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Annet J.H. Maathuis, Ellen G. van den Heuvel, Margriet H.C. Schoterman, and Koen Venema

Galacto-oligosaccharides have prebiotic activity in a dynamic *in vitro* colon model using a ^{13}C labeling technique [1,2,3]

Annet J.H. Maathuis[4], Ellen G. van den Heuvel[5], Margriet H.C. Schoterman[6], Koen Venema[4*].

[4] TNO Earth, Environmental and Life Sciences, Research Group Pharmacokinetics and Human Studies, P. O. Box 360, 3700 AJ Zeist, the Netherlands

[5] FrieslandCampina Research, P. O. Box 87, 7400 AB, Deventer, the Netherlands

[6] FrieslandCampina Domo, P. O. Box 1551, 3800 BN, Amersfoort, the Netherlands

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Author list for indexing: Maathuis, van den Heuvel, Schoterman, Venema

* Address for correspondence: koen.venema@tno.nl

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Abbreviations used: BCFA, branched-chain fatty acids, CFU, colony-forming units, FOS, fructo-oligosaccharides, GOS, galacto-oligosaccharides, I-Chip, intestinal chip, LC-MS, liquid chromatography- mass spectrometry, rRNA, ribosomal RNA, SIEM, standard ileal efflux medium, SIP, stable isotope probing, TIM-2, TNO *in vitro* model of the colon.

ABSTRACT

Galacto-oligosaccharides (GOS) are considered to be prebiotic, although the contribution of specific members of the microbiota to GOS fermentation and the exact microbial metabolites that are produced upon GOS fermentation are largely unknown. We aimed to determine this using uniformly ^{13}C-labeled GOS. The normal (control) medium, unlabeled or ^{13}C-labeled GOS was added to a dynamic, validated, *in vitro* model of the large-intestine (TIM-2) containing an adult-type microbiota. Liquid-chromatography mass-spectrometry was used to measure incorporation of ^{13}C-label into metabolites. 16S-rRNA stable isotope probing coupled to a phylogenetic microarray was used to determine label incorporation in microbial biomass. The primary members within the complex microbiota that were directly involved in GOS fermentation were shown to be *Bifidobacterium longum, B. bifidum, B. catenulatum, Lactobacillus gasseri* and *L. salivarius*, in line with the prebiotic effect of GOS, although some other species incorporated ^{13}C-label also. GOS fermentation led to an increase in acetate (+49%) and lactate (+23%) compared to the control. Total organic acid production was 8.50 and 7.52 mmol per gram carbohydrate fed, for the GOS and control experiments, respectively. At the same time the cumulative production of putrefactive metabolites (branched-chain fatty acids and ammonia) was reduced by 55%. Cross-feeding of metabolites from primary GOS-fermenters to other members of the microbiota was observed. The main finding of this study is direct proof of the prebiotic activity of GOS. In addition, its high potential for use in combination with certain probiotic strains for synbiotic purposes was shown as well.

INTRODUCTION

The benefits of a healthy, well balanced gut microbiota include increased colonization resistance, stimulation of the immune system, and production of butyrate (1). A healthy gut microbiota should be dominated by beneficial bacteria such as bifidobacteria and lactobacilli. The composition of the microbiota can be influenced by external factors, for example by the feeding of prebiotics (1). Prebiotics are "non-digestible 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" (2), thereby improving host health. Examples of prebiotics are galacto-oligosaccharides, fructo-oligosaccharides and inulin (3). Fermentation of prebiotics leads to the production of short-chain fatty acids which are beneficial to the host, for example by providing energy for the host epithelial cells (4), inhibiting pathogen growth (3), and increasing mineral absorption (5, 6). Also, a role of prebiotics in obesity and other metabolic disorders has been proposed (7). On the other hand, the microbiota can produce toxic compounds. For example by shifting from carbohydrate to protein fermentation resulting in the formation of branched-chain fatty acids (8), hydrogen sulfide, ammonia and phenolics (9, 10). The fermentation of prebiotics has been shown to reduce production of these putrefactive compounds (10). Current studies on the physiological properties and health benefits of prebiotics are focused on immunomodulation, preventing diarrhea, their role in metabolic disorders, and increasing calcium absorption (5, 7, 11).

Although the ultimate model for determining functional food effects is the human being, this 'model' has various drawbacks, such as ethical constraints and limitations in sampling from the bowel. Use of dynamic laboratory *in vitro* models, which closely simulate the successive kinetic conditions in the gastro-intestinal tract,

including an active microbiota, allows comparison of different ingested products under identical and standardized conditions. The TNO *in vitro* model of the colon (TIM-2) has been validated using data from sudden-death individuals (12-16).

A phylogenetic micro-array platform such as the human Intestinal Chip [or I-Chip; (17, 18)] enables fast and efficient analyses of microbial populations, allowing changes in composition that occur in the microbiota as a consequence of differences in diet and/or differences in health status, to be detected.

Use of stable-isotope labeled substrates (primarily ^{13}C) allows one to unambiguously identify which metabolites are produced from a specific substrate, by analyzing the presence of ^{13}C-atoms in the metabolites using LC-MS (19). By measuring the ^{13}C-incorporation in microbial biomass (e.g. 16S-rRNA), using Stable Isotope Probing, micro-organisms that ferment the substrate can be identified (19, 20).

Galacto-oligosaccharides are chains of galactose with a terminal glucose produced through the enzymatic conversion of lactose with the enzyme β-galactosidase. They occur naturally in human milk (11, 21, 22). Consumption of GOS is believed to lead to several beneficial effects such as reduced risk of infections, immune-modulating effects and improvement of mineral absorption (21, 23). These could be attributed to the bifidogenic effect of GOS. However, limited knowledge is available on the mechanisms in the gut that underlie these effects.

The aim of this study was to establish the prebiotic effect of (^{13}C-labeled) GOS. An additional aim was to predict which probiotic bacteria have high potential for use in combination with GOS for synbiotic purposes. This was studied in TIM-2, simulating the *in situ* situation in the human adult colon. Production of (^{13}C-labeled) microbial metabolites was measured using LC-MS. Changes in the composition of the microbiota were measured using SIP coupled to the use of the I-Chip.

MATERIALS AND METHODS

Test products. Universally ^{13}C-labeled- and unlabeled GOS were supplied by Royal FrieslandCampina Domo (Amersfoort, The Netherlands). ^{13}C-labeled GOS was enzymatically produced from ^{13}C-labeled lactose (Omicron Biochemicals, Inc., USA). Labeled and unlabeled GOS (Vivinal® GOS) were purified such that monosaccharides and most of the lactose were removed, as these components are digested and absorbed in the small intestine (composition: galactose, glucose, lactose and oligosaccharides were 0.2, 0.3, 1.0 and 98.5% respectively).

Test system. The study was performed in TNO's *in vitro* model of the proximal large intestine, TIM-2, as described in detail earlier (12, 14-18). Briefly, the model consisted of glass units, each with a flexible wall inside. Peristaltic movements were achieved by pumping water (37°C) at regular intervals into the space between the glass unit and the flexible walls. To simulate the pH of the proximal colon, the pH was set at 5.8 and controlled by secretion of 2M NaOH into the system. Water and fermentation products were removed from the lumen through the dialysis system, consisting of semi-permeable hollow fibers. This maintained physiological concentrations of microbial metabolites and prevented accumulation of metabolites to toxic levels. The model contained an inlet system for the delivery of food. The system was kept anaerobic by flushing with nitrogen gas, which allowed for the growth of a dense, complex microbiota, comparable to that found in the proximal colon of humans (12, 15, 16).

Origin of the microbiota. To create a standardized microbiota, fresh stools of eight healthy Dutch adult volunteers (age range 25-57 y; 3 male; 5 female) were obtained

(after signing an informed consent). These were pooled and used as an inoculum in a fed batch fermentor simulating the caecum conditions as described earlier (16) except that the method was optimized through the use of 200 g of pooled fresh stools instead of 20 g. The standardized microbiota was aliquoted, snap-frozen in liquid nitrogen and stored at -80 ºC until it was used as inoculum in TIM-2 (16).

Standard Ileal Efflux Medium. As a standard medium fed to TIM-2, SIEM (Tritium Microbiology, Eindhoven, The Netherlands) was used as described earlier (24). SIEM contained the following carbohydrates: pectin, xylan, arabinogalactan, amylopectin, starch, reflecting the major components in a normal Western diet. SIEM was dosed at 62 mL/day using a syringe. Other details are described elsewhere (15, 17). Total carbohydrates supplied by SIEM was 7.4 g/d, whereas 10 g/d was used in the GOS experiments.

Conduct of the TIM-2 experiments. At the start of the experiment each TIM-2 unit was inoculated with 30 mL of the standardized microbiota and 80 mL of two times diluted SIEM. Normal SIEM was fed continuously through the food container. The microbiota was allowed to adapt to the model and SIEM for 16 h (**Fig. 1**). After this adaptation period there was a 2 hour starvation period to allow fermentation of all available standard carbohydrates in the system. This period was neither intended to nor sufficient to remove all non-labeled microbial metabolites (not shown). Thereafter the long-term study started (a 3 d test period; n=2), in which the five standard carbohydrates present in SIEM were replaced by unlabeled purified GOS. Samples were taken from the lumen and from the dialysate starting after the starvation period (set as time-point 0) and after 24, 48 and 72 hours of feeding GOS. After 24

and 48 h a total lumen sample of 25 mL was removed from the system to simulate passage of chyme from the proximal to the distal colon. During this 3 day period the microbiota was allowed to adapt to GOS. Thereafter, a short-term study started (*n*=1), in which 1 gram of ^{13}C-labeled GOS was supplied to TIM-2 (Fig. 1), following another starvation period of 2 h to allow fermentation of all unlabeled GOS. The ^{13}C-labeled GOS was added as a bolus and was the only substrate. No SIEM was added during the subsequent 8 h. Samples were taken from both lumen and dialysate starting after the starvation period (time-point 74) and 1, 2, 4 and 8 h after the addition of the ^{13}C-labeled GOS. A third long-term TIM-2 experiment was performed as a control (fed with the standard carbohydrates from SIEM, instead of GOS; *n*=1) to compare the effect of GOS with that of the control on the production of health-beneficial and toxic metabolites and on the microbiota composition (Fig. 1).

Analytical methods. SCFA (acetate, propionate, *n*-butyrate) and BCFA (*i*-butyrate and *i*-valerate) analyses were performed as described earlier (15). Samples for ammonia and lactate analyses were analyzed enzymatically as described earlier (15), and were automated on a Roche Cobas Mira Plus autoanalyzer (Roche, Almere, the Netherlands). Concentrations of all metabolites in the samples were calculated based on calibration curves, using a series of standard solutions with known concentrations.

^{13}C-labeled SCFA and ^{13}C-lactate. ^{13}C-SCFA and ^{13}C-lactate were measured essentially according to Meesters et al. (25), with some modifications. LC-MS analysis was performed on an LTQ Orbitrap system consisting of a Surveyor AS Autosampler, and a Surveyor MS pump, equipped with an LTQ Orbitrap mass detector (Thermo Fisher Scientific, San Jose, USA). Separation was carried out using

an IOA-2000 ion-exchange column (150x6.5 mm, 8 um, Grace Alltech, Breda, the Netherlands) at 90°C with an isocratic flow of 0.15 mmol/L benzoic acid in water (0.6 mL/min). Detection was carried out using atmospheric pressure chemical ionization in the negative ionization mode.

