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ORIGINAL ARTICLE

Dietary Red Meat Aggravates Dextran Sulfate Sodium-Induced Colitis in Mice Whereas Resistant Starch Attenuates Inflammation

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Abstract

Background Although a genetic component has been identified as a risk factor for developing inflammatory bowel disease, there is evidence that dietary factors also play a role in the development of this disease.

Aims The aim of this study was to determine the effects of feeding a red meat diet with and without resistant starch (RS) to mice with dextran sulfate sodium (DSS)-induced colitis.

Methods Colonic experimental colitis was induced in Balb/c mice using DSS. The severity of colitis was evaluated based on a disease activity index (based on body-weight loss, stool consistency, rectal bleeding, and overall condition of the animal) and a histological score. Estimations were made of numbers of a range of different bacteria in the treatment pools of caecal digesta using quantitative real-time PCR.

Results Consumption of a diet high in red meat increased DSS-induced colitis as evidenced by higher disease activity and histopathological scores. Addition of RS to the red meat diet exerted a beneficial effect in acute DSS-induced colitis. Subjective analysis of numbers of a range of bacterial targets suggest changes in the gut microbiota abundance were induced by red meat and RS treatments and these changes could contribute to the reported outcomes.

Conclusions A dietary intake of red meat aggravates DSS-induced colitis whereas co-consumption of resistant starch reduces the severity of colitis.

Keywords Inflammation · Resistant starch · Red meat · Gut microbiota · Dextran sulfate sodium

Abbreviations

DSS	Dextran sulfate sodium
RS	Resistant starch
RM	Red meat
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CRC	Colorectal cancer
SCFA	Short chain fatty acids
AIN	American Institute of Nutrition
Hi-maize	High amylose maize starch
DAI	Disease activity index
SRB	Sulfate-reducing bacteria
aps	Adenosine-5-phosphosulfate reductase gene

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Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis (UC), result from complex interactions between environmental and genetic factors [1].

Journal : Large 10620 | Dispatch : 19-8-2013 | Pages : 8
Article No. : 2844 | ☐ LE | ☐ TYPESET
MS Code : DDSJ-D-13-00383 | ☑ CP | ☑ DISK

Patients experience chronic relapsing symptoms that include abdominal pain, diarrhoea, rectal bleeding and anaemia resulting from intestinal inflammation, oedema and ulceration [2]. Although a genetic component has been identified as a risk factor for developing IBD [3], there is evidence that dietary factors may play a role in the development of IBD [4]. The incidence of IBD is high in western countries and is on the increase in low-incidence areas such as southern Europe and Asia, as well as developing countries that are now adopting a westernised diet [5] [6].

A typical western diet is rich in red and processed meat and poor in fruits and vegetables. Red meat has been identified by the World Cancer Research Fund as a convincing cause of colorectal cancer (CRC) [7] patients with IBD also have a greater risk of developing CRC [8, 9]. In contrast, a high intake of dietary fibre, fruit, or vegetables may be protective against the development of IBD [10] and also CRC [7, 11]. Short-chain fatty acids (SCFAs) are products that are derived from fermentation of unabsorbed dietary fibre and starch in the colon. The SCFA "butyrate" is important for colonic integrity as it is the principal energy source for the colonic epithelium, inhibits growth of cancer cells in vitro and forces a more normal differentiated phenotype [12, 13]. A deficiency of SCFAs in the intestinal lumen is often related with epithelium atrophy and inflammation. In UC, an overall impaired butyrate metabolism has been reported in several studies [14]. In a rodent model of dextran sulfate sodium (DSS)-induced colitis, oral administration of sodium butyrate has been shown to improve mucosal lesions and attenuate the inflammatory profile of the intestinal mucosa and local lymph nodes [15]. Also, Morita et al. [16] reported a protective effect of resistant starch (RS) in the form of high-amylose cornstarch on trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats where enhancement of mucosal protection was exerted possibly due to large bowel SCFA production.

The DSS-induced colitis mouse model provides an experimental model that displays many symptoms similar to those seen in human UC, such as diarrhoea, bloody faeces, body weight loss, mucosal ulceration, and shortening of the colorectum [17]. In the present study, we determined the effects of feeding a diet high in red meat with and without RS on DSS-induced colitis in Balb/c mice.

Methods

Animals and Diets

Thirty-two male Balb/c mice were obtained from the Animal Resource Centre, Perth, Western Australia, and housed in controlled conditions of 22 ± 2 °C (SD), 80 ± 10 % humidity, and 12-h light/dark cycle. Mice were acclimatized for a minimum of 1 week before commencement of the trial. Mice were then divided into four groups ($n = 8$) and fed one of four experimental diets (Table 1) for a period of 12 days. The experimental diets were modified forms of the AIN-76a standard for purified diets for rats and mice. The first group "Control" consumed the modified AIN-76a diet. The second group "RS" consumed high amylose maize starch (Hi-maize® 260; National Starch and Food innovation, Bridgewater, NJ, USA) at a level of 10/100 g diet. The third group "RM" consumed cooked red meat at a level of 30/100 g diet. The fourth group "RM + RS" consumed cooked red meat at a level of 30/100 g diet and high amylose maize starch at a level of 10/100 g diet.

High amylose maize starch (Hi-maize 260), was used as the source of resistant starch and was supplied by the National Starch and Chemical Company. Hi-maize 260 has been shown to contain approximately 50 % resistant starch [18] and was added at a level of 10/100 g diet; therefore, a total of 5 % resistant starch was added to the diet. This proportion of starch consumed as RS in this RS-containing diet is feasible in the context of the human diet and is not likely to create any serious problem of side effects such as flatulence and bloating [19]. Lean, minced rump steak was purchased, cooked at medium temperature on a gas hotplate with continuous mixing to prevent the meat from burning, and oven-dried overnight before grinding to powder. Total nitrogen level of the cooked/dried red meat

Table 1 Composition of experimental diets

Ingredient	Control	RS	RM	RM + RS
Casein	20	20	0	0
Red meat	0	0	30	30
Corn starch	15	5	15	5
Sucrose	37.93	37.93	31.13	31.13
High amylose maize starch[a]	0	10	0	10
Sunflower seed oil	16.8	16.8	16.8	16.8
Lard[b]	3.2	3.2	0	0
α-cellulose	2	2	2	2
L-cysteine	0.3	0.3	0.3	0.3
Choline	0.12	0.12	0.12	0.12
Mineral mix[c]	3.5	3.5	3.5	3.5
Vitamin mix[c]	1	1	1	1
Methionine	0.15	0.15	0.15	0.15

[a] High amylose maize starch (Hi-maize 260™) used as the source of resistant starch

[b] AIN-76 vitamin and mineral mixtures

[c] Lard was added to the Control and RS diets to balance each diet for saturated fat and to give a total fat content of 20 %

Journal : Large 10620 Dispatch : 19-8-2013 Pages : 8
Article No. : 2844
MS Code : DDSJ-D-13-00383
☐ LE ☑ CP ☐ TYPESET ☑ DISK

was determined by the Dumas method [20], and the total protein content was calculated to be 73 %. Saturated fat content of the red meat was 6 % when analysed by a standard fat extraction method [21]. Moisture content of the meat was found to be 10 % by weighing known amounts of meat product and drying overnight to calculate moisture lost from the sample. Final diet preparations were placed into air-sealed containers and stored at 4 °C, with fresh food in the mouse cage bowls replaced daily.

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

Induction of Colitis

Experimental colitis was induced by adding DSS (molecular weight 36-50 kDa; MP Biomedicals) to the drinking water at a level of 3 % for the first 5 days of the study. All mice received standard tap water from day 6 to day 12 of the study.

Tissue Collection

Mice were anesthetised with a 10 % ketamine and 10 % metotomodine solution at 75 mg/kg and decapitated 7 days after DSS treatment. After dissection, the colon was removed and placed into a 10 % buffered formalin solution containing 3.6 % formaldehyde for 24 h and transferred to 70 % ethanol for histologic processing. Tissue was rehydrated through gradient alcohols and embedded in paraffin wax for histological assessment.

