

# Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum* NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study<sup>1-3</sup>

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## ABSTRACT

**Background:** Galactooligosaccharides are selectively fermented by the beneficial member of the colonic microflora contributing to the health of the host.

**Objective:** We assessed the prebiotic potential of a novel galactooligosaccharide produced through the action of  $\beta$ -galactosidases, originating from a probiotic *Bifidobacterium bifidum* strain, against a galactooligosaccharide produced through the action of an industrial  $\beta$ -galactosidase and a placebo.

**Design:** Fifty-nine healthy human volunteers participated in this study. Initially, the effect of the matrix on the prebiotic properties of a commercially available galactooligosaccharide (7 g/d) was assessed during 7-d treatment periods with a 7-d washout period in between. During the second phase, 30 volunteers were assigned to a sequence of treatments (7 d) differing in the amount of the novel galactooligosaccharide (0, 3.6, or 7 g/d). Stools were recovered before and after each intervention, and bacteria numbers were determined by fluorescent in situ hybridization.

**Results:** Addition of the novel galactooligosaccharide mixture significantly increased the bifidobacterial population ratio compared with the placebo ( $P < 0.05$ ), whereas 7 g/d of the novel galactooligosaccharide significantly increased the bifidobacterial ratio compared with the commercial galactooligosaccharide ( $P < 0.05$ ). Moreover, a significant relation ( $P < 0.001$ ) between the bifidobacteria proportion and the novel galactooligosaccharide dose (0, 3.6, and 7 g/d) was observed. This relation was similar to the effect of the novel galactooligosaccharide on the prebiotic index of each dose.

**Conclusions:** This study showed that galactooligosaccharide mixtures produced with different  $\beta$ -galactosidases show different prebiotic properties and that, by using enzymes originating from bifidobacterial species, an increase in the bifidogenic properties of the prebiotic product is achievable. *Am J Clin Nutr* 2008;87:785-91.

**KEY WORDS** *Bifidobacterium*, galactooligosaccharides, prebiotic, human fecal flora, intestinal microflora, functional food

## INTRODUCTION

The human gastrointestinal tract, especially the colon, is a relatively underexplored microbial ecosystem, offering a good

opportunity for the development of dietary interventions targeting disease reduction risk and maintenance of good health. Modifying the colonic microflora through both dietary probiotic (1) and prebiotic (2) strategies has attracted much research and product developments. Probiotics aim to target exogenous bacteria into the colonic microflora, whereas the introduction of nondigestible food ingredients that aim to enhance the growth or the metabolic activity of potentially health-promoting indigenous bacteria is the prebiotic approach. In both cases, the target organisms are usually bifidobacteria and lactic acid bacteria.

Although any food ingredient that escapes digestion in the upper gastrointestinal tract has the potential to act as a prebiotic, only certain carbohydrates were shown to provide convincing evidence in favor of this. Many different nondigestible carbohydrates have claimed to exert prebiotic properties; however, only fructooligosaccharides and galactooligosaccharides were tested in vivo for all the requirements for the current criteria of a successful prebiotic (3, 4). Bouhnik et al (4) compared the prebiotic properties of several candidate prebiotics in vivo and reported that galactooligosaccharides, after 7 d of administration, showed a prebiotic effect and a stronger bifidogenic effect than did short chain fructooligosaccharides (4).

Galactooligosaccharides are produced through the action of  $\beta$ -galactosidases on lactose, and, depending on the source of the  $\beta$ -galactosidase, different synthetic product mixtures are formed. Given that  $\beta$ -galactosidase enzymes from different microorganisms display differing rate constants for conversion of specific glycosidic linkages, it is anticipated that synthetic mixtures produced through enzymes from probiotic organisms will confer selectivity on those specific probiotics when then fermented by the colonic microflora. This hypothesis was tested in vitro for both bifidobacteria (5) and lactobacilli (6), suggesting that the development of prebiotics with enhanced selectivity is feasible.

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**TABLE 1**  
Volunteer characteristics

	Phase 1 (n = 29)	Phase 2 (n = 30)
Age (y)	32.5 (19–55) <sup>1</sup>	36.3 (21–59)
Sex		
M	13	12
F	16	18
Microflora (log <sub>10</sub> cells/g feces)		
Total cells	10.62 ± 0.23 <sup>2</sup>	10.58 ± 0.24
<i>Bifidobacterium</i>	9.32 ± 0.22	9.21 ± 0.28
<i>Bacteroides-Prevotella</i>	9.69 ± 0.26	9.58 ± 0.31
<i>Clostridium perfringens</i> – <i>hystolyticum</i> subgroup	8.62 ± 0.33	8.65 ± 0.32
<i>Lactobacillus-Enterococcus</i>	8.53 ± 0.26	8.63 ± 0.28

<sup>1</sup>  $\bar{x}$ ; range in parentheses (all such values).

<sup>2</sup>  $\bar{x} \pm$  SD (all such values).