Stable Isotope Probing. RNA-Stable Isotope Probing was performed as described earlier (12, 26, 27). After fractionation the samples were analyzed with the I-Chip (see below). Based on the presence of ribosomal RNA in the heavy fractions (fractions 1 and 2) at t2 and/or t4 a subset of the I-Chip-signals was further investigated. This subset corresponded to those microbes that had fermented ^{13}C-GOS and had incorporated the ^{13}C-label in their rRNA. The signals in all fractions were summed, and the individual signals were expressed as a percentage ratio of this sum.

Micro-array analysis of the microbiota. The composition of the microbiota was analyzed using the I-Chip as described earlier (17, 18). The I-Chip contains roughly 400 probes, some for phylogenetic groups (*e.g. Actinomycetes*), some for group-level detection (*e.g.* all *Bifidobacterium* species) and some for detection of individual species (*e.g. Bifidobacterium longum*). Taxonomic selection was confirmed and expanded based on massive parallel sequencing of 16S rDNA. For each species represented on the microarray a unique short oligonucleotide sequence (approximately 20 nt) from within the 16S rDNA was used, for which just one nucleotide mismatch already resulted in an absence (or very strong decrease) of signal after hybridization. DNA was isolated from 200 mg of the luminal samples at the start and at the end of the long-term TIM-2 experiments as described earlier (17, 18). The rRNA from the density gradient fractions of the short-term experiments was

converted to cDNA as described earlier (26). Hybridization signals were quantified by calculating the mean of all pixel values of each spot and calculating the local background of each spot. For each spot a signal to background ratio was calculated. Spots having a minimal number of observations of more than double their local background were selected for further analyses (17, 18).

RESULTS

Long-term study

Production of beneficial microbial metabolites. The absolute amount of SCFA (acetate, propionate and *n*-butyrate) produced in TIM-2 was higher after 72 hours in the two experiments with GOS (231 and 203 mmol, *n*=2) compared to the control experiment (144 mmol, *n*=1). To exclude differences in amount of carbohydrate feeding in the control (7.4 g/day; *n*=1) versus the GOS experiments (10 g/day; *n*=2), the values were corrected to express SCFA production in mmol/g of carbohydrate fed to TIM-2. Even then the production of SCFA was higher on GOS (**Table 1, Fig. 2**). In the GOS experiments the ratio of acetate to the total SCFA was higher (70%) compared to control (52%), while the ratio of propionate was lower (7% for GOS and 19% for control) and the proportion of butyrate was slightly lower (24% and 29% for GOS and control, respectively).

When also taking lactate production into account, the total amount of organic acids (SCFA + lactate) per gram of carbohydrates fed was on average 8.50 mmol and 7.52 mmol in the GOS and control experiments, respectively (Table 1). No difference was observed in the ratio between L- and D-lactate (Table 1). The amount of organic acids produced in this long-term experiment is the sum of the acids originating from carbohydrates and those originating from protein. In the short-term study described below, it was possible to determine the exact amount of SCFA and lactate produced from the labeled substrate, due to ^{13}C incorporation into the metabolites.

Production of putrefactive microbial metabolites. Because the BCFA (*i*-butyrate and *i*-valerate) and ammonia come from protein fermentation, they cannot be expressed as 'per gram carbohydrate', but are expressed as absolute amounts. Total BCFA

206 production was highest for the control experiment (0.3 mmol and 3.0 mmol for the
207 GOS and control experiment, respectively; **Table 2**). In addition the cumulative
208 amount of ammonia produced in the control experiment was more than double that
209 produced in the GOS experiments (Table 2).
210
211 *Microbiota composition assessed using the I-Chip.* Not all probes on the I-Chip gave
212 a signal above background noise, meaning that these micro-organisms are not present
213 above the level of detection of the method (approximately 10^7 CFU/g). The I-Chip is
214 semi-quantitative due to the different sequences (G-C content) of each probe.
215 Hybridization intensity does not necessarily reflect abundance, because these G-C
216 differences result in different hybridization efficiencies. However, by comparing
217 signals before and after addition of GOS, an absolute change in numbers of microbial
218 species can be determined. The shift in signals over time from the two TIM-2
219 experiments with GOS was compared to the shift in signals over time measured for
220 the control experiment. Data is expressed as those probes that showed an increase or
221 decrease in signal compared to control (**Fig. 3**). The different shades of green signify
222 2, 10, 20, 40 or 60-fold higher signals compared to the control, whereas the different
223 shades of red indicate 2, 10, or 20-fold lower signals compared to the control. GOS
224 increases several *Bifidobacterium* species as well as all bifidobacterial species
225 recognized by the overall bifidobacterial probe (group-probe). *B. bifidum* and *B.*
226 *catenulatum* were most affected (Fig. 3). GOS also increased several *Lactobacillus*
227 species, specifically *L. gasseri* and *L. salivarius*. Not only beneficial microbes were
228 induced, but also for example *Enterobacteriaceae* and *Klebsiella*. Species/groups that
229 were reduced compared to the control include different *Bacteroides* species,
230 *Eubacterium halii*, *Prevotella* and *Lactococcus* (Fig. 3).

