Physiological and Bifidogenic Effects of Prebiotic Supplements in Infant Formulae

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ABSTRACT

Objectives: This randomized controlled trial involving 110 healthy neonates studied physiological and bifidogenic effects of galactooligosaccharides (GOS), oligofructose, and long-chain inulin (fructooligosaccharides, FOS) in formula.

Methods: Subjects were randomized to Orafti Synergy1 (50 oligofructose:50 FOS) 0.4 g/dL or 0.8 g/dL, GOS:FOS (90:10) 0.8 g/dL, or a standard formula according to Good Clinical Practice guidelines. A breast-fed group was included for comparison. Outcome parameters were weight, length, intake, stool characteristics, crying, regurgitation, vomiting, adverse events, and fecal bacterial population counts. Statistical analyses used nonparametric tests.

Results: During the first month of life, weight, length, intake, and crying increased significantly in all of the groups. Regurgitation and vomiting scores were low and similar. Stool frequency decreased significantly and similarly in all of the formula groups but was lower than in the breast-fed group. All of the prebiotic groups maintained soft stools, only slightly harder than those of breast-fed infants. The standard group had significantly harder stools at weeks 2 and 4 compared with 1 ($P < 0.001$ and $P = 0.0279$). The total number of fecal bacteria increased in all of the prebiotic groups (9.82, 9.73, and 9.91 to 10.34, 10.38, and 10.37, respectively, log10 cells/g feces, $P = 0.2298$) and more closely resembled the breast-fed pattern. Numbers of lactic acid bacteria, bacteroides, and clostridia were comparable. In the SYN1 0.8 g/dL and GOS:FOS groups, Bifidobacterium counts were significantly higher at D14 and 28 compared with D3 and were comparable with the breast-fed group. Tolerance and growth were normal.

Conclusions: Stool consistency and bacterial composition of infants taking SYN1 0.8 g/dL or GOS:FOS–supplemented formula were closer to the breast-fed pattern. There was no risk of dehydration.

Key Words: galactooligosaccharides, gastrointestinal microbiota, infants, inulin, oligofructose, pediatrics, prebiotics, stool consistency

Infants' gastrointestinal (GI) microbiota, an important constituent of the gut defense barrier, is an obvious target for the development of functional foods because the gut microbiota affects immunological homeostasis of the host (1,2). Before birth, the GI tract is generally considered to be sterile. Massive and rapid colonization of the neonatal digestive tract by microorganisms is part of the adaptation to extrauterine life. Early bacterial colonization plays a crucial role in the development of the innate and adaptive immune system (3). Bacterial–epithelial cell cross-talk is necessary early in life for the development of normal host defenses (4). Colonizing bacteria (ie, bifidobacteria) stimulate enterocytes or lymphoid elements, promoting a T-helper 1 response and restoring a balance toward tolerance (5,6). The first 2 days after birth are characterized by the predominance of a few bacteria, for example, *Escherichia coli* and *Enterococcus*. At the end of the first week, an equilibrium is found depending on the type of feeding.

Eighty-five percent of breast-fed infants harbor bifidobacteria as predominant microorganisms. Mother's milk is the infant's natural and optimal nutrition. In addition to its numerous nutritional and psychological advantages, human milk protects against infections (7) and the development of atopy (8). Human milk stimulates the growth of bifidobacteria because of its high oligosaccharides (10–12 g/L) content (9). These oligosaccharides are predominantly neutral, low-molecular-weight molecules, whose composition depends on the Lewis blood group of the mother. Exclusively breast-fed infants harbor a bifidus-predominant GI microbiota (10). Upon the introduction of formula, the microbiota diversifies, which is reflected by alterations of stool color, consistency, and odor. Formula-fed babies harbor a varied microbiota consisting of bifidobacteria, *E coli*, and bacteroides (11–13). After weaning, the fecal microbiota resembles the adult (climax) microbiota (14).

A major role of the GI microbiota is the protection of the newborn against invasion by pathogenic microorganisms. However, various dietary and environmental stresses, infections, and antibiotics may cause changes in the gut microbiota throughout life. A new trend has therefore arisen to produce foods that stimulate the growth or activity of beneficial microorganisms in the GI tract. Pro- and prebiotics are dietary supplements designed to modulate the composition of the gut microbiota in a manner deemed to confer health advantages on the host (15,16). Prebiotics are selective substrates for beneficial bacteria in the colon that are neither hydrolyzed nor absorbed in the upper GI tract, and are able to alter

the colonic microbiota in favor of a healthier composition. Examples of prebiotics include galactooligosaccharides (GOS) and inulin-type fructans: oligofructose (DP 2–8) and long-chain inulin (DP 2–60) (fructooligosaccharides, FOS) (17). The first prebiotics added to infant food in Europe were a combination of 10% inulin with 2 to 60 fructose monomers (called FOS for phonetic reasons) and 90% galactooligosaccharides 2 to 7 monomers (called GOS). Studies with concentrations of 0.4 and 0.8 g GOS:FOS added to 100 mL of infant formula demonstrated bifidogenic effects (18). The higher-concentration GOS:FOS softened stools but showed no side effects (1,19,20). Orafti Synergy1 (SYN1) (Beneo-Orafti, Oreye, Belgium) is a combination of chicory inulin molecules with selected chain lengths, enriched by a specific fraction of oligofructose produced by partial enzymatic hydrolysis of chicory inulin. It consists of 50:50 oligofructose and long-chain inulin.

The objective of the present study was to assess physiological parameters (safety and tolerance) and bifidogenic effects of different doses of SYN1 on the fecal microbiota of healthy neonates in comparison with standard formula, GOS:FOS–supplemented formula, and breast milk.

METHODS

Study Design

A 28-day prospective, randomized, double-blind, 2-center clinical trial including 5 different feeding groups was conducted by the Division of Pediatric Gastroenterology and Nutrition, Queen Paola Children's Hospital ZNA, Antwerp, in 2 neonatology units: the maternity units of the Middelheim Hospital ZNA and University Hospital Antwerp, Belgium. The internal review boards of both institutions reviewed and accepted the protocol and informed consents. Inclusion was conditional on obtaining informed consent from both parents.

Study Population

One control group consisted of breast-fed babies. Mothers of exclusively breast-fed babies were approached to participate in this control group. To strictly avoid any interference with the mother's decision for breast-feeding, only those mothers who had decided to use formula during their stay in the maternity unit were approached for recruitment to the study. Inclusion in the study was possible up to the fifth day of the infant's life. Subjects were recruited before discharge and were followed at home. Only term, healthy babies with exclusive formula feeding and normal feeding behavior were included. Infants were excluded from the study if they were born by cesarean section, and/or had respiratory, neurological, or GI problems, infections, use of antibiotics by the baby (including eye or nose drops) or by mother in the breast-fed group, fever, feeding problems, the need for therapeutic formulae (semi-elemental, hydrolyzed) or formula thickeners or cereals. All of the parents or legal guardians gave signed informed consent.

Test Products

All of the infant formulae used in the present study were prepared especially for the trial. The test product was produced by a company specializing in infant nutrition, under the authority of Beneo-Orafti. The test formula was based on the mandatory composition according to Directive 91/321/EEC on infant formulae and follow-on formulae. All of the products and production facilities complied with European Union quality directives. The basic formula to which the different prebiotics were added was a standard

infant formula with a composition identical to commercial products available in Belgium.

The GOS was a commerical mixture (Vivinal, Domo, Amersfoort, the Netherlands) that contains approximately 60% β-GOS and 40% digestible sugars (lactose, glucose, galactose). The GOS are composed of 1 terminal glucose unit that is essentially β(1-4)-linked with 1–7 galactose molecules. In the GOS:FOS formula, GOS was mixed in a proportion of 90:10 with long-chain inulin (Orafti HP) of β(2-1)-linked fructans starting essentially with a terminal glucose unit and 2 to 60 fructosyl residues.

Orafti Synergy1 (SYN1) is a patented 50:50 mixture of Orafti HP and oligofructose (β(2-1)-linked fructooligosaccharides with less than 9 fructose moieties and partially containing a terminal glucose unit.

GOS:FOS was added to a standard formula compliant with Directive 91/321/EEC to 0.8 g/dL. SYN1 was added to the same standard formula with either 0.4 or 0.8 g/dL.

Study products were packed in 450-g tin cans with appropriate instructions for use imprinted on them. The products were blind-coded and Beneo-Orafti kept the code until the end of the study. The infant formulae were provided to the parents free for the duration of the study. Parents of breast-fed babies received free diapers for a cost equaling the price of infant formula provided to the formula-fed groups.

Infants were randomly assigned to 1 of 4 formula-fed groups: standard formula without enrichment (control; n = 21), standard formula enriched with SYN1 0.4 g/dL (n = 21), standard formula enriched with SYN1 0.8 g/dL (n = 20), or standard formula enriched with GOS:FOS (90:10) 0.8 g/dL (n = 19). A breast-fed control group (n = 29) was also included.

Procedure

After inclusion, subjects were randomized to 1 of 4 formula groups. The goal was to include 20 subjects per group. The sponsor kept randomization codes and blind-coded the formula tins into 4 "color" groups. The study coordinator (S.S.) kept a computer-generated randomization list. Upon inclusion, subjects were sequentially allocated to a food color. The code could be broken only in cases of serious adverse event upon the request of the investigator. Because such events did not occur, all of the codes were broken after the study was terminated and statistical analyses were performed.

The test formulae were offered for every meal for 28 days. The subjects did not alter their usual feeding regimen during the study period. Each bottle was prepared by the mother according to European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines (21) as follows: 90 mL of water was heated in a bottle and 3 spoonfuls of 5 g (15 g/100 mL, 15% solution) formula powder were added and mixed by shaking.

Primary outcomes were to obtain physiological parameters and data on the fecal microbiota. Data were collected from the subjects upon enrollment in the study, and at days 14 and 28 of the study. A case report form was completed for each subject, including initials; date of birth; sex; gestational age; Appearance, Pulse, Grimace, Activity, Respiration (APGAR) score (at 1–10 minutes); antibiotic intake by mother for 4 weeks before delivery; perinatal data including mother's group B *Streptococcus* screening; mode of delivery (this was a double check because cesarean section was an exclusion criterion); biometry (weight, length, head circumference) at birth, and at days 14 and 28 of the study. A physical examination was performed at enrollment. Parents were called at the end of weeks 1 and 3 and visited at home at the end of weeks 2 and 4 of the study.

Parents agreed to keep a diary. They recorded the following information on days 1, 2, 3, and 12, 13, 14, and 26, 27, 28: formula intake, stool characteristics (frequency and consistency), crying behavior, and the occurrence of regurgitation (stomach contents returning into the mouth) or vomiting (forceful ejection of stomach contents through the mouth). Stool consistency was rated on a 1-to-4 scale with pictograms (1 = watery, 2 = soft, 3 = formed, 4 = hard). Crying behavior was rated as 1 = practically not crying, 2 = crying with respect to feeding, or 3 = crying excessively. Regurgitation was rated as 1 = no episodes, 2 = 1 or 2 episodes, or 3 = more than 2 episodes per day. Vomiting was rated as 1 = no episodes, 2 = 1 episode, or 3 = more than 1 episode per day. Any adverse events were recorded. Adverse event was defined as any untoward event in a study subject who received an investigational product and was handled according to Good Clinical Practice guidelines. Subjects were free to withdraw from the study at any time.

Stool samples were collected on day 3 and after 2 and 4 weeks of feeding (days 14 and 28). Plastic foil was put in the diapers to collect the stools, which were then kept frozen ($-20°C$).

Enumeration of Fecal Bacteria

Frozen fecal samples were transported to the University of Reading, where they were thawed on ice and processed for fluorescence in situ hybridization (FISH) analysis according to Waldram et al [22]. The nucleic acid stain DAPI (4′,6-diamidino-2-phenylindole) was used to enumerate total number of bacteria (DAPI). Cy3-labeled probes (Bif164, Bac303, Chis150, and Lab150) were used to enumerate specific bacterial populations: bifidobacteria (Bif164), bacteroides (Bac303), clostridia (Chis150), and lactic acid bacteria (Lab150). All of the probes were obtained from Sigma-Genosys (St Louis, MO). Details for the probes used in the present study can be found in Table 1; hybridization and washing conditions were as described in the literature.

Statistical Analyses

Statistical analyses were subcontracted to the independent Centre for Statistics, Limburgs Universitair Centrum, 3590 Diepenbeek, Belgium. Anthropometric data were summarized by their mean and standard deviation. Respective homogeneity of groups was tested using both nonparametric and parametric methods. For nonparametric tests, the Kruskal-Wallis test was used for group homogeneity, and in the case of significance, the Mann-Whitney test was used for pairwise group comparison. Multiway ANOVA was used as a parametric alternative to test for group homogeneity

while accounting for baseline covariates. This model is based on the normality and constant variance assumption. The normality assumption was investigated using the Shapiro-Wilk test and the Kolmogorov-Smirnov test. The Levene test was used to investigate the constant variance assumption.

To account for their nonnormal distribution, data for stool frequency and consistency were described as the median and interquartile ranges. Nonparametric and parametric methods were also used. For nonparametric tests, the Friedman test was used to test homogeneity of the groups and pairwise comparison in case of differences. For parametric methods, generalized estimating equations (23) were used with different distributions in the exponential family, depending on the response being analyzed. An exchangeable working correlation matrix was used.

All of the tests were performed on a ⟨level of 5%; *P* values <0.05 were considered significant. SAS/STAT version 9.1.3 software (SAS Institute, Cary, NC) was used.

RESULTS

Study Population

Figure 1 is a flowchart of enrolled infants and the number of infants who continued in the study providing data for the individual measurements at the end of the study. The clinical data of enrolled subjects are reviewed in Table 2. The number of male children was slightly higher than the number of female children; this trend was consistent across all of the feeding groups. Generally, most mothers neither had antibiotic intake 4 weeks before delivery nor tested positive for group B Streptococcus. The average weight, length, and head circumference at birth and gestational age were similar across all of the feeding groups. Some children were withdrawn from the study, thus leading to missing values (Table 3). The number of dropouts by day 28 is represented by the number of missing anthropometry data. Some parents did not fill out the diary correctly, leading to higher numbers for missing information on food intake. Because no measurements were obtained for food intake for children on breast milk, the food intake values are missing for all of the children in this group. Because missing values reduce the sample size of the data, nonparametric tests were used to test within-group homogeneity over time for all of the parameters reported below.

Adverse Events

No serious adverse events occurred throughout the study period. Infants were withdrawn from the study (dropouts) because of symptoms of gastroesophageal reflux, regurgitation, or hunger, for which their primary pediatrician recommended formula change.

TABLE 1. Details for the FISH stain and probes used in the present study (bacteriological marker and target bacteria)

Stain/probe name	Target bacteria
DAPI	Stain for all entities (eg, viruses, yeasts, bacteria) containing double-stranded DNA; used to enumerate total bacteria
Bac303	Most *Bacteroides* and *Prevotella* species, all *Parabacteroides* species, *Barnesiella viscericola,* and *Odoribacter splanchnicus*
Bif164	Most *Bifidobacterium* species and *Parascardovia denticolens*
Chis150	Most members of *Clostridium* cluster I and all of cluster II (including *C histolyticum* and *C perfringens*)
Lab158	Lactic acid bacteria: most *Lactobacillus* species, all *Vagococcus, Melisococcus, Tetragenococcus, Enterococcus, Catelicoccus, Pediococcus, Paralactobacillus,* and *Oenococcus* species, *Lactococcus lactis,* and most *Weissella* and *Leuconostoc* species

