2).[11] The SCFA butyrate has generated the most interest as it may be protective against colorectal cancer.[12,13] Although butyrate is the primary energy source for colonic epithelium,[14] it inhibits growth of cancer cells in vitro and forces a more normal differentiated phenotype.[12] In addition, it is a potent pro-apoptotic agent[15] which might aid removal of cells with damaged DNA. Colonic production of butyrate by fermentation is associated with reduced rate of aberrant crypt foci[16] and tumor mass in an animal model, provided that fermentation is active in the distal colon.[17]

Resistant starch may also protect through broader mechanisms associated with fibre such as alterations of gut microbiota to a more beneficial state,[18] reducing bile acid metabolism,[19] increasing faecal bulk, decreasing transit time and reducing pH levels in colonic lumen.[11] All these effects might contribute to protection and colorectal cancer.

This study investigated the effects of feeding increasing concentrations of the RS_2 high amylose cornstarch (HAS) to rats on chemically-induced colorectal cancer. We explored the relationship between effect of RS on SCFA production and cellular processes of relevance to carcinogenesis, specifically epithelial proliferation and apoptosis. The purpose was to determine if protection occurred as a result of active fermentative production of butyrate.

MATERIALS AND METHODS

Animals and diets. A total of 90 male Sprague-Dawley rats, five weeks of age, were obtained from the Animal Resource Centre, Perth, Western Australia. Animals were divided randomly into three experimental groups and housed 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.[20] Each group of animals was fed an experimental diet based on the control diet (Table 1). Choline, methionine, minerals, and vitamins were added as previously.[21] The first group "control" consumed a diet containing no added fibre or resistant starch. The second group "10% HAS" were fed a diet that contained 100 g/kg raw high amylose maize starch (HAS). The third group "20% HAS" were fed a diet that contained 200 g/kg HAS. High amylose maize starch (Hi-maize® 958) a RS_2 was used as the source of resistant starch and was supplied by National Starch and Chemical Company, Bridgewater, New Jersey, USA. High amylose maize starch was added to the diets at the expense of an equal amount of digestible cornstarch.

Table 1 **Composition of experimental diets (g/100g diet).[1,2]**

Ingredient	Control	10% HAS	20% HAS
Casein	20.00	20.00	5.00
Corn starch	46.15	36.15	26.15
High amylose maize starch	-	10.00	20.00
Corn oil	18.00	18.00	18.00
Sucrose	10.95	10.95	10.95
dl-Methionine	0.3	0.3	0.3
Choline	0.1	0.1	0.1
Mineral mix[2]	3.5	3.5	3.5
Vitamin mix[2]	1.0	1.0	1.0

[1]High amylose maize starch used as source of resistant starch. [2]AIN-76 vitamin and mineral mixtures.[20]

Experimental procedure. After four weeks on experimental diets each rat received s.c. injections of azoxymethane (15 mg/kg body weight) Sigma Chemical Co., St Louis, MO) once weekly for two weeks and then maintained on their dietary regimen until sacrifice (25 weeks post second AOM injection). The rats in each group were weighed once weekly. In the fourth week of the experiment fifteen rats from each treatment group were placed in metabolic cages to measure faecal output, food intake and collect urine. Fresh faecal samples were collected from each rat and 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 last three days of the experimental period by gently handling the rats until they produced a faecal sample.

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 caecal and colonic contents collected for SCFA measurements. Distal and proximal colonic content and caecal digesta were collected and 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. The distal portion was collected from lower 1/3 of colon while the proximal portion was taken from "herring bone" area. The small intestine and colon were examined for intestinal tumors, and the location and number of tumors were assessed with a dissection microscope (magnification 20x) and recorded.

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

Colonic tumors. Using a light microscope (magnification 40x), the colon were scored for tumor number and location by an independent observer who was unaware of dietary treatment as (described previously in ref. 22). Tumors were removed and embedded in paraffin (5 μm) for histopathological analysis. All tumors were examined histologically and evaluated by an independent observer based on the criteria of Pozharisski.[23] Adenoma was characterized

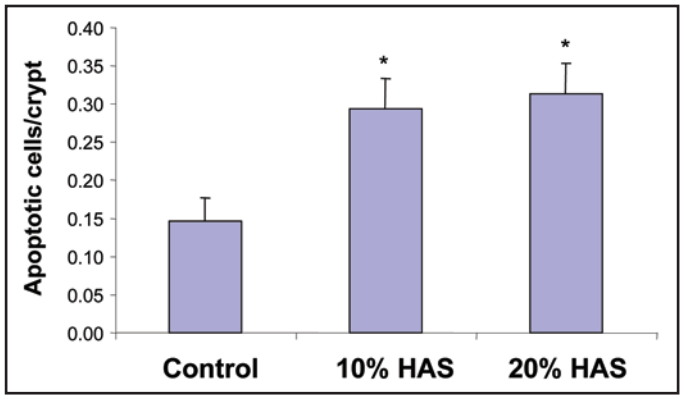

Figure 1. Influence of RS on apoptosis in mucosa of distal colon of AOM-treated rats (n = 15). Values are mean ± SEM. *p < 0.01 compared with Control group.

by expansion of the mucosa layer, reduction in goblet cell number, cellular dysplasia, moderate loss of mucosal architecture by glandular growth and lack of invasion through the basement membrane. Adenocarcinoma was identified from the following characteristics: typical cytological change, prominent cellular atypia, loss of cell polarity, marked distortion of glandular architecture and invasion.[23]

Epithelial cellular processes. Colon sections (0.5 cm x 0.5 cm) in 70% ethanol were cut from distal segments of the colon free of neoplasms 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 cell shrinkage, presence of condensed chromatin and sharply delineated cell borders surrounded with a clear halo as reported previously.[24]

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, USA) was placed on the slides (1/500 dilution) and incubated overnight at room temperature. A Level 2 Ultra Streptavidin detection system (Signet Laboratories, Inc, USA) was used utilising biotinylated goat anti-mouse as the secondary antibody. The slides were counterstained for 3 min with haematoxylin. In all cases, an independent observer who was unaware of the experimental dietary treatment determined the quantification of proliferative cells. The labelling index (LI), which is calculated as the number of positive cells in divided by the total number of cells in each crypt column multiplied by 100.

SCFA analysis. SCFA including acetate, propionate and butyrate were determined in the caecal, colon contents and faeces of rats as (described previously in ref. 21).

Statistical analysis. The effect of RS on tumor incidence and number were analysed used log binomial generalized linear models. Poisson regression models were used to analyse differences between treatments for tumor numbers in the colon. For analysis of SCFA concentrations, apoptosis and cell proliferation a One-way analysis of variance with Ryan-Einot-Gabriel-Welsch multiple stepdown post-hoc procedure was undertaken. Data are presented as the mean (SEM) for each treatment group. Means with the same letter are not statistically significantly different at $p \leq 0.05$. The relationship between caecal, colonic and faecal parameters with apoptosis, cell proliferation and colon tumor incidence was determined using the Bivariate Correlation procedure utilising Pearson's correlation coefficient. A value of $p < 0.05$ was used as the criterion of significance. The statistical package Stata 8 was used for all analyses.

Table 2 **Effect of resistant starch and indigestible protein on weight gain, feed intake and faecal output in rats[1,2]**

	Treatment Group		
	Control	10% HAS	20% HAS
Weight gain (g/30 wk)	570.5 (9.2)	530.0 (16.9)	539 (12.3)
Daily water intake (ml)	24.3 (0.8)	28.7 (2.2)	27.5 (2.2)
Daily food intake (g/d)	18.0 (0.4)	18.2 (0.8)	17.7 (0.7)
Caecal measurements			
pH	7.0 (0.03)[a]	6.6 (0.05)[b]	6.3 (0.05)[c]
Tissue weight (g)	0.6 (0.01)[a]	0.9 (0.02)[b]	1.1 (0.03)[c]
Contents weight (g)	1.6 (0.09)[a]	2.3 (0.11)[b]	3.8 (0.20)[c]
Faecal measurements			
pH	7.2 (0.04)[a]	6.9 (0.01)[b]	6.5 (0.03)[c]
Faecal output (g/d)	0.6 (0.1)[a]	1.4 (0.1)[b]	2.1 (0.2)[c]

