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Dietary resistant and butyrylated starches have different effects on the faecal bacterial flora of azoxymethane-treated rats

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Abstract

Epidemiological studies have suggested that dietary fibre lowers the risk of colorectal cancer, which may be due to increased butyrate production from colonic fermentation of a type of fibre, resistant starch (RS). The present study investigated the effects of dietary RS and butyrylated RS on the faecal microbiota of rats treated with azoxymethane. A total of four groups of nine rats were fed diets containing either standard maize starch (low-amylose maize starch (LAMS), low RS), LAMS with 3% tributyrin (LAMST), cooked 10% high-amylose maize starch (HAMS, high RS) or cooked 10% butyrylated HAMS (HAMSB). Faecal samples were examined by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments. Multivariate analysis demonstrated no differences between faecal microbiota before treatment but revealed differences in DGGE patterns between diet groups, with the exception of the two low-RS groups (LAMS and LAMST). Subsequent analysis identified eleven DGGE bands contributing significantly to the differentiation between diets. These phylotypes belonged to Clostridiales (five), *Lactobacillus* (one) and Bacteroidetes (five) lineages. Rats fed HAMS had increased concentration of propionate in their distal colonic digesta and developed faecal populations containing *Ruminococcus bromii*-like bacteria. HAMSB increased propionate and butyrate concentrations in distal colonic digesta and was associated with the appearance of two non-butyrate-producing bacteria, *Lactobacillus gasseri* and *Parabacteroides distasonis*. In conclusion, supplementation with specific dietary RS leads to changes in faecal microbiota profiles that may be associated with improved bowel health.

Key words: Bacteria: Colorectal cancer: Rats: Resistant starch: Butyrate

Dietary and lifestyle factors account for approximately 70% of colorectal cancer, and an optimal dietary approach may reduce the incidence of this disease[1]. Consumption of resistant starch (RS) improves the colonic environment by softening stools, increasing faecal bulk, decreasing faecal pH and increasing luminal SCFA concentrations[2,3]. The main SCFA produced by colonic fermentation of RS are acetate, propionate and butyrate, and these SCFA have important roles in the maintenance of bowel health. Butyrate, which is the preferred energy source for colonocytes[4], may be protective against large-bowel cancer[5] by enhancing apoptosis, inhibiting proliferation and inducing differentiation of cancer cells *in vitro*[6,7]. Acylated starches have been shown to deliver butyrate to the large bowel of rats[8,9] and humans[10]. The esterified butyrate is released by colonic bacteria and is absorbed or utilised by colonocytes or gut microbes.

The aim of the present study was to investigate the effects of dietary RS and butyrylated RS on the faecal microbiota of rats treated with the carcinogen azoxymethane (AOM). Rats were also fed diets containing low RS and low RS with added tributyrin to supply butyrate systemically from small-intestinal absorption. Faecal samples were collected from the rats throughout the study to explore the effects of specific diet components on measures of bowel health. Gaining insight into the behaviour and metabolic activity of these microbial populations, the influence of diet and the development of cancer may provide opportunities to modulate colorectal cancer risk.

Experimental methods

The present study used faecal samples collected from a subset of animals from a larger trial, which is described by Clarke *et al.*[11]. The study involved male Sprague–Dawley rats (198 (SEM 2) g), which were randomly allocated to four groups of nine rats and fed experimental diets. These rats were treated identically to the rats in the larger study, and were fed experimental diets based

Abbreviations: AOM, azoxymethane; DGGE, denaturing gradient gel electrophoresis; HAMS, high-amylose maize starch; HAMSB, butyrylated high-amylose maize starch; LAMS, low-amylose maize starch; LAMST, low-amylose maize starch with 3% tributyrin; RS, resistant starch.

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on a control AIN-93G[12] diet which contained 530 g standard low-amylose maize starch, 200 g casein, 70 g maize oil and 50 g α-cellulose/kg of diet. Choline, methionine, minerals, vitamins and antioxidant were added as described previously[10]. The amount of standard maize starch in the diet was reduced to allow for the addition of experimental starches and tributyrin. The experimental diets were control (low-amylose maize starch (LAMS), low RS), LAMS with 3 % tributyrin (LAMST, low RS), cooked 10 % high-amylose maize starch (HAMS, high RS) or cooked 10 % butyrylated HAMS (HAMSB, high RS). The latter was manufactured by National Starch Food Innovation (Bridgewater, NJ, USA) and had a degree of substitution of 0·25 (i.e. 0·25 of the hydroxyl groups on each starch D-glucopyranosyl unit were derivatised or replaced by substituent acids). HAMS and HAMSB were cooked with water and spray-dried (to minimise crystallite formation), as described previously[13], before addition to the diets. This process was used to mimic the condition of starches consumed in foods by humans, as cooking changes the production and delivery of butyrate to the colon by starches[10]. All diets were prepared regularly at the Commonwealth Scientific and Industrial Research Organisation and stored at 4°C. The rats were fed a fresh diet daily. After weeks 4 and 5 of feeding the experimental diets, rats were injected with 15 mg/kg of AOM subcutaneously (Sigma Chemical Company, St Louis, MO, USA). One rat was euthanased early due to events unrelated to the experimental procedures (from the LAMS group in week 9). All procedures involving animals were approved by the Commonwealth Scientific and Industrial Research Organisation Human Nutrition Animal Ethics Committee and complied with the Australian code of practice (NHMRC, 2004).

Faecal specimens were collected at week 0 (AIN-93G base diet), week 4 (after 4 weeks of the experimental diets, before AOM injections) and at weeks 7, 11, 15, 19, 21, 27, 29 and 31 of the experiment. Each specimen was collected from the anus of the rat and consisted of a faecal pellet of approximately 0·1 g. The samples were immediately placed in NaCl–Tris–EDTA buffer (10 mM-Tris–HCl (pH 8·0); 1 mM-EDTA; 100 mM-NaCl) and stored at −20°C. A total of 336 rat faecal specimens were collected. Analysis of pH and liquid phase-free SCFA concentrations were undertaken in duplicate as described previously[9]. At the end of the study, the rats were anaesthetised, and tissue and digesta samples were collected for analysis[11]. Inadequate digesta was available for distal colonic pH and SCFA concentration determinations in some rats (n 6 for the LAMS and LAMST groups; n 7 for the HAMS and HAMSB groups).

DNA extractions from rat faecal specimens, denaturing gradient gel electrophoresis (DGGE) and sequencing of DGGE bands were performed as described previously[14]. Briefly, DNA was extracted using a matrix-based method, and universal bacterial 16S rRNA gene primers were used to amplify approximately 500 bp of the 16S rRNA gene using the forward primer 907f (5′-AAACTCAAAGGAATTGACGG-3′[15]) and the GC-clamped reverse primer 1392rc (5′-CGCCCGCCGCGCCCCGCCCGGCCCGCCGCCCCCGCCCCACGGGCGGTGTGTRC-3′[16]). The resulting PCR amplicons were subjected to DGGE using a 30–75 % denaturing gradient and 6 % acrylamide gel. For each sample, 15 μl of the PCR product was loaded per track and electrophoresed at 110 V for 16 h at 60°C. Gels were stained for 30 min in 1 × SYBR Gold nucleic acid stain (Molecular Probes, Eugene, OR, USA). The gels were then destained in 100 ml of MilliQ water and photographed under UV light (DigiDoc System; Bio-Rad Laboratories, Hercules, CA, USA).

GraphPad Prism version 4.0 (www.graphpad.com; GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analyses on final day measures and tumour indices[11]. Multivariate statistical analysis of DGGE-banding patterns was performed using the Primer 6 package (PRIMER-E Limited, Plymouth, UK), with the assumption that each DGGE band represents one phylotype. Multidimensional scaling plots demonstrating the relatedness of individual samples from specific time points were calculated using the Bray–Curtis similarity calculation on square root-transformed data[17,18]. Differences between diet groups on the basis of DGGE-banding patterns were calculated with a one-way analysis of similarity routine[19] and tested at $\alpha = 0{\cdot}01$; this gives a value for the similarity of two groups, where a value of 0 indicates no difference between groups and a value of 1 indicates no similarity. Similarity percentage analysis was used to identify the DGGE bands that primarily accounted for the dissimilarity between different diet groups, identified using one-way analysis of similarity, at week 27[19]. Analysis of the variation between rats within a diet group, for each week, was calculated using the dispersion sequence with the MVDISP routine. This calculated the average rank dissimilarity within each of the groups, whereby the higher the value, the more dispersed the samples are within a group.

The Shannon–Wiener index (H')[20] was calculated for each DGGE profile, also taking into account relative band intensities. The index describes how the intensity of bands is distributed across each profile. Analysis of the variation in diversity over the course of the trial was performed using a linear mixed model allowing for random variation between rats and also between weeks within rats. Variability was normally distributed, so transformation was not performed.

Following similarity percentage analysis, DGGE bands of interest were extracted and re-amplified as described previously[21]. Sequencing and phylogenetic analysis of DGGE bands were performed using the ARB software package[22], as described previously[23]. Sequences from the present study were deposited within the Genbank database under accession numbers DQ839222–DQ839231 and EU703763.

Results

Growth rates were similar in all of the experimental groups, and there were no significant differences in final rat body weights between the dietary treatments (Table 1). Large-bowel tumour incidence (i.e. the proportion of rats/group with tumours), tumour number and surface area did not differ between the subgroups of animals used in this trial, although there were differences between dietary groups in these indices when data from the main study were

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Table 1. Final measures of rats fed diets containing low-amylose maize starch (LAMS), LAMS with 3 % tributyrin (LAMST), high-amylose maize starch (HAMS) and butyrylated HAMS (HAMSB)

(Mean values with their standard errors, *n* 6–9)

	LAMS		LAMST		HAMS		HAMSB	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Final body weight (g)	648	38	665	39	666	30	620	15
Distal colonic digesta pH	6·97	0·05	6·85	0·07	6·66	0·05	6·77	0·06
Distal colon SCFA concentration (mM)								
Acetate	60·5	5·1	47·9	5·0	57·8	5·1	56·4	3·8
Propionate	14·5*†	1·5	12·2‡§	1·7	26·2	3·5	31·6	3·2
Butyrate	12·2*	2·5	8·1‡	1·1	12·6*	1·7	22·3	2·1
Large-bowel tumour indices								
Average tumour area‖	11·5	6·8	18·0	7·9	26·8	17·0	8·8	6·9
Number of tumours/rat‖	1·0	0·5	0·78	0·3	1·0	0·4	0·44	0·2
Incidence of tumours (%)¶	62·5		55·6		55·6		44·4	
Tumour type								
Adenoma**	6		7		7		4	
Adenocarcinoma**	2		0		2		0	

* Mean values were significantly different from HAMSB ($P<0·01$).
† Mean values were significantly different from HAMS ($P<0·05$).
‡ Mean values were significantly different from HAMSB ($P<0·001$).
§ Mean values were significantly different from HAMS ($P<0·01$).
‖ Analysed using non-parametric Kruskal–Wallis ANOVA; *n* 8 (LAMS) or *n* 9.
¶ Analysed using Pearson's χ^2 test.
** Analysed using contingency comparison between groups.

analysed[11]. There was no effect of diet on the proportion of adenomas that progressed to adenocarcinomas. Acetate concentrations and pH were not affected by diet in the distal colon; however, propionate concentrations were higher in rats fed the high-RS diets compared with the low-RS diets ($P<0·05–0·001$). Distal butyrate concentrations were higher in the rats fed HAMSB ($P<0·01$) than for the other groups, and were similar in the HAMS, LAMS and LAMST groups (Table 1).

DGGE analysis was performed on samples collected from six to nine individual rats on each diet at the ten sampling points. Between weeks 5 and 33, DGGE bands were detected in individual samples, and between weeks 4 and 7, the faecal DGGE profiles diverged rapidly according to the diet consumed (see Table S1 of the supplementary material available at http://www.journals.cambridge.org/bjn), with the exception of the two low-RS diets (LAMS and LAMST), which were significantly different only in the week 7 sample (see Table S1 of the supplementary material available at http://www.journals.cambridge.org/bjn). For the remainder of the trial, three distinct profiles were observed: the LAMS and LAMST samples grouped together, and the HAMS and HAMSB groups formed divergent populations that were distinctly different from each other (Fig. 1). Analysis of the inter-diet variability using dispersion sequence analysis revealed a greater degree of variability in the low-RS diets (LAMST: 1·19 (SEM 0·245)) and LAMS: 1·16 (SEM 0·207)) than in the high-RS diets (HAMSB: 0·73 (SEM 0·312) and HAMS: 0·97 (SEM 0·19)) over the course of the dietary intervention.

At week 0, there was no significant difference in microbial diversity between the diet groups as measured by the Shannon index (H' 2·47–2·64). After 4 weeks of dietary treatment, the microbial populations showed a reduction in diversity, and the mean H' values for all diet groups decreased significantly compared with week 0 samples (H' 1·46–2·00, $P<0·001$). By week 7, the microbial diversity increased (H' 1·90–2·65, $P<0·0001$), and was no longer significantly different from week 0 samples for any of the diets; after this time point, diversity did not change significantly.

The phylogenetic affiliation of characteristic bands revealed significant differences between the composition of the predominant phylotypes in each of the three diets, with the low-RS diets characterised by bands 58, 82, 36 and 13, comprising two members of Clostridiaceae and two members of Bacteroidetes (see Fig. S1 of the supplementary material available at http://www.journals.cambridge.org/bjn). Rats consuming HAMS were characterised by bands 13, 19 and 27 which grouped with *Ruminococcus bromii* (96, 95 and 96 % similarity, respectively), and band 58 which grouped within a clade of uncultivated members of Bacteroidetes (see Fig. S1 of the supplementary material available at http://www.journals.cambridge.org/bjn). Rats consuming HAMSB were characterised by six bands. One of these, band 47, contained a mixed sequence and was not included in the analysis. Bands 65, 71 and 75 were grouped within the Bacteroidetes, and were all related to *Parabacteroides distasonis* (98, 98 and 96 % similarity, respectively); band 54 was related to *Phascolarctobacterium* (98 % similarity); and band 60 was related to *Lactobacillus gasseri* (99 % similarity). Of these, bands 65, 71 and 75 related to *P. distasonis* were only detected in the HAMSB diet, whereas all the dominant bands found in the HAMSB diet, including 54 and 60, were also detected at low levels (weak bands; see Fig. S2 of the supplementary material available at http://www.journals.cambridge.org/bjn) in the HAMS diets.

Signature phylotypes for each diet group were identified using similarity percentage analysis, revealing eleven key DGGE bands that had >10 % contribution to the similarity of individual rats within different diet groups (see Fig. S1

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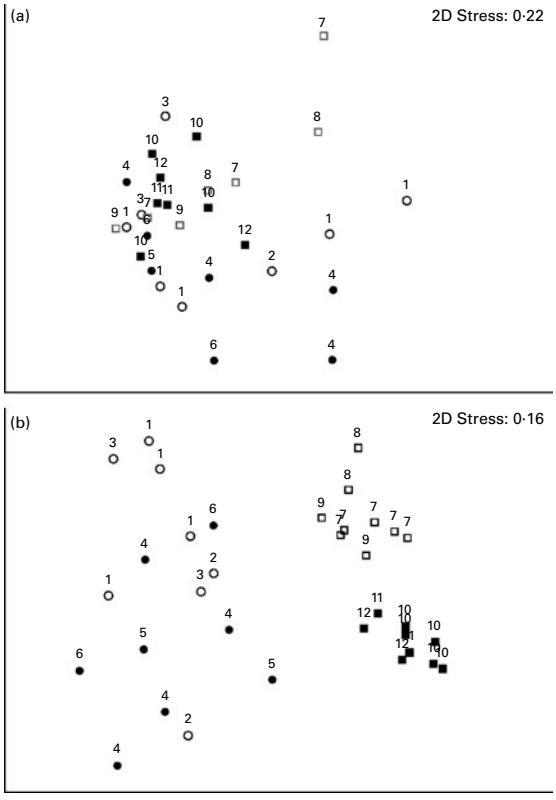

Fig. 1. Multidimensional scaling plots, based on the Bray–Curtis similarity calculation on square root-transformed data, demonstrating the similarity of faecal bacteria profiles of rats fed diets containing low-amylose maize starch (LAMS, ○), LAMS with 3% tributyrin (LAMST, ●), high-amylose maize starch (HAMS, □) and butyrylated HAMS (HAMSB, ■) at (a) weeks 0 and (b) week 27; numbers next to data points show the cages.

of the supplementary material available at http://www.journals.cambridge.org/bjn). One phylotype (band 36) significantly contributed to the dissimilarity between the low- and high-RS diets. The phylogenetic affiliation of characteristic bands revealed significant differences between the composition of the predominant phylotypes in each of the three diets, with the low-RS diets characterised by bands 58, 82, 36 and 13, HAMS associated with bands 13, 19, 27 and 58 and HAMSB were characterised by bands 54, 60, 65, 71 and 75; the latter three were only found on the HAMSB diet (see Fig. S2 of the supplementary material available at http://www.journals.cambridge.org/bjn).

