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Fermentation of Fructooligosaccharides and Inulin by Bifidobacteria: a Comparative Study of Pure and Fecal Cultures

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The utilization of fructooligosaccharides (FOS) and inulin by 55 *Bifidobacterium* strains was investigated. Whereas FOS were fermented by most strains, only eight grew when inulin was used as the carbon source. Residual carbohydrates were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection after batch fermentation. A strain-dependent capability to degrade fructans of different lengths was observed. During batch fermentation on inulin, the short fructans disappeared first, and then the longer ones were gradually consumed. However, growth occurred through a single uninterrupted exponential phase without exhibiting polyauxic behavior in relation to the chain length. Cellular β-fructofuranosidases were found in all of the 21 *Bifidobacterium* strains tested. Four strains were tested for extracellular hydrolytic activity against fructans, and only the two strains which ferment inulin showed this activity. Batch cultures inoculated with human fecal slurries confirmed the bifidogenic effect of both FOS and inulin and indicated that other intestinal microbial groups also grow on these carbon sources. We observed that bifidobacteria grew by cross-feeding on mono- and oligosaccharides produced by primary inulin intestinal degraders, as evidenced by the high hydrolytic activity of fecal supernatants. FOS and inulin greatly affected the production of short-chain fatty acids in fecal cultures; butyrate was the major fermentation product on inulin, whereas mostly acetate and lactate were produced on FOS.

Bifidobacteria constitute a significant portion of the intestinal microflora and have beneficial effects on their host (52). These obligate anaerobes (43) compete with other species of intestinal flora and transient organisms for nutrients and attachment sites in the gut (45). Bifidobacteria produce lactic and acetic acids which acidify the large intestine and restrict putrefactive and potentially pathogenic bacteria (46). Bifidobacteria also play an important role in immunostimulation (10, 18), anticarcinogenic activity (21), human pathogen growth inhibition (47), vitamin and amino acid production (8, 12, 30), and the reduction of the conversion of primary bile salts to secondary bile salts (21, 29).

Like most intestinal bacteria, bifidobacteria are saccharolytic: they obtain carbon and energy through fermentation of host and dietary carbohydrates. Bifidobacteria catabolize a variety of mono- and oligosaccharides (9, 43) released by glycosyl hydrolases acting on nondigestible plant polysaccharides or host-derived glycoproteins and glycoconjugates (44). Fructooligosaccharides (FOS) and inulin occur naturally in many foods of vegetable origin, such as onions, Jerusalem artichokes, asparagus, leeks, and garlic. They consist of mixtures of fructose moieties linked by β-(2→1)-glycosidic bonds with a terminal glucose unit. FOS have a degree of polymerization (DP) of 2 to 10 and can be produced from sucrose by transfructosylation and from inulin by controlled hydrolysis. Inulin, extracted from chicory roots, has a more heterogeneous DP, ranging from 3 to 60 (40). FOS and inulin transit through the stomach and small intestine, where they are neither absorbed nor degraded, and reach the colon, where they are fermented by resident bacterial groups and promote the proliferation of bifidobacteria. Thus, FOS and inulin are effective prebiotics, defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health (16).

The fermentation of oligo- and polysaccharides in the colon is the result of intestinal microbial metabolic activity. On transit through the large bowel, unabsorbed carbohydrates such as FOS and inulin are hydrolyzed to their respective sugars. These sugars are fermented to short-chain fatty acids (SCFA) and biomass by the complex bacterial flora (11, 24). SCFA are absorbed by the perfused human colon in a concentration-dependent manner (42) and are the major respiratory fuels for colonocytes, supplying up to 60 to 70% of their energy needs (23, 28). SCFA also stimulate the growth of colorectal mucosal cells, retard mucosal atrophy, and decrease the risk of malignant transformation in the colon. Butyrate has been shown to be particularly effective in decreasing the risk of malignant transformation of the colon (14, 34).

Human in vivo trials have established that the addition of FOS or inulin to the diet leads to an increase in bifidobacteria (4, 5, 15, 27), and several studies have described in vitro fermentation of FOS in pure cultures of *Bifidobacterium* (17, 22, 25, 31, 36, 38, 53, 55). Nevertheless, relatively little is known regarding the influence of differing DP of fructofuranosides on fermentation capability. A single in vitro study on three *Bifido-*

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bacterium strains suggested that short chains were fermented at a higher rate than longer FOS and resulted in a higher biomass yield (37). Despite the bifidogenic effect of FOS and inulin, a rigorous analysis of the ability of several *Bifidobacterium* strains to ferment fructans of differing lengths has not yet been published. The present study seeks to fill this gap by comparing the growth of 55 strains of *Bifidobacterium*, which are representative of most species of human and animal origin, on FOS to that on inulin.

Unlike previous studies (25, 31, 32, 36, 55), where complex undefined media were used, semisynthetic medium was used here to compare the behavior of *Bifidobacterium* on FOS to that on inulin, based on the exact composition of the sugars. Pure culture experiments were conducted to reveal differences in the growth and degradation kinetics of bifidobacteria and in the activity and location of β-fructofuranosidase (EC 3.2.1.7). β-Fructofuranosidase is the enzyme that hydrolyzes fructose moieties from the terminal β-2,1 positions and is involved in fructan and sucrose hydrolysis (13, 31, 54).

The impact of FOS and inulin as carbon sources was studied in fecal cultures in order to compare bifidogenic effects and SCFA production patterns. SCFA measurements have previously been largely confined to fecal samples, which are inadequate because less than 5% of bacterially derived SCFA appears in feces as a result of colonic absorption (33, 49). Fecal cultures, on the other hand, are an in vitro model of the human colon that circumvent the absorption and utilization of SCFA by colonocytes. Thus, the use of fecal cultures in this study allowed for the direct comparison of the contributions of FOS and those of inulin to SCFA production.

MATERIALS AND METHODS

Strains. *Bifidobacterium* strains were obtained from ATCC and DSMZ culture collections (American Type Culture Collection, Rockville, Md., and Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, respectively) or from the Institute of Agricultural Microbiology of the University of Bologna Collection (V. Scardovi Collection) or were isolated from feces of healthy volunteers who had followed a probiotic-free diet for 1 month.

Chemicals and media. The following commercial preparations of prebiotic fructans with selected DP distributions were investigated: Raftilose Synergy, Raftiline HP, and Raftilose P95 (Orafti, Tienen, Belgium). Raftilose Synergy and Raftilose P95 were utilized as sources of FOS. Raftilose Synergy is a mixture of FOS and inulin, where FOS is the major component and inulin represents a minor portion. Raftilose P95 is composed of 95% FOS (DP of 3 to 10). Raftiline HP (inulin) is long-chain inulin (average DP, 25; FOS with DP of <5, absent). All other chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated.

The fermentation of carbohydrates by pure *Bifidobacterium* cultures was tested in semisynthetic medium (SM), which contained the following (in grams per liter): Casamino Acids (Difco Laboratories, Sparks, Md.), 15; yeast nitrogen base (Difco Laboratories), 6.7; ascorbic acid, 10; sodium acetate, 10; $(NH_4)_2SO_4$, 5; urea, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 7H_2O$, 0.007; NaCl, 0.01; Tween 80, 1; cysteine, 0.5 (with the pH adjusted to 7.0 and autoclaved for 30 min at 110°C). Glucose, FOS (Raftilose Synergy), or inulin was autoclaved separately and added to the sterile basal medium to obtain the concentration of 10 g liter^{-1}. Fecal culture experiments were carried out with a complex medium (FM medium), which contained the following (in grams per liter): yeast extract, 5; ascorbic acid, 10; sodium acetate, 10; $(NH_4)_2SO_4$, 5; urea, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 7H_2O$, 0.007; NaCl, 0.01; Tween 80, 1; hemin 0.05; cysteine, 0.5; FOS (Raftilose P95) or inulin as test carbohydrates, 10 (pH 7.0).

Isolation from feces. Isolation from feces was achieved by homogenizing fresh samples (10%, wt/vol) with prereduced half-strength Wilkins-Chalgren anaerobe broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). The homogenates were filtered through a 100-μm metal sieve to remove large food particles, serially diluted in the same medium, and plated on the *Bifidobacterium*-selective raffinose-bifidobacterium (RB) medium (20). Plates were incubated anaerobically at 37°C for 48 h. All preparations were done in an anaerobic cabinet (Anaerobic System, model 2028; Forma Scientific Co., Marietta, Ohio) under an 85% N_2, 10% CO_2, 5% H_2 atmosphere. Attribution of the colonies isolated on RB agar to the genus *Bifidobacterium* was attained by assaying the activity of fructose-6-phosphate phosphoketolase, the key enzyme of *Bifidobacterium* carbohydrate metabolism (3). In order to confirm that new isolates belonged to this genus, colonies were picked for amplification with the 16S rRNA gene primer set Bif164/Bif662 specific for this genus, according to the method described previously by Kok et al. (26), to identify the proper 523-bp amplicon. The taxonomy was confirmed to the species level for the strain *Bifidobacterium* sp. ALB 1 by automated ribotyping.

Culture conditions. Pure *Bifidobacterium* strains were subcultured anaerobically in MRS broth (Difco Laboratories) containing 0.5 g liter^{-1} L-cysteine HCl. Cells from the 24-h MRS culture were inoculated (2%, vol/vol) into 10 ml of SM containing 10 g liter^{-1} of carbon source (glucose, FOS, or inulin). The cultures were incubated anaerobically at 37°C for 48 h and were propagated three times in the same medium. Growth was determined by measuring the final pH and any increase in optical density at 600 nm (OD_{600}). Data concerning growth on glucose, FOS, and inulin were compared using Student's *t* test on paired samples. Differences were considered statistically significant when P was ≤0.05. The supernatants of 21 randomly selected pure cultures were analyzed to determine degradation of the fructans. To evaluate whether FOS-induced enzymes were involved in the fermentation of long-chain inulin, bifidobacteria subcultured in SM containing 10 g liter^{-1} glucose were inoculated (2%, vol/vol) into SM containing 10 g liter^{-1} inulin and into SM containing 10 g inulin liter^{-1} plus 0.15 g FOS (Raftilose P95) liter^{-1}. Respective turbidity was compared after 48 h to evaluate the induction.

pH-controlled batch culture experiments were performed in the SM containing FOS and in the SM containing inulin in a BM-PPS3 bioreactor (Bioindustrie Mantovane, Porto Mantovano, Italy) with a 2-liter working volume. The temperature was kept constant at 37°C. Culture pH was continuously measured (Mettler Toledo InPro 3030/325) and was kept at pH 6.5 by the automatic addition of 4 M NaOH. Anaerobic conditions were maintained by sparging the culture with filter-sterilized nitrogen (Millex filter type GS, 33 mm) at 0.05 vol/vol/min. The culture was constantly stirred (300 rpm). The fermenter was inoculated (10%, vol/vol) with exponential-phase precultures grown in the same medium. To determine the biomass dry weight, the cells contained in 10 ml of fermentation broth were filtered onto preweighed cellulose nitrate membrane filters (Sartorius 11307-47-N, 0.2 μm), washed with distilled water, dried at 105°C for 24 h, and weighed. The dry weight/OD_{600} ratio was 0.39 g liter^{-1}of dry biomass per absorbance unit.

