

## Oligofructose and long-chain inulin: influence on the gut microbial ecology of rats associated with a human faecal flora

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Dietary incorporation of fermentable, indigestible fructans may be of benefit to gastrointestinal health by providing short-chain fatty acids, stimulating the proliferation of bifidobacteria or lactobacilli and suppressing potential pathogenic organisms in the gut. We tested the hypothesis that the effects of fructans on caecal, colonic and faecal short-chain fatty acid concentration and microflora composition depend on their chain length. Germ-free rats associated with a human faecal flora were randomly assigned to one of four treatments as follows: (1) commercial standard diet as a control (Con); (2) Con+50 g short-chain oligofructose/kg (OF); (3) C+50 g long-chain inulin/kg (lcIN); or (4) Con+50 g OF–lcIN/kg (Mix OF–lcIN). Changes in bacterial population groups in response to feeding these diets were investigated with 16S rRNA-targeted probes applied in *in situ* hybridization. Mix OF–lcIN- and lcIN-containing diets resulted in larger numbers of caecal, colonic and faecal bacteria of the *Clostridium coccoides*–*Eubacterium rectale* cluster than Con (10.6 and 10.3 v. 9.5 log<sub>10</sub>/g wet wt), whereas OF alone did not affect this bacterial group in caecum, colon or faeces. A bifidogenic effect was only observed in the colon and faeces of OF-treated rats. More lactobacilli were found in caecal and colonic contents of Mix OF–lcIN-fed rats and in faeces of OF-fed rats compared with Con. Mix OF–lcIN and OF led to significantly smaller numbers of caecal, colonic and faecal bacteria belonging to the *Clostridium histolyticum* and *C. lituseburensis* groups than Con (6.8 and 6.9 v. 7.9 log<sub>10</sub>/g wet wt). Counts of total bacteria, *Bacteroides*–*Prevotella* and *Enterobacteriaceae* did not differ between the groups. OF and/or lcIN-containing diets significantly increased the caecal and colonic concentration of butyrate and its relative molar proportion. Only lcIN-containing diets resulted in a higher faecal concentration of butyrate than Con. Higher molar proportions of faecal butyrate were observed with all diets that had been supplemented with OF and/or lcIN. Stimulation of butyrate production could be of interest for the prevention of ulcerative colitis and colon cancer.

### Butyrate: Oligofructose: Inulin: Intestinal microflora: Gnotobiotic rats

The recent commercialization of inulin (IN) and oligofructose (OF) as food ingredients and fat replacers has served to focus much research on the influence of these substrates on the intestinal ecosystem and colonic function (Roberfroid, 2000). IN and OF occur in plants such as chicory, leek, onion, garlic, artichoke and asparagus at high levels (Roberfroid & Delzenne, 1998; Van Loo *et al.* 1995). IN and OF are not digested by mammalian enzymes in the alimentary tract, thus contributing to the amount of fermentable carbohydrates in caecum and colon (Alles *et al.* 1996; Roberfroid, 1997). Fermentation of OF and IN in the gut may favour the production of short-chain fatty acids (SCFA) such as acetate, propionate and butyrate and

sometimes of lactate (Cummings & Macfarlane, 1997). The role of SCFA in the physiology of the colon is well known. Besides being involved in colonic Na and water absorption, SCFA are an important energy source for the colonocytes with butyrate being their preferred substrate and have a trophic effect on the colonic mucosa (Roediger, 1995). Butyrate also contributes to the maturation of colonic epithelium, to regeneration of the mucosa in the case of atrophy (Sakata, 1987), induces cell differentiation and stimulates apoptosis of cancerous cells (Hague *et al.* 1996). Therefore, butyrate is thought to play an important role as a preventative agent in colon carcinogenesis and/or ulcerative colitis. Based on these effects the stimulation of bacterial

**Abbreviations:** Con, control; DP, degree of polymerization; HFA, human flora-associated; IN, inulin; lc, long-chain; Mix OF–lcIN, oligofructose–long-chain inulin (1:1, w/w); OF, oligofructose; SCFA, short-chain fatty acid.

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butyrate formation by dietary means is of particular interest. Animal studies have shown that the absolute concentration and molar proportion of butyrate and their distribution along the intestine varied between different sources of oligosaccharides (Campbell *et al.* 1997). Based on these findings, it may be hypothesised that specific IN-type fructans may be especially effective in raising the butyrate concentration in the colon.

Interest in IN and OF for human nutrition also stems from the prebiotic properties of these carbohydrates. Prebiotics are defined as non-digestible food ingredients, that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of potentially health-promoting bacteria in the large intestine (Gibson & Roberfroid, 1995; Van Loo *et al.* 1999). Previous *in vitro* and *in vivo* studies have demonstrated that IN and OF selectively stimulate the growth of bifidobacteria or lactobacilli, both of which are considered to be beneficial to the host (Hidaka *et al.* 1986; Wang & Gibson, 1993; Gibson *et al.* 1995; Bouhnik *et al.* 1996, 1999; Campbell *et al.* 1997; Djouzi & Andrieux, 1997; Kleessen *et al.* 1997; Sghir *et al.* 1998; Kruse *et al.* 1999; Le Blay *et al.* 1999; Menne *et al.* 2000). These bacteria are recognised for creating conditions unfavourable for the growth of potentially pathogenic organisms, such as enterobacteria and certain clostridia (Gibson & Wang, 1994; Roberfroid, 1997), and for forming potentially toxic or carcinogenic compounds (Buddington *et al.* 1996).

Taking into account all these aspects, there is a need to understand better the role of IN and OF in modulating the interactions between intestinal microflora and health. With a view toward an increasing butyrogenic effect in the gut the question arises as to whether the chemical and structural composition of the fructose-based non-digestible carbohydrates, e.g. differences in chain length, may influence the composition of the intestinal microflora and the microbial fermentations products in various segments of the gastrointestinal tract. The purpose of this study was therefore to compare effects of OF, long-chain (lc) IN and a mixture of these fructans on caecal, colonic and faecal SCFA concentration, pH, total large bowel wt and wall wt, and gut microflora in human flora-associated (HFA) rats.

## Materials and methods

### Fructans

Raftilose<sup>®</sup> P95 and Raftiline<sup>®</sup> HP which contained mainly OF (950 g/kg) and IN (995 g/kg) respectively, were supplied by the Raffinerie Tirlémontoise, Tienen, Belgium. Raftilose (OF), which is produced by partial enzymatic hydrolysis of chicory IN, is a mixture of  $\beta$  (2–1) fructans with a degree of polymerization (DP) ranging between 3 and 7 (average DP of 4). Raftiline<sup>®</sup> HP is lc chicory IN devoid of fructans with the lower DP. The DP of Raftiline<sup>®</sup> HP ranges between 10 and 60 (average DP of 25). This choice of test substrates thus allowed us to investigate effects of DP on microflora composition and SCFA concentration.

### Animals and diets

All treatments and diets were approved by the Ministry of Nutrition, Agriculture, and Forestry, Brandenburg, Germany (permission 48-3560-0/3). Twenty-four male 5-week-old germ-free Wistar rats (inbred strain AVN-Ipcv; DIfE, Bergholz-Rehbrücke, Germany) with an initial body weight of 122 (SD 9) g were randomly divided into four groups of six animals. Rats were maintained in positive-pressure isolators (Metall & Plastik, Radolfzell, Germany) equipped with a sterile water supply and housed in macrolon cages on wire grates at a temperature of  $22 \pm 2^\circ\text{C}$ , relative humidity  $55 \pm 5\%$  and on a 12 h light–dark cycle (07.00–19.00 hours). They were given free access to water and a commercial standard diet (Altromin fortified<sup>®</sup> 1310; Altromin, Lage, Germany) with the following composition (according to the manufacturers) (g/kg): crude protein 225, crude fat 50, ash 65, moisture 135, N-free extract 480. Gross energy was 12.5 MJ/kg diet. Rats were associated with bacteria by applying 3 ml 1:10 dilution of fresh human faeces from a healthy male subject (age 30 years). Ten days after inoculation, the rats were divided into four groups. During a 7 d adaptation period treatments were as follows: (1) control diet (Altromin fortified<sup>®</sup> 1310) (Con); (2) control diet+25 g Raftilose<sup>®</sup> P95/kg (OF); (3) control diet+25 g Raftiline<sup>®</sup> HP/kg (lcIN); (4) control diet+25 g Raftilose<sup>®</sup> P95 – Raftiline<sup>®</sup> HP (1:1, w/w)/kg (Mix OF–lcIN). Subsequently, the first group was fed on Con for 8 d (experimental period). The other three groups received the diet containing 50 g fructans (OF, lcIN, Mix OF–lcIN)/kg. The diets were sterilized by irradiation at 25 kGy in plastic bags. Food intake was determined daily and body weight was recorded every 2 d.

Total dietary fibre content in the experimental diets was determined by the enzymic–gravimetric Association of Analytical Chemists' method (Prosky *et al.* 1988). In this method, fat-free material was treated with heat-stable  $\alpha$ -amylase, and then enzymically digested with protease and amyloglucosidase to remove protein and starch. Enzyme digesta was treated with four volumes ethanol (780 ml/l) to precipitate soluble dietary fibre. Total residue was filtered, washed with ethanol (780 ml/l), ethanol (960 ml/l) and acetone, dried and weighed. Residue value was corrected for protein and ash. The dietary fibre content of Con (without added fructans) was 87 g/kg diet. For the other experimental diets, Con was partially replaced by fructans, resulting in a total dietary fibre content of 109.8 and 132.6 g/kg diet during the adaptation and experimental periods respectively.

### Sampling

Fresh faecal samples were collected at day 7 of the experimental period. For microbiological enumeration 0.5 g specimen was fixed as described previously (Kruse *et al.* 1999). About 0.2 g sample was immediately frozen at  $-80^\circ\text{C}$  for analysis of lactate. For analysing SCFA, 0.2 g specimen was prepared as described by Kruse *et al.* (1999). The residual material was used for pH measurement and DM estimation. At the end of the experimental period, rats were killed by decapitation. The caecum and colon were

aseptically removed from the abdominal cavity and dissected free from fat and mesentery. Caecal and colonic contents were collected, weighed, pH measured, and aliquots were immediately processed for bacterial enumeration, analysis of lactate and SCFA, and DM estimation. Following removal of the intestinal contents, the tissues were cleaned with cold PBS, blotted and weighed to determine caecal and colonic wall weight.

#### Bacterial enumeration

Total bacteria and bacterial groups in the caecum, colon and faeces were enumerated by fluorescent *in situ* hybridization using the following 16S/23S 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) Erec 482 (S-\*Erec-0482-a-A-19) specific for most of the clostridia and eubacteria belonging to the *Clostridium coccoides-Eubacterium rectale* group (Franks *et al.* 1998); (4) the bifidobacterial probe Bif 164 (S-G-Bif-0164-a-A-18) (Langendijk *et al.* 1995); (5) Lab 158 (S-G-Lab-0158-a-A-20) for nearly all species of the genera *Lactobacillus* and *Enterococcus* in the gut (Harmsen *et al.* 1999); (6) Efs (L-\*Efs-0343-a-A-18) to distinguish gut enterococci, i.e. *Enterococcus faecalis* among the Lab 158 (Beimfohr *et al.* 1993); (7) Ec 1531(L-S-Eco-1531-a-A-21) specific for a number of *Enterobacteriaceae* (e.g. *Escherichia coli*, *Klebsiella pneumonia*) (Poulsen *et al.* 1995); (8) Chis 150 (S-\*Chis-0150-a-A-23) and (9) Clit 135 (S-\*Clit-0135-a-A-19) to detect bacteria of the *Clostridium histolyticum* group and the *C. lituseburense* group (Franks *et al.* 1998). The nonsense probe Non 338 was used as a control to monitor non-specific oligonucleotide binding (Amann *et al.* 1995). The oligonucleotides were purchased 5' labelled with the indocarbocyanine dye Cy3 (Interactiva Biotechnologie GmbH, Ulm, Germany).

Fluorescent *in situ* hybridization was done as described by Manz *et al.* (1993) with some modifications. For homogenization, fixed samples were treated for 4 min in a Uniprep-Gyrotor (InViTek GmbH, Berlin, Germany) and spotted on single wells of 3-aminopropyltriethoxysilane-coated slides (2 g 3-aminopropyltriethoxysilane in 99 ml methanol), and allowed to air-dry. To improve permeabilization of the cell envelope, samples were treated with 10  $\mu$ l lysozyme buffer (100 mM-Tris-HCl (pH 8.0), 50 mM-EDTA, 1 mg lysozyme (130 000 U/mg; Boehringer, Mannheim, Germany) and incubated on ice for 10 min. Hybridizations were performed for 16 h at 46°C (50°C) in humid chambers after addition of 10  $\mu$ l hybridization buffer (0.9 M-NaCl, 0.1 g SDS/l and 10 mM-Tris-HCl, pH 7.4) containing 5 ng probe/ $\mu$ l. After washing the hybridization slides in hybridization buffer for 20 min at 48°C (52°C), and subsequent treatment with the SlowFade<sup>®</sup> Antifade Kit (Molecular Probes, Leiden, The Netherlands) they were examined with a Carl Zeiss Axioplan epifluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a 100 $\times$ 1.30 Plan Neofluar Ph3 oil immersion objective, an HBO 100 W/3 Hg lamp, the filter block 15, and a 12-bit

cooled CCD camera (Sensicam 370 KL, PCO Kelheim, Germany). Images were taken and fluorescent cells were counted with the image analysis system KS400 Release 3.0 (Carl Zeiss). The microbial counts are expressed as log<sub>10</sub> organisms/g wet wt.

#### Short-chain fatty acids and lactate

SCFA were extracted as described previously by Kruse *et al.* (1999). Samples of 1  $\mu$ l were injected into a GC (HP 5890 A; Hewlett Packard GmbH, Waldbronn, Germany) equipped with a flame-ionization detector and a capillary column (25 m $\times$ 0.23 mm) impregnated with 20 M Carbowax (Hewlett Packard GmbH). The carrier gas was He at a column flow rate of 12 ml/min with a split ratio of 1:10. The column temperature was 125°C. Isobutyric acid served as an internal standard. Lactate was determined enzymically using L- and D-lactate dehydrogenases (Kit 1112821; Boehringer Mannheim). For DM estimation intestinal samples were freeze-dried to constant weight under vacuum.

#### Statistics

The Statistical Package for Social Science (SPSS for Windows, version 8.0; SPSS Inc., Chicago, IL, USA) was used to determine if variables differed among treatment groups. Two-way ANOVA was performed to assess the effects of diet (Con, OF, lcIN, Mix OF-lcIN), of segment (caecum, colon and faeces) and of the interaction between diet and segment (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. In the tables, results are presented as mean values with their standard errors. Data were checked for normality before statistical analysis was performed (Zar, 1984). Bacteriological results were transformed-log<sub>10</sub> to improve the homogeneity of variance. Differences were considered to be significant at  $P < 0.05$ .

## Results

#### Feed intake and body-weight gain

Rats were generally in good health throughout the experiment. There were no differences in feed intake or body-weight gain among the treatment groups. The mean feed intake in the experimental period was 23.1–23.7 g/d (SEM 0.4). The mean body-weight gain of the rats ranged from 14.2 to 14.8 g/2 d (SEM 0.6). There were no growth differences at any interval throughout the study (results not shown).

#### Total large bowel content, wall weight, DM, pH and faecal excretion

Addition of OF and lcIN to the diet led to greater wet content and wall weight of the caecum compared with control (Con) rats (Table 1). Wet content of the colon was higher ( $P < 0.05$ ) in rats consuming the lcIN diet than in

**Table 1.** Caecal and colonic content and wall weights, DM, pH and faecal output in rats associated with a human faecal flora†  
(Mean values for six rats per group)

Segment	Diet‡				Pooled SEM
	Control	OF	lcIN	Mix OF–lcIN	
Wet content (g)					
Caecum	5.76	7.78	7.24	8.34*	0.51
Colon	2.44	2.87	3.85*	2.60	0.25
Wall weight (g)					
Caecum	1.30	1.64	1.51	1.87*	0.05
Colon	1.16	1.51	1.32	1.42	0.05
pH					
Caecum	6.40	5.71*	5.65*	6.31	0.08
Colon	6.25	5.75*	5.61*	6.00	0.06
Faeces	6.07	5.92	6.16	6.19	0.05
DM (g/kg)					
Caecum	235.6	195.9	202.0	216.0	5.1
Colon	275.6	246.4	234.2	228.8	4.2
Faeces	345.5	275.1	293.2	264.9*	10.8
Faecal output (g wet wt/d)	11.00	12.97*	12.67*	13.34*	0.22

OF, oligofructose (Raftilose® P95; Raffiniere Tirlemontoise, Tienen, Belgium); lcIN, long-chain inulin (Raftiline® HP; Raffiniere Tirlemontoise); Mix OF–lcIN, OF–lcIN (1:1, w/w).

Mean values were significantly different from those for the control group: \*  $P < 0.05$ .

† For details of diets and procedures, see p. 292.

‡ Differences among treatment groups were analysed by one-way ANOVA followed by Scheffe's test for multiple comparison.

rats on the Con diet. Colonic wall weights were unaffected by diets. The percentage faecal DM was lower ( $P < 0.05$ ) in rats fed Mix OF–lcIN than in rats on the Con diet (Table 1). Caecal and colonic pH was lower ( $P < 0.05$ ) in rats consuming OF or lcIN diet compared with the Con diet. No significant differences among groups were noted in faecal pH. Faecal output was higher in rats consuming fructan-containing diets than Con due to increased water excretion.

#### Bacterial enumeration

The effects of treatments on caecal, colonic and faecal microflora of rats are presented in Table 2. Total bacterial counts/g wet weight did not differ, but there were differences with respect to counts of certain bacterial groups. Caecal, colonic and faecal counts of bacteria belonging to the *Clostridium coccooides*–*Eubacterium rectale* cluster were significantly ( $P < 0.05$ ) higher in rats consuming Mix OF–lcIN than in the rats consuming Con. Bacteria of this group also tended ( $P = 0.062$ ) to be higher in rats fed lcIN than in rats fed Con but did not differ in rats fed OF. The OF-containing diet resulted in higher numbers of bifidobacteria ( $P < 0.05$ ) in the colon than Con and in a trend ( $P = 0.052$ ) to higher counts in the faeces, whereas the numbers observed in the caecum were not statistically different. In contrast, rats consuming the lcIN-containing diet had fewer ( $P < 0.05$ ) bifidobacteria in the caecum compared with rats receiving the Con diet. The numbers of bifidobacteria were unaffected by Mix OF–lcIN treatment. Rats fed Mix OF–lcIN, however, had higher ( $P < 0.05$ ) caecal and colonic numbers of *Lactobacillus* compared with Con. In contrast, rats consuming OF displayed higher ( $P < 0.05$ ) numbers of *Lactobacillus* and *Enterococcus* in faeces compared with Con-fed rats. Mix OF–lcIN and OF led to smaller ( $P < 0.05$ ) numbers of bacteria belonging to the

*Clostridium histolyticum* and *C. lituseburensense* groups than Con. No significant differences among groups were noted in numbers of *Bacteroides*–*Prevotella* and *Enterobacteriaceae*.

#### Short-chain fatty acids and lactate

Both the diet and the site of sampling in the gut (segment) affected the concentration of SCFA, and there were significant interactions between the effects (Table 3). The SCFA concentrations were always higher in caecal contents than in faeces ( $P < 0.05$ ). The caecal and colonic concentrations of butyrate were higher ( $P < 0.05$ ) in all rats consuming OF and/or lcIN than in rats consuming Con. Only lcIN-containing diets (lcIN, Mix OF–lcIN), however, affected the faecal concentration of butyrate ( $P < 0.05$ ). Moreover, rats fed lcIN had a higher ( $P < 0.05$ ) caecal propionate concentration than Con. More acetate was found in colonic contents of OF-fed rats and in faeces of Mix OF–lcIN-fed rats ( $P < 0.05$ ). The molar ratios of SCFA in the intestinal samples differed with the diet (Table 4). The butyrate proportions were higher in all rats fed OF and/or lcIN than in those fed Con diet. Lower proportions of acetate ( $P < 0.05$ ) were found in the caecal contents of lcIN-fed rats and in the faeces of Mix OF–lcIN-fed rats compared with Con. Moreover, OF and Mix OF–lcIN resulted in lower colonic propionate proportions. Caecal and faecal concentrations of lactate were unaffected by treatments (Table 3).

#### Discussion

Feeding trials with HFA rats were performed to investigate the influence of OF and/or lcIN on caecal, colonic and faecal microbiota and microbial fermentation products. Gnotobiotic rats were used for these investigations because intestinal



**Table 2.** Effects of dietary oligofructose and inulin on caecal, colonic and faecal bacterial counts ( $\log_{10}$  counts/g wet weight) in rats associated with a human faecal flora†  
(Mean values for six rats per group)

Segment	Diet				Pooled SEM	Statistical significance of effect of: <i>P</i>		
	Control	OF	lcIN	Mix OF–lcIN		Diet	Segment	Diet × Segment
<b>Total bacteria</b>								
Caecum	10.5	10.6	10.5	10.8	0.05	0.21	0.35	0.97
Colon	10.7	10.7	10.7	10.8	0.06			
Faeces	10.6	10.5	10.6	10.8	0.07			
<b><i>Bacteroides–Prevotella</i></b>								
Caecum	10.1	10.1	9.6	9.7	0.11	0.07	0.08	0.48
Colon	9.9	10.0	9.6	9.7	0.08			
Faeces	10.2	9.9	10.0	9.6	0.07			
<b><i>Clostridium coccoides–Eubacterium rectale</i> cluster</b>								
Caecum	9.6	9.9	10.2	10.6*	0.10	0.0001	0.51	0.79
Colon	9.5	9.8	10.4	10.6*	0.11			
Faeces	9.4	9.9	10.3	10.6*	0.09			
<b><i>Clostridium histolyticum</i> group and <i>C. lituseburensense</i> group</b>								
Caecum	8.1	6.8*	7.5	7.1*	0.15	0.0001	0.41	0.69
Colon	7.7	7.1	7.4	6.5*	0.15			
Faeces	8.0	7.0*	7.4	6.9*	0.14			
<b><i>Bifidobacterium</i></b>								
Caecum	8.6	9.2	7.4*	9.0	0.17	0.0001	0.19	0.003
Colon	8.5	9.7*	8.4	8.7	0.13			
Faeces	8.7	9.6	8.2	8.3	0.15			
<b><i>Lactobacillus</i></b>								
Caecum	8.0	8.6	8.4	9.5*	0.18	0.0001	0.20	0.011
Colon	8.3	8.9	8.7	9.8*	0.16			
Faeces	8.0	9.2*	8.2	8.7	0.17			
<b><i>Enterococcus</i></b>								
Caecum	7.6	8.0	7.0	7.5	0.20	0.001	0.42	0.48
Colon	7.5	8.0	6.9	7.7	0.15			
Faeces	7.0	8.6*	7.5	8.1	0.17			
<b><i>Enterobacteriaceae</i></b>								
Caecum	7.5	7.2	7.8	7.7	0.13	0.45	0.18	0.46
Colon	7.5	7.5	7.7	7.4	0.09			
Faeces	8.1	7.9	7.8	7.5	0.14			

OF, oligofructose (Raftilose® P95; Raffiniere Tirlemontoise, Tienen, Belgium); lcIN, long-chain inulin (Raftiline® HP; Raffiniere Tirlemontoise); Mix OF–lcIN, OF–lcIN (1:1, w/w).

Mean values were significantly different from those of the control group: \*  $P < 0.05$ .

† For details of diets and procedures, see p. 292.

contents except faeces cannot be obtained easily from healthy human subjects without the application of invasive techniques which cannot be justified for ethical reasons. The HFA rat is a model allowing accessibility to caecal and colonic contents. This model was validated for fermentation studies of non-digestible fructans (Djouzi & Andrieux, 1997). The similarity of the resultant flora to that of human faeces has been investigated at bacteriological and metabolic levels (Mallet *et al.* 1987; Rumney & Roland, 1992). In our present study, rats fed the control diet kept the major characteristics of the human donor in terms of bacterial population (e.g. numbers of bacteria belonging to the dominant flora: *Clostridium coccoides–Eubacterium rectale* cluster, *Bacteroides–Prevotella*, bifidobacteria) and metabolic profile (Tables 2–4). The HFA rat model facilitates studies of the interaction between dietary fructans and the human gut flora, since it is easier to control and manipulate animal diets than those of human subjects.