231

232 *Short-term study*

233 *^{13}C- metabolite production*. ^{13}C-labeled SCFA and ^{13}C-lactate were measured using
234 LC-MS. The different isotopomers are indicated by their molecular mass (=M) + a
235 number indicating the number of ^{13}C-atoms in the molecule. For example, M+2-
236 propionate means two ^{13}C-atoms in a C_3-molecule of propionate, whereas M+3-
237 propionate indicates that all carbon-atoms were ^{13}C-labeled. The peak of fully, M+3
238 labeled lactate was produced after approximately 2 hours (**Fig. 4A**). Most (95%) of
239 the labeled lactate found was the M+3 lactate isotopomer, but also some partly
240 labeled isotopomers of lactate were produced. Comparable to lactate, the amount of
241 fully labeled acetate (M+2) produced also reached a plateau after approximately 2
242 hours (**Supplemental Fig. 1A**). Partly labeled acetate was also produced (**Fig. 4B**).
243 The major isotopomer of ^{13}C-propionate was the fully labeled isotopomer (M+3),
244 whereas most of the ^{13}C-butyrate was produced as M+2 (Fig. 4B and **Supplemental**
245 **Fig. 1B and 1C, respectively**).

246

247 *Stable isotope probing*. After density-gradient centrifugation and fractionation (26)
248 samples were analyzed using the I-Chip. The signals in all fractions (1 [heavy] to 8
249 [light]) were summed, and the individual signals were expressed as the percentage
250 ratio of this sum. This made it possible to see shifts in the density of the rRNA,
251 indicative of incorporation of the ^{13}C-label in the RNA. Probes, that corresponded to
252 the microbes that had incorporated the label in their rRNA, were investigated in more
253 depth (Fig. 3; probes indicated with a +). For *B. bifidum* (**Fig. 5A**), the amount of
254 rRNA in the different fractions was expressed over time. On time-point 0, when no
255 label was added yet, most of the rRNA was present in fraction 4. Two hours after

addition of the ^{13}C-labeled GOS, rRNA was also found in the heavy fractions 1 and 2. Four hours after addition of the label, rRNA was still found in fractions 1 and 2. However, eight hours after addition of the ^{13}C-labeled GOS, rRNA was no longer detected in fraction 1 (Fig. 5A). This indicated dilution of the incorporated ^{13}C-atoms in the biomass. Altogether, this is indicative of *B. bifidum* being able to incorporate the ^{13}C-label into its biomass, indicating that *B. bifidum* was directly stimulated by GOS. Other representative results are shown for the family-probe *Enterobacteriaceae* and *L.* gasseri (**Fig. 5B** and **5C**, respectively). Several bifidobacteria (*B. catenulatum, B. bifidum* and *B. longum* - in decreasing order of importance) and lactobacilli (*L. gasseri* and *L. salivarius*) were fermenting the ^{13}C-GOS (based on the incorporation of ^{13}C in their rRNA; Fig. 3 and **Supplemental Fig. 2**). Of these, *B. bifidum, B. catenulatum, L. gasseri* and *L. salivarius* were also stimulated in the long-term experiments (Fig. 3). Also the genus or group-specific probes for bifidobacteria and lactobacilli showed a shift in signal towards the high-density fractions. The remaining probes of species which also incorporated ^{13}C in their rRNA, but to a lower extent, correspond to a multitude of microbes, namely the *Bacteroidetes* group-probe, *Collinsella aerofaciens*, *Enterococcus hirae*, *Eubacterium siraerum*, the *Enterobacteriaceae*-group probe, *Vagococcus fluvialis*, *Clostridium disporicum* and a probe covering *Salmonella Typhi* and *Klebsiella pneumonae* (data not shown).

DISCUSSION

It is not clear whether in the gut microbiota bifidobacteria are stimulated directly or indirectly by GOS, or which specific species of bifidobacteria are stimulated. Nor is it exactly known which microbial metabolites are produced. Although there are numerous reports on the bifidogenic nature of GOS (see (21) for a recent review and (28-31) for other recent material), the evidence that bifidobacteria (and lactobacilli) are stimulated by GOS is indirect. Therefore, we studied this using a stable-isotope approach, allowing the unambiguous identification of microbes involved in fermentation of the labeled substrates and the metabolites that are produced from it. We have shown, in previous experiments, that shifts in the composition of the microbiota in TIM-2 over a period of 3 days are predictive for what appears over longer periods of time (16). SIP allows linking of the phylogenetic identity of a micro-organism with its ecological function in a complex microbial community (12, 26). SIP results were interpreted in combination with the phylogenetic micro-array I-Chip (17, 18). qRT-PCR using the 16S rRNA gene as the target gene for amplification showed that there is a linear relationship between the signal on the I-Chip and the number of cells present over at least four orders of magnitude (unpublished data; R^2=0.93). Label incorporation was highest after 2-4 hours, indicating fast fermentation of GOS. After 8 hours there was already dilution of the incorporated ^{13}C-atoms in the biomass. Based on the incorporation of ^{13}C in their rRNA, *B. bifidum, B. catenulatum, L. gasseri* and *L. salivarius* especially fermented the ^{13}C-GOS. However, ^{13}C incorporation in the rRNA was found for a multitude of other microbes, albeit to a lesser extent. Examples are *Klebsiella* and *Enterobacteriaceae*, which are micro-organisms very commonly found in the gut, consisting of harmless as well as potentially harmful strains (32). The increase in these micro-organisms was relatively

small compared to the increase seen for the beneficial micro-organisms *Lactobacillus* and *Bifidobacterium*. From the species/groups that were reduced compared to the control, *Prevotella* specifically can be considered an "unhealthy" micro-organism, especially in the oral cavity (33). Thus, we hypothesize that the microbiota composition on GOS shifted the colonic environment towards a more healthy milieu. The strength of the combination of SIP and the I-Chip is that it allowed for the exact identification of those species that fermented GOS because only those micro-organisms incorporate the label.