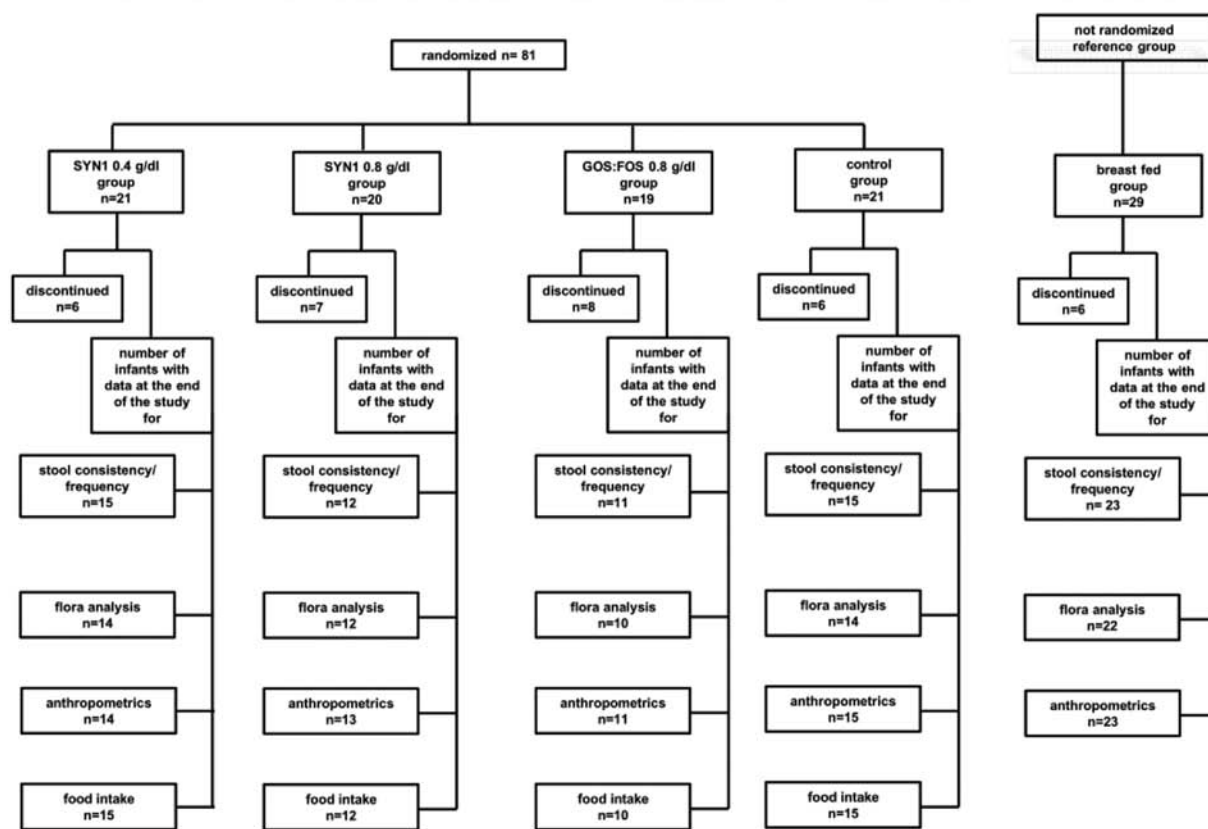DAPI = 4′,6-diamidino-2-phenylindole.

FIGURE 1. Enrolled infants, dropouts, and data available for day 28.

The number of withdrawn subjects was evenly distributed over the feeding groups, suggesting that the adverse events were not related to the use of FOS.

Anthropometry

Length, weight, and head circumference of the children at birth were comparable between the different supplementary groups. Also, supplementation of formulae had no influence on growth (length and weight gain) of children during the period of the study. Weight at birth was positively associated with length at birth and head circumference (all $P < 0.05$, multiple ANOVA, Kruskal-Wallis). Weight increased significantly with time (days) and was similar across all of the feeding groups ($P > 0.05$); thus, there was no difference in the growth (weight) pattern between the feeding groups (Figs. 2 and 3). Length gain during the study was positively associated with weight at birth and weight gain during the study ($P < 0.05$). However, length gain during the study was negatively associated with length at birth.

Food Intake

Food intake increased significantly in weeks 2 and 4 compared with week 1 ($P < 0.001$, Friedman test) and was comparable in all formula-fed infants.

TABLE 2. Clinical data of infants and mothers enrolled in the study

	Feeding group				
Response	SYN1 0.4 g/dL	GOS:FOS	SYN1 0.8 g/dL	Breast-fed	Control
No. of children (M/F)	21 (12/9)	19 (10/9)	20 (11/9)	29 (18/11)	21 (12/9)
Gestational age, wk	39 ± 0.85	38.63 ± 1.01	39.10 ± 1.07	39.24 ± 1.15	39.38 ± 0.80
Weight at birth, kg	3.52 ± 1.32	3.42 ± 1.56	3.52 ± 1.86	3.49 ± 1.69	3.46 ± 1.84
Length at birth, cm	50.83 ± 1.32	50.50 ± 1.56	50.30 ± 1.86	50.85 ± 1.69	50.26 ± 1.84
Head circumference, cm	34.83 ± 1.10	34.66 ± 1.56	34.75 ± 1.14	34.83 ± 1.16	34.53 ± 1.26
Mothers taking antibiotics (yes/no)	2/19	1/18	3/17	1/28	2/19
Mother's GBS[*] (+/−)	6/15	3/16	7/13	2/27	1/20

Data are presented as mean ± standard deviations or frequencies. FOS = fructooligosaccharides; GOS = galactooligosaccharides; SYN1 = Synergy1.
[*]GBS, group B *Streptococcus*.

TABLE 3. Number (percentage) of children with missing information

Group	Length at day 28	Weight at day 28	Food intake at baseline	Food intake (wk 2)	Food intake (wk 4)
SYNI 0.4 g/dL	7 (0.33)	7 (0.33)	4 (0.19)	5 (0.24)	6 (0.29)
GOS:FOS	8 (0.42)	8 (0.42)	6 (0.32)	8 (0.42)	9 (0.47)
SYNI 0.8 g/dL	7 (0.35)	7 (0.35)	9 (0.45)	9 (0.45)	8 (0.40)
Breast-feeding	6 (0.21)	6 (0.21)	29 (100)	29 (100)	29 (100)
Control	6 (0.29)	6 (0.29)	4 (0.19)	5 (0.24)	6 (0.29)

The first 2 columns represent the number of dropouts at the end of the study. Abbreviations same as Table 2.

Crying Behavior, Regurgitation, and Vomiting

Crying increased from week 1 to week 4 for all of the groups similarly but remained low. Regurgitation and vomiting scores were low and similar for all of the groups.

Stool Frequency

Stool frequency was similar in all of the formula-fed groups, whether or not they received prebiotics. Breast-fed infants had the highest stool frequency. Notably, stool frequency decreased over time (Friedman test, multivariate models) (Fig. 4).

Stool Consistency

On average, children in the breast-fed group had the softest stools and children in the control group had the hardest stools (Fig. 5). Children in all of the prebiotic-supplemented groups had significantly softer stools than children in the control group. The control group had significantly harder stools at week 2 and week 4 compared with week 1 ($P < 0.001$ and $P = 0.0279$, respectively). Stool consistency was comparable between the SYN1 0.8 g/dL and GOS:FOS groups. Thus, the ordering of the groups from best stool consistency to worst was breast-fed, SYN1 0.8 g/dL and GOS:FOS, SYN1 0.4 g/dL, and control (Friedman test).

Microbial Composition of the Fecal Microbiota

The number of available stool samples for microbial analysis was satisfactory for these kinds of studies (Table 4) Missing stool samples were equally distributed among all of the groups. Some

groups of fecal bacteria were below the detection limit (≤ 6.19, $\log_{10}$ cells/g feces) of FISH for some of the infants included in the study (Table 5); consequently, no FISH counts were obtained for these bacteria.

Because of the small sample sizes, when investigating differences within groups over time, all of the tests were based on nonparametric methods. The Friedman test was used to investigate homogeneity over time within each group. In the case of significance, Wilcoxon signed rank test was used for within-group pairwise comparison of measurements taken at different sample days.

To address the problem relating to stool samples not yielding counts on FISH, the following considerations were taken into account.

Breast-fed infants were not included in the statistical analysis because they were not randomly allocated to the group. Comparisons with breast-fed data are thus of a descriptive nature. Total DAPI in all of the samples were above the detection limit. Standard deviations were below 0.6 log. No further mathematical imputation

FIGURE 3. Mean (± 1 standard deviation) weight gain by each feeding group after (A) 2 weeks and (B) 4 weeks of feeding regimen. Feeding group: 1, Synergy1 0.4 g/dL; 2, galactooligosaccharides:fructooligosaccharides; 3, Synergy1 0.8 g/dL; 4, control; 5, breast-fed.
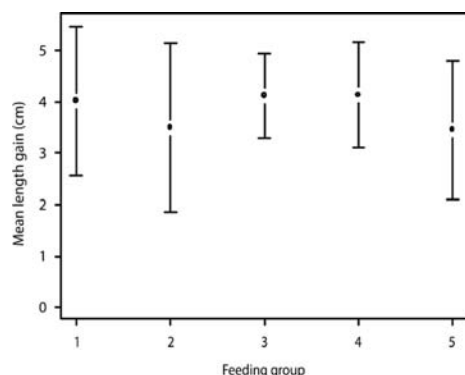

FIGURE 2. Mean (± 1 standard deviation) length gain by each feeding group after 4 weeks of feeding regime. Feeding group: 1, Synergy1 0.4 g/dL; 2, galactooligosaccharides:fructooligosaccharides; 3, Synergy1 0.8 g/dL; 4, control; 5, breast-fed.
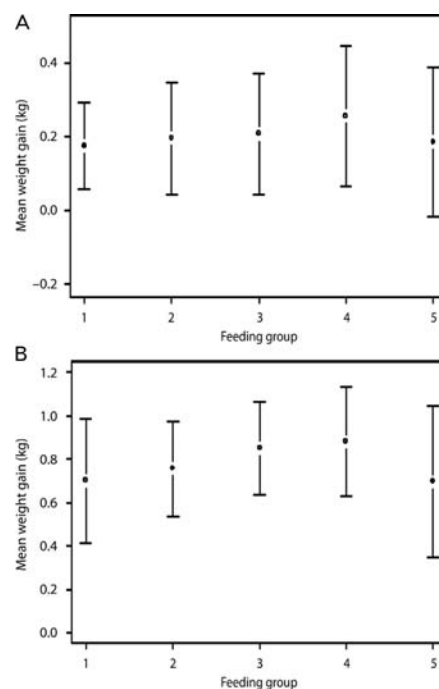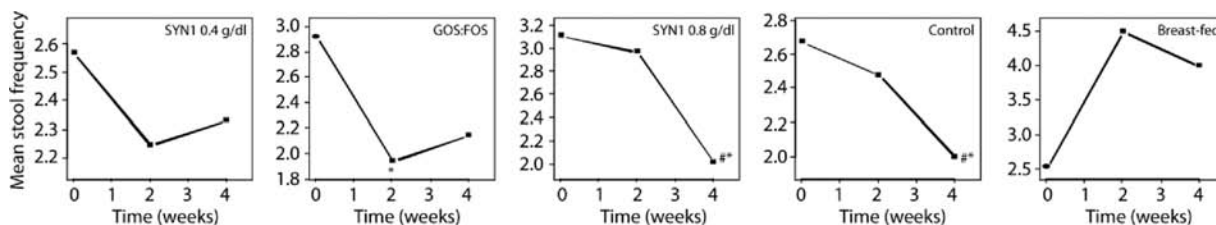
FIGURE 4. Mean stool frequency over the course of each different feeding regimes. *Indicates a significant difference ($P < 0.05$) for week 4 compared with week 1, # indicates a significant difference ($P < 0.05$) for week 4 compared with week 2.

was needed. There was an increase in DAPI on days 14 and 28 compared with day 3 for SYN1 0.4 g/dL, SYN 0.8 g/dL, and GOS: FOS groups, unlike the control group that showed no changes in DAPI over time. However, all of the groups had similar DAPI counts.