Histopathological Anlaysis

Colon sections (5 μm) were stained with haematoxylin and eosin and were independently and randomly coded so that dietary groups were not known to the pathologist. Eight randomly selected fields (magnified ×100) were viewed under a light microscope, and each section was graded and averaged according to the method described by Cooper et al. [22]. The severity of mucosal injury was graded as follows: grade 0, normal—intact colonic crypt; grade 1, slight—cystic dilatation of crypts; grade 2, mild—loss of basal 1/3 of crypts; grade 3, moderate—loss of basal 2/3 of crypts; grade 4, severe—loss of entire crypt with surface epithelium remaining intact.

Disease Activity Index Assessment

Mice were scored daily using a Disease Activity Index (DAI) based on weight loss, stool consistency, rectal bleeding, and overall condition of the animal [23].

Each of these elements was scored on a 0–3 scale, with 0 representing no disease symptom and 3 representing severe disease symptom. Weight loss was scored as 0 representing no weight loss compared to the original weight, 1 representing a weight loss of less than 5 %, two representing a weight loss of between 5 and 10 %, and three representing a weight loss of more than 10 % of the original weight. The grading of each variable was scored from 0 to 3. Data are the sum of scores for four independent variables.

Bacterial Quantification

Caecal digesta collected from each mouse was combined into treatment group pools (insufficient material was available for individual analysis). DNA was extracted from 0.25 g of each pool using the repeated bead beating plus column method of Yu and Morrison [24] and then used for estimation of numbers of target bacteria using quantitative real-time PCR (qRT-PCR). Each pool was analysed in quadruplicate. PCR reactions were carried out on a CFX Connect 96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) in a volume of 10 μl. Each reaction contained 1 μl DNA template, 5 μl SsoFast EvaGreen Supermix, 0.2 μl bovine serum albumin (0.5 μl DMSO used for SRB_APS reaction), primers (according to references below) and PCR-grade water. Bacterial assays were performed according to previous publications: *Akkermansia muciniphila*, *Bifidobacterium* spp. (84 °C step before fluorescence acquisition performed in this study), *Clostridium coccoides* group, *Clostridium leptum* group, *Escherichia coli* and *Faecalibacterium prausni* [25]; SRB_*aps* and total bacteria [26]; *Entercoccus* spp. and *Parabacteroides distasonis* [27]; *Ruminococcus bromii* (conditions like *F. prausnitzii* except 30 s annealing) primers used were he-10F and he-10R from [28]; *Ruminococcus gnavus* (conditions like *F. prausnitzii* except 58 °C annealing) [29]; *Bacteroides–Prevotella* (conditions like *R. gnavus* except 45 s annealing and 700 nM primer) [30]. A series of eight tenfold dilutions of a sample-derived standard for each amplicon were analysed with samples to estimate bacterial abundance and PCR efficiency. It was not possible to examine differences between groups using statistical tests due to the lack of sample replication (due to the requirement for sample pooling).

Statistical Analysis

Qualitative DAI and semi quantitative histological severity scores were analysed using the non-parametric Kruskal–Wallis test with pairwise comparisons. For all analyses, $P < 0.05$ was considered significant. All data are expressed as the geometric mean ± standard error of the mean (SEM). Statistical comparisons were made using IBM SPSS for Windows software package V20.0 (Chicago, IL).

Journal : Large 10620 | Dispatch : 19-8-2013 | Pages : 8
Article No. : 2844 | LE | TYPESET
MS Code : DDSJ-D-13-00383 | CP | DISK

Results

Clinical Symptoms and DAI

No mortality was observed in the control treatment, whereas 1 mouse died from each of the RS and RS + RM groups and 3 mice died from the red meat group. The DAI score was monitored daily over the 12 days (5 days DSS treatment followed by 7 days tap water). DAI scores are shown in Fig. 1 and statistical outcomes in Table 2. Significant differences in DAI scores were evident as early as day 2 with the RM treatment group having significantly higher scores than all other treatment groups; this pattern was maintained to day 5. There were no differences seen on days 6 between the different groups. On day 7, the RM group was significantly higher than the RS group. On day 8, the RM group was significantly higher than the Control and RS groups. On days 9 and 10, the RM group displayed higher DAI compared to the RS group. No differences were observed on days 11 and 12.

Histopathology Analysis

Histologically, the DSS model of colitis is characterized by a disruption in crypt architecture, reduced crypt area and increased inflammatory infiltrate. Figure 2 shows a representative samples from each dietary group. Mice consuming the red meat diet (RM) had significantly higher histological severity scores than the Control group and the RS group (Fig. 3).

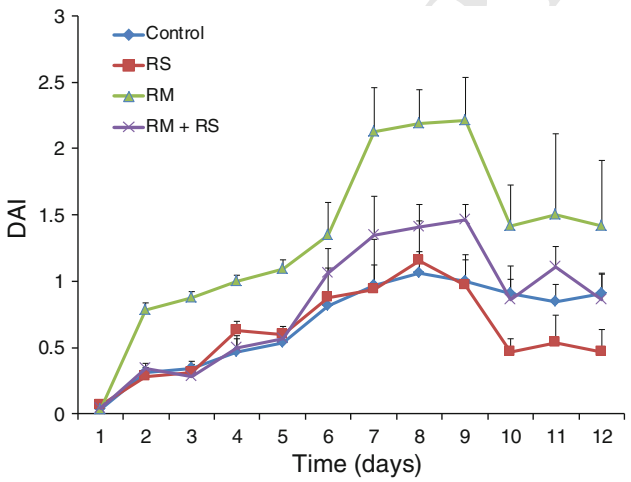

Fig. 1 Disease activity index (DAI) scores in mice monitored daily over the 12 days (5 days DSS treatment followed by 7 days tap water)

Table 2 Disease activity index (DAI) scores during the 12 days mice were fed the different diets

	Control	RS	RM	RM + RS
D1	0.03 ± 0.03	0.06 ± 0.04	0.03 ± 0.03	0.03 ± 0.03
D2	0.31 ± 0.04 a	0.28 ± 0.03 a	0.78 ± 0.06 b	0.34 ± 0.05 a
D3	0.34 ± 0.04 a	0.31 ± 0.06 a	0.87 ± 0.04 b	0.28 ± 0.03 a
D4	0.47 ± 0.10 a	0.62 ± 0.07 a	1.00 ± 0.05 b	0.50 ± 0.10 a
D5	0.53 ± 0.10 a	0.59 ± 0.07 a	1.10 ± 0.07 b	0.56 ± 0.08 a
D6	0.81 ± 0.11	0.88 ± 0.22	1.34 ± 0.25	1.06 ± 0.18
D7	0.97 ± 0.16 ab	0.94 ± 0.38 a	2.13 ± 0.33 b	1.34 ± 0.30 ab
D8	1.06 ± 0.17 a	1.15 ± 0.30 a	2.19 ± 0.25 b	1.40 ± 0.18 ab
D9	1.00 ± 0.21 ab	0.97 ± 0.23 a	2.21 ± 0.32 b	1.46 ± 0.11 ab
D10	0.90 ± 0.13 ab	0.68 ± 0.10 a	1.35 ± 0.31 b	1.10 ± 0.16 ab
D11	0.84 ± 0.16	0.54 ± 0.20	1.50 ± 0.61	1.10 ± 0.15
D12	0.90 ± 0.19	0.46 ± 0.18	1.42 ± 0.49	0.86 ± 0.20

Rows with different letters are significantly different at $P < 0.05$

Caecal Bacterial Analysis

Estimations were made of numbers of a range of different bacteria in caecal digesta were quantified using qRT-PCR and are shown in Table 3. The caecal digesta from mice in each treatment group was pooled to provide sufficient material for DNA extraction and analysis. Although statistical analyses were not possible due to a lack of sample replication, a subjective comparison of means suggests a combination of red meat and RS treatment resulted in reduced numbers of *C. coccoides*, *Enterococcus* spp. and *E. coli* relative to other groups, and effects of red meat treatment on *F. prausnitzii*, *P. distasonis*, *A. muciniphila*, *Bifidobacteria* and the *C. leptum* group.