[1]Mean (SEM), n = 30. See methods for each group's dietary composition. [2]One-way analysis of variance with Ryan-Einot-Gabriel-Welsch multiple stepdown post-hoc procedure. Means with a different superscript are statistically significantly different at $p \leq 0.05$.

RESULTS

Body weights, food intake and water intake. There were no significant differences in the body weight gain (g/30 wk), water or food intake between the groups (Table 2).

Caecal and faecal parameters. The wet weight of caecal contents increased as the HAS content of the diet increased (Table 2). This was accompanied by an increase in caecal tissue weights and a reduction in the pH of caecal contents. Faecal pH showed a similar pattern of change to that in the caecum. Faecal output in the rats increased as the HAS content of the diet increased (Table 2).

Colonic neoplasms. The effects of diet on incidence (proportion of rats who develop neoplasia), type of neoplasm (adenoma or cancer) in the colon and number of neoplasms per colon are shown in Table 3. There was a significant decrease in the incidence of all colonic neoplasms with the 20% HAS compared to the Control group. There was a 50% decrease in colon cancer incidence when HAS was incorporated into to the diet irrespective of dose compared to Control group ($p < 0.01$). Both doses of HAS significantly decreased the number of cancers per rat colon (tumor burden) compared to the Control group ($p < 0.05$).

Effects of diet on apoptosis and cell proliferation in colon. The frequency of apoptotic cells which were detected by haematoxylin staining are shown in Figure 1. There was a significant increase in the number of apoptotic cells in the distal colonic crypts of the HAS groups compared with the Control group ($p < 0.01$).

Cell proliferation was evaluated by assessing the PCNA staining in the distal colonic crypts, and Figure 2 shows the PCNA labelling index for the different groups. Both doses of HAS significantly lowered the PCNA labelling index compared to the Control group ($p < 0.01$).

Table 3 **Effect of resistant starch on the proportion and number of azoxymethane-induced colonic neo plasms**

Dietary Group	Control n = 30	10% HAS n = 30	20% HAS n = 30
Proportion of rats developing neoplasia (%)[1,3]			
All Colonic Neoplasms	63 (9)	40 (9)	37 (9)[a]
adenomas	10 (5)	13 (8)	13 (8)
cancer	57 (11)	27 (10)[b]	27 (10)[b]
Number of neoplasms per rat colon[2]			
all colonic neoplasms	0.73 (0.11)	0.50 (0.12)	0.50 (0.16)
adenomas	0.10 (0.06)	0.17 (0.08)	0.20 (0.10)
cancer	0.63 (0.11)	0.33 (0.11)[a]	0.30 (0.10)[a]

[1]Proprtion (SE) and using log binomial generalized linear model. [2]Mean (SEM) and using Poisson regression model. [3]Compared to Control: [a]$p \leq 0.05$, [b]$p \leq 0.01$.

Table 4 **Effects of resistant starch and indigestible protein on caecal, proximal and distal colon and faecal SCFA concentrations (μmol/g) in rats[1,2]**

