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Effect of a Synbiotic Yogurt on Levels of Fecal Bifidobacteria, Clostridia, and Enterobacteria

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While ingestion of synbiotic yogurts containing *Bifidobacterium animalis* subsp. *lactis* and inulin is increasing, their effect on certain microbial groups in the human intestine is unclear. To further investigate this, a large-scale, crossover-design, placebo-controlled study was utilized to evaluate the effect of a synbiotic yogurt containing *B. animalis* subsp. *lactis* Bb-12 and inulin on the human intestinal bifidobacteria, clostridia, and enterobacteria. Fecal samples were collected at 14 time points from 46 volunteers who completed the study, and changes in the intestinal bacterial levels were monitored using real-time PCR. Strain Bb-12 could not be detected in feces after 2 weeks of washout. A live/dead PCR procedure indicated that the Bb-12 strain detected in the fecal samples was alive. A significant increase ($P < 0.001$) in the total bifidobacterial numbers was seen in both groups of subjects during the final washout period compared to the prefeeding period. This increase in total bifidobacteria corresponded with a significant decrease ($P < 0.05$) in numbers of clostridia but not enterobacteria. No significant differences in numbers of bifidobacteria, clostridia, or enterobacteria were observed between the probiotic and placebo groups during any of the feeding periods. However, subgrouping subjects based on lower initial bifidobacterial numbers or higher initial clostridial numbers did show corresponding significant differences between the synbiotic yogurt and placebo groups. This was not observed for a subgroup with higher initial enterobacterial numbers. While this synbiotic yogurt can increase bifidobacterial numbers and decrease clostridial numbers (but not enterobacterial numbers) in some individuals, it cannot modulate these microbial groups in the majority of individuals.

The human large intestine is host to a wide variety of bacteria, with bifidobacteria being prominent members of this complex ecosystem. These are (often irregularly) rod-shaped Gram-positive, anaerobic, non-spore formers that utilize glucose via a pathway that involves a diagnostic enzyme, fructose-6-phosphate phosphoketolase (F6PPK). As bifidobacteria are generally believed to contribute to good intestinal health, attempts have been made to increase their numbers in the intestine by including them in certain foods as probiotics. They are frequently included in yogurts together with prebiotics, such as inulin or fructooligosaccharides, the combination of which can be referred to as a synbiotic yogurt. Bifidobacteria are implicated in the prevention and treatment of diarrhea, development and maintenance of a healthy microbiota in low-weight preterm infants, and stimulation of certain immune responses (25, 28). It is believed that counts of bifidobacteria begin to decline during old age, coinciding with a proliferation of other bacterial groups, including clostridia and members of the *Enterobacteriaceae* family (11, 25, 43). Consequently, increasing bifidobacterial counts may be advantageous for controlling the proliferation of certain undesirable bacteria.

Clostridia are toxin producers and have been implicated in many intestinal ailments, such as nosocomial diarrhea, antibiotic-associated diarrhea, necrotizing enterocolitis, and gastrointestinal (GI) infections (5). The genus *Clostridium* substantially consists of about 140 different species. It has been divided into 19 clusters based on the phylogenetic analysis of the 16S rRNA gene. Cluster I is the largest cluster, consisting of most of the pathogenic species of *Clostridium*, including *C. tetani*, *C. botulinum*, and *C. perfringens* (41). Gastrointestinal diseases caused by clostridia are mostly a result of an imbalanced gut microbiota, which disrupts the protective effects of the GI flora (21). This imbalance can be precipitated by antibiotic treatment, stress, immunocompromization, and old age (reviewed in references 6 and 10).

Escherichia species such as *Escherichia coli* belong to the family *Enterobacteriaceae* and are normal inhabitants of human and animal guts. They are Gram-negative, rod-shaped, facultative anaerobes with excellent survival outside the gut and are frequently utilized as indicators of fecal contamination. While most *E. coli* strains are nonpathogenic, commensal bacteria, certain strains are harmful and opportunistic due to the presence of virulence factors that support their ability to induce infection (15, 16). The most recognized of these strains is enterohemorrhagic *E. coli* (EHEC) due to the prevalence of *E. coli* O157:H7 in food-borne disease outbreaks (16, 27). While it has been proposed that a low level of commensal strains may be beneficial (35), their role is undefined; thus, there is an increasing interest in the role of the intestinal microbiota in maintaining overall and GI health (8, 9).

