Assessment of the safety, tolerance, and protective effect against diarrhea of infant formulas containing mixtures of probiotics or probiotics and prebiotics in a randomized controlled trial[1−4]

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ABSTRACT

Background: Probiotics and prebiotics are considered to be beneficial to the gastrointestinal health of infants.

Objective: The objective was to evaluate infant formulas containing probiotics and synbiotics (combinations of probiotics and prebiotics) for safety and tolerance.

Design: In a prospective, controlled, double-blind, randomized trial, healthy full-term infants were exclusively fed a control formula or study formulas containing *Bifidobacterium longum* BL999 (BL999) + *Lactobacillus rhamnosus* LPR (LPR), BL999 + LPR + 4 g/L of 90% galactooligosaccharide/10% short-chain fructooligosaccharide (GOS/SCFOS), or BL999 + *Lactobacillus paracasei* ST11 (ST11) + 4 g/L GOS/SCFOS from ≤2 to 16 wk of age (treatment period). Safety and tolerance were assessed based on weight gain during the treatment period (primary outcome) as well as recumbent length, head circumference, digestive tolerance, and adverse events (secondary outcomes), which were evaluated at 2, 4, 8, 12, 16, and 52 wk of age.

Results: Two hundred eighty-four infants were enrolled. During the treatment period, difference in mean weight gain between control and study formula groups in both the intention-to-treat and per-protocol populations were within the predefined equivalence boundaries of ±3.9 g/d, indicating equivalent weight gain. Secondary outcomes did not show significant differences between groups during the treatment period.

Conclusion: Infants fed formulas containing probiotics or synbiotics show a similar rate in weight gain compared with those fed a control formula and tolerate these formulas well. *Am J Clin Nutr* 2008;87:1365–73.

INTRODUCTION

Breastfed infants are generally healthier than formula-fed infants, especially with respect to their ability to fend off infections (1). Some of the health benefits of human milk have been attributed partly to factors that modulate the development of a normal gut microbiota (1). These factors, which include complex oligosaccharides, are thought to selectively stimulate the growth of bacteria considered to be beneficial, such as bifidobacteria and lactobacilli (2, 3), and inhibit the growth of potentially pathogenic bacteria (4, 5).

Hence, the development of improved infant formulas has focused on emulating the beneficial effects of breast milk by, among other approaches, supplementing formulas with specific probiotics or oligosaccharides (prebiotics) that selectively stimulate the growth or metabolic activity of potentially beneficial indigenous bacteria such as bifidobacteria. A number of clinical studies in which the formula of infants was supplemented with probiotics suggest that some probiotics may indeed have beneficial effects in managing and preventing gastrointestinal (GI) infections and diarrhea (6–10), prevent the onset of allergy, and be useful in the treatment of atopic disease (11, 12). The potential benefits of prebiotics are multifaceted; in addition to enhancing growth of indigenous bacteria, prebiotics are fermented in the large intestine, yielding short-chain fatty acids (SCFAs). These create an acidic environment that inhibits growth of potentially pathogenic bacteria (13) and also serve as an energy source for the gut epithelium (14). Furthermore, it has also been suggested that SCFAs aid the absorption of magnesium, calcium, and iron (15, 16).

The combination of probiotics and prebiotics (synbiotics) has been proposed to have a synergistic effect by both ensuring survival of delivered probiotics and stimulating the growth of selected indigenous bacteria (13, 17). However, clinical studies showing the effects of feeding synbiotics to infants are scarce. A recent study (18) has shown that a standard infant formula supplemented with a mixture of a probiotic (*Bifidobacterium longum* BL999) with the prebiotics galactooligosaccharide (GOS) and short-chain fructooligosaccharide (SCFOS) is safe and well tolerated by healthy infants. In the current study, we aimed to systematically evaluate 3 formulas containing different mixtures of the probiotics *B. longum* BL999, *Lactobacillus rhamnosus* LPR, and *Lactobacillus paracasei* ST11 as well as a mixture of

probiotics and the prebiotics GOS and SCFOS for their safety and effect on the incidence of diarrhea in infants. The benefit of *L. rhamnosus* LPR in the prevention and treatment of diarrhea is well documented in clinical studies (19) and has been reviewed (9, 20, 21). *B. longum* BL999 is a human GI tract isolate that is currently consumed as part of some milk-based products. It has been used safely in clinical studies in both infants and adults and has been shown to transiently colonize the gut of infants after antibiotic treatment without causing any side effects (22–24). Finally, *L. paracasei* ST11, an isolate from a healthy infant, has also been safely used in infants and has been shown to be useful in ameliorating nonrotavirus-induced diarrhea (25, 26).

SUBJECTS AND METHODS

Subjects

Subjects were healthy infants recruited from mothers who had chosen not to breastfeed and had decided to feed their infants exclusively with formula from the time of enrollment until infants were at least 16 wk old. To be included in the study, infants had to be healthy, full-term (gestational age between 37 and 42 wk), ≤14 d old, singletons, and weigh between 2500 and 4500 g. Infants were excluded from the study if they had major deformities or cardiovascular, GI, renal, neurological, or metabolic illnesses; had required hospital admission for intensive care or for ≥3 d; were born to mothers with diabetes; or if their parents were expected to have difficulty complying with the feeding regimen.

This study was conducted in accordance with the principles and rules of the Declaration of Helsinki and adhered to Good Clinical Practice guidelines of the International Conference on Harmonization. It was approved by the Ethics Committee of Grenoble (7 April 2004), and written informed consents were signed by parents or legal guardians before enrollment.

Trial design

The current study was a prospective, double-blind, reference-controlled, parallel-group, randomized trial, conducted in 5 centers in France: CHU of Grenoble; Hôpital de la Croix Rousse, Lyon; Service de Néonatologie, Maternité Régionale, Nancy; Hôpital d'Enfants Armand Trousseau, Paris; and Service de Néonatologie, Hôpital de La Conception, Marseille. The total treatment period lasted ≈8 mo (15 October 2004 to 21 June 2005), and each infant was treated for 14–16 wk.

On enrollment, infants were assigned into 1 of 4 groups by block randomization using sex and center as strata. Formulas were coded by the sponsor, and both the investigators and the infants' parents were blinded to the formulas. Each group received either a control formula lacking any probiotics or prebiotics, or one of 3 study formulas containing different mixtures of probiotics or synbiotics: *B. longum* BL999 and *L. rhamnosus* LPR (group BL999 + LPR); *B. longum* BL999, *L. rhamnosus* LPR, and GOS/SCFOS, (group BL999 + LPR + GOS/SCFOS); or *B. longum* BL999, *L. paracasei* ST11, and GOS/SCFOS (group BL999 + ST11 + GOS/SCFOS). Baseline measurements were taken at enrollment, and parents received the corresponding formulas for their infants, along with instructions on preparation and feeding. During the treatment period (0–4 mo) infants were fed ad libitum exclusively with their allocated formulas starting on the day of enrollment (1–14 d of age) through 112 d (16 wk) of age. Follow-up visits took place when infants were 11–17, 28–42, 49–63, 77–91, 105–119, and ≈365 d old (2, 4, 8, 12, 16 and 52 wk, respectively). The observational period consisted of data from 16 to 52 wk (4 to12 mo).

Parents kept daily records of the infants' intake of formula, supplementary foods, and any medication or treatments. Three days before and following a visit, parents also recorded stool characteristics, frequency of flatulence, and behavior of infants. At each visit investigators took anthropomorphic measurements; reviewed compliance (based on diet records kept by parents and by comparing the number of cans of formula issued with the number remaining at each visit), intake, and infants' tolerance (based on records kept by the parents); and assessed any incidents of morbidity.

Formulas and bacterial strains

All of the formulas were powdered starter formulas intended for full nutritional support of infants from birth to 6 mo and contained the same amounts of protein, carbohydrate, fat, vitamins, minerals, and long-chain polyunsaturated fatty acids. The control formula was unsupplemented formula (Nan; Nestec SA, Konolfingen, Switzerland), whereas the study formulas were control formula supplemented with mixtures of probiotic bacteria strains or probiotics and prebiotics (**Table 1**). The probiotic strains were *B. longum* BL999 (ATCC: BAA-999 designation BB536, Morinaga, Japan), *L. rhamnosus* LPR (CGMCC 1.3724), and *L. paracasei* ST11 (CNCM I-2116), with the number of colony-forming units in each formula shown in Table 1. The prebiotic mixture (0.4 g/100 mL) contained 90% GOS and 10% SCFOS. All formulas were manufactured according to Good Manufacturing Practices and packaged by the sponsor.

TABLE 1
Probiotic and prebiotic composition of formulas in the different study groups

Formula group	*B. longum* BL999[1]	*L. rhamnosus* LPR[1]	*L. paracasei* ST11[1]	90% GOS/10% SCFOS[2]
	CFU/100mL	*CFU/100mL*	*CFU/100mL*	*g/100mL*
Control	—	—	—	—
BL999 + LPR	1.29×10^8	6.45×10^8	—	—
BL999 + LPR + GOS/SCFOS	1.29×10^8	6.45×10^8	—	0.4
BL999 + ST11 + GOS/SCFOS	2.58×10^8	—	2.58×10^8	0.4

[1] Colony forming unit (CFU) per 100 mL of reconstituted formula.
[2] Grams per 100 mL of reconstituted formula.

Measurements

The primary outcome was weight gain in infants fed the study formulas from ≤14 to 112 d of age. The secondary outcomes were recumbent length, head circumference, symptoms of digestive tolerance (GI symptoms, which included frequency of flatulence, colic, spitting up, vomiting, and diarrhea and stool characteristics), and frequency of adverse events (AEs) during the treatment period. Additionally, incidence of diarrhea, antibiotic intake, and hospitalization during the observational period were assessed. Data for the treatment period were based on AE forms filled out by investigators and, for the observational period, on each infant's personal health record book (carnet de santé) completed by the practitioner.

