# Cooked navy and black bean diets improve biomarkers of colon health and reduce inflammation during colitis

Claire Zhang[1,2], Jennifer M. Monk[1,2], Jenifer T. Lu[1,2], Leila Zarepoor[1,2], Wendy Wu[1], Ronghua Liu[1], K. Peter Pauls[3], Geoffrey A. Wood[4], Lindsay Robinson[2], Rong Tsao[1] and Krista A. Power[1,2]*

[1]Guelph Food Research Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, ON, Canada N1G 5C9
[2]Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, ON, Canada N1G 2W1
[3]Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada N1G 2W1
[4]Department of Pathobiology, University of Guelph, Guelph, ON, Canada N1G 2W1

## Abstract

Common beans contain non-digestible fermentable components (SCFA precursors) and phenolic compounds (phenolic acids, flavonoids and anthocyanins) with demonstrated antioxidant and anti-inflammatory potential. The objective of the present study was to assess the *in vivo* effect of cooked whole-bean flours, with differing phenolic compound levels and profiles, in a mouse model of acute colitis. C57BL/6 mice were fed a 20% navy bean or black bean flour-containing diet or an isoenergetic basal diet (BD) for 2 weeks before the induction of experimental colitis via 7 d dextran sodium sulphate (DSS, 2% (w/v) in the drinking-water) exposure. Compared with the BD, both bean diets increased caecal SCFA and faecal phenolic compound concentrations ($P < 0.05$), which coincided with both beneficial and adverse effects on colonic and systemic inflammation. On the one hand, bean diets reduced mRNA expression of colonic inflammatory cytokines (IL-6, IL-9, IFN-$\gamma$ and IL-17A) and increased anti-inflammatory IL-10 ($P < 0.05$), while systemically reduced circulating cytokines (IL-1$\beta$, TNF$\alpha$, IFN$\gamma$, and IL-17A, $P < 0.05$) and DSS-induced oxidative stress. On the other hand, bean diets enhanced DSS-induced colonic damage as indicated by an increased histological injury score and apoptosis (cleaved caspase-3 and FasL mRNA expression) ($P < 0.05$). In conclusion, bean-containing diets exerted both beneficial and adverse effects during experimental colitis by reducing inflammatory biomarkers both locally and systemically while aggravating colonic mucosal damage. Further research is required to understand the mechanisms through which beans exert their effects on colonic inflammation and the impact on colitis severity in human subjects.

**Key words: Colitis: Inflammation: Black beans: Navy beans: Colon health: Dextran sodium sulphate: SCFA**

It is becoming increasingly evident that many human chronic diseases are linked to the health of the gastrointestinal tract. Gut health is characterised by many factors including a functioning intact gut barrier, a balanced gut microbiota composition, and absence of diseases such as inflammatory bowel disease (IBD) and colon cancer[1]. Maintaining a healthy gut involves a symbiotic relationship between the gut microbiota and the host immune system, thereby preserving the integrity of the intestinal barrier and minimising inappropriate inflammatory responses[1,2]. Dysfunction or imbalances in gut health lead to chronic human diseases, such as IBD, obesity, colon cancer, diabetes and neurological disorders[3–6]. Therefore, finding methods to maintain gut health in healthy individuals and re-establishing gut health in diseased individuals are of current interest.

Common beans (*Phaseolus vulgaris*) are a type of pulse crop with a long history of human health benefits, including gut health[7–9]. Specifically, beneficial changes in the gut microbiota profile and activity[10–12], as well as reduced risk of colon cancer in humans and experimental models[13–18], have been reported to be associated with bean consumption. Beans contain an array of bioactive components, namely fermentable non-digestible components and phenolic compounds, which may play a role in the gut health-promoting potential of beans[19–21]. Major bean components, non-starch polysaccharides, resistant starches and oligosaccharides, are fermented by the microbiota in the large intestine generating SCFA: acetate; propionate; butyrate[11,12]. Butyrate exerts direct effects on colon health as it is an important energy source for colonocytes enabling proper growth and function

of the epithelium[22–24], supports healthy commensal bacterial growth[25], increases mucus secretion via the stimulation of Muc2 (mucin 2) expression[26], and exerts anti-inflammatory effects, in part, through the modulation of inflammation-related cell signalling pathways and enhancement of gut barrier integrity[23,24,27–32]. Phenolic compounds present in common beans are also known to induce an array of biological effects, including reduction of colon cancer growth[17,33–36] and oxidative stress[37,38], attenuation of immune responses[38–42], enhancement of gut barrier integrity[43,44], and modulation of the microbiota community structure and activity[33,45–47]. Therefore, with the diverse gut modulatory effects of bioactives, gastrointestinal diseases, in addition to colon cancer, may also be beneficially modulated by common beans.

Inflammation plays a major role in the development of a number of colon-associated diseases, including IBD and colon cancer[48,49]. Colitis is a form of IBD associated with microbiota dysbiosis[50–54], elevated oxidative stress[55–58], compromised gut barrier integrity and function (permeability, tight-junction protein expression, Toll-like receptor signalling, anti-microbial peptide production and goblet cell function)[59–63], and defects in the intestinal immune and inflammatory responses (NF-κB signalling, cytokine expression and immune cell infiltration)[59,64]. Current therapeutic interventions, including anti-inflammatory agents, immunosuppressants and antibiotics, aim to reduce inflammation during disease relapse and promote disease remission[65–67]. However, patients often suffer from side effects, fail to respond and also become resistant to these therapies[68,69]. Therefore, alternative or complementary therapies, including dietary components, are being studied for their effectiveness in reducing colitis symptoms and disease progression[70–73].

Therefore, the primary objective of the present study was to examine the gut health-promoting potential of cooked common beans (i.e. consumption of the whole food rather than individual bioactives) in experimental colitis in mice. Although the effects of beans on colitis have not been studied previously, increased consumption of either fermentable non-digestible components or SCFA has been reported to reduce inflammation in both IBD patients and animal colitis models[29,74–80]. Furthermore, consumption of pure phenolic compounds or phenolic-rich food extracts has been shown to attenuate disease biomarkers through reduction of colonic oxidative stress, reducing immune cell infiltration, modulating NF-κB activation and reducing the secretion of pro-inflammatory cytokines[40–42,81–83]. In addition, two bean varieties with striking differences in their seed coat colour (navy and black beans) were utilised in the present study. Flavonoids, including flavonols, anthocyanins and tannins are largely responsible for the different pigmentations observed in beans[84], and generally darker beans have more diverse flavonoid profiles at higher concentrations compared with the light-coloured beans[21,85,86]. Therefore, by comparing the effects of these varieties, a secondary objective was to determine whether bean phenolic profiles affect the colitis phenotype.

## Materials and methods

### Preparation of bean flours and diets and experimental design

Navy beans (ACUG 10-B2) and black beans (ACUG 10-6) were provided by Dr Ali Navabi, University of Guelph, Bean Breeding Program. Whole beans were soaked overnight in 250 ml of distilled water at room temperature and cooked in a slow cooker (Hamilton Beach) on high with 0·3 % (w/v) baking powder (Kraft Canada, Inc.) for 7 h. Cooked beans and the remaining cooking water were homogenised, and the resulting bean paste was freeze-dried by the Guelph Food Technology Centre (Guelph) to produce bean flour. Proximate analyses and analyses of the crude fibre content of bean flours were carried out by Maxxam Analytics (Table S1, available online). In the present study, three isoenergetic experimental diets were used: basal diet (BD); BD supplemented with 20 % navy bean (NB) flour; BD supplemented with 20 % black bean (BB) flour, which were prepared by Harlan Laboratories in accordance with the AIN-93G diet formulation with 5 % maize oil being substituted for 5 % soyabean oil, and cellulose content increased from 5 to 7 %, to ensure that the total dietary fibre content was equal across the diets (Table 1). The soluble fibre contents of the BD, NB diet and BB diet were analysed by Maxxam Analytics and determined to be 0·4, 0·8 and 1·5 g/100 g dry weight, respectively.