While there is no legal definition for probiotics in the United States, they are generally referred to as live microbial feed supplements that, when ingested in sufficient amounts, can affect the host beneficially (29). Bifidobacteria are widely used as probiotics in foods. *B. animalis* is the most common species used in foods, as it has the highest tolerance to oxygen and acids. The continued adaptation of this species to the fermented dairy environment resulted in the evolution of a newly adapted bacterium that was initially classified as a new species, *B. lactis* (24). However, owing to very close genetic similarities to *B. animalis*, it was accepted as a

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TABLE 1 Means of ages, weights, and heights for study participants

Parameter	Group A	Group B	*P* value
No. of subjects	22	24	
Mean age (yr)	30.3 ± 9.7	30.9 ± 13.8	0.87
Mean wt (lb)	166 ± 46.4	145 ± 26	0.06
Mean ht (ft)	5.7 ± 0.33	5.49 ± 0.28	0.48

subspecies of *B. animalis* (4). *B. animalis* subsp. *lactis* strain Bb-12 is a common commercial probiotic used in foods and is exclusively supplied by Chr. Hansen Inc., a worldwide supplier of fermentation cultures. Studies on Bb-12 ingestion have shown it to have many potential health benefits. These include maintaining the intestinal microbial balance (reviewed in reference 32), diarrhea prevention (37), stimulation of the phagocytic activity in peripheral blood samples (38), and treatment of atopic eczema in infants (14). However, given small subject numbers and limitations of culturing methods, further studies are needed to evaluate the effect of this widely used probiotic culture on the intestinal microbiota. The purpose of this study was to examine the effects of a yogurt supplemented with inulin and strain Bb-12 (~10^9 to 10^{10} CFU/serving) consumed daily by a large number of healthy subjects on intestinal bifidobacterial, clostridial, and enterobacterial numbers using a probe-based real-time PCR approach.

MATERIALS AND METHODS

Bacteria and culture conditions. *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Chr. Hansen Inc., Horsholm, Denmark), *B. longum* strain DJO10A (13, 19), *Clostridium perfringens* (Diez laboratory, University of Minnesota), and *E. coli* (Invitrogen, Carlsbad, CA) were used in this study. *E. coli* and *B. longum* strain DJO10A were used as negative controls for strain Bb-12-specific TaqMan real-time PCR. All cultures were streaked on agar plates as follows: strains Bb-12 and DJO10A on BIM-25 (26); *C. perfringens* on RCM (reinforced clostridial medium) (2); and *E. coli* on LB. Strains Bb-12 and DJO10A were cultured anaerobically in broth cultures using BliM plus Fe (13) at 37°C for 48 h, *C. perfringens* was cultured anaerobically in RCM at 37°C for 24 h in anaerobic jars, and *E. coli* was cultured aerobically in LB medium at 37°C overnight.

Human subjects, study design, and sample collection. Prior to initiating the study, approval for the study design was obtained from the Institutional Review Board (IRB) of the University of Minnesota. Fifty-two healthy volunteers participated in this study, with 46 of them completing it. The average age, height, and weight for the group of 46 subjects were 31 years, 5 feet 6 in., and 155 lb, respectively. The detailed demographic data are presented in Table 1. The inclusion criteria for this study were that subjects were adults with no known allergies or intolerance to dairy foods and had not consumed foods containing bifidobacteria for at least 2 months prior to the start of the study. To determine the latter criterion, subjects were specifically asked whether they had consumed any dairy products that had the term "bifidus" or "bifidobacteria" or any word beginning with "bifid" on its label. Subjects were asked to maintain their usual diet during the study, with no intake of products containing bifidobacteria with the exception of what was given to them within the study. The subjects were randomly assigned to two groups, A and B, with 26 subjects in each. However, only 46 of the total initial number of subjects completed the study. Consequently, group A finished with 24 subjects and group B with 22 subjects.

The study was a double-blind, crossover, placebo-controlled, randomized-feeding trial. It was divided into five consecutive periods: a prefeeding period (1 week), followed by a feeding period (3 weeks), a washout period (4 weeks), a second feeding period (3 weeks), and a final washout period (4 weeks). The prefeeding period was a control period, during which the subjects were not given any yogurt drink. Fecal samples were collected at the start and at the end of the week. During the first feeding period, the subjects consumed daily either 94 g of placebo, which consisted of milk acidified to pH 4.2 with lactic acid, or 94 g of a drinkable yogurt containing 10^9 to 10^{10} CFU of strain Bb-12 and 1 g of inulin per serving. The yogurt was prepared with skim milk and a standard yogurt starter blend consisting of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, together with the *B. animalis* subsp. *lactis* Bb-12 culture, and had a final pH of 4.2. Both products were flavored with sucrose and strawberry. The shelf life of the yogurt was set as 50 days at 4°C, corresponding to the lower level (10^9 CFU/serving) of strain Bb-12. Fecal samples were collected at the end of each of the 3 weeks. The subjects consumed neither the yogurt nor the placebo during the subsequent washout period. Single fecal samples were collected at the end of every 2 weeks. Participants were given 50-ml Falcon tubes containing 10 ml of sterile phosphate-buffered saline (PBS) buffer and a sterile plastic spoon and were asked to fill the tube to the 40-ml mark with feces from the midstream defecation period. The tubes were mixed thoroughly and centrifuged to collect a fixed amount of pelleted stool. During the second feeding period, there was a crossover of the feeding design. Again, fecal samples were collected at the end of each of the 3 weeks. The final washout period lasted for 4 weeks. Neither placebo nor probiotic yogurt was consumed during that period. Fecal samples were collected at the end of each week. In all, 14 fecal samples were collected from each subject. For every collection, ~5 g of feces was collected in 10 ml of phosphate buffer (pH 7) and immediately frozen at −20°C. The study design and sample collection strategy are depicted in Fig. 1.