Inocula for fecal cultures were prepared from the fresh feces of seven healthy volunteers (four men and three women) who had followed a pre-/probiotic-free diet for 1 month and had not been treated with antibiotics for at least 3 months. Fecal samples were homogenized in the anaerobic cabinet and diluted 100-fold in prereduced half-strength Wilkins-Chalgren anaerobe broth (Oxoid). Anaerobic serum bottles containing 40 ml of FM medium and FOS or inulin as a carbon source were inoculated with 0.4 ml of the fecal suspension and incubated at 37°C for 24 h. Final pH was measured, and residual oligo- and polysaccharides were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Intestinal bacterial groups were enumerated using specific fluorescence in situ hybridization (FISH) technique commercial kits (Microscreen B.V.; Microbial Diagnostics, Groningen, The Netherlands) for the *Lactobacillus* group (*Lactobacillus* 10-ME-H006), for the *Bifidobacterium* genus (*Bifidobacterium* 10-ME-H001), for the *Bacteroides* genus (*Bacteroides*/*Prevotella* 10-ME-H008), for the *Clostridium butyricum* group (*Clostridium butyricum* 10-ME-H009), and for the *Escherichia coli* group (*Escherichia coli* 10-ME-H004). Each sample was enumerated in triplicate. Depending on the number of fluorescent cells, 30 to 100 microscopic fields were counted and averaged. Bifidobacteria were also counted by plating the culture on selective RB agar plates where raffinose was replaced by FOS or inulin.

β-Fructofuranosidase assays. *Bifidobacterium* cells from exponential growth on SM containing glucose, FOS, or inulin were harvested by centrifugation at 6,000 × *g* for 10 min at 0°C, washed twice in Z buffer (0.1 M phosphate buffer, pH 7, 10 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CaCl_2$), and resuspended. Optical density (OD_{600}) was adjusted to 0.7, corresponding to about 10^8 cells ml^{-1}, yielding approximately 70 μg protein ml^{-1} (41). Cells were permeabilized by mixing 0.5 ml of suspension with 0.5 ml of Triton X-100 (5%, vol/vol, in Z buffer) and incubating at 37°C for 10 min. The reaction mixture, composed of 300 μl cell suspension, 300 μl Z buffer, and 400 μl sucrose (20%, wt/vol), was incubated at 40°C for 25 min, and thereafter, 1 ml Rochelle solution (10 g liter^{-1} 3,5-dinitrosalicylic acid, 300 g liter^{-1} K-Na tartrate·$4H_2O$, 0.4 M NaOH) was

added and then boiled for 5 min. The sample was diluted with 10 ml double-distilled water, and absorbance was read at 540 nm. A linear calibration curve was obtained with 10 solutions of glucose plus fructose (1:1), ranging from 0 to 100 mM. Specific activity was expressed as enzymatic units per milligram of protein, where 1 unit was defined as the amount of enzyme required to release 1 micromole of reducing sugar per minute from sucrose.

Extracellular β-fructofuranosidase was assayed by evaluating fructan hydrolytic activity in the supernatants of bifidobacterial and fecal cultures, both grown on FOS and inulin. The supernatant was filtered (0.22-μm cellulose acetate syringe filter; Albet Filalbet, Barcelona, Spain) and split into two portions: one portion was supplemented with 0.5 g liter^{-1} FOS and the other was supplemented with 0.5 g liter^{-1} inulin. Hydrolysis of fructans by extracellular enzymes was monitored by HPAEC-PAD after 24 h of anaerobic incubation at 37°C.

Carbohydrate analysis. The residual soluble oligo- and polysaccharides in the pure and fecal cultures were measured with HPAEC-PAD. Carbohydrate analyses were performed with a model 4000i gradient module and pulsed amperometric detection (Dionex, Sunnyvale, Calif.). The detector used the following detection waveform: E1, 0.10 V (t_1 = 0.50 s); E2, 0.60 V (t_2 = 0.08 s), E3, −0.60 V (t_3 = 0.05 s) (versus a silver-silver chloride reference electrode and a gold working electrode). The integration of the signal occurred between 0.30 and 0.50 s. Samples were injected using a Rheodyne model 9125 nonmetal (peek) injection valve with a peak sample loop of 10 μl (Cotati, Calif.). Separations were performed at room temperature on a Dionex CarboPac PA100 column connected to the associated guard column. Chromatographic data were collected and plotted using the Dionex AI-450 chromatography workstation. Mobile phase was composed of high-performance liquid chromatography-grade water (eluent 1), 0.6 M sodium hydroxide (eluent 2), and 0.5 M sodium acetate (eluent 3), according to the following time and composition program (eluents 1, 2, and 3 were expressed as percent [vol/vol]): 0 to 5 min, 89, 10, and 1; 5.1 to 45 min, 50, 20, and 30; 45.1 to 95 min 0, 25, and 75; 95.1 to 105 min, 0, 25, and 75. The flow rate was set at 0.8 ml min^{-1}. All mobile phases were sparged and kept under pressure with helium in order to prevent the absorption of carbon dioxide and the subsequent production of carbonate, which would act as a displacing ion and shorten the retention times. Since 1-kestose was eluted at about 21 min, the chromatographic peaks with DPs higher than 3 were assigned based on the generally accepted assumptions that the retention time of a homologous series of carbohydrates increases as the DP increases and that each successive peak represents a fructan that has one fructose more than the fructan of the previous peak. Under these optimized conditions, the selective separation of both low-molecular-weight and high-molecular-weight fructan mixtures in the whole molecular weight range was achieved.

SCFA analysis. Qualitative profiles of short-chain fatty acids in fecal cultures were obtained by capillary zone electrophoresis and indirect UV detection at 214 nm according to a method described previously by Corradini et al. (6). Peaks were identified by comparing migration times and spiking samples with known quantities of standard solutions of formiate, succinate, acetate, lactate, propionate, and butyrate.

RESULTS

Growth of *Bifidobacterium* pure cultures on FOS and inulin. Growth on glucose, FOS, or inulin was analyzed for 55 *Bifidobacterium* strains, representing 11 species, by measuring the OD_{600} and the pH after 48 h of anaerobic fermentation (Fig. 1). The higher the OD_{600} was, the lower the final pH, although this was not a linear correlation. Almost all strains grew abundantly on glucose and FOS, but only eight strains were able to grow on inulin. The paired-sample *t* test on OD_{600} and on pH values revealed that growth was significantly better on glucose ($P \leq 0.05$). Among the 13 strains of animal origin, only *B. thermophilum* ATCC 25866 grew on inulin. Based on these findings, no relationship between species, origin, and the ability to ferment inulin was found. Induction experiments excluded the possibility that FOS could induce the expression of enzymes that enable the fermentation of inulin. Thus, the presence of a suboptimal amount of FOS in the medium containing inulin did not enable the growth of the 47 strains that didn't ferment inulin.