The production of mainly fully ^{13}C-labeled isotopomers of the microbial metabolites indicates they are produced by direct fermentation of the uniformly labeled GOS. Production of partly labeled isotopomers might indicate an indirect pathway, so called cross-feeding, where several microbes cooperate in the production of the metabolite (12). Most (95%) of the detected lactate and acetate isotopomers were fully labeled, but also some partly-labeled isotopomers were produced. For example, M+1 acetate was detected, which is indicative of the Wood-Ljungdahl pathway, which couples a labeled CO_2 to an unlabeled CO_2 (through formate). The major butyrate isotopomer was produced as M+2. This can be produced by coupling of a fully-labeled and an unlabeled acetate in a cross-feeding reaction, or by conversion of lactate to butyrate by butyrogenic microbes, such as *Clostridium* species (34). Due to the fact that ^{13}C-lactate and ^{13}C-acetate production reached their plateaus after 2 hours we concluded that the ^{13}C-GOS was completely fermented after 2 hours, in agreement with the label incorporation in the biomass. However, even after 8 hours, label was still being incorporated in ^{13}C-labeled propionate and butyrate. The production of these did not start directly after intake of the ^{13}C-labeled GOS, but only after a delay of approximately 1 hour, again indicative of cross-feeding. The production of lactate

corresponded perfectly to the stimulation of bifidobacteria and lactobacilli by GOS. In addition, the acetate production can be explained (in part) by its production by bifidobacteria, as this genus produces almost equal amounts of lactate and acetate during fermentation of carbohydrates (35). Thus, the use of a stable-isotope labeled substrate uniquely allowed the determination of the food-web occurring in a complex mixture of microbes such as the colonic microbiota.

SCFA and lactate are metabolites considered beneficial for the host, because they can be used by the cells of the body as an energy source (4, 8, 36). Furthermore, SCFA reduce the pH in the colon which is known to inhibit pathogen growth (3). The absolute amounts of SCFA and lactate produced in TIM-2 were higher in the long-term experiment with GOS compared to the control experiment. When corrected for the amount of substrate added, there still was more acid production upon GOS fermentation (8.5 mmol/g) compared to the control (7.5 mmol/g). Lactate only accumulates when fermentation is fast, whereas otherwise it is converted into other SCFA (primarily propionate and butyrate) and does not accumulate (34). Thus, lactate accumulation during the GOS experiments corroborates that GOS was fermented quickly. The amount of organic acids produced in the long-term study was the sum of acids originating from carbohydrates and those originating from protein and other fermentable substrates. Only in the experiment with the ^{13}C-labeled GOS could the exact amount of SCFA and lactate produced from the substrate be distinguished from those produced from other, unlabeled substrates such as protein. BCFA and ammonia are toxic metabolites produced only from protein fermentation, a process which is generally believed to be putrefactive (8, 9, 37-39). This usually occurs when there fermentable carbohydrate is no longer present. 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. In the long-term study, total BCFA production was highest for the control experiment, indicating higher protein fermentation in these experiments. This is corroborated by the fact that, in the control experiment, the cumulative amount of ammonia produced was more than double that of the GOS experiments. Thus, we show that the addition of 10 g/day of GOS results in suppression of protein fermentation, as has been shown earlier for GOS, FOS and inulin *in vitro* as well as *in vivo* (15, 40).

Previously, we have tested fermentation of ^{13}C-labeled starch (12), and ^{13}C-inulin (41) in the same *in vitro* model. The cumulative production of the different metabolites in the experiments with these three ^{13}C-labeled substrates is compared in **Table 3**. Because lactate was not measured quantitatively during the inulin fermentation, exact comparison with GOS for this metabolite cannot be made. However based on estimated peak-areas of GC analyses, lactate was produced in similar amounts as acetate in experiments with unlabeled inulin (unpublished data). On starch there was negligible lactate production. Together this indicates that GOS produced the highest amount of lactate. Butyrate production was highest on starch, as expected, as starch is considered to lead to high butyrate amounts in the colon (42). The sum of all metabolites measured was in the same range, considering that lactate was not quantitatively measured. Inulin was shown to primarily stimulate a species related to *Dorea longicatena* in the same *in vitro* model when using SIP (41). In addition, RNA of *Bifidobacterium adolescentis* was found to be present in the fractions with high density, corroborating the bifidogenic nature of inulin. However, the GOS used in this study had a clearer bifidogenic effect than the inulin previously used. Interestingly, *B. adolescentis* seems to play a role in potato starch fermentation also, albeit a minor one

(12). It appears that GOS, in comparison to inulin and starch, is able to stimulate the infant-type bifidobacteria (*B. bifidum* and *B. longum*) rather than the adult-type (43), even when a microbiota originating from adults was used in these experiments. Each labeled experiment was performed as a single experiment (n=1), because of the high reproducibility of the computer-controlled system. However, confirmation of these experiments, also *in vivo*, is required.

In conclusion, GOS is specifically fermented by bifidobacteria and lactobacilli based on the fact that they show the highest incorporation of ^{13}C-label in 16S-rRNA. Coupled to production of labeled lactate and acetate, this unambiguously corroborates the conclusion that GOS is a prebiotic. Potentially, these results allow for selection of species or strains from the intestinal microbiota that could be used together with GOS as synbiotics. From the results obtained, *B. catenulatum*, *B. bifidum* or *L. gasseri* seem particularly suitable for this application.

