British Journal of Nutrition (2003), **89**, 597–606
DOI: 10.1079/BJN2002827

Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats

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(Received 29 May 2002 – Revised 14 November 2002 – Accepted 2 December 2002)

The effects of fructans in the diet on the mucosal morphometry (height of villi, depth of the crypts, number of goblet cells), the thickness of the epithelial mucus layer and the histochemical composition of intestinal mucosubstances in the distal jejunum and the distal colon were investigated by comparing germ-free (GF) rats, rats harbouring *Bacteroides vulgatus* and *Bifidobacterium longum* (diassociated (DA) rats), and rats with a human faecal flora (HFA). The rats were fed either a commercial standard diet (ST) or ST + (50 g oligofructose (OF)–long-chain inulin (lcIN))/kg. Changes in total bacteria, bifidobacteria and *Bacteroides–Prevotella* in response to feeding these diets were investigated by fluorescent *in situ* hybridization with 16S rRNA-targeted probes both in intestinal contents (lumen bacteria) and tissue sections (mucosa-associated bacteria). The OF–lcIN-containing diet resulted in higher villi and deeper crypts in bacteria-associated, but not in GF rats. In DA and HFA rats, the colonic epithelial mucus layer was thicker and the numbers of the goblet cells were greater than in GF rats. These effects were enhanced by the OF–lcIN-containing diet. In both dietary groups, bacterial colonization of GF rats caused an increase in neutral mucins in the distal jejunum and colon. Bacteria-associated rats had more acidic mucins in the colon than GF rats, and the OF–lcIN-containing diet stimulated sulfomucins as the predominant type of acidic mucins, while sialomucins dominated in the ST-fed groups. The number of mucosa-associated bifidobacteria detected in the colon of DA and HFA rats was greater with OF–lcIN than ST (4·9 and 5·4 *v.* 3·5 and 4·0 $\log_{10}/mm^2$ mucosal surface respectively), whereas the number of luminal bifidobacteria was only affected by fructans in DA rats. *Bacteroides* did not differ between the groups. The stabilisation of the gut mucosal barrier, either by changes in the mucosal architecture itself, in released mucins or by stimulation of mucosal bifidobacteria with fructans, could become an important topic in the treatment and prophylaxis of gastrointestinal disorders and health maintenance.

Bifidobacteria: Fructan: Intestinal microflora: Mucin: Mucosa: Gnotobiotic rats

Non-digestible fructans that occur naturally in various food plants, such as chicory, onion and leek (Van Loo *et al*. 1995), have been claimed to benefit the health of the host by selectively stimulating the growth and/or activity of one or a limited number of potentially health-promoting bacteria in the colon, such as bifidobacteria (prebiotics) (Gibson & Roberfroid, 1995; Roberfroid *et al*. 1998; Van Loo *et al*. 1999). Bifidobacteria are generally considered to be useful in promoting intestinal health by prevention of colonization of potential pathogens by lowering intestinal pH through increased fermentation products, e.g. acetate and lactate, or producing inhibitory substances such as bacteriocins (Gibson & Wang, 1994), restoration of the intestinal flora following antibiotic therapy (Korshunov *et al*. 1985) and stimulation of the immune system (Sekine *et al*. 1985). *In vitro* and *in vivo* studies indicate that oligofructose (OF) and inulin (IN) selectively stimulate the growth of these organisms in the intestinal content or faeces of human subjects or rats (Wang & Gibson, 1993; Gibson *et al*. 1995; Campell *et al.* 1997; Djouzi & Andrieux, 1997; Kleessen *et al*. 1997; Sghir *et al*. 1998; Kruse *et al*. 1999). However, little information exists on effects of fructans on the composition, metabolism and health-related significance of bacteria existing at or near the mucosa surface or in the

Abbreviations: AB, Alcian Blue; DA, diassociated; EML, epithelial mucus layer; GF, germ-free; HFA, human flora-associated; IN, inulin; lc, long-chain; OF, oligofructose; PAS, periodic acid–Schiff; ST, standard.

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mucus layer. Mucins and/or fructans may be important growth substrates for these mucosa-associated species. Mucins are high molecular mass glycoproteins that are synthesized and secreted by the goblet cells. They form a gel-like layer on the mucosal surface, and provide favourable conditions for the resident microflora by forming a specific microenvironment (Forstner, 1978). The amount and composition of the mucus layer reflects an equilibrium between mucus secretion on the one hand and its erosion and degradation by bacteria on the other (Deplancke & Gaskins, 2001). Diet and microflora may also alter the distribution and composition of intestinal mucins (Sharma *et al*. 1995). The quantity of mucin secreted and the relative number of goblet cells are increased in rats during high dietary fibre intake (Satchithanandam *et al*. 1990; Schmidt-Wittig *et al*. 1996). Supplementing the diet with galacto-oligosaccharide or IN modifies the goblet-cell number and mucin types in conventional or human flora-associated (HFA) rats (Meslin *et al*. 1993; Fontaine *et al*. 1996). Based on these findings, it may be hypothesized that specific IN-type fructans are especially effective in raising mucus secretion for microbial colonization. In addition, such fructans may affect the mucin types (neutral mucins, sialomucins and sulfomucins) and/or the mucosal architecture in the small or large intestine either directly or by changing the intestinal flora.

The purpose of the present study was, therefore, to investigate the effects of fructans (OF–long-chain (lc) IN) on the mucosal morphometry (height of villi, depth of crypts, number of goblet cells), the thickness of the epithelial mucus layer (EML) and the histochemical composition of the mucins in the distal jejunum and the distal colon of rats. To study the distribution of bacteria in the intestinal lumen (lumen flora) and on the mucosa (mucosa-associated flora), fluorescent *in situ* hybridization with 16S rRNA-targeted oligonucleotide probes was applied in intestinal contents and in mucus-preserved tissue sections for the detection of total bacteria, *Bifidobacterium* and *Bacteroides–Prevotella*. In light of the interest in prebiotics, the presence of bifidobacteria was of particular interest. In order to distinguish the effects of the fructans from those mediated by the bacterial flora, we compared germ-free (GF), diassociated (DA) (*Bacteroides vulgatus* and *Bifidobacterium longum*) and HFA rats.

Materials and methods

Fructans

Raftilose® P95 and Raftiline® HP, which contain mainly OF (950 g/kg) and IN (995 g/kg) respectively, were supplied by Raffinerie Tirlemontoise (Tienen, Belgium). Raftilose (OF), which is produced by partial enzymatic hydrolysis of chicory IN, is a mixture of ß (2-1) fructans with a mean degree of polymerization of 4 (range 3–7). Raftiline® HP, with a mean degree of polymerization of 25 (range 10–60), is chicory lc-IN devoid of fructans with the lower degree of polymerization. Raftilose® P95 and Raftiline® HP were used as a mixture (1:1, w/w).

Animals, diets and experimental design

The experiment was performed in accordance with the guidelines of the Ministry of Nutrition, Agriculture, and Forestry, Brandenburg, Germany (permission no. 48-3560-0/3). Thirty-six male 5-week-old GF Wistar rats (inbred strain AVN-Ipcv; German Institute of Human Nutrition (DIFE), Bergholz-Rehbrücke, Germany) with an initial body weight of 118 (sem 8) g were randomly divided into six groups of six animals. Two rat groups each were inoculated *per os* with bacteria by applying 3 ml ten-fold diluted fresh human faeces from a healthy male subject (age 31 years) (HFA rats), or by applying on three successive days first, 1 ml *Bacteroides vulgatus* DSM1447 culture (8×10^9 bacteria/ml), and second, 1 ml *Bifidobacterium longum* ATCC 15707 culture (5×10^8 bacteria/ml) (DA rats). Fresh cultures of the latter organisms were obtained in Schaedler anaerobic broth (Oxoid, Wesel, Germany). Human faecal inoculum was characterized by fluorescent *in situ* hybridization (*n*/g wet faeces): total bacteria 5×10^{10}, *Bacteroides–Prevotella* 1×10^{10}, bifidobacteria 8×10^8. Two rat groups were kept germ-free (GF rats). Rats were maintained in positive-pressure isolators (Metall & Plastik, Radolfzell, Germany) equipped with a sterile water supply and housed individually in macrolon cages on wire grates at a temperature of 22 ± 2°C, 55 ± 5 % relative humidity and a 12 h light–dark cycle (lights on 07.00–19.00 hours). They were given free access to water and a commercial standard diet (ST) (Altromin fortified® 1310; Altromin, Lage, Germany) sterilized by irradiation (25 kGy) and containing (g/kg): crude protein (N × 6·25) 232, crude fat 51, ash 64, moisture 109, N-free extract 468. The dietary fibre content of the diet was 83 g/kg diet, as determined by the enzymatic–gravimetric method of the Association of Analytical Chemists (Prosky *et al*. 1988), and was derived from coarsely milled cereal products. Ten days after inoculation, when the microbial flora had stabilized, one group each of HFA, DA and GF rats was allotted to one of two dietary treatments for 28 d (experimental period). Dietary treatments were ST (Altromin fortified® 1310) or ST+(50 g Raftilose® P95–Raftiline® HP (OF–lcIN 1:1, w/w))/kg. Food intake was determined daily and body weight was recorded every 2 d.

Sampling and tissue preparations

At the end of the experimental period, rats were killed by decapitation. The small intestine and colon were removed aseptically from the abdominal cavity and dissected free from fat and mesentery. The distal jejunum was sampled at 200 mm proximal to the caecal border. The specimen (80 mm) was placed on a cooled glass dish and cut open along its longitudinal axis to obtain an exact orientation of crypts and villi. The specimen from the distal colon was taken 30 mm proximal to the rectum and cut open as described earlier. For microbiological enumeration, the intestinal content of these samples was carefully collected without scraping the mucosa and was fixed immediately in fresh paraformaldehyde (40 ml/l PBS, pH 7·4) as described previously (Kleessen *et al*. 1999). For stabilization of the

mucous gel layer, tissue samples were immersed in cooled Carnoy's solution (ethanol–acetic acid–chloroform (6:3:1, by vol.)) for 2 h at 4°C (Matsuo *et al*. 1997). They were then placed in 100 % ethanol, cleared in xylene and embedded in Histoplast (Hypercenter® XP; LifeScience International GmbH, Thermo Shandon, Frankfurt Main, Germany) according to routine procedures, and stored at 4°C prior to preparation of samples with cross-sectional thicknesses of 2 μm (fluorescent *in situ* hybridization) or 3 μm (histochemistry). Three blocks of embedded tissue from the distal jejunum and distal colon of each animal were sectioned. Sections were placed onto coated microscope slides (Superfrost*/Plus; Menzel-Gläser, Braunschweig, Germany). Prior to fluorescent *in situ* hybridization, dewaxed sections were post-fixed in fresh paraformaldehyde (40 ml/l PBS) for 5 min.