With respect to prebiotics, an increase in bifidobacteria and/ or lactobacilli is considered to be beneficial in infants. Breast-fed infants are commonly accepted as the criterion standard, whereas formulae without prebiotic supplement (here, the control formula) are the (negative) benchmark. A false-positive effect for the test substances could easily and falsely be derived if the control groups had more data close, but still below the detection limit, than the test substances. In the present study, all 3 test formulae provided Bif164 counts above the detection limit at day 14 and day 28 (day 3 is not relevant for the efficacy assessment) and only the control group had 2 (resp. 1) sample(s) below. Thus, to avoid any false positive, an imputation of 6.19 in the control group is the highest theoretically positive FISH count, but the actual value would have been lower. This would lead to a masking of a bifidogenic effect rather than to suggest a false increase. Thus, this approach can be considered as the most conservative with respect to Bifidobacteriaceae.

An increase in mean *Bifidobacterium* (Bif164) counts was observed over time within all of the feeding groups (Table 6). Although the rate of increase was similar between the prebiotic-supplemented and breast-fed groups, it was slower for the control group. Significant changes were indicated between the SYN1 0.8 g/dL and GOS:FOS groups and control, but not for SYN 0.4 g/dL. For the SYN1 0.8 g/dL and GOS:FOS groups, there was a significant increase in Bif164 counts on days 14 and 28 compared with day 3. No significant changes in Bif164 counts

were observed between days 14 and 28 for any of the feeding groups (Fig. 6). Noteworthy is that the absolute means of the control group on days 14 and 28 were 1 log lower than the 0.8 g/dL supplemented groups. The standard deviation of the computed control group was still slightly higher than those of the supplemented groups. The top dose prebiotics approached the breast-fed controls on days 14 and 28.

For lactic acid bacteria (Lab158, LAC), the number of samples with FISH counts below the detection limit was highest in all of the feeding groups. For control, SYN1 0.4 g/dL, and GOS:FOS similar numbers of fecal samples were below the detection limit on days 14 and 28. The SYN1 0.8 g/dL on day 28 provided only 1 value below the detection limit. Thus, computation of 6.19 for lower values should be equally applicable to all of the groups and not provide false-positive or false-negative results. Assuming that an increase in lactobacilli is considered beneficial, 1 value below the detection limit in contrast to 5 to 8 in the other test groups with SYN1 0.8 g/dL would lead to false-negative results and can thus be considered acceptable.

Based on this analysis, only the SYN 0.4 g/dL group shows an increasing trend in LAC count without reaching significance. For the other test groups, the LAC count remained essentially stable during the whole period (Table 6). There is no statistically significant change in counts over time within all of the formula groups and no difference between groups on all days. Lowest LAC counts were observed with the breast-fed group. Means of all of the test groups were close and with similar standard deviation. This indicates that there was in fact no difference, and the statistical approach can be considered appropriate.

BAC and CHIS can be considered undesirable microbes. A decrease would point toward a favorable effect and an increase toward an unfavorable effect of the test substances. Thus, false positives would occur, if an artificial decrease between days 14/28 and day 3 and/or significant false lower values compared with the control formula would be generated. Low counts in breast-fed babies should be considered the gold target.

In fact, most occurrences below the detection limit associated with the lowest mean with about 1 log standard deviation were found with breast-fed bacteria. The unsupplemented control formula led to the highest means instead, followed by the SYN1 0.4 g/dL group. GOS:FOS and SYN1 0.8 g/dL were in between with standard deviations in the range of the control. Standard deviation suggests that the imputation of 6.19 for values below the detection limit is appropriate. No significant changes over time and between groups were found. A computation inserting "0" for the control group was dismissed because it led to an extraordinary high standard deviation of 3 to 4 log, which is not considered plausible and would have masked any true significance.

FIGURE 5. Mean stool consistency over the course of each different feeding regimen. Stool consistency score: 1, watery; 2, soft; 3, formed; 4, hard. *Different from control group in week 2; #different from control group in week 4. Feeding group (from top): (1) control, (2) Synergy1 0.4 g/dL; (3) galactooligosaccharides:fructooligosaccharides; (4) Synergy1 0.8 g/dL; (5) breast-fed.

DISCUSSION

In our study population, more than 90% of mothers start breast-feeding. The high incidence of breast-feeding makes clinical
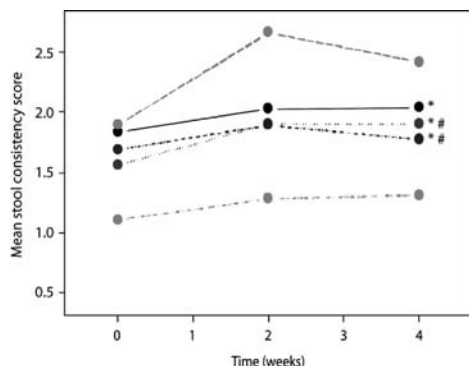
TABLE 4. Stool analysis

	SYN1 04 g/dL	SYN1 08 g/dL	GOS:FOS	Control	Breast-fed
No. infants[*] (total infants)	18 (21)	15 (20)	14 (19)	16 (21)	26 (29)
No. collected samples day 3[†]	18	11	13	14	22
No. collected samples day 14[†]	16	12	10	15	22
No. collected samples day 28[†]	14	12	10	14	22
No. infants with only 2 samples[‡]	2	4	1	3	4
No. infants with only 1 sample[‡]	2	3	4	1	4

This table contains the number of children for which stool samples were available (of total subjects/group) for analysis in total and per day. FOS = fructooligosaccharides; GOS = galactooligosaccharides; SYN1 = Synergy1.
[*] Number of infants with fecal samples (providing either 1, or 2, or 3 fecal samples). [†] Number of analyzed samples at the respective time point (day 3, 14, or 28). [‡] Number of infants of whom only 1 or 2 instead of 3 fecal samples were analyzed.

studies such as the present study not a trivial undertaking because it is difficult to recruit neonates who are exclusively formula-fed early after birth. However, the challenge to improve formulae in such a way that the natural bifidus-predominant microbiota of the neonate remains preserved is worthwhile because many mothers discontinue breast-feeding, at least partially during the first weeks, and not everywhere is the incidence of maternal feeding so favorable (24).