All experimental procedures were approved by the institutional animal care committee (University of Guelph; Animal Use Protocol# 10R067) in accordance with the guidelines of the Canadian Council of Animal Care. Male C57BL/6 mice aged 5 weeks were purchased from Charles River, housed three to four mice per cage under controlled conditions (temperature: 23±2°C; humidity: 30±5%; light: 12 h light−12 h dark cycle) and acclimatised for 1 week with free access to the BD and water. Mice were assigned to one of the three dietary groups (BD *n* 26, NB *n* 13 and BB *n* 13) such that the group mean body weights (BW) were similar (*P*>0·05) and were fed these diets for 2 weeks during which food intake and BW were recorded. After 2 weeks,

**Table 1.** Diet composition*

| Ingredients (g/kg diet) | Diets | | |
| --- | --- | --- | --- |
| | BD | NB | BB |
| Bean flour | 0 | 200 | 200 |
| Casein | 200 | 146 | 141 |
| L-Cystine | 3·0 | 3·0 | 3·0 |
| Maize starch | 378 | 284 | 284 |
| Maltodextrin | 132 | 132 | 132 |
| Sucrose | 100 | 100 | 100 |
| Maize oil | 70·0 | 66·0 | 67·0 |
| Cellulose | 70·0 | 21·4 | 25·4 |
| Mineral mix (AIN-93G-MX) | 35·0 | 35·0 | 35·0 |
| Vitamin mix (AIN-93-VX) | 10·0 | 10·0 | 10·0 |
| Choline bitartrate | 2·5 | 2·5 | 2·5 |
| TBHQ | 0·014 | 0·014 | 0·014 |

BD, basal diet; NB, navy bean; BB, black bean; TBHQ, tertiary butylhydroquinone.
* All diets are isoenergetic and contain 20 % protein, 7 % fat and 7 % fibre.

fresh faecal samples were snap-frozen and stored at $-80°C$ for later analysis of phenolic content and antioxidant activity. Experimental colitis was induced by administering 2% (w/v) dextran sodium sulphate (DSS; molecular weight 36 000–50 000; MP Biomedicals) in the drinking-water for 7 d. Half of the mice in the BD group ($n$ 13) were given DSS-free drinking-water and served as controls. All mice were allowed free access to food and water *ad libitum* throughout the study period.

During DSS exposure, food intake and water consumption were monitored. Additionally, mice were monitored daily for disease activity index (DAI), assessed by measuring BW, stool consistency and blood content in the stool. BW loss was calculated as the percentage difference compared with BW at the start of DSS exposure. Stool consistency and stool blood content were measured in fresh faecal samples using the scoring system summarised in Table S2 (available online). Beckman Coulter Hemoccult Sensa test (Fisher Scientific) was used to detect occult blood. Scores for BW loss percentage, stool consistency and stool blood were each on a scale from 0 to 3, with 0 representing the normal/healthy condition and 3 representing the worst disease outcome.

All mice were killed on day 7 of DSS exposure by cervical dislocation, blood was collected by cardiac puncture and serum was stored at $-80°C$. Colons were excised (from the caeco-colonic junction to the rectum), measured for length, weighed and flushed before removing 0·5 cm sections of the proximal and distal colon, which were snap-frozen in liquid $N_2$ and stored at $-80°C$. The remaining colon was Swiss-rolled and fixed in 10% buffered formalin solution for later histological analysis. Spleen, caecum and caecal contents were also weighed and stored at $-80°C$.

### Colon histopathology and immunohistochemistry

Paraffin-embedded colon Swiss rolls were sectioned (5 μm) and stained with haematoxylin and eosin or Alcian blue/Nuclear Fast Red for histological scoring of colitis severity. From the proximal to the distal colon, a crypt erosion score (1–6) and a goblet cell/mucin depletion score (1–6) were assigned using haematoxylin and eosin- or Alcian blue/Nuclear Fast Red-stained sections, respectively (see Table S3 (available online) for a description of the scoring system). Each score was then multiplied by the percentage of colon area affected, such that the most damaged colon would have a maximum score of 600. The final histological score was obtained by averaging the above crypt erosion and goblet cell depletion scores. All colon sections were viewed (200–400✕ magnification) and scored blindly using a BX51 microscope (Olympus Canada, Inc.) equipped with an Olympus DP72 Digital Camera system, and the scoring system was developed in consultation with a veterinary pathologist (G. A. W.). This scoring method was also validated by correlating the scores with colonic myeloperoxidase (MPO) levels and colon weight:length ratio, which are well-known biomarkers of colonic inflammation[87,88].

To determine the level of apoptosis within the colon epithelium of DSS-exposed mice ($n$ 7–8 per dietary group),

immunohistochemistry was performed using the active caspase-3 antibody (cleaved caspase-3 (ASP175) (#9661); Cell Signaling Technology). Briefly, formalin-fixed, paraffin-embedded sections of colon Swiss rolls were deparaffinised in xylene and rehydrated in graded ethanol, and antigens were retrieved in 10 mM-citrate buffer (pH 6·0) in a microwave for 15 min. The sections were incubated in a solution of 3% $H_2O_2$ for 10 min to inhibit endogenous peroxidase activity. The sections were then blocked with 5% normal goat serum in Tris-buffered saline with Tween-20 for 1 h at room temperature. Primary antibody (1:300) was prepared in SignalStain Antibody Diluent (#8112) (Cell Signaling Technology) and added to the sections overnight at 4°C in a humidified chamber. Washed sections were then treated with the Signal-Stain Boost IHC Detection Reagent (HRP, Rabbit) #8114 (Cell Signaling Technology) for 30 min in a humidified chamber, followed by treatment with a liquid diaminobenzidine + substrate chromogen system (Dako Canada) for 3·5 min. The sections were counterstained with haematoxylin, dehydrated and coverslipped using Permount (Fisher Scientific). Negative control sections were treated the same as described above, except primary antibody was replaced with antibody diluent.

For the quantification of apoptosis, the sections were viewed blindly (400✕ magnification) using a BX51 microscope (Olympus Canada, Inc.) equipped with an Olympus DP72 Digital Camera system. The numbers of positive cells (brown staining) were counted throughout the entire colon, which was further divided into the proximal and mid–distal colon sections. Furthermore, apoptosis within areas showing intact crypt architecture was quantified separately from apoptosis within areas identified as ulcers (no crypt architecture remaining). The length of the entire colon, as well as that of the proximal and mid–distal colon, was measured, and apoptosis is expressed as the number of positive cells/mm colon.

### Colonic myeloperoxidase and glutathione peroxidase assays

Distal colon tissue samples were homogenised in a lysis buffer (200 μl/10 mg colon) containing 28 μg aprotinin/ml, 1 μg leupeptin/ml and 1 mM-phenylmethanesulfonyl fluoride in a Tris buffer with 10% glycerin and 5 mM-EDTA, and the protein concentration of each sample was determined using a colorimetric Bio-Rad protein assay (Bio-Rad). Colonic MPO levels were measured by ELISA (Hycult Biotech) following the manufacturer's instructions, and the final values were corrected for protein concentration. Glutathione peroxidase (GPx) activity in colon lysates was measured using the Chemical GPx Kit (Cayman Chemical) as per the manufacturer's instructions, and enzyme activity is expressed as GPx units/mg protein.

### Serum cytokine analysis

Serum concentrations of IL-10, IL-1β, IL-6, IL-17A TNF-α and interferon-γ (IFN-γ) were simultaneously assayed using the Bio-PlexPro Assay (Bio-Rad). All samples were run in

duplicate using the Bio-Plex 200 System (Bio-Rad) according to the manufacturer's instructions.

### Colonic mRNA expression analysis

Proximal colon RNA was isolated by TRIzol/chloroform extraction and purified using the RNeasy kit (Qiagen). Samples were DNase treated using the RNase-Free DNase kit (Qiagen), and RNA quality was assessed using Experion RNA Analysis Kits (Bio-Rad). Total RNA (1 µg) was converted to complementary DNA using the $RT^2$ First Strand Kit (Qiagen), and quantitative real-time PCR was carried out on the ViiA7 Real-Time PCR System (Life Technologies) using the Mouse Inflammatory Response and Autoimmunity PCR Arrays (PAMM-077Z, Qiagen). Data were analysed using the $\Delta\Delta C_T$ method to determine the expression level of each transcript normalised to that of the housekeeping genes provided on the arrays. All data were normalised to the relative expression level of each gene of interest in healthy mice that consumed the BD.

### Caecal SCFA analyses

The concentrations of caecal SCFA (acetate, propionate and butyrate) were determined by GC as described by Sugawara & Miyazawa[89] with slight modifications. Caecal contents were first freeze-dried, and after homogenisation[89], the resultant supernatant was filtered through a 0·2 µm polyvinylidene difluoride filter (Chromatographic Specialties Inc.), and 1 µl of the filtrate was used for injection into the GC system (Hewlett Packard 5890 Series II) using J & W GC columns (CP WAX 52 CB 30 × 0·53 (1·0); Agilent Technologies). Injector and detector temperatures were maintained at 240 and 280°C, respectively. The oven temperature, after an initial period of 1 min at 75°C, was increased to 180°C at a rate of 6°C/min, then increased by 10°C/min and held at 230°C for 6 min. The final oven temperature was increased by 2°C/min and kept at 240°C for 5 min. The peaks were identified by comparing their retention times with acetate, propionate and butyrate GC standards (Sigma-Aldrich). Data collection was managed using the HPCHEM software developed by Agilent Technologies.

### Phenolic compound extraction

Total phenolics were extracted from the NB and BB flours and all the three experimental diets (1 g) using the microwave-assisted extraction method[90]. The supernatant from the NB and BB flour phenolic extracts (2 ml) was hydrolysed with 1 ml of 6 M-HCl at 85°C for 2 h for later anthocyanin profile determination (Fig. S1, available online). Faecal phenolic compounds were extracted as described by Espín *et al.*[91].

### Total phenolic content and total flavonoid content assays

The Folin−Ciocalteu method was used to determine the total phenolic content in both diet and faecal extracts as described by Li *et al.*[92], and it is expressed as mg gallic acid equivalents per sample (g). Total flavonoid content in diet and faecal extracts was determined using a colorimetric method as described by Zhishen *et al.*[93] with slight modifications. Briefly, 100 µl of catechin (0−100 mg/l) or sample extracts were added to a 2 ml microcentrifuge tube containing 400 µl of double-distilled water and 30 µl of 0·75 M-NaNO₂. After 5 min, 30 µl of 0·75 M-AlCl₃ were added, followed by the addition of 200 µl of 1 M-NaOH 1 min later. The mixtures were diluted with 240 µl of double-distilled water and vortexed, and absorbance was read at 510 nm using a microplate reader (PowerWave XS2; BioTek). Total flavonoid content is expressed as mg catechin equivalents per sample (g).

### Antioxidant activity assay

Antioxidant activity was measured using the ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays as described elsewhere[92].

### Statistical analyses

All data were analysed by one-way ANOVA (main effect: diet) with the exception of food intake, BW and BW change percentage, which were analysed by two-way ANOVA (main effects: diet and time). The Student−Newman−Keuls, least-squares means or Dunn non-parametric tests were used for all *post hoc* analyses. Differences were considered significant with $P < 0.05$, and all values are expressed as means with their standard errors. Pearson's correlation analyses were carried out to examine the relationship between histological scores and colonic MPO activity or colon weight:length ratio. Statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, Inc.), Sigma Plot 12.0 (Systat Software, Inc.) and SAS system (SAS Institute) for Windows (version 9.0).

## Results

### Phenolic content, antioxidant activity and anthocyanin profile

Phenolic content in both the experimental diets and mouse faecal samples collected before DSS exposure was determined. The BB diet and BB group faecal samples had the highest phenolic content (total phenolic content and total flavonoid content) and antioxidant activity (FRAP and ORAC), followed by the NB diet and NB group faecal samples and then the BD and BD group faecal samples, which had the lowest values in all measurements (Table 2). Although ORAC was undetectable in the BD, faecal samples collected from the BD group exhibited a detectable ORAC, possibly due to the presence of other faecal or microbiota-derived phenolic compounds. The anthocyanin profile of both the NB and BB flours revealed five anthocyanin compounds (delphinidin, cyaniding, petunidin, pelargonidin and malvinidin; Fig. S1, available online), which were identified only in the BB flour.

**Table 2.** Phenolic compound content (total phenolic content (TPC) and total flavonoid equivalents (TFC)) and antioxidant activity (ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC)) in the diet and mouse faecal extracts

(Mean values with their standard errors)*

| Assays | BD | | NB | | BB | |
|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM |
| TPC (mg GAE/g) | | | | | | |
| Diet | 0.13[c] | 0.0 | 0.24[b] | 0.0 | 0.56[a] | 0.0 |
| Faeces | 0.48[c] | 0.03 | 0.65[b] | 0.02 | 0.96[a] | 0.08 |
| TFC (mg CE/g) | | | | | | |
| Diet | 0.00[c] | 0.00 | 0.02[b] | 0.00 | 0.10[a] | 0.004 |
| Faeces | 0.13[c] | 0.01 | 0.28[b] | 0.01 | 0.32[a] | 0.02 |
| FRAP (μM-AAE/g) | | | | | | |
| Diet | 0.10[c] | 0.01 | 0.75[b] | 0.03 | 2.98[a] | 0.2 |
| Faeces | 1.57[c] | 0.1 | 2.05[b] | 0.1 | 3.75[a] | 0.3 |
| ORAC (μM-TE/g) | | | | | | |
| Diet | 0.0[c] | 0 | 21[b] | 2 | 28[a] | 2 |
| Faeces | 41[c] | 4 | 71[b] | 7 | 128[a] | 11 |

BD, basal diet; NB, navy bean; BB, black bean; GAE, gallic acid equivalents; CE, catechin equivalents; AAE, acetic acid equivalents; TE, Trolox equivalents.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0.05$).
* For the diet analysis, $n$ 3 per dietary group; for the faecal analysis, $n$ 6–8 per dietary group.

## Dextran sodium sulphate-induced colitis disease activity index

Before DSS exposure, there was no difference in food intake or BW between the dietary groups ($P>0.05$; Table S3, available online). During DSS exposure, mice continuously reduced their intake of food and water compared with the BD-fed healthy control mice; however, there were no significant differences between the DSS-exposed groups (Fig. 1). Throughout the DSS exposure period, mice were monitored daily for phenotypic changes (BW loss, stool consistency and stool blood content), which collectively comprise the DAI score (Fig. 1). After 1 d of DSS exposure, all the dietary groups exhibited an increased DAI score compared with the healthy age-matched control mice (DAI = 0), but there were no differences between the DSS-treated dietary groups. Only on day 3 of DSS exposure, the BB + DSS group exhibited an improved DAI score compared with the NB + DSS group; however, it still did not differ from the BD + DSS group ($P<0.05$). At all the other time points throughout the DSS exposure period, there was no difference in DAI scores between the DSS-treated dietary groups.