Histochemistry

Dewaxed sections were stained with haematoxylin and eosin for the demonstration of the cellular composition of the gut tissues. To characterize different mucins in the goblet cells, serial sections were subjected to the following histological procedures: (1) the periodic acid–Schiff (PAS) reaction for studying unsubstituted α-glycerol rich neutral mucins (red); (2) 1 % Alcian Blue (AB), pH 2·5, for localization of the carboxylated and/or sulfated types of acidic mucins (blue); (3) 1 % AB, pH 1·0, for the selective characterization of strongly sulfated sulfomucins (blue) (Romeis, 1989). The mucin that was stained by AB pH 2·5, but not stained by AB pH 1·0, was classified as sialomucin in the present study; (4) the combined 1 % AB pH 2·5 and PAS staining distinguishes neutral mucin from sialomucin and also from weakly stained sulfomucin. A purple colour indicates a mixture of neutral and acidic mucins, and a deep purple colour is obtained when neutral mucins are predominant (Filipe, 1979). To ensure comparability between the different groups all sections were stained in one batch (Varistain XY; Thermo Shandon GmbH) in order to exclude differences due to technical manipulations. Semiquantitative staining intensities were expressed as: 0, unreactive; 1, weak reactivity; 2, moderate reactivity; 3, intense reactivity.

Histological morphometry

Computerized morphometric measurements were made of the following: (1) villus height (μm, measured from the tip to the base of villus); (2) crypt depth (μm, measured from the bottom of the crypt to the crypt entrance); (3) number of goblet cells of the crypt (counted on both sides of the crypt) was carried out on blinded slides using an Axioplan2 imaging microscope equipped with a charge-coupled device camera (Axiocam, Carl Zeiss, Jena, Germany) and the image analysis system KS400 (Carl Zeiss, Jena, Germany). Morphology was assessed on haematoxylin and eosin stained sections by light microscopy on twenty-five longitudinally oriented villi and crypts. The thickness of the EML for each rat was measured on three AB pH 2·5–PAS-stained sections at 300 μm intervals with a minimum of six points on each section, and was defined as the distance between the apical mucosal surface and the outermost layer of the surface mucus gel on the luminal side. Additional intestinal sections were used for histochemical assessment of goblet cells stained for mucin as described earlier.

Fluorescent in situ *hybridization and oligonucleotide probes*

Total bacteria and bacterial groups in the intestinal content (lumen bacteria) and in the tissue sections (mucosa-associated bacteria) of the distal jejunum and distal colon were enumerated by fluorescent *in situ* hybridization as described previously (Kleessen *et al*. 2001, 2002) using the following 16S rRNA targeted oligonucleotide probes: (1) an equimolar mixture of five *Bacteria*-directed probes EUB 338, EUB 785, EUB 927, EUB 1055, EUB 1088 (Amann *et al*. 1995; Kleessen *et al*. 1999), referred to as EUB mix, to detect all bacteria; (2) Bac 303 to detect the *Bacteroides–Prevotella* cluster (Manz *et al*. 1996); (3) the bifidobacterial probe Bif 164 (S-G-Bif-0164-a-A-18) for species of *Bifidobacterium* (Langendijk *et al*. 1995). The non-sense probe Non 338 was used as a control to monitor non-specific oligonucleotide binding (Amann *et al*. 1995). The fluorescing cells were viewed with an Axioplan epifluorescence microscope (Carl Zeiss) equipped with the filter set 15. Digital images were captured with a 12-bit cooled charge-coupled device camera (Sensicam 370 KL; PCO Kelheim, Germany) and processed with the image analysis system KS400, version 3.01 (Carl Zeiss). The oligonucleotides were purchased 5′-labelled with the indocarbocyanine dye Cy3 (Thermo Hybaid GmbH, Ulm, Germany).

Statistical analysis

Results are expressed as mean values with their standard errors. Rat was the experimental unit. Statistical analyses were conducted with the Statistical Package for Social Science (SPSS for Windows, version 8.0; SPSS Inc., Chicago, IL, USA) to determine if variables differed among treatment groups. Results were checked for normality before statistical analysis was performed (Zar, 1984). Data obtained from the different segments (distal jejunum and distal colon) were analysed separately by two-way ANOVA to assess the effects of diet (ST, OF–lcIN), of bacteria or rat model (GF, DA, HFA) and of the interaction between diet and rat model (Wilcoxon & Wilcox, 1964). When the ANOVA indicated that significant treatment effects had occurred, the least significant difference test was used to locate differences between treatment means. Before statistical analysis of the bacteriological results, the cell counts were transformed to $\log_{10}$ numbers (Wilcoxon & Wilcox, 1964). Differences were considered to be significant at $P < 0{\cdot}05$.

Results

Feed intake and body-weight gain

Rats were generally in good health throughout the experiment. Feeding the various diets did not result in

any differences in feed intake or in body-weight gain in GF, DA and HFA rats. The mean feed intake in the experimental period was 24·1–25·3 (sem 0·4) g/d. The mean body-weight gain of the rats ranged from 13·6–14·4 (SEM 0·5) g/2 d. There were no growth differences throughout the study (results not shown).

Bacterial enumeration

The presence of bacteria in the intestinal content and on the mucosal surface (Fig. 1) of the distal jejunum and distal colon are shown in Tables 1 and 2. Bacteria were calculated per g wet weight (lumen-associated flora) or per mm^2 epithelial surface area (mucosa-associated flora). The surface area was defined as the area between the apical mucosal surface and a line 10 μm parallel to the surface on the lumen side. Bacteria were counted at ten randomly selected points in each section.

The total numbers of bacteria were unaffected by diet, but there were differences with respect to counts of bifidobacteria. Counts of bifidobacteria in the colonic content were higher in DA rats fed OF–lcIN than in those fed ST or in HFA rats, whereas the numbers observed in the jejunal contents were not statistically different. In contrast, both DA and HFA rats consuming the OF–lcIN-containing diet displayed greater ($P<0·05$) counts of mucosa-associated bifidobacteria in the colon than ST. However, they were only detected on the jejunal mucosa of the DA rats consuming the fructans, but not in the other groups. No significant differences among treatment groups were noted in numbers of *Bacteroides* in DA rats. In contrast, a slight trend towards lower counts of *Bacteroides–Prevotella* was observed in the colon of HFA rats fed OF–lcIN compared with those fed ST, but these organisms were not detected on the jejunal mucosa of the fructan-fed rats.