DNA extraction. DNA was extracted from pure cultures by the use of a Qiagen MiniPrep DNA extraction kit protocol with slight modifications. A 1-ml aliquot of culture was centrifuged (1,350 rpm for 10 min in a Hermle Labnet Z 233 MK centrifuge with rotor model C-0230-2A) to reduce cells to pellets. The cells were resuspended in 340 μl of ASL buffer and subjected to 95°C for 5 min. Proteinase K and buffer AL were subsequently added directly to the heat-treated cells in ASL buffer. The rest of the Qiagen protocol was followed according to the manufacturer's instructions. DNA was extracted from feces by the use of a Qiagen MiniPrep DNA extraction kit according to the manufacturer's instructions. The DNA was then quantified using a Nanodrop spectrophotometer and stored at −20°C.

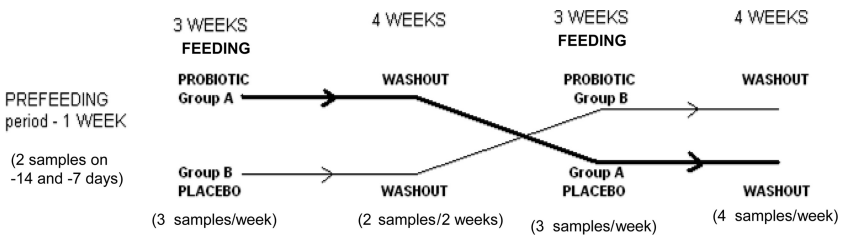

FIG 1 Representation of the study design and fecal collection. The study was divided into 5 periods: a prefeeding period, a feeding period, a washout period, a crossover feeding period, and a final washout period. Fecal samples are represented by the sterile tubes containing buffer and collection spoon given to subjects.

TABLE 2 Primers and probes used for quantitative real-time PCR

Target	Primer or probe	Sequence (5′→3′)	Concn (nM)	Reference or source
Bb-12[a]	F-Bal 23	GGT GGT CTG GTA GAG TAT ACC G	900	Chr. Hansen Inc.
	R-Bal 23	GGC GAC TTG CGT CTT G	900	
	P-Bal 23	CGC CCA CGA CCC GCA AG	200	
Enterobacteria	F-En	CATGCCGCGTGTATGAAGAA	900	12
	R-En	CGGGTAACGTCAATGAGCAAA	900	
	P-Bal 23	CGC CCA CGA CCC GCA AG	200	
Bifidobacterium genus	F-TAQ	GCG TCC GCT GTG GGC	300	34
	R-TAQ	CTT CTC CGG CAT GGT GTT G	300	
	P-TAQ	TCC ACC GGC ACC AAG AAC GC	200	
Clostridium cluster I	F-CI	TAC CHR AGG AGG AAG CCA C	300	40
	R-CI	GTT CTT CCT AAT CTC TAC GCA T	300	
	P-CI	GTG CCA GCA GCC GCG GTA ATA CG	200	

[a] These primers are specific for *B. animalis* subsp. *lactis*.

Quantification of *B. animalis* subsp. *lactis* Bb-12 and total bifidobacteria in feces by using TaqMan real-time PCR. Specific primers and TaqMan probes used for amplification in TaqMan real-time PCR are listed in Table 2. Probes were labeled with the fluorescent dye 6-carboxyfluorescein (FAM) and the quencher 6-carboxytetramethylrhodamine (TAMRA). Seven 10-fold dilutions with known cell numbers (ranging from 10^2 to 10^9 CFU/ml) of strain Bb-12 were used to spike fecal samples that were free of strain Bb-12, and the DNA extracted was used in real-time PCR to generate a standard curve for quantification of strain Bb-12. DNA from a pure culture of bifidobacteria diluted in seven 10-fold dilutions with known cell numbers (ranging from 10^2 to 10^9 CFU/ml) was also used as the template in TaqMan real-time PCR to generate standard curves for quantification of total bifidobacteria.

Quantification of clostridia and enterobacteria in feces by the use of TaqMan real-time PCR. Specific primers and probes for *Clostridium* cluster I and enterobacteria used for amplification in real-time PCR are given in Table 2. Probes were labeled with the fluorescent dye FAM and black hole quencher (BHQ). Seven 10-fold dilutions with known cell numbers (ranging from 10^2 to 10^9 CFU/ml) of *C. perfringens* and *E. coli* were used to extract DNA. These DNAs were then used as templates in real-time PCR to generate standard curves for quantification of *Clostridium* cluster I and enterobacteria.

