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Inulin and fructo-oligosaccharides have divergent effects on colitis and commensal microbiota in HLA-B27 transgenic rats

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Abstract

Modulation of intestinal microbiota by non-digestible carbohydrates may reduce inflammation in inflammatory bowel disease (IBD). The aim of the present study was to assess the effects of inulin and fructo-oligosaccharides (FOS) on intestinal microbiota and colitis in HLA-B27 transgenic rats, a well-validated rodent model for IBD. In this study, 4-week-old rats were fed 8 g/kg body weight inulin or FOS for 12 weeks, or not. Faeces were collected at 4 and 16 weeks of age; and caecal samples were collected at necropsy. The effects of inulin and FOS on chronic intestinal inflammation were assessed using a gross gut score, histology score and levels of mucosal IL-1β. Intestinal microbiota were characterised by quantitative PCR and denaturing gradient gel electrophoresis. Colitis was significantly reduced in all FOS-fed rats compared to the control diet, whereas inulin decreased chronic intestinal inflammation in only half the number of animals. Quantitative analysis of caecal microbiota demonstrated that inulin increased the numbers of total bacteria and the *Bacteroides-Prevotella-Porphyromonas* group, FOS increased bifidobacteria, and both fructans decreased *Clostridium* cluster XI. In the faecal samples, both inulin and FOS decreased total bacteria, *Bacteroides-Prevotella-Porphyromonas* group, and *Clostridium* clusters XI and XIVa. FOS increased *Bifidobacterium* spp., and mediated a decrease of gene copies of Enterobacteriaceae and *Clostridium difficile* toxin B in faeces. SCFA concentrations in the faecal and caecal samples were unaffected by the diets. In conclusion, FOS increased the abundance of *Bifidobacterium* spp., whereas both fructans reduced *Clostridium* cluster XI and *C. difficile* toxin gene expression, correlating with a reduction of chronic intestinal inflammation.

Key words: Colitis: Fructo-oligosaccharides: Inulin: Denaturing gradient gel electrophoresis: Quantitative PCR: *Clostridium difficile*

Inflammatory bowel disease (IBD), encompassing ulcerative colitis (UC) and Crohn's disease, is a group of chronic intestinal disorders associated with uncontrolled inflammation within the gastrointestinal tract[1]. The pathogenesis of IBD is attributed to an interaction of genetic, immune and environmental factors[1]. Intestinal micro-organisms and their products play an important role in the initiation and perpetuation of chronic intestinal inflammation[2]. Inflammation occurs in areas with the highest number of luminal bacteria[3]. Broad-spectrum antibiotics[4] and surgical diversion of the faecal stream can prevent disease recurrence in Crohn's disease[5]. Moreover, microbial imbalance, called 'dysbiosis', is observed in IBD patients[6]. IBD patients have greater numbers of mucosa-associated intestinal bacteria compared to non-IBD controls[7]. A reduced abundance of Bacteroidetes and Firmicutes species and an increase of virulent *Escherichia coli* were observed in the mucosa-associated microbiota of IBD patients[7–10]. The role of microbiota in IBD was further confirmed in animal models for IBD. HLA-B27/human β2-microglobulin transgenic (TG) rats raised under specific pathogen-free environment spontaneously develop chronic colitis mimicking IBD, and arthritis. However, these TG rats raised in germ-free conditions fail to develop inflammation, but introduction of (specific) bacteria induces colitis[4,11,12]. Similarly, other rodent models of colitis remain disease-free in the absence of bacteria[13].

Modification of the gastrointestinal microbiota may restore the balance of the host bacteria and reduce inflammation.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; DP, degree of polymerisation; DSS, dextran sodium sulphate; FOS, fructo-oligosaccharides; GGS, gross gut score; IBD, inflammatory bowel disease; PC, principal component; qPCR, quantitative PCR; TG, transgenic; UC, ulcerative colitis.

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Non-digestible carbohydrates such as β-fructans stimulate the growth of specific resident bacteria in the gut and thus allow manipulation of host microbiota. Inulin and fructo-oligosaccharides (FOS) are linear β (2 → 1) linked fructans. Inulin has a degree of polymerisation (DP) between 10 and 60, whereas FOS has a DP varying between 2 and 10. Dietary inulin and FOS are not hydrolysed by mammalian enzymes, but are readily fermented by the bacterial community in the caecum and colon, and favour the growth of intestinal bifidobacteria[14,15]. These β-fructans occur at high levels in plants such as chicory, asparagus, leek, onions, garlic and Jerusalem artichokes, and are also produced by *Lactobacillus* spp. in cereal fermentations[16,17].

Studies in animal models for IBD indicate that a combination of inulin and FOS reduce chronic intestinal inflammation. Treatment of HLA-B27 TG rats with inulin and FOS reduced inflammation in conjunction with an increase of caecal *Lactobacillus* spp. and *Bifidobacterium* spp.[18,19]. Inulin and FOS alone or in combination with two strains of *Bifidobacterium infantis* also reduced inflammation in dextran sodium sulphate (DSS)-induced colitis in rats[20]. However, FOS exacerbated DSS-induced colitis in mice fed a purified diet[21]. Small clinical studies in patients with active UC reported reduced colonic inflammation after oral therapy with *Bifidobacterium longum* in combination with the prebiotics inulin and FOS[22], whereas a reduction of the inflammatory faecal marker calprotectin was reported in active UC patients administered inulin plus FOS[23]. However, the use of FOS plus inulin mixture was ineffective in a large randomised, placebo-controlled trial in patients with mild-to-moderately active Crohn's disease[24]. Whereas studies in animal models and some of the clinical trials indicate that prebiotics show promise in the treatment or prevention of IBD, the divergent outcomes of studies clearly demonstrate that additional data on the mechanisms of action of non-digestible carbohydrates are required before these can be recognised as a valid tool in the management of IBD.