To our knowledge, this is the first study which exploits the fluorescent *in situ* hybridization-technology using a panel of ten different 16S/23S rRNA targeted oligonucleotide probes for determining gut bacteria in HFA rats on a

quantifiable basis. This method has made it possible to enumerate bacterial populations without prior cultivation. In particular the occurrence of obligately anaerobic bacteria such as *Eubacterium* sp. may be considerably underestimated when cultivation-based enumeration techniques are used. Therefore, fluorescent *in situ* hybridization leads to a major improvement in our current knowledge of the composition of the intestinal microflora (Welling *et al.* 1997).

The feeding of OF and/or lcIN to HFA rats resulted in a number of changes in caecal, colonic and faecal variables, most notably, variations in microflora composition (Tables 1–4). One important finding of the study concerns the increase in bacteria belonging to the *Clostridium coccoides–Eubacterium rectale* cluster in rats consuming lcIN-containing diets compared with Con. During the period of Mix OF–lcIN ingestion, these organisms increased as a proportion of the total flora from 13% to 63%, displacing *Bacteroides–Prevotella* as the predominant micro-organisms in the caecum. It is tempting to speculate that the selective stimulation of the *Clostridium coccoides–Eubacterium rectale* group by lcIN or Mix OF–lcIN, but

**Table 3.** Effects of dietary oligofructose and long-chain inulin on caecal, colonic and faecal concentrations ( $\mu\text{mol/g}$  wet wt) of short-chain fatty acids (SCFA) and lactate in rats associated with a human faecal flora†  
(Mean values for six rats per group)

Segment	Diet				Pooled SEM	Statistical significance of effect of: <i>P</i>		
	Control	OF	lcIN	Mix OF–lcIN		Diet	Segment	Diet × Segment
<b>Total SCFA</b>								
Caecum	84.4	99.4*	103.8*	98.3*	1.9	0.0001	0.0001	0.09
Colon	68.6	87.1*	81.9	85.8*	2.1			
Faeces	59.3	61.9	63.7	59.2	1.6			
<b>Acetate</b>								
Caecum	49.8	55.4	45.9	51.2	1.3	0.006	0.0001	0.004
Colon	41.1	50.8*	42.5	46.7	1.2			
Faeces	37.4	35.1	35.6	27.6*	1.3			
<b>Propionate</b>								
Caecum	21.1	22.8	32.5*	19.1	0.8	0.01	0.0001	0.06
Colon	18.2	17.8	21.3	16.8	0.7			
Faeces	14.7	15.5	14.4	15.8	0.4			
<b>Butyrate</b>								
Caecum	13.4	21.3*	25.4*	28.0*	1.3	0.0001	0.0001	0.3
Colon	9.3	18.6*	18.1*	22.3*	1.2			
Faeces	7.1	11.4	13.6*	15.7*	0.9			
<b>Lactate</b>								
Caecum	5.8	9.2	8.8	8.7	0.7	0.5	0.05	0.2
Colon	4.5	7.6	7.7	6.9	0.4			
Faeces	3.6	4.9	4.6	4.9	1.1			

OF, oligofructose (Raftilose® P95; Raffiniere Tirlemontoise, Tienen, Belgium); lcIN, long-chain inulin (Raftiline® HP; Raffiniere Tirlemontoise); Mix OF–lcIN, OF–lcIN (1:1, w/w).

Mean values were significantly different from those of the control group: \*  $P < 0.05$ .

† For details of diets and procedures, see p. 292.

not by OF alone, is based on a highly efficient enzyme system for the utilization of lcIN which enables members of the *Clostridium coccooides*–*Eubacterium rectale* group to compete successfully with other bacterial groups in the gut ecosystem.

Moreover, it is noteworthy that the feeding of OF-containing diets resulted in a significant decrease of bacteria, belonging to the *Clostridium histolyticum* or *C. lituseburens* groups, e.g. *C. perfringens*. A high

proportion of these organisms may be pathogenic, e.g. through their proteolytic capabilities and toxin production. An increased SCFA-production through bacterial fermentation of non-digestible carbohydrates such as OF or lcIN and the resulting decrease in caecal and colonic pH (Table 1) is an accepted mechanism for the inhibition of pathogens (Hentges, 1967). However, Gibson & Wang (1994) pointed out that acidity may not be the sole mechanism of inhibition. The authors showed by fermentation experiments that even

**Table 4.** Effects of dietary oligofructose and long-chain inulin on caecal, colonic and faecal molar ratios of acetate, propionate and butyrate in rats associated with a human faecal flora†‡  
(Mean values for six rats per group)