Morphology of villi and crypts

Morphological measurements of distal jejunal and colonic tissue (Table 3) showed responses to bacterial association and to consumption of fructans-containing diet. Villus height and crypt depth were significantly higher ($P<0·05$) in HFA-rats consuming OF–lcIN than in rats consuming ST or in GF rats. Higher villi ($P<0·05$) were

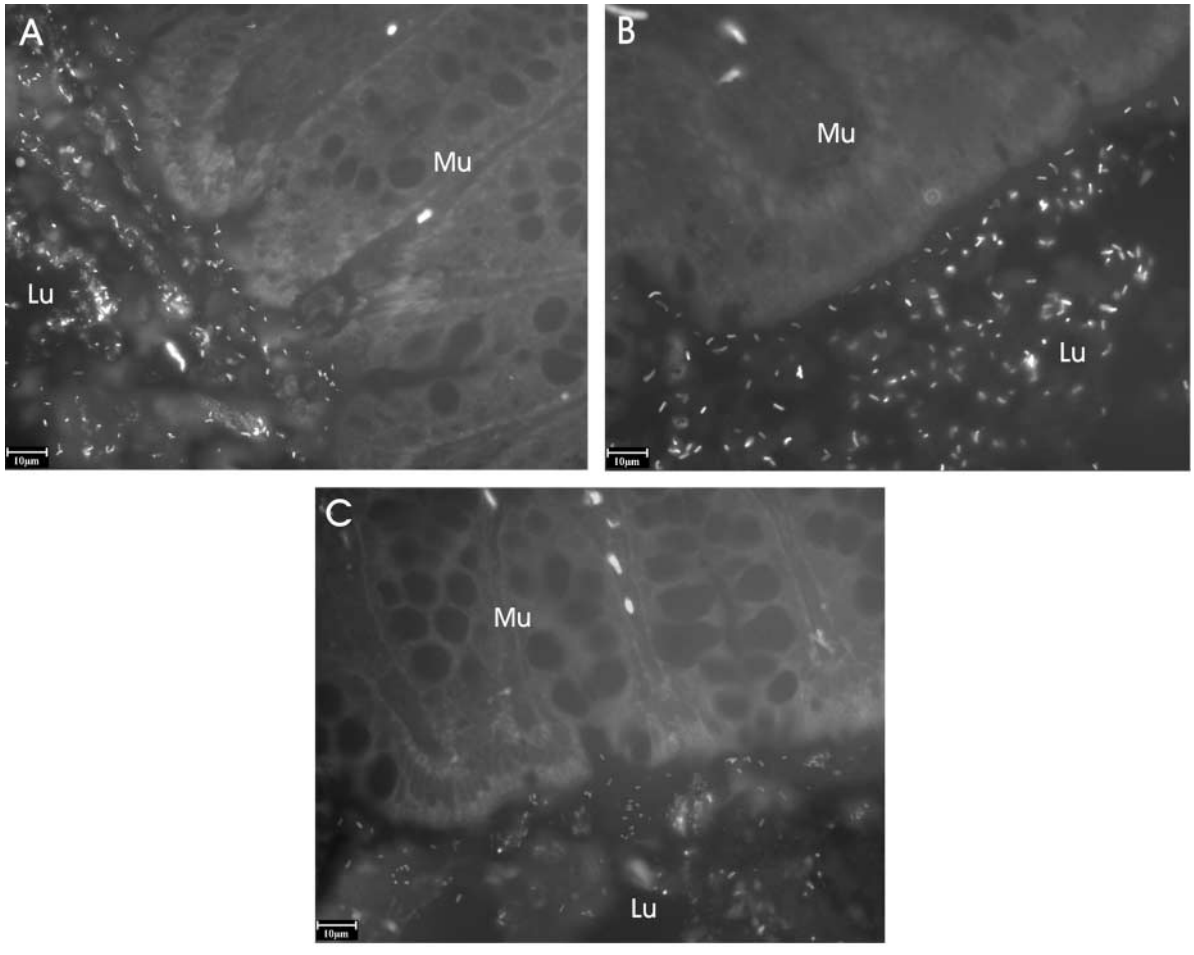

Fig. 1. Fluorescent *in situ* hybridization of tissue sections from colonic mucosal samples of human flora-associated rats fed a standard diet supplemented with oligofructose and long-chain inulin. Epifluorescence images of bacteria that hybridized with 16S rRNA-targeted oligonucleotide probes: a mixture of *Bacteria*-directed probes (EUB mix) to detect all bacteria (A), the bifidobacterial probe Bif 164 for species of *Bifidobacterium* (B) and Bac 303 to detect the *Bacteroides–Prevotella* cluster (C). The oligonucleotides were 5'-labelled with the indocarbocyanine dye Cy3 (Thermo Hybaid GmbH, Ulm, Germany). Mu, mucosa; Lu, lumen. For details of diets and procedures, see p. 598.

Table 1. Numbers of bacteria in the intestinal content of the distal jejunum and the distal colon of diassociated (DA) and human flora-associated (HFA) rats fed a standard diet (ST) or a standard diet supplemented with fructan†

(Mean values for six rats per group)

	Rat model (log_{10} counts/g wet weight)			Statistical significance of effect: *P*		
	DA§	HFA‖	Pooled SEM	Diet	Rat model	Diet × Rat model
Distal jejunum						
Total bacteria						
ST	7·0	7·7	0·20	0·29	0·056	0·94
OF–lcIN¶	7·2	7·9	0·22			
Bifidobacterium						
ST	6·8	6·6	0·22	0·15	0·21	0·57
OF–lcIN¶	6·9	6·9	0·25			
Bacteroides						
ST	6·6	5·9	0·20	0·97	0·052	0·78
OF–lcIN¶	6·7	5·9	0·21			
Distal colon						
Total bacteria						
ST	10·4	10·7	0·12	0·15	0·08	0·54
OF–lcIN¶	10·6	10·8	0·14			
Bifidobacterium						
ST	9·0	9·4	0·17	0·005	0·69	0·041
OF–lcIN¶	10·0*	9·6	0·20			
Bacteroides						
ST	10·2	10·0	0·17	0·25	0·05	0·074
OF–lcIN¶	10·4	9·5	0·16			

OF, oligofructose; lc, long-chain; IN, inulin.
Mean value was significantly different from that of the ST group: *$P < 0·05$.
† For details of diets and procedures, see p. 598.
§ Germ-free rats were inoculated sequentially with *Bacteroides vulgatus* DSM1447 and *Bifidobacterium longum* ATCC 15707 cultures.
‖ Germ-free rats were associated with bacteria of fresh human faeces from a healthy male subject.
¶ Supplemented diet contained 50 g fructans (25 g OF (Raftilose® P95, Raffinerie Tirlemontoise, Tienen, Belgium) + 25 g lcIN (Raftiline® HP; Raffinerie Tirlemontoise))/kg.

also found in the distal jejunum of OF–lcIN-fed DA rats compared with ST-fed rats. In contrast, feeding the fructans-containing diet for 4 weeks did not alter the jejunal and colonic mucosal morphometry in GF rats.

Intestinal mucins

GF rats had only a thin and discontinuous mucus layer on the jejunal epithelium, whereas the mucus layer on the colonic epithelium was of homogeneous thickness and almost completely continuous. Both bacterial association and diet affected the thickness of the EML and/or the number of the goblet cells per crypt in the intestine (Table 4). In DA and HFA rats, colonic EML was thicker and the number of the goblet cells was greater than in GF rats. Compared with ST diet, OF–lcIN resulted in higher number of goblet cells in the jejunal and colonic mucosa of both DA and HFA rats, whereas a thicker EML was only observed in the HFA rats (Fig. 2). In GF rats, however, fructans had no effect on the thickness of EML and numbers of goblet cells both in the distal jejunum and the distal colon.

Mucin histochemistry, using AB and/or PAS staining, demonstrated that both intestinal bacteria and diet have an influence on mucin composition of the EML and in the goblet cells in the rat gut (Table 5). In both dietary groups, bacterial colonization of GF rats caused an increase in neutral mucins in the distal jejunum and colon, but the colonic mucosa of HFA rats displayed the highest PAS-staining intensity. Bacteria-associated rats also revealed more acidic mucins in their colon than GF rats. However, sulfomucins were the predominant type of acidic mucins in the colonic mucosa of OF–lcIN-fed DA and HFA rats, while sialomucins dominated in the colon of ST-fed groups (AB pH 2·5 positive, AB pH 1·0 negative–weak positive). Moreover, OF–lcIN resulted in more sialomycins in the jejunum of GF rats.

Discussion

The present study shows, for the first time, changes in both the mucosal architecture, released mucins and mucosa-associated bacteria induced by an OF–lcIN-containing diet in DA and HFA rats (Tables 1–5).

The gnotobiotic rat model was chosen for these investigations because intestinal contents and mucus samples cannot be obtained easily from healthy human subjects without the application of invasive techniques. The DA rat is a simple model to evaluate the colonization of the gastrointestinal tract by two defined populations of the human faecal flora as well as bacteria–host interactions. The HFA rat is a model simulating the human conditions more closely than the DA rat. The similarity of the HFA rat flora to that of human faeces has been proved previously (Kleessen *et al.* 2001). In our present study, rats fed ST retained characteristics of the human donor in terms of special bacterial populations (log_{10}/g wet faeces); namely, 10 d after inoculation, log counts of total

Table 2. Numbers of mucosa-associated bacteria in the distal jejunum and the distal colon of diassociated (DA) and human flora-associated (HFA) rats fed a standard diet (ST) or a standard diet supplemented with fructan†

(Mean values for six rats per group)

	Rat model (log_{10} cells/mm^2 mucosal surface)			Statistical significance of effect: *P*		
	DA§	HFA‖	Pooled SEM	Diet	Rat model	Diet × Rat model
Distal jejunum						
Total bacteria						
ST	2·5	3·1	0·19	0·78	0·40	0·06
OF–lcIN¶	3·0	2·8	0·15			
Bifidobacterium						
ST	nd	nd		–	–	–
OF–lcIN¶	2·3	nd	0·17			
Bacteroides						
ST	2·4	2·5	0·18	–	–	–
OF–lcIN¶	2·0	nd	0·17			
Distal colon						
Total bacteria						
ST	5·4	5·6	0·21	0·49	0·32	0·76
OF–lcIN¶	5·5	5·8	0·21			
Bifidobacterium						
ST	3·5	4·0	0·17	0·0001	0·20	0·10
OF–lcIN¶	4·9*	5·4*	0·18			
Bacteroides						
ST	5·2	5·3	0·20	0·78	0·26	0·042
OF–lcIN¶	5·5	4·8	0·13			

OF, oligofructose; lc, long-chain; IN, inulin; nd, not detected.
Mean values were significantly different from those of the ST group: *$P<0·05$.
† For details of diets and procedures, see p. 598.
§ Germ-free rats were inoculated sequentially with *Bacteroides vulgatus* DSM1447 and *Bifidobacterium longum* ATCC 15707 cultures.
‖ Germ-free rats were associated with bacteria of fresh human faeces from a healthy male subject.
¶ Supplemented diet contained 50 g fructans (25 g OF (Raftilose® P95; Raffinerie Tirlemontoise, Tienen, Belgium) + 25 g lcIN (Raftiline® HP; Raffinerie Tirlemontoise))/kg.

bacteria were 10·6, *Bacteroides–Prevotella* 10·3 and bifidobacteria 9·0.

