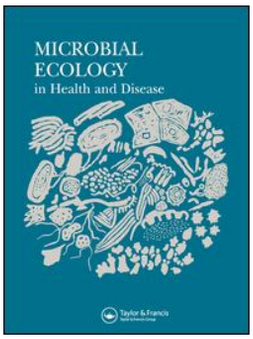

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ORIGINAL ARTICLE

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The Effect of Various Inulins and *Clostridium difficile* on the Metabolic Activity of the Human Colonic Microbiota *in vitro*

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The influence of inulins with different average degree of polymerization (ranging from 3 to 25) on the metabolic activity of the human colonic microbiota with or without the addition of *Clostridium difficile* was investigated *in vitro*. The *in vitro* system used was a dynamic, computer-controlled model that simulates the conditions of the proximal part of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. The addition of inulin stimulated the formation of the total amount of short-chain fatty acids acetate, propionate and butyrate up to 50%, and lactate >10-fold for short-chain inulin, while the formation of ammonia and the branched-chain fatty acids *iso*-butyrate and *iso*-valerate was suppressed. Ammonia formation was suppressed by about 30% and that of *iso*-butyrate and *iso*-valerate was almost completely suppressed. These effects became much more pronounced when *C. difficile* was present in the system. The introduction of *C. difficile* caused a stimulation of the production of the protein fermentative metabolites ammonia, branched-chain fatty acids and the phenolic compounds indole, phenol and *p*-cresol. This stimulatory effect of *C. difficile* was almost completely prevented by the addition of inulins. Thus, these results indicate a potential of inulins to shift the metabolic activity of the human colonic microbiota towards the production of less potentially toxic metabolites, both under normal conditions and under conditions with a disturbed microbiota (with a high level of *C. difficile*). *Key words:* inulin, metabolic activity, *Clostridium difficile*, *in vitro* model, colonic microbiota.

INTRODUCTION

Inulin is a reserve carbohydrate found in many plants and vegetables (1). It occurs for instance in wheat, onions, garlic and chicory. The inulin content ranges from <1% in banana, 1–4% in wheat, 1–7% in onion and leek to 15–20% in chicory (2). The carbohydrate polymer is composed of β-2,1-linked fructosyl moieties mostly with a terminal glucose. The number of fructosyl residues (degree of polymerization, DP) extends from 3 to 250, mainly depending on the plant species.

For about 15 years inulin has also been available as an ingredient for foods. It is extracted from chicory roots and purified (see for instance ref. 3). These types of inulin have a DP of 2–60, with an average ranging from 9 to 25. Short-chain inulins can be prepared enzymatically from sucrose with fructosyltransferases, or with an endo-inulinase from long-chain inulin (4). All types of inulin are used in a large variety of foodstuffs, both for their technological and nutritional benefits. The technological benefits include fat and sugar replacement (in combination with high intensity sweeteners), low caloric bulking agent, texturing and water-binding agent, and filler/binder in tablets. Based on these properties it finds applications in, for instance, dairy products, in bread and other bakery products, in confectionery and ice, and in low-fat or zero-fat spreads. The nutritional benefits arise from the fact that inulin resists digestion in the human small intestine. Therefore it reaches the colon intact. In the first part of the colon, the caecum or proximal colon, it is fermented by the microbiota to short-chain fatty acids (SCFAs; acetate, propionate and butyrate), lactate and gas. The absorption of the SCFAs, and subsequent metabolism by the host, salvages some of the original energy in inulin. However, only about 1.5 kcal/g is delivered from inulin (compared with 3.9 kcal/g from fructose) which explains the low caloric value (5).

Consumption of inulins leads to an increase of *Bifidobacterium* and *Lactobacillus* species in the human faecal colon microbiota (6, 7). This is the so-called prebiotic activity of inulin: it increases the number and/or activity of specific microorganisms in the gastrointestinal tract presumed to be health-promoting (8). Apparently the growth of these bacteria in the colon is stimulated by inulins, as has also been found *in vitro* (9). This increase has been implicated in causing an inhibition of growth of pathogens, and other physiological effects both locally and systemically. Among these effects is the increased synthesis of vitamins,

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an increase in mineral absorption from the colon, a lowering of serum lipids, or the stimulation of immune functions (3, 10).

SCFAs can be used by the cells of the body. Butyrate is considered a health-promoting metabolite, which functions as the major energy source for epithelial cells of the colon (11, 12). Butyrate may play a role in the inhibition of colon carcinogenesis and in the regression of colitis (12, 13). Acetate and propionate are used systemically in the body, especially in the liver (12). Lactate is metabolized by the muscle tissue (and will also be used to a large extent by the colonic microbiota). The branched-chain fatty acids (BCFAs) *iso*-butyrate and *iso*-valerate are produced by fermentation of the amino acids valine and leucine, respectively (14). The latter products may have a negative impact on health and can cause liver problems (12). Proteolytic fermentation can also lead to other (potentially) toxic components, such as ammonia and phenolic compounds (14). Ammonia is toxic to the colonic epithelium and promotes colon cancer in rats (15). In addition, ammonia is a (potential) liver toxin and has been implicated in the onset of neoplastic growth (14, 16, 17). The production of phenolic compounds by intestinal bacteria has been associated with a variety of disease states in humans, including schizophrenia (14). While the role of phenols in cancer is unclear, nitrosation of dimethylamine, by nitrite, is stimulated by phenol, and the interaction of phenol with nitrite forms diazoquinone, which is mutagenic in the Ames test. Other potential (pro)carcinogens can also arise from the metabolic conversions of hepatically detoxified compounds that are secreted into the gastrointestinal tract by the liver via bile. For instance, bacterial β-glucuronidase can release carcinogens from hepatically derived glucuronic acid conjugates (13). Deconjugated bile acids, products of bacterial bile acid metabolism, have been implicated in the events that lead to colon cancer (18). Although a healthy person can handle a limited amount of these toxic metabolites, the balance of health-promoting and toxic metabolites produced by the colonic microbiota is thought to be important for a healthy colon (19). In a disturbed microbiota, e.g. as the result of antibiotic treatment, this balance can be shifted towards more toxic metabolites.

The objective of this study was to examine the effect of the addition of inulins of different DP on the activity of the human colonic microbiota *in vitro*. To introduce a disturbance of the microbiota we used *C. difficile* and we investigated whether the addition of the various inulins was able to suppress the effects of the introduction of *C. difficile*. This microorganism is the causative agent of antibiotic-associated diarrhoea (20) and pseudomembranous colitis (21), both known to be linked to disturbances of the colonic microbiota. Infection with *C. difficile* is a major problem in hospitals and leads to significant morbidity and mortality (21).

MATERIALS AND METHODS

Materials

Inulins were provided by Sensus Operations (Roosendaal, The Netherlands). Frutafit® IQ with an average DP of 9 (DP9-inulin; 84.9% > DP5) and Frutafit® TEX! with an average DP of 25 (DP25-inulin; 99.6% > DP5) are chicory-based materials. Short-chain inulin with an average DP of 3 (DP3-inulin; Actilight®; 5% = monomers, DP2; 38.5% = DP3; 46% = DP4; 7.7% = DP5; 2.1% > DP5), produced by enzymatic synthesis from sucrose, was obtained from Eridania Béghin-Say (Neuilly sur Seine, France). All chemicals used were of the highest analytical grade.

Strains and faecal flora

C. difficile ATCC 17857, a human isolate, was used in the experiments. It was cultivated anaerobically in Schaedler bouillon at 37°C and added to the *in vitro* large intestinal model to a final number of 5×10^8–1×10^9 cells per experiment.

Faecal microbiota samples were donated by 10 healthy, adult volunteers (employees of TNO Nutrition and Food Research; average age 27 years). They were non-smokers and had not used antibiotics or laxatives at least 3 months before the donation, nor had they used probiotic bacteria 3 weeks before donation. Faecal samples were collected into a gas-tight bag, in a plastic container containing an Anaerocult® strip to create anaerobic conditions. Within 10 min the material was transferred into an anaerobic cabinet. For the production of a standardized, cultivated faecal microbiota, which was used for the *in vitro* experiments, a mixture was made using about 2 g faeces from each individual. This sample was cultivated in a 5-l fermentor at 37°C and pH 5.8. Using fed-batch fermentation a final volume of 5l was obtained in approximately 40 h. The fermentation medium was the artificial ileal delivery medium as described by Gibson et al. (22), with some modifications (see below). Anaerobic conditions were maintained by flushing the fermentor with gaseous nitrogen. Adequately sized samples were snap-frozen in liquid nitrogen (with glycerol 20% v/v as cryoprotective agent) and stored at −80°C until further use.

Experiments in the in vitro model of the proximal colon (TIM-2)

Details of the *in vitro* model (TNO Intestinal Model, TIM-2, Fig. 1) can be found in Minekus et al. (23) and Venema et al. (24). In short, the proximal colon model consists of four glass units with a flexible wall inside (peristaltic compartments). Water of body temperature (37°C) was pumped into the space between the glass jacket and the flexible wall,

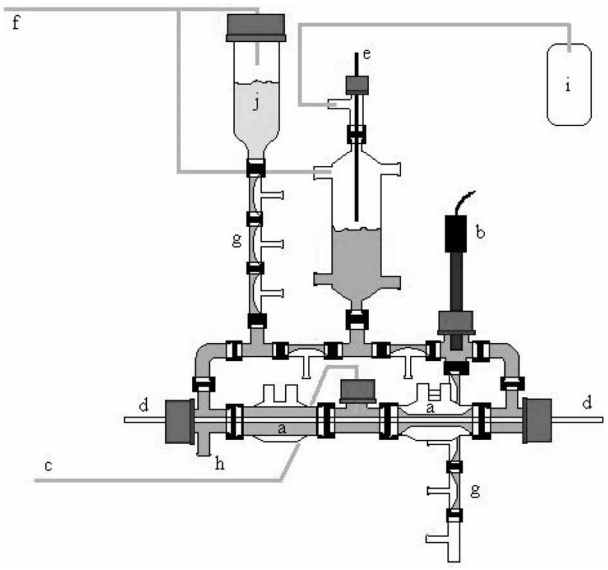

Fig. 1. Schematic representation of the large intestinal model TIM-2. a, peristaltic compartments containing fecal matter; b, pH electrode; c, alkali pump; d, dialysis liquid circuit with hollow fibres; e, level sensor; f, N_2 gas inlet; g, inlet or outlet valves; h, sampling port; i, gas outlet; j, 'ileal delivery' container.

causing the microbiota to be mixed and moved. The sequential squeezing of the walls, controlled by a computer, caused a peristaltic wave forcing the material to circulate through the loop-shaped system. Appropriate electrolyte and metabolite concentrations in the lumen were maintained with a dialysis system consisting of hollow fibres, running through the lumen of the reactor, through which dialysis liquid was pumped. The model further contained an inlet system for delivery of the artificial ileal delivery medium, and a level sensor to maintain the luminal content at a set level. The system was kept anaerobic by flushing with gaseous nitrogen. At the start of each experiment the model was inoculated with approximately 30 ml of the standard, cultivated faecal microbiota. The microbiota was allowed to adapt to the model conditions for 16 h, after which the experiments started.

All experiments were performed in duplicate. Addition of the various inulins started after the adaptation period. The inulins were added to the standard ileal delivery medium (see below) and fed to the system at a rate of 10 g of inulin per day. In the experiments with *C. difficile*, the strain was introduced after the adaptation period in a single dosage of 5×10^8–10^9 cells. In all experiments the pH was maintained at 5.8 by titration with 2 M NaOH and the temperature was kept at 37°C. Every 24 h a 45-ml sample was removed from the model to simulate passage of material from the proximal part to the distal colon.

In these luminal samples and samples of the dialysate taken at the same moment in time the concentrations of the various compounds as described below were determined. With these data and the known volume of the dialysate, the cumulative production of SCFAs, BCFAs, lactate and ammonia in time was calculated. The presented data are the average of duplicate experiments, and were all within 10% of each average.

Ileal delivery medium

Gibson et al. (22) described a medium which simulates the material passing the ileo-caecal valve in humans (ileal delivery). This medium was modified for the experiments in TIM-2 concerning the following components (g/l): 4.7 pectin, 4.7 xylan, 4.7 arabinogalactan, 4.7 amylopectin, 23.5 casein (all from Sigma-Aldrich, Zwijndrecht, The Netherlands), 39.2 starch (BDH, Amsterdam, The Netherlands), 17 Tween 80 (Merck, Amsterdam, the Netherlands), 23.5 bactopeptone (Oxoid, Haarlem, the Netherlands), 0.4 bile (Oxoid).

Dialysate

Dialysis liquid contained (per litre): 2.5 g $K_2HPO_4.3H_2O$, 4.5 g NaCl, 0.005 g $FeSO_4.7H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.45 g $CaCl_2.2H_2O$ (all from Merck), 0.05 g bile (Oxoid) and 0.4 g cysteine.HCl (BDH), plus 1 ml of a vitamin mixture containing (per litre): 1 mg menadione, 2 mg D-biotin, 0.5 mg vitamin B12, 10 mg pantothenate, 5 mg nicotinamide, 5 mg *p*-aminobenzoic acid and 4 mg thiamine (all from Sigma-Aldrich).

Analytical methods

SCFAs/BCFAs. Samples were centrifuged (12 000 rpm, 5 min) and a mixture of formic acid (20%), methanol and 2-ethyl butyric acid (internal standard, 2 mg/ml in methanol) was added to the clear supernate. According to the method described by Jouany (25), a 0.5-µl sample was injected on a GC-column (Stabilwax-DA, length 15 m, ID 0.53 mm, film thickness 0.1 µm; Varian Chrompack, Bergen op Zoom, The Netherlands) in a Chrompack CP9001 gas chromatograph using an automatic sampler (Chrompack liquid sampler CP9050; Varian Chrompack).

Lactate. Samples for lactate analysis were centrifuged as described above. In the clear supernatant both L- and D-lactate were determined enzymatically (based on Boehringer, UV-method, Cat. No. 1112821) by a Cobas Mira plus autoanalyzer (Roche, Almere, The Netherlands).

Ammonia. Ammonia was measured with an ammonia-selective electrode (Orion, Beverly, USA), after adjusting the sample with an ionic strength adjustor to bring the pH above 12. The high pH turned all NH_4^+ ions into NH_3. The electrode measured the released gas, and concentrations in the samples were determined via comparison with a series of standard solutions with known concentrations.

Phenolic compounds. Phenolic compound analyses were performed on a gas chromatograph (GC) according to

methods described previously (26, 27), with minor modifications. Chloroform was used for extraction of the phenolic compounds. After mixing well, samples were centrifuged (12 000 *g* for 5 min), and the chloroform phase was diluted with methanol (1:10). Then 100 µl of the internal standard (*p*-nitrophenol, 10 mM) were added to the diluted sample. Of the obtained mixture, 0.5 µl was loaded onto a WCOT Fused Silica GC column (Varian Chrompack) using the automatic sampler.

Determination of C. difficile

All handling was carried out in an anaerobic cabinet. Serial 10-fold dilutions were made in peptone/physiological saline (Oxoid) and were spread on CD agar (CM0601; Oxoid) with a *C. difficile* selective supplement (SR096; Oxoid) for enumeration of *C. difficile*. Plates were incubated anaerobically for 3 days at 37°C. The *C. difficile* strain used here (ATCC 17857) could be discriminated from other bacteria growing on CD agar owing to its large colony size.

RESULTS

SCFA production

Both total SCFA production and SCFA ratios were used for the description of the metabolic activity of a microbiota. A straight line of acid production is expected when a continuous production of SCFAs occurs (see Fig. 2A and B). The main SCFAs produced were acetate, propionate and butyrate (Fig. 2 and Table I). Valerate could hardly be detected in the experiments without *C. difficile*; under these circumstances the cumulative production of this compound remained below 1 mmol in 112 h. The total cumulative production as well as the SCFA ratios at the end of the experiments are given in Table I. Compared with the control experiment, the addition of inulins increased the total amount of SCFAs produced and the relative amount of propionate to 25% for DP9-inulin and 34% for DP3-inulin (Table I). The percentage of butyrate decreased to about 15% with DP3-inulin. The total amount of SCFAs increased with increasing chain length of the inulins (Table I).

The main effect of the addition of only *C. difficile* to the model was a suppression of the propionate production (Table I; Fig. 2B), which also resulted in the total production of SCFAs being less. This latter effect could be reversed by the addition of DP9- or DP25-inulin (210 mmol versus 244 respectively 250 mmol; see Table I). The lowest level of SCFA production was found with DP3-inulin and *C. difficile* (Table I). Higher levels of valerate were detected under the conditions with *C. difficile*, but they remained very low compared with the other SCFA (maximally about 4 mmol in 112 h were produced; data not shown).

Lactate production

Without inulins added there was almost no lactate produced, but it is clear from Table II that the addition of any type of inulin stimulated lactate production. The production was slightly larger with shorter chain length of the inulins. There was almost no difference in lactate production with or without the addition of *C. difficile*.

BCFA production

The BCFAs *iso*-butyrate and *iso*-valerate arise from deamination of valine and leucine, respectively. Their production by the human colonic microbiota in these experiments was enhanced following the addition of *C. difficile* (Fig. 3B) compared with the control (Fig. 3A): it resulted in a two-fold increase of *iso*-valerate production. Both without and with the pathogen added, the production of BCFAs was strongly reduced by the addition of inulins (Fig. 3C and D, respectively; only shown for DP9-inulin). Similar effects were observed with DP3- and DP25-inulin (data not shown).

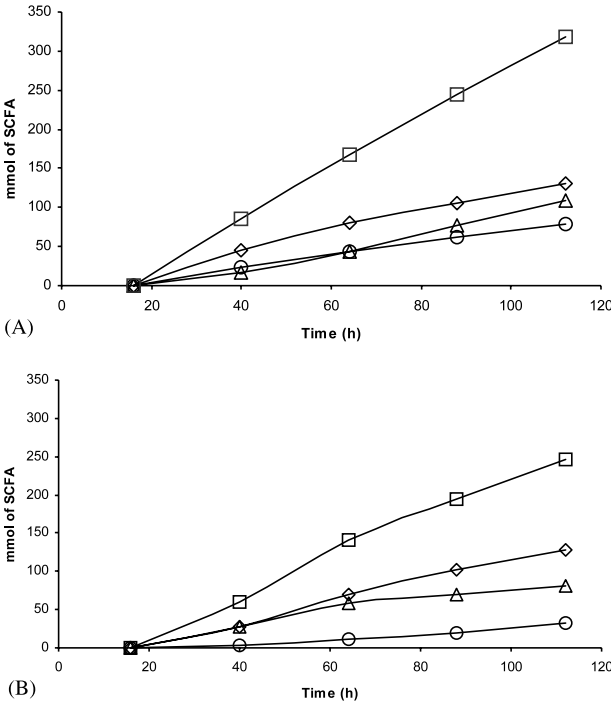

Fig. 2. Cumulative SCFA production after the addition of DP9-inulin and in the absence (A) and presence of *C. difficile* (B). The cumulative production of acetate (◇), propionate (○), *n*-butyrate (△) and of the sum of these (□) in time is shown. The additions (inulin and/or *C. difficile*) were made to the system at 16 h. The inulins were added at a rate of 10 g/day. Samples were taken at the times indicated, SCFAs were determined as described and the cumulative production was calculated.

Table I

The effect of inulins of various DP on the total cumulative SCFA production and SCFA ratios after 112 h of fermentation in TIM-2, in the absence (−C) or presence (+C) of C. difficile

	Control		DP3-inulin		DP9-inulin		DP25-inulin	
SCFA	−C	+C	−C	+C	−C	+C	−C	+C
Total production (mmol)	230	210	271	144	318	244	338	250
Acetate (%)	45.6	61.8	51.0	47.9	41.2	52.9	47.6	43.6
Propionate (%)	18.7	0.6	34.3	20.8	24.8	13.5	27.0	13.2
Butyrate (%)	35.7	37.6	14.7	31.3	34.0	33.6	25.4	43.2

At 16 h *C. difficile* (single dosage) was added to the system and the addition of inulins was started at 10 g/day. After 112 h samples were taken, analysed for SCFA content as described, and the cumulative SCFA production and percentage of individual SCFAs at 112 h were calculated.

Ammonia production

As with the BCFA production, the ammonia production in the experiments without *C. difficile* (35 mmol) was suppressed by the addition of inulins by about 30% after 112 h. Introduction of *C. difficile* resulted in a strong stimulation of ammonia formation (Fig. 4). This stimulation was suppressed completely by the addition of DP25-inulin, as shown in Fig. 4. The described effects were identical for DP3- and DP9-inulin (data not shown), indicating that the effect is independent of the average chain length of the fructan.

Phenolic compounds

The production of phenolic compounds (indole, *p*-cresol and phenol; skatole could not be detected) was low under all conditions tested. Their presence could only be shown in the lumen samples and not in the dialysis fluid. Hence cumulative production could not be calculated for these components. After the introduction of *C. difficile* the concentration of indole increased from 612 to 620 μmol/l and only under this condition were *p*-cresol (143 μmol/l) and phenol (17 μmol/l) detected. In none of the experiments in which inulins were added, were the phenolic compounds present in detectable amounts (data not shown). These results indicated that the various inulins are able to diminish the production of these compounds.

C. difficile content

In all the experiments where *C. difficile* was added to the *in vitro* system, their number remained at the starting value (data not shown). Also the content of other microbiological groups did not change (data not shown).

DISCUSSION

The TNO *in vitro* model has been developed and validated using data from sudden death individuals (11, 23). Both with respect to composition and with respect to metabolic activity, a faecal inoculum in the model has been shown to simulate the data from these sudden death individuals very well. In addition, we have performed experiments in the model using different microbiotas from dogs. The microbiotas originated from the caecum and from faecal material. The microbiotas maintained in TIM-2 originating from either the caecum or faeces were shown to have the same composition and microbial activity as those of the microbiota when freshly obtained from the caecum, supporting the hypothesis that the microbiota in TIM-2 does develop to resemble that of the caecum (unpublished data). We hypothesize that this is also the case for a human microbiota. A human microbiota could be maintained stably in TIM-2 for up to 3 weeks on the standard pre-digested food, without changes in microbial composition or activity (23).

In the experiments without *C. difficile*, the addition of inulins suppressed the formation of BCFA (Fig. 3), ammonia (Fig. 4) and phenolic compounds (data not shown), while at the same time SCFA and lactate production was stimulated (Fig. 2 and Table II, respectively). Since BCFAs, ammonia and phenolic compounds are products typical of breakdown and fermentation of proteins it can be

Table II

The effect of inulins with various DP on the cumulative production of lactate after 112 h of fermentation in TIM-2 in the absence (−C) or presence (+C) of C. difficile

	Control		DP3-inulin		DP9-inulin		DP25-inulin	
	−C	+C	−C	+C	−C	+C	−C	+C
Lactate (mmol)	6	7	165	183	109	104	87	110

At 16 h *C. difficile* (single dosage) was added to the system and the addition of inulins was started at 10 g/day. After 112 h samples were taken, analysed for lactate content as described and the cumulative lactate production was calculated.

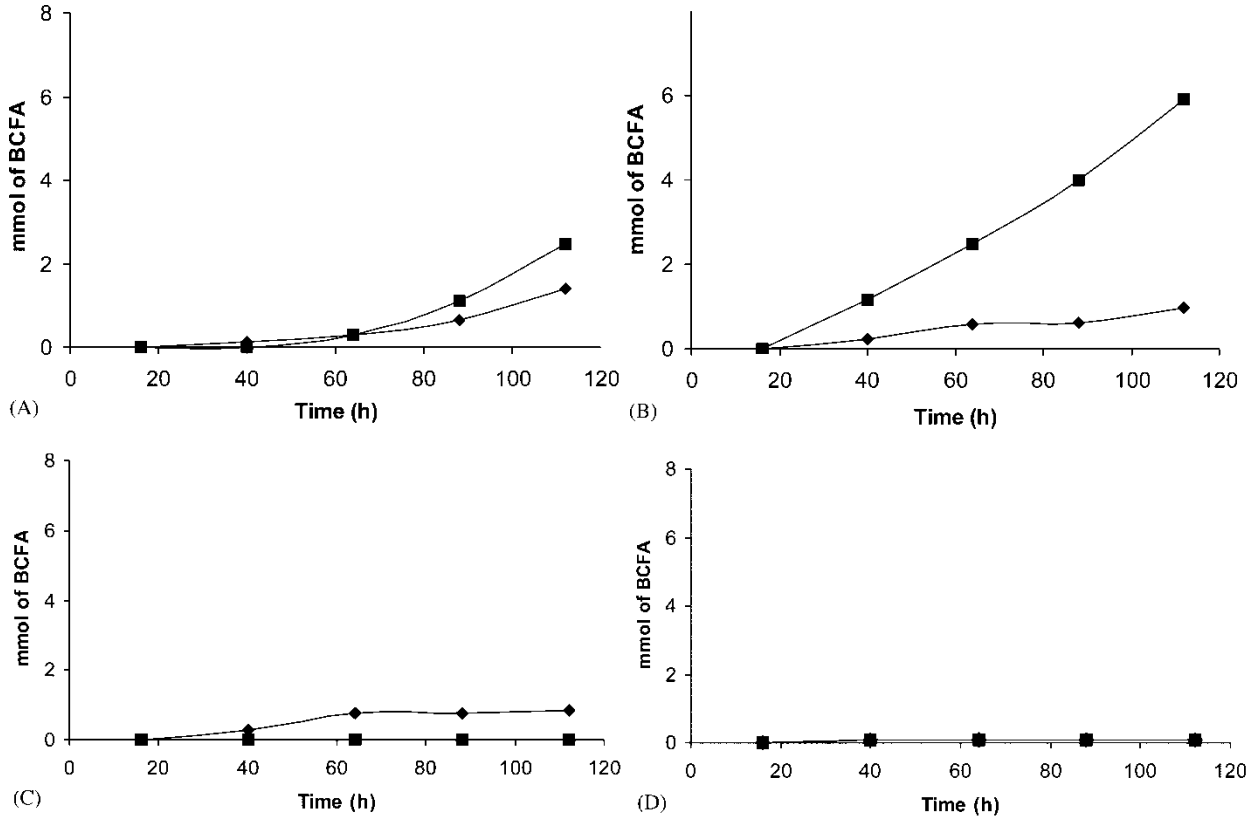

Fig. 3. Cumulative production of *iso*-butyrate (◆) and *iso*-valerate (■) in time during control experiments (A), with the addition of *C. difficile* (B), DP9-inulin (C), or both DP9-inulin and *C. difficile* (D). The additions as described were made to the system at 16 h. DP9-inulin was added at a rate of 10 g/day. Samples were taken at the times indicated, BCFAs were determined as described and the cumulative production was calculated.

concluded that inulins inhibit the proteolytic activity of the colonic microbiota and stimulate the saccharolytic activity, as indicated by the increased SCFA and lactate production. With *C. difficile* introduced into the model, the effects of inulin became even more pronounced. The increase in proteolytic activity caused by *C. difficile* was suppressed to a large extent by the addition of the inulins, irrespective of the chain length. These results corroborate the hypothesis that fermentable fibres, such as inulin, decrease the amount of protein fermentation products by repressing protein fermentation itself. In an *in vitro* batch incubation, a reduction of phenol and *p*-cresol production by 63% and 78%, respectively, was observed during fermentation of a readily fermentable source of carbohydrate (starch) by human faecal bacteria (26). Instead of being fermented, the amino acids and ammonia are incorporated into the bacterial biomass; Levrat et al. (28) observed an increase in the use of ammonia by the microbiota of rats after the addition of inulin to their diet.

Our results demonstrate clearly the potential of inulins to shift the metabolic activity of the human colonic microbiota into a direction resulting in the production of potentially less toxic products, without major changes in the composition of this microbiota. For the inulins with varying DPs grossly similar effects were found for the potential to induce changes in the metabolism of the colonic microbiota in this system. So the prebiotic activity (i.e. the ability to bring about a metabolic shift) of the various inulins seems to be relatively independent of the chain length. This may seem surprising in view of the differences reported for the bifidogenic effects of inulins in relation to chain length, with DP3-inulin suggested to be more effective than DP25-inulin (29). However, in the present study we could not find a major difference for the changes in metabolic activity brought about by the different inulins. Our hypothesis is that the complex microbiota present in TIM-2 is equally able to ferment long-chain inulin as short-chain inulin. We propose that endo-inulinase activity quickly reduces long-chain inulin into short-chain molecules. It may well be that different species are responsible for the fermentation of different inulins, but that the overall metabolic effect is the same.

The introduction of only a small number of *C. difficile* bacteria (only about 1% or less of the total bacterial population was added) caused a major shift in the overall metabolism of the colonic microbiota towards proteolysis. The fact that the number of *C. difficile* did not change after inulin addition is probably due to the constant pH of the system, as May et al. (30) suggested that a decrease in pH is necessary to inhibit *C. difficile* growth. Apparently, the

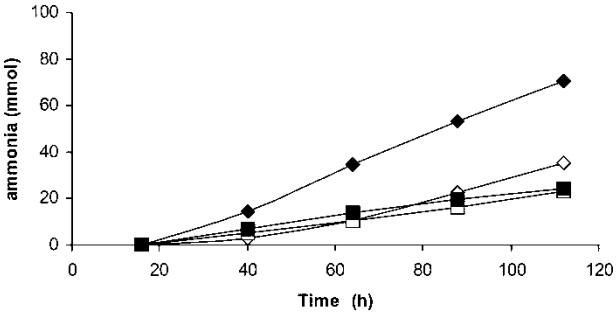

Fig. 4. The effect of the addition of DP25-inulin (□, ■) compared to the control (◇, ◆) on the cumulative production of ammonia in the absence (open symbols) or presence (closed symbols) of *C. difficile*. At 16 h *C. difficile* was introduced to the system and the addition of DP25-inulin was started at a rate of 10 g/day. Samples were taken at the times indicated, ammonia was determined as described and the cumulative ammonia production was calculated.

metabolic balance, disturbed by the introduction of *C. difficile*, cannot be restored by the microbiota alone, since there was no normalization of the metabolism during the time course of these experiments without addition of inulins. For instance, the cumulative production of ammonia in time remained virtually unchanged (Fig. 4). Apparently, the addition of, for example inulins, is required to shift the metabolism from proteolysis to saccharolysis. Similar data were found by Terada et al. (31) following lactulose consumption by human volunteers. Lactulose consumption suppressed the formation of proteolytic products in faeces (31). In addition, Hidaka et al. (32) found similar results in a human study: consumption of short-chain inulins (DP3-inulin) led to a decrease in the faecal concentrations of BCFAs and phenolic compounds.

As we used samples of a standardized faecal microbiota (grown in a fed-batch fermentor) as inoculum, the results of the different experiments can be properly compared. We have previously shown that the microbial composition and activity of the inoculum did not differ substantially from that of the fresh faecal microbiota ((23, 24), and data not shown). Therefore we presume that the results as presented reflect the possible *in vivo* effects of inulins or a *C. difficile* infection.

The mechanism causing the differences in metabolic activity between the *C. difficile*-containing microbiota with and without the addition of inulin is postulated to be that the inulins also provide a suitable carbon source for the *C. difficile* bacteria, thus preventing protein fermentation. The *C. difficile* strain used in this study was able to ferment the inulins used in this study (unpublished observations) and it is known that *C. difficile* VPI 10463 can grow on short-chain inulins (31). Since clostridia are involved in butyrate production, the observation of a higher relative amount of butyrate with DP3- and DP-25 inulin in combination with *C. difficile* is in accordance with the proposed mechanism described above.

The cause of the decreased propionate production after addition of *C. difficile* or its restoration after inulin addition remains obscure. It could well be that the increased lactate formation following the addition of inulins (Table II) leads to an increased propionate formation (33), but the reduction of propionate production remains unexplained.

The results show that even in a microbiota originating from healthy humans, a low intake of proteins (approximately 5 g/day in the present study) can result in the production of potentially toxic or harmful metabolites. Since the model represents the proximal colon, this indicates that even in a region of the gut where protein fermentation is of minor importance these substances can be produced. Inulins can reduce this production, whereas addition of *C. difficile* can stimulate it. It is of general interest to determine whether individuals with chronic bowel problems, such as inflammatory bowel disease patients, show evidence of an altered metabolic function of the microbiota in the large intestine and whether this is related to the onset or progression of the disease. Dietary interventions in these patients may have the same effect on the metabolic activity of the microbiota as that shown for healthy individuals. We are currently investigating this aspect.

The relevance of these findings is that the prebiotic action of inulin clearly is not limited to a mere (bifidogenic) change in the composition of the colonic microbiota, but includes a shift in metabolic activity; from proteolysis to saccharolysis. It is certainly a feature of the *in vitro* system employed in this study that such an effect can be found. *In vivo*, adequate sampling is impossible, since one can only take faecal samples from which most of the fermentation products (SCFAs, ammonia) will already have been absorbed by the body (34, 35). It is therefore not surprising that others could not find a change in faecal SCFAs after consumption of inulin by human volunteers, despite a significant bifidogenic change in bacterial composition of the faecal microbiota (6, 34). Similarly, we have found no changes in faecal SCFAs after lactulose treatment, whereas the SCFA production in TIM-2, simulating the proximal colon, did change (unpublished observations).

The effect on metabolic activity seems much more relevant for any potential health implication of the use of inulins in food than a mere shift in bacterial composition. Model studies such as those reported here may prove to be an important tool for establishing the effects of (potential) prebiotic ingredients on the metabolic activity of the colonic microbiota. The model had already been used with cultivated human microbiota from healthy individuals to simulate conditions in the proximal colon (24). From the present study it can be concluded that this *in vitro* model of the large intestine can also be used to study the effects of prebiotics in the gastrointestinal tract of humans infected with a pathogen or with a disturbed microbiota.

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