Investigators measured the weight of naked infants on calibrated electronic scales. Measurements were taken twice during each visit (at the beginning and end of the visits) to the nearest 10 g, and the mean was reported. Investigators also measured recumbent length to the nearest 2 mm on standardized length boards and head circumference to the nearest 2 mm with standard measuring tapes. All investigators had the same brand of scales, length boards, and measuring tapes.

Mean formula intake (mL/d) and measurements of digestive tolerance were calculated based on the parents' records. Digestive tolerance consisted of records of stool frequency and consistency (hard, formed, soft, or liquid); frequency of flatulence (occasional if ≤5/d or often if >5/d); frequency of vomiting and colic (none, once, or more than twice per day); and the occurrence of spitting up. Stool frequency was reported as mean occurrence/d, and all other measurements of tolerance were reported as the percentage of occurrence of a symptom/characteristic. The investigators assessed AEs and the records of digestive tolerance at each visit.

An incident of diarrhea was recorded for infants with at least 1 diarrhea episode. Diarrhea was defined as the occurrence of 3 or more loose or watery stools in 24 h, and an episode of diarrhea was considered to have ended when there were 2 consecutive nonwatery stools or no stools in 24 h.

AEs

AEs were defined as illnesses or signs or symptoms of illnesses (including abnormal laboratory measurements) that occurred or worsened during the course of the study, and they were assessed based on inquiries to the parents and on their daily records. All AEs were recorded and evaluated by the investigators for causality and severity. AEs were assessed as serious if they were life threatening, caused permanent harm, resulted in hospitalization or extension of in-patient hospital treatment, or were considered to be medically relevant by the investigator. All other AEs were categorized as nonserious. AEs were coded using the Medical Dictionary for Regulatory Activities, and analysis was performed for all enrolled subjects.

Statistics

Sample-size calculation was based on the primary outcome, weight gain during the first 4 mo of life (14–112 d). The objective was to show equivalence in weight gain using the $\Delta = 3.9$ g/d margin of equivalence (a convention used in previous trials) (18). This is the largest acceptable difference, and any difference greater than this was considered to be clinically relevant. To show weight gain as significant on an α-level of 5%, the two-sided 90% CI of the treatment difference had to fall entirely in the −3.9 to +3.9 g/d range. The expected SD of weight gain was taken from a previous trial, with $\sigma = 6.1$ g/d (18). The sample size was calculated with $n_i = 55$ per group to maintain a power of 90%. Taking a dropout rate of 25% into account, 296 infants had to be enrolled. The control group was made up of infants recruited for this study and was an entirely different group from infants in the study by Puccio et al (18). The sample-size calculation was performed with PASS 2000 (Number Cruncher Statistical Systems, Kaysville, UT).

For the treatment period (0–4 mo), data from all randomly assigned infants were used in the intention-to-treat (ITT) analysis. The per-protocol (PP) analysis also included only data from the treatment period but excluded data from subjects if, during the study, they had a life-threatening event, were hospitalized >3 days, were off the study formula >3 consecutive days, were fed >1 bottle/wk of a different formula, or took complementary food. Primary outcome was analyzed in both the ITT and PP populations. Secondary outcomes were analyzed in the PP population, and safety in the ITT population. For the observational period (4–12 mo), only data from infants who followed up to 12 mo were analyzed.

Changes in weight (weight gain), recumbent length, and head circumference were analyzed by a mixed model correcting for sex and center. The mixed model describes the development of weight, length, and head circumference over time by a quadratic curve, taking into account each subject's intercept and slope (random effects). The mixed model was inquired for the slope in the middle (day 63) of the treatment period (day 14 to day 112). The slope at day 63 equaled the weight gain, calculated by difference in weight divided by the difference in age (from day 14 to day 112). The mixed model was used rather than the analysis of variance (ANOVA) because it does not have the problem of meeting the exact target ages (which varied because of differences in the time of visit); takes into account all weight measurements (at 14, 28, 56, and 112 days); and is robust against dropouts. CI of treatment differences were adjusted for multiple comparisons according to Bretz et al (27). Weight-for-age, length-for-age, and BMI-for-age *z*-scores were calculated based on the WHO Child Growth Standard (28), which is based on data from exclusively breastfed infants.

Formula intake was analyzed by a mixed model, and a general treatment effect was assessed by a likelihood ratio test. Stool consistency, flatulence, colic, spitting up, vomiting, incidence of diarrhea, antibiotic intake, and hospitalization were analyzed by logistic regression, and treatment effects were presented as odds ratios. Multiple comparisons were adjusted according to Hommel (29). For stool frequency, differences between treatment groups were analyzed by ANOVA and adjusted for multiple tests by Tukey-Kramer. Incidence of infants experiencing at least 1 AE was analyzed by logistic regression, correcting for center.

