Manipulation of the gut microbiota using resistant starch is associated with protection against colitis-associated colorectal cancer in rats

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Abstract

This study evaluated whether dietary resistant starch (RS) and green tea extract (GTE), which have anti-inflammatory and anticancer properties, protect against colitis-associated colorectal cancer (CAC) using a rat model, also investigated potential mechanisms of action of these agents including their effects on the gut microbiota. Rats were fed a control diet or diets containing 10% RS, 0.5% GTE or a combination of the two (RS + GTE). CAC was initiated with 2 weekly azoxymethane (AOM) injections (10 mg/kg) followed by 2% dextran sodium sulphate in drinking water for 7 days after 2 weeks on diets. Rats were killed 20 weeks after the first AOM. Colon tissues and tumours were examined for histopathology by H&E, gene/protein expression by PCR and immunohistochemistry and digesta for analyses of fermentation products and microbiota populations. RS and RS + GTE (but not GTE) diets significantly (P < 0.05) decreased tumour multiplicity and adenocarcinoma formation, relative to the control diet. Effects of RS + GTE were not different from RS alone. RS diet caused significant shifts in microbial composition/diversity, with increases in Parabacteroides, Barnesiella, Ruminococcus, Marvinbryantia and Bifidobacterium as primary contributors to the shift. RS-containing diets increased short chain fatty acids (SCFA) and expression of the SCFA receptor GPR43 mRNA, and reduced inflammation (COX-2, NF-κB, TNF-α and IL-1β mRNA) and cell proliferation P < 0.05. GTE had no effect. This is the first study that demonstrates chemopreventive effects of RS (but not GTE) in a rodent CAC model, suggesting RS might have benefit to patients with ulcerative colitis who are at an increased risk of developing CRC.

Introduction

Inflammatory bowel disease (IBD), which includes Crohn’s disease and Ulcerative Colitis, is a chronic and relapsing inflammatory disorder of the gastrointestinal tract and is one of the three high-risk conditions predisposing to colorectal cancer (CRC), along with heritable familial CRC, such as familial adenomatous polyposis and Lynch syndrome (HNPCC). Individuals with long-standing IBD have a 10–20-fold greater risk of developing colitis-associated colorectal cancer (CAC) compared to the general population (1). Clinically, IBD often affects younger individuals, persists life-long, affects the patient’s quality of life and increases the risk of developing CRC. As a consequence, endoscopic surveillance is recommended for patients with long-standing IBD as a mean of CRC prevention. CRC takes an average of 10–15 years to develop, thus providing ample time...
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AOM</td>
<td>azoxymethane</td>
</tr>
<tr>
<td>ADC</td>
<td>adenocarcinoma</td>
</tr>
<tr>
<td>AD</td>
<td>adenoma</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>CAC</td>
<td>colitis-associated colorectal cancer</td>
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<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
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<tr>
<td>DAI</td>
<td>disease activity index</td>
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<tr>
<td>DSS</td>
<td>dextran sodium sulfate</td>
</tr>
<tr>
<td>ECGG</td>
<td>(-)-epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>GPR43</td>
<td>G-protein-coupled receptor 43</td>
</tr>
<tr>
<td>GTE</td>
<td>green tea extract</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>SCFA</td>
<td>short chain fatty acid</td>
</tr>
<tr>
<td>TNT</td>
<td>total number of tumours</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
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<tr>
<td>RS</td>
<td>resistant starch</td>
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and opportunity to identify and/or develop safe and effective agents that can reverse, inhibit or halt further progression to cancer, which could be an important complement to the endoscopic surveillance in this high risk population (2).