## Caecal SCFA concentrations

There was a reduction in caecal weight (tissue + caecal contents) in the BD + DSS group compared with that in both the BD-fed group and the NB diet-fed and BB diet-fed DSS-treated groups (Table 3). Analysis of caecal contents revealed that DSS exposure (BD + DSS) reduced the total SCFA concentrations relative to the BD-alone dietary treatment, and this was attenuated by both the NB and BB diets, which had increased total SCFA concentrations (Table 3). Analysis of the specific SCFA species revealed that both bean diets significantly increased caecal acetate, propionate and butyrate concentrations relative to both the BD + DSS and BD-alone dietary treatments ($P<0.05$; Table 3).

## Colon histology and biomarkers of immune cell infiltration and oxidative stress

Phenotypic changes in colon size (i.e. increased colon weight: length ratios) were apparent in all the DSS-treated mice compared with the healthy control mice (Fig. 2), which were further increased in mice that consumed the bean-containing diets compared with those in the BD + DSS
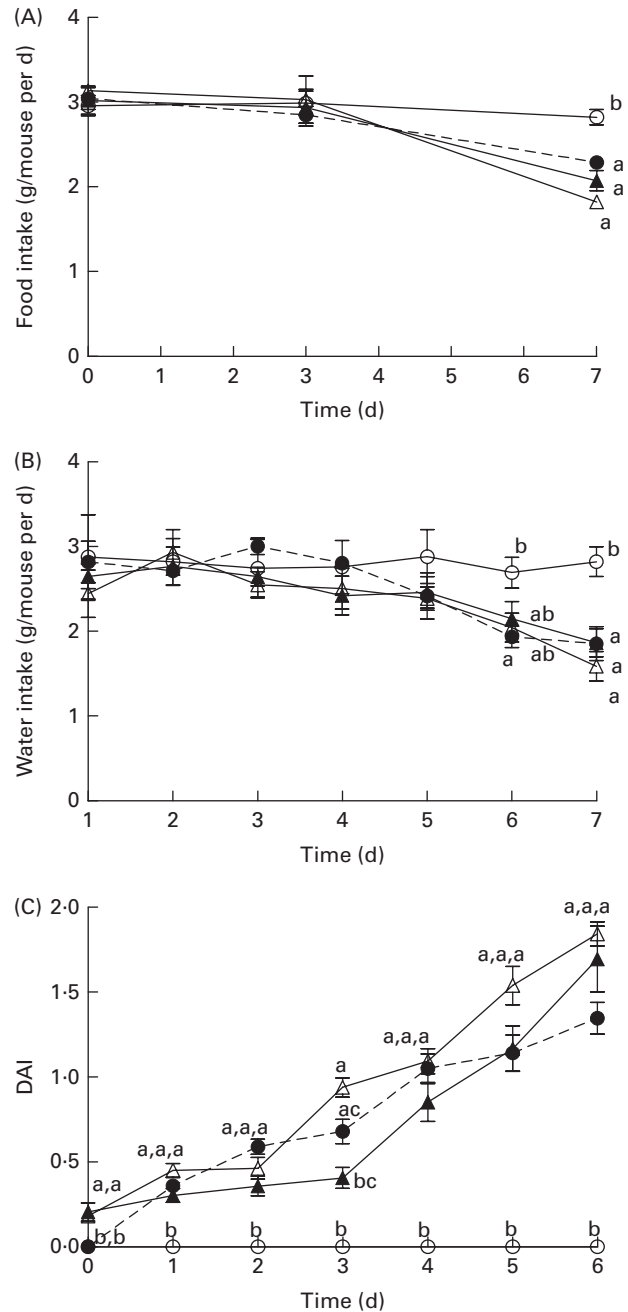


**Fig. 1.** Time course (7 d) of changes in (A) food intake, (B) water intake and (C) the disease activity index (DAI) score in healthy mice fed the BD (○) and colitic mice fed the BD + DSS (●), NB + DSS (Δ) and BB + DSS (▲) diets. Values are means ($n$ 13 per dietary group), with their standard errors represented by vertical bars. [a,b,c] Mean values with unlike superscript letters were significantly different at each time point (day of DSS exposure) ($P<0.05$; Kruskal–Wallis test followed by Dunn's multiple comparison test). BD, basal diet; NB, navy bean; BB, black bean; DSS, dextran sodium sulphate.

**Table 3.** Effect of the bean diets on caecal weight and SCFA concentrations in colitic mice

(Mean values with their standard errors; *n* 13 per dietary group)

| | BD | | BD + DSS | | NB + DSS | | BB + DSS | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| Caecal weight (g) | 0·21[a] | 0·01 | 0·12[b] | 0·005 | 0·17[a,b] | 0·02 | 0·19[a] | 0·02 |
| Acetate (µmol/g DW) | 73·27[b,c] | 7·02 | 25·21[c] | 8·52 | 163·3[a] | 23·32 | 154·7[a,b] | 16·08 |
| Propionate (µmol/g DW) | 17·23[b] | 1·77 | 4·41[c] | 1·33 | 36·19[a] | 2·95 | 38·36[a] | 4·33 |
| Butyrate (µmol/g DW) | 20·65[b] | 2·36 | 4·50[c] | 1·7 | 29·09[a] | 2·18 | 31·61[a] | 3·74 |
| Total SCFA (µmol/g DW) | 111·1[b] | 10·73 | 34·11[c] | 11·5 | 228·6[a] | 27·27 | 224·7[a] | 23·14 |

BD, basal diet; DSS, dextran sodium sulphate; NB, navy bean; BB, black bean; DW, dry weight.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

group ($P < 0.05$). Colon tissue sections of the DSS-treated mice displayed an increase in crypt distortion and, as expected, had multiple erosive lesions (indicative of increased colonic damage) compared with those of the healthy control mice (representative images for all groups are shown in Fig. 2). Furthermore, the distribution of histological damage induced by DSS was similar in the proximal colon, mid-colon and distal colon (Fig. S2, available online). Both the NB + DSS and BB + DSS groups exhibited an increase in the total colon histological score compared with the BD + DSS group, indicating an aggravation of DSS-induced colonic damage (Fig. 2). Although the histological damage score was highest for the proximal colon and mid-colon sections of the bean diet-fed mice, a significant difference was observed only in the scores for the mid-colon section, wherein the NB diet-fed mice had significantly higher histological damage scores compared with the DSS control mice (Fig. S2, available online).

Distal colon GPx activity was increased in all the DSS-exposed groups compared with that in the healthy control mice, but it did not differ between the dietary groups, indicating that DSS-induced oxidative stress in the distal colon was not modulated by the bean diets (Fig. 2). Similarly, the distal colon MPO level (indicative of neutrophil infiltration) was increased in all the DSS-treated mice compared with that in the healthy control mice; however, it did not differ between the dietary groups (Fig. 2). Additionally, a significant positive correlation existed between histological scores and colon weight:length ratio ($r$ 0·5313; $P = 0.0192$) and colonic MPO levels ($r$ 0·6242; $P = 0.0033$), confirming that these biomarkers are reflective of increased colonic inflammation and damage.