Discussion

The findings of the current investigation demonstrate that a diet high in red meat can increase the severity of DSS-induced colitis in mice whereas co-consumption of RS appears to reduce the severity of red meat-induced effects. Mice consuming the red meat diet alone demonstrated increased morbidity and mortality, heightened histological damage in the colon and enhanced DAI scores (from day 2 to day 5). Addition of resistant starch appeared to protect against DSS-induced colitis, as it was observed that mice fed RS together with red meat had fewer mortalities, the enhancement of DAI by red meat through day 2 to day 5 was ameliorated and the histopathology score was not significantly different from controls.

Epidemiological evidence suggests that diet plays a role in IBD [6]. Incidence rates of IBD have increased over the years in populations adopting a westernised diet [5, 6].

Journal : Large 10620 Dispatch : 19-8-2013 Pages : 8
Article No. : 2844 LE TYPESET
MS Code : DDSJ-D-13-00383 CP DISK

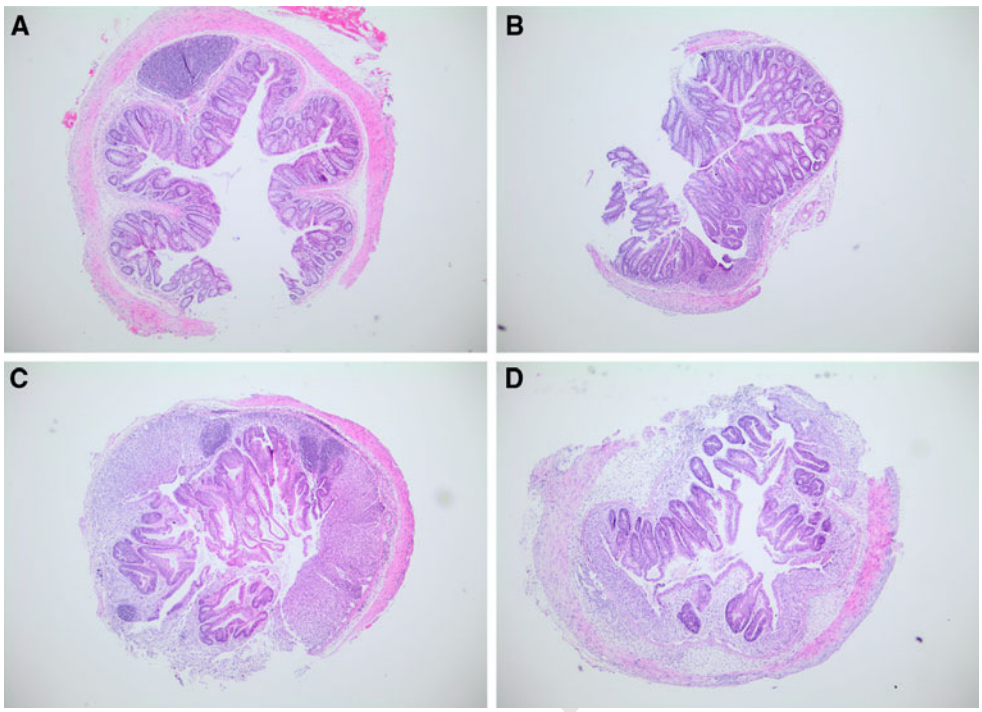

Fig. 2 Histological analysis of DSS treated mice. **a** Colon section from control fed mouse showing normal architecture (HE, ×100); **b** colon section from RS fed mouse showing normal architecture (HE, ×100); **c** colon section from red meat (RM) fed mouse showing crypt inflammation and moderate damage (HE, ×100); **d** colon section from RM + RS fed mouse showing inflammation and minor damage (HE, ×100), the tissue damage was less severe than in C damage (HE, ×100)