There is also growing evidence that a Western diet and lifestyle contributes significantly to the increased incidence of these conditions. CRC rates are approximately 13 times higher in African Americans than in rural Africans, and there are profound differences in the gut microbiota compositions of the two populations that are at least partly linked to differences in the diet (3). Disruptive changes in the gut microbiota (dysbiosis) and perturbations of intestinal host-microbe interactions are observed in IBD and CRC, and may play pivotal roles in their pathogenesis. Studies consistently reveal a reduced bacterial diversity in IBD and often show lower numbers of key bacteria responsible for production of short chain fatty acids (SCFA) such as butyrate (4,5). In CRC, changes in the gut microbiota become increasingly dysbiotic as adenoma (AD) progresses to carcinoma, and some microbial groups are particularly associated with advanced stages of the cancer (3). Based on these observations it is feasible that some foods or dietary patterns could be used to treat IBD and prevent CRC and CAC through their capacity to modulate the gut microbiota (i.e. by enriching beneficial bacteria).

Dietary fibres have shown particular promise for the prevention of CRC and attenuation of colonic inflammation (6). A number of studies, notably the European Prospective Investigation into Cancer and Nutrition (EPIC), have clearly shown a reduced CRC risk associated with increased fibre consumption (7). While the underlying mechanisms are likely to be multifactorial, and include dilution of toxins through stool bulking, the production of SCFA as a consequence of microbial fermentation of fibres is frequently cited as a major potential contributor to the protective effect of fibre (8). Resistant starch (RS) refers to starch that resists digestion in the small intestine and enters the large intestine (9) where it undergoes fermentation producing SCFA. Of the SCFA produced, butyrate is of particular importance as it plays a significant role in maintaining intestinal function and integrity, modulates immune and inflammatory responses and is linked to the attenuation of carcinogenesis in animal models (10). RS has been shown to protect against colitis and CRC in experimental animal studies (11–14) as well as beneficially regulating several biomarkers associated with CRC in humans consuming high levels of red meat (linked to increased risk of CRC) (15) and in patients with CRC (16). However, human trials evaluating the effectiveness of RS in treating CRC have been limited and have provided inconsistent results (17); most studies have targeted patients with hereditary CRC (familial adenomatous polyposis, HNPCC). To date, there have been no studies to evaluate the preventive effect of RS on CAC; our knowledge of the impacts of RS in individuals with IBD is also very limited.

Other dietary components, such as a range of polyphenolic compounds, have also been identified as having anti-inflammatory and antitumourigenic effects. Green tea is the most common beverage consumed worldwide and its main polyphenolic component ‘(-)-epigallocatechin-3-gallate’ (EGCG) has been shown to be protective against both colitis and CRC in animal models (18). The potential benefits of combining RS with green tea in CRC prevention have not been studied. The possible interaction between RS and GTE is of interest due to the likelihood of their co-occurrence in the diet, particularly in Asian countries, where the incidence of CRC is relatively low compared with Western countries.

In this study, we have used a rat model of CAC induced through treatment with azoxymethane (AOM) and dextran sodium sulphate (DSS), and tested whether addition of RS or a green tea extract (GTE) to the diet, alone or in combination, attenuates the formation of tumours. In addition, we have examined the impacts of the dietary treatments on gut microbiota populations, microbial fermentation products and expression of inflammatory cytokines, cancer biomarkers and cell proliferation, with a view to understanding their roles in mediating any effects.

Materials and methods

Chemicals and reagents

AOM and GTE were purchased from Sigma-Aldrich Pty. Ltd. GTE (P1204) contains 65% catechin including 34.5% ECGG. High amylose maize starch (Bylon VII) was supplied by The Ingredion, Bridgewater, NJ, USA. DSS (molecular weight 36–50 kDa) was purchased from MP Biomedicals. Primary antibodies (monoclonal/polyclonal) to p-catenin (E5, #SC-7963), Ki-67 (MIB-5, #M7248) and Apc-H3 (lys9/lys14, #9677) were from Santa Cruz Biotechnology, Abcam and Cell Signalling.

