Clinical Pediatrics
2015, Vol. 54(4) 359–370

DOI: 10.1177/0009922814553433
cpj.sagepub.com
SAGE

Effect of an α-Lactalbumin-Enriched Infant Formula Supplemented With Oligofructose on Fecal Microbiota, Stool Characteristics, and Hydration Status: A Randomized, Double-Blind, Controlled Trial

Susan Wernimont, PhD, RD[1], Robert Northington, PhD[1], Martin J. Kullen, PhD[2], Manjiang Yao, MD, PhD[1], and Jodi Bettler, MA, RD[1]

Abstract

Aims. To evaluate the impact of oligofructose (OF)-supplemented infant formula on fecal microbiota, stool characteristics, and hydration. *Methods*. Ninety-five formula-fed infants were randomized to α-lactalbumin-enriched control formula (CF) or identical formula with 3.0 g/L OF (EF) for 8 weeks; 50 infants fed human milk (HM) were included. *Results*. Eighty-four infants completed the study, 70 met per-protocol criteria. Over 8 weeks, bifidobacteria increased more in EF than CF group (0.70 vs 0.16 $\log_{10}$ bacterial counts/g dry feces, $P = .008$); EF was not significantly different from HM group ($P = .32$). EF group stool consistency was intermediate between CF and HM groups; at week 8, EF group had softer stools than CF (5-point scale: 1 = hard, 5 = watery; consistency score 3.46 vs 2.82, $P = .015$) without significant differences in stool frequency. Physician-assessed hydration status was normal for all infants. *Conclusions*. Infant formula with 3.0 g/L OF promoted bifidobacteria growth and softer stools without adversely affecting stool frequency or hydration.

Keywords

infant formula, prebiotic, α-lactalbumin, oligofructose, bifidobacteria, microbiota, stool consistency, stool frequency, hydration

Introduction

Human milk (HM) is recognized as the gold standard in infant nutrition, containing an array of nutrients and biofactors that promote health and well-being[1] in part through modulation of the intestinal microbiota. Following birth, the infant colon is rapidly populated by a variety of bacteria. Differences in the fecal microbiota of HM-fed and formula-fed (FF) infants have been reported; the microbiota of HM-fed infants is dominated by *Bifidobacterium* spp. with lower proportions of *Bacteroides* spp. and *Clostridium* spp. compared with FF infants.[2-4] In addition, compared with FF infants, those fed HM typically have softer, more frequent stools.[5,6]

Bioactive components of HM that may provide benefits to infants include whey proteins such as α-lactalbumin as well as complex oligosaccharides. HM is abundant in α-lactalbumin, a high-quality, easy-to-digest whey protein that comprises 25% to 35% of total HM protein.[7] Furthermore, α-lactalbumin[8,9] and peptides derived from the digestion of this protein[10] have been shown in vitro to stimulate the growth of bifidobacteria, while intact α-lactalbumin may inhibit the in vitro growth of potential pathogens.[11,12] In addition to α-lactalbumin, HM is abundant in complex oligosaccharides with a range of biological activities related to gastrointestinal health and host–microbe interactions. These oligosaccharides function as prebiotics, antiadhesive antimicrobials, and modulators of intestinal epithelial cell responses and immune function.[13] While more than 100 such oligosaccharides have been identified in HM, these compounds are

[1]Nestlé Nutrition, King of Prussia, PA, USA
[2]DuPont Nutrition & Health, Wilmington, DE, USA

Corresponding Author:
Susan Wernimont, Nestlé Nutrition, 3000 Horizon Drive, Suite 100, King of Prussia, PA 19406, USA.
Email: susan.wernimont@rd.nestle.com

virtually absent from infant formula.[13] In order to more closely mimic the intestinal microbiota and stool characteristics of HM-fed infants, infant formula manufacturers have added plant-derived fibers, including oligofructose (OF), to infant formulas. However, because of the osmotic effects of fiber, concerns have been raised about the potential for these fiber-supplemented formulas to adversely affect infant hydration status.[14]

Oligofructose is an oligosaccharide fiber which is not digested in the small intestine but is utilized predominantly by colonic bacteria such as bifidobacteria as a fuel source. Because of these characteristics it is classified as a prebiotic.[15-17] Although previous clinical trials have evaluated oligosaccharide mixtures that included OF, relatively few have evaluated the effects of OF provided in the absence of other complex carbohydrates such as inulin or galactooligosaccharides in exclusively FF healthy term infants.[18-21] Two such studies found little impact on fecal bacterial groups[18,20] although OF-supplemented formula was fed for only 1 to 2 weeks. Moreover, Euler et al[20] analyzed fecal microbiota using quantitative culture, a less sensitive method than currently available techniques. Nonetheless, the investigators did observe a decline in the proportion of infants positive for *Clostridium difficile* toxin in the groups that received OF-supplemented formula.[20] Using fluorescent in situ hybridization (FISH), a more sensitive method to analyze fecal microbiota, Yao et al[21] reported higher fecal bifidobacteria counts in infants fed formulas supplemented with a combination of OF and a high *sn*-2 palmitate fat blend compared with infants who received a control formula without OF or high *sn*-2 palmitate; however, in this study, *sn*-2 palmitate alone was also found to promote the growth of fecal bifidobacteria compared with the control formula.