Segment	Diet				Pooled SEM	Statistical significance of effects of: <i>P</i>		
	Control	OF	lcIN	Mix OF–lcIN		Diet	Segment	Diet × Segment
<b>Acetate</b>								
Caecum	59.1	55.8	44.2*	51.9	1.2	0.0001	0.2	0.01
Colon	59.6	58.3	52.0	54.7	1.0			
Faeces	62.8	56.4	56.0	47.0*	1.6			
<b>Propionate</b>								
Caecum	25.0	22.9	31.3	19.5	1.0	0.002	0.2	0.01
Colon	26.8	20.4*	25.9	19.4*	0.8			
Faeces	25.2	25.3	22.7	26.7	0.8			
<b>Butyrate</b>								
Caecum	15.9	21.3	24.5*	28.6*	1.2	0.0001	0.06	0.8
Colon	13.6	21.3*	22.1*	25.9*	1.1			
Faeces	12.0	18.3*	21.3*	26.3*	1.4			

OF, oligofructose (Raftilose® P95; Raffiniere Tirlemontoise, Tienen, Belgium); lcIN, long-chain inulin (Raftiline® HP; Raffiniere Tirlemontoise); Mix OF–lcIN, OF–lcIN (1:1, w/w).

Mean values were significantly different from those of the control group: \*  $P < 0.05$ .

† For details of diets and procedures, see p. 292.

‡ Molar ratio, mol/100 mol short-chain fatty acids (acetate+propionate+butyrate).

at a neutral pH level clostridia were inhibited when co-cultured with *Bifidobacterium infantis*. Therefore, the production of inhibitory substances such as bacteriocins by the bifidobacteria is assumed. Feeding of OF-containing diets also resulted in a stimulation of *Lactobacillus* (Table 2). The increase in lactobacilli detected in rats fed Mix OF–lcIN was, however, restricted to caecal and colonic contents and in rats fed OF to the faeces. A similar observation was made by Le Blay *et al.* (1999) using fructooligosaccharides in a long-term experiment with conventional rats. The expected bifidogenic effects, however, were only restricted to the colon and faeces of the OF-treated rats. These observations are seemingly in contradiction to previous own investigations using IN in human subjects (Kleessen *et al.* 1997; Kruse *et al.* 1999), *in vitro* work by others (Wang & Gibson, 1993), *in vivo* studies in HFA rats (Djouzi & Andrieux, 1997), and studies on human subjects (Gibson *et al.* 1995). These discrepancies may be explained as follows: First, in all these studies, IN with an average DP of 8–10 and not lcIN with an average DP of 25 has been used. Second, bifidobacteria do not have a highly efficient enzyme system for the utilization of lcIN (Roberfroid *et al.* 1998). Third, it is still an open question as to whether the initial number of bifidobacteria in the faeces influences the extent of stimulation of bifidobacteria in response to the consumption of IN (Roberfroid *et al.* 1998; Rao, 1999). In our present study, the high level of bifidobacteria both in the human faecal inoculum ( $9.0 \log_{10}/g$  wet faeces) and in the initial faecal samples of HFA rats ( $8.7 \log_{10}/g$  wet faeces) may be responsible for the lack of increase in bifidobacterial numbers in response to lcIN (Table 2).

The marked differences in rat caecal, colonic and faecal microflora of rats in response to feeding of OF and/or lcIN confirm that modifications in the chain length of fructans have the potential to change the composition of the intestinal microflora. Despite the evidence that the gut flora composition is affected by the administration of OF or lcIN, it is difficult to identify those bacterial species that are responsible for these changes. This difficulty arises from the fact that complex carbohydrates, such as lcIN, are metabolized in a process based on cross-feeding by the microflora (Gibson, 1999).

Although it is generally accepted that OF and IN are not metabolized by rat digestive enzymes in the small intestine and thus reach the caecum and colon, a partial bacterial hydrolysis and fermentation of these non-digestible fructans, at least of OF, in the distal part of the small intestine of the HFA rats cannot be excluded. This view is supported by the ileal numbers of lactobacilli which tended to be higher in rats fed OF ( $2 \times 10^8/g$  wet wt) than in control rats ( $5 \times 10^7/g$  wet wt), but this difference was not significant (results not shown).

Several authors have reported that feeding OF or IN increases tissue weights and contents of the caecum (Andrieux *et al.* 1991; Campbell *et al.* 1997; Le Blay *et al.* 1999). Accordingly, we observed higher caecal contents and wall weights in rats fed OF and/or lcIN than in Con. This means that, on the one hand OF or lcIN are substrates, and thus nutrients for bacterial metabolism, and thereby cause an increase in bacterial mass. This is supported by higher numbers of total organisms in caecal digesta in the rats fed

these diets as compared with Con diet. On the other hand, it has been suggested that fermentable polysaccharides or oligosaccharides cause marked caecal enlargement associated with an increase in mucosal cell proliferation. It has been argued that this trophic effect is due to increased bacterial metabolism leading to a reduction in intralumen pH, or a direct stimulating effect of SCFA, mainly butyrate (Sakata, 1987). Increased production of SCFA within the large bowel may influence intestinal mucosal proliferation by increasing proglucagon mRNA expression and stimulating glucagon-like peptide-2 secretion (Massimino *et al.* 1998; Tappenden *et al.* 1998).