Animals

A total of 100 male Sprague-Dawley (SD) rats, age (4-week-old) and weight (~100g ± 10g) matched were obtained from the Animal facility, Adelaide University, Australia. The animal study closely followed the Guide for the Care of Laboratory Animals, and was approved by the Animal Welfare Committee at Flinders University (# 799/11), Australia. Rats were acclimatized for 2 weeks before the four experimental diets were started. Rats were divided randomly into four equal experimental groups of n = 25 (with comparable initial body weights), housed in plastic cages (two rats per cage) and maintained in a temperature and humidity-controlled animal facility with a 12-h light/dark cycle at 22 ± 2°C temperature and 40 ± 10% humidity. Rats were given free access to water and food at all times, weighed weekly and were monitored closely for clinical signs of ill health throughout the study (during the period of DSS treatment, rats were given free access to DSS in drinking water).

Diets

The experimental diets were based on the modified American Institute of Nutrition (AIN)-76A diet and contained 20% sunflower oil by weight to ‘humanize’ the fat contribution to energy intake to ~35 kcal (19). The first group ‘Control diet’ consumed the modified AIN-76A diet; the second group ‘RS diet’ consumed high amylose maize starch at a level of 20g/100g diet; the third group ‘GTE diet’ consumed GTE at a level of 0.5g/100g diet. The fourth group ‘RS+GTE diet’ consumed high amylose maize starch at

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a level of 20 g/100 g diet and GTE at a level of 0.5 g/100 g diet. All diets were prepared fresh, pelleted and stored at −20°C until used. High amylose maize starch (a type RS2) was used as the source of RS, it is a natural and unmodified, food-grade ingredient manufactured and supplied by Ingredion TM. This high amylose maize starch contains approximately 50% RS (20). Details of the experimental diets are provided in Supplementary Table 1, available at Carcinogenesis Online. Doses of 0.5% GTE contain 172.5 mg EGCG/100 g diet and provide 25.9 mg EGCG/rat/day, which when calculated on a per body weight basis would provide the equivalent intake in an adult human of 3–4 cups of green tea per day.

Induction of colitis-associated colon cancer in rats

After 2 weeks on experimental diets, all rats received two subcutaneous injections of AOM (10 mg/kg body weight) 1 week apart, followed by 2% (W/V) DSS in drinking water for 7 days. Rats remained on their experimental diets throughout the study until euthanized by CO2 asphyxiation 20 weeks after the last AOM injection (Figure 1A). At termination, the colon was removed, rinsed in saline and collected for tumour analysis. Colon length was measured in centimetres and colon tumours (>0.5 cm) were cut into two parts, half of each tumour was placed in RNAlater (Ambion) for RNA isolation and the other half was fixed with neutralized 10% buffered formalin for histopathological evaluation and immunostaining. Caecal and faecal samples were collected for measuring pH and SCFA; faecal samples were also stored at −80°C before subsequent gut microbiota analysis. A distal segment of normal-appearing colon (2 cm) was dissected for histological evaluation of inflammation by haematoxylin and eosin (H&E) staining, goblet cells by periodic acid-Schiff stain and for immunohistochemistry of β-catenin, acetylated histone H3 (Ac-H3) and cell proliferation (Ki-67).

Disease activity index assessment and inflammation

Rats were scored daily using a Disease Activity Index (DAI) based on weight loss, stool consistency, rectal bleeding and overall condition of the animal during the week of DSS treatment and 1 week after the treatment, or weekly over the course of the experiment. Normal-appearing colons were stained with H&E and analysed for the degree of inflammation in a blinded fashion for all colons as done previously (11) (for details see Supplementary Material and Methods section, available at Carcinogenesis Online).

Histopathology of tumours

The colons were stained with methylene blue and scored for the number of tumours to determine the tumour incidence and tumour multiplicity per animal. The size of identified tumours was determined by measuring the largest diameter of the lesion using a Caliper. The size of tumours was further grouped into small (1–3 mm), medium (4–6 mm) and large (>7 mm) tumours. Due to the presence of large numbers of tumours in the colon of most animals, three tumours within a 5-cm section of the distal end of each colon were embedded in paraffin blocks for histopathological examination after staining with H&E. Some rats from the control diet either had no tumours or very few tumours, and in these cases none or only one or two tumours from each rat were assessed by H&E. Tumours were categorized as adenoma (AD) or adenocarcinoma (ADC) based on their histopathological features as described previously (12).