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STATEMENT OF AUTHOR'S CONTRIBUTIONS TO MANUSCRIPT

M. H. C. S., E. G. v.d. H. and K. V. designed research. A. J. H. M. conducted research and analyzed the data. A. J. H. M. and K.V. wrote the paper. K. V. had primary responsibility for final content. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1 Set-up of the experiments performed in TIM-2. ⬇: moment of sampling; ⇩: start of the short-term experiment.

Figure 2 Daily cumulative production of short-chain fatty acids (SCFA) expressed as per gram carbohydrate fed. Data are presented as individual values: GOS: *n*=2 (**A**); control: *n*=1 (**B**).

Figure 3 Probes on the I-Chip which show differential hybridization in the long-term TIM-2 experiments with GOS. Bacteria that build in the ^{13}C label in their biomass were measured by stable isotope probing (SIP) and are indicated with a plus-symbol. r1 and r2: the two individual long-term experiments with unlabeled GOS.

Figure 4 Cumulative amount of the ^{13}C-labeled lactate (**A**) and SCFA isotopomers (**B**) produced in TIM-2 after the 8 h short-term experiment; *n* = 1. In Supplemental Figure 1 of the Online Supporting Material additional kinetic graphs for acetate, propionate and butyrate are shown.

Figure 5. Distribution of the rRNA over the different fractions and over time for *Bifidobacterium bifidum* (**A**), *Enterobacteriaceae* (**B**), and *Lactobacillus gasseri* (**C**) after density-gradient ultracentrifugation. In Supplemental Figure 2 of the Online Supporting Material additional graphs are shown for *B. catenulatum*, *B. longum* and *L. salivarius*.

TABLE 1 Organic acid production in TIM-2 after 72 h expressed as per gram carbohydrate added[1]

	acetate	propionate	*n*-butyrate	lactate	% L- and D-lactate	total SCFA+lactate
			mmol/g			
GOS	5.50, 4.49	0.49, 0.49	1.71, 1.69	1.09, 1.45	64.9% L; 35.1% D	8.78, 8.22
Control	3.38	1.21	1.9	1.03	66.5% L; 33.5% D	7.52

[1]Data are presented as individual values (GOS: *n*=2; control *n*=1). In Supplemental Table 1 of the Online Supporting Material the total cumulative amount of SCFA and lactate produced over the 72 h TIM-2 experiment is shown.

TABLE 2 Absolute cumulative BCFA and ammonia production after 72 hours[1]

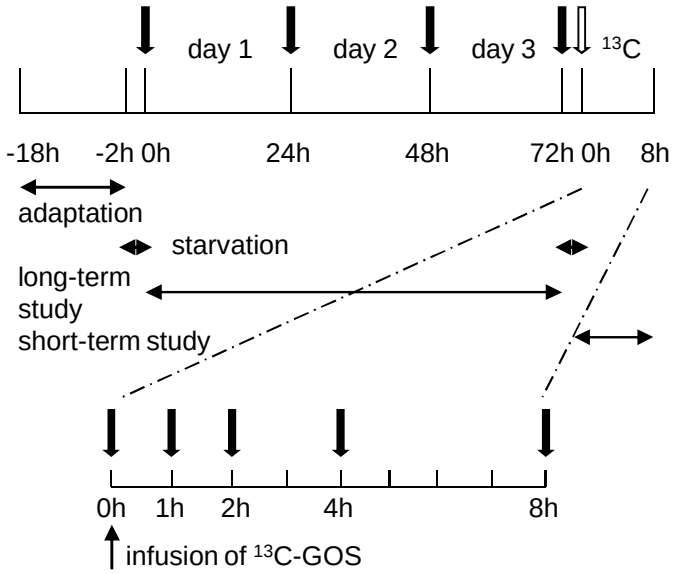
	i-butyrate	*i*-valerate	ammonia
		mmol	
GOS	0.21, 0.19	0.13, 0.10	22.1, 22.0
Control	0.88	2.16	47.0

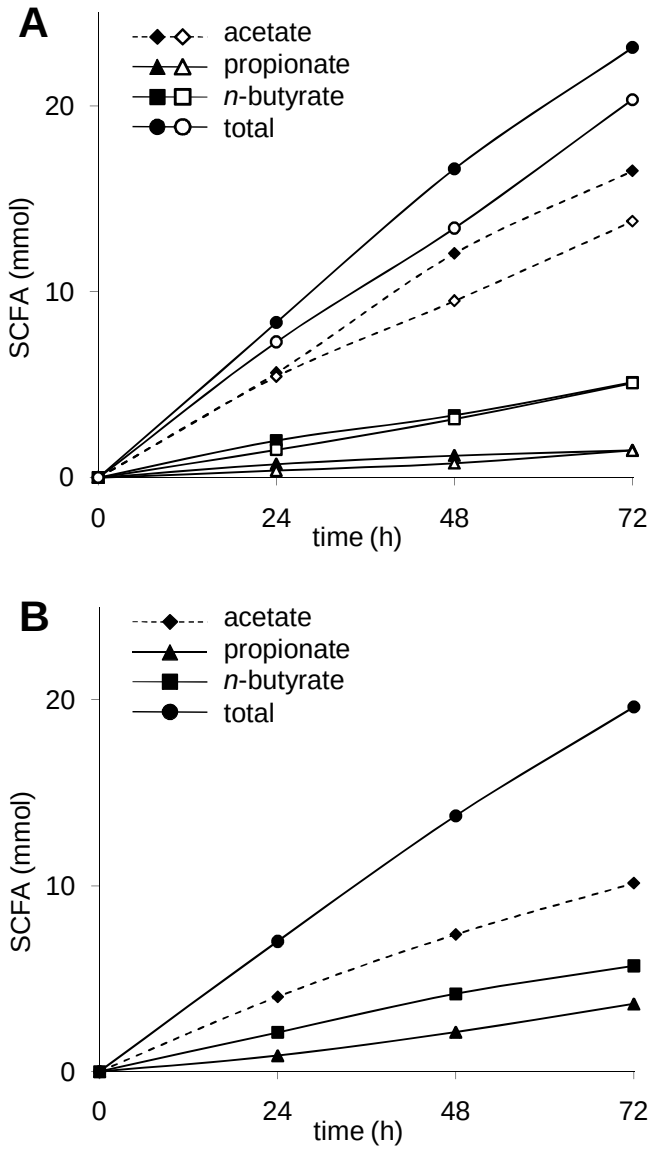
[1]BCFA = branched-chain fatty acids. Data are presented as individual values (GOS: *n*=2; control *n*=1).

TABLE 3 Cumulative amount of the different ^{13}C-labeled metabolites produced from ^{13}C-labeled substrates[1]

	acetate	propionate	*n*-butyrate	lactate	reference
			mmol		
^{13}C - starch	~ 5	~ 1	~ 2	not measured	(12)
^{13}C - inulin	~ 4	~ 2	~ 0.4	not measured	(41)
^{13}C - GOS	~ 3.2	~ 0.5	~ 1.1	~ 5.1	current study

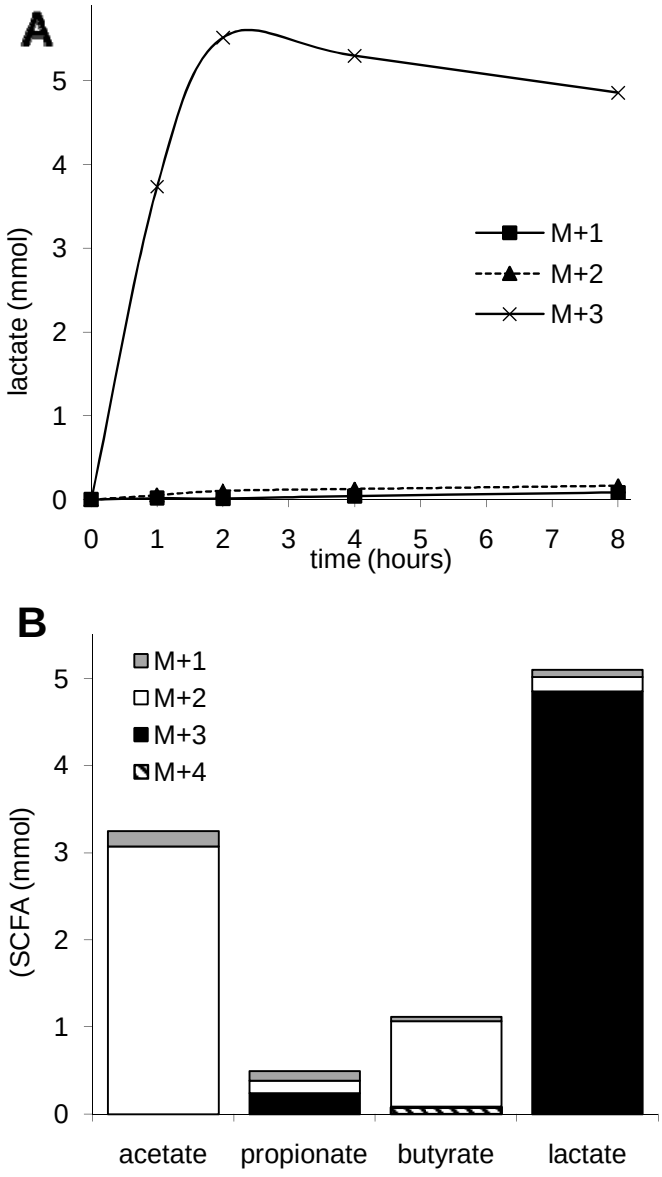
[1] The same inoculum was used for the experiments with starch and inulin. The experiments with GOS were done at a later point in time and a different microbiota was used.