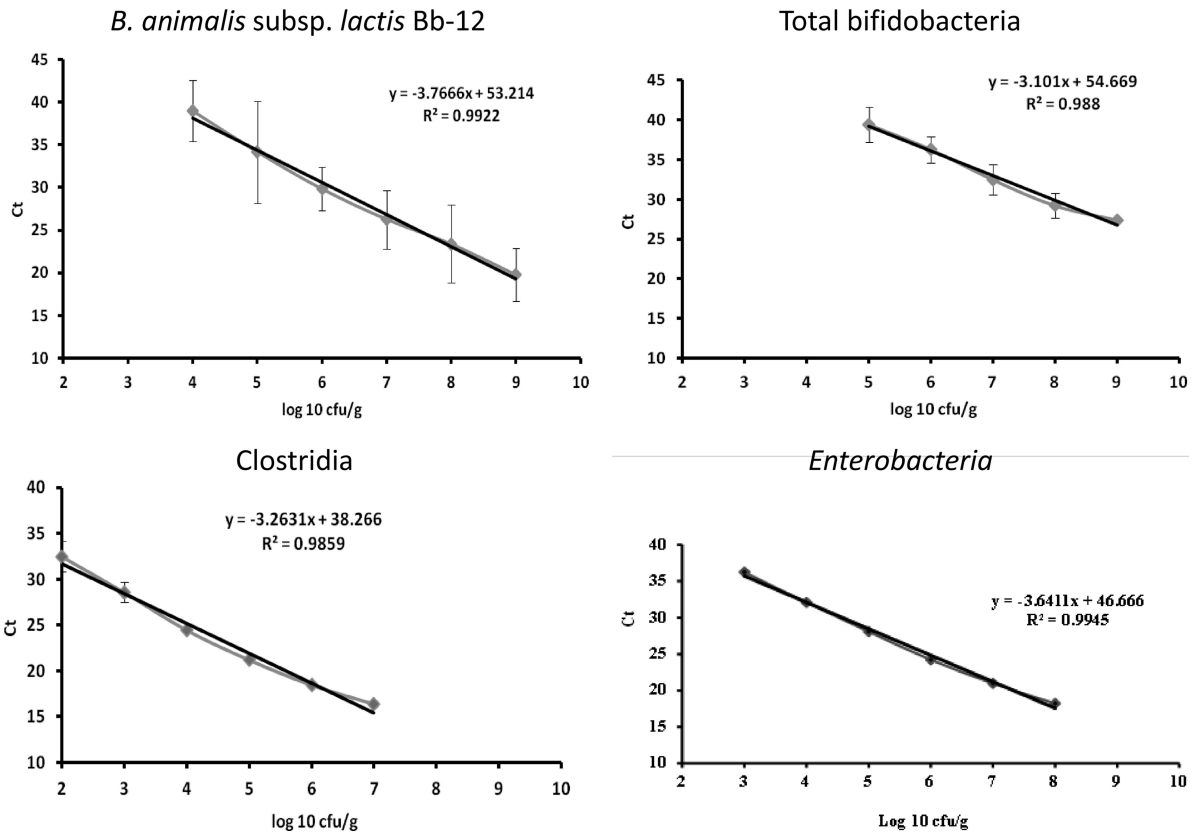

FIG 2 C_T standard curves for real-time PCR quantitative analysis of *B. animalis* subsp. *lactis* Bb-12, total bifidobacteria, clostridia, and enterobacteria.

TABLE 3 Quantitative real-time PCR data for strain Bb-12 in the feces of 10 subjects before and after EMA treatment

Sample	C_T before EMA	Amt of Bb-12 (log 10 CFU/g) before EMA	C_T after EMA	Amt of Bb-12 (log 10 CFU/g) after EMA	% viable Bb-12 in feces
1	31.93	5.65	32.50	5.50	97.35
2	32.38	5.53	33.63	5.20	94.03
3	31.86	5.67	53.21	0.00	00.00
4	33.25	5.30	34.00	5.10	96.23
5	31.44	5.78	32.61	5.47	94.64
6	33.48	5.24	34.38	5.00	95.42
7	32.42	5.52	53.21	0.00	00.00
8	33.85	5.14	35.81	4.62	89.88
9	32.08	5.61	32.53	5.49	97.86
10	32.31	5.55	32.42	5.52	99.46

Real-time PCR. Real-time PCR amplifications were performed on an ABI 7500 real-time PCR machine. Each reaction was carried out in duplicate in a volume of 25 μl using 96-well optical-grade ABI plates. The temperature settings for the different bacteria were as follows: one cycle of 95°C (10 min) and 40 cycles of 95°C (15 s) and 60°C (1 min) for strain Bb-12; one cycle of 95°C (10 min) and 40 cycles of 95°C (15 s) and 58°C (1 min) for total bifidobacteria; one cycle of 95°C (10 min) and 45 cycles of 95°C (15 s), 63°C (30 s), and 72°C (45 s) for clostridia; and one cycle of 95°C (10 min) and 45 cycles of 95°C (15 s) and 60°C (1 min) for enterobacteria. The C_T (cycle at which the signal crosses a threshold) values were plotted as a linear function of the base 10 logarithm of the number of respective bacterial cells in the culture as determined by plate counts. These standard curves were then used to quantify the fecal samples with unknown cell concentrations collected during the study. Three negative controls, consisting of a no-template control and two controls for nontarget bacteria, were used to validate the specificity of each real-time PCR procedure.