## Colonic mRNA expression

All gene expression data were normalised to the expression levels in the healthy control mice (BD alone). As expected, DSS exposure led to the differential expression of 55 % of the genes assessed within the Mouse Inflammatory Response and Autoimmunity PCR Array pathway compared with the gene expression in the healthy control mice (BD *v.* BD + DSS; Table S4, available online). The magnitude of the change in gene expression between the dietary groups exposed to DSS is summarised in Table 4. Compared with the BD + DSS dietary treatment, both bean diets reduced the colonic mRNA expression of the inflammatory cytokines *IL-6*, *IFN-γ* and *IL-22* ($P < 0.05$) and up-regulated the

expression of the anti-inflammatory cytokine *IL-10* ($P = 0.01$). Additionally, both bean diets differentially affected the mucosal expression of genes involved in neutrophil chemotaxis by decreasing the expression of chemokine (C–X–C motif) ligand 1 (*Cxcl1*) and *Cxcl5* ($P = 0.008$ and $P = 0.037$, respectively) and increasing the expression of *IL-8* ($P = 0.04$). The mucosal mRNA expression of Toll-like receptor 4 (*Tlr4*) was modestly increased ($P = 0.003$) and the mRNA expression of Fas ligand (*FasL*) was also up-regulated by both bean diets ($P = 0.001$). Only the BB diet reduced the colonic mRNA expression of *IL-17A* compared with the BD + DSS dietary treatment ($P = 0.042$). Conversely, only the NB diet increased the mRNA expression of chemokine (C–C motif) ligand (*Ccl12*) and CCAAT/enhancer binding protein (C/EBP), β (*Cebpb*) ($P = 0.033$ and $P = 0.041$, respectively) and decreased the expression of *IL-9* ($P = 0.047$) compared with the BD + DSS dietary treatment.

## Colonic cleaved caspase-3 expression

The level of apoptosis in the colonic epithelium of the DSS-exposed mice was determined by cleaved caspase-3 immunostaining of sections from colon Swiss rolls. As shown in Fig. 3, colon tissue sections of the NB diet-fed and BB diet-fed DSS-exposed mice exhibited an increase in the number of apoptotic cells within crypts throughout the entire length of the colon, compared with those of the BD-fed DSS-exposed mice ($P < 0.05$). When the colon was divided into either the proximal or the mid–distal half, it was evident that apoptosis was increased in crypts from both locations of the colon in the NB diet-fed mice, compared with that in the BD-fed DSS-exposed group ($P < 0.05$). While this pattern was similar for the BB diet-fed mice, apoptosis was significantly higher only in the proximal colon of the BB diet-fed mice, compared with that in the BD-fed DSS-exposed mice (Fig. 3). Cleaved caspase-3 immunostaining was also assessed within ulcerated areas in the colon tissue sections; however, there was no significant difference in the mean number of apoptotic cells/mm ulcer between the BD-fed (16·09 (SEM 3·54)), NB diet-fed (30·66 (SEM 4·76)) and BB diet-fed (24·07 (SEM 3·65)) DSS-exposed groups ($P > 0.05$).

## Systemic response to dextran sodium sulphate exposure

DSS exposure increased the serum concentrations of all cytokines assessed compared with those of the healthy control

| | Colon size (mg/mm) | | Histological score | | MPO (ng/mg) | | GPx activity (units/mg protein) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| BD | 1·5[c] | 0·1 | 0·0 | 0·0 | 158·2[b] | 18·46 | 474[b] | 39 |
| BD+DSS | 2·4[b] | 0·07 | 294·5[b] | 10·5 | 2541[a] | 389·9 | 791[a] | 96 |
| NB+DSS | 3·0[a] | 0·1 | 406·6[a] | 20·9 | 6492[a] | 2357 | 802[a] | 111 |
| BB+DSS | 2·9[a] | 0·08 | 395·1[a] | 20·8 | 4525[a] | 1070 | 716[a] | 61 |
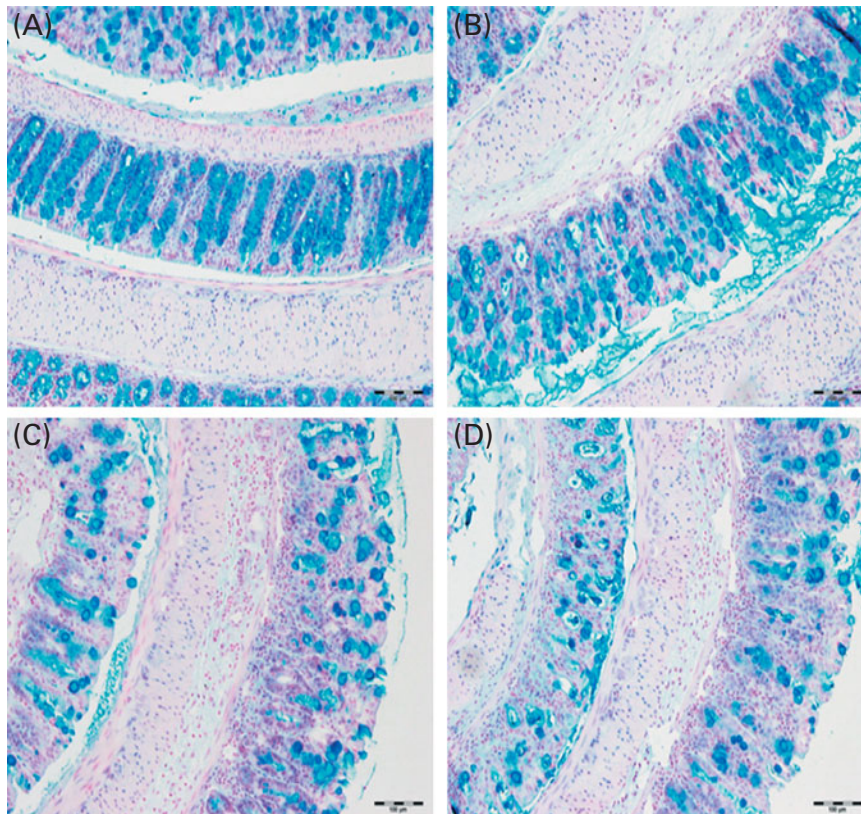


**Fig. 2.** Colitis severity as indicated by colon size, histological damage, myeloperoxidase (MPO) level and antioxidant (glutathione peroxidase, GPx) activity in healthy mice fed the basal diet (BD) and dextran sodium sulphate (DSS)-induced colitic mice fed the BD, navy bean (NB) diet and black bean (BB) diet. Colon size (n 12–13 per dietary group) expressed as colon weight (mg)/colon length (mm). Histological scores reflect both the degree of damage and the percentage of area damaged in the entire length of the colon (n 7 per dietary group). MPO levels and GPx activity (n 10–12 per dietary group). Values are means with their standard errors. [a,b,c] Mean values within a column with unlike superscript letters were significantly different for each analysis (P<0·05). Representative colon images of tissue sections of the (A) BD, (B) BD + DSS, (C) NB + DSS and (D) BB + DSS groups stained with Alcian blue/Nuclear Fast Red (200×; scale bar 100 μm).

mice (Fig. 4; P<0·05). In the DSS-treated mice, the serum concentrations of the inflammatory cytokines IL-1β, IFN-γ, TNFα and IL-17A were reduced by both the NB and BB diets (Fig. 4(A)–(D); P<0·05), whereas the circulating concentrations of IL-6 and IL-10 did not differ between the dietary groups (P>0·05; Fig. 4(E) and (F)). Additionally, serum ORAC in the BD + DSS group was significantly lower than that in the healthy control mice, indicating a decrease in antioxidant capacity following DSS exposure, which was attenuated by both bean diets (Fig. 5). Similarly, splenic tissue weights were increased in response to DSS exposure (BD + DSS v. BD alone), which was attenuated in both the NB + DSS and BB + DSS groups (Fig. 5).

## Discussion

The present study assessed the effect of diets supplemented with 20 % cooked common bean flours (navy and black bean varieties) on the DSS-induced acute colitis phenotype. It was hypothesised that bean diets would beneficially modulate colonic inflammation in response to DSS due to the fermentable fibre components (i.e. resistant starch, oligosaccharides and non-starch polysaccharides)[19,20] and phenolic compound content of beans[21], which are known to reduce colonic inflammation and improve colon health[38–41,75,79,80]. Common beans have been investigated previously for their role as modulators of gut health, primarily in reducing colon cancer risk[9,13,14,17,20,94]; however, the present study is the

**Table 4.** Dextran sodium sulphate (DSS)-induced fold changes in proximal colon mRNA expression*

(Mean values with their standard errors)

| Genes | BD + DSS Mean | SEM | NB + DSS Mean | SEM | BB + DSS Mean | SEM | P |
|---|---|---|---|---|---|---|---|
| Ccl12 | 5.7[a] | 1.0 | 14.5[b] | 4.5 | 5.5[a] | 1.4 | 0.033 |
| Cxcl1 | 19.6[a] | 5.7 | 5.7[b] | 1.5 | 3.8[b] | 0.7 | 0.008 |
| Cxcl5 | 75.0[a] | 15.6 | 19.2[b] | 5.6 | 10.5[b] | 2.3 | 0.037 |
| Cebpb | 1.0[a] | 0.07 | 3.2[b] | 0.9 | 2.2[a,b] | 0.7 | 0.041 |
| FasL | 0.7[a] | 0.2 | 2.3[b] | 0.3 | 1.6[b] | 0.4 | 0.001 |
| IL-10 | 1.7[a] | 0.4 | 5.6[b] | 1.3 | 6.0[b] | 3.1 | 0.038 |
| IFN-γ | 6.1[a] | 2.6 | 2.7[b] | 1.0 | 2.3[b] | 0.6 | 0.032 |
| IL-22 | 88.6[a] | 9.4 | 49.1[b] | 10.6 | 29.7[b] | 2.2 | 0.036 |
| IL-6 | 396.6[a] | 56.8 | 79.8[b] | 18.7 | 56.4[b] | 10.5 | 0.003 |
| IL-8 | 0.9[a] | 0.2 | 3.0[b] | 0.2 | 3.0[b] | 0.8 | 0.044 |
| IL-9 | 3.2[a] | 0.7 | 1.5[b] | 0.5 | 1.8[a,b] | 0.5 | 0.047 |
| IL-17A | 7.4[a] | 1.4 | 4.4[a,b] | 2.4 | 1.3[b] | 0.2 | 0.042 |
| Tlr4 | 0.4[a] | 0.08 | 1.0[b] | 0.08 | 1.0[b] | 0.2 | 0.003 |

BD, basal diet, NB, navy bean; BB, black bean; Ccl, chemokine (C–C motif) ligand; Cxcl, chemokine (C–X–C motif) ligand; Cebpb, CCAAT/enhancer binding protein (C/EBP), β; FasL, Fas ligand; IFN-γ, interferon-γ; Tlr, Toll-like receptor.

[a,b] Mean values within a row with unlike superscript letters were significantly different (P < 0.05).

* Values reported are fold changes in the colonic mRNA expression of the DSS-treated mice (n 6–7 per dietary group) normalised to the expression level of the BD-fed healthy control mice (n 4). The effect of diet on DSS-induced changes in mRNA expression for each gene detected on the Mouse Inflammatory Response and Autoimmunity PCR Arrays (PAMM-077Z; Qiagen) was analysed by one-way ANOVA, and only the results obtained for genes that were differentially expressed between the dietary groups in response to DSS are reported.

first to demonstrate their role in the modulation of colitis and colitis-associated systemic inflammation.

In the present study, critical mediators of colonic and systemic inflammation and colonic mucosal damage were clearly

modulated by the bean diets in mice exposed to DSS. Evidence of a reduced local (colonic) inflammatory response to DSS in the bean diet-fed groups was supported by the reduced mRNA expression of the pro-inflammatory cytokines IL-6 and IFN-γ and the up-regulated mRNA expression of the anti-inflammatory cytokine IL-10 (Table 4). The bean-associated anti-inflammatory phenotype was extended systemically, as circulating concentrations of the inflammatory cytokines IL-1β, IFN-γ, TNF-α and IL-17A were reduced (Fig. 4), serum antioxidant capacity was preserved and spleen hypertrophy was attenuated (Fig. 5) in the NB and BB dietary groups. This effect could be interpreted to reflect a reduced magnitude in the inflammatory response generated following exposure to DSS, as circulating cytokine concentrations represent a spillover into the extravascular compartment wherein inflammatory responses are generated[95]. On the other hand, the bean diets enhanced DSS-induced colon histological damage (Fig. 2) and increased the mRNA expression of FasL (apoptosis mediator) (Table 4) and the number of apoptotic cells in the colon (Fig. 3), indicating the aggravation of DSS-induced colitis. Overall, the present results demonstrate that bean diets beneficially modulate some biomarkers of colonic and systemic inflammation while simultaneously enhancing DSS-induced colonic damage and apoptosis.

The beneficial effects observed following the consumption of bean diets may have been driven by the SCFA, in particular, butyrate – a microbial fermentation end product of non-digestible bean components[11,12]. Mechanistically, butyrate is an important energy source for the maintenance

(A)

| Group | Entire colon Mean | SEM | Proximal colon Mean | SEM | Mid and distal colon Mean | SEM |
|---|---|---|---|---|---|---|
| BD+DSS | 14.38[b] | 1.82 | 10.86[b] | 1.45 | 15.05[b] | 2.73 |
| NB+DSS | 34.88[a] | 3.01 | 36.56[a] | 5.94 | 37.2[a] | 5.02 |
| BB+DSS | 25.41[a] | 2.52 | 34.32[a] | 7.11 | 22.27[ab] | 3.44 |

(B)



BD+DSS          NB+DSS
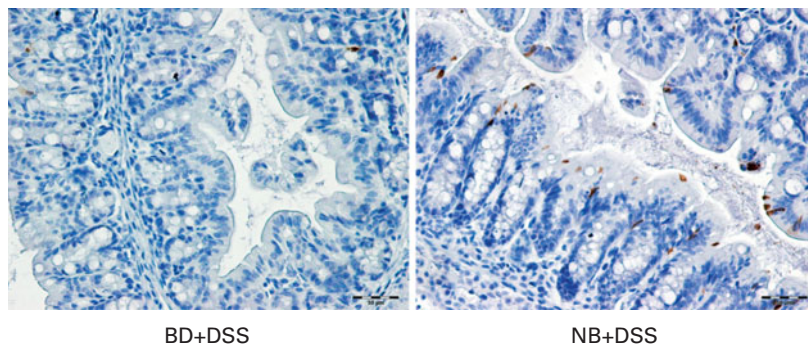
**Fig. 3.** Cleaved caspase-3-positive apoptotic cells in the colonic epithelium of the dextran sodium sulphate (DSS)-exposed mice. Cleaved caspase-3 expression was analysed by immunohistochemistry in the colon sections of the DSS-treated mice fed the basal diet (BD), navy bean (NB) diet or black bean (BB) diet. (A) Total number of positive cells/mm colon within crypt-containing areas along the entire colon, proximal colon, and mid and distal colon. Values are means with their standard errors. [a,b] Mean values within a column with unlike superscript letters were significantly different (P < 0.05; Kruskal–Wallis followed by Dunn's multiple comparison test). (B) Representative images of the proximal colon of the DSS-exposed BD-fed, NB diet-fed and BB diet-fed mice, wherein brown-stained cells demonstrate cleaved caspase-3 immunostaining (500×; scale bar 50 μm).
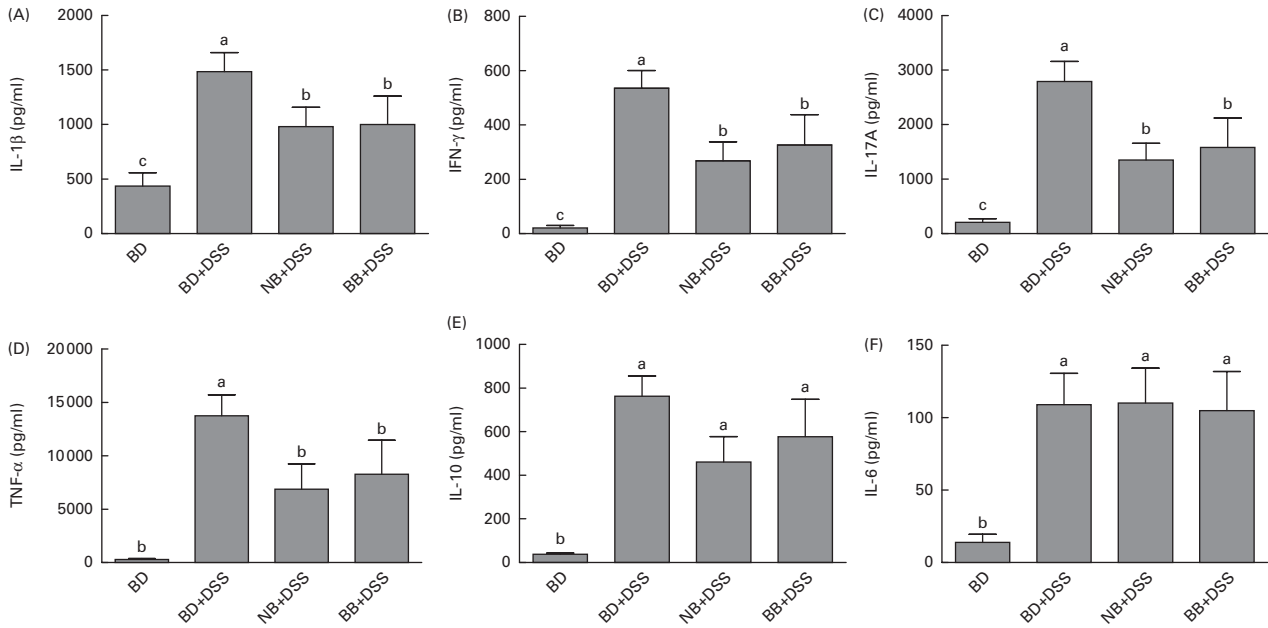
**Fig. 4.** Serum cytokine concentrations of (A) IL-1β, (B) interferon-γ (IFN-γ), (C) IL-17A (D) TNF-α, (E) IL-10 and (F) IL-6 in healthy mice fed the basal diet (BD) and dextran sodium sulphate (DSS)-induced colitic mice fed the BD, navy bean (NB) diet and black bean (BB) diet. Values are means, with their standard errors represented by vertical bars. [a,b,c] Mean values with unlike superscript letters were significantly different ($P < 0.05$).

and growth of colonocytes, enhancing the integrity of the gut barrier and exerting direct effects within the colon epithelium through the modulation of inflammation-related signalling pathways, in part, through the inhibition of histone deacetylase activity[22–24]. In the present study, the caecal concentrations of SCFA (acetate, propionate and butyrate) were increased by the bean diets (Table 3). Furthermore, proximal colon samples of the bean diet-fed mice exhibited reduced mRNA expression of *IFN-γ*, *IL-6*, and *Cxcl5*, as well as increased expression of *IL-10* (Table 4), pro- and anti-inflammatory mediators previously been shown to be modulated by butyrate[96–98]. Interestingly, although the bean diets reduced the proximal colon mRNA expression of the chemokines *Cxcl1* and *Cxcl5*, which are involved in the recruitment of immune cells to the inflamed colon[99], distal colon MPO levels (biomarker of neutrophil infiltration) did not differ

significantly between the DSS-exposed groups (Fig. 2). Generally, SCFA are more concentrated in the proximal colon and gradually deplete as the faeces moves through the colon[100]. Therefore, if the bean-induced changes in chemokine mRNA expression in the proximal colon were mediated by butyrate, this effect may not have persisted in the distal regions of the colon, where butyrate concentrations are low.

Beans are also a rich source of phenolic compounds[21,85,86], and it was hypothesised that higher phenolic content and antioxidant activity may result in lower oxidative stress and, therefore, an attenuation of colitis symptoms. Both IBD and DSS-induced animal models of colitis are associated with increased colonic and systemic biomarkers of oxidative stress[55–58,101,102]. Oxidative stress results from the increased production of reactive oxygen species and reactive nitrogen species, produced mainly by activated inflammatory cells,
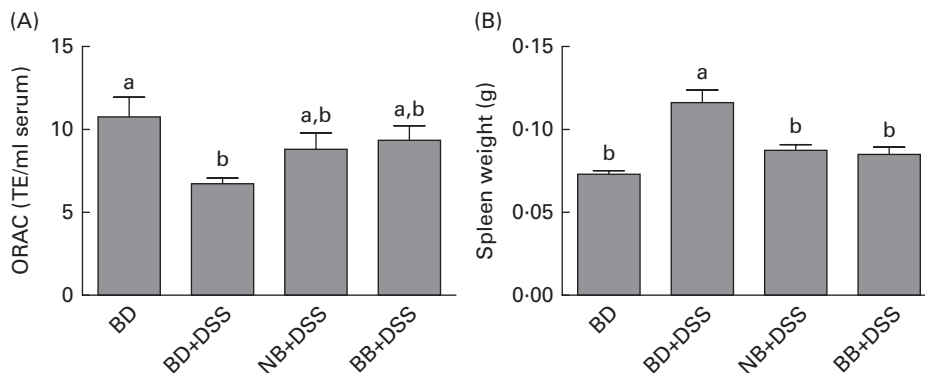


**Fig. 5.** Systemic effects of the bean diets on spleen weight and serum antioxidant status in healthy mice fed the basal diet (BD) and dextran sodium sulphate (DSS)-induced colitic mice fed the BD, navy bean (NB) diet and black bean (BB) diet. (A) Serum antioxidant capacity measured by oxygen radical absorbance capacity (ORAC, *n* 5–6 per dietary group). (B) Spleen weight (*n* 11–13 per dietary group). Values are means, with their standard errors represented by vertical bars. [a,b] Mean values with unlike superscript letters were significantly different ($P < 0.05$). TE, Trolox equivalents.

which aggravate colonic damage and perpetuate the inflammatory cycle[102,103]. Therefore, antioxidants are currently being investigated for their role in the reduction of oxidative stress in colitis[37,104–106]. Both bean diets contained higher phenolic compound content (increased total phenolic content and total flavonoid content) and antioxidant potential (increased FRAP and ORAC) compared with the BD (Table 2). In both instances, the BB diet had the highest phenolic compound load and antioxidant activity, which could be attributed to the addition of anthocyanins that were present only in the BB diet (Fig. S1, available online). Moreover, faecal phenolic compound content and antioxidant activity were elevated in mice that consumed both the bean-containing diets compared with those in mice that consumed the BD (Table 2), with the highest levels being detected in samples obtained from mice that consumed the BB diet, thereby demonstrating that these compounds were reaching the distal colon and could exert direct effects during colitis.