Previously, we applied this approach of exploiting  $\beta$ -galactosidases from probiotics to produce a novel galactooligosaccharide mixture designed to specifically improve *Bifidobacterium* numbers (B-GOS) (7). With the use of an in vitro model of the human colon and an in vivo pig-feeding trial (8), we have shown the increased bifidogenic activity of this novel galactooligosaccharide. Here, we now extend the study to an intervention trial with healthy human volunteers to assess and evaluate the prebiotic potential of the novel galactooligosaccharide at 2 different doses on the human colonic microflora. Furthermore, we attempted to test the hypothesis that the galactooligosaccharide mixture synthesized through enzymes from *Bifidobacterium*

*bifidum* NCIMB 41171 (B-GOS) would display a higher bifidogenic effect than would a galactooligosaccharide mixture produced through enzymes from *Bacillus circulans* ATCC 4516.

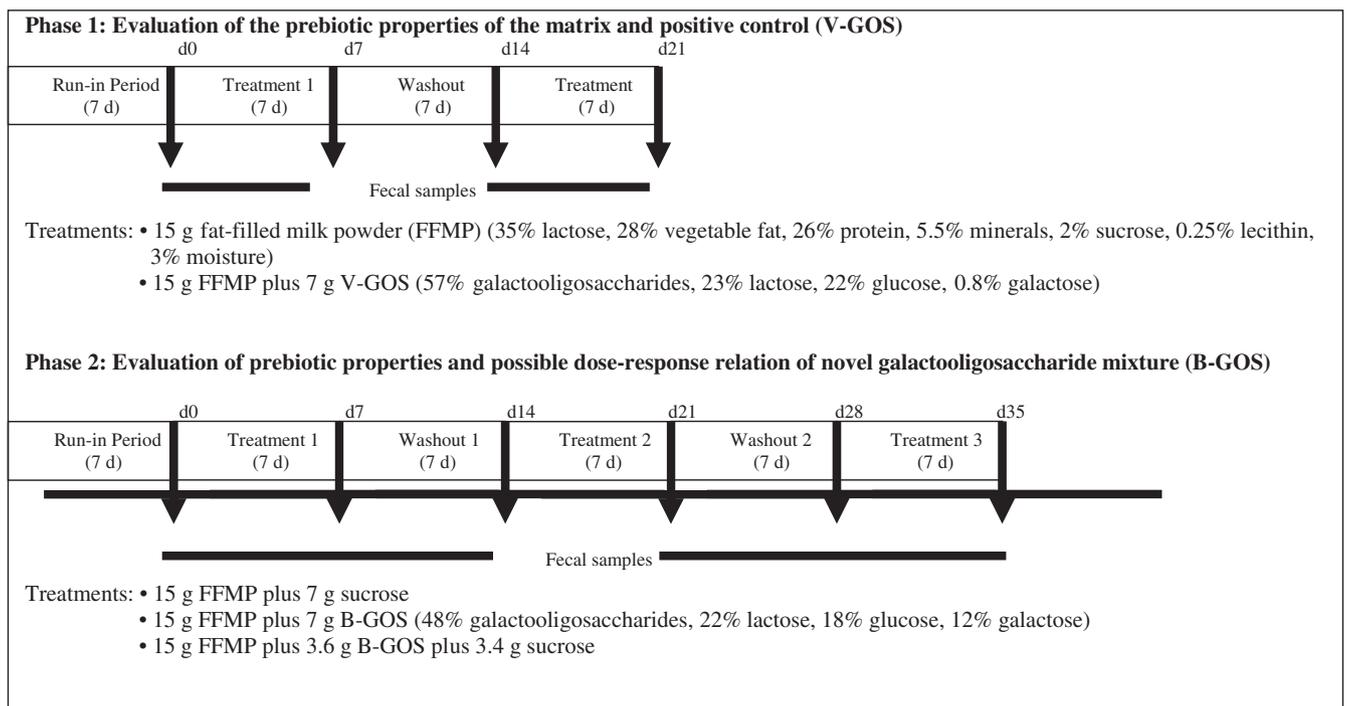
## SUBJECTS AND METHODS

### Subjects

Fifty-nine healthy human volunteers (25 men and 34 women; average age: 34.4 ± 6.7 y) participated in the study (Table 1). Written consent was obtained from all volunteers, and the study protocol was reviewed and approved by the Norwich District Research Ethics Committee. Volunteers were assessed before the start of the trial for good health and were selected according to certain exclusion and inclusion criteria. Inclusion criteria for participation in the study were signed consent form, age of 18–60 y inclusive, good general health, absence of allergy to milk products, and absence of gastrointestinal disorders (eg, chronic constipation, diarrhea, inflammatory bowel disease, inflammatory bowel syndrome, or other chronic gastrointestinal complaints). Volunteers taking probiotics, prebiotics, synbiotics, or antibiotics within 2 mo before the study were also excluded. Volunteers were instructed not to consume such products during the study and not to alter their usual diet or fluid intake.