Determination of numbers of live *B. animalis* subsp. *lactis* Bb-12 cells in feces. Ethidium monoazide bromide (EMA) binds covalently to DNA upon exposure to light and has been validated to differentiate between live and dead cells by the use of PCR (36, 39). To validate this procedure for strain Bb-12 in feces, a 1-ml aliquot of a fully grown culture (8.3×10^8 CFU/ml) of strain Bb-12 was subjected to heat killing at 100°C for 10 min and incubated overnight at 4°C. Two 250-mg aliquots of feces with no detectable *B. animalis* subsp. *lactis* were spiked with 1 ml of either dead or live cells. EMA (100 μg/ml) was added to these samples, and the mixture was incubated in the dark at 4°C for 1 h. After incubation, the fecal samples were placed on ice and exposed for 1 h to 600 W of halogen light located at a distance of 20 cm. The samples were then used to extract DNA for real-time PCR. To differentiate between live and dead cells in fecal samples collected during the study, 100 μg of EMA/ml was added to them and the fecal DNA isolation procedure outlined above was repeated.

Statistical analysis. For statistical analysis, SPSS software was used. Independent-sample *t* tests were used for between-subject analysis. Paired-sample *t* tests were used for within-subject analysis.

RESULTS

Enumeration of *Bifidobacterium animalis* subsp. *lactis* Bb-12, total bifidobacteria, clostridia, and enterobacteria in feces. Standard curves were generated by plotting real-time PCR C_T values obtained for each bacterial group against the initial number of cells in the culture (Fig. 2). The curves were found to be linear, with R^2 values > 0.98, over the ranges of 10^5 to 10^9 CFU/ml for total bifidobacteria (10^5 was therefore taken as the limit of detection); 10^4 to 10^9 CFU/ml for strain Bb-12 (10^4 was taken as the limit of detection); 10^2 to 10^7 CFU/ml for clostridia (10^2 was taken as the limit of detection); and 10^3 to 10^8 CFU/ml for enterobacteria (10^3 was taken as the limit of detection). These curves were then applied to quantify the totals of intestinal bifidobacteria, *Clostridium* cluster I, enterobacteria, and strain Bb-12 in the fecal samples collected from subjects during the study. This revealed the variations in cell numbers of strain Bb-12, total bifidobacteria, clostridia, and enterobacteria in each subject over the study period.

Detection and viability of strain Bb-12 in feces. The Bb-12 primers did not detect any *B. animalis* subsp. *lactis* in fecal samples collected during the prefeeding period except for the first sample of one subject. As the second fecal sample was not positive, the subject was allowed to continue in the study. Strain Bb-12 was detected in the feces of 70% of subjects during the period in which they were ingesting the supplemented yogurt but in only 2 out of 22 subjects (9%) after 1 week of washout and none after 2 weeks. To evaluate viability using a live/dead PCR approach, a control experiment with fecal samples spiked with live or dead cells of strain Bb-12 was used to validate the procedure. This procedure was applied to 10 fecal samples that had tested positive for strain Bb-12 from 10 different subjects and revealed that strain Bb-12 was alive in 8 out of the 10 samples, substantiating its ability to survive gastric transit (Table 3).

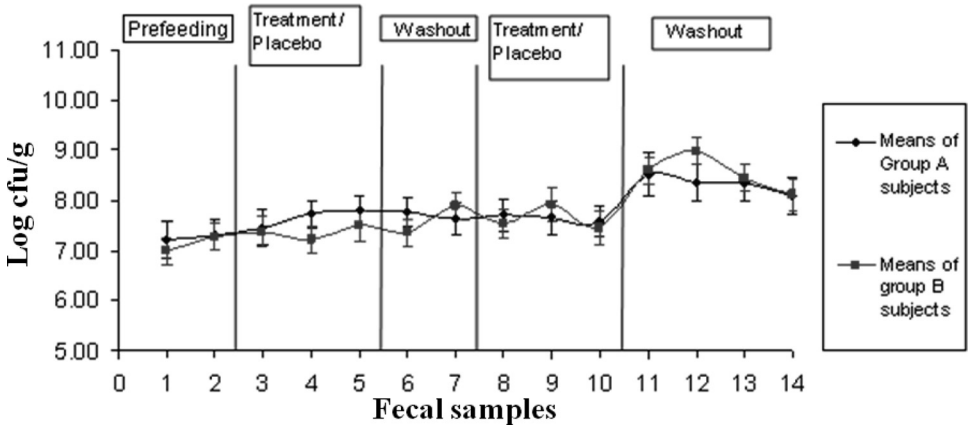

FIG 3 Mean numbers of total bifidobacteria for all group A subjects who consumed probiotic first and all group B subjects who consumed placebo first at different time points during the study.