	Treatment Group		
	Control	10% HAS	20% HAS
Caecal content			
Total SCFA	89.2 (4.6)[a]	122.2 (6.6)[b]	127.2 (8.6)[b]
Acetate	56.1 (3.2)[a]	80.6 (5.0)[b]	84.9 (6.0)[b]
Propionate	19.3 (1.0)[b]	26.9 (1.8)[c]	10.4 (1.0) [a]
Butyrate	8.7 (0.8)[a]	10.3 (0.7)[a]	26.3 (2.5) [b]
Proximal colon			
Total SCFA	63.1 (9.4)[a]	116.6 (7.0)[b]	83.3 (4.0)[a]
Acetate	40.0 (6.6)[a]	82.1 (5.5)[b]	57.9 (3.3)[a]
Propionate	13.0 (2.2)[b]	21.7 (2.3)[c]	5.8 (1.0)[a]
Butyrate	6.1 (0.8)[a]	9.9 (1.1)[a]	16.8 (2.3) [b]
Distal colon			
Total SCFA	41.0 (5.4)[a]	101.1 (12.3)[b]	89.1 (8.7)[b]
Acetate	27.0 (4.1)[a]	63.0 (8.9)[b]	58.6 (6.4)[b]
Propionate	6.9 (1.1)[a]	21.6 (2.5)[b]	7.2 (1.1)[a]
Butyrate	3.8 (0.5)[a]	12.3 (1.8)[b]	19.5 (3.0)[b]
Faeces			
Total SCFA	35.3 (2.9)[a]	64.2 (7.4)[b]	64.6 (6.8)[b]
Acetate	22.2 (2.0)[a]	39.9 (5.6)[b]	40.4 (5.6)[b]
Propionate	6.2 (0.7)[a]	13.2 (1.4)[b]	8.6 (1.1)[a]
Butyrate	3.9 (0.5)[a]	6.9 (1.1)[ab]	10.8 (1.6)[b]

[1]Mean (SEM), n = 30. See methods for each group's dietary composition. [2]One-way analysis of variance with Ryan-Einot-Gabriel-Welsch multiple stepdown post-hoc procedure. Means with a different superscript are statistically significantly different at $p \leq 0.05$.

Effect of the diets on colonic carbohydrate fermentation. Table 4 summarizes the SCFA concentrations in caecum, proximal and distal colonic content and faeces of rats fed the experimental diets.

The SCFA concentrations in the caecum were approximately twice those of the faeces. Caecal total SCFA concentration was elevated in the HAS groups compared with Control. The analysis of the individual SCFA showed that HAS groups had significantly higher caecal acetate concentrations compared to Control. Caecal propionate concentration was highest in the 10% HAS group followed by Control and lowest in the 20% HAS group. Butyrate concentration in the caecum was the highest in the 20% HAS group with a two-fold elevation compared to the 10% HAS and Control groups.

The SCFA concentrations in the proximal colonic content were slightly lower than that in the caecum. Proximal total SCFA and acetate concentration's was highest in the 10% HAS compared to control and 20% HAS group. Proximal propionate concentration was highest in the 10% HAS group followed by the Control and then the 20% HAS group. Butyrate concentration in the proximal colon was highest in the 20% HAS compared to the Control and 10% HAS groups.

In the distal colon, total SCFA and acetate concentration were elevated in the HAS groups compared with the Control group. Distal propionate was elevated in the 10% HAS groups compared with the Control and 20% HAS groups. Butyrate concentration in the distal colonic content was significantly higher in the 10% HAS and 20% HAS groups with a 3-fold and 5-fold increase respectively compared with the Control group.

In the faeces, total SCFA, acetate and propionate were all significantly elevated in the HAS groups compared with the Control group. Butyrate concentration in the faeces was significantly higher in the 20% HAS group compared to the Control group.

Association of caecal, colonic and faecal parameters with apoptosis, cell proliferation and colon cancer incidence. Apoptosis in the distal colon was found to be significantly associated with a number of parameters in the caecum and faeces after controlling for the effect of the different diets (Table 5). Significant relationships were seen between caecal pH ($p < 0.05$) and caecal total SCFA ($p < 0.01$), caecal acetate ($p < 0.01$) and caecal butyrate ($p < 0.05$) with distal apoptosis. Relationships for faecal total SCFA and acetate with apoptosis in the distal colon were also observed. A strong relationship was seen for caecal pH and cell proliferation ($p < 0.01$) while a weak negative relationship was observed for faecal total SCFA and faecal butyrate with cell proliferation ($p < 0.05$) (Table 5).