Age of terminating assigned formulas was analyzed by ANOVA. Treatment differences between control and experimental groups were determined and *P* values were adjusted according to the Dunnett test. ANOVA and logistic regression were performed with SAS (SAS Institute Inc, Cary, NC) and the mixed models with R 2.1.1 (The R Foundation for Statistical Computing, Vienna, Austria).
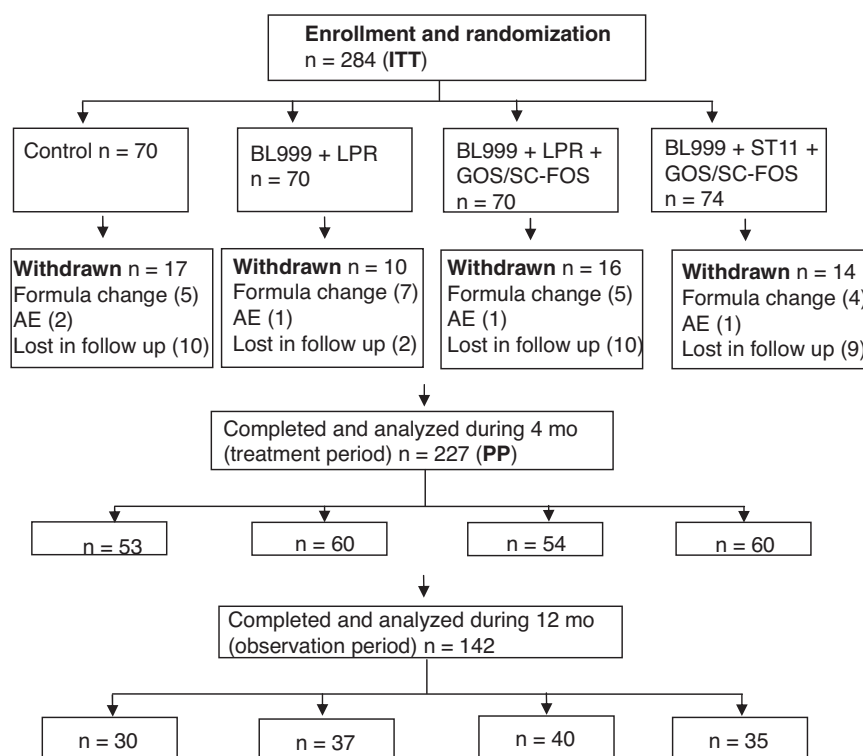
FIGURE 1. Flow chart of progression of infants during the study.

RESULTS

Subjects

Two-hundred eighty-four healthy infants were enrolled and constituted the ITT population. A total of 57 infants (20%) dropped out by the end of the treatment period (at 4 mo of age), resulting in a PP population of 227. Dropouts were due to change of formula ($n = 21$), adverse events ($n = 5$), and loss in follow-up ($n = 31$) (**Figure 1**). From 4 to 12 mo of age (observational period), an additional 85 infants were lost to follow-up. There were no significant differences in the dropout rates between groups (Kruskal-Wallis test, $P > 0.1$). Baseline characteristics, other than the mode of birth, were comparable between groups (**Table 2**). More natural births were reported in the BL999 +

LPR + GOS/SCFOS group compared with the other groups ($P = 0.034$).

Formula intake and growth

Infants in the different groups consumed similar quantities of the study formulas during the treatment period (0–4 mo) (**Figure 2**). The age at which feeding with the assigned formulas was terminated was significantly later in the BL999 + LPR group compared with the control group (mean age 6.56 ± 1.2 mo compared with 5.84 ± 0.7 mo, $P = 0.045$). However, there were no significant differences between the other study formula groups and the control group.

Weight gain during the treatment period was equivalent among infants in the different formula groups (**Table 3**). A

TABLE 2
Baseline demographic data of infants by treatment group (per protocol data set $n = 227$)[1]

	Control ($n = 53$)	BL999 + LPR ($n = 60$)	BL999 + LPR + GOS/SCFOS ($n = 54$)	BL999 + ST11 + GOS/SCFOS ($n = 60$)	P values (chi-square test or ANOVA)
Sex: Boy/Girl					
n	25/28	29/30	28/26	28/32	0.95
(%)	47/53	49/51	52/48	47/53	
Birth	42/10	46/12	51/2	44/14	0.025
Natural/Cesarean (%)	81/19	79/21	96/4	76/24	
Gestational age (wk)	39.7 ± 1.3	39.5 ± 1.2	39.5 ± 1.2	39.5 ± 1.1	0.38
Weight at birth (kg)	3.4 ± 0.3	3.4 ± 0.4	3.4 ± 0.5	3.4 ± 0.4	1.0
Length at birth (cm)	$50.0 \pm .9$	49.8 ± 1.9	50.3 ± 1.8	49.8 ± 2.0	0.57
Head circumference at birth (cm)	34.8 ± 1.3	34.6 ± 1.3	34.9 ± 1.4	34.6 ± 1.3	0.42