The finding of nondigestible oligosaccharides in breast milk inspired the addition of prebiotics to infant formulae to conserve a bifidus-predominant microbiota. Indeed, the addition of a commercial mixture of GOS and long-chain inulin (90:10) to infant formulae was proven to be bifidogenic (20,25). In addition, other effects of prebiotic supplementation have been described such as an increase in the number of fecal lactobacilli (20,25,26), a change in stool short-chain fatty acid profile closer to the breast-fed pattern

TABLE 5. Stool analysis (number of samples for which FISH counts of specific groups of bacteria could not be obtained [ie, numbers of bacteria were below the detection limit of the method])

Probe	Feeding group	Day 3	Day 14	Day 28
Bac303	SYN1 0.4 g/dL	1	1	2
	GOS:FOS	3	1	2
	SYN1 0.8 g/dL	4	2	2
	Breast-fed	8	9	6
	Control	5	5	2
Bif164	SYN1 0.4 g/dL	1	0	0
	GOS:FOS	1	0	0
	SYN1 0.8 g/dL	1	0	0
	Breast-fed	1	0	0
	Control	1	2	1
Chis150	SYN1 0.4 g/dL	3	2	2
	GOS:FOS	3	2	4
	SYN1 0.8 g/dL	4	3	4
	Breast-fed	15	15	18
	Control	3	2	2
Lab158	SYN1 0.4 g/dL	11	7	5
	GOS:FOS	7	7	8
	SYN1 0.8 g/dL	4	6	1
	Breast-fed	18	14	15
	Control	7	8	7

FOS = fructooligosaccharides; GOS = galactooligosaccharides; SYN1 = Synergy1.

(27), a decreasing number of potential fecal pathogens (28), and an increase in fecal sIgA (29). Infants receiving GOS:FOS–enriched formula have fewer GI and respiratory infections (30), and may be protected against atopic dermatitis after 6 months (31) and manifestations of atopy even after 2 years (32). The window did allow for breast-feeding before inclusion should be taken into account and may be crucial. In the present study infants could be included up to the fifth day; in other studies the window was larger: up to 2 weeks (31).

In the present study we first examined the physiological effects of various prebiotic supplements, whereas the GOS:FOS formula essentially served as a control prebiotic formula. Tolerance and safety profiles were excellent for all of the supplements. Subjects dropped out solely because caretakers wanted to try another formula because of common reflux symptoms or hunger. Changing formula is unfortunately common practice in Belgium (33). There was no excessive crying, regurgitation, or vomiting. Anthropomorphic measures were similar for all of the feeding groups. In all of the prebiotic-supplemented groups, stool consistency improved, implying that stools became softer, more resembling stools produced by breast-fed infants. It is known that GOS:FOS softens stools (20) without affecting water balance at a concentration of 0.8 g/dL (34). In the present study, the softness of stools produced by infants receiving SYN1 0.8 g/dL scored between the breast-fed and GOS:FOS groups. Thus, based on the results we can conclude that SYN1 feeding at the tested dose did not indicate an increased risk of dehydration.

Subsequently we examined the bifidogenic effects of various prebiotic supplements. Stool samples were examined using the FISH technique, which is a state-of-the art method. Quantitative culture independent methods as FISH and real-time quantitative polymerase chain reaction are highly accurate and are considered current key techniques for the assessment of intestinal microbiota (35). An alternative method for the identification and quantification of microbes is the classical culture technique. However, this technique has several shortcomings, in particular the lack of selectivity. The problem we encountered with samples below the detection limit was most likely caused by conservation factors rather than the method used.

The study lasted for 4 weeks and was aimed at investigating the dosage-related bifidogenic effect of SYN1 on the flora of healthy neonates compared with the currently used prebiotic GOS:FOS, standard formula, and breast milk. The responses represent counts of different bacteria measured in log10 cells/g feces. The number of bacteria for some samples was below the detection limit of 6.19 on a log10 cells/g scale. Thus, the number of bacteria could not be obtained for such samples and 6.19, which corresponds to the detection limit, was inserted and considered conservative for bifidobacteria and lactobacilli and appropriate for

TABLE 6. Mean and standard deviations for DAPI (MDapi and SDapi), BAC (MBac and SBac), BIF (MBif and SBif), Chis (MChis and SChis), and Lac (MLac and SLac) by supplemented group (Group) and time (Days)

Group	Days	MDapi	SDapi	MBac	SBac	MBif	SBif	MChis	SChis	MLac	SLac
SYN1 0.4 g/dL	3	982	59	785	143	842	130	731	116	690	93
	14	10.26[*]	26	792	125	930	121	779	122	715	107
	28	10.34[*]	26	804	117	908	130	778	144	741	114
GOS:FOS	3	991	35	689	93	845	137	721	103	691	82
	14	10.27[*]	40	698	126	9.36[*,†]	126	797	149	678	95
	28	10.37[*]	40	745	133	9.88[*]	80	782	147	659	87
SYN1 0.8 g/dL	3	973	45	685	69	832	155	689	95	715	89
	14	10.19[*]	43	742	120	9.49[*,†]	131	760	127	695	82
	28	10.38[*]	32	761	113	9.74[*]	80	770	124	726	84
Breast-feeding	3	945	48	717	97	859	92	650	61	645	72
	14	993	49	670	59	948	78	650	66	667	84
	28	1015	37	730	103	956	87	645	73	657	69
Control	3	986	24	721	126	844	134	702	95	702	87
	14	994	44	734	130	847	160	788	115	694	88
	28	1016	33	771	108	875	139	821	128	705	98

FOS = fructooligosaccharides; GOS = galactooligosaccharides; SYN1 = Synergy1.
[*] Indicates difference of group means for a given time from group mean on day 3. [†] Indicates difference between a group mean and the mean of the control group.