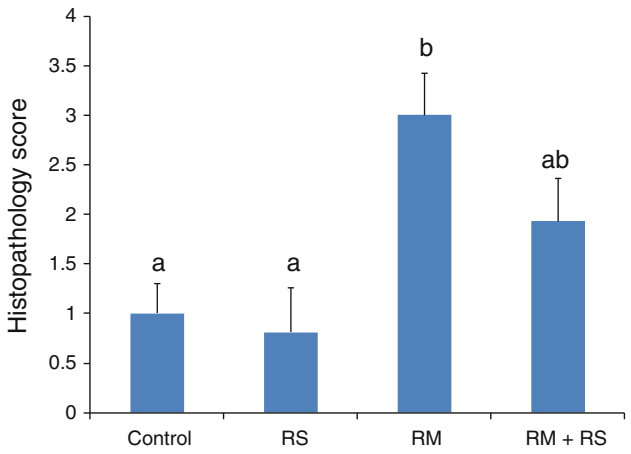

Fig. 3 Histopathology severity scores. *Bars* with different superscript letters are significantly different at $P < 0.05$

Animal protein, particularly red meat, [31–33], has been singled out as a possible risk factor as contributing to the development of IBD. Plausible explanations of why increased red meat intake may contribute to IBD may include increased delivery of amino acids and heme to the colon where they undergo fermentation and metabolism by the colonic microbiota [34], which results in the generation of potentially toxic substances such as ammonia, amines, N-nitroso compounds, phenols, cresols and hydrogen sulfide [35, 36]. Increased dietary heme from haemoglobin in red meat can form reactive oxygen species, [37] Sesink et al. [38] showed that dietary heme increases luminal cytotoxicity which causes damage to the colonic epithelium. Previous animal studies by us have also shown that red meat consumption results in a thinning of the colonic mucus layer and increases damage to the colonocytes in the form of DNA strand breaks [39] or promutagenic adducts [40].

Mice consuming RS along with the red meat diet had reduced clinical signs of colitis when compared to mice fed only the red meat diet. Furthermore, the RM + RS mice did not differ from the control mice for either histopathological severity or daily DAI scores. There are a number of potential reasons why RS may improve or prevent colonic inflammation. RS is the portion of starch that resists digestion in the small intestine and enters the large bowel, and so contributes to total dietary fibre intake [41, 42]. In the large bowel, RS is fermented by the microbiota, resulting in the production of butyrate which improves colonic physiology [42, 43] as well as providing a major source of energy for the growth of microorganisms [44]. Although SCFA levels were not measured in the current study, we have previously reported significant increases in total SCFA and butyrate in mice and rats consuming similar dietary RS levels to those used in the present study [40, 45]. Additional studies have also shown that adding RS to a diet high in red meat profoundly alters protein and carbohydrate fermentation in a manner that can be interpreted as constituting a more favourable luminal environment [40, 46–48].

The gut bacterial population profiles of individuals with IBD are altered compared to healthy individuals, including a reduced overall diversity of microbes [28, 29, 49]. In this

Journal : Large 10620 | Dispatch : 19-8-2013 | Pages : 8
Article No. : 2844 | LE | TYPESET
MS Code : DDSJ-D-13-00383 | CP | DISK