Measurement of pH and SCFA

Faecal and caecal samples were measured by gas chromatography as previously reported (15) (for details see Supplementary Material and Methods section, available at Carcinogenesis Online).
Measurement of the gut microbiota
DNA was extracted from rat faeces from control and RS groups using bead beating followed by the PowerMag™ Microbiome RNA/DNA Isolation Kit (MO BIO Laboratories, Inc) optimized for epMotion™ platforms (for details see Supplementary Material and Methods section, available at Carcinogenesis Online) (21).

MiSeq Illumina sequencing was used to determine bacterial community composition. To relate the changes in the microbiota to other endpoints, distance-based linear models (DISTLM) and distance-based redundancy analysis (dbRDA) were used. To further explore their relationship with changes in the microbiota, extreme groups were identified and the differences in the microbiota between these extreme groups were analysed. These analyses were performed at MR DNA (www.mrdnalab.com, Shallowater, TX) following their guidelines on library preparation and sequence classification. Primers amplifying the V4-V5 region of the 16S rRNA gene (530F and 926R) were used (for details see Supplementary Material and Methods section, available at Carcinogenesis Online) (22).

Immunohistochemical analysis
The detailed procedures for immunohistochemical analysis of Ki-67, β-catenin and acetylated histone H3 (Ac-H3) are reported previously (23) and are shown in more details in the Supplementary Material and Methods section.

Quantitative real-time PCR (RT-PCR)
Total RNA was extracted from normal-appearing colons using a QIAGEN RNaseasy Mini Kit (Qiagen, Germany) and cDNA was synthesised from 3 μg total RNA for each sample using a QIAGEN Quantitect Reverse Transcription Kit. Primer details are shown in Supplementary Table 2, available at Carcinogenesis Online and PCR were conducted as described previously (23) (for details see Supplementary Material and Methods section, available at Carcinogenesis Online).

Statistical analyses
All statistical analyses were performed using IBM SPSS version 22.0 (Chicago, IL), Stata MP13.1 (TX, USA) and Primer 6 (Plymouth, UK). The percentages of AD and ADC among selected tumours were reported and the differences of proportion were tested using two-sample test of proportions. Differences between groups in the number of AD and ADC were assessed using simple differences between treatments within a phylogenetic level and distance based redundancy analysis (dbRDA) was used to determine changes in the microbial structure between dietary groups. Negative binomial regression was used to compare tumour incidence across dietary groups. Poisson regression was used to compare tumour incidence across dietary groups. Negative binomial regression was used to compare tumour incidence across dietary groups.

Results

Body weight
There was no significant difference in food consumption between the groups (data not shown). Body weights of rats did not differ between dietary groups until week 20 of the study, after which rats fed the control diet weighed significantly less than rats fed the RS, GTE and RS+GTE diets (Figure 1B).

Disease activity and inflammation
In the week following DSS treatment, typical symptoms and signs of acute inflammation were observed in rats fed the control diet as indicated by the DAI, but were less evident in rats fed the RS and RS + GTE diets, as shown by the significantly lower DAI, P < 0.05 (Supplementary Figure 1A, available at Carcinogenesis Online). The RS-containing diets also significantly increased colon length (Supplementary Figure 1B, available at Carcinogenesis Online) and lowered the inflammation score (Figure 1D), P < 0.05, and restored the architecture of the colonic lamina propria (Figure 1E). The GTE diet had no significant effect on DAI, inflammation score and colon length compared to rats fed the control diet. Although DAI from all rats returned to baseline level 2 weeks after DSS, an increased DAI was observed in some rats towards the end of the study which was mostly likely associated with the development of colon tumours due to weight loss and fecal bleeding (Figure 1C). Colonic goblet cell number and size, indicators of mucus secretion and the health of the mucus barrier, were unaffected by dietary intervention (Supplementary Figure 2, available at Carcinogenesis Online).