Carbohydrate analysis. The consumption of FOS and inulin was determined in triplicate samples for 21 randomly selected strains after 48 h of incubation in semisynthetic medium. A strain-dependent capability to degrade fructans of different lengths was observed. Figure 2 presents the HPAEC-PAD patterns of the SM containing 10 g liter^{-1} FOS or inulin before and after 48 h of incubation with *B. thermophilum* ATCC 25866. The analysis of the spent broths showed that the peaks corresponding to oligosaccharides and inulin had disappeared or had shrunk. Both profiles indicated the preferential consumption of oligosaccharides and inulin chains eluted before 70 min and a delayed consumption of longer chains. In agreement with this observation, *B. thermophilum* ATCC 25866 grew on both FOS and inulin (Fig. 1), being the sole strain of animal origin able to hydrolyze and ferment inulin. Figure 3 presents the residual carbohydrate after growth of *B. infantis* ATCC 27920 on FOS. This was one of the strains with the lowest biomass yields on FOS. Its consumption of fructans was limited to one of the lightest chains, which is consistent with the poor growth reported in Fig. 1. Coherently, *B. infantis* ATCC 27920 could not grow on inulin. Figure 4 shows the residual carbohydrate of *Bifidobacterium* sp. ALB 1 grown on inulin. This strain showed the greatest degradative capability and also fermented the longest chains of inulin and did not exhibit stringent selectivity based on DP. The similarity value between the EcoRI ribotyping profile of *Bifidobacterium* sp. ALB 1 and the reference strains of RiboPrinter database (DUP) indicated that this strain should be referred to *B. adolescentis* species (data not shown).

In order to correlate the consumption of FOS and inulin with growth kinetics, pH-controlled batch fermentations of *B. adolescentis* MB 239 and *Bifidobacterium* sp. ALB 3 were carried out on FOS and inulin, respectively. The medium composition ensured that the carbon source became limiting towards the end of growth and that idiophase began when carbohydrates were depleted or could not be further fermented. *B. adolescentis* MB 239 (Fig. 5) grew on FOS with a constant specific growth rate (μ = 0.60 h^{-1}) and a calculated yield of 0.037 g biomass/g FOS and shifted to the idiophase when FOS were depleted. Comparison of HPAEC-PAD elution patterns revealed that most FOS were consumed simultaneously during the growth phase. Interestingly, the specific growth rate of *B. adolescentis* MB 239 was much lower on the same medium when it contained either glucose or fructose as the carbon source (μ = 0.19 and 0.15 h^{-1}, respectively) (1). *Bifidobacterium* sp. ALB 3, when grown on inulin, exhibited a constant specific growth rate (μ = 0.21 h^{-1}). Comparison of the elution patterns showed that growth stopped when all peaks with a retention time lower than 70 min disappeared (Fig. 6). The short fructans were fermented first, and then the longer ones were gradually consumed. However, growth occurred during a single uninterrupted exponential phase without exhibiting polyauxic behavior in relation to DP.

β-Fructofuranosidase activity. β-Fructofuranosidase activity was analyzed in permeabilized cells of 21 *Bifidobacterium* strains after 6 h of growth on glucose, FOS, or inulin. Under the assay conditions, the strains presented the activities described in the legend of Fig. 7. For 17 strains, little or no enzyme activity was detected for growth on glucose (average, 0.27 U mg^{-1}; standard deviation, 0.2 U mg^{-1}). By contrast, the