### Experimental design

The study (Figure 1) consisted of 2 parts; phase 1 investigated the suitability of the matrix in studying prebiotic effects on the colonic microflora of human volunteers against a well-documented prebiotic galactooligosaccharide mixture (3, 9),



**FIGURE 1.** Study design of and nutritional information on the treatments administrated throughout the study. Each treatment was preweighed and delivered in individual containers. Volunteers were asked to mix the material with 175 mL water before use. B-GOS, novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum*; V-GOS, galactooligosaccharide mixture produced through enzymes from *Bacillus circulans* available commercially as Vivinal GOS.

consisting of galactooligosaccharides in  $\beta 1 \rightarrow 4$  and  $\beta 1 \rightarrow 6$  linkages [Vivinal GOS (V-GOS); Friesland, Zwolle, Netherlands]. Phase 2 consisted of a dose-response study of a novel galactooligosaccharide mixture (B-GOS), which methylation analysis (10) has shown to consist of galactooligosaccharides in mainly  $\beta 1 \rightarrow 3$  as well as  $\beta 1 \rightarrow 4$  and  $\beta 1 \rightarrow 6$  linkages (data not shown), and sucrose as the placebo. Both parts of the study were conducted through a blind-coded crossover design. During phase 1, 29 volunteers were randomly assigned to vegetable fat-filled milk powder (FFMP; 15 g; Cowbell International, Bryanston, South Africa) or vegetable FFMP (15 g) plus 7 g V-GOS. Volunteers consumed the treatment for a 7-d period, which was followed by a 7-d washout period before switching to the other treatment. Phase 2 aimed to evaluate the prebiotic potential of the novel GOS mixture (B-GOS) (11) and possible dose-response effect of it on the colonic microflora with the use of 1 placebo and 2 B-GOS treatments (high- and low-dose B-GOS). For this purpose, 30 volunteers were randomly assigned to a sequence consisting of the following 3 treatments: vegetable FFMP (15 g) plus 7 g sucrose, or vegetable FFMP (15 g) plus 3.6 g B-GOS plus 3.4 g sucrose, or vegetable FFMP (15 g) plus 7 g B-GOS. Each treatment was consumed for 7 d followed by a 7-d washout period. All treatments were preweighed and provided in individual containers, and volunteers were asked to mix the materials with water (175 mL) immediately before consumption at any time during the day.

### Fecal sample collection and preparation

Fecal samples were collected on day 0 (baseline) and at the end of each treatment and washout period (Figure 1). Freshly voided fecal samples were collected in sterile plastic pots, kept cool in insulated boxes until transfer to the laboratory where they were analyzed immediately (<2 h from stool emission). Samples were diluted 1 in 10 (wt:wt) in phosphate-buffered saline [(PBS); 0.1 mol/L; pH 7.0] and homogenized in a Stomacher 400 (Seward, Norfolk, United Kingdom) for 2 min at normal speed.

### Bacterial enumeration

Fecal homogenates were subjected to fluorescent in situ hybridization, with the use of synthetic oligonucleotide probes, targeting specific regions of the 16S rRNA molecule and labeled with the fluorescent dye Cy3 as previously described by Rycroft et al (12). Briefly, samples were fixed overnight (4 °C) in 4% (wt:vol) paraformaldehyde. Fixed cells were centrifuged at  $1500 \times g$  for 5 min at 25 °C and washed twice in 1 mL filtered PBS. The washed cells were resuspended in 150  $\mu$ L PBS and stored in ethanol (1:1 by vol) at -20 °C. After overnight hybridization with each probe, the fixed cells were washed and vacuum filtered (2- $\mu$ m polycarbonate isopore membrane filter; Millipore UK Ltd, Watford, United Kingdom). They were then mounted onto a glass slide with 20  $\mu$ L of slowfade (Molecular Probes, Leiden, The Netherlands) and enumerated with the use of the Fluor 100 lens (Eclipse 400 epifluorescent microscope; Nikon, Kingston upon Thames, United Kingdom). The probes used were Bif164 for *Bifidobacterium* genus (13), His150 for the *Clostridium perfringens*-*histolyticum* subgroup (14), Bac303 for *Bacteroides-Prevotella* (15), and Lab158 for *Lactobacillus-Enterococcus* spp (16). 4,6-Diamidino-2-phenylindole was used for the enumeration of total cell counts. All probes were provided by MWG-Biotech (London, United Kingdom).

### Intestinal isolates

To identify which of the beneficial members of the commensal microflora would better respond to the galactooligosaccharide administration, fecal samples from the participating volunteers were used to isolate bifidobacteria and lactobacilli strains for further in vitro fermentation tests.

Altogether, 132 bifidobacteria and lactobacilli isolates were collected from Beerens (17) and Rogosa agars (Oxoid Ltd, Basingstoke, United Kingdom) by picking representatives of all clearly distinct colony morphotypes from fecal samples of the healthy human volunteers. The isolates were checked for purity and were tentatively identified on the basis of typical microscopic structure by Gram stain and the API 20A (bioMerieux, Hazelwood, MO) commercial identification system. Thirty-four isolates were further identified by 16S rRNA gene sequencing as previously described (18).

Briefly, total bacterial DNA was extracted with the use of an Insta-Gene-Matrix kit (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom) according to the manufacturer's instructions. The 16S rRNA gene was then amplified by polymerase chain reaction (PCR) with the use of conserved primers close to the 3' and 5' ends of the gene.

The PCR products were then purified with the use of a QIAquick PCR purification kit (QIAGEN, Crawley, United Kingdom), and between 300 and 600 nucleotides proximal to the 5' end of the rRNA were sequenced with the use of a Taq Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and a model 377A automatic DNA sequencer (Applied Biosystems). Generated sequences were compared with 16S rRNA gene sequences available in the GenBank/EMBL (<http://www.ebi.ac.uk>) database with the FASTA program.

### Batch culture fermentations

Isolated bacteria were stored on beads at -70 °C and when needed grown on Beerens or Rogosa agar and incubated at 37 °C in an anaerobic cabinet (10% H<sub>2</sub>; 10% CO<sub>2</sub>; 80% N<sub>2</sub>). After incubation, individual colonies were removed, checked for purity with Gram staining, and subcultured onto fresh agar. After a second incubation, cells were subcultured into Wilkins-Chalgren broth (Oxoid Ltd) and incubated for 24 h. These cultures, at a concentration of 1% (by vol), were then used to inoculate 100-mL batch culture fermenters. The fermenters were previously filled with prereduced basal nutrient medium (2 g peptone water l<sup>-1</sup>, 2 g yeast extract l<sup>-1</sup>, 0.1 g NaCl l<sup>-1</sup>, 0.04 g K<sub>2</sub>HPO<sub>4</sub> l<sup>-1</sup>, 0.04 g KH<sub>2</sub>PO<sub>4</sub> l<sup>-1</sup>, 0.01 g MgSO<sub>4</sub>·7H<sub>2</sub>O l<sup>-1</sup>, 0.01 g CaCl<sub>2</sub>·6H<sub>2</sub>O l<sup>-1</sup>, 2 g NaHCO<sub>3</sub> l<sup>-1</sup>, 2 mL Tween 80 L<sup>-1</sup>, 0.02 g Hemin l<sup>-1</sup>, 10  $\mu$ L vitamin K<sub>1</sub> l<sup>-1</sup>, 0.5 g Cysteine.HCl l<sup>-1</sup>, 0.5 g bile salts l<sup>-1</sup>). Broth pH was adjusted to 6.8 and, after sterilization at 121 °C for 15 min, left overnight in the anaerobic cabinet. Just before inoculation, a 1/10 dilution of glucose, V-GOS, or B-GOS in distilled water was prepared, filter-sterilized, and added to the broth at a final concentration of 1% (wt:vol).

### Statistical analysis

Paired *t* tests were used to compare the changes in the bacterial population proportions from baseline to the end of each treatment for each of the bacterial groups monitored in the in vivo study.

**TABLE 2**

Bacterial proportions of the monitored fecal microflora components and prebiotic index (PI) score of the treatments used in the healthy human volunteers during the study period<sup>1</sup>

Treatment	Proportions of counted bacterial groups				PI <sup>2</sup>
	<i>Bifidobacterium</i>	<i>Bacteroides-Prevotella</i>	<i>Clostridium perfringens-histolyticum</i> subgroup	<i>Lactobacillus-Enterococcus</i>	
Phase 1 (n = 29 subjects)					
FFMP					
Baseline	5.18 ± 1.13	11.34 ± 2.90	1.05 ± 0.29	0.95 ± 0.28	
End of 7-d treatment	5.04 ± 1.46	11.27 ± 8.75	1.02 ± 0.77	0.98 ± 0.26	-0.16 ± 0.12 <sup>a</sup>
FFMP + 7 g V-GOS					
Baseline	5.00 ± 1.07	12.47 ± 3.06	0.81 ± 0.22	0.75 ± 0.23	0.18 ± 0.08 <sup>b</sup>
End of 7-d treatment	5.98 ± 1.13 <sup>3</sup>	10.36 ± 3.27 <sup>3</sup>	0.82 ± 0.27	0.69 ± 0.18	
Phase 2 (n = 30 subjects)					
FFMP + 7 g sucrose					
Baseline	4.54 ± 1.17	10.45 ± 3.31	1.14 ± 0.21	1.29 ± 0.31	
End of 7-d treatment	4.02 ± 1.76	10.26 ± 2.19	1.19 ± 0.27	1.31 ± 0.47	-0.13 ± 0.19 <sup>a</sup>
FFMP + 3.6 g B-GOS + 3.4 g sucrose					
Baseline	4.07 ± 0.92	10.28 ± 2.81	0.99 ± 0.20	1.17 ± 0.27	
End of 7-d treatment	5.36 ± 1.12 <sup>3</sup>	10.72 ± 2.57	1.20 ± 0.26 <sup>3</sup>	1.13 ± 0.32	0.12 ± 0.09 <sup>b</sup>
FFMP + 7 g B-GOS					
Baseline	4.05 ± 1.06	10.07 ± 2.34	1.13 ± 0.19	1.07 ± 0.21	
End of 7-d treatment	6.74 ± 1.19 <sup>4</sup>	10.53 ± 2.69	1.35 ± 0.28	1.13 ± 0.34	0.40 ± 0.13 <sup>c</sup>

<sup>1</sup> All values are  $\bar{x} \pm SD$ . FFMP, vegetable fat-filled milk powder (35% lactose, 28% vegetable fat, 26% protein, 5.5% minerals, 2% sucrose, 0.25% lecithin, 3% moisture; Cowbell International, Bryanston, South Africa); V-GOS, galactooligosaccharide mixture produced through enzymes from *Bacillus circulans* available commercially as Vivinal GOS; B-GOS, novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum*.

<sup>2</sup> Calculated in accordance with Palfram et al (19) as  $PI = [(Bif_e/Bif_b)/(Total_e/Total_b)] + [(Lab_e/Lab_b)/(Total_e/Total_b)] - [(Bac_e/Bac_b)/(Total_e/Total_b)] - [(Clos_e/Clos_b)/(Total_e/Total_b)]$ , where  $Bif_e$ ,  $Lab_e$ ,  $Bac_e$ ,  $Clos_e$ , and  $Total_e$  are *Bifidobacterium*, *Lactobacillus-Enterococcus*, *Bacteroides-Prevotella*, *Clostridium perfringens-histolyticum* subgroup, and total numbers at the end of the treatment period, respectively;  $Bif_b$ ,  $Lab_b$ ,  $Bac_b$ ,  $Clos_b$ , and  $Total_b$  are *Bifidobacterium*, *Lactobacillus-Enterococcus*, *Bacteroides-Prevotella*, *Clostridium perfringens-histolyticum* subgroup, and total numbers at baseline, respectively. Values within each phase of the study with different superscript letters are significantly different,  $P < 0.05$ . The PI values were analyzed by ANOVA with 3 fixed effects (treatment, period of treatment, and sequence of treatment).

<sup>3,4</sup> Significantly different from beginning of treatment (paired  $t$  test): <sup>3</sup> $P < 0.05$ , <sup>4</sup> $P < 0.001$ .

For the prebiotic index (PI) scores of each treatment for each subject, the following equation was used (19):

$$PI = [(Bif_e/Bif_b)/(Total_e/Total_b)] + [(Lab_e/Lab_b)/(Total_e/Total_b)] - [(Bac_e/Bac_b)/(Total_e/Total_b)] - [(Clos_e/Clos_b)/(Total_e/Total_b)] \quad (1)$$

where  $Bif_e$  is *Bifidobacterium* numbers at the end of the treatment period,  $Bif_b$  is *Bifidobacterium* numbers at baseline,  $Lab_e$  is *Lactobacillus-Enterococcus* numbers at the end of the treatment period,  $Lab_b$  is *Lactobacillus-Enterococcus* numbers at baseline,  $Bac_e$  is *Bacteroides-Prevotella* numbers at the end of the treatment period,  $Bac_b$  is *Bacteroides-Prevotella* numbers at baseline,  $Clos_e$  is *C. perfringens-histolyticum* subgroup numbers at the end of the treatment period,  $Clos_b$  is *C. perfringens-histolyticum* subgroup numbers at baseline,  $Total_e$  is total number of bacteria at the end of the treatment period, and  $Total_b$  is total number of bacteria at baseline.

The PI values were then analyzed by an analysis of variance (ANOVA) model with repeated measurements taking into account the crossover design. In the ANOVA model, treatment, period (day 0 and day 7), and sequence of treatments were introduced as fixed effects and volunteer score as a random effect. Reported  $P$  values are 2 sided, and no statistically significant interaction between sequence and treatment or between period and treatment was found. In vitro fermentation data were analyzed by one-factor ANOVA. All analyses were performed with

the use of SPSS for WINDOWS version 10.0 (SPSS Inc, Chicago, IL). Differences were considered significant at  $P < 0.05$ .

## RESULTS

The typical fecal microflora composition of the healthy human volunteers who participated in either 1 of the 2 phases of the study did not show any significant differences ( $P > 0.10$ ) at the beginning of each phase in terms of *Bifidobacterium*, *Bacteroides-Prevotella*, *C. perfringens-histolyticum* subgroup, and *Lactobacillus-Enterococcus* populations, as well as the total numbers of bacteria (Table 1), as monitored with the use of fluorescent in situ hybridization. During both phases, at day 0 *Bacteroides-Prevotella* and *Bifidobacterium* were numerically predominant among the bacterial populations monitored in the fecal samples, whereas the *C. perfringens-histolyticum* subgroup and *Lactobacillus-Enterococcus* showed lower but similar numbers between them.

### Phase 1

During phase 1 of the study, none of the bacterial numbers of the monitored fecal microflora showed any significant difference (Table 2), compared with the baseline numbers, when vegetable FFMP was used as treatment. A significant increase ( $P < 0.05$ ) was found for changes in fecal bifidobacteria after the 7-d consumption of FFMP supplemented with 7 g V-GOS/d. No changes could be seen for the *C. perfringens-histolyticum* subgroup and the *Lactobacillus-Enterococcus* proportions, whereas the *Bacteroides-Prevotella* group showed a significant decrease ( $P$

< 0.05) after the treatment compared with baseline numbers. The volunteers did not report any adverse symptoms attributable to the consumption of either the FFMP or the V-GOS-supplemented treatment.

## Phase 2

During phase 2 of the study, no significant differences in baseline values were found among the 3 treatments ( $P = 0.29$ ) (Table 2). *Bacteroides-Prevotella* and *Lactobacillus-Enterococcus* did not exhibit any significant variation throughout phase 2 of the study. The placebo treatment (FFMP supplemented with 7 g sucrose) did not show any effect on either the bifidobacterial or the *C. perfringens-histolyticum* subgroup.

Supplementation of FFMP with 3.6 g B-GOS showed a significant increase in the bifidobacteria ( $P < 0.05$ ) and the *C. perfringens-histolyticum* subgroup ( $P < 0.05$ ) compared with baseline values. Supplementation of FFMP with 7 g B-GOS significantly increased the bifidobacteria count ( $P < 0.01$ ), whereas the *C. perfringens-histolyticum* subgroup was not significantly affected ( $P = 0.07$ ) compared with the baseline values.

The volunteers did not report any adverse symptoms when the 3.6-g B-GOS treatment was administered. Two of the 30 volunteers reported abdominal discomfort and diarrhea when the 7-g B-GOS treatment was consumed, but overall both preparations were well tolerated by the volunteers.

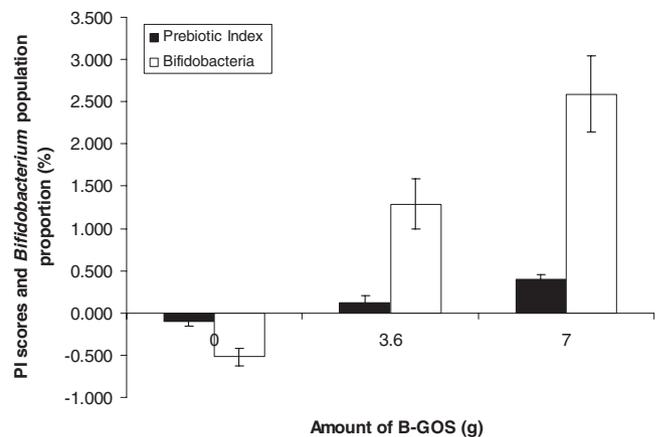
## Prebiotic index

In an attempt to quantify the prebiotic effect of the galactooligosaccharides used in this study, the PI equation was used (19) (Table 2). According to this equation during the 7-d treatment FFMP ( $-0.16 \pm 0.12$ ) and FFMP supplemented with 7 g sucrose ( $-0.13 \pm 0.19$ ) were found not to have any prebiotic properties. FFMP supplemented with either V-GOS (7 g:  $0.18 \pm 0.08$ ) or B-GOS (3.6 g:  $0.12 \pm 0.09$ ; 7 g:  $0.40 \pm 0.13$ ) showed prebiotic properties. The highest PI value found with 7 g B-GOS was significantly different from FFMP supplemented with 3.6 g B-GOS and from FFMP supplemented with 7 g sucrose ( $P < 0.001$ ).

Moreover, the PI value of FFMP supplemented with 7 g sucrose, FFMP supplemented with 3.6 g B-GOS and 3.4 g sucrose, and FFMP supplemented with 7 g B-GOS followed a linear relation ( $r^2 = 0.753$ ). This seems to be based on the effect of the B-GOS dose on changes of the bifidobacterial proportion in the fecal microflora, which seemed to follow a similar relation ( $r^2 = 0.796$ ) (Figure 2).

## Fermentation studies

Thirty-four of the initial 132 fecal isolates, identified by microscopic structure and the API 20A identification system as *Bifidobacterium* or *Lactobacillus* species were further characterized by partial 16S rRNA gene sequencing. Twenty-two were identified as belonging to the *Bifidobacterium* genus, with 7 isolates being identified as *B. bifidum*, 5 as *Bifidobacterium adolescentis*, 4 as *Bifidobacterium longum*, 3 as *Bifidobacterium animalis (lactis)*, and 3 as *Bifidobacterium infantis*. Of the 12 lactobacilli isolates, 5 were identified as *Lactobacillus casei*, 3 as *Lactobacillus rhamnosus*, 2 as *Lactobacillus acidophilus*, and 2 as *Lactobacillus reuteri*. From the bifidobacteria isolates those identified as *B. bifidum* ( $P < 0.001$ ), *B. infantis* ( $P < 0.05$ ), *B. longum* ( $P < 0.05$ ), and *B. animalis* ( $P < 0.01$ ) were found to



**FIGURE 2.** Dose-response effects of the supplementation with a novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum* (B-GOS) on the prebiotic index (PI) score ( $r^2 = 0.753$ ) and the proportion of the *Bifidobacterium* population ( $r^2 = 0.796$ ) after 7 d of administration to healthy human volunteers ( $n = 30$ ).

have significantly higher growth rates in the presence of B-GOS than were those with glucose as the carbon source, whereas *B. bifidum* ( $P < 0.01$ ) and *B. longum* ( $P < 0.05$ ) showed significantly higher growth rates in the presence of B-GOS than in the presence of V-GOS (Table 3). Among the lactobacilli isolates only *L. reuteri* ( $P < 0.05$ ) and *L. acidophilus* ( $P < 0.05$ ) showed increased growth rates in the presence of V-GOS than with glucose as the carbon source (Table 3).

## DISCUSSION

The aim of this double-blind, randomized, placebo-controlled, crossover study was to determine the bifidogenic effect of a novel galactooligosaccharide mixture produced from lactose with the use of galactosidases located on *B. bifidum* NCIMB 41171 cells. Because the choice of food matrix was reported to affect the fermentation of prebiotic products (4) and thereby influence outcome, the study initially evaluated the suitability of vegetable FFMP as a matrix. To achieve this, major bacterial groups of the fecal microflora of healthy human volunteers were monitored after two 1-wk periods during which the volunteers consumed either FFMP (matrix) or the FFMP supplemented with a known galactooligosaccharide prebiotic mixture (V-GOS; 7 g) as positive control. The choice of a relatively short administration period (7 d) was because in previous studies with similar oligosaccharide products (20–22), this treatment period was sufficient to display a significant effect on fecal bifidobacterial counts. Each treatment period was followed by a 1-wk washout period to avoid the effect of one treatment being carried over to the next and was shown to be long enough as determined from the ratio values of the monitored bacterial groups at the baseline of each treatment during each phase of the study (Table 2).

The FFMP treatment did not show any significant effect on any of the bacterial groups assessed during the study, whereas supplementation with 7 g V-GOS significantly increased the ratio of bifidobacterial population ( $P < 0.05$ ) and significantly decreased the ratio of the *Bacteroides-Prevotella* group ( $P < 0.05$ ). Because similar results for this galactooligosaccharide mixture were previously reported (4, 23), we conclude that the vegetable FFMP matrix not only did not have any bifidogenic



TABLE 3

Growth rates of the intestinal *Bifidobacterium* and *Lactobacillus* isolates from fecal samples of healthy human volunteers in batch cultures in the presence of 1% (wt:wt) glucose, novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum* (B-GOS), or galactooligosaccharide mixture produced through enzymes from *Bacillus circulans* available commercially as Vivinal GOS (V-GOS) as carbon source<sup>1</sup>

Species	Sequence similarity <sup>2</sup>	Isolates	Growth rate		
			Glucose	V-GOS	B-GOS
	%	no.		$\mu/h$	
<i>Bifidobacterium adolescentis</i>	97	5	0.32 ± 0.03 <sup>3</sup>	0.38 ± 0.03	0.37 ± 0.03
<i>Bifidobacterium bifidum</i>	99	7	0.59 ± 0.04 <sup>a</sup>	0.63 ± 0.05 <sup>a</sup>	0.89 ± 0.06 <sup>b</sup>
<i>Bifidobacterium infantis</i>	98	3	0.41 ± 0.03 <sup>a</sup>	0.45 ± 0.05 <sup>a,b</sup>	0.51 ± 0.04 <sup>b</sup>
<i>Bifidobacterium longum</i>	98	4	0.36 ± 0.04 <sup>a</sup>	0.42 ± 0.04 <sup>a</sup>	0.55 ± 0.05 <sup>b</sup>
<i>Bifidobacterium animalis</i> (lactis)	97	3	0.38 ± 0.02 <sup>a</sup>	0.54 ± 0.04 <sup>b</sup>	0.62 ± 0.04 <sup>b</sup>
<i>Lactobacillus reuteri</i>	100	2	0.42 ± 0.05 <sup>a</sup>	0.53 ± 0.04 <sup>b</sup>	0.51 ± 0.05 <sup>a,b</sup>
<i>Lactobacillus acidophilus</i>	97	2	0.42 ± 0.04 <sup>a</sup>	0.67 ± 0.05 <sup>b</sup>	0.48 ± 0.04 <sup>a</sup>
<i>Lactobacillus casei</i>	99	5	0.37 ± 0.03	0.43 ± 0.05	0.41 ± 0.04
<i>Lactobacillus rhamnosus</i>	100	3	0.35 ± 0.04	0.35 ± 0.05	0.38 ± 0.03

<sup>1</sup> Values (of 3 replicates) within the same row with different superscript letters are significantly different,  $P < 0.05$  (one-factor ANOVA).

<sup>2</sup> The sequences were submitted as BLAST searches at the EMBL website (<http://www.ebi.ac.uk>), and anything  $>97\%$  recognition was accepted as identity.

<sup>3</sup>  $\bar{x} \pm SD$  (all such values).

properties, but neither did it mask any bifidogenic properties of the test ingredient and therefore was suitable for use in phase 2 of the study.

During phase 2 of the study sucrose was used as a placebo and as a supplementation for the low-dose treatment to maintain the osmotic characteristics of the preparations at similar values and because of its lack of prebiotic properties. No significant effect was observed on the *Bacteroides-Prevotella* and *Lactobacillus-Enterococcus* group proportions during any of the treatment periods. The addition of sucrose to FFMP did not show any significant effect on either the proportion of *Bifidobacterium* or the *C. perfringens-histolyticum* subgroup, suggesting that the sucrose-FFMP preparation had the same effect as the FFMP preparation on the bacterial groups of the colonic microflora monitored. The addition of the novel galactooligosaccharide mixture showed a significant increase in the proportion of the *Bifidobacterium* group between the baseline and the end of the treatment period for both the low-dose B-GOS ( $P < 0.05$ ) and the high-dose B-GOS ( $P < 0.001$ ). Moreover, the effect on this bacterial group during the 3 treatments (sucrose, low-dose B-GOS, and high-dose B-GOS) followed a linear relation ( $r^2 = 0.796$ ), indicating a clear dose-response effect (Figure 2).

