Butyrylated starch protects colonocyte DNA against dietary protein-induced damage in rats

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Dietary resistant starch (RS), as a high amylose maize starch (HAMS), prevents dietary protein-induced colonocyte genetic damage in rats, possibly through the short-chain fatty acid (SCFA) butyrate produced by large bowel bacterial RS fermentation. Increasing butyrate availability may improve colonic health and dietary high amylose maize butyrylated starch (HAMSB) is an effective method of achieving this goal. In this study, rats (n = 8 per group) were fed diets containing high levels (25%) of dietary protein as casein with 10 or 20% dietary HAMS and HAMS. Colonocyte genetic damage was measured by the comet assay and was 2-fold higher in rats fed 25% protein than those fed 15% protein (P < 0.001). Concurrent feeding of 25% protein and either HAMS or HAMSB lowered genetic damage significantly relative to a low-RS high-protein control diet. The 20% HAMSB diet was twice as effective as 20% HAMS in opposing genetic damage. Large bowel digesta butyrate was significantly increased in rats fed 20% compared with 10% HAMS and in rats fed 20% compared with 10% HAMSB. The levels were significantly higher in the HAMSB groups relative to the HAMS groups. Hepatic portal venous SCFA were higher in rats fed HAMS and highest in those fed HAMSB. Caecal digesta ammonia was increased by HAMSB and correlated negatively with digesta pH. Ammonia is cytotoxic and lower digesta pH could lower its absorption, possibly contributing to lower genetic damage. Delivery of butyrate to the large bowel by HAMSB could reduce colorectal cancer risk by preventing diet-induced colonocyte genetic damage.

Introduction

Diet has been implicated as a risk factor for both colorectal cancer (CRC) and inflammatory bowel diseases. Dietary fibre has been suggested as a possible protective agent against these non-infectious large bowel diseases although the results of epidemiological studies are inconsistent (1,2) possibly due to the diverse nature of dietary fibre (3). Resistant starch (RS) is dietary starch that escapes digestion in the small intestine and enters the large bowel where it is nearly all fermented by the commensal bacteria (4,5). Short-chain fatty acids (SCFA) are major products of this fermentation and the major acids found in adults are acetate, propionate and butyrate. All three exert a number of general beneficial actions on the viscera but butyrate is believed to have a particularly important role in maintaining colonic health and function by activating apoptosis and cell cycle arrest to protect against oncogenesis. Strategies to enhance the levels of butyrate within the colon include increased RS consumption as its fermentation is believed to yield butyrate preferentially (6). Limited population survey data suggest that RS is protective against CRC (7). This is consistent with the hypothesis that butyrate may contribute to improved colonic health and there are data showing that raising large bowel butyrate by diet can oppose CRC induced by a genotoxic agent in rats (8).

Whereas RS might improve bowel health, an early case-control study (9) and a meta-analysis of international population studies (10) suggested that greater total protein intakes increased CRC risk. Specific sources of dietary protein have been linked to CRC risk and a recent meta-analysis of large prospective population studies concluded that greater red and processed meat consumption increased CRC risk dose dependently (11).

We have shown previously that colonocyte DNA damage, measured as single-strand breaks (SSB), was higher in rats fed diets containing higher levels of various dietary proteins including casein, red meat or a soy protein isolate (12,13). More recently, we have shown that both red and white (chicken) meat increased both SSB and double-strand breaks although the level of damage was substantially lower with white meat (14).

While the body of evidence supports the role of butyrate in the promotion of large bowel health, it is not conclusive. Further, the effects of long-term butyrate delivery on protein-induced large bowel damage remain to be established. We have developed acylated starches as vehicles for the sustained delivery of specific SCFA to the large bowel (15–17). This technology offers the opportunity to examine the effects of individual SCFA on large bowel health. In this study, we compare the effects of high amylose maize butyrylated starch (HAMSB) and high amylose maize starch (HAMS) on colonocyte genetic damage and indices of large bowel health in rats fed diets containing low and high levels of protein.

Materials and methods

Animals and diets

Forty-eight adult male Sprague–Dawley rats (200 ± 20 g) were purchased from the Animal Resource Centre, Murdoch University, Perth, Western Australia. They were housed in wire-bottomed cages, four rats per cage, in a room of controlled temperature (23 ± 1°C) and lighting (lights on at 08:00–20:00 h). The rats were allowed free access to commercial rat diet (Ridgley Agriproducts, Murray Bridge, South Australia, Australia) food and water. After a 7 day adaptation period, the animals were divided into six groups (n = 8 per group) and were fed the experimental diets for 4 weeks. The diet has been described previously and is based on diet AIN-93G (18) (Table I). Two of these groups served as low protein (LP) and high protein (HP) controls, respectively. The diet of the former contained 15% and the latter 25% casein (Murray Goulburn Co. Ltd., Melbourne, vic., Australia). In these control diets, all the starch was highly digestible low amylose maize starch (LAMS). The diets of the remaining groups contained 25% casein and 10 or 20% by weight of either HAMS or HAMSB (Table I). The latter was prepared by National Starch and Chemical Co. and had a degree of substitution of 0.23. The test starches were cooked prior to incorporation into the diets to simulate normal human consumption conditions (19). Experimental procedures were undertaken with the approval of the animal ethics committees of CSIRO Human Nutrition and the University of Adelaide and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th ed (20).
significance was determined using Tukey’s post hoc test. Comparisons between dietary protein level and carbohydrate source were analysed by two-way analysis of variance. The relationship between caecal and faecal parameters and colonic DNA damage were determined by linear regression analysis. A value of \( P < 0.05 \) was taken as the criterion of significance.

### Results

#### Animal and tissue measurements

Body weight gain, water intakes and urine output of the rats were not significantly different between the controls and any treatment group (data not shown). Food intakes of rats fed higher protein with 20% HAMSB were significantly lower than all other groups for days 3 and 4 of the trial but not at any other time (data not shown).

### Table I. Composition of experimental dietsa

<table>
<thead>
<tr>
<th>Experimental diets (g/Kg)</th>
<th>LP control</th>
<th>10% HAMS</th>
<th>20% HAMS</th>
<th>10% HAMSB</th>
<th>20% HAMSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAMSBd</td>
<td>—</td>
<td>100</td>
<td>100</td>
<td>200</td>
<td>—</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Vitamins</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Minerals</td>
<td>35</td>
<td>35</td>
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</tbody>
</table>

### Statistical methods

Values are represented as means and standard error of the means unless otherwise stated. Statistical analyses were performed using Graphpad Prism 4.0 (Graphpad Software, San Diego, CA). Where appropriate, the effects of diet or large bowel site and their interactions were evaluated using one-way and significance was determined using Tukeys post hoc tests. Comparisons

### Sample collection and analytical procedures

Individual rat body weights and pooled cage food intakes were measured daily. At the conclusion of the experimental period, rats were exsanguinated under halothane anaesthesia. The lengths of the small and large intestines were measured and caecal, colonic, liver and spleen weights were recorded. Digesta from the caecum, proximal and distal colon was sampled for SCFA (16). Digesta SCFA pools were calculated for each region of the large bowel by multiplying the SCFA concentration by the total digesta weight. Portal vein plasma SCFA were determined by diethyl ether extraction as described by Murase et al. (21).

1. A 6 cm segment of colon was removed from each rat at a point 3 cm from the distal end of the colon and colonocytes were isolated immediately. These cells were used for the measurement of DNA strand breaks using the single-cell gel electrophoresis (comet) assay (Trevigen, Gaithersburg, MD) as described previously (22). Slides were examined under a fluorescent microscope (Olympus BX-41, Olympus Corp., Tokyo, Japan) using ×20 objective magnification. Comet tail moment is the product of tail length and the fraction of DNA in the tail and was calculated for 50 cells per rat. The measure was calculated by Comet Score v1.5 image processing and analysis software (TriTek Corp., Summerduck, VA). Tissue samples from the distal colon were taken into 10% buffered formalin (Sigma Chemical Co., St Louis, MO) for routine histological processing. Colonocyte apoptosis was assessed in formalin-fixed distal colonic tissue segments using haematoxylin-stained sections (8). Briefly, this was measured by characteristic morphological changes associated with apoptosis such as cell shrinkage, nuclear condensation and blebbing and the formation of apoptotic bodies. Thirty separate crypt columns (one side of a crypt) were assessed and the apoptotic index was calculated as the apoptotic cells per crypt column divided by the total cells in the column multiplied by 100.

Total ammonia concentration was determined using the indophenol blue colorimetric reaction using the digesta supernatants collected for SCFA analysis. Digesta samples were pipetted onto a 96-well plates (5 μl per well) in triplicate followed by 45 μl of distilled water and 100 μl reagent A (10 g/l phenol and 50 mg/l sodium nitroprusside) and reagent B (5 g/l sodium hydroxide and 400 mg/l sodium hypochlorite). The plate was then incubated for 1 h at 30°C in the dark and then read using a plate reader at two wavelengths, \( \lambda_1 (630 \text{ nm}) \) to measure the indophenol blue and \( \lambda_2 (480 \text{ nm}) \) to measure turbidity of the samples. The actual absorbance was calculated as \( \lambda_1 - \lambda_2 \) to remove interference of the colour of the samples. Concentrations were calculated using the mean absorbance and the formula generated from an ammonium chloride standard curve.

#### Statistical methods

Values are represented as means and standard error of the means unless otherwise stated. Statistical analyses were performed using Graphpad Prism 4.0 (Graphpad Software, San Diego, CA). Where appropriate, the effects of diet or large bowel site and their interactions were evaluated using one-way and significance was determined using Tukeys post hoc tests. Comparisons
Butyrylated starch protects colonocyte DNA

Small intestinal and colon lengths were unaffected either by high dietary protein, HAMS or HAMSB diet compared with controls (data not shown). The caecal tissue weight was significantly greater in rats fed 10% HAMSB \( (P < 0.01 \text{ compared with LP LAMS (control)}) \) and 20% HAMSB \( (P < 0.001 \text{ compared with LP LAMS control, HP LAMS control, HP-10% HAMS and HP-20% HAMS and } P < 0.01 \text{ compared with HP-10% HAMSB}) \). However, there was no significant effect of dietary treatment on colon tissue weight (Table II).

**Large bowel digesta weight, pH and SCFA**

Caecal digesta wet weight was unaffected by diet except in rats fed 20% HAMSB where it was significantly higher than all other groups. There was no effect of diet on digesta mass in the proximal colon. Caecal and colonic digesta \( pH \) values were significantly lower in rats fed 10 or 20% dietary HAMSB than in those fed the LP and HP LAMS control diets \( (P < 0.05) \) and to those fed the HP LAMS control in the distal colon \( (P < 0.05) \). Caecal \( pH \) in rats fed the 20% HAMSB was significantly lower than in the 10% HAMS \( (P < 0.01) \) and 20% HAMS \( (P < 0.05) \) groups. There were no significant differences in \( pH \) between the groups fed 10 or 20% HAMSB throughout the large bowel (Table II).

Large bowel digesta acetate pools were significantly greater in rats fed 10 or 20% HAMSB compared with those fed the LP control, HP control or 10% HAMS diets in the caecum and distal colon \( (P < 0.01) \). Caecal acetate pools of rats fed 20% HAMSB were greater than the rats fed all other diets \( (P < 0.05) \). Distal colonic acetate pools of rats fed 20% HAMSB were significantly greater than rats fed LP control and HP control diets \( (P < 0.01) \). No significant differences were observed in the proximal colon (Table III).

Caecal digesta propionate pools were significantly greater in rats fed 10 or 20% HAMSB than in those rats fed any other diet \( (P < 0.05) \). Distal colonic propionate pools were significantly greater in rats fed 20% HAMSB than those fed the LP control or HP LAMS control diets \( (P < 0.01) \). Propionate pools in distal colonic digesta of rats fed 10 or 20% HAMSB were greater than the pools of rats fed the LP and HP control diets \( (P < 0.001) \) and the 10% HAMS diet \( (P < 0.01) \). No significant differences were observed in the proximal colon (Table III).

Large bowel digesta butyrate pools were significantly greater in rats fed 10 or 20% HAMSB or 20% HAMS compared with the LP controls, HP controls \( (P < 0.05) \) and rats fed 10 or 20% HAMSB were significantly greater than 10% HAMS \( (P < 0.05) \), in the caecum and distal colon. Caecal butyrate pools of rats fed 10% HAMSB were greater than the rats fed 20% HAMS \( (P < 0.05) \) and the caecal butyrate pools in the rats fed 20% HAMSB were greater than all other diets \( (P \text{ at least } 0.01) \). The overall significance observed in the proximal colon could not be attributed to differences in specific diets by a post hoc test (Table III).

**Hepatic portal venous SCFA**

Acetate concentrations in the portal vein were greater in rats fed 20% HAMSB compared with the LP LAMS control \( (P < 0.05) \) but no other differences were significant. Feeding 10 or 20% HAMSB significantly increased the portal venous propionate concentration compared with the LP or HP control fed rats \( (P \text{ at least } 0.01) \). Concentrations in rats fed 10% HAMSB were greater than in those fed 10% HAMS \( (P < 0.05) \). Portal vein plasma butyrate concentrations were significantly greater in rats fed 20% HAMS or 10 or 20% HAMSB than in rats fed the LP or HP LAMS control or the 10% HAMS diets \( (P < 0.01) \) (Figure 2).

**Genetic damage**

Genetic damage, as measured by the comet assay, was 2-fold higher in rats fed the 25% protein control diet compared with those fed 15% protein dietary \( (P < 0.001) \). Concurrent feeding of 25% protein with either HAMS or HAMSB lowered genetic damage significantly in a dose-dependant manner with 20% HAMS being twice as effective as 20% HAMS. There appeared to be a dose effect as the level of DNA damage in that damage tended to be lower in the 20% HAMSB group than in the 10% HAMS group (not statistically significant). DNA damage in rats fed higher dietary protein with 20% HAMSB rats was the same as in rats fed the LP control diet (Figure 3). There were significant negative correlations between comet tail moment and caecal acetate \( (r^2 = 0.035, P < 0.0001) \), propionate \( (r^2 = 0.31, P < 0.0001) \) and butyrate \( (r^2 = 0.33, P < 0.0001) \). Distal colonic acetate \( (r^2 = 0.24, P < 0.01) \) propionate \( (r^2 = 0.13, P < 0.05) \) and butyrate \( (r^2 = 0.12, P < 0.05) \) also correlated negatively with tail moment. Tail moment was correlated negatively with hepatic portal venous total SCFA \( (r^2 = 0.144, P < 0.02) \), propionate \( (r^2 = 0.144, P < 0.02) \) and butyrate \( (r^2 = 0.194, P < 0.005) \) concentrations.

**Colonocyte apoptosis**

Colonocyte apoptosis as measured morphologically was not significantly affected by increased dietary protein or concurrent feeding of either HAMS or HAMSB (LP LAMS = 0.01 ± 0.01; HP LAMS =

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**Table II.** Caeco-colonic tissue weights and digesta weights and \( pH \) of rats fed experimental diets for 28 days

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>LP control</th>
<th>HP control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caecum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue weight</td>
<td>0.53</td>
<td>0.65</td>
</tr>
<tr>
<td>10% HAMS</td>
<td>0.79</td>
<td>0.88b</td>
</tr>
<tr>
<td>20% HAMSB</td>
<td>1.05</td>
<td>1.31b</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>7.10</td>
<td>7.07</td>
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<tr>
<td>Tissue weight</td>
<td>4.44</td>
<td>4.44</td>
</tr>
<tr>
<td>pH</td>
<td>0.40</td>
<td>0.44</td>
</tr>
<tr>
<td>Distal colon</td>
<td>6.56</td>
<td>6.08b</td>
</tr>
<tr>
<td>Tissue weight</td>
<td>0.79a</td>
<td>0.67</td>
</tr>
<tr>
<td>pH</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>Total colon</td>
<td>1.11</td>
<td>1.11</td>
</tr>
<tr>
<td>Tissue weight</td>
<td>1.34</td>
<td>1.24</td>
</tr>
<tr>
<td>( pH )</td>
<td>1.11</td>
<td>1.11</td>
</tr>
</tbody>
</table>

Data for each gut region were analysed by one-way analysis of variance. For clarity, data are represented as mean and pooled SEM and significance shown as \( P < 0.05 \). Significant differences are indicated by superscripts.

- \(^a\) Compared with LP control.
- \(^b\) Compared with HP control.
- \(^c\) Compared with HP-10% HAMS.
- \(^d\) Compared with HP-20% HAMS.
- \(^e\) Compared with HP-10% HAMSB.
Digesta ammonia

Caecal digesta ammonia concentrations were significantly greater in rats fed either 10 or 20% HAMSB (P < 0.001). There was no significant effect of protein supplementation on ammonia concentration (Figure 4). There was a significant negative correlation between caecal ammonia concentration and pH (r^2 = 0.40, P < 0.0001) for all rats and comet tail moment (r^2 = 0.28, P < 0.001) for rats fed the HP diets and a positive correlation between caecal ammonia concentration and caecal butyrate concentration (r^2 = 0.73, P < 0.0001).

Discussion

The present data confirm previous studies which showed that higher dietary protein increases colonocyte genetic damage as measured by the comet assay (12,13,23,24). It is important to note that all these data were obtained without treatment with a genotoxic agent (such as azoxymethane (AOM)) so that the changes reflect responses to diet alone (rather than an exogenous carcinogen). Genetic damage was ~100% higher in rats fed the HP diet with highly digestible LAMS (HP control) than in rats fed the LP diet with the same starch. This is the same order of increase as noted previously by Toden et al. (23) in our laboratory, who also reported a similar dose-dependant lowering of genetic damage when the rats were fed 10 and 20% HAMS (24). The dose-dependant reduction in SSB seen with dietary HAMSB was approximately twice as large as that seen with HAMS. We interpret this as reflecting the greater ability of HAMSB to deliver butyrate to the large bowel resulting in large caecal butyrate pools. Indeed, the number of SSB in rats fed the 20% HAMSB diet was the same as in rats fed the LP control diet. Collectively, these data support the hypothesis that butyrate supply is...
Butyrate has been shown to inhibit proliferation and induce differentiation in rats fed high-RS diets following administration of AOM (8). Removing cells with genetic damage, potentially protecting against apoptosis through a histone hyperacetylation-mediated pathway (30) is through inhibition of histone deacetylase (29) resulting in increased HAMS or HAMSB i.e. when butyrate was elevated. Some of this effect is possible that the increase in exogenous butyrate provided by HAMSB independent of fermentation may apply selection pressures against starch fermenting bacteria such as Ruminococcus bromii (44), which are known to use ammonia as a nitrogen source (45). However, in vitro batch fermentations using caecal inoculum from post-weaned piglets demonstrated significant increases in ammonia concentration following the addition of sodium butyrate (46). This effect was dependent on the dose of sodium butyrate and significant increases were observed in 4 h which is more rapid than would occur through selection pressure on bacterial populations. The results from our study clearly indicate that the increased caecal ammonia was not associated with increased DNA damage. A greater understanding of ammonia fluxes under differing dietary conditions and the influence of HAMSB on large bowel microflora are required. Nevertheless, it is important to note that fermentable carbohydrate shifts nitrogen excretion from urine to faeces (47), consistent with the current findings.

The data obtained in this study confirm and extend earlier reports and show that feeding of RS either as HAMS or HAMSB protects colonocytes from genetic damage in rats induced by high dietary protein. This protection may be due to direct effects of colonic butyrate, it is also more effective inducing these other potentially beneficial changes.

In this study, the HAMS and HAMSB were heated with water to approximate to the cooking of food that leads to increased gelatinization and loss of amylose resistance. This is especially important for HAMS, which is more susceptible to the effects of cooking than HAMSB (16,19,34) leading to lower large bowel SCFA compared with the uncooked starch. The increased total SCFA of HAMSB reflects the passage and fermentation to the large bowel of undigested acylated starch, which resists structural breakdown during cooking (35).

Data on the true ileal digestibility of dietary proteins in rats appear to be unavailable but a study in humans with ileostomy showed that it was ~85% for a range of protein sources (36). This means that a significant fraction of ingested protein escapes into the large bowel and this would be expected to increase with higher dietary protein. Undigested dietary protein is fermented by colonic microbes leading to the formation of cytotoxic agents including phenols, cresols, amines and ammonia (37). Previous studies have shown that feeding RS lowers the concentrations of these agents (23,38) contributing to a more favourable luminal environment. This would be expected to lower colonocyte DNA exposure to potentially damaging compounds. It was anticipated that dietary HAMS and HAMSB would lower digesta ammonia. However, caecal concentrations rose in proportion to the butyrate pool. Recently, Duncan et al. (39) showed that both faecal ammonia and SCFA fell in volunteers consuming HP, low carbohydrate diets, consistent with the present observations. Ammonia is a known cytotoxic agent and carcinogen (40) and is a mediator of colonocyte damage (41). Free ammonia can diffuse into colonocytes but only when unprotonated (42) and while the apical surface of colonocytes is known to buffer to neutrality by SCFA–HCO3 exchange (43) potentially increasing ammonia absorption, the significantly lower pH observed in rats fed HAMSB may have negated this effect. The differences in pH persisted throughout the colon, suggesting that any dietary protection against the cytotoxicity due to ammonia was maintained in the regions where DNA damage was assessed. It is possible that the increase in exogenous butyrate provided by HAMSB independent of fermentation may apply selection pressures against starch fermenting bacteria such as Ruminococcus bromii (44), which are known to use ammonia as a nitrogen source (45). However, in vitro batch fermentations using caecal inoculum from post-weaned piglets demonstrated significant increases in ammonia concentration following the addition of sodium butyrate (46). This effect was dependent on the dose of sodium butyrate and significant increases were observed in 4 h which is more rapid than would occur through selection pressure on bacterial populations. The results from our study clearly indicate that the increased caecal ammonia was not associated with increased DNA damage. A greater understanding of ammonia fluxes under differing dietary conditions and the influence of HAMSB on large bowel microflora are required. Nevertheless, it is important to note that fermentable carbohydrate shifts nitrogen excretion from urine to faeces (47), consistent with the current findings.

The data obtained in this study confirm and extend earlier reports and show that feeding of RS either as HAMS or HAMSB protects colonocytes from genetic damage in rats induced by high dietary protein. This protection may be due to direct effects of colonic butyrate either in maintaining cellular integrity or in the deletion of genetically damaged cells that could otherwise progress to malignancy. However, we were unable to show any differences in apoptosis, suggesting that the latter appears to be unlikely. Alternatively, the lower digesta pH levels in rats fed HAMSB may reduce the absorption of ammonia and contribute to the lowering of genetic damage. The greater effectiveness of HAMSB relative to HAMS is consistent with the suggestion that large bowel butyrate is a significant contributor to improved colonic health with the potential to lower CRC risk.

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References