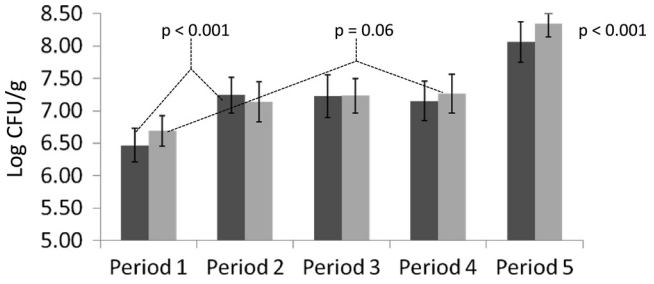

FIG 4 Fecal bifidobacterial levels in subjects subgrouped on the basis of bacterial numbers $< 10^8$ CFU/g in the prefeeding period. Period 1, prefeeding period; period 2, first supplemented yogurt feeding period; period 3, first washout period; period 4, crossover feeding period; period 5, final washout period. Group A is represented by the darker shading. *P* values represent differences between the yogurt feeding and final washout periods compared to the prefeeding period.

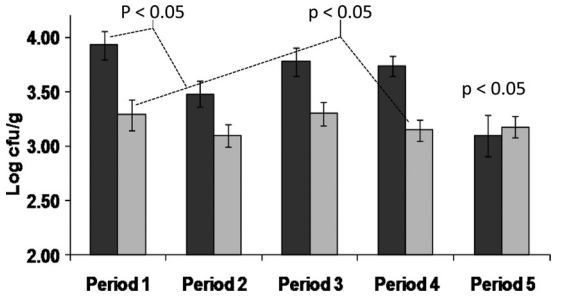

FIG 6 Fecal *Clostridium* (cluster I) levels in subjects subgrouped on the basis of bacterial numbers > 3.5 $\log_{10}$ CFU/g in the prefeeding period. Period 1, prefeeding period; period 2, first supplemented yogurt feeding period; period 3, first washout period; period 4, crossover feeding period; period 5, final washout period. Group A is represented by the darker shading. *P* values represent differences between the yogurt feeding and final washout periods compared to the prefeeding period.

Total bifidobacterial counts in fecal samples. Prior to the feeding trial, the total bifidobacterial counts (as estimated by real-time PCR) in individual stool samples ranged from the detection limit of 5.0 $\log_{10}$ CFU/g to 9.0 $\log_{10}$ CFU/g, the mean bifidobacterial count being 7.2 $\log_{10}$ CFU/g. During the two feeding periods, no statistically significant difference was seen between the placebo and the probiotic groups. However, there was a fluctuating increase in the bifidobacterial numbers within the two groups (Fig. 3). The final washout period was marked with a significant increase in the bifidobacterial population in both groups compared to the prefeeding period ($P < 0.001$). However, a subgroup of subjects who had bifidobacterial counts $< 10^8$ CFU/g in their feces during the prefeeding period harbored a significant increase ($P < 0.001$ for group A and $P = 0.06$) when ingesting the synbiotic yogurt (Fig. 4).

Clostridial counts in fecal samples. The initial clostridial counts in fecal samples were found to be 3.36 $\log_{10}$ CFU/g (the values ranged from the limit of detection, 2.0 $\log_{10}$ CFU/g, to 5.0 $\log_{10}$ CFU/g). The only statistically significant difference in clostridial numbers between the placebo and yogurt groups occurred during the second feeding period ($P < 0.05$) (Fig. 5). However, there was no reduction in numbers within each group in comparison to the prefeeding periods. There was a decrease in the clostridial numbers in the final washout period compared with the prefeeding levels in both groups. This reduction was not statistically significant for the whole washout period but was significant for the final fecal sample ($P < 0.001$ for group A and $P < 0.05$ for group B). This reduction was significant ($P < 0.05$) for the whole final washout period when subjects were subgrouped on the basis of high clostridial counts (>3.5 $\log_{10}$ CFU/g) in the prefeeding period (Fig. 6). This subgroup also exhibited a significant decrease in numbers of clostridia ($P < 0.05$) during the period in which the members of the subgroup were ingesting the supplemented yogurt compared to their prefeeding levels, which was consistent with the concomitant increase in total bifidobacterial numbers.

Enterobacterial counts in fecal samples. The initial mean *Enterobacterium* count in fecal samples was found to be 5.68 $\log_{10}$ CFU/g (the values ranged from 3.5 $\log_{10}$ CFU/g to 8.2 $\log_{10}$ CFU/g). There was no statistically significant decrease in *Enterobacterium* numbers between the synbiotic yogurt and placebo groups or between any group period and the corresponding prefeeding period (Fig. 7). However, subjects with initial *Enterobacterium* counts > 5.5 $\log_{10}$ CFU/g feces showed a decrease in mean *Enterobacterium* counts during the feeding study and this decrease was statistically significant ($P < 0.001$) for both groups (Fig. 8). However, there were no significant differences between the results seen with the synbiotic yogurt and placebo feeding periods even for this subgrouping.