Inulin and FOS differ in their effects on caecal, colonic and faecal microbiota of rats that were colonised with human microbiota[25]. Likewise, dietary β-glucans differing in their molecular weight exerted different effects on the intestinal microbiota in pigs[26]. However, past studies on dietary intervention to prevent colitis in rodent models provided only a partial characterisation of intestinal microbiota[18,20]. Moreover, no studies have been performed using β-fructans with different DP in a well-validated, spontaneous colitis model. It was therefore the aim of the present study to compare the effect of inulin and FOS on intestinal inflammation and to determine how changes in the profile of caecal and faecal microbiota correlate with colitis reduction in HLA-B27 TG rats, the model in which this prebiotic mixture was previously shown to be beneficial. Inflammation was assessed by a validated gross gut score (GGS), histology score, and by quantification of pro-inflammatory mucosal IL1-β concentration.

Materials and methods

Experimental design and sample collection

HLA-B27 TG rats, a validated colitis model, were used in this study. Animals were randomly assigned to three different treatment groups: (1) commercial standard diet as a control (5053 PicoLab® Rodent Diet 20; Lab Diet, Inc.); (2) FOS (average DP 4, Orafti P-95; Raffinerie Tirlemontoise); (3) inulin (average DP 25, Orafti HP). Then, 8 g/kg body weight of the respective fructans were mixed with the standard diet, a dose previously found to be optimal for its colitis-reducing effects[27]. Feed was provided *ad libitum* and the average feed consumption of the rats was 20 g of standard diet/d. The body weight of the animals was measured every 2 weeks and the fructan addition to the diet was adjusted according to the body weight. The number of animals included in the study was as follows: control group – thirteen females and nine males, FOS group – three females and nine males and inulin group – seven females and eight males. Treatment started at 4 weeks of age (before colitis occurred) and continued until 16 weeks of age. Faecal samples were collected from each group at 4 and 16 weeks of age. All rats were euthanised at 16 weeks of age. At necropsy, caecal and colonic tissues and their contents were taken for histology, quantification of mucosal IL-1β, as well as for microbiota analysis. All samples were immediately frozen and stored at −80°C. Animal trials were approved by the University of Alberta Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines.

Assessment of treatment effects on inflammation

Caecal and colon tissues were fixed and stained with haematoxylin and eosin, as previously described[4]. Blinded evaluation of caecal and colonic microscopic inflammation was performed using a validated histology score ranging from 0 to 4[4]. Histology score included the following parameters: (1) number of inflammatory and goblet cells, (2) mucosal thickening, (3) infiltration of submucosal cells and (4) destruction of the architecture of the intestinal epithelium. The degree of macroscopic inflammation was further assessed using a validated GGS based on a scale from 0 to 4, as previously described[4]. Caecal inflammation evaluated by GGS included the criteria (1) number of caecal nodules, (2) severity of mesenteric contractions, (3) severity of adhesions and (4) extent of caecal wall thickening. The mucosal IL1-β concentration in the caecal and colonic homogenates was quantified by a rat-specific IL1-β ELISA[11], using a commercial DuoSet ELISA Development System kit (R&D Systems, Inc.). Recombinant rat IL-1β with known concentration was used as standard for the ELISA assay and 2-fold dilutions of the standard were run on each plate. Results were calculated using total protein for normalisation of the targeted cytokine measurement and presented as μg IL-1β/g protein.

Genomic DNA extraction and quantitative PCR analysis

Bacterial DNA was extracted from the caecal and faecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, Inc.).

Quantity and quality of DNA were checked on a NanoDrop spectrophotometer system ND-1000, version 3.3.0 (Thermo Fisher Scientific, Inc.). Before PCR analysis, samples were diluted to contain comparable DNA concentrations.

Quantitative PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). The primers and probes based on 16S rRNA gene sequences were chosen to target total bacteria, *Bacteroides-Prevotella-Porphyromonas* spp., the dominant group of Gram-negative bacteria, *Clostridium* clusters IV and XIVa, the main Gram-positive bacterial groups in intestinal microbiota, as well as clostridial clusters I and XI, which include pathogens (Table 1). Genes encoding *Clostridium difficile* toxin B and *Clostridium perfringens* α toxin were quantified by qPCR to specifically identify toxin-producing organisms in these clusters (Table 1). Microbiota analysis further included the Enterobacteriaceae family, which also includes pathogens, and bacteria known to have beneficial effect on the host such as *Bifidobacterium* spp. and *Lactobacillus-Pediococcus-Leuconostoc-Weissella* spp. (*Lactobacillus* group; Table 1). Samples were analysed in duplicate in MicroAmp Fast Optical ninety-six-well reaction plates capped with MicroAmp Optical Adhesive Film (Applied Biosystems). The PCR reaction mixtures consisted of 12·5 μl Fast SYBR Green Master Mix (Applied Biosystems), 0·4 μM of each primer (Table 1), 2 μl of template DNA of caecal or faecal samples and sterile Milli-Q water to final volume of 25 μl. The cycling programme was as follows: initial denaturation at 95°C for 5 min; forty cycles of 95°C for 15 s, primer annealing at the optimal temperatures (Table 1) for 30 s, and extension at 72°C for 30 s. Melting curves were obtained by a stepwise increase of the temperature from 60 to 95°C (at 10 s/0·5°C). Melting-curve data were analysed to verify amplification of the correct targeted PCR products. For quantification with the gene-specific primers and the group-specific primers for *Clostridium* cluster XI, TaqMan assays were performed. Amplifications were carried out in a total volume of 25 μl, containing 12·5 μl TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), 0·4 μM of each primer (Table 1), 1 μl of TaqMan probe (100 nM), 2 μl of template DNA of caecal or faecal samples and 7·5 μl sterile Milli-Q water. These amplification conditions were as follows: one cycle at 50°C for 2 min; one cycle at 95°C for 10 min; forty-five cycles of 95°C for 20 s, primer annealing at the optimal temperatures (Table 1) for 30 s, and extension at 72°C for 45 s. Fluorescent emission was measured at the extension step for the SYBR Green assays and at the primer annealing step for TaqMan assays. For generation of standard curves, 10-fold serial dilutions of purified and quantified PCR products were used. The standard curves of the individual qPCR assays were obtained by PCR using primers listed in Table 1 and genomic DNA isolated from *Clostridium perfringens* ATCC 13124, *Clostridium difficile* 75, or DNA extracted from the faecal samples. Individual reactions of the standard curves were run in duplicate on each plate for the respective bacterial

Table 1. Primers and PCR conditions

Target group/gene	Oligonucleotide sequence (5′–3′)	T_m (°C)	Product size (bp)	Reference or source
Domain bacteria (total bacteria)	F: CGGYCCAGACTCCTACGGG R: TTACCGCGGCTGCTGGCAC	63	200	Lee *et al.*[50]
Lactobacillus-Pediococcus-Leuconostoc group	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG GC clamp-CACCGCTACACATGGAG*	63	341	Walter *et al.*[51] Heilig *et al.*[52] This study
Bacteroides-Prevotella-Porphyromonas group	F: GGTGTCGGCTTAAGTGCCAT R: CGGAYGTAAGGGCCGTGC GC clamp-CGGAYGTAAGGGCCGTGC*	60	140	Rinttila *et al.*[53] This study
Enterobacteriaceae family	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	63	195	Bartosch *et al.*[54]
Bifidobacterium spp.	F: TCGCGTCYGGTGTGAAAG R: CCACATCCAGCRTCCAC	60	243	Rinttila *et al.*[53]
	g-Bifid F: CTCCTGGAAACGGGTGG g-Bifid R GC: GC clamp-GGTGTTCTTCCCGATATCTACA†	65	596	Matsuki *et al.*[55]
Clostridium cluster I	F: ATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT	58	120	Rinttila *et al.*[53]
Clostridium cluster IV	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA GC clamp-CTTCCTCCGTTTTGTCAA*	60	239	Matsuki *et al.*[56] This study
Clostridium cluster XIVa	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	58	438–441	Matsuki *et al.*[55]
Clostridium cluster XI	F: ACGCTACTTGAGGAGGA R: GAGCCGTAGCCTTTCACT FAM-GTGCCAGCAGCCGCGGTAATACG-BHQ	58	139	Song *et al.*[57]
Clostridium perfringens α toxin	F: GCTAATGTTACTGCCGTTGA R: CCTCATTAGTTTTGCAACC 6FAM-GCGCAGGACATGTTAAGTTTG-TAMRA	55	109	Messelhäusser *et al.*[58]
Clostridium difficile toxin B	398CLDs: GAAAGTCCAAGTTTACGCTCAAT 399CLDas: GCTGCACCTAAACTTACACCA FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA	58	177	Van den Berg *et al.*[59]

T_m, melting temperature; F, forward; R, reverse.
* GC clamp sequence – CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG.
† GC clamp sequence – CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC.

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group. The detection limit was 10^2 copy numbers/g caecal content or faeces for the group-specific primers and primers for *C. difficile* toxin B and *C. perfringens* α toxin.

Analysis of faecal microbiota using PCR-denaturing gradient gel electrophoresis

A total of four sets of group-specific primers targeting regions of the bacterial 16S rRNA gene were used to investigate the diversity of *Lactobacillus* group, *Bifidobacterium* spp., *Bacteroides* group and *Clostridium* cluster IV by PCR-DGGE. PCR specific for bifidobacteria was performed using primers g-Bifid F and g-Bifid R-GC (Table 1) in a Gene Amp PCR System 9700 (Applied Biosystems). To assess diversity in the *Lactobacillus* group, *Bacteroides* group, and *Clostridium* cluster IV in faecal microbiota by DGGE, the respective group-specific primer sets were employed at the appropriate annealing temperature (Table 1). PCR products were then used as templates in a second PCR, performed with the same primer pairs with attached GC clamp to the 5′ end of the reverse primer (Table 1). Amplicons were checked by electrophoresis in 2% (w/v) agarose gel before DGGE analysis.

PCR fragments were analysed by DGGE with a DCode Universal Mutation Detection System (Bio-Rad) on a 6% (w/v) polyacrylamide gel (37·5:1 acrylamide–bisacrylamide). *Bifidobacterium*-specific amplicons were separated in 40–70% denaturing gradient (100% corresponds to 7 M-urea and 40% (w/v) formamide). Amplicons obtained with primers targeting *Lactobacillus* and *Bacteroides* groups, and *Clostridium* cluster IV were separated in a denaturing gradient from 30% to 55%. Electrophoresis was performed in buffer containing 40 mM-Tris, 20 mM-acetic acid, 1 mM-EDTA at a constant voltage of 130 V and a temperature of 60°C for 4 h. Gels were stained with SYBR Safe 1 × solution (Invitrogen) for 1 h and 30 min and photographed by UV transillumination. DGGE profiles were compared using Bionumerics software (version 4.01, Applied Maths) and similarities were expressed based on Pearson correlation coefficients. All gels were normalised using a reference sample with bands distributed throughout the whole gel.

DNA sequencing

A total of five samples were randomly chosen and amplified in duplicates with group-specific primers for bifidobacteria (Table 1). Samples were analysed by DGGE, and the DGGE bands obtained with primers g-Bifid F and g-Bifid R-GC were sequenced by service of Macrogen. Sequences were compared to those in the Ribosomal Database Project (rdp.cme.msu.edu/seqmatch). The GenBank accession numbers for the sequences are HQ283419, HQ283420, HQ283421, HQ283422 and HQ268606.

Analysis of SCFA

Caecal contents and faeces (100 mg) were mixed with 300 μl water, vortexed vigorously and centrifuged for 15 min at 20 800 ***g***. The supernatant (100 μl) was mixed with 300 μl 7% perchloric acid, incubated at 4°C overnight and the precipitates were removed by centrifugation for 5 min at 20 800 ***g***. SCFA concentration was determined using HPLC (Agilent 1200 Series; Agilent Technologies, Inc.) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories). The column was eluted with 5 mM-H_2SO_4 at a temperature of 70°C and a flow rate of 0·4 ml/min. Detection of the SCFA was achieved employing UV (210 nm) and refractive index detectors. External standards were used to calculate the concentration of the SCFA.

Statistical analysis

Data analysis was performed using the mixed procedure (PROC MIXED) of the Statistical Analysis Systems (SAS Insti-

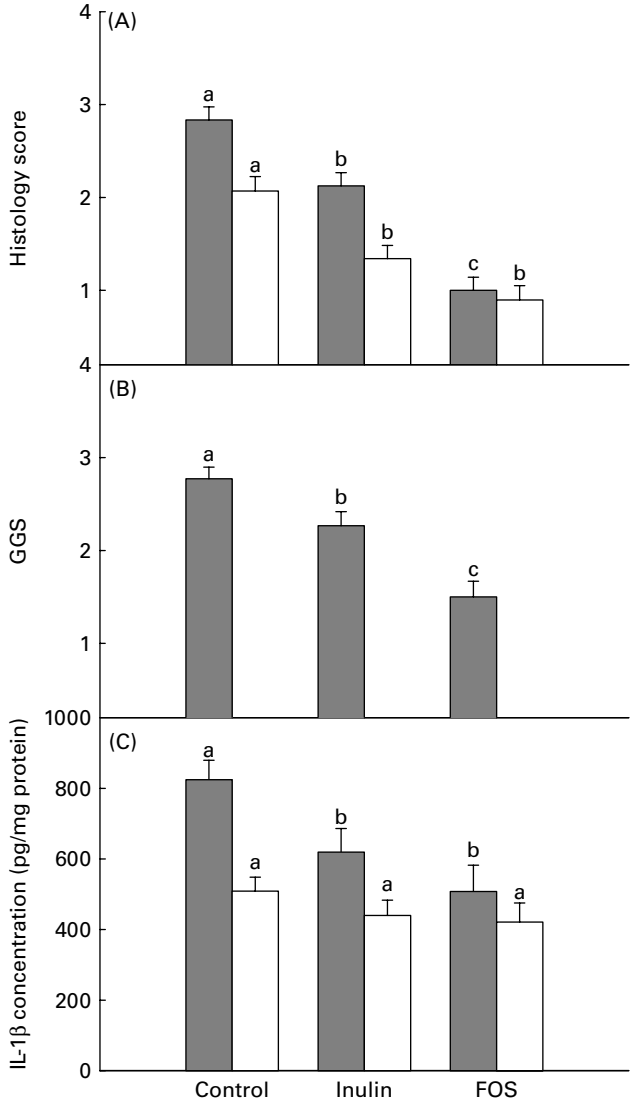

Fig. 1. (A) Histology score, (B) gross gut score (GGS) and (C) IL-1β concentration of caecal (■) and colonic (□) tissue samples collected from HLA-B27 transgenic rats. The rats were either treated with inulin or fructo-oligosaccharides (FOS), or not. Values are least-square means, with their standard errors represented by vertical bars. [a,b,c] Least-square mean values (obtained from the same tissue) with unlike letters were significantly different ($P \leq 0{\cdot}05$).

tute, Inc.). The differences in faecal microbiota and SCFA between groups were evaluated employing repeated-measures ANOVA. There were two fixed effects – time and treatment, whereas the random variation was individual rats. The variance and covariance associated with the responses over time were assumed by compound symmetry structure. To detect differences between groups, caecal bacterial populations, SCFA and caecal tissue inflammation were analysed using randomised block design, in which treatments were fixed effects and animals were random effects. Df were estimated by the Kenward–Rogers method and the probability of difference was used to test differences between least-square means of different treatments. Results are expressed as least-square means with their standard errors. A P value of $<0{\cdot}05$ was considered statistically significant.

Principal component analysis and linear discriminant analysis were performed using JMP software (version 8.0.1, SAS Institute, Inc.). The loading plot is a graphical representation of the amount of variation within the data set and shows the correlation of the individual variables of the first two principal components (PC1 and PC2). The correlations between mucosal inflammation and bacterial populations were assessed by Spearman's correlation test using GraphPad Prism version 5.00 (GraphPad Software).

Results

Effect of inulin and fructo-oligosaccharides on chronic intestinal inflammation

The evaluation of mucosal IL-1β concentration and histology scores indicated that the caecum was more inflamed in comparison with colonic tissue in rats on a control diet (Fig. 1(A)–(C)). Histology scores and GGS showed that inulin and FOS reduced inflammation in the caecum and colon (Fig. 1(A) and (B)). The concentration of the pro-inflammatory cytokine IL-1β was decreased in caecal tissue of animals treated with inulin ($P=0{\cdot}024$) or FOS ($P<0{\cdot}001$) *v.* control rats, further confirming a protective effect on intestinal inflammation by β-fructans treatment (Fig. 1(C)). However, GGS and histology scores indicated that the intestinal inflammation in FOS-treated rats was less severe compared to inulin-treated animals (Fig. 1(A) and (B)).

Quantification of bacterial populations by quantitative PCR

To determine the dietary impact of the different treatments on caecal and faecal microbiota of HLA-B27 TG rats, qPCR was performed (Figs. 2 and 3). Faecal and caecal samples were analysed to allow the comparison of samples from the same