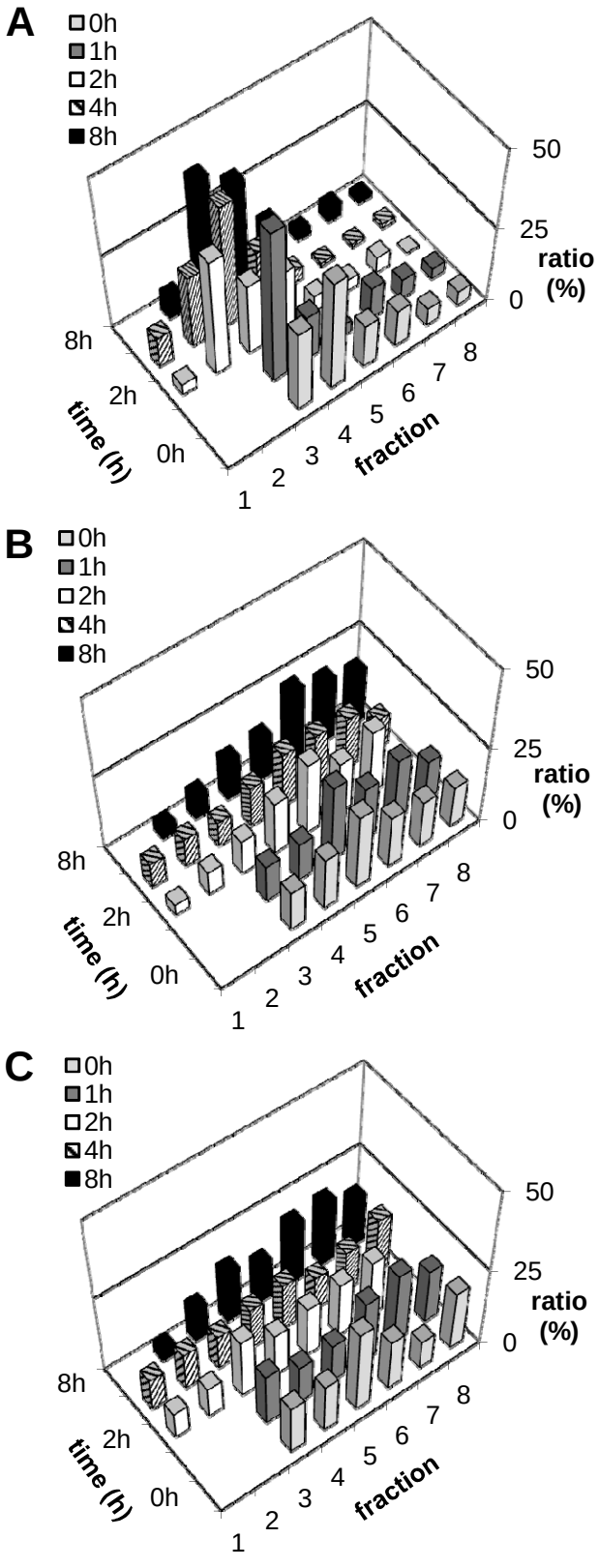



A
acetate
propionate
n-butyrate
total
SCFA (mmol)
0
10
20
0
24
48
72
time (h)
B
acetate
propionate
n-butyrate
total
SCFA (mmol)
0
10
20
0
24
48
72
time (h)

	GOS r1	GOS r2	SIP		GOS r1	GOS r2	SIP
Archeaegroup				*Enterobacteriacae*			+
Bacillus subtilis				*Enterococcus hirae*			+
Bacillus subtilis				*Enterococcus* species			
Bacteriodes species				*Eubacterium* group 01			
Bacteroides distasonis				*Eubacterium halii*			
Bacteroides fragilis				*Eubacterium rectale*			
Bacteroides group				*Eubacterium romicigenerans*			
Bacteroides ovatus				*Eubacterium romicigenerans*			
Bacteroides prevotella group				*Eubacterium siraeum*			+
Bacteroides thetaiotaomicron				*Fibrobacter succinogenes*			
Bacteroides vulgatus				*F.nucleatum* subsp. *nucleatum*			
Bacteroidetes			+	*Fusobacterium prausnitzii*			
Betaproteobacteria				*Fusobacterium prausnitzii*			
Bifidobacteriaceae			+	*Haloanaerobiales*			
Bifidobacterium adolescentis				*Klebsiella* group 01			
Bifidobacterium adolescentis				*Lactobacillaceae*			+
Bifidobacterium adolescentis				*L.casei / L.paracasei*			
B.adolescentis / B.angulatum				*Lactobacillus gasseri*			+
Bifidobacterium angulatum				*Lactobacillus gasseri*			+
Bifidobacterium animalis				*Lactobacillus* group 02			
Bifidobacterium animalis				*Lactobacillus sakei*			
Bifidobacterium bifidum			+	*Lactobacillus salivarius*			+
Bifidobacterium bifidum			+	*Lactococcus* group 01			
Bifidobacterium breve				*Lactococcus lactis*			
Bifidobacterium breve				*Lc.lactis* subsp. *cremoris*			
Bifidobacterium catenulatum				*Microbacteriaceae*			
Bifidobacterium catenulatum			+	*Micrococcaceae*			
B.catenulatum group			+	*Mollicutes*			
Bifidobacterium dentium				*Nitrospirae*			
Bifidobacterium dentium				*Prevotella bivia*			
Bifidobacterium group 01				*Prevotella* group 08			
Bifidobacterium group 02			+	*Ruminococcus albus*			
Bifidobacterium longum				*Ruminococcus bromii*			
Bifidobacterium longum				*Ruminococcus bromii*			
Bifidobacterium longum			+	*Ruminococcus clostridium* grp 01			
Bifidobacterium species			+	*Ruminococcus obeum*			
Campylobacter coli / jejuni				*Ruminococcus productus*			
Clostridium disporicum			+	*Salmonella Typhi / Kl.pneumonae*			+
Clostridium paraputrificum				*Staphylococcus aureus*			
Collinsella aerofaciens			+	*Staphylococcus saprophyticus*			
Collinsella aerofaciens				*Streptococcus* group 04			
Coriobacteriaceae				*Streptococcus* group			
E.cloacae / Serratia marcesens				*Vagococcus fluvialis*			+
E.cloacae / Ser.marc/ Sal.Typhi				*Vagococcus* species			+
E.cloacae / Ser.marc/ Sal.Typhi				*Yersinia enterocolitica*			

Fold lower	:	<2	<10	<20	Fold higher	:	>2	>10	>20	>40	>60


A
lactate (mmol)
0
1
2
3
4
5
M+1
M+2
M+3
0
1
2
3
4
5
6
7
8
time (hours)
B
(SCFA (mmol)
M+1
M+2
M+3
M+4
0
1
2
3
4
5
acetate
propionate
butyrate
lactate


A
0h
1h
2h
4h
8h
50
25
ratio (%)
0
8h
2h
0h
time (h)
1
2
3
4
5
6
7
8
fraction
B
0h
1h
2h
4h
8h
50
25
ratio (%)
0
8h
2h
0h
time (h)
1
2
3
4
5
6
7
8
fraction
C
0h
1h
2h
4h
8h
50
25
ratio (%)
0
8h
2h
0h
time (h)
1
2
3
4
5
6
7
8
fraction