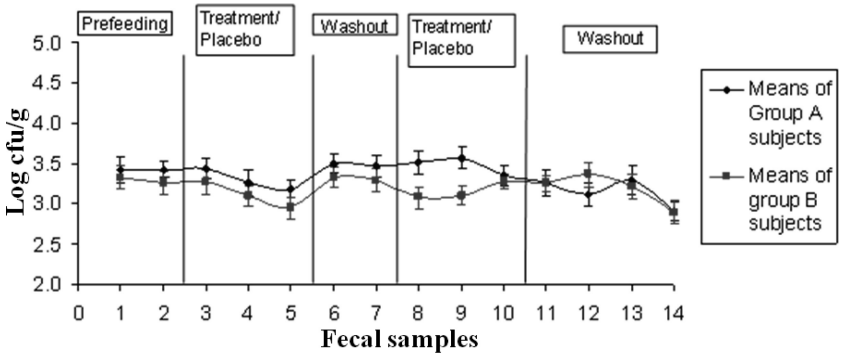

FIG 5 Mean numbers of *Clostridium* cluster I for all group A subjects who consumed probiotic first and all group B subjects who consumed placebo first at different time points during the study. Values below the level of detection (10^2 CFU/g) have been equated to the value of the limit of detection of 10^2 CFU/g for mean calculations. The bars represent standard errors.

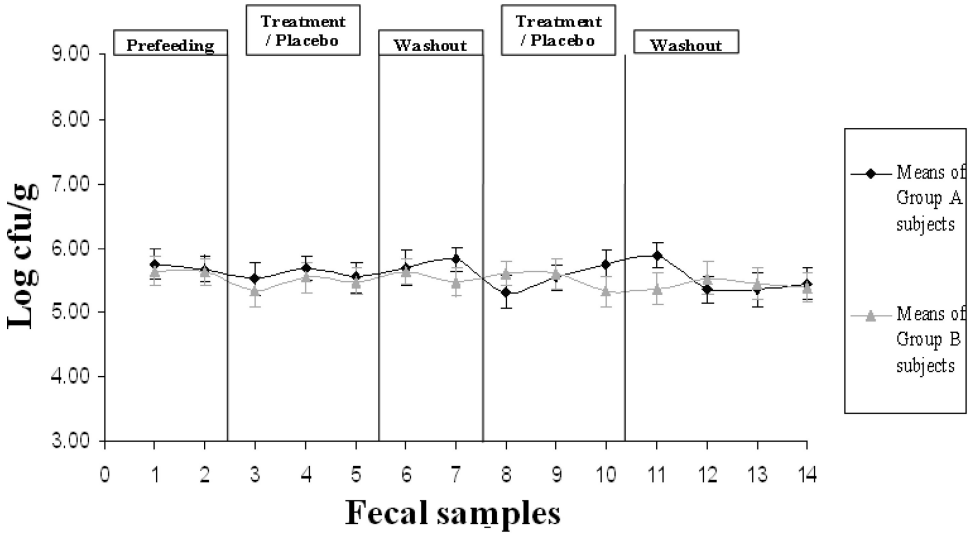

FIG 7 Mean numbers of enterobacteria for all group A subjects who consumed probiotic first and all group B subjects who consumed placebo first at different time points during the study. Values below the level of detection (10^3 CFU/g) have been equated to the value of the limit of detection of 10^3 CFU/g for mean calculations. The bars represent standard errors.

DISCUSSION

The impact of the intestinal microbial community on human health is currently an expanding field of research. There is currently insufficient evidence to evaluate whether commonly used bifidobacteria, such as strains of *B. animalis* subsp. *lactis*, have a modulatory impact on the intestinal microbiota. In this study, we evaluated the effect of *B. animalis* subsp. *lactis* Bb-12 and inulin administered in a drinkable yogurt on the numbers of intestinal bifidobacteria, clostridia, and enterobacteria in 46 human volunteers.

As subjects were selected on the basis of not having consumed yogurt-type foods for 2 months, their initial fecal samples were negative for any *B. animalis* subsp. *lactis*. One subject's initial fecal sample tested positive, suggesting that that subject had unknowingly ingested it, as has also been found to have occurred in other studies (23, 30) and is plausible given the vast variety of available foods harboring this culture. The percentage of subjects who tested positive for strain Bb-12 during probiotic yogurt consumption in this study was consistent with studies that used comparable levels of this strain (7) and with the correlation between dosage and subsequent recovery of this probiotic from fecal samples (18). The rapid disappearance of Bb-12 from fecal samples following cessation of feeding is consistent with other published clinical studies (7, 22). Some studies have seen persistence in one or two subjects for a week or two longer (42). However, this may have been due to variations in subject reliability or to inadvertent consumption of other foods containing bifidobacteria rather than persistence of the species.