Infants fed formula supplemented with 3.0 g/L OF were found to have softer stools compared with infants fed formula supplemented with 1.5 g/L OF[20] and less constipation compared with infants fed a control formula without OF or a formula supplemented with 1.5 g/L OF.[19] Furthermore, infants fed formula supplemented with 3.0 g/L OF and a high *sn*-2 palmitate fat blend had softer stools compared with a control formula without OF or high *sn*-2 palmitate; although the stool characteristics reported in this comparison represent the combined effects of both *sn*-2 palmitate and OF, the authors noted that stools were softer without an increase in stool frequency.[21] In contrast to these results, Euler et al[20] found that infants receiving formula supplemented with 3.0 g/L OF experienced an increase in the number of stools per day compared with presupplementation (infants receiving formula supplemented with 1.5 g/L OF experienced a decrease in the number of stools per day during the same period). In addition, infants receiving formula with 3.0 g/L OF experienced a greater frequency of "looser stools" compared with the group receiving formula with 1.5 g/L OF; together these findings raised questions about whether OF may potentially affect infant hydration status.[14] Although studies of OF-supplemented infant formulas have reported good tolerance to these formulas (based on collection of adverse events and other safety data), none included a detailed evaluation of infant hydration status. In addition, differences in study formula composition (including the presence of other ingredients that may affect stool characteristics or microbiota), durations of OF supplementation, and microbiota detection methodologies suggest further evaluation of the effects of OF supplementation alone on fecal microbiota, stool characteristics, and hydration status in healthy term infants is warranted.

Here we report the effects of a bovine milk–based, α-lactalbumin-enriched infant formula supplemented with 3.0 g/L OF on intestinal microbiota, stool characteristics, and hydration status in healthy term infants. The present investigation arose from a study that utilized quantitative culture techniques to evaluate the effects of α-lactalbumin enriched formula with and without OF on fecal bifidobacteria concentrations.[22] While that analysis was limited due to the low sensitivity of the quantitative culture method, the study design and methodology provided an opportunity to explore the effects of OF-supplemented infant formula on fecal microbiota in more detail using FISH, a method with greater sensitivity to fecal flora differences. It was hypothesized that infants fed OF-supplemented formula would exhibit differences in fecal bifidobacteria and stool consistency compared to infants fed formula without OF, and would be more similar to a HM-fed reference group included in the study, while maintaining adequate hydration status. In addition, two noninvasive indicators of intestinal disease, fecal *C difficile* toxin and calprotectin, were evaluated as secondary outcomes.

Methods

Study Design

A multicenter, prospective, randomized, double-blind, controlled study was conducted at 10 outpatient pediatric offices in the United States from May 2005 to April 2006. The study was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki. Institutional review boards approved the study protocol for each study site, and written informed consent of parents/guardians was obtained.

Both FF and HM-fed infants were recruited to participate in this 8-week study. Infants aged 1 to 13 days were recruited to participate in a screening visit; those who met inclusion criteria were eligible to participate in the baseline study visit (Study Day 0), which was planned between 5 and 14 days of age. At the baseline visit, FF infants (n = 95) were randomized to receive 1 of 2 term infant formulas for 8 weeks: a bovine milk–based α-lactalbumin-enriched control formula (CF), or an identical formula supplemented with 3.0 g/L OF (EF). A nonrandomized reference group of healthy term HM-fed infants (n = 50) was included; these infants continued to be fed HM throughout the study. Study participants, care providers, and those assessing outcomes were blinded to the feeding group assignment through the use of coded feeding groups. Study formulas were randomly assigned based on 4 equal-sized color-coded feeding groups, each corresponding to 1 of the 2 study feedings. A biostatistician with no involvement in trial conduct used a computer algorithm designed specifically for this study to generate a randomization schedule for each site in which study numbers were randomly assigned in blocks of 6, comprised of 2 study numbers for HM-fed infants and one study number for each of the 4 formula color codes. Study coordinators at each site assigned a unique study number and a corresponding treatment group to infants sequentially from the randomization schedule, depending on whether the infant was HM-fed or FF. The entire block of 6 study numbers was dispensed before moving to the next block.