Mechanistically, however, the role of bean phenolics as antioxidants in the colon tissue was not supported in the present study. DSS is known to increase oxidative stress in the colon tissue, thereby increasing tissue antioxidant enzyme activity (i.e. GPx)[107,108]. The present study supports previous findings, demonstrating that DSS exposure increases colonic GPx activity and MPO levels (enzyme involved in free-radical generation)[102] in the colon tissue (Fig. 2). Similarly, serum ORAC, a measure of systemic antioxidant capacity, was reduced in DSS-exposed mice fed the BD (Fig. 5), indicating the depletion of endogenous antioxidants potentially due to elevated oxidative stress[101,109]. However, colonic GPx activity in the bean diet-fed groups did not differ from that in the DSS control mice, indicating that the bean phenolics were not acting as antioxidants in the colon tissue. On the other hand, the bean diet-fed DSS-exposed mice had serum ORAC that did not differ from that of the healthy control mice, indicating improved or sustained systemic antioxidant capacity (Fig. 5). Whether this indicates an increase in circulating antioxidants due to phenolic-rich bean consumption or a general reduction in systemic inflammation, and thus oxidative stress, in the bean diet-fed mice is currently unknown. However, the reduction of serum cytokine concentrations (Fig. 4) and attenuation of spleen hypertrophy (Fig. 5) induced by the bean diets indicate an overall reduction of systemic inflammation.

Phenolic compounds are also known to induce a myriad of cellular effects, beyond that of their antioxidant potential. One mechanism through which bean phenolic compounds exert an anti-inflammatory effect is the modulation of NF-κB signalling and subsequent expression of inflammatory mediators such as IL-6, TNF-α and IL-1β[38–41,110–117]. Interestingly, despite the BB diet having a higher phenolic content, both the NB and BB diets exhibited similar effects on inflammatory cytokine expression by reducing the colonic mRNA levels of *IL-6* (Table 4) and the circulating concentrations of both TNF-α and IL-1β (Fig. 4). However, compared with the BD + DSS dietary treatment, only the BB diet reduced the colonic mRNA expression of *IL-17A*, indicating an additional

anti-inflammatory benefit that could be attributed to the presence of anthocyanins, which have been shown to reduce colitis severity[118,119]. Collectively, our data demonstrate that bean phenolic compounds may account, at least in part, for the observed reduction of pro-inflammatory cytokine concentrations, which contributes to the decrease in the severity of the colitis phenotype.

On the other hand, the bean diets also induced a significant increase in the colonic architectural damage caused by DSS (Fig. 2). While the mechanisms by which beans mediated this response are not yet clear, both bean diets up-regulated the proximal colon mRNA expression of *FasL* compared with that in the DSS control group (Table 4). FasL targets cells to undergo apoptosis via the trimerisation of the Fas receptor and subsequent recruitment of caspase 8, which in turn activates caspase-3, leading to apoptosis[120]. We also demonstrated an increase in the number of apoptotic (cleaved caspase-3) cells within the colon crypts of the bean diet-fed mice compared with that in the DSS control group (Fig. 3). An increase in apoptosis can result in reduced gut barrier integrity, impaired wound healing and delayed epithelial restitution, which would lead to increased or prolonged damage caused by DSS[121–124]. Thus, increased apoptosis may have played a role in the bean diet-mediated increase in colonic damage observed in the present study.

Currently, there is no evidence to suggest that cooked bean-supplemented diets would induce toxic or adverse effects in the colon. Although raw beans are known to contain a number of anti-nutritional components (i.e. lectins and protease inhibitors) that may adversely affect gut health, the method used for preparing the cooked bean flours (i.e. soaking and heating) in the present study might have reduced their content in the animal diets[8,125,126]. Furthermore, many animal studies of longer duration and higher doses of cooked beans have been conducted with no reports of adverse health effects, reduced dietary intake or growth rate[17,127–129]. The present results also confirm that the bean diets were well tolerated by the mice, as the growth rate and diet intake did not differ between the dietary groups (Table S3, available online). This may suggest that this phenomenon was related to the disease model and/or the colitis induction system employed in the present study, in particular, the use of DSS to induce colitis.

Increased colonic damage may be caused by increased DSS exposure; however, we did not observe differences in water intake during DSS exposure (Fig. 1), indicating that the intake of DSS did not differ between the dietary groups. On the other hand, there may have been differences in the delivery of DSS to the colon. In fact, earlier studies have reported that the consumption of bean-supplemented diets increases gastrointestinal transit time in rodents[130]. Therefore, the increased gastrointestinal transit time might have prolonged the exposure of the luminal contents to the intestinal tract, including DSS, thereby inducing greater mucosal damage in the bean diet-fed mice. Although the diets used in the present study contained equal macronutrient and total dietary fibre levels (Table 1), the bean diets contained an array of non-digestible fermentable components, which may have

British Journal of Nutrition

increased intestinal lumen viscosity, water-holding capacity of the digesta and intestinal transit time[131]. This potential caveat in the study of complex food matrixes in the DSS-induced colitis model requires further investigation, which we are currently exploring. Future studies assessing the dose and timing of bean consumption relative to colitis onset may be useful for determining the balance between the beneficial and adverse effects elicited by the bean diets in relation to the severity of the colitis phenotype. For example, the anti-inflammatory effects of beans may reduce the severity of colon inflammation upon initiation, but continued consumption during colitis relapse may ultimately aggravate disease symptoms.

In summary, dietary incorporation of whole cooked beans improved the DSS-induced colitis phenotype by reducing inflammatory mediator expression both locally within the inflamed colon and systemically, an effect attributable to the bean bioactive components, namely fermentation-derived SCFA and/or phenolic compounds. The amount of evidence supporting an anti-inflammatory role of diet-derived bioactives and extracts is increasing[132,133]; however, there is a lack of information regarding the impact of consuming whole foods. In the present study, whole beans were cooked in the same manner as commonly prepared domestically, thereby providing an additional advantage when translating the outcomes to the clinical setting. However, in the present study, to incorporate cooked beans into the experimental diets, a freeze-drying step was utilised. Freeze-drying is known to alter the physical characteristics of starch, leading to a potential increase in retrograded (i.e. resistant) starch content in the experimental bean diets and production of SCFA[11,134], thereby producing a potential limitation in translating the present results to those expected from consuming domestically processed beans. Furthermore, the bean supplementation level used in the present study (20 %) may be achievable for human consumption[135]; however, future dose–response studies are required to determine the potential health benefits of lower bean supplementation levels. In clinical practice, a combination of intervention strategies designed to reduce the inflammatory response, control IBD symptoms and prolong remission is commonly employed[66]. Dietary intervention represents an additional component that may be effective in combination with pharmaceuticals at reducing disease severity, and in this context, increased consumption of beans may prove beneficial. On the other hand, the bean-supplemented diets led to an increase in DSS-induced mucosal damage, an effect which we hypothesise to be caused by an interaction between the bean diets and the DSS. Therefore, a potential limitation may exist in the use of this colitis model when examining the role of complex food matrices in colonic inflammation; however, this hypothesis requires further study.

## Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114513004352

## Acknowledgements

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