One important finding of the present study concerns the increase in mucosal bifidobacteria in rats consuming the OF–lcIN-containing diet compared with ST. Such stimulation was not observed in the intestinal lumen of the HFA rats. The latter observation is consistent with previous microbiological results obtained in HFA rats fed similar diets (Kleessen *et al.* 2001). These results suggest that specific bacterial populations that occupy an

Table 3. Villus height and crypt depth in the distal jejunum and the distal colon of germ-free (GF), diassociated (DA) and human flora-associated (HFA) rats fed a standard diet (ST) or a standard diet supplemented with fructan†

(Mean values for six rats per group)

	Rat model				Statistical significance of effect: *P*		
	GF	DA§	HFA‖	Pooled SEM	Diet	Rat model	Diet × Rat model
Distal jejunum							
Villus height (μm)							
ST	245·2	283·9	298·2	6·5	0·0001	0·0001	0·0001
OF–lcIN¶	236·5	310·4*	375·6*	7·1			
Crypt depth (μm)							
ST	102·6	112·2	116·8	5·1	0·01	0·01	0·160
OF–lcIN¶	110·8	122·7	148·7*	4·2			
Distal colon							
Crypt depth (μm)							
ST	166·8	192·5	203·6	5·7	0·003	0·001	0·958
OF–lcIN¶	180·9	235·9	276·1*	5·5			

OF, oligofructose; lc, long-chain; IN, inulin.
Mean values were significantly different from those of the ST group: *$P<0·05$.
† For details of diets and procedures, see p. 598.
§ Germ-free rats were inoculated sequentially with *Bacteroides vulgatus* DSM1447 and *Bifidobacterium longum* ATCC 15707 cultures.
‖ Germ-free rats were associated with bacteria of fresh human faeces from a healthy male subject.
¶ Supplemented diet contained 50 g fructans (25 g OF (Raftilose® P95; Raffinerie Tirlemontoise, Tienen, Belgium) + 25 g lcIN (Raftiline® HP; Raffinerie Tirlemontoise))/kg.

Table 4. Thickness of the epithelial mucus layer and the numbers of goblet cells of the distal jejunum and the distal colon of germ-free (GF), diassociated (DA) and human flora-associated (HFA) rats fed a standard diet (ST) or a standard diet supplemented with fructan†

(Mean values for six rats per group)

	Rat model				Statistical significance of effect: *P*		
	GF	DA§	HFA‖	Pooled SEM	Diet	Rat model	Diet × Rat model
Distal jejunum							
Thickness of mucus layer (μm)							
ST	10·48	19·67	16·30	2·93	0·01	0·2	0·393
OF–lcIN¶	13·58	22·60	24·55*	3·02			
Goblet cells (*n*) per crypt							
ST	11·0	14·2	16·8	1·95	0·001	0·01	0·279
OF–lcIN¶	13·2	21·8*	23·3*	2·06			
Distal colon							
Thickness of mucus layer (μm)							
ST	18·22	45·50	43·38	2·75	0·0001	0·0001	0·079
OF–lcIN¶	22·42	55·58	62·22*	3·30			
Goblet cells (*n*) per crypt							
ST	16·4	22·3	24·1	1·78	0·0001	0·009	0·65
OF–lcIN¶	19·2	27·3*	32·3*	1·55			

OF, oligofructose; lc, long-chain; IN, inulin.
Mean values were significantly different from those of the ST group: *$P < 0·05$.
† For details of diets and procedures, see p. 598.
§ Germ-free rats were inoculated sequentially with *Bacteroides vulgatus* DSM1447 and *Bifidobacterium longum* ATCC 15707 cultures.
‖ Germ-free rats were associated with bacteria of fresh human faeces from a healthy male subject.
¶ Supplemented diet contained 50 g fructans (25 g OF (Raftilose® P95; Raffinerie Tirlemontoise, Tienen, Belgium) + 25 g lcIN (Raftiline® HP; Raffinerie Tirlemontoise))/kg.

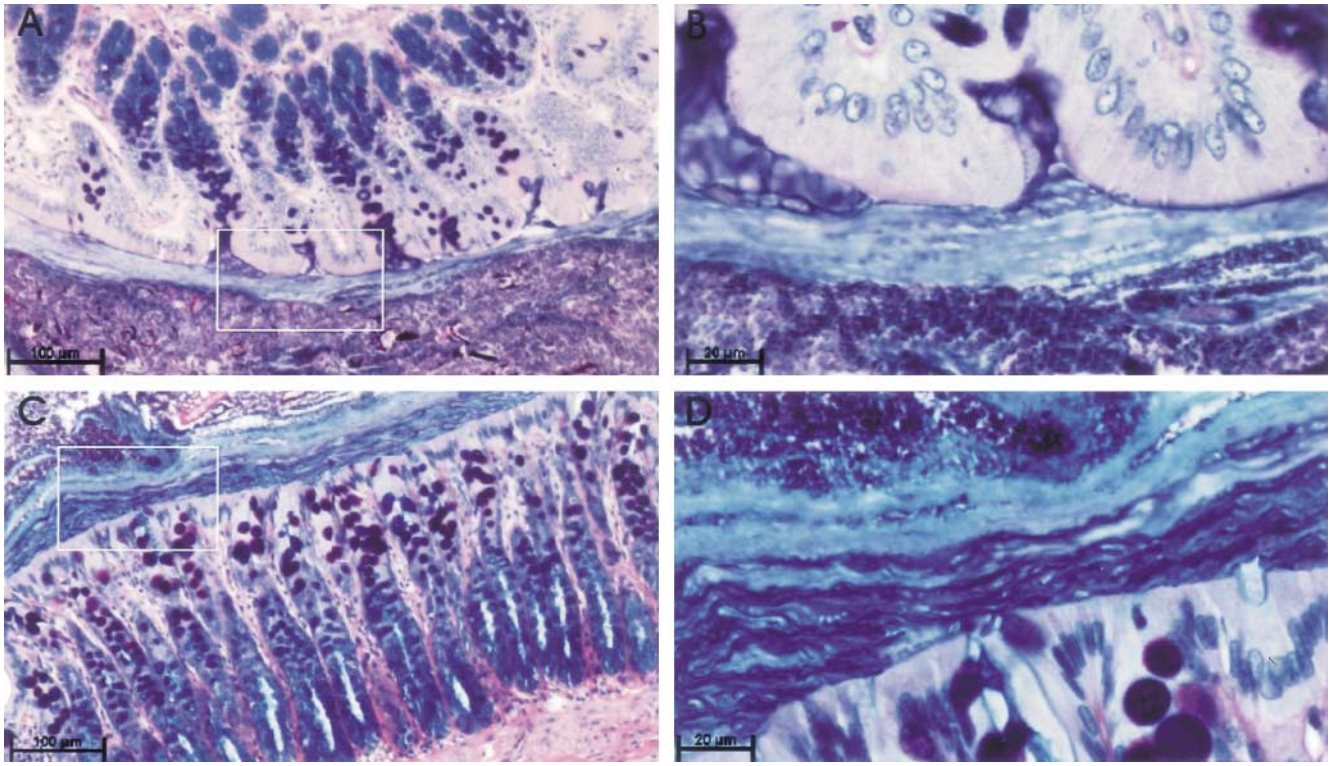

Fig. 2. Histological analysis of mucins in the colon of human flora-associated rats fed a standard diet ((A) and (B)) or a standard diet supplemented with oligofructose and long-chain inulin ((C) and (D)). A combination of Alcian Blue, pH 2·5 and periodic acid–Schiff staining was used to distinguish acidic (dark blue) and neutral (red) mucins. A purple colour indicates the presence of both acidic and neutral mucins. The epithelial mucus layer was thicker in fructans-fed rats. (A) and (C), magnification × 200; (B) and (D), higher magnification of the areas inside the boxes in (A) and (C): magnification × 630. For details of diets and procedures, see p. 598.

Table 5. Mucin distribution in the epithelial mucus layer and in the goblet cells from the distal jejunum and the distal colon of germ-free (GF), diassociated (DA) and human flora-associated (HFA) rats fed a standard diet (ST) or a standard diet supplemented with fructan*†‡
(Values for six rats per group)

	GF rats		DA§		HFA‖	
	Epithelial mucus layer	Goblet cells	Epithelial mucus layer	Goblet cells	Epithelial mucus layer	Goblet cells
Distal jejunum						
PAS						
ST	1	0–1	2	2	1–2	1
OF–lcIN¶	0–1	0–1	1–2	1	1–2	1–2
AB pH 2·5						
ST	1–2	2	1–2	1	1	1
OF–lcIN¶	2	2–3	1–2	2	1	1
AB pH 1·0						
ST	0–1	0–1	1	0–1	1	0–1
OF–lcIN¶	0–1	0–1	0–1	0–1	1	0–1
Distal colon						
PAS						
ST	1	0–1	1–2	2	3	2
OF–lcIN¶	0–1	0–1	1–2	2	3	3
AB pH 2·5						
ST	0–1	1	2	2	3	2
OF–lcIN¶	1	1	2–3	3	3	3
AB pH 1·0						
ST	1	0–1	0–1	1	1	0–1
OF–lcIN¶	1	1	2	2	2–3	3

PAS, periodic acid–Schiff; OF, oligofructose; lc, long-chain; IN, inulin; AB, Alcian Blue.
* For details of diets and procedures, see p. 598.
† Serial sections were subjected to the following three stainings for the identification of mucin components: (1) the PAS reaction for unsubstituted α-glycerol rich neutral mucins; (2) AB pH 2·5 to identify acidic mucins; (3) AB pH 1·0 to differentiate sulfated mucins.
‡ Results are expressed as intensity of reaction: 0, no reactivity; 1, weak reactivity; 2, moderate reactivity; 3, intense reactivity.
§ Germ-free rats were inoculated sequentially with *Bacteroides vulgatus* DSM1447 and *Bifidobacterium longum* ATCC 15707 cultures.
‖ Germ-free rats were associated with bacteria of fresh human faeces from a healthy male subject.
¶ Supplemented diet contained 50 g fructans (25 g OF (Raftilose® P95; Raffinerie Tirlemontoise, Tienen, Belgium) + 25 g lcIN (Raftiline® HP; Raffinerie Tirlemontoise))/kg.

ecological niche such as the epithelial surface or the mucus layer are distinct from those in the gut lumen. Support for this view comes from Poxton *et al*. (1997) and Macfarlane *et al*. (2000), who reported differences between luminal bacteria and bacterial populations intimately associated with the mucosal surface. These authors found that *Bacteroides*, particularly *B. vulgatus* and *B. thetaiotaomicron*, and also bifidobacteria, such as *B. adolescentis*, are the major organisms associated with the mucosal surface in the gut. It is generally accepted that bacteria differ in their capacity to colonize the mucus layer or the mucosal epithelium. Moreover, adhesion to the intestinal mucus supports persistence in the human gut ecosystem. He *et al*. (2001) suggested that the mucosal adhesion of bifidobacteria might be strain-specific and dependent on substrate availability such as mucins or fructans. According to Bernet *et al*. (1993), protein moieties seem to facilitate bifidobacterial adhesion to colonic epithelial cells (Caco-2 cells). In these investigations, adherent *B. longum*, *B. breve* and *B. infantis* variously inhibited cell-association and invasion of different gut pathogens, such as *Escherichia coli*, *Salmonella typhimurium* and *Yersinia pseudotuberculosis*. In view of these findings, the high numbers of bifidobacteria observed in our present study in the mucosal mucus layer of fructans-fed HFA rats lead us to speculate that these organisms may play a role in the protection of the mucosal epithelium. However, further investigations are needed to define the protective nature of *Bifidobacterium* on the colonic mucosa. The finding that the mucus layer of the rats fed the ST diet was less colonized by bifidobacteria may be related to the development of a less adherent *Bifidobacterium* population or to changes in the mucus composition of this dietary group and hence in reduced *Bifidobacterium* adhesion. In these rats, bacteria of the *Bacteroides–Prevotella* group were the most prevalent.

The presence of either a simple flora of only two different species (DA rat) or a complex human faecal flora (HFA rat) resulted in a number of changes in the mucosal architecture, most notably, variations in the EML (Tables 3–5). Moreover, these alterations were stimulated by dietary intake of fructans. The finding that the fructans-containing diet increased jejunal villus height and crypt depth in bacteria-associated rats but not in GF rats suggests that OF–lcIN does not affect the epithelial morphometry in the jejunum directly. A similar observation was made in the colon of HFA rats where crypts were elongated. Thus, this change in mucosal architecture in bacteria-associated rats seems to constitute an indirect effect of the fructans and their fermentation by intestinal bacteria. The resulting fermentation products are mainly short-chain fatty acids, with butyrate being the preferred substrate of the colonocytes and having a trophic effect on the mucosa (Sakata, 1987; Tappenden *et al*. 1998). This would be consistent with the stimulation of short-chain fatty acids formation observed previously in HFA rats fed OF and/or lcIN-containing diets (Kleessen *et al*. 2001). However, it should be mentioned that the rats in our present study were fed a basic diet of natural ingredients, which was coarsely

ground and contained 83 g dietary fibre/kg, mainly of cereal origin. Therefore, substrate interactions between fructans and fibre fermentation cannot be excluded.

Besides the changes in the mucosal architecture, bacteria-associated rats had more goblet cells per crypt and an increased mucin layer in response to fructans. The observed changes are in agreement with studies in conventional and heteroxenic rats fed dietary fibre- or galactooligosaccharide-containing diets (Meslin *et al*. 1993; Sharma & Schumacher, 1995; Schmidt-Wittig *et al*. 1996). Such a selective advantage for goblet cell differentiation might be related to an influence on the stem cell population or on the immature crypt cells committed to be goblet cells (Laboisse *et al*. 1996).

In accordance with the findings of other authors (Meslin *et al*. 1993; Sharma *et al*. 1995; Fontaine *et al*. 1996; Meslin *et al*. 1999), our present results, shown in Table 5, also confirm that the intestinal mucin types differ with the intestinal region investigated and with the bacterial status of the rats. Neutral mucosubstances were the predominant type seen in the small intestine, whereas acid mucosubstances were predominant in the colon of bacteria-associated rats. Moreover, it is noteworthy that the histological staining of the different mucins performed in our present study demonstrated alterations in the proportions of sulfo- and sialomucins in the colonic mucus of DA and HFA rats fed the different diets. Feeding of OF–lcIN-containing diet stimulated sulfomucins as the predominant type of acidic mucins, while sialomucins dominated in the ST-fed groups. Whether this finding is of physiological relevance remains to determined. However, it has been suggested that acidic mucins protect against bacterial translocation because sulfated mucins, in particular, appear less degradable by bacterial glycosidases and host proteases (Deplancke & Gaskins, 2001). Moreover, a variety of intestine diseases are accompanied by changes in the mucin composition. While inflammatory bowel disease is associated with decreased amounts of sialo- and sulfomucins, mucins isolated from colon cancer tissue appear to contain increased amounts of negatively charged mucin glycoproteins (Corfield *et al*. 1995; Rhodes, 1997; Brockhausen *et al*. 1998). Thus, alterations in the amounts of sulfomucins and sialomucins by fructans could have important effects on the susceptibility of epithelial mucins to attack by bacterial enzymes (Rhodes, 1997).

These results suggest that specific IN-type fructans in the diet not only change the mucosal architecture and the number of goblet cells in gnotobiotic rats, but also alter the composition of the mucins in the epithelial cells and EML. Changes in the mucin content or composition at the jejunal mucosa might be involved in modification of small intestinal nutrient absorption, whereas alterations in the distal colon might reflect different responses to specific bacterial populations or metabolite.

Given that treatment with fructans is associated with stabilizing the gut mucosal barrier via the stimulation of mucosa-associated bifidobacteria, it is probable that such dietary manipulations could be of benefit in both the protection of the intact intestinal epithelium and the therapy of a disturbed mucosal barrier. The definition of exact mechanisms underlying the claimed health-promoting effects of mucosal bifidobacteria will be crucial for understanding their protective role.

Acknowledgements

We want to express our thanks to Susanne Dietrich, Ines Grüner, Renate Herzog, Ute Lehmann and Elke Thom for taking care of the animals. The expert technical assistance of Sabine Schmidt and Elisabeth Meyer is acknowledged. The authors thank Dr Gerhard Dongowski for determination the total dietary fibre content of experimental diets. This work was supported by European Research Project FAIR-CT-97-3035.

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