For colon cancer incidence there was a significant relationship with caecal pH ($p < 0.05$), while negative relationships were observed with total distal SCFA ($p < 0.05$), distal acetate and distal butyrate concentrations ($p < 0.05$) (Table 5).

DISCUSSION

In the present study, we provide clear evidence that consumption of a diet rich in RS suppresses AOM-induced colon tumor development, as assessed in terms of tumor incidence and multiplicity in male Sprague-Dawley rats. This protection was achieved by feeding a diet containing 10% or 20% high amylose cornstarch. This might have important implications for humans because the proportion of starch consumed as RS in the 10% HAS 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.[11,25]

RS in the form of high amylose starch appears to be an excellent substrate for the colonic microflora, this was evidenced in the present study by promoting fermentation throughout the large bowel. In the distal colon, the site where tumors predominate,[26] total SCFA concentrations including butyrate were markedly increased with HAS feeding. Butyrate is considered to be protective against colon cancer.[27] Studies in vivo have indicated that providing the colonic production of butyrate through fermentation is active in the distal colon then

Table 5 **Correlations between apoptosis and cell proliferation and colon cancer incidence with selected faecal and caecal parameters[1]**

	Apoptosis		Cell Proliferation		Colon Cancer (%)	
	(r)	p-value	(r)	p-value	(r)	p-value
Caecum						
pH	-0.34	<0.05	0.59	<0.01	0.24	0.03
Total SCFA (µmol/g)	0.42	<0.01	-0.20	0.34	-0.18	0.10
Acetate	0.42	<0.01	-0.21	0.33	-0.19	0.09
Propionate	0.10	0.52	0.10	0.64	-0.07	0.56
Butyrate	0.31	<0.05	-0.32	0.13	-0.11	0.31
Proximal colon						
Total SCFA	0.24	0.31	-0.15	0.71	-0.27	0.15
Acetate	0.32	0.19	-0.27	0.48	-0.25	0.18
Propionate	-0.40	0.87	-0.03	0.94	-0.13	0.51
Butyrate	0.26	0.29	0.14	0.72	-0.26	0.16
Distal colon						
Total SCFA	0.33	0.13	-0.01	0.97	-0.38	0.02
Acetate	0.31	0.15	-0.05	0.88	-0.36	0.04
Propionate	0.18	0.4	-0.04	0.92	-0.17	0.32
Butyrate	0.26	0.24	0.21	0.54	-0.37	0.03
Faeces						
pH	-0.31	0.07	0.54	<0.05	0.14	0.39
Total SCFA	0.35	<0.05	-0.46	<0.05	-0.05	0.79
Acetate	0.33	<0.05	-0.42	0.06	-0.02	0.92
Propionate	0.21	0.21	-0.17	0.48	-0.08	0.62
Butyrate	0.25	0.14	-0.46	<0.05	-0.0.7	0.66

[1]The relationship between caecal, colonic and faecal variables with apoptosis[2] and cell proliferation[2] and colon tumor incidence[3] was done by partial correlation controlling for different diets. [2]n = 15; [3]n = 30.

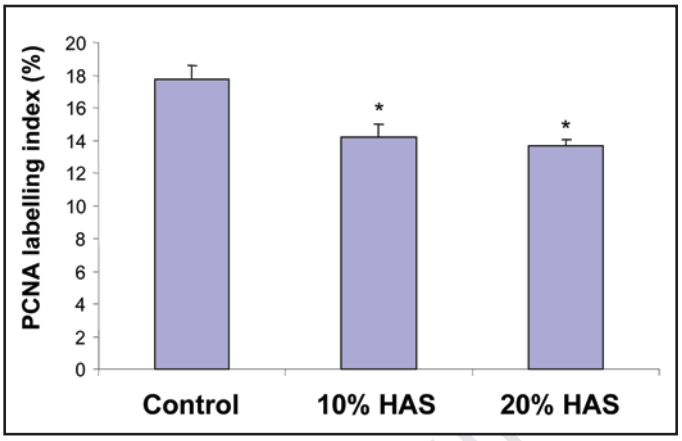

Figure 2. Influence of RS on PCNA labelling index in mucosa of distal colon of AOM-treated rats (n = 15). Values are mean ± SEM. *p < 0.01 compared with Control group.