Neoplastic lesions
Neoplastic lesions, ranging from small (<3 mm) to large (>7 mm) and from dysplasia, AD to ADC, were observed (Figure 2A). Histological examination revealed thickened intestinal walls, crypt distortion and inflammation within the mucosa that occasionally extended to the muscularis mucosa. Most of the tumours were located in the mid to distal region of the colon, with only occasional tumours observed in the proximal colon. A total of 74, 61, 71 and 58 tumours (within 5 cm of section of the distal end of each colon) were randomly selected from the control, RS, GTE and RS + GTE diets, respectively and examined for the occurrence of AD or ADC. A significantly reduced proportion and number of ADC tumours were present in rats fed RS or RS + GTE diets relative to rats fed the control diet (Table 1). Tumour histology is shown in Figure 2C. RS diets also had a significant inhibitory effect on tumour multiplicity compared with the control diet P < 0.001 (Table 1). Effects of diets on small (<3 mm), medium (4-6 mm) and large (>7 mm) tumours were also analysed separately (Figure 2B). The RS-containing diets resulted in significantly lower numbers of medium and large tumours relative to the control diet, P < 0.01. The RS+GTE diet did not differ from RS alone in effect on tumour size.

SCFA
In rats fed the RS-containing diets, but not the GTE diet, concentrations of acetate, butyrate and propionate were significantly increased, and pH significantly lowered in the cecal and distal colon digesta when compared with rats fed the control diet (Table 2). A significant interaction was observed between RS and GTE for fecal pH (P = 0.04), but no interactions were observed for cecal/faecal SCFA levels and caecal pH.

Gut microbiota
A phylogenetic approach was taken to analyse differences in digesta microbial structure from the rats fed the control and
RS diets only (Supplementary Table 3, available at Carcinogenesis Online). Sequencing of the V4-5 region of the 16S rRNA gene of the digesta bacterial community yielded a mean of 67234 [28252–113688] reads across the two diet groups. As a proportion of reads the majority belonged to the Firmicutes and Bacteroidetes phyla but the ratio of Firmicutes to Bacteroidetes changed significantly with the RS diet (control diet 7.5 [1.4–38] and RS diet 2.0 [0.97–5.2], P = 0.009). Significant changes in the microbial composition occurred from phylum through to species level (P < 0.0001). Feeding the RS diet resulted in a decrease in bacterial diversity at the phylum level to the species level (P < 0.05) using the Shannon index. However, the inverse Simpson index only showed a lowering of diversity to family level (P < 0.006).

An in-depth analysis performed at the genus level found that the RS diet significantly altered the populations of 21 genera when compared with rats fed the control diet (Figure 3A), seven of these increased by at least 3-fold. SIMPER analysis identified Parabacteroides, Barnesiella, Allobaculum, Ruminococcus, Marvinbryantia and Bifidobacterium as being the primary contributors to the dissimilarity in bacterial populations between the RS and control diets, contributing to 16 % of the difference, and all being increased in response to RS.

DISTLM and dBRDA analysis showed that acetate, propionate, DAI, inflammation score, colon length, pH, ADC and total number of tumours (TNT) were significantly related to the microbiota changes while butyrate was not (Figure 3B).

Furthermore, extreme groups were identified which were outside (below or above) the 95% confidence intervals for both parameters tested. The size of these extreme groups varied between 9 and 13 observations. Correlations between fermentation parameters and TNT are shown in Supplementary Table 4, available at Carcinogenesis Online. The following correlations between TNT and acetate (r = −0.52; P < 0.001), propionate (r = −0.48; P = 0.001) and pH (r = 0.58; P < 0.001) were further explored. Bilophila and Anaerotruncus were associated with all the extreme groups whereas Turicibacter and Aerococcus were linked to the extreme groups for propionate and acetate/pH, respectively.