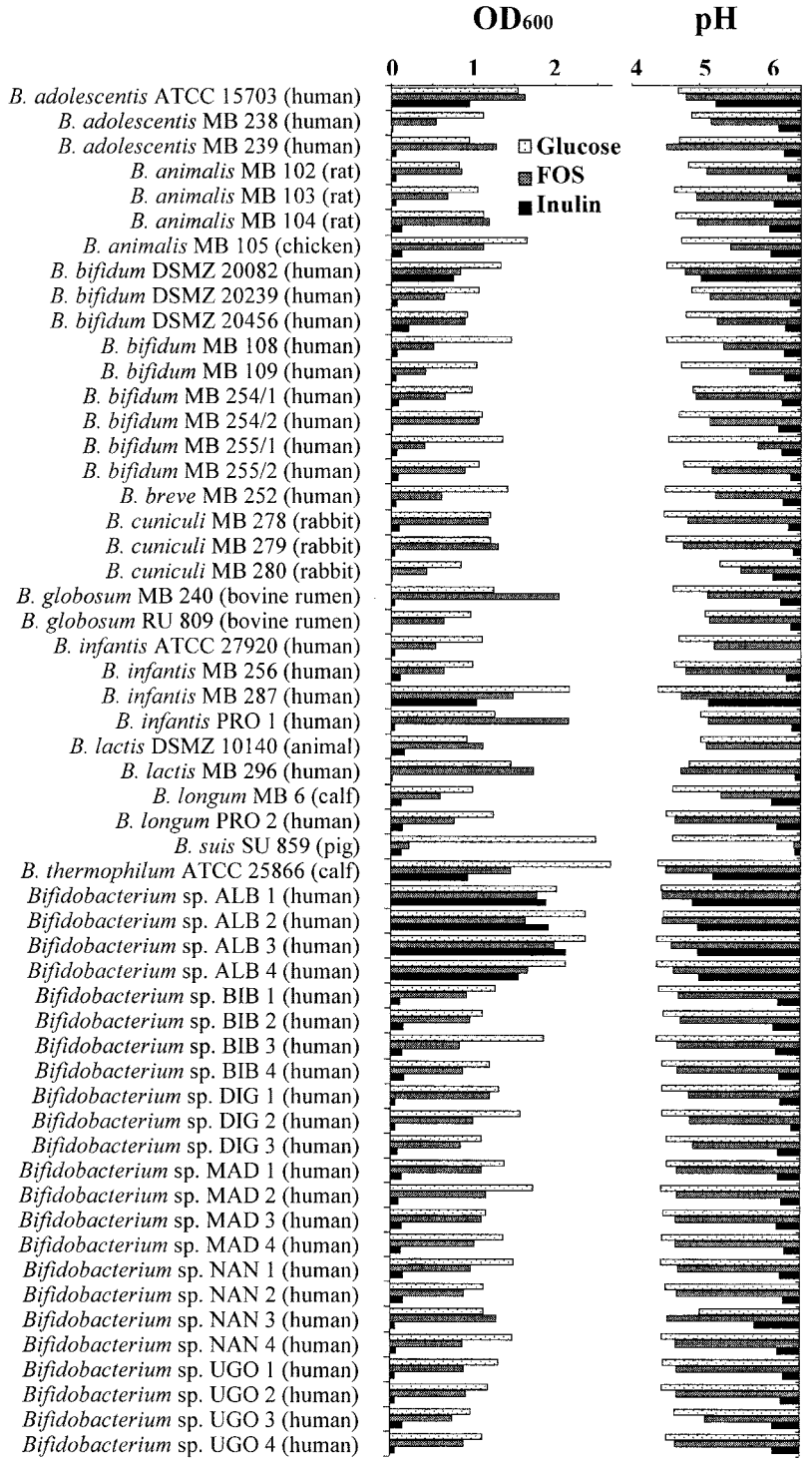

FIG. 1. Species, strains, and sources of 55 bifidobacteria examined for the ability to ferment glucose, FOS, and inulin. Optical density (OD_{600}) and pH were measured after a 48-h incubation in SM containing 10 g liter^{-1} glucose, FOS, or inulin as the sole carbon source. The results are mean values from three separate experiments (standard deviations were always less than 0.27 for OD_{600} and 0.15 for pH).

activity expressed by cultures growing on FOS was significantly higher ($P \leq 0.05$; average, 2.30 U mg^{-1}), despite the wide strain-to-strain variability (standard deviation, 1.4 U mg^{-1}). Only four strains (*B. adolescentis* MB 239, *B. animalis* MB 102, *B. animalis* MB 104, and *B. adolescentis* ALB 1) produced high levels of β-fructofuranosidase on glucose, and the same high activity was obtained (ranging from 1.64 to 3.18 U mg^{-1}) during growth on glucose and on fructooligosaccharides. The presence of inulin instead of FOS did not significantly affect β-fructofuranosidase activity in the strains able to ferment

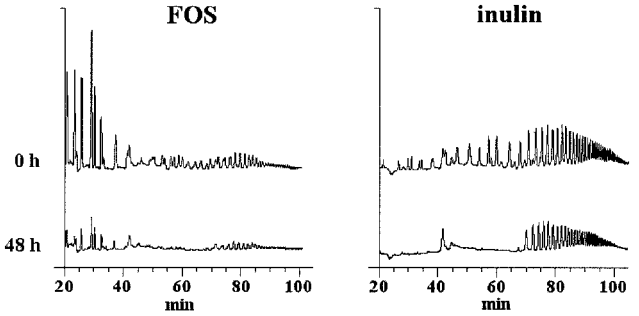

FIG. 2. Comparison of HPAEC-PAD patterns of SM containing 10 g liter^{-1} FOS or 10 g liter^{-1} inulin at the inoculation and after 48 h of incubation with *B. thermophilum* ATCC 25866. The peaks correspond to the selectively eluted fructans with increasing DP.

inulin. Furthermore, no correlation was observed between the final turbidity reached on FOS or inulin and the specific β-fructofuranosidase activity achieved during growth (R^2 coefficients of 0.31 and 0.29 for FOS and inulin, respectively).

Enzyme location experiments were carried out to define whether bifidobacteria produced extracellular enzymes that hydrolyze FOS or inulin. These tests monitored the changes in the carbohydrate composition of the supernatant, which was supplemented with FOS versus inulin, using HPAEC-PAD. The techniques normally used for the direct assay of β-fructofuranosidase could not be applied to the supernatant (2, 35) because the medium contained high levels of reducing agents which interfered with the detection of the reducing moieties released. Four strains were tested: *B. infantis* PRO 1 and *B. bifidum* DSMZ 20456 were able to grow on FOS but not on inulin, whereas *B. adolescentis* ALB 1 and *B. thermophilum* ATCC 25866 fermented both FOS and inulin. The supernatant of *B. infantis* PRO 1 and *B. bifidum* DSMZ 20456 did not present any hydrolytic activity against FOS or inulin. In fact, HPAEC-PAD profiles revealed that FOS and inulin, added to the supernatants after growth, were not broken down during 24 h of incubation (data not shown). The presence of extracellular β-fructofuranosidase activity in *B. adolescentis* ALB 1 was found to be dependent on the type of sugar used as the growth substrate. When *B. adolescentis* ALB 1 was grown on

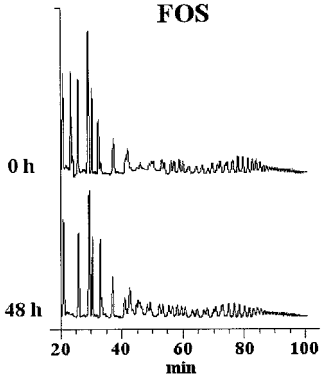

FIG. 3. Comparison of HPAEC-PAD patterns of SM containing 10 g liter $^{-1}$ FOS at the inoculation and after 48 h of incubation with *B. infantis* ATCC 27920.

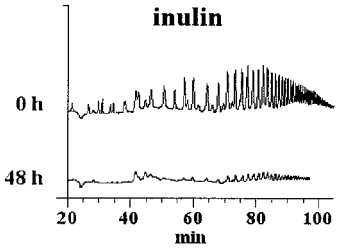

FIG. 4. Comparison of HPAEC-PAD patterns of SM containing 10 g liter^{-1} inulin at the inoculation and after 48 h of incubation with *B. adolescentis* ALB 1.

FOS, no extracellular hydrolytic activity against FOS or inulin was detected. In fact, the HPAEC-PAD profiles of the FOS and of the inulin added to the supernatants remained unchanged after 24 h of incubation. In contrast, when *B. adolescentis* ALB 1 was grown on inulin, the supernatant hydrolyzed both FOS and inulin completely, as demonstrated by the disappearance of all of the HPAEC-PAD peaks. *B. thermophilum* ATCC 25866 produced extracellular β-fructo-

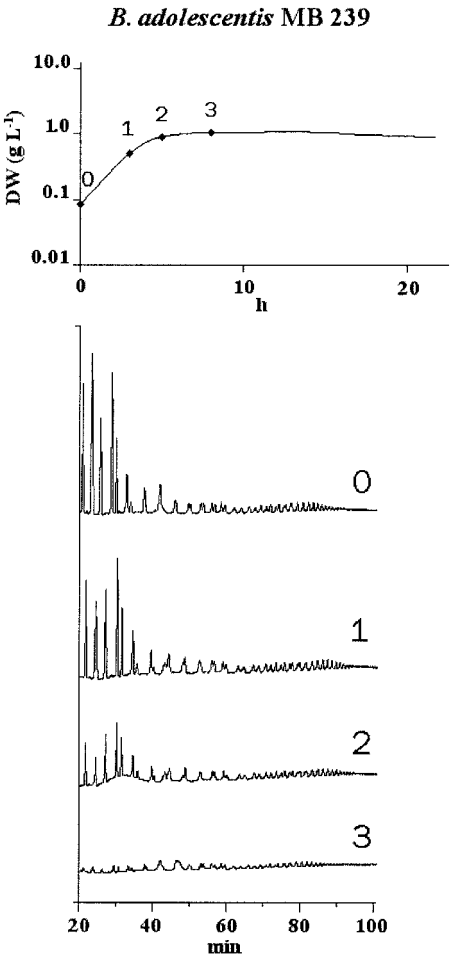

FIG. 5. Small-scale batch fermentations with controlled pH of *B. adolescentis* MB 239 in SM containing 10 g liter^{-1} FOS as the sole carbon source; growth curve and changes in fructan composition monitored by HPAEC-PAD are shown. DW, dry weight.

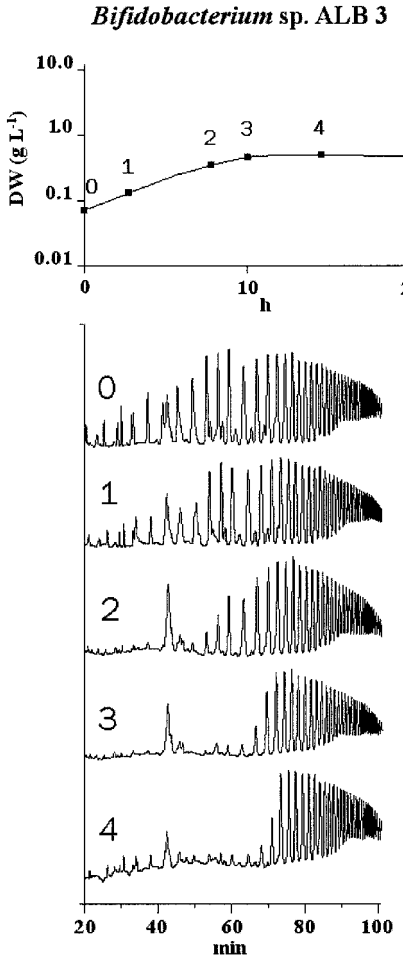

FIG. 6. Small-scale pH-controlled batch fermentations of *Bifidobacterium* sp. ALB 3 in SM containing 10 g liter^{-1} inulin as the sole carbon source; growth curve and changes in fructan composition monitored by HPAEC-PAD are shown. DW, dry weight.

furanosidase which completely hydrolyzed the FOS and inulin added to the supernatants. This occurred regardless of which fructan mixture was used as a growth substrate. The supernatants of all fecal cultures, as demonstrated by HPAEC-PAD profiles, were able to hydrolyze FOS and inulin, regardless of which carbon source was available during growth (data not shown).

Effects of FOS and inulin on fecal cultures: growth of bifidobacteria, pH, and SCFA. In order to compare the growth of bifidobacteria on FOS to that on inulin in mixed fecal cultures, strictly anaerobic fermentation vessels were inoculated with fecal samples. The FM medium contained all the nutrients that support the growth of intestinal bacteria and FOS (Raftilose P95) or inulin (Raftiline HP) as the sole carbon source. The amount of carbohydrates brought by inocula was negligible because of the 1:10,000 dilution of the fecal matter. During growth of fecal cultures on either FOS or inulin, the oligosaccharides were completely depleted after 6 h of incubation, whereas the peaks corresponding to the long fructans did not shrink. After 24 h, the fermentation of inulin resulted in the complete consumption of all the carbohydrates (data not shown).

Table 1 presents the enumeration, by FISH technique, of *Bifidobacterium*, *E. coli*, *Clostridium butyricum*, *Lactobacillus*, and *Bacteroides* groups in fecal cultures containing FOS compared to inulin before and after 24 h of anaerobic incubation at 37°C. Data are reported as average values of seven samples ± standard deviations. Clostridia, lactobacilli, coliforms, and *Bacteroides*, enumerated by FISH, increased ($P \leq 0.05$) on both FOS and inulin, although no statistically significant difference was detected between the two carbon sources.

Bifidobacteria were also enumerated by plating onto RB-modified medium containing FOS or inulin as the sole carbon source. Enumeration by FISH and by counting colonies on selective FOS plates gave consistent results: mean concentration increased by 2.9 and 2.7 orders of magnitude in fecal cultures grown on FOS and on inulin, respectively. Enumeration on selective inulin plates of bifidobacteria grown in fecal cultures containing FOS compared to inulin was 3 orders of magnitude lower than enumeration by FISH ($P \leq 0.05$). Thus, few bifidobacteria of the fecal inocula fermented inulin when the growth of other intestinal bacteria was inhibited by the selective medium. These results confirmed that most bifidobacteria in fecal cultures containing inulin as the carbon source were not able to use the long fructans when the hydrolytic activity of the other intestinal bacteria was excluded.