[1] Number of infants and percentages or mean ($\pm$SD). More natural births were reported in the BL999 + LPR + GOS/SCFOS group compared to the other groups (test on proportions: $P = 0.034$).
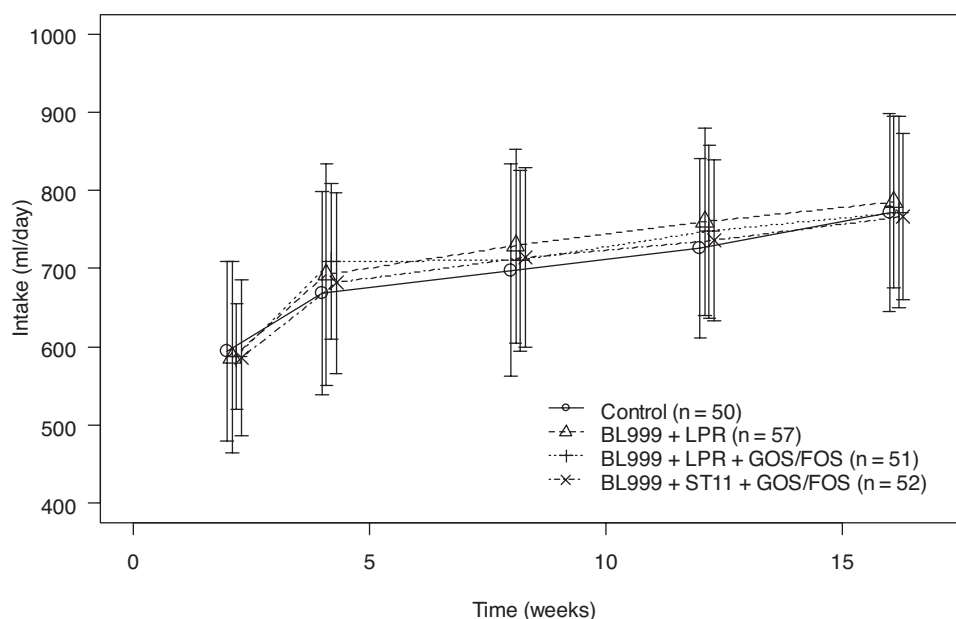
FIGURE 2. Volume of daily formula intake ($\bar{x} \pm$ SD) during the treatment period (PP population).

comparison of the mean weight gain per day of infants fed each of the study formulas with that of infants fed the control formula (PP population) showed a treatment effect of 1.41 (90% CI: −0.77 to 3.59) in the BL999 + LPR group; 0.82 (90% CI: −1.40 to 3.05) in the BL999 + LPR + GOS/SCFOS group; and 1.64 (90% CI: −0.53 to 3.81) in the BL999 + ST11 + GOS/SCFOS group. In all cases the two-sided 90% CI interval lay between −3.9 and + 3.9 g/d, indicating equivalent weight gain in infants fed the control and study formulas. Furthermore, the upper bound of the two-sided 90% CI was above −3 g/d in all comparisons, fulfilling the safety criteria of the American Academy of Pediatrics (30). Analysis of the ITT data set showed similar results (data not shown).

Mean changes in length, head circumference, and BMI measurements during the treatment period were not different between all of the study formula groups and the control group ($P >$ 0.05 for all comparisons, Table 3). A comparison of weight and length z-scores with the WHO multicenter growth reference (28) showed that, among infants who completed the study to 12 mo, z-scores were close to 0 at all times during the study (**Figure 3**). Z-scores were not different from the WHO growth reference for all groups in the PP population followed through the treatment period (0–4 mo), and no significant differences were observed

between the control and any of the study formula groups (data not shown). Comparison with Euro-growth reference for head circumference (31, 32) (WHO standards for head circumference were unavailable) also showed that z-scores were not different from the standard (data not shown).

Safety and tolerance

The incidence of diarrhea during the treatment period was not different between the control and study formula groups (**Table 4**). However, during the observation period, the incidence of diarrhea was significantly lower in the BL999 + LPR group compared with the control group (5/37 compared with 13/30). The frequency of antibiotic treatment or hospitalization during this period did not vary among groups (Table 4).