Stool samples were collected at baseline and after 1, 2, 4, and 8 weeks of exclusive study formula or exclusive HM feeding and analyzed for selected fecal microbiota using FISH. The primary endpoint was the concentration of fecal bifidobacteria at week 8. Secondary endpoints were fecal concentrations of *Bacteroides*, clostridia, *Enterobacteriaceae, Lactobacillus/Enterococcus*, and *Staphylococcus* at week 8. Other secondary endpoints included fecal *C difficile* toxin and calprotectin, as well as stool frequency and consistency, hydration status, infant weight, and number of wet diapers per day. Caregivers recorded stool frequency (number of stools per day) and consistency (5-point scale: 1 = hard, 2 = firm, 3 = soft, 4 = loose, 5 = watery) on 24-hour diary cards, which were collected at baseline, week 1, week 2, week 4, and week 8; fecal *C difficile* toxin and calprotectin were measured at the same time points. Hydration status was assessed by study physicians at the screening, week 4, and week 8 visits. A set of 5 common clinical parameters that reflect infant hydration status was established prior to study start and consistently used by investigators across all study sites to evaluate hydration. These parameters included mucous membranes, skin turgor, body weight, formula intake, and number of wet diapers per day. Study physicians performed a physical examination of the infants at the screening and week 8 visits to monitor overall health status. Anthropometric measurements (weight, length, and occipital frontal circumference) were collected at the screening and week 8 visits; weight was also collected at baseline. Study coordinators interviewed caregivers during clinic visits at week 1, week 2, week 4, and week 8 and by phone at week 3, week 6, and poststudy. Interview questions assessed any changes in infant health, and whether the infant or breastfeeding mother started on any new medications. Study coordinators also determined whether the infant continued to be exclusively fed study formula or HM. Safety was assessed by monitoring the incidence of adverse events, defined as any unintended change in pathology or anatomic, metabolic, or physiologic functioning temporally associated with enrollment in the study; this definition included events that occurred both during the trial and within 15 days after the last day of study feeding. At each visit/assessment, adverse events observed by the investigator or reported by the parent/caregiver were recorded, regardless of whether they were related to the study treatment; investigators were instructed to thoroughly report all adverse events.

Study Population

All infants were required to have been either exclusively FF or HM-fed from birth. Infants were not enrolled in the study if they were presently receiving or had received prohibited medications which could potentially impact study endpoints such as stool consistency or fecal microbiota. These medications included antibiotics; antifungals, except topical; suppositories; bismuth-containing medications; herbal supplements; and/or any medication that could neutralize or suppress gastric acid secretion. In addition, infants were not enrolled in the study if they had a congenital abnormality, evidence of significant disease, or a history of feeding intolerance or allergy to cow's milk protein. Finally, HM-fed infants were not enrolled in the study if their mothers were presently receiving or had received postpartum antibiotics or antifungal medications (except topical).

Study Formulas

Study formulas were bovine milk–based term infant formulas enriched with α-lactalbumin (Wyeth® S-26® GOLD; Askeaton, Ireland), or an identical formula supplemented with 3.0 g/L OF (Orafti® P95, BENEO-ORAFTI, Tienen, Belgium). Formulas were supplied in 454-g cans in powdered form and were identified by 2 of

Table 1. Macronutrient Composition of Study Formulas.

	CF	EF
Energy, kcal/L	672	672
Protein (total), g/L	14	14
α-lactalbumin, g/L	2.2	2.2
Carbohydrate (total), g/L	73	75
Lactose, g/L	73	71.5
Oligofructose,[a] g/L	0.0	3.0
Fat, g/L	36.0	36.0

Abbreviations: CF, control formula (a whey-dominant infant formula enriched with α-lactalbumin); EF, CF supplemented with 3.0 g/L oligofructose.
[a]Oligofructose contributes 2.0 kcal/g.

4 colored labels (yellow, orange, green, blue) with a corresponding letter code on the label (formula Y, formula O, formula G, formula B). When reconstituted according to label directions, the formulas provided 672 kcal/L. The macronutrient composition of the study formulas is described in Table 1. Compliance was documented through the completion of daily study formula feeding records and formula accountability records.

Fecal Analysis

Stool specimens were collected by the caregivers in preweighed tubes containing liquid phosphate buffered saline + 2% gelatin. Following collection the samples were shipped to the analytical laboratory under refrigeration. For the FISH analyses, the samples were prepared, fixed with ethanol, and stored at −20°C or below until subjected to hybridization. The analysis was conducted using Syto® BC green fluorescent nucleic acid stain (Molecular Probes S34855; Grand Island, NY) and commercially synthesized Cy5 5′ end labeled oligonucleotide probes (Operon Biotechnologies; Huntsville, AL); FISH targets and probes are shown in Table 2. Data acquisition was performed using a BD FACSCalibur™ flow cytometer (Becton Dickinson; Franklin Lakes, NJ). Bacterial counts were corrected for autofluorescence and reported as counts per gram dry fecal weight.

For analysis of *C difficