MAJOR ARTICLE

Microbiological Effects of Consuming a Synbiotic Containing *Bifidobacterium bifidum*, *Bifidobacterium lactis*, and Oligofructose in Elderly Persons, Determined by Real-Time Polymerase Chain Reaction and Counting of Viable Bacteria

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Background. Because of changes in gut physiology, immune system reactivity, and diet, elderly people are more susceptible to gastrointestinal infections than are younger adults. The gut microflora, which provides a natural defense against invading microorganisms, changes in elderly people with the development of potentially damaging bacterial populations, which may lead to alterations in bacterial metabolism and higher levels of infection.

Methods. A randomized, double-blind, controlled feeding trial was done with 18 healthy elderly volunteers (age, >62 years) using a synbiotic comprising *Bifidobacterium bifidum* BB-02 and *Bifidobacterium lactis* BL-01 (probiotics) together with an inulin-based prebiotic (Synergy 1; Orafti). Real-time PCR was employed to quantitate total bifidobacteria, *B. bifidum*, and *B. lactis* in fecal DNA before, during, and after synbiotic consumption. Counting all viable anaerobes, bifidobacteria, and lactobacilli and identification of bacterial isolates to species level was also done.

Results. Throughout feeding, both bifidobacteria species were detected in fecal samples obtained from all subjects receiving the synbiotic, with significant increases in the number of copies of the 16S rRNA genes of *B. bifidum, B. lactis,* and total bifidobacteria, compared with the control week and the placebo group. At least 1 of these species remained detectable in fecal samples 3 weeks after feeding in individuals that had no fecal *B. bifidum* and/or *B. lactis* in the control week, indicating that the probiotics persisted in the volunteers. Counting of viable organisms showed significantly higher total numbers of fecal bifidobacteria, total numbers of lactobacilli, and numbers of *B. bifidum* during synbiotic feeding.

Conclusion. Synbiotic consumption increased the size and diversity of protective fecal bifidobacterial populations, which are often very much reduced in older people.

The human colonic microbiota is affected by host, microbiological, dietary, and environmental factors and can vary greatly in composition between individuals. The microbiota is generally viewed as being stable during adult life [1, 2], during which it plays an important role in host physiology and metabolism [3], and it provides a natural defense against invading pathogens [4]. However, gut function changes with age, and this is often accompanied by an increased incidence of gastrointestinal infection [5]. This may result from changes in host physiology, immune system reactivity, and diet, but it also may be caused by shifts in the bacterial population in the large bowel. Some studies have indicated that bifidobacterial levels decrease in older people, in whom there are increased levels of clostridia and enterobacteria [6–11].

Although there is debate concerning the benefits of probiotics for adults with normal, healthy gut ecosystems, use of probiotics may prevent traveler's diarrhea, antibiotic-associated diarrhea, and acute diarrhea or improve recovery from disease [12]. Lactobacilli and bifidobacteria are the most frequently used probiotics

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in humans, and there is evidence that some of these organisms can increase resistance to gut infections by inhibiting pathogens [13] and improving host immunity [14]. Inulin-type fructans are commonly used prebiotics, which have been defined as nondigestible dietary components that selectively stimulate the growth and/or activities of bacteria in the large bowel [15]. A number of investigations have shown that oligofructose promotes the growth of bifidobacteria [16, 17]; therefore, consumption of a probiotic in combination with a suitable prebiotic (synbiotic) can result in synergistic effects, improving survival of the probiotic by providing a readily available substrate for fermentation and by increasing autochthonous bifidobacteria levels.

Therefore, probiotics and prebiotics offer attractive strategies to reduce unfavorable changes in the aging gut and to maintain a more "healthy" intestinal microbiota, to help maintain bowel function and to reduce susceptibility to infection in elderly persons. The aim of this double-blind, randomized, controlled feeding trial was to study the effects of ingestion of a synbiotic containing 2 bifidobacterial species (*Bifidobacterium bifidum* BB-02 and *Bifidobacterium lactis* BL-01) and oligofructose on the composition of intestinal bifidobacteria and *Lactobacillus* populations in older people.

METHODS

Subjects. A total of 18 healthy female volunteers aged >62 years with no recent history of gastrointestinal disease participated in the study. All volunteers were living in the Tayside area of East Scotland. No antibiotics or laxatives were taken 2 months before or during the study, and volunteers maintained their usual diet. All volunteers gave written informed consent to the protocol, which was approved by the Tayside Committee on Medical Research Ethics. The volunteers were randomized into 2 groups by sealed envelope, and neither volunteers nor investigators knew the code during the investigation. The mean age of the volunteers in the placebo group was 71 years (range, 63–85 years), and the mean age of volunteers in the synbiotic group was 73 years (range, 68–90 years).

Experimental design of the feeding trial. The study lasted for 8 weeks and was divided into 3 consecutive phases: a prefeeding period (1 week), followed by the feeding period (4 weeks) and a postfeeding period (3 weeks). During the feeding phase, the synbiotic group received supplements which consisted of 6 g of Raftilose Synergy1 (Orafti) and a gelatin capsule containing 100 mg of a freeze-dried probiotic containing ~3.5×10^{10} cfu each of *B. bifidum* strain BB-02 and *B. lactis* BL-01 (Rhodia). Raftilose Synergy1 is a combination of chicory inulin enriched by a fraction of chicory oligofructose produced by partial enzymic hydrolysis of chicory inulin. The placebo group received 6 g of maltooligosaccharides, which are completely digested and absorbed in the small bowel, together with a gelatin capsule containing potato flour. The oligofructose or maltooligosaccharides together with the capsules were taken with a cold drink twice per day after meals, to reduce killing of the probiotic by gastric acid. We analyzed fecal samples from week 1 (i.e., the prefeeding period), weeks 3–5 (i.e., the feeding period), weeks 6 and 8 (i.e., the postfeeding period). All fecal samples were received as planned, except for week 5, when a sample was not received from 1 person in the placebo group. All fecal specimens were processed within 60 min. Viable cell counts were done on material received in week 1 (the prefeeding period), week 4 (the feeding period), and week 8 (the postfeeding period).

Isolation and enumeration of fecal bacteria. Specimens of fresh feces were homogenized (10% wt/vol) in 0.1 mol/L sodium phosphate buffer (pH, 6.5) containing 80 mg/L pectin (citrus), 150 mg/L starch (soluble), 80 mg/L xylan (oatspelt), and 80 mg/L mucin (partially purified porcine gastric mucin), before being serially diluted 10-fold in half-strength anaerobic peptone water. Samples were spread onto selective agar plates and incubated in an anaerobic chamber (H_2, 10%; CO_2, 10%; N_2, 80%) for 48 h at 37°C. Total anaerobe counts were done using Wilkins-Chalgren agar, and bifidobacteria and lactobacilli levels were enumerated using Beerens medium [18] and Rogosa agar, respectively.

Identification of bacterial isolates by analysis of cellular fatty acid methyl esters. For identification of bifidobacteria and lactobacilli, the predominant colony types were identified and subcultured on Wilkins-Chalgren agar. All isolates were identified to species level on the basis of their cellular fatty acid profiles using the Microbial Identification System (MIDI). Fatty acid methyl esters were extracted from bacterial cell mass obtained from a 30-mL overnight culture grown anaerobically in peptone–yeast extract–glucose broth according to the manufacturer's instructions. Methylated fatty acids were identified by gas chromatography as described elsewhere [8], and bacterial identification was performed by comparison of the cellular fatty acid profile with the standard MIDI library for anaerobes, Moore version 3.9 (available at http://www.midi-inc.com/pages/databases.html).

DNA extraction from fecal specimens. DNA was extracted from 2 mL of fecal slurry (10% wt/vol) using the Quiagen Stool Kit (Quiagen) with a modified protocol for cell lysis. Cells were collected by centrifugation (20,000 *g* for 10 min) and were resuspended in 1.4 mL of stool lysis buffer. Three hundred fifty mg of 0.1-mm glass beads (BioSpec) were added, and the cells were mechanically disrupted using a Mini-Beadbeater (BioSpec) for 2 min at maximum speed. The suspension was subsequently heated for 5 min at 95°C, and the bead-beating step was repeated for 2-min. Cell debris was removed by centrifugation at 5000 *g* for 2 min. After lysis, DNA-damaging substances and PCR inhibitors were removed using InhibitEX tab-

lets (Quiagen). The protein digests and DNA purification were done using QIAamp spin columns, in accordance with the manufacturer's instructions.

Real-time PCR. Real-time PCR was performed using an iCycler iQ apparatus (Bio-Rad) with iCycler Optical System Interface software, version 2.3 (Bio-Rad). Each reaction was done in duplicate in a volume of 20 μL, using 96-well optical grade PCR plates (Bio-Rad). Amplification reactions were done using the iQ SYBR Green Supermix (Bio-Rad) containing 3 mmol/L $MgCl_2$, 20 mmol/L Tris HCl (pH, 8.4), 50 mmol/L KCl, 200 μmol/L each deoxynucleoside triphosphate, SYBR Green I, 10 nmol/L fluorescein, 0.625 U iTaq DNA polymerase mixed with the selected primers set (table 1) at a concentration of 0.5 μmol/L for each primer, and 1.6 μL of the respective template DNA or water. Amplifications were done with the following temperature profiles: 1 cycle at 95°C for 3 min, 35 cycles of denaturation step at 95°C for 30 s, primer annealing at the optimal temperature for 30 s (optimal temperatures for each set of primers are shown in table 1), and 1 final cycle at 95°C for 30 s. For the Bflact2/Bflact5 primer set, an additional elongation step at 72°C for 10 s was done after primer annealing. Finally, melt-curve analysis was done by slow heating of the PCR reactions from 55°C to 95°C (1°C per cycle of 10 s) with simultaneous measurement of SYBR Green I signal intensity. Quantitation was done using standard curves made from known concentrations of plasmid DNA containing the respective amplicon for each set of primers.

Chemicals. The probiotic strains were a gift from Cultech. Synergy 1 and maltooligosaccharides were gifts from Orafti. Unless stated otherwise, all chemicals were purchased from Sigma Chemical. Bacteriological culture media were obtained from Oxoid.

Statistical analyses. Logarithms of bacterial counts and rRNA gene copy values were used to achieve normal distribution, and mean values ($\pm$SDs) were calculated. Because the profile of fecal bacterial species can vary greatly between the individuals, the mean values and SDs presented in the tables were calculated only for the individuals who had the particular species present in their stool samples. However, for statistical tests, negative results were included as the value 0 to consider changes in cell numbers and prevalence of the bacterial species in a volunteer group. SPSS software, version 10.0.7, was employed; independent Student's *t* test was used to compare dietary groups, and the Bonferroni test was used for comparisons of the control week with the feeding and postfeeding periods. Differences were considered to be statistically significant at $P < .05$.

RESULTS

Counts of total viable anaerobes and bifidobacteria and lactobacilli in feces. Total anaerobe counts in individual stool samples ranged between 9.0 and 11.0 $\log_{10}$ cfu/g (wet weight). Mean bacterial counts in the synbiotic and placebo groups (table 2) were similar—approximately 10.0 $\log_{10}$ cfu/g (wet weight)—and remained unchanged in the different dietary periods. *Lactobacillus* counts ranged from 3.0 to 9.2 $\log_{10}$ cfu/g (wet weight). Mean total numbers were higher during synbiotic feeding compared to the placebo (table 3). The most frequently isolated species were *Lactobacillus paracasei, Lactobacillus sharpeae,* and *Lactobacillus salivarius.* However, *Lactobacillus* carriage varied markedly between the volunteers, as well as within the different dietary periods for a given individual.

Bifidobacteria were isolated from all stool samples, with counts ranging from 5.7 to 10.3 $\log_{10}$ cfu/g (wet weight). *Bifidobacterium adolescentis, Bifidobacterium angulatum,* and *Bi-*

Table 1. Primer sets used for real-time PCR quantitation of fecal bifidobacteria in elderly volunteers.

Target organism, primer set	Primer sequence	Product size, bp	Annealing temperature, °C	Reference
Bifidobacterium bifidum				
BiBIF-1F	5′-CCA CAT GAT CGC ATG TGA TTG-3′	278	62	[19]
BiBIF-2R	5′-CCG AAG GCT TGC TCC CAA A-3′	…	…	…
Bifidobacterium lactis				
BlactF	5′-CCC TTT CCA CGG GTC CC-3′	194	65	[20]
BlactR	5′-AAG GGA AAC CGT GTC TCC AC-3′	…	…	…
Bifidobacterium lactis				
Bflact2	5′-GTG GAG ACA CGG TTT CCC-3′	680	65	[21]
Bflact5	5′-CAC ACC ACA CAA TCC AAT AC-3′	…	…	…
Bifidobacterium genus				
Bif164F	5′-GGG TGG TAA TGC CGG ATG-3′	457	59	[22]
Bif601R	5′-TAA GCC ATG GAC TTT CAC ACC-3′	…	…	[23][a]

[a] Modified.

Table 2. Counts of viable fecal anaerobes and bifidobacteria in elderly volunteers during synbiotic or placebo feeding.

	Synbiotic group (n = 9)						Placebo group (n = 9)					
	Prefeeding period		Feeding period		Postfeeding period		Prefeeding period		Feeding period		Postfeeding period	
Organism(s)	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]
All anaerobes	9	10.3 ± 0.5	9	10.1 ± 0.3	9	10.0 ± 0.2	9	10.3 ± 0.5	9	9.9 ± 0.4	9	10.0 ± 0.8
Bifidobacterium adolescentis	4	8.9 ± 0.4	6	9.0 ± 0.9	5	9.0 ± 0.8	4	8.5 ± 0.8	6	8.3 ± 0.6	6	8.3 ± 0.6
Bifidobacterium angulatum	5	8.8 ± 0.6	7	9.0 ± 0.8	7	8.2 ± 0.6[b]	5	8.3 ± 1.2	5	7.7 ± 0.9	1	7.5
Bifidobacterium bifidum	3	8.4 ± 1.3	5	8.8 ± 0.6[b]	6	8.7 ± 0.7[b]	ND	…	ND	…	ND	…
Bifidobacterium boum	ND	…	ND	…	ND	…	1	5.7	1	7.9	ND	…
Bifidobacterium breve	1	6.6	ND	…	1	7.8	1	8.8	ND	…	1	7.3
Bifidobacterium catenulatum	ND	…	ND	…	1	8.4	ND	…	ND	…	ND	…
Bifidobacterium dentium	4	9.0 ± 0.4	5	8.1 ± 1.2	3	7.7 ± 0.7	2	7.4 ± 0.1	4	7.3 ± 1.0	5	7.5 ± 0.7
Bifidobacterium lactis	1	8.5	1	8.0	1	7.7	1	6.3	2	7.3 ± 1.3	1	6.7
Bifidobacterium longum	2	9.0 ± 0.8	1	9.6	3	8.4 ± 1.2	1	8.4	ND	…	3	7.6 ± 0.8
Bifidobacterium pullorum	ND	…	1	9.9	1	7.6	ND	…	ND	…	1	8.1
All bifidobacteria	9	9.3 ± 0.4	9	9.4 ± 0.8[b]	9	9.0 ± 0.7[b]	9	8.6 ± 1.0	9	8.3 ± 0.8	9	8.1± 0.8

NOTE. The prefeeding period was week 1, the feeding period was week 4, and the postfeeding period was week 8. ND, not detected.

[a] Results are expressed as mean $\log_{10}$ cfu/g (wet weight) of feces ± SD.

[b] Indicates a statistically significant difference ($P < .05$), compared with the placebo group.

Table 3. Counts of viable fecal lactobacilli in elderly volunteers during synbiotic and placebo feeding.

Lactobacillus species	Synbiotic group (n = 9)						Placebo group (n = 9)					
	Prefeeding period		Feeding period		Postfeeding period		Prefeeding period		Feeding period		Postfeeding period	
	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]	No. of subjects with organism present	Organism count, mean ± SD[a]
L. acidophilus	1	4.7	ND	…	ND	…	1	6.7	1	4.5	1	3.8
L. alimentarius	ND	…	ND	…	ND	…	1	5.0	ND	…	ND	…
L. casei	1	5.5	1	3.6	ND	…	1	5.0	ND	…	1	4.6
L. cateneforme	1	8.0	1	9.1	1	7.9	ND	…	ND	…	ND	…
L. coryneformis subsp. *coryneformis*	ND	…	ND	…	ND	…	ND	…	2	4.7 ± 1.6	ND	…
L. delbruekii subsp. *lactis*	1	7.0	1	7.1	ND	…	ND	…	1	5.0	2/9	4.9 ± 0.1
L. fermentum	ND	…	ND	…	ND	…	ND	…	ND	…	1	5.2
L. gasseri	ND	…	2	7.6 ± 1.3	1	6.9	ND	…	ND	…	ND	…
L. kefir	ND	…	ND	…	ND	…	ND	…	1	5.3	ND	…
L. mali	ND	…	1	5.5	1	5.3	1	6.5	1	4.3	1	5.4
L. oris	2	5.7 ± 1.3	1	8.7	ND	…	ND	…	1	8.0	ND	…
L. paracasei subsp. *paracasei*	2	5.5 ± 0.8	ND	…	2	5.6 ± 1.3	4	5.6 ± 0.7	2	4.7 ± 0.3	1	5.0
L. plantarum	1	5.7	ND	…	ND	…	ND	…	ND	…	1	3.6
L. reuteri	ND	…	ND	…	ND	…	1	8.7	1	5.3	1	5.5
L. rhamnosus	ND	…	ND	…	1	5.2	ND	…	1	4.3	ND	…
L. rimae	ND	…	ND	…	ND	…	ND	…	ND	…	1	8.3
L. rogosae	ND	…	ND	…	1	8.0	ND	…	ND	…	ND	…
L. salivarius subspecies *salicinius*	ND	…	3	6.1 ± 0.9	2	3.8 ± 1.1	ND	…	1	5.3	ND	…
L. sharpeae	1	6.8	3	5.8 ± 1.7	1	7.3	1	4.5	2	4.2 ± 0.1	1	4.8
Lactobacillus species D10	ND	…	ND	…	2	8.2 ± 0.1	ND	…	ND	…	2	7.1 ± 0.3
All lactobacilli	8	6.1 ± 1.1	9	6.8 ± 1.7[b]	9	6.6 ± 1.8	8	6.0 ± 1.4	8	5.4 ± 1.2	8	5.9 ± 1.5
L. lactis	ND	…	1	6.5	ND	…	ND	…	ND	…	2	5.8 ± 0.7

NOTE. The prefeeding period was week 1, the feeding period was week 4, and the postfeeding period was week 8. ND, not detected.

[a] Results are expressed as mean $\log_{10}$ cfu/g (wet weight) of feces ± SD.

[b] Indicates a statistically significant difference ($P < .05$), compared with the placebo group.

Table 4. Results of real-time PCR quantitation of rRNA genes of all bifidobacteria using primer sets Bif164 and Bif601.

Subject group, subject, statistic	Prefeeding period: week 1	Feeding period			Postfeeding period	
		Week 3	Week 4	Week 5	Week 6	Week 8
Placebo group						
P1	10.4	10.9	10.8	10.4	11.0	10.6
P2	9.9	9.6	10.5	10.8	9.5	9.2
P3	8.7	8.2	8.1	8.4	8.8	8.0
P4	7.6	8.9	11.0	9.1	8.5	9.1
P5	10.5	10.3	10.0	9.8	9.9	10.3
P6	7.8	9.6	7.9	8.4	6.1	8.2
P7	9.7	10.7	9.8	9.5	9.5	9.2
P8	7.9	10.4	8.6	8.2	8.1	9.0
P9	9.5	9.7	9.5	NS	9.3	9.5
Mean ± SD	9.1 ± 1.1	9.8 ± 0.9	9.6 ± 1.2	9.3 ± 1.0	9.0 ± 1.4	9.3 ± 0.8
Prevalence[a]	9/9	9/9	9/9	8/8	9/9	9/9
P for week 1 vs. other weeks[b]	NA	1.000	1.000	1.000	1.000	1.000
Synbiotic group						
S1	10.1	10.6	10.9	10.5	10.3	10.5
S2	9.7	10.4	11.6	10.0	10.5	9.9
S3	9.2	10.9	10.5	10.5	10.9	10.1
S4	10.1	10.7	9.7	9.8	9.5	10.6
S5	8.1	9.8	9.9	8.8	9.6	8.8
S6	10.7	9.8	10.9	10.9	10.0	10.5
S7	10.2	10.3	10.8	11.0	10.6	10.6
S8	10.7	10.1	11.2	11.1	10.5	10.8
S9	10.5	10.7	11.0	10.8	10.2	10.5
Mean ± SD	9.9 ± 0.8	10.4 ± 0.4	10.7 ± 0.6	10.4 ± 0.7	10.2 ± 0.5	10.2 ± 0.6
Prevalence[a]	9/9	9/9	9/9	9/9	9/9	9/9
P						
Week 1 vs. other weeks	NA	1.000	.144	1.000	1.000	1.000
Between dietary groups[c]	.106	.089	.019	.024	.017	.011

NOTE. Data are $\log_{10}$ 16S rRNA gene copies/g (wet weight), unless otherwise indicated. NA, not applicable; NS, not studied.

[a] No. of subjects with specific 16S rRNA genes present/no. of subjects tested.

[b] The Bonferroni test was used for pair-wise multiple comparison of the mean values for the control week and for the other dietary weeks.

[c] The independent Student's *t* test was used to compare the mean values between the dietary groups.

fidobacterium dentium predominated (table 2); *Bifidobacterium breve, Bifidobacterium longum,* and *Bifidobacterium pullorum* were occasionally found; and *Bifidobacterium boum* and *Bifidobacterium catenulatum* were detected in only a few stool samples. Total bifidobacterial counts were significantly higher during synbiotic feeding, compared to placebo feeding, and they remained this way after feeding. An increase of ⩾1 order of magnitude in the count of *B. angulatum* or *B. adolescentis* during feeding was observed almost twice as often in individuals taking the synbiotic, compared with placebo recipients (data not shown). However, significant differences between the synbiotic and the placebo groups were only found with *B. angulatum* counts in the postfeeding period, as a result of the fact that this organism was detected in only 1 placebo recipient but in 7 synbiotic recipients. In the synbiotic group, *B. bifidum* was detected in 3 of 9 volunteers before feeding, and the rate increased to 5 of 9 during the feeding period and to 6 of 9 during the postfeeding period. *B. bifidum* was not isolated from placebo recipients at any time. Mean counts of this bacterium were significantly higher in the synbiotic group during the feeding and postfeeding periods than in the placebo recipients. *B. lactis* was detected infrequently, and no diet-induced changes were observed.

Quantitation of fecal bifidobacteria levels using real-time PCR. With use of primers specific for the genus *Bifidobacterium,* significantly higher copy numbers of target DNA were found in the synbiotic group during the feeding period (weeks 4 and 5) and during the postfeeding period (weeks 6 and 8) (table 4). Bifidobacterial 16S rRNA gene copies were detected in each stool sample using real-time PCR. When specific primers were used to target *B. bifidum* (table 5) and *B. lactis* (table 6), both species were found in all stool samples obtained from

Table 5. Results of real-time PCR quantitation of rRNA genes of *Bifidobacterium bifidum* using primer sets BiBIF-1 and BiBIF-2.

Subject group, subject, statistic	Prefeeding period: week 1	Feeding period			Postfeeding period	
		Week 3	Week 4	Week 5	Week 6	Week 8
Placebo group						
P1	ND	ND	ND	ND	ND	ND
P2	ND	ND	ND	ND	ND	ND
P3	ND	ND	ND	ND	ND	ND
P4	ND	ND	ND	ND	ND	ND
P5	8.3	9.9	8.9	9.1	9.5	8.4
P6	ND	ND	ND	ND	ND	ND
P7	ND	9.4	ND	ND	ND	ND
P8	ND	8.9	ND	ND	ND	ND
P9	ND	ND	ND	NS	ND	ND
Mean ± SD	8.3	9.4 ± 0.5	8.9	9.1	9.5	8.4
Prevalence[a]	1/9	3/9	1/9	1/8	1/9	1/9
P for week 1 vs. other weeks[b]	NA	1.000	1.000	1.000	1.000	1.000
Synbiotic group						
S1	10.1	10.1	10.4	10.0	9.5	10.1
S2	8.3	9.9	10.0	9.3	9.4	8.7
S3	ND	7.9	7.5	8.1	9.7	ND
S4	6.5	9.8	8.5	8.0	7.7	6.6
S5	ND	8.6	8.9	7.6	7.7	6.5
S6	10.2	9.4	10.7	10.6	9.9	9.8
S7	ND	9.2	10.1	10.1	10.1	9.7
S8	ND	8.7	10.0	9.8	9.9	9.8
S9	9.3	10.0	10.3	10.2	9.7	7.7
Mean ± SD	8.9 ± 1.5	9.3 ± 0.8	9.6 ± 1.1	9.3 ± 1.1	9.3 ± 0.9	8.6 ± 1.5
Prevalence[a]	5/9	9/9	9/9	9/9	9/9	8/9
P						
Week 1 vs. other weeks	NA	.008	.003	.007	.008	.369
Between dietary groups[c]	.045	.001	.000	.000	.000	.000

NOTE. Data are $\log_{10}$ 16S rRNA gene copies/g (wet weight), unless otherwise indicated. NA, not applicable; ND, not detected; NS, not studied.

[a] No. of subjects with specific 16S rRNA genes present/no. of subjects tested.

[b] The Bonferroni test was used for pair-wise multiple comparison of the mean values for the control week and for the other dietary weeks.

[c] The independent Student's *t* test was used to compare the mean values between the dietary groups.

synbiotic recipients during the feeding period. During the control week, *B. bifidum* was detected in 5 of 9 synbiotic recipients, compared with only 1 of 9 placebo recipients. However, a significant increase in the prevalence and numbers of this species during synbiotic feeding was observed. Three weeks after the feeding period had stopped, *B. bifidum* rRNA genes were still detectable in fecal samples obtained from 3 volunteers (subjects S5, S7, and S8) who did not harbor this species in their indigenous fecal population.

A significant increase in the number of *B. lactis* rRNA gene copies was found during synbiotic feeding, which contrasted with the infrequent detection of this organism with cultivation techniques. Moreover, *B. lactis* rRNA genes were found in 3 fecal samples during week 8 (obtained from subjects S1, S6, and S8) but not during the control week. There was also a marked increase in prevalence of *B. lactis* when placebo feeding started, and a second set of primers was used to check whether primer set BlactF/BlactR reacted in a nonspecific way with fecal DNA. However, the numbers of rRNA gene copies found with primer set Bflact2/Bflact5 were very similar in each individual sample (data not shown).

DISCUSSION

This study showed that consumption of synbiotics by elderly people modified their fecal bifidobacteria communities. *B. bifidum* and *B. lactis* rRNA genes were detected by real-time PCR in all fecal samples during synbiotic feeding. *B. bifidum* and/

Table 6. Results of real-time PCR quantitation of rRNA gene levels for *Bifidobacterium lactis* using primer sets BlactF and BlactR.

Subject group, subject, statistic	Prefeeding period: week 1	Feeding period			Postfeeding period	
		Week 3	Week 4	Week 5	Week 6	Week 8
Placebo group						
P1	6.9	6.9	6.8	6.9	8.1	ND
P2	ND	ND	7.2	6.4	ND	ND
P3	ND	6.5	ND	ND	7.5	6.3
P4	ND	ND	ND	6.9	ND	ND
P5	ND	8.7	6.8	7.5	6.9	ND
P6	7.1	6.5	7.3	8.9	7.7	7.5
P7	ND	7.8	ND	ND	ND	ND
P8	ND	6.9	ND	ND	ND	ND
P9	ND	6.1	8.3	NS	6.1	ND
Mean ± SD	7.0 ± 0.2	7.0 ± 0.9	7.3 ± 0.6	7.3 ± 0.9	7.3 ± 0.8	6.9 ± 0.9
Prevalence[a]	2/9	7/9	5/9	5/8	5/9	2/9
P for week 1 vs. other weeks[b]	NA	1.000	1.000	1.000	1.000	1.000
Synbiotic group						
S1	ND	9.6	8.5	8.5	7.4	6.6
S2	ND	8.4	8.7	7.9	8.0	ND
S3	ND	8.9	8.6	7.8	7.7	ND
S4	ND	9.2	8.0	7.7	6.1	ND
S5	8.5	7.6	8.0	7.8	8.0	7.8
S6	ND	8.1	8.8	8.7	7.7	6.7
S7	ND	7.4	8.0	7.7	8.5	ND
S8	ND	7.6	7.9	7.5	8.4	6.7
S9	ND	9.1	9.0	9.0	9.0	ND
Mean ± SD	8.5	8.4 ± 0.8	8.4 ± 0.4	8.1 ± 0.5	7.9 ± 0.8	6.9 ± 0.6
Prevalence[a]	1/9	9/9	9/9	9/9	9/9	4/9
P						
Week 1 vs. other weeks	NA	.000	.000	.000	.000	.740
Between dietary groups[c]	.670	.017	.004	.016	.062	.346

NOTE. Data are $\log_{10}$ 16S rRNA gene copies/g (wet weight), unless otherwise indicated. NA, not applicable ND, not detected; NS, not studied.

[a] No. of subjects with specific 16S rRNA genes present/no. of subjects tested.

[b] The Bonferroni test was used for pair-wise multiple comparison of the mean values for the control week and for the other dietary weeks.

[c] The independent Student's *t* test was used to compare the mean values between the dietary groups.

or *B. lactis* were also found in some volunteers, 3 weeks after feeding stopped, but not in the control week, indicating that the organisms not only survived transit of the gastrointestinal tract, but they persisted in the colonic ecosystem. It has been reported previously that long-term persistence in the gut of a *B. longum* strain could occur, but that it depended on the volunteer [24]. In other studies that have used different bifidobacterial strains, the probiotics exhibited tolerance to gastrointestinal transit but disappeared from the gut after feeding stopped [25–28].

In contrast to the placebo group, *B. bifidum* was detected relatively frequently in the synbiotic recipients during the control week, but synbiotic consumption increased the counts of this species significantly. This was particularly evident in individuals who had, at the beginning of the study, little or no *B. bifidum* in their fecal samples, whereas in volunteers with high initial counts of this organism, no increase was detectable. This correlates with previous studies that have reported difficulties in increasing initial high numbers of lactic acid bacteria in the gut by ingestion of probiotics [29, 30].

During the feeding period, *B. lactis* counts were significantly lower in placebo recipients than in the synbiotic group, but the bacterium was detected frequently in the placebo group using real-time PCR, although there is currently no evidence that this species is autochthonous to the human gut. Nonspecific reactions of the PCR primers with fecal DNA are an unlikely explanation for this observation, because 2 different sets of primers [20, 21] were used to detect the bacterium, and

similar results were obtained with all of the fecal samples. *B. lactis* strains different than the type strain *B. lactis* DSM 10140 have been previously found in samples of human feces [21, 31], and it is possible that this species has been overlooked in cultivation studies of fecal bifidobacteria. This notion is supported by the work described here, in which *B. lactis* was only found occasionally by cultivation with selective agars, possibly because it grew as very small colonies, which made detection and isolation difficult. It is possible that other dietary sources were responsible for the presence of *B. lactis* in the placebo group, because all volunteers (except for 1 person) stated that they regularly ate yogurt, and *B. lactis* can be detected in normal yogurts that are not explicitly labeled as containing bifidobacteria (R. Zink, personal communication).

B. bifidum and *B. lactis* were detected less frequently with use of selective agars than with real-time PCR; nevertheless, an increase in prevalence and cell numbers of *B. bifidum* occurred in the synbiotic group. The limitations of cultivation-based techniques have often been criticized, yet they permit counting of viable cells, which are the essential component of an effective probiotic [32]. PCR amplification of chromosomal DNA cannot distinguish between nonviable and viable cells, and therefore, dead bacteria may have been detected using the real-time PCR approach, leading to overestimations of their population size [33]. In fecal samples in which *B. bifidum* and *B. lactis* were detected by cultivation with selective agars, cell counts showed trends similar to the 16S rRNA gene copy numbers found with real-time PCR (data not shown), but the counts were, on average, 1 order of magnitude lower. Another reason why rRNA gene copy numbers were higher than cell numbers is that rRNA operons vary widely in bacteria, and between 2 to 5 rRNA operons have been found in different species belonging to the genus *Bifidobacterium* [34].

In the present study, volunteers ingested oligofructose together with the probiotic capsules to improve the transit tolerance and adhesion properties of the probiotics [35] and also to stimulate growth of indigenous bifidobacterial populations [16, 17, 36]. Apart from *B. bifidum* cell counts, no major differences were found between the 2 volunteer groups with respect to the types of bifidobacterial species isolated. Nevertheless, some volunteers in the synbiotic group had higher levels of *B. adolescentis* and *B. angulatum* (data not shown), which contributed, in part, to the significantly increased total bifidobacteria count. The few reports in the literature on synbiotic feeding have failed to observe any stimulating effect of prebiotics, such as galactooligosacharides [28, 29, 37] or inulin [38], on bifidobacteria, when given without probiotics. It has been shown that other bacteria, including staphylococci, enterococci, bacteroides, clostridia, and lactobacilli, have the potential to ferment inulin-type fructans [39], and the small but significant increases in lactobacilli levels with use of the synbiotic in this study suggest that other gastrointestinal bacteria had been utilizing oligofructose.

Fecal bifidobacterial levels were within the range of those reported previously in elderly people [10, 11]. In contrast to other studies that used counts of viable organisms, in which bifidobacteria were detected in only 50%–86% of stool samples obtained from older people [7, 9–11], we found these organisms in 100% of the fecal samples using real-time PCR and plate counts of viable organisms, indicating that the participants of our study still had relative high levels in bifidobacteria. Old age is often associated with increased ill health, and it is known that detrimental changes in the gut microflora are more prevalent in hospitalized elderly patients than in older people who are healthy, whereas increased use of antibiotics in older members of the community further contributes to degenerative changes in the gut ecosystem [6]. However, in this study, the synbiotic was shown to modify the composition of intestinal bifidobacterial populations in the healthy elderly volunteers, demonstrating that it has potential to be of particular benefit to individuals with more unbalanced gut ecosystems.

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References

1. Bornside GH. Stability of the human fecal flora. Am J Clin Nutr **1978**; 31:S141–4.
2. Finegold SM, Sutter VL, Mathisen GE. Normal indigenous intestinal flora. In: Hentges DJ, ed. Human intestinal microflora in health and disease. New York: Academic Press, **1983**:3–31.
3. Macfarlane GT, Gibson GR. Metabolic activities of the normal colonic flora. In: Gibson SAW, ed. Human health: the contribution of microorganisms. London: Springer Verlag, **1994**:17–52.
4. Wells CL, Maddaus MA, Jechorek RP, Simmons RL. Role of intestinal anaerobic bacteria in colonization resistance. Eur J Clin Microbiol Infect Dis **1988**; 7:107–13.
5. Lovat LB. Age related changes in gut physiology and nutritional status. Gut **1996**; 38:306–9.
6. Bartosch S, Fite A, Macfarlane GT, McMurdo M. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effect of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol **2004**; 70:3575–81.
7. Gavini F, Cayuela C, Antoine JM, et al. Differences in the distribution of bifidobacterial and enterobacterial species in human fecal microflora of three different (children, adults, elderly) age groups. Microb Ecol Health Dis **2001**; 13:40–5.
8. Gorbach SL, Nahas L, Lerner PI, Weinstein L. Studies of intestinal microflora. I. Effects of diet, age, and periodic sampling on numbers of fecal microorganisms in man. Gastroenterology **1967**; 53:845–55.
9. Hopkins MJ, Sharp R, Macfarlane GT. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut **2001**; 48: 198–205.
10. Mitsuoka T, Hayakawa K, Kimura N. Die Faekalflora bei Menschen.

II. Mitteilung: Die Zusammensetzung der Bifidobakterienflora der verschiedenen Altersgruppen. Zentralbl Bakteriol [Orig A] **1974**; 226: 469–78.
11. Mutai M, Tanaka R. Ecology of *Bifidobacterium* in the human intestinal flora. Bifid Micro **1987**; 6:33–41.
12. Elmer GW, McFarland LV. Biotherapeutic agents in the treatment of infectious diarrhea. Gastroenterol Clin North Am **2001**; 30:837–54.
13. Yamazaki S, Machii K, Tsuyuki S, Momose H, KawashimaT, Ueda K. Immunological responses to monoassociated *Bifidobacterium longum* and their relation to prevention of bacterial invasion. Immunology **1985**; 56:43–50.
14. Sekine K, Toida T, Saito M, Kuboyama M, Kawashima T, Hashimoto Y. A new morphologically characterized cell wall preparation (whole peptidoglycan) from *Bifidobacterium infantis* with a higher efficacy on the regression of an established tumour in mice. Cancer Res **1985**; 45: 1300–7.
15. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr **1995**; 125: 1401–12.
16. Gibson GR, Wang X. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J Appl Bacteriol **1994**; 77:412–20.
17. Gibson GR, Wang X. Enrichment of bifidobacteria from human gut contents by oligofructose using continuous culture. FEMS Microbiol Lett **1994**; 118:121–7.
18. Beerens H. Elective and selective isolation medium for *Bifidobacterium* spp. Lett Appl Microbiol **1990**; 11:155–7.
19. Matsuki T, Watanabe K, Tanaka R, Fukuda M, Oyaizu H. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene–targeted species-specific primers. Appl Environ Microbiol **1999**; 65:4506–12.
20. Malinen E, Kassinen A, Rinttila T, Palva A. Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rRNA gene-targeted oligonucleotide probes in quantification of selected fecal bacteria. Microbiology **2003**; 149:269–77.
21. Ventura M, Reniero R, Zink R. Specific identification and target characterization of *Bifidobacterium lactis* from different environmental isolates by a combined multiplex-PCR approach. Appl Environ Microbiol **2001**; 67:2760–5.
22. Langendijk PS, Schut F, Jansen GJ, et al. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl Environ Microbiol **1995**; 61:3069–75.
23. Bernhard AE, Field KG. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Appl Environ Microbiol **2000**; 66:1587–94.
24. Fujiwara S, Seto Y, Kimura A, Hashiba H. Intestinal transit of an orally administered streptomycin-rifampicin–resistant variant of *Bifidobacterium longum* SBT2928: its long-term survival and effect on the intestinal microflora and metabolism. J Appl Microbiol **2001**; 90:43–52.
25. Bouhnik Y, Pochart P, Marteau P, Arlet G, Goderel I, Rambaud JC. Fecal recovery in humans of viable *Bifidobacterium* sp. ingested in fermented milk. Gastroenterology **1992**; 102:875–8.
26. Brigidi P, Vitali B, Swennen E, Altomare L, Rossi M, Matteuzzi D. Specific detection of *Bifidobacterium* strains in a pharmaceutical probiotic product and in human feces by polymerase chain reaction. Syst Appl Microbiol **2000**; 23:391–9.
27. Kullen MJ, Amann MM, O'Shaughnessy W, O'Sullivan DJ, Busta FF, Brady U. Differentiation of indigested bifidobacteria by DNA fingerprinting demonstrates the survival of an unmodified strain in the gastrointestinal tract of humans. J Nutr **1997**; 127:89–94.
28. Malinen E, Matto J, Salmitie M, Alander M, Saarela M, Palva A. PCR-ELISA II: Analysis of *Bifidobacterium* populations in human fecal samples from a consumption trial with *Bifidobacterium lactis* Bb-12 and a galacto-oligosaccharide preparation. Syst Appl Microbiol **2002**; 25: 249–58.
29. Alander M, Mättö J, Kneifel W, et al. Effect of galacto-oligosaccharide supplementation on human fecal microflora and on survival and persistence of *Bifidobacterium lactis* Bb-12 in the gastrointestinal tract. Int Dairy J **2001**; 11:817–25.
30. de Champs C, Maroncle N, Balestrino D, Rich C, Forestier C. Persistence of colonization of intestinal mucosa by a probiotic strain, *Lactobacillus casei* subsp. *rhamnosus* Lcr35, after oral consumption. J Clin Microbiol **2003**; 41:1270–3.
31. Ventura M, Zink R. Rapid identification, differentiation, and proposed new taxonomic classification of *Bifidobacterium lactis*. Appl Environ Microbiol **2002**; 68:6429–34.
32. Fuller R. Probiotics in man and animals. J Appl Bacteriol **1989**; 66: 365–78.
33. Josephon P, Gerba CP, Pepper IL. Polymerase chain reaction detection of nonviable bacterial pathogens. Appl Environ Microbiol **1993**; 59: 3513–5.
34. Klappenbach JA., Saxman PR, Cole JR, Schmidt TM. rrndb: The ribosomal RNA operon copy number database. Nucleic Acids Res **2001**; 29:181–4.
35. He F, Ouwehand AC, Hashimoto H, Isolauri E, Benno Y, Salminen S. Adhesion of *Bifidobacterium spp*. to human intestinal mucus. Microbiol Immunol **2001**; 45:259–62.
36. Wang X, Gibson GR. Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. J Appl Bacteriol **1993**; 75:373–80.
37. Tanaka R., Takayama H, Morotomi M, et al. Effects of administration of TOS and *Bifidobacterium breve* 4006 on the human fecal flora. Bifid Micro **1983**; 2:17–24.
38. Bouhnik Y, Flourie B, Andrieux C, Bisetti N, Briet F, Rambaud JC. Effects of *Bifidobacterium* sp. fermented milk ingested with or without inulin on colonic bifidobacteria and enzymatic activities in healthy humans. Eur J Clin Nutr **1996**; 50:269–73.
39. Roberfroid MB, Delzenne NM. Dietary fructans. Annu Rev Nutr **1998**; 18:117–43.