protection against tumorigenesis is achieved.[17] Furthermore, high butyrate producing substrates[16] and delivery of butyrate directly to the distal colonic mucosa[28] have been linked to protection against the initial stages of colon carcinogenesis. The present study supports a role of butyrate in colorectal cancer protection as a significant negative correlation was observed between butyrate concentration in the distal colon and colonic tumor incidence.

In our study apoptosis was significantly increased in the distal colon of the HAS groups 25 weeks after the last AOM injection. Apoptosis is an important regulatory process in the protection against the development of cancer. Apoptosis provides an innate cellular defence against oncogenesis by processes that include removal of cells with genomic instability that have developed during oncogenesis[29] and by deletion of cells suffering DNA insult from genotoxic agents such as carcinogens.[30] Analysis of the present data showed that total SCFA, acetate and butyrate concentrations in the caecum correlated with apoptosis in the distal colon. This is consistent with the in vitro data that show apoptosis is induced by SCFA (acetate, propionate and butyrate, with butyrate being the most effective.[31] Few in vivo studies examining tumorigenesis with intervention by RS or other fermentable substrates have investigated the colonic apoptotic response at this late time point. Bauer-Marinovic et al.[8] however did observe a significant increase in apoptosis in rats fed hydrothermally treated RS_3. In this particular study apoptosis was measured in the colon of rats that had received an intensive and prolonged carcinogen schedule, namely 20 s.c. injections of DMH where the rats were killed one week after the last DMH injection. Previous studies from our laboratory have shown that fermentable substrates such as HAS or wheat bran can increase the acute apoptotic response to a genotoxic carcinogen, whereby apoptosis is measured 6 h after carcinogen injection, furthermore this increase correlates with the concentration of butyrate.[24,32] Increased apoptosis during initiation events might enhance the elimination of mutated cells that might otherwise progress to malignancy.[33] Such an effect is likely to further contribute to how RS acts to protect against colorectal tumorigenesis. Our results also showed reduced cell proliferation in the distal colon of the rats fed the RS containing diets. Increased cell turnover may enhance the risk of mutations which can lead to an increased risk of developing colorectal cancer.[34] Similar reductions in cell proliferation were observed in rats fed a RS_3[8] or the carbohydrate oligofructose[35] which also demonstrated protection against colorectal tumorigenesis. It is likely that the increased SCFA production via fermentation of starch in the colon contributes to the homeostatic maintenance of the colonic epithelium, this effect is likely to play a role in protection against colon tumorigenesis in the present study.

RS may also protect through broader mechanisms associated with fermentation. RS has prebiotic properties.[36,37] Previous studies in rats have shown that as little as 10% HAS in the diet is capable of stimulating the production of desirable bacterial species like lactobacilli and bifidobacteria.[21] RS in the current study was able to induce positive changes in the luminal microenvironment such as acidification of digesta and increased faecal bulk both which may contribute to enhanced colonic health for the host.[11] It has been reported that RS is also important in maintaining colonic mucus barrier.[38,39] The mucus layer covering the colonic mucosa is considered the first line of defence against harmful products arising from the luminal content.[40] Breakdown of mucosal barrier integrity is often observed in diseases such as inflammatory bowel disease which is a documented risk factor for colon cancer.[41] RS through enhanced production of SCFA, particularly butyrate, may be a means by which the colonic mucus barrier is maintained.

In conclusion, the present results show that feeding RS as HAS suppresses colorectal cancer induced by AOM exposure in rats. We observed increased SCFA production including butyrate from the fermentation of the RS, as well as enhanced apoptosis and reduced

cell proliferation in the colonic epithelium. These findings support the hypothesis that starch that is resistant to digestion in the small intestine can positively influence the colonic luminal environment and protect against colorectal cancer.

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