Gene expression
Expression of genes related to inflammation (COX-2, NF-KB, IL-1β and TNF-α) were significantly reduced by the RS-containing diets relative to the control diet, P < 0.05 (Figure 4). The GTE diet significantly reduced the expression of COX-2, but did not affect TNF-α, IL-1β or NF-KB. The expression of GPR43, a receptor for SCFA, was significantly increased by the RS diet relative to the control diet, P < 0.05.

Genetic and epigenetic biomarkers
Cell proliferation was significantly inhibited by the RS-containing diets when compared with the control diet P < 0.05, but not by the GTE diet (Supplementary Figure 3, available at Carcinogenesis Online). Tumours showed enhanced nuclear translocation of β-catenin whereas β-catenin was mainly present in cell membranes of normal-appearing colonic epithelium. With regard to Ac-H3 staining, tumours showed reduced expression of Ac-H3, with enhanced expression of Ac-H3 in normal-appearing colonic epithelium. However, there was no significant difference between the dietary intervention and the control diet in regard to β-catenin membrane and/or nuclear staining. Similarly, there were no effects of diets on Ac-H3 within colonic crypts.
Table 1. Effects of RS, GTE and their combination on adenoma (AD), adenocarcinoma (ADC) and tumour multiplicity in rats

<table>
<thead>
<tr>
<th>Diet</th>
<th>No of rats</th>
<th>Tumor multiplicity</th>
<th>No (%) of analysed tumours classified as AD</th>
<th>No (%) of analysed tumours classified as ADC</th>
<th>IRR [95%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>0.87 [0.58, 1.29]*</td>
<td>1.6</td>
<td>1.7</td>
<td>1.72 ± 2.2</td>
</tr>
<tr>
<td>RS</td>
<td>25</td>
<td>0.9 [0.58, 1.29]</td>
<td>1.2</td>
<td>1.2</td>
<td>1.72 ± 2.2</td>
</tr>
<tr>
<td>GTE</td>
<td>25</td>
<td>0.87 [0.58, 1.29]</td>
<td>0.8</td>
<td>0.8</td>
<td>1.72 ± 2.2</td>
</tr>
<tr>
<td>RS + GTE</td>
<td>25</td>
<td>0.87 [0.58, 1.29]</td>
<td>0.8</td>
<td>0.8</td>
<td>1.72 ± 2.2</td>
</tr>
</tbody>
</table>

Due to the presence of large numbers of tumours in the colon of most animals, three tumours within a 5-cm section of the distal end of each colon were stained with H&E for histopathological examination. IRR, incidence rate ratio, derived from a negative binomial regression. Groups with different lowercase letters are significantly different at *P* < 0.05 level (two-sample test of proportions). Groups with different uppercase letters are significantly different at *P* < 0.05 level (pairwise comparisons after Bonferroni adjustment).
Table 2. Effect of RS, GTE and their combination on faecal and caecal pH and SCFA (µmol/g) concentrations in rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RS</th>
<th>GTE</th>
<th>RS + GTE</th>
<th>Interaction of RS and GTE (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caecal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate (µmol/g)</td>
<td>36.8 ± 2.0</td>
<td>46.7 ± 1.8*</td>
<td>34.7 ± 1.8</td>
<td>41.2 ± 1.8*</td>
<td>0.41</td>
</tr>
<tr>
<td>Propionate (µmol/g)</td>
<td>11.2 ± 1.0</td>
<td>22.8 ± 1.1*</td>
<td>10.6 ± 0.6</td>
<td>19.3 ± 1.1*</td>
<td>0.13</td>
</tr>
<tr>
<td>Butyrate (µmol/g)</td>
<td>11.2 ± 1.0</td>
<td>16.3 ± 0.6*</td>
<td>11.2 ± 1.0</td>
<td>16.1 ± 0.9*</td>
<td>0.94</td>
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<tr>
<td>pH</td>
<td>6.7 ± 0.1</td>
<td>6.0 ± 0.1*</td>
<td>6.7 ± 0.1</td>
<td>6.0 ± 0.1*</td>
<td>0.61</td>
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<tr>
<td>Faecal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate (µmol/g)</td>
<td>22.7 ± 2.1</td>
<td>35.4 ± 2.5*</td>
<td>22.6 ± 3.1</td>
<td>36.9 ± 1.6*</td>
<td>0.74</td>
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<tr>
<td>Propionate (µmol/g)</td>
<td>7.8 ± 0.6</td>
<td>17.8 ± 1.6*</td>
<td>8.0 ± 1.4</td>
<td>17.5 ± 1.1*</td>
<td>0.82</td>
</tr>
<tr>
<td>Butyrate (µmol/g)</td>
<td>8.0 ± 1.1</td>
<td>14.1 ± 1.5*</td>
<td>7.3 ± 1.2</td>
<td>17.7 ± 1.2*</td>
<td>0.09</td>
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<tr>
<td>pH</td>
<td>6.6 ± 0.1</td>
<td>6.0 ± 0.1*</td>
<td>6.8 ± 0.1</td>
<td>5.5 ± 0.1*</td>
<td>0.04</td>
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</tbody>
</table>