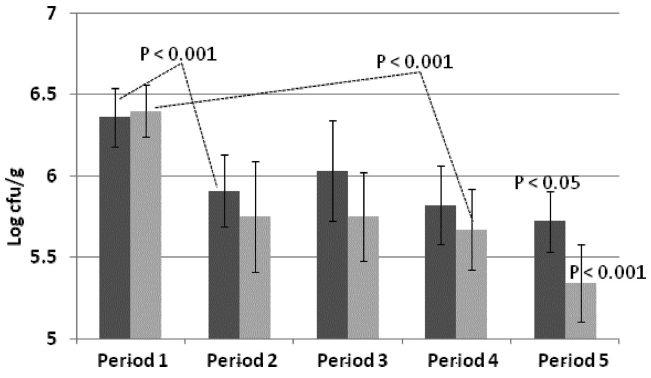

FIG 8 Fecal enterobacterial levels in subjects subgrouped on the basis of bacterial numbers > 5.5 $\log_{10}$ CFU/g in the prefeeding period. Period 1, prefeeding period; period 2, first supplemented yogurt feeding period; period 3, first washout period; period 4, crossover feeding period; period 5, final washout period. Group A is represented by the darker shading. *P* values represent differences between the yogurt feeding and final washout periods compared to the prefeeding period.

While the use of real-time PCR to monitor the intestinal flora facilitates studies using a larger number of subjects, it is usually limited by lack of data on the viability of the ingested culture. Some studies have included viable plate counts with real-time PCR (3). To address this limitation without including viable plate counts, which would limit the number of subjects that could be used, as fresh feces would be needed, we utilized a live/dead cell differentiation procedure to show that strain Bb-12 cells detected in feces retained their viability. As this procedure was demonstrated in this study to differentiate between live and dead cells of strain Bb-12 directly in feces, it indicated that the strain Bb-12 cells were viable in the majority of subjects at the time of freezing the samples.

The average number of bifidobacteria in human feces in adults has been reported to be ~7.5 $\log_{10}$ CFU/g (23, 38). This was consistent with the total bifidobacterial counts in this study. Bifidobacterial numbers have been found to vary with age, with infants normally harboring larger numbers (~10^{10} CFU/g of feces) (17, 31). Elderly people and those suffering from bowel diseases such as cancer have been reported to have lower numbers of bifidobacteria in their feces (33). In this study, older subjects (age > 50 years) were found to have lower numbers of bifidobacteria in their prefeeding fecal samples, and while the sample size was small, it is consistent with this trend. Some studies have reported an increase

in bifidobacterial numbers during probiotic intake (23, 38). In our study, no significant differences between the synbiotic and the placebo groups during yogurt consumption were observed. This differs from another study using Bb-12, which did find a difference (1). However, that study had a subject group of just 10 compared to the 46 subjects in our study, indicating the probability of greater interindividual variability between subjects in this study. When subjects were subdivided on the basis of initial lower bifidobacterial numbers, an increase in total numbers of bifidobacteria during periods of probiotic yogurt consumption became evident (Fig. 4). An increase in the total numbers of bifidobacteria in subjects with low starting numbers of bifidobacteria has been observed previously (20, 22). Given that the total numbers of fecal microbes were not estimated in any of these studies, the possibility that such a result is linked to the fluctuations observed cannot be ruled out.

A significant increase in numbers of bifidobacteria for all groups in the final washout period was very evident and has not been seen in other studies. This significant increase in the final washout period was not due to irregularities with the real-time PCR quantification, as subject participation was staggered and quantification of samples was conducted randomly rather than sequentially. This may suggest that in a long probiotic feeding study, such as this 15-week study, some subjects may change their overall eating habits, given that they are constantly cognizant of the association of fermented foods and a favorable intestinal environment. This increase in bifidobacterial numbers corresponded with a significant decrease in numbers of clostridia but not in numbers of enterobacteria. An inverse correlation between bifidobacterial and clostridial numbers has been observed previously in bifidobacterium feeding studies (23, 33).

Several overall conclusions can be drawn from the study. Strain Bb-12 survived passage through the gastrointestinal tract but did not persist in the gut. Total numbers of bifidobacteria increased and clostridia (but not enterobacteria) decreased at the end of the study for all subjects irrespective of when they consumed the synbiotic yogurt or placebo (acifidied milk). This novel finding may reflect a general change of eating habits of subjects over the course of a long probiotic feeding study. It was also evident that subjects that had lower starting bifidobacterial levels or higher starting clostridial levels benefited the most (by increasing total numbers of bifidobacteria or decreasing numbers of clostridia) from ingesting the supplemented yogurt. However, there were no significant differences in enterobacterial numbers between the synbiotic yogurt and placebo feeding periods even in the subgroup of subjects that had higher initial levels of enterobacteria. These data would therefore support the idea that ingestion of this supplemented yogurt does not statistically modulate enterobacterial numbers but may modulate total bifidobacterial and clostridial numbers in people with either below-average levels of bifidobacteria or above-average levels of clostridia.

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