Discussion

The present study examined the effects of HAMSB, a butyrylated starch designed to promote large-bowel health by rapidly delivering butyrate to the colon, on the faecal microbiota of rats. HAMSB was found to be particularly effective at raising butyrate concentrations in the distal colon, which is the site of most degenerative large-bowel disease in humans[24]. Although the effects on tumour indices or type were not significant in the subset of rats sampled for the present study, HAMSB reduced the incidence and number of large-bowel tumours/rat in the main study[11]. Based on these findings, it is unlikely that factors arising from differences in tumour burden (for instance, amount of luminal serosanguineous exudate produced by ulcerated colonic tumours) were responsible for the differences in faecal microbiota seen in rats in this present study.

Dietary HAMSB sponsored the dominance of a bacterial phylotype related to *L. gasseri*, an organism that may have potential as a probiotic[25]. The HAMSB microbiota was also characterised by a phylotype related to *Phascolarctobacterium faecium,* which is an anaerobe that produces propionic acid from succinate but is not known to ferment carbohydrates[26]. The prevalence of a phylotype related to *P. faecium* may be a result of cross-feeding of succinic acid produced by *P. distasonis*-related phylotypes, which also predominated in the faeces of HAMSB rats. *P. distasonis*-related phylotypes were only detected in faeces from rats fed the HAMSB diet, suggesting that these bacteria may be readily adaptable and capable of deacylating and utilising the starch backbone for growth. *P. distasonis* has been found to predominate in the faeces of volunteers consuming HAMSB in two independent clinical trials (JM Clarke, unpublished results). However, *in vitro* experiments need to be conducted to verify the specific role of *P. distasonis* in HAMSB hydrolysis and fermentation. The increase in distal colonic digesta butyrate concentration in the HAMSB group is likely to be the result of the release of esterified butyrate, rather than fermentative in origin.

The rats consuming HAMS differed from the other diets due to the predominance of bands grouping with *R. bromii,* and propionogenic bacteria including a clade of uncultivated members of the Bacteroidetes. The former is an organism known to be involved in amylolysis, requiring NH_4^+ as a nitrogen source and involved in assimilatory sulphate reduction[27]. Indeed, *R. bromii* and related phylotypes have been shown to be one of the major colonisers of starch particles during *in vitro* fermentations[28], while phylotypes closely related to this group have been shown to increase in dominance in response to high-RS diets in healthy humans[14].

The increased propionate concentration measured in samples from the HAMS diet was unexpected as dietary RS is usually reported to increase butyrate production, although propionate can be produced in preference to butyrate[2]. Digesta pH may be a determining factor, as increased large-bowel digesta propionate concentrations have been reported in rats fed a diet containing 10% HAMS. However, in rats fed 20% HAMS, the digesta pH fell to 6·5, and butyrate was the dominant SCFA produced[27]. The distal colonic digesta pH in rats in the present study were higher than 6·5, and the present results suggest that the microbiota of the HAMS group was dominated by propionogenic organisms, typically members of the *Bacteriodes/Prevotella* rather than butyrogenic bacteria.

At the commencement of the trial, there were no significant differences in rat faecal microbial populations between cages and future dietary groups. The greater degree of population variability in the low-RS diets suggests that these diets have a less stringent effect on the composition of the dominant colonic microbiota than the HAMS and HAMSB diets. The lack of significant difference in community composition between the two LAMS-based diets at most sampling points during the intervention was expected, as the tributyrin in the

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LAMST diet would be absorbed in the small intestine[29] and would not affect colonic flora. The difference at week 7 following AOM treatment was unexpected but may have been the result of the AOM, which could have affected the microbial population. Differences in diversity between the LAMS- and HAMS-based diets are assumed to be a result of the high levels of RS in the two HAMS-based diets. It is unclear whether the differences between HAMS and HAMSB reflect differing ability of bacteria to deacylate HAMSB and hence access the starch as a substrate, or are a direct result of the released butyrate. The diversity findings were supported by Shannon index analysis suggesting that faecal bacteria populations capable of quickly utilising the new substrates became predominant, decreasing bacterial diversity in response to a strong selection for bacteria capable of degrading the major nutrients in the new diet. However, the microbial populations were robust and diversity was restored, as complex relationships were established after 4–7 weeks on a constant diet.

In conclusion, supplementation with dietary RS with and without butyrylation leads to changes in faecal microbiota profiles that may be associated with health benefits. The potential health benefits associated with the increased butyrate level observed on the HAMSB diet are well known. However, the cholesterol-lowering benefit of increased propionate levels[30,31] as seen on both RS diets is still being substantiated. This study supports the suggestion that dietary intervention with specific RS may be used to modify the large-bowel microbiota with the potential to improve colonic health.

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