Values are mean ± SEM, n = 25. Interaction between RS and GT was tested in ANOVA by including the interaction term of RS and GT in the model.
*Significant effect of experimental diets, compared with the control diet, P < 0.001 (by one way ANOVA).

Figure 3. Effect of RS on colonic microbiota. Genera with significant different percentage presence in faecal samples. Fold change between control and RS diets is presented in parentheses () with a minus sign indicating a reduction (A). Microbial structure at Genus level using distance based redundancy analysis (dbRDA) plot and Distance-based linear models (DistLM) using the Best selection with R² as the selection criteria (B). Vectors showing the relation (direction) and strength of selected variables. Marginal tests table show the significance of the relationship between the variable and the microbiota and the % of variation in the microbiota explained by each variable () . RS, resistant starch.

development of CRC (3). This is to some extent also observed in the present study, with many genera previously been linked to CRC, including Allobaculum, Blautia, Clostridium, Christensenella, Olsenella, Escherichia and Fusobacterium (30,31), having a lower prevalence on the RS diet compared to the control diet.

RS feeding increased the abundance of bacteria associated with RS fermentation, including Parabacteroides, Ruminococcus and Bifidobacterium. However, we also observed large increases in bacteria not directly involved in RS fermentation [Anaeroprobacter (>13-fold increase)] or associated with CRC [Marvinbryantia (>10-fold)]. These bacteria may help in preventing inflammation (32) or in mucosa regeneration (33). The current study found that the expression of some genes related to inflammation (COX-2, NF-KB, IL-1β and TNF-α) were significantly reduced by feeding the RS diet. Such findings are supported by microarray data from pigs in which RS provoked major changes in colonic gene expression that are involved in inhibition of inflammatory pathways and suppression of immune response (25). In a previous study, Barnesiella was reported to cure vancomycin-resistant Enterococcus in mice (34). The same competition between these genera was observed in this study with an increase of Barnesiella (>7-fold) and a decrease in Enterococcus (>7-fold) in response to RS. This is noteworthy, as species of Enterococcus have been implicated in CRC. These results suggest RS may drive gut microbiota populations away from dysbiosis and potentially fuelling inflammation and/or carcinogenesis, toward a more beneficial profile.