The SCFA produced in fecal cultures grown on FOS and inulin were quantitatively and qualitatively different. The SCFA profiles of two cultures after 24 h of incubation are depicted in Fig. 8. Inulin led to a remarkable accumulation of butyric acid and lower amounts of acetic, lactic, and propionic acids. On the contrary, lactic and acetic acids were the major products of FOS fermentation. Butyric acid also appeared, even if in low amounts, but propionic acid was not present. The different final SCFA composition of fecal cultures grown on FOS with respect to those grown on inulin explained the different pHs reached on the two sugar mixtures (pH 4.2 ± 0.3 and 4.8 ± 0.5, respectively).

DISCUSSION

The gastrointestinal tract is a complex ecosystem containing up to 10^{12} bacteria/g of intestinal content. This large population of bacteria plays a key role in normal gut function, human health, and well-being (51). There is great interest in the manipulation of intestinal microflora aimed at increasing the number of bifidobacteria and lactic acid bacteria and the production of SCFA in the colon. To date, probiotics and prebiotics are predominantly used in food, and their application in medicine is on the rise (7, 19). Another means of modifying the composition of the intestinal microbiota is the use of targeted synbiotics, which are combinations of selected living bacteria and unabsorbed substrates that they are able to metabolize.

Despite the wide use of fructans as functional food ingredients and their well-studied prebiotic activity, very little is known about the relationship between the chain length of fructans and the ability of bifidobacteria to ferment them. To compare their growth on glucose, on FOS, and on inulin, a collection of 55 *Bifidobacterium* strains was screened. Perhaps the most striking observation of this study was that all of the

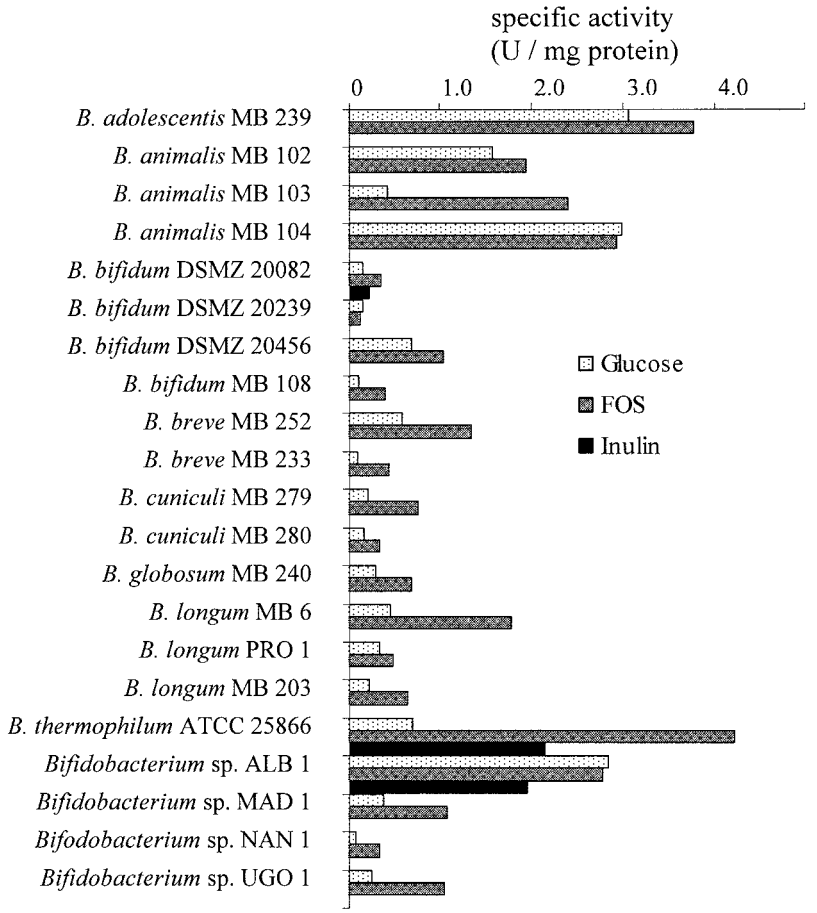

FIG. 7. β-Fructofuranosidase activity detected in permeabilized cells of 21 *Bifidobacterium* pure cultures during the growth phase on glucose, on FOS, and on inulin as the sole carbon source. The enzymatic unit is defined as the amount of enzyme required to release 1 micromole of reducing sugar per minute.

strains fermented FOS, whereas most of them failed to grow on inulin. In agreement with previous studies, bifidobacteria preferred short-chain FOS as a substrate for growth (17, 31, 32). Although a few reports stated that animal strains can metabolize inulin better than human ones (31, 32), our findings excluded any relationship between the strain origin and its ability to ferment inulin. Of the 13 animal strains, only *B. thermophilum* ATCC 25866, isolated from bovine rumen, grew on inulin. Considerable differences in fructan utilization patterns were detected among the strains. This opens up a new perspective on the selection of optimal probiotic strains for synbiotic formulations. The most remarkable differences in degradation profiles emerged for the longest fructan chains. For instance, among the eight strains fermenting inulin, only *B. adolescentis* ALB 1 consumed the longest inulin chains.

Unexpectedly, the specific growth rates of pH-controlled pure cultures grown on FOS and on inulin were constant and excluded any polyauxic growth in relation to the degree of polymerization. The specific growth rates of *B. adolescentis* MB 239 were higher on FOS than on glucose and fructose, in agreement with the evidence that in many *Bifidobacterium* species, growth rates are higher on oligosaccharides than on their monomeric constituents (1, 17, 22, 50). This observation suggested adaptation to an environment naturally poor in

TABLE 1. Enumeration by FISH technique of *Bifidobacterium*, *E. coli*, *Clostridium butyricum*, *Lactobacillus*, and *Bacteroides* groups in fecal cultures containing FOS or inulin, before and after 24 h of anaerobic incubation at 37°C

Time of cell harvest (h)[a]	Culture supplement	Cell concn ($\log_{10}$ ml^{-1})[b]				
		Bifidobacterium	*E. coli*	*Clostridium butyricum*	*Lactobacillus*	*Bacteroides*
0	None	5.1 ± 0.4	4.2 ± 0.3	3.7 ± 0.6	3.9 ± 0.2	5.2 ± 0.3
24	FOS	8.0 ± 0.3	6.9 ± 0.4	6.7 ± 0.7	7.6 ± 0.1	7.2 ± 0.3
24	Inulin	7.8 ± 1.0	6.8 ± 0.6	6.8 ± 0.8	7.3 ± 0.3	7.4 ± 0.5

[a] Hours of anaerobic incubation.
[b] Data are reported as average values of seven samples ± standard deviations.

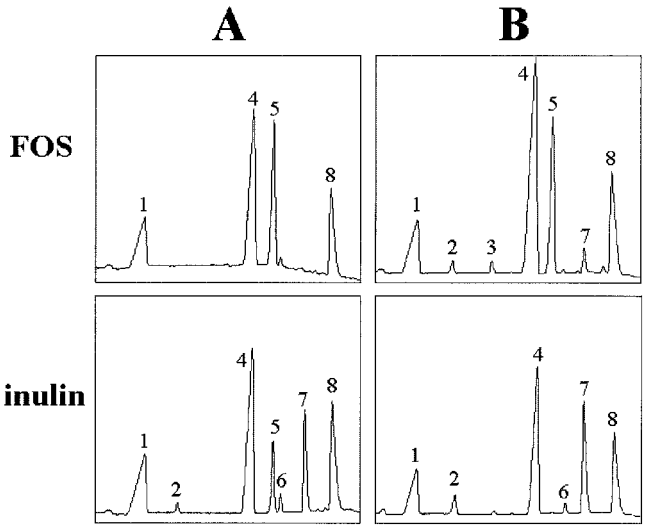

FIG. 8. SCFA profiles of two fecal cultures (A and B) after 24 h of incubation on FOS and on inulin as the sole carbon sources. Peak identification is as follows: sulfate, 1; formiate, 2; succinate, 3; acetate, 4; lactate, 5; propionate, 6; butyrate, 7; ascorbate, 8.

monosaccharides, where the high rate of the metabolism of oligo- and polysaccharides confers a competitive advantage with respect to other bacterial groups.

In all strains, the presence of FOS as the carbon source caused the production of high levels of intracellular β-fructofuranosidases, even if strain-to-strain differences were substantial. The level of intracellular specific activity was not related to the final biomass yield or to the capability of fermenting inulin. Glucose repression of the enzyme was marked for most of the strains tested, with the exception of four strains, which produced high amounts of β-fructofuranosidases even during growth on the monosaccharide.

The various responses of bifidobacteria to fructans of different lengths were described not only in terms of their fermentation capability but also in terms of their ability to induce differently located enzymes. Experiments were conducted to determine whether bifidobacteria produced extracellular enzymes that were able to hydrolyze FOS or inulin. Results indicated that the ability of a strain to grow on inulin as the sole carbon source is related to the production of extracellular enzymes that hydrolyze long-chain fructans. This finding is in agreement with the current paradigm that most bifidobacteria possess only inducible cell-associated β-fructofuranosidases (38); strains lacking extracellular hydrolytic enzymes first transport FOS into the cell and then hydrolyze them. Thus, the presence of extracellular β-fructofuranosidases may provide a basis for selection of new probiotic strains.

The failure of most bifidobacteria to grow on inulin seemed contradictory to its accepted bifidogenic effect in vivo. However, the fermentation of oligo- and polysaccharides in the colon is the result of a complex sequence of metabolic pathways carried out by numerous species. The presence of extracellular hydrolytic enzymes supports mutual metabolic and nutritional dependencies among the mixed population in the intestine. Therefore, a physiological study of bifidobacteria on FOS and on inulin would be of limited use unless these studies were supported by data describing their response to fructans of different DP in mixed cultures. In fecal cultures, primary degraders hydrolyzed inulin, as confirmed by the high hydrolytic activity detected in the supernatants, and supplied mono- or oligosaccharides (FOS) for bifidobacteria to grow. It is important that bifidobacteria grew similarly on FOS and on inulin and did not undergo a significant disadvantage in fermenting the products of primary degraders and competing as scavengers of partially hydrolyzed substrates.

Particularly exciting was the finding that in fecal cultures, FOS and inulin strongly affected the products of fermentation; different types and amounts of SCFA were produced. Butyrate was the major fermentation product during growth on inulin, whereas acetate and lactate were produced when FOS were present. These findings reflect the consequence of the different effects of FOS and inulin on microflora composition and activity.

Due to the lack of a suitable detection method for the major butyrate and propionate producers of the human gut, such as bacteria related to the genera *Eubacterium*, *Roseburia*, *Faecalibacterium*, and *Coprococcus* (39, 52), FISH enumeration did not show significant differences between inulin and FOS as carbon sources in fecal cultures. Furthermore, a recent study on the prebiotic effect of galactooligosaccharides has demonstrated that despite the availability of excellent tools for the analysis of bacterial communities, changes in metabolic activities can be detected, whereas changes in the composition of the microbial population cannot (48).

Our observations indicated that the nutritional relationships between the members of the colonic microflora are neither well known nor predictable. In particular, we observed that structurally related carbohydrates of different lengths determined markedly different physiological responses.

One of the most highly sought-after targets for prebiotic development is increased colonic persistence, which can result in saccharolytic metabolism and a lowered pH extended to the distal colon. Our study provided new perspectives on more extensive use of inulin as a prebiotic, due to the extremely beneficial SCFA produced in fecal cultures and to its high DP, which can provide a long-lasting effect throughout the colon. This study also suggests that a better understanding of *Bifidobacterium* metabolic activity will help to identify the probiotic strains which can better compete for nutrients and will also be a contribution to the definition of optimal synbiotic combinations.

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