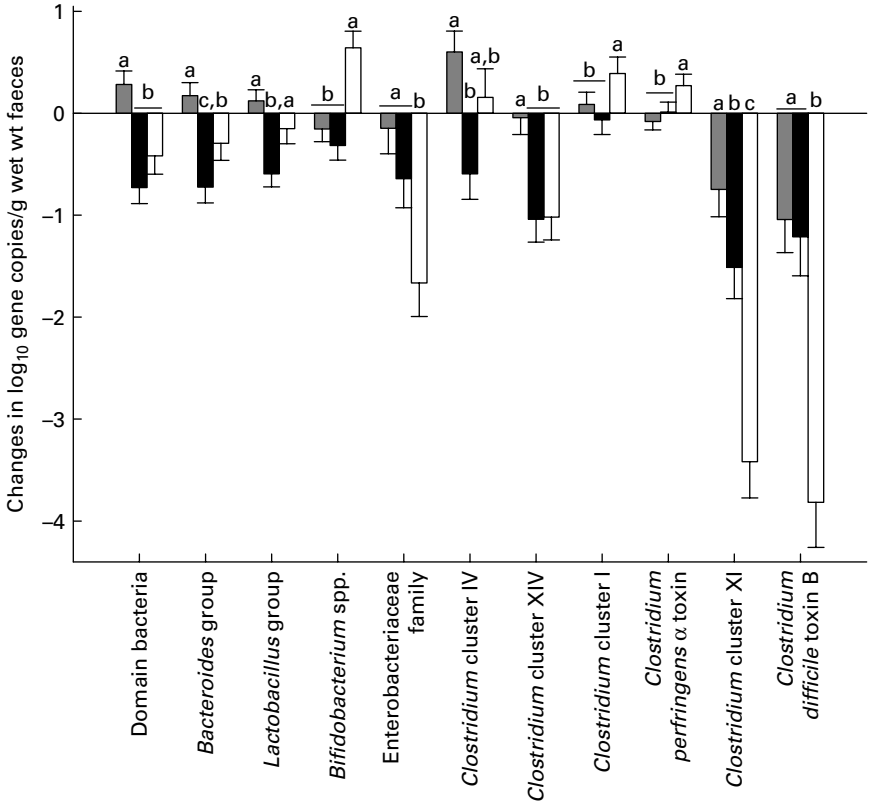

Fig. 2. Quantification of bacterial populations of faecal samples (▩, control; ■, inulin; □, fructo-oligosaccharides) collected from HLA-B27 transgenic rats before and at the end of the fibre treatments. Values are change in $\log_{10}$ copy numbers of 16S rDNA (week 16 − week 4) least-square means, with their standard errors represented by vertical bars. ^{a,b,c} Least-square mean values (obtained with the same primer pairs) with unlike letters were significantly different ($P\leq 0{\cdot}05$).

animal over time (faecal samples, Fig. 2) as well as the analysis from the intestinal site with the highest degree of inflammation (caecal samples, Fig. 3). Studying faecal microbiota, treatment with both fructans decreased the numbers of total bacteria and the *Bacteroides* group. Dietary intervention with fructans reduced *Clostridium* cluster XI and cluster XIVa by more than one log (Fig. 2). FOS treatment increased the numbers of bifidobacteria and *Clostridium* cluster I ($P<0{\cdot}001$); however, FOS-fed animals also showed significantly decreased copy numbers of Enterobacteriaceae ($P=0{\cdot}023$) and *C. difficile* toxin B ($P<0{\cdot}001$). Inulin specifically mediated the reduction of the *Lactobacillus* group ($P=0{\cdot}03$) and clostridial cluster IV ($P<0{\cdot}001$). Animals harboured high copy numbers of 16S rRNA genes of organisms belonging to the clostridial cluster I. This cluster comprises commensal fibrolytic and butyrate-producing bacteria as well as toxinogenic species such as *C. perfringens*[28]. The low abundance of gene copy numbers of *C. perfringens* α toxin indicates very low numbers of toxinogenic *C. perfringens*. However, gene copy numbers of *C. difficile* toxin B were equivalent to the copy numbers of 16S rRNA genes of *Clostridium* cluster XI, which demonstrated that *C. difficile* was the main representative of that clostridial cluster (Figs. 2 and 3).

The effects of inulin and FOS treatments on the caecal microbiota were less pronounced compared to their effects on the faecal microbiota (Fig. 3). Compared to caecal samples from control animals, inulin increased the numbers of 16S rRNA gene copies of total bacteria ($P=0{\cdot}006$) and organisms from the *Bacteroides-Prevotella-Porphyromonas* group ($P=0{\cdot}008$), but reduced organisms in *Clostridium* cluster XI ($P<0{\cdot}001$) (Fig. 3). FOS treatment increased the numbers of caecal bifidobacteria by one log ($P<0{\cdot}001$), whereas *Clostridium* cluster IV ($P=0{\cdot}015$) and *Clostridium* cluster XI ($P<0{\cdot}001$) were decreased *v.* rats in the control group. The numbers of caecal bifidobacteria and organisms belonging to clostridial clusters I and IV in FOS-treated animals were also significantly different in comparison to rats treated with inulin.

PCR-denaturing gradient gel electrophoresis profiles

To determine whether quantitative changes in intestinal microbiota were accompanied by qualitative changes, PCR-DGGE analysis was performed using primers targeting four phylogenic groups whose abundance was altered by dietary intervention with fructans. The cluster analysis for DGGE-profiles obtained with group-specific primers targeting the *Bacteroides* group is shown in Fig. 4. DGGE patterns were separated in two main clusters. The upper cluster mainly consisted of inulin- and FOS-treated animals, whereas the lower cluster contained mainly control animals (Fig. 4). This result indicates that diet-induced changes in the abundance were indeed associated with qualitative changes in composition of bacterial taxa in the *Bacteroides* group. However, patterns generated with primers specific for the *Lactobacillus* group