During the treatment period, stool frequency was significantly higher among infants in the BL999 + LPR + GOS/SCFOS group compared with those in the control group ($\bar{x}$ frequency 2.1/d compared with 1.6/d, $P = 0.03$). Although there was not a significant difference in stool frequency between the other study formula groups and the control group, infants in the BL999 + ST11 + GOS/SCFOS group tended to have higher but not significantly different stool frequencies than those in the control group (mean 2.0 versus 1.6/d, $P = 0.06$). Furthermore, infants in

TABLE 3
Mean ($\pm$SD) weight gain and changes in length, head circumference, and body mass index (BMI) during the treatment period (0–4 mo) in the per protocol population[1]

	Boys				Girls			
Formula group	C ($n = 25$)	B + L ($n = 29$)	B + L + G/F ($n = 28$)	B + S + G/F ($n = 28$)	C ($n = 28$)	B + L ($n = 30$)	B + L + G/F ($n = 26$)	B + S + G/F ($n = 32$)
Weight gain (g/d)	30.9 ± 6.1	31.9 ± 6.0	31.5 ± 5.9	32.1 ± 5.9	26.9 ± 6.0	27.9 ± 6.0	27.5 ± 6.0	28.1 ± 6.0
Length (mm/mo)	32.6 ± 3.6	33.4 ± 3.7	33.1 ± 3.7	34.2 ± 3.6	31.2 ± 3.7	32.0 ± 3.8	31.7 ± 3.6	32.7 ± 3.8
Head circumference (mm/mo)	18.4 ± 2.3	18.7 ± 2.4	17.9 ± 2.3	18.3 ± 2.3	16.7 ± 2.4	17.0 ± 2.4	16.2 ± 2.3	16.6 ± 2.4
BMI (kg · m⁻² · mo⁻¹)	1.1 ± 0.4	1.2 ± 0.4	1.2 ± 0.4	1.2 ± 0.4	0.9 ± 0.4	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.4

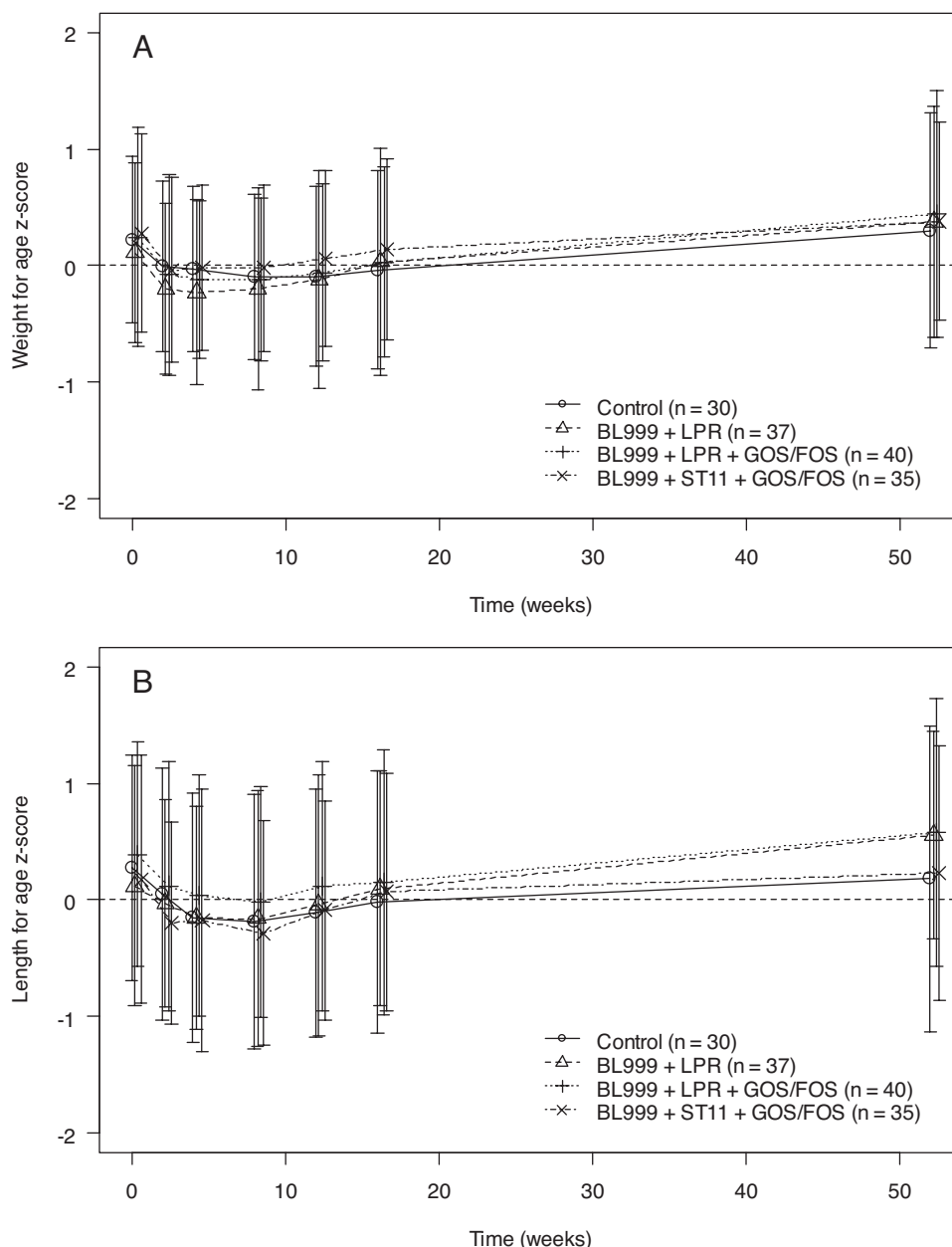[1] C, Control; B + L, BL999 + LPR; B + L + G/F, BL999 + LPR + GOS/SCFOS; B + S + G/F, BL999 + ST11 + GOS/SCFOS.