Table 3 Numbers of target bacteria in treatment pools of cecal digesta

Target	Numbers per g digesta (Mean)			
	Control	RS	RM	RM + RS
Akkermansia muciniphila	1.72×10^8	1.15×10^8	3.99×10^6	1.18×10^7
Bacteroides–Prevotella group	3.58×10^8	4.9×10^8	1.13×10^9	NA
Bifidobacterium spp.	1.99×10^8	2.47×10^8	3.63×10^5	8.92×10^5
Clostridium coccoides group	7.07×10^8	1.33×10^9	5.39×10^8	5.17×10^5
Clostridium leptum group	1.08×10^8	1.98×10^8	1.44×10^6	9.21×10^6
Enterococcus spp.	2.38×10^7	9.20×10^6	1.83×10^6	1.72×10^5
Escherichia coli	5.09×10^8	1.58×10^8	1.94×10^9	5.70×10^5
Faecalibacterium prauznitzii	1.35×10^7	2.14×10^7	3.30×10^5	2.31×10^6
Parabacteroides distasonis	3.90×10^7	3.81×10^7	6.05×10^6	1.59×10^5
Ruminococcus bromii	9.15×10^4	1.82×10^5	NA	3.91×10^4
Ruminococcus gnavus	6.73×10^6	1.06×10^7	1.02×10^5	2.98×10^5
SRB_*aps*	1.48×10^7	1.93×10^7	NA	NA

SRB Sulfate-reducing bacteria, *aps* adenosine-5-phosphosulfate reductase gene, *NA* not available

study, we have examined effects of treatment on gut populations of a range of bacteria that are implicated in IBD or bowel health broadly. Our targets included *F. prausnitzii*, *R. bromii*, *P. distasonis*, the *C. coccoides* group and the *C. leptum* group that are associated with fermentation of complex carbohydrates and production of SCFA, and generally thought to provide benefit. Numbers of the latter two groups are also low in IBD [49], and *F. prausnitzii* is of additional interest due to its anti-inflammatory effects and lower numbers in the gut of individuals with colitis [50, 51]. Populations of *A. muciniphila* and *R. gnavus*, which contribute to mucus turnover and are altered in IBD [29], were also examined. Other targets were accepted markers of bowel health (*Bifidobacterium* spp. and *Lactobacillus* spp.), bacteria often associated with poor health outcomes (*E. coli*, *Enterococcus* spp.), and some groups that may play a role in health (*Bacteroides–Prevotella*, sulfate-reducing bacteria). Although the contribution of bacteria to the increased colonic damage that can occur in response to diets high in red meat is poorly defined, it is likely that they have a role as many of the products that are produced in the gut following bacterial fermentation of proteins are toxic. Similarly, bacteria are implicated in the protection against dietary protein-induced increases in colonic DNA damage that occurs in response to consumption of RS, primarily because production of SCFA via bacterial action, especially of butyrate, correlates strongly with protection [52]. In our study, we were unable to carry out definitive analyses of the gut microbiota due to constraints related to the amount of digesta available. However, our subjective analysis of numbers of a range of bacterial targets suggests there were changes in the abundance of some bacteria in response to red meat and RS treatments, and these changes could contribute to the reported outcomes. Some of the changes suggested by our limited microbial data, such as drops in numbers of *A. muciniphila* and bacteria belonging to the *C. coccoides* and *C. leptum* groups in response to the red meat diet in DSS-treated mice, are also observed in the large bowel mucosa of humans with Crohn's disease [29, 49], suggesting similar mechanisms may be at play in our DSS animal model of colitis.

In conclusion, dietary red meat worsens the histopathology, inflammatory indicators and clinical signs in DSS-induced colitis, whereas resistant starch added to a high red meat diet reduces the severity of colitis. Changes in the gut microbiota by consumption of red meat and resistant starch may play a role in the modulation of the severity of the DSS-induced colitis. Further studies are required to elucidate the mechanisms involved in the worsening of colitis by red meat and beneficial effects of resistant starch in a suitable model.

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Conflict of interest None.

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Journal : Large 10620 | Dispatch : 19-8-2013 | Pages : 8
Article No. : 2844 | LE | TYPESET
MS Code : DDSJ-D-13-00383 | CP | DISK

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Journal : Large 10620 | Dispatch : 19-8-2013 | Pages : 8
Article No. : 2844 | ☐ LE | ☐ TYPESET
MS Code : DDSJ-D-13-00383 | ☑ CP | ☑ DISK

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Article No. : 2844 | ☐ LE | ☐ TYPESET
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