BAC and CHIS. This approach is conservative for bifidobacteria only for the control group 6.19, which is higher than actual detected values and thus prone to more false-negative than false-positive statistical results. For lactic acid bacteria, the number below the detection limit was equally distributed among the groups for which statistics were applied apart from only 1 value in the SYN 0.8 g/dL group at day 28. Because a lower number is rather prone to mask a true-positive effect rather than to indicate a false positive, this approach can also be considered conservative for lactobacilli. The results are given in Table 6. The total number of bacteria increased similarly over time in all groups. All of the values were above the detection limit.

It is known from the literature that the colonization of the aseptic intestine of the newborn, which begins at birth and continues in a stepwise manner, depends on several factors such as mode of delivery, environmental factors, bacterial interactions, the host

FIGURE 6. Differences in *Bifidobacterium* (Bif164) counts among the 5 feeding groups over time. Feeding group: white, Synergy1 0.4 g/dL; light gray, galactooligosaccharides:fructooligosaccharides; gray, Synergy1 0.8 g/dL; black, breast-fed; dark gray, control. [*]Significantly different from day 3; [#] significantly different from the control group.

itself, and feeding. Hence, the colonization progress of the intestine and composition of the microflora vary substantially between individual babies at the species level (10). This is supported by the data in our study: significant changes were observed between the 0.8 g/dL supplemented groups with SYN1 and GOS:FOS and the control groups, which came closer to the breast-fed group (no statistics applied). Only in these supplemented groups was a significant increase from baseline (day 3) observed—to an extent also found in the breast-fed group.

LAC had the highest number of samples with counts below the threshold. No significant changes over time and between groups were observed. Lowest means were observed with the breast-fed group. Inserting the theoretical highest possible value without generating significances should not suggest false relevance for this parameter.

BAC and CHIS were lowest in breast-fed children and highest in control-fed infants (CHIS) or SYN1 0.4 g/dL (BAC). Imputation of 6.19 at the relevant days 14/28 had to be applied for 1 to 2 values in these groups only. Thus, the absence of statistical significance can be considered a robust result. The 0.8 g/dL feeding groups were in between (no significance in treatment or time). Thus, it can be concluded that a risk of an increase in these bacterial groups that are considered less desirable was excluded.

Clostridia belonging to the *C histolyticum/C lituseburense* group (Chis150) are only minor components of the fecal flora of newborn infants. They were detected in some, mainly formula-fed, infants at proportions varying from 0% to 1% of total bacteria, with 1 exception at 2.2% (10). Lactobacilli and enterococci were detected in early samples at proportions varying from 0% to 4.6%. After the first early days, the relative numbers did not exceed 0.7% (10). The above descriptions of the neonatal microbiota (10,36) fully support the results of our study.

The bifidogenic effect of SYN1 has not been examined previously in infants. In the present study the SYN1 0.8 g/dL and GOS:FOS groups showed an increase in BIF counts over time. Also, SYN1 0.8 g/dL and GOS:FOS groups had higher BIF counts than SYN1 0.4 g/dL and control groups on day 14.

Administration of a 70:30 mixture of oligofructose and inulin (2.25 g/day for 3 weeks) to toddlers receiving an antibiotic treatment led to faster restoration of bifidobacteria (37), and
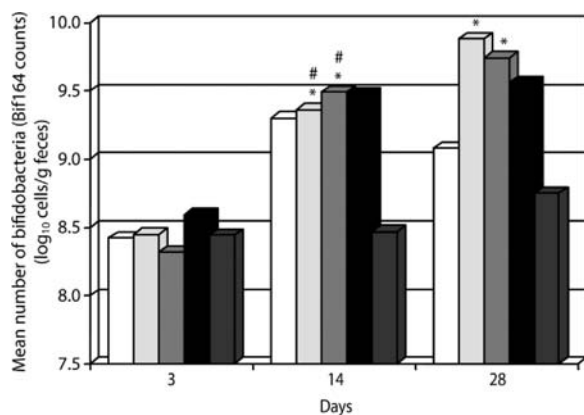
770

oligofructose alone (2 g/day for 3 weeks) to toddlers in childcare was bifidogenic (38). Here we demonstrated that SYN1 0.8 g/dL and GOS:FOS 0.8 g/dL elicited levels of fecal bifidobacteria that were comparable to the levels found in breast-fed babies after 2 weeks of supplementation. This clarifies why a trial of 1-week oligofructose (1.5 and 3 g/day) is too short to influence microbiota composition (39).

The adequate dose for SYN1 appears to be 0.8 g/dL because 0.8 g/dL resulted in softer stools than SYN 0.4 g/dL, and although Bif164 increased in the SYN 0.4 g/dL group, the change was not significantly different from the control group.

In conclusion, the addition of prebiotics SYN1 or GOS:FOS to standard formula was well tolerated and led to normal thriving. Formula enrichment with 0.8 g/dL of prebiotics led to softer stools, with a fecal consistency closer to those of breast-fed infants. Levels of fecal bifidobacteria from infants supplemented with SYN1 or GOS: FOS at a concentration of 0.8 g/dL were comparable to levels of bifidobacteria found in stools of breast-fed infants. This favorable bifidogenic effect mirrors only one of the many advantages of breast milk, which remains the criterion standard for feeding infants.

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