FIGURE 3. Weight- and length-for-age *z*-scores ($\bar{x} \pm$ SD), relative to WHO standards, of the subset of infants who completed the study to 12 mo. Treatment period of 2–16 wk and observation period of 52 wk.

the BL999 + LPR + GOS/SCFOS group had higher stool frequency compared with those in the BL999 + LPR group, but this difference was not significant ($\bar{x}$ 2.1/d compared with 1.7/d, $P = 0.07$).

During the treatment period, liquid stools occurred significantly more frequently in the BL999 + ST11 + GOS/SCFOS group than in both the control group (odds ratio = 3.17, 95% CI: 1.59 to 6.30, $P = 0.005$) and the BL999 + LPR group (odds ratio = 2.79, 95% CI: 1.48 to 5.29, $P = 0.008$). The difference in the frequency of liquid stools between any of the other groups was not significant. Furthermore, the frequency with which the other stool consistencies (soft, formed, hard) occurred in all groups

were not significantly different. The frequency with which flatulence, colic, spitting up, and vomiting occurred were not significantly different in the control and study formula groups ($P > 0.1$ for all).

The occurrence of at least 1 serious AE (SAE) was reported in 24 infants (**Table 5**), and at least 1 AE was reported in 184 infants during treatment period. There were no significant differences in the frequency of either SAEs or AEs between the different groups. Two SAEs involving cow's milk protein allergy was assessed as certainly and possibly related to the study formula (both in the BL999 + LPR group). Two others, a case of diarrhea and a case of gastroesophageal reflux disease, were considered to

TABLE 4
Incidence (*i*) of diarrhea during the treatment period (0–4 mo) in the ITT population and during the observational period (4–12 mo) in infants who were followed up to 1 y

Period	Condition	Control		BL999 + LPR		BL999 + LPR + GOS/SCFOS		BL999 + ST11 + GOS/SCFOS	
		n	*i* (%)	*n*	*i* (%)	*n*	*i* (%)	*n*	*i* (%)
Treatment	Diarrhea	59	3 (5)	64	4 (6)	58	4 (6)	66	3 (4)
Observational	Diarrhea	30	13 (43)	37	5 (13)[1]	39	10 (25)	35	8 (22)
Observational	Antibiotics	30	14 (46)	37	18 (48)	39	16 (42)	35	18 (51)
Observational	Hospitalization	30	1 (3)	37	1 (2)	39	2 (5)	35	0 (0)

[1] Significant difference with control group, logistical regression $P = 0.03$.

be probably related to the formula (both in the control group). The rest of the SAEs (except 4 whose causality were not assessed by the investigators) were considered to be unrelated or unlikely to be related to the study products. Most (78%) of the AEs were respiratory and GI problems (including allergies) and infections.

DISCUSSION

In this study we evaluated the safety of 3 infant formulas that were supplemented with probiotics or synbiotics. The primary outcome, weight gain following 14–16 wk of formula feeding (treatment period), was equivalent in the control and study formula groups. Similarly, changes in length, head circumference, and BMI between 0 and 4 mo of age were similar in all study groups. Employing additional measures of safety and tolerance, we also evaluated the occurrence of various GI symptoms, including diarrhea and the need for antibiotic treatment or hospitalization during the treatment (0–4 mo) and observational (4–12 mo) periods. During both periods infants in all groups grew normally; weight-for-age and length-for-age *z*-scores indicated growth rates comparable to WHO growth standards. Because the WHO growth standards are based on healthy, exclusively breastfed infants, these results are a good indication of the nutritional sufficiency of these formulas, though no direct comparison of the value of these formulas with breastfeeding can be

made based on our data. Although not statistically significant, the differences in *z*-scores for length at 12 mo suggest that there might be a difference in the effect of the 2 formulas containing LPR compared with the control. However, our study was not designed to detect this difference, and future studies with significantly more subjects (424 per group to see a difference of 0.87 mm/mo, the difference between the BL999 + LPR and control groups) will have to be performed to determine whether there is a real effect of LPR on length. Furthermore, all of the comparisons in weight gain between study formula and control groups showed a trend toward greater weight gain in the former. To see a difference in weight-for-age *z*-scores of 0.1 (which was the treatment difference between BL999 + LPR and control group after 1 year) as significant with a power of 80%, a new trial with 2654 infants per group would have to be conducted. A recent study (33) specifically evaluating the growth of infants fed formula containing *L. rhamnosus* has shown that these infants gained weight at a significantly faster rate compared with those who were fed a control formula. Whether *L. rhamnosus* leads to increased weight gain needs to be confirmed, but these results indicate its safety in healthy infants.