Many of the benefits of RS have been linked to the SCFA derived from its large bowel fermentation. A recent study suggested that dietary fibre protected against CRC in a microbiota and butyrate-dependent manner (29). Others demonstrated lower levels of butyrate-producing bacteria from CRC patients compared to healthy participants (35), and an inverse correlation between faecal butyrate levels and tumour size in CRC (36). In this study we demonstrated that RS feeding significantly increased genera involved in butyrogenesis and increased the production of acetate, butyrate and propionate. But, we did not find a strong inverse relationship between faecal butyrate...
and tumours, indicating other fermentation metabolites of RS may also play protective roles in this model. Instead, we found strong inverse correlations between tumour (ADC and TNT) and acetate and/or propionate as well as pH. Based on those correlations we identified specific and common bacterial genera across the extreme groups for acetate, propionate and pH, and specifically, we found *Bilophila* and *Anaerotruncus* were reduced on the RS diet. *Bilophila* can utilise taurine (a bile acid), leading to hydrogen sulphide production which promotes colitis in mice (37). *Anaerotruncus*, recently isolated from human stool, is of clinical significance after it was found to cause bacteraemia (38). Further studies are required to investigate the roles of these genera in CRC development.

SCFA can modulate cell functions either through the activation of G-protein coupled receptors (GPRs), or by inhibiting histone deacetylase (39). GPR43 is expressed in intestinal epithelial cells and certain innate immune cells, but is markedly reduced in human CRC (40) and in a colitis model (41). A recent study shows the beneficial effects of a high-fibre diet (yielding high levels of acetate) involves the activation of GPR43 (42). We found that RS significantly increased colonic GPR43 expression, indicating activation of GPR43 may play a role in maintaining intestinal homeostasis. Given that acetate and propionate had strong inverse correlations with tumour outcomes, it suggests that their protective effects could be through modulation of Th cells and immune function, as interactions between acetate-propionates and GPR43 have been shown to induce anti-inflammatory effects (43).

We found that acetylation of histone H3 was reduced in AOM/DSS-induced tumours, but feeding RS did not increase H3 histone acetylation levels. Although our results were not in agreement with the others studies, in which naturally occurring HDAC inhibitors such as butyrate have been shown to reverse the hypoacetylation of histone 3 (44), our data suggests that the protective effect of RS from this study may, in part, be associated with acetate and propionate, as acetate in particular has been shown to have anti-inflammatory effects (45). In addition, we showed that AOM/DSS-induced tumours had increased β-catenin nuclear translocation. However, RS feeding affected neither β-catenin membrane nor nuclear staining, suggesting the effects of RS against CAC from this study do not involve WNT inhibition.

Green tea might protect against CRC and CAC but experimental animal studies and human trials have yielded conflicting results, some reported protection of GTE against CRC or CAC (44), while others did not (46). We sought to determine if consumption of GTE could provide independent or additional benefits to those that might be achieved with dietary RS but we did not see this (47). Some studies showed feeding high level of GTE to animals can increase the multiplicity of tumours (48). The dose of GTE (0.5%) used in the present study was within the range that has been shown to be protective in a rodent cancer model (49) and which had a modest effect in our previous rat study using the AOM model (23). The discrepancy with respect to green tea’s anticancer efficacy may depend on the different type, effective dose and treatment length of green tea and the models.
used for the studies. More studies involving animal models and human trials are needed if we are to explore the chemopreventive potential of green tea. Of note, combining RS with GTE did not show any further benefit in preventing CAC than RS alone, which was in contrast to a in vitro study, where a combination of ECCG and sodium butyrate was found to be more effective in inhibiting colony formation in RKO CRC cells than either ECCG or butyrate alone (44). There is interest in the combination of several chemopreventive or dietary agents (50), but the results from our study suggest that this combination is not as promising as might be predicted from prior studies.

In conclusion, we report a protective role of RS in CAC which is related to significant shifts in the gut microbiota, production of SCFA and increased expression of GPR43 as well as decreased expression of inflammatory cytokines and cell proliferation. GTE was not effective in either preventing or limiting the development of CAC, and the combination of RS with GTE did not offer any improved efficacy against CAC relative to RS alone. Thus, proceeding to test dietary RS for CRC prevention in subjects affected by IBD should be considered.

**Supplementary material**

Supplementary Materials and Methods, Tables 1–4 and Figures 1–3 can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)

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