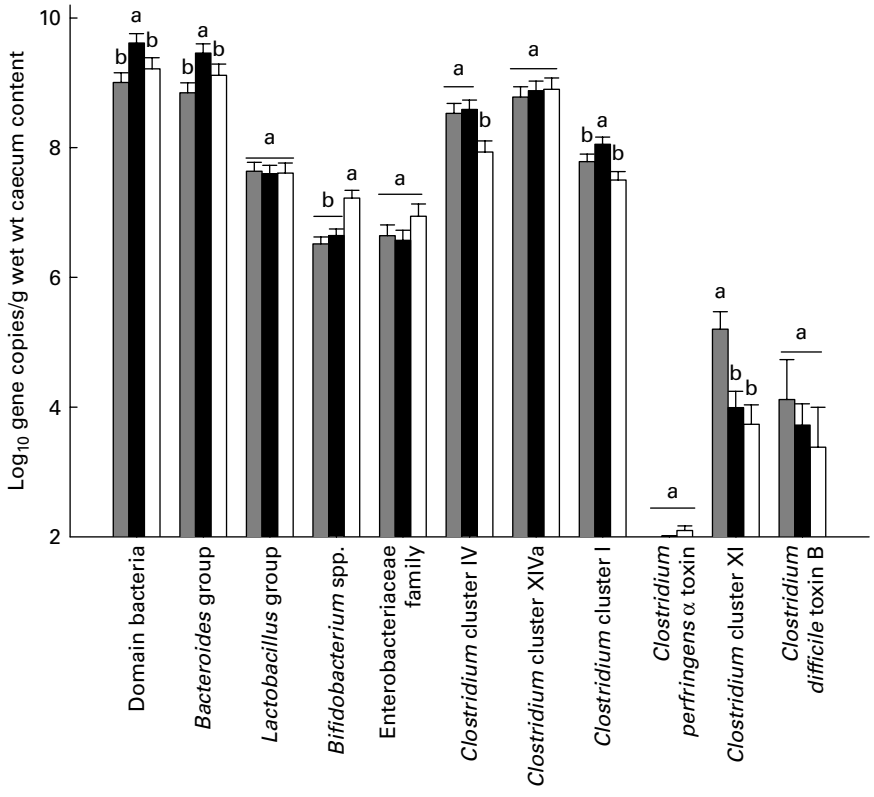

Fig. 3. Quantitative PCR analysis of bacterial populations of caecal samples (▩, control; ■, inulin; □, fructo-oligosaccharides) collected from HLA-B27 transgenic rats at 16 weeks of age. Values are least-square means, with their standard errors represented by vertical bars. [a,b,c] Least-square mean values (obtained with the same primer pairs) with unlike letters were significantly different ($P\leq 0{\cdot}05$).

and the clostridial cluster IV did not segregate according to the different diets (Fig. S1, supplementary material for this article can be found at http://www.journals.cambridge.org/bjn). Amplicons obtained with primers specific for *Bifidobacterium* spp. migrated as a single fragment on DGGE gels, indicating the presence of one *Bifidobacterium* species only in each animal (data not shown). Sequence analysis of five amplicons (one FOS-fed animal, two animals each from the inulin and control groups) was performed to identify bifidobacteria on species level; four sequences matched *Bifidobacterium animalis* with > 99% identity; one sequence matched *Bifidobacterium pseudolongum* (99% identity).

Caecal and faecal SCFA

SCFA composition in the faecal and caecal samples collected from HLA-B27 TG rats at the end point of carbohydrate treatments (16 weeks) did not differ significantly between the diets (data not shown). The total SCFA concentration of the faecal samples collected at 4 and 16 weeks of age was 54·8 (SEM 8·4) and 68·5 (SEM 5·8) μmol/g, respectively, for the control group, 45·8 (SEM 9·4) and 78·5 (SEM 6·4) μmol/g, respectively, for the inulin group, and 46·7 (SEM 8·7) and 77·2 (SEM 7·4) μmol/g, respectively, for the FOS group. The increase in total SCFA over the treatment period was significant for inulin- and FOS-treated animals ($P=0·023$ and $P=0·018$, respectively), but not for the control group. Acetate was the major product (about 70% of the total SCFA); faecal acetate significantly increased with age for all groups (data not shown).

Correlations among bacterial populations, SCFA and degree of inflammation for caecum and faeces

Correlations among the individual variables of intestinal bacterial populations, SCFA, and the degree of caecal inflammation were initially performed by PC analysis. The animals in the three treatment groups clustered separately in the linear discriminant analysis, indicating a differential effect of the two fructans on intestinal microbiota and intestinal inflammation (data not shown). Loading plots are depicted in Fig. 5(A; caecum samples) and Fig. 5(B; faecal samples) to indicate correlations between variables. In the caecal samples, a cluster located on the upper left quadrant included *Clostridium* cluster XI, IL-1β concentration, GGS, histology score, propionate and butyrate (Fig. 5(A)). *Bifidobacterium* spp., Enterobacteriaceae, *C. perfringens* α toxin, acetate and total SCFA formed clustered opposite to the first cluster, indicating a negative correlation. A third cluster containing total bacteria, *Bacteroides* and *Lactobacillus* groups and clostridial clusters I, IV, cluster XIVa was located in the upper right quadrant of the loading plot (Fig. 5(A)). In the loading plot for faecal samples (Fig. 5(B)), *Bifidobacterium* spp., *Clostridium* cluster I and *C. perfringens* α toxin, as well as acetate, propionate, butyrate and total SCFA formed a cluster negatively influenced by PC2. *Clostridium* cluster XI and *C. difficile* toxin B positively correlated with inflammation markers such as GGS, histology score and IL-1β concentration; and together formed a second cluster positively influenced by PC2 and negatively related to the first cluster. Total bacteria, the *Bacteroides* and *Lactobacillus* groups, and the clostridial clusters IV and XIVa were separated as a third cluster, which was positively correlated mainly to PC1.

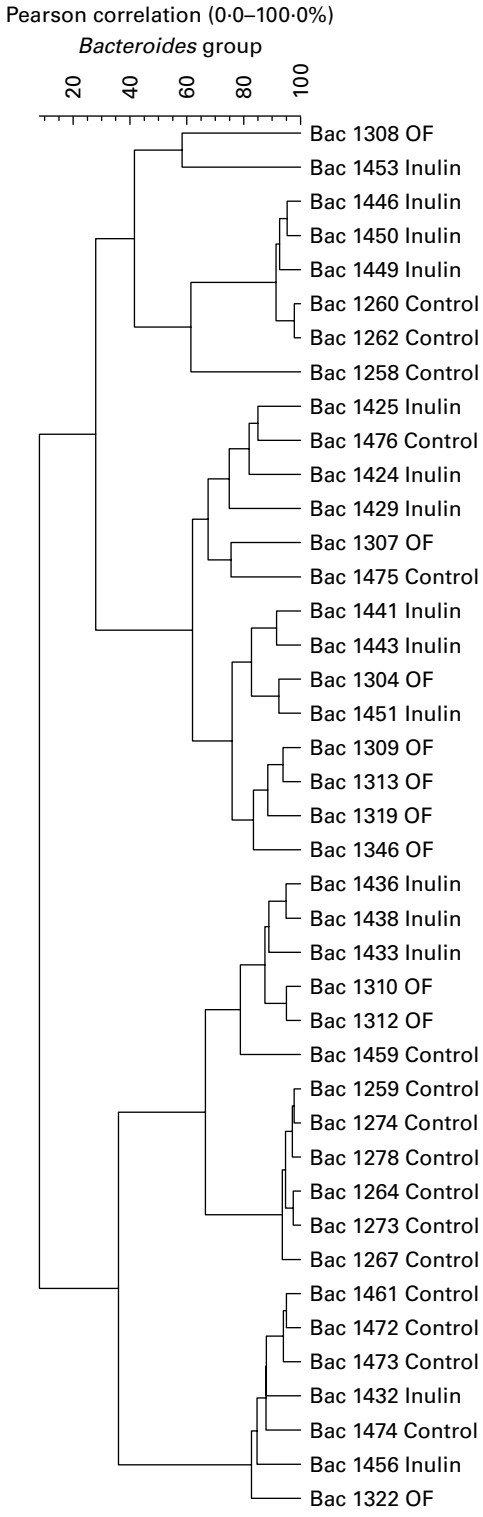

Fig. 4. Pearson correlation coefficient of denaturing gradient gel electrophoresis (DGGE) profiles of faecal communities of the *Bacteroides-Prevotella-Porphyromonas* group. DNA extracted from the faecal samples collected at the end point (16 weeks of age) was used as a template.

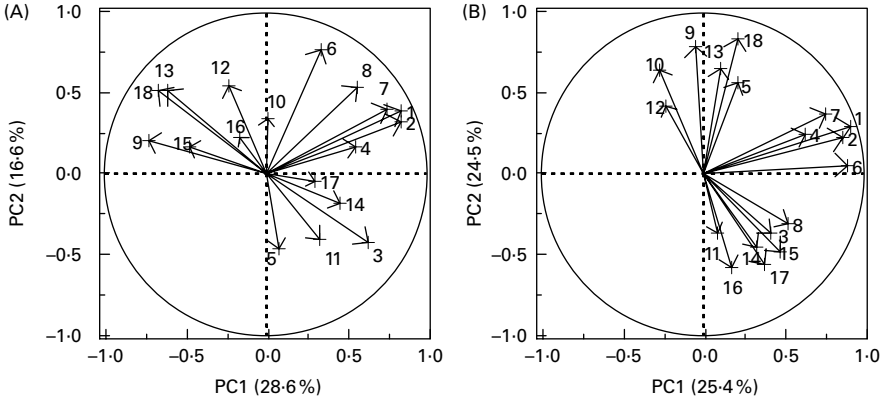

Fig. 5. Loading plots of the first two principal components (PC1 and PC2) for (A) caecum and (B) faeces show correlations among copy numbers for bacterial groups, SCFA, gross gut score (GGS) and IL-1β concentration. 1 – total bacteria; 2 – *Bacteroides-Prevotella-Porphyromonas* group; 3 – *Bifidobacterium* spp.; 4 – *Lactobacillus-Pediococcus-Leuconostoc-Weissella* group; 5 – Enterobacteriaceae family; 6 – *Clostridium* cluster IV; 7 – *Clostridium* cluster XIVa; 8 – *Clostridium* cluster I; 9 – *Clostridium* cluster XI; 10 – *Clostridium difficile* toxin B; 11 – *Clostridium perfringens* α toxin; 12 – IL-1β concentration; 13 – GGS; 14 – acetate; 15 – propionate; 16 – butyrate; 17 – total SCFA; 18 – histology score.

The associations between the variables were further analysed by Spearman's correlation analysis to determine which bacterial groups or metabolites were associated with chronic intestinal inflammation (Table 2). The analysis revealed significantly negative correlations of *Bifidobacterium* spp. with inflammation markers both in caecum and faeces. However, *Clostridium* cluster XI and genes coding for toxin B of *C. difficile* were positively correlated with inflammation (Table 2). Surprisingly, the correlations between *Clostridium* cluster XIVa and GGS (r 0·29 and P=0·044) and histology score (r 0·39 and P=0·006) were positive.

Discussion

Dietary interventions with non-digestible fermentable carbohydrates such as inulin and FOS present a potential tool for the prevention or treatment of IBD. An understanding of their protective mechanisms of action, which probably involves an altered composition and activity of intestinal microbiota, is required in order to determine chain length, structure, and linkages type of non-digestible carbohydrates that effectively improve colitis. To our knowledge, this is the first study to determine whether dietary inulin and FOS, differing in their DP, have divergent effects on the composition of intestinal microbiota in a well-validated colitis model. Different from intervention studies using healthy animals[25], intestinal microbiota in colitis models are also affected by intestinal inflammation[29]. In our study design, faecal microbiota were analysed over time to evaluate the effect of dietary intervention and inflammation; caecal microbiota were analysed at the time of killing of the animals to obtain insight into the composition of microbiota in the intestinal compartment exhibiting the highest degree of inflammation.

Treatment with a mixture of inulin and FOS reduced colitis in HLA-B27 TG rats[18]. Other studies in rats reported protective effects of β-fructans inulin on DSS-induced colitis[30], and trinitrobenzene sulphonic acid-induced colitis[31,32]. However, not all studies using fructans have reported positive outcomes[33]. A direct comparison of β-fructans differing in their molecular weight has not been reported in a colitis model. Both fructans had anti-inflammatory effects, but the effects of FOS on caecal inflammation were more pronounced compared to inulin.

Table 2. Correlations (r) between abundance in bacterial groups and mucosal inflammation indicated by IL-1β concentration, gross gut score (GGS) and histology score in caecal or faecal samples* (Correlation coefficients and P values)

	IL-1β concentration (pg/mg protein)		GGS		Histology score	
Bacterial groups	*r*	*P*	*r*	*P*	*r*	*P*
Caecum						
Bifidobacterium spp.	−0·18	0·278	−0·44†	0·002	−0·60†	0·001
Clostridium cluster XI	0·22	0·127	0·53†	0·001	0·59†	0·001
Faeces						
Bifidobacterium spp.	−0·15	0·299	−0·33†	0·019	−0·25	0·079
Clostridium cluster XIVa	0·04	0·809	0·29†	0·044	0·39†	0·006
Clostridium cluster XI	0·29†	0·041	0·42†	0·002	0·64†	0·001
Clostridium difficile toxin B	0·39†	0·041	0·42†	0·003	0·43†	0·002

* Correlations are assessed by Spearman's correlation test.
† Coefficients with P<0·05

Prevention of chronic inflammation was previously associated with increased numbers of intestinal lactobacilli and bifidobacteria[18]. The present study confirmed that the abundance of bifidobacteria was consistently and negatively correlated with chronic intestinal inflammation. FOS but not inulin increased the numbers of caecal and faecal bifidobacteria. FOS did not alter the diversity of bifidobacteria; *B. animalis* was predominant in all animals. This divergent effect on the abundance of bifidobacteria corresponded to the differential effect of inulin and FOS on chronic intestinal inflammation and probably relates to the preferential metabolism of FOS by most *Bifidobacterium* spp. A majority of bifidobacteria metabolise FOS by oligosaccharide transport and hydrolysis by intracellular β-fructofuranosidases[15,34]. Only few bifidobacteria possess extracellular enzymes to degrade polymeric β-fructans such as inulin[34,35].

Intestinal bacteria that are more abundant than bifidobacteria may also contribute to protection. FOS also increased the caecal abundance of the *Bacteroides* group. DGGE showed that this group, which ferments a wide range of non-digestible carbohydrates, is also qualitatively influenced by inulin and FOS. The *Bacteroides* group plays a controversial role in IBD. *Bacteroides vulgatus* induced colitis in gnotobiotic HLA-B27 TG rats[4,11], and are found more frequently in colonic biopsies taken from UC patients compared to healthy subjects[36,37]. However, other strains of *B. vulgatus* protected against *E. coli*-induced colitis in gnotobiotic IL-2-deficient mice[38]. A diet rich in resistant starch that is associated with a high faecal concentration of SCFA and a reduced risk of IBD also resulted in a high abundance of organisms in the *Bacteroides* group[39].

The abundance of Enterobacteriaceae in other colitis models positively correlated with host-mediated inflammation[40]; and pathogenic bacteria of the Enterobacteriaceae family such as pathogenic *E. coli* frequently are more abundant in IBD patients in comparison to healthy individuals[8,10,29]. However, HLA-B27 TG rats mono-associated with *E. coli* did not develop colitis[11] and *E. coli* Nissle 1917 even maintained remission in patients with UC[41], indicating that some Enterobacteriaceae are protective. In this study, PC and correlation analyses of intestinal microbiota and inflammation markers did not reveal a consistent association of Enterobacteriaceae with inflammation or protection.

Chronic intestinal inflammation consistently correlated with the abundance of *Clostridium* cluster XI as well as the abundance of genes encoding for the *C. difficile* toxin B. Fructans substantially decreased the gene copy numbers of the *Clostridium* cluster XI in faecal microbiota. This decrease was matched by a reduction of gene copy numbers of the *C. difficile* toxin B. *C. difficile* is the major cause of nosocomial diarrhoea, and growth and toxin production of *C. difficile* is favoured by dysbiosis[42]. Patients with UC are prone to infections with *C. difficile*, which further increases morbidity and even mortality in UC patients who are also frequently on immunosuppressive drugs[43,44]. Independent of any colitis-reducing effect of dietary β-fructans in patients, a reduction of *C. difficile* overgrowth thus justifies their therapeutic use.

SCFA – acetate, propionate and butyrate – are the major end-products of intestinal fermentation of β-fructans[45]. Lactate formation by lactobacilli and bifidobacteria increase SCFA through cross-feeding of butyrate-producing bacteria[46]. Butyrate is the main fuel for colonocytes and also promotes the down-regulation of pro-inflammatory cascades in experimental and human IBD[30]. The protective effects of bifidobacteria in an animal model for enteric infection were recently attributed to acetate formation[47]. The protective effect of a non-purified diet in DSS-induced colitis in mice when compared to a purified diet also correlated to high intestinal concentration of SCFA[21]. Stimulation of SCFA production by β-fructans may thus contribute to their anti-inflammatory effect. In this study, linear discriminant indicated a negative correlation between faecal SCFA and inflammation markers. However, more than 90 % of SCFA produced in the intestine are absorbed in the caecal and colonic epithelial cells[48,49]. Therefore, SCFA levels in the faeces of rats are a poor indicator of intestinal carbohydrate fermentation[18,25,48].

In summary, despite the wide use of FOS and inulin as functional food ingredients and their well-studied prebiotic activity, very little is known about the relationship between their DP and their effect on intestinal microbiota or host health[25]. Our results demonstrate that FOS and inulin differ in colitis reduction and also vary in their effects on the intestinal microbiota in HLA-B27 TG rats. The abundance of *Bifidobacterium* spp. and *Clostridium* cluster XI correlated negatively and positively, respectively, to chronic intestinal inflammation. The protective effects of intestinal microbiota in IBD may relate to the formation of SCFA in the large intestine; however, additional studies are required to confirm a protective role of SCFA in experimental colitis and in clinical studies.

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