Interestingly, whereas during the treatment period there were no differences in the frequency of symptoms of GI intolerance, diarrhea, and other AEs between infants in any of the study

TABLE 5
Number (%) of serious adverse events (SAE) presented by preferred term based on coding by MedDRA

SAE	Control (*n* = 70)	BL999 + LPR (*n* = 70)	BL999 + LPR + GOS/SCFOS (*n* = 70)	BL999 + ST11 + GOS/SCFOS (*n* = 74)
			n (%)	
Gastroenteritis		1 (1.4)		
Gastroesophageal reflux disease	1 (1.4)			
Diarrhea	1 (1.4)	2 (2.9)	1 (1.4)	
Milk allergy		2 (2.9)		
Vomiting	1 (1.4)			2 (2.7)
Febrile infection	1 (1.4)	1 (1.4)		
Surgery			1 (1.4)	
Pyrexia	2 (2.9)			
Rectal hemorrhage		1 (1.4)		
Pyelonephritis		1 (1.4)		1 (1.4)
Bronchiolitis		2 (2.9)	3 (4.3)	1 (1.4)
Cough		1 (1.4)		
Drug toxicity	1 (1.4)			
Inguinal hernia			2 (2.9)	
Total	7 (10)	11 (15.7)	7 (10)	4 (5.4)

[1] Data refer to the intent-to-treat population during the treatment (0–4 mo) period. A total of 24 infants had one or more SAE.

formula groups and those in the control formula group, at the 1-year follow-up, infants in the group that received the formula containing *B. longum* BL999 and *L. rhamnosus* LPR (BL999 + LPR) had significantly fewer incidents of diarrhea. It is intriguing and, to our knowledge, novel that the decrease in the incidence of diarrhea was observed several months after infants had stopped taking the probiotic-supplemented formula. Even though infants in the BL999 + LPR group continued to take their assigned formula longer than infants in the control group, they had stopped taking the probiotic-supplemented formula by the time they were, on average, 6.5 ± 1.2 mo old. Therefore, this finding suggests that the effect of the probiotics in this group may have lasted an average of 5.5 ± 0.7 mo after the termination of administration. Because other possible confounding factors during the observational period (such as diet) were not studied here, it is not possible to attribute our observation solely to the effect of probiotics.

There are numerous studies demonstrating the efficacy of probiotics in treating or preventing diarrhea (6, 7, 9, 20, 21), including during antibiotic treatment (34). However, the effect of probiotics following an extended period after intake has been terminated has not yet been reported. Although our results will have to be confirmed in future studies aimed specifically at addressing the long-term effects of probiotics (including microbiological analyses of stool samples during treatment and control for confounding factors), they do suggest that when intake of probiotics occurs early in an infant's life (2 wk or younger), there may be a long-term beneficial effect on the incidence of diarrhea. It is interesting to speculate that administering probiotics very early in the infants' lives, when their GI microbiota and immune system were still not fully developed, may have influenced their microbiota and primed their immune system. By being recognized as "nonself" by the immune system, probiotics could have acted as adjuvants, increasing the mucosal immune system activity and natural defenses, thereby affecting health at a later period. Alternatively, administering probiotics at this early stage may have influenced the development of the gut immune system and the maturation of the gut itself, which may then have contributed to the improved ability of infants to resist diarrhea later on. Unfortunately, microbiological analysis was not performed in this study so we cannot begin to address these possibilities here, but it will be an important aspect of future investigations. It is surprising that a similar effect among infants in the BL999 + LPR + GOS/SCFOS group was not observed. This finding may suggest a specific effect of either or both probiotic strains (*B. longum* BL999 or *L. rhamnosus* LPR). We hypothesize that in the BL999 + LPR + GOS/SCFOS group, the prebiotics mixture may have dampened the effect of the probiotics seen in the BL999 + LPR group by stimulating the growth of other components of the microbiota, thereby effectively reducing the relative proportion of the probiotics among the GI microbiota or hiding the role of a probiotic in the imprinting of the neonatal immune system. A recent publication by Ziegler et al (35) showed that a formula supplemented only with prebiotics led to significantly higher incidence of diarrhea in the short term (during 30–120 d of feeding) compared with an unsupplemented formula. This finding suggests that supplementation with specific probiotics may be more beneficial than stimulating the growth of a broader population of bacteria.

The tendency for infants fed prebiotics to have an increase in overall stool frequency and in the frequency of liquid stools, though statistically significant, bears little clinical relevance because these frequencies were all within the normal range for infants. Previous studies have shown that GOS/FOS causes a slight increase in stool frequency (36, 37) and frequency of loose stools (18, 35, 37, 38). However, it was also shown that there was no correlation between the increase in stool frequency and lower weight gain in at least 1 study (18).

Thus, this study confirms the safety of different mixtures of probiotics and synbiotics. It also raises the possibility that the specific mixture containing *B. longum* BL999 and *L. rhamnosus* LPR may have a prolonged effect in reducing the incidence of diarrhea.

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