It should be noted, that OF and/or lcIN were included in the diets also at the expense of dietary fibre as shown by the Association of Analytical Chemists' method (Prosky *et al.* 1988). The bulking capacity of dietary fibre might have influenced the faecal weight in this study.

The high number of bacteria detected by fluorescent *in situ* hybridization in the caecum of rats reflects a considerable fermentation capacity in the caecum. Butyrate was particularly increased in rats fed Mix OF–lcIN or lcIN and considerably less in rats fed the Con diet. This is consistent with previous studies using OF or IN both *in vitro* (Gibson & Wang, 1994) and *in vivo* (Campbell *et al.* 1997). The same applies to the molar proportion of butyrate to total SCFA. The molar proportion of colonic and faecal butyrate was also elevated in those rats that were fed any of the three supplemented diets. However, in contrast to the caecum and colon the increase in butyrate concentration in faeces was only detected in rats consuming lcIN-containing diets and was not paralleled by an increase in the total SCFA concentration. It is conceivable that the observed effect was not due to a reduced fermentation of fructans in the distal part of the colon but to an increased absorption of SCFA during the passage through the colon (Cummings, 1981; Cummings & Macfarlane, 1991) and a shift of bacterial metabolic activity towards butyrate production. This would be consistent with the observed elevation of bacteria belonging to the *Clostridium coccoides*–*Eubacterium rectale* group. This phylogenetic group contains eubacteria and clostridia belonging to the XIVa cluster of gram-positive bacteria as defined by Collins *et al.* (1994), e.g. species such as *Eubacterium contortum*, *E. hadrum*, *E. ramulus*, *E. rectale* or *Clostridium coccoides*, *C. clostridiiformes* and *C. xylanolyticum* (Franks *et al.* 1998). The molecular characterisation of butyrate-producing species from faecal samples of human volunteers by Barcenilla *et al.* (2000) has shown that most of the butyrate-producing isolates are related to the *Clostridium coccoides*–*Eubacterium rectale* group.

The presence of butyrate in the colon may be important for the prevention of colon cancer (Weaver *et al.* 1988) or ulcerative colitis (Simpson *et al.* 2000). Cell culture studies show that the presence of butyrate at physiological concentrations enhances growth of normal cells and inhibits that of malignant ones (Velazquez *et al.* 1996). These actions are effected by a variety of mechanisms including promotion of DNA repair and differentiation of tumour cells (Smith *et al.* 1998). The induction of apoptosis in malignant cells is considered as a major effect of butyrate. A significant reduction in aberrant crypt numbers (the earliest

preneoplastic lesions) was observed in azoxymethane-treated rats fed lCN- and OF-containing diets when compared with azoxymethane-treated control rats fed on a standard diet (Reddy *et al.* 1997). Ulcerative colitis may also relate to butyrate availability. Colonocytes isolated from colitis patients have an impaired capacity to oxidise butyrate *in vitro* (Burke *et al.* 1997). Faecal butyrate concentration is higher in colitis patients than in controls (Treem *et al.* 1994). These data suggest that there is a defect in butyrate metabolism which could be overcome through stimulation of butyrate formation by microbial fermentation of non-digestible carbohydrates such as resistant starch or fructans (Jacobasch *et al.* 1999).

It should be mentioned that the HFA rats in our study were fed a basic diet of natural ingredients, which was coarsely ground and contained 87 g dietary fibre/kg of mainly cereal origin. Therefore, substrate interactions between OF and/or lCN and fibre fermentation may have occurred in the caecum and colon, as was assumed previously for resistant starch and dietary fibre (Cummings *et al.* 1996; Morita *et al.* 1999). Such interactions might protect fructans from full fermentation in the caecum or proximal colon by slowing down the rate of fermentation. Moreover, Roberfroid *et al.* (1998), demonstrated *in vitro* that IN chains with DP > 10 are fermented on average half as fast as OF with DP < 10. It may therefore be possible to control the caecal and colonic fermentation by modifying the chain length of dietary IN. Such dietary manipulations increase the butyrate production in the colon and may thereby contribute to the prevention of certain intestinal diseases such as ulcerative colitis and cancer.

To our knowledge, this is the first study which shows that fructan-containing diets increase the number of a bacterial population (*Clostridium coccoides*–*Eubacterium rectale* cluster) other than bifidobacteria or lactobacilli. To define the role of this group of organisms and to know their metabolic properties will be crucial for understanding their role in colonic metabolism and gut health. Whether organisms of this cluster have any health-promoting properties beyond their ability to produce butyrate needs to be established.

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