A significant increase ( $P < 0.05$ ) in the *C. perfringens-histolyticum* subgroup could be seen after ingestion of the low-dose B-GOS preparation. This increase did not affect the bacterial ratio at the baseline of each treatment, and, although displayed, it was not significant ( $P = 0.07$ ) during the ingestion of the high-dose preparation. Overall, the novel galactooligosaccharide mixture was well tolerated during both the low- and high-dose treatments. Two of the 30 volunteers reported abdominal discomfort and diarrhea when the high-dose preparation was ingested, but this could have been the result of the osmotic pressure of unfermented oligosaccharides. Of interest, both of the volunteers were among the ones with the lower initial bifidobacterial counts (data not shown).

To quantify the overall prebiotic effect of the treatments used in this study, the PI described by Palfram et al (19) was applied. This equation, based on the changes in the proportion of the 4

bacterial groups monitored in this study, provides a quantitative score for the prebiotic effect of the test ingredient. Although the PI is based on only 4 bacterial groups, the significant changes in the monitored bacterial groups during this study were mainly limited to the *Bifidobacterium* group and suggest that the PI score could offer a representative prebiotic evaluation of the tested ingredients. The PI scores during the first phase of the study confirmed the prebiotic properties of V-GOS ( $0.18 \pm 0.08$ ) compared with the PI score of the placebo ( $-0.16 \pm 0.12$ ;  $P < 0.05$ ), with part of the prebiotic properties attributed to a significant reduction in the *Bacteroides-Prevotella* group proportion (Table 2). During the second phase of the study the PI scores of the 2 treatments containing the novel galactooligosaccharide mixture were significantly higher than the placebo ( $-0.13 \pm 0.19$ ), with the 3.6-g dose scoring  $0.12 \pm 0.09$  ( $P < 0.05$ ) and the 7-g dose scoring  $0.40 \pm 0.13$  ( $P < 0.001$ ). Moreover, the PI score of the 7-g B-GOS treatment was significantly higher than the PI score of the 3.6-g B-GOS treatment ( $P < 0.05$ ). The PI scores of the B-GOS treatments followed a linear relation attributed only to the increase in the *Bifidobacterium* group (Figure 2). This finding allows us to assume that our hypothesis of developing more selective prebiotics with bifidobacterial enzymes is valid.

To further evaluate the hypothesis and because we could not monitor the microflora changes during the study at species values in vivo, we isolated and identified 22 *Bifidobacterium* and 12 *Lactobacillus* strains from a healthy human volunteer and studied in vitro their ability to grow in the presence of the novel GOS mixture (B-GOS) compared with glucose and the GOS mixture (V-GOS) used in the in vivo study. The isolated *Bifidobacterium* species were *B. bifidum*, *B. adolescentis*, *B. longum*, *B. animalis*, and *B. infantis*, which are in accordance with previously detected *Bifidobacterium* species in fecal samples of European adults (24, 25), and the isolated lactobacilli were *L. reuteri*, *L. casei*, *L. acidophilus*, and *L. rhamnosus*. The addition of B-GOS (1% wt:wt) showed, with the exception of *B. adolescentis*, a significant increase in the growth rate of the isolated bifidobacterial strains compared with glucose ( $P < 0.05$ ) and a significant increase for *B. bifidum* and *B. longum* compared with V-GOS ( $P$



< 0.05). Although in vitro data cannot be readily generalized as being valid for the in vivo situation, *B. bifidum* is the species to which the bifidobacterial strain used for the production of B-GOS belongs, and galactosidases isolated from *B. longum* have shown high (75%) similarities of amino acid sequence to the galactosidases isolated from *B. bifidum* (National Centre for Biotechnology Information Database).

In conclusion, the novel galactooligosaccharide mixture, as a supplement to the Western diet, exerted a prebiotic and more specifically bifidogenic effect in a dose-response relation in healthy human volunteers at doses of 3.6 and 7 g/d. Although the 3.6-g dose showed a significant increase in the *C. perfringens-histolyticum* subgroup, the 7-g/d dose seems to be more preferable because the effect on the *C. perfringens-histolyticum* subgroup was eliminated and a higher bifidogenic effect was noted. Moreover, the effect of a galactooligosaccharide mixture produced with enzymes from *B. bifidum* showed significantly higher prebiotic and bifidogenic effects than did a galactooligosaccharide mixture produced with enzymes from *B. circulans*. This indicates that manufacturing of prebiotic oligosaccharides with higher selectivity toward specific bacterial groups is possible.

The author's responsibilities were as follows—GRG: study design; KI'A: sample preparation; FD: data collection; GT and JV: data analysis; GT: review of data; KI'A and GT: test substance preparation; GT, GRG, FD, and JV: writing of the manuscript. None of the authors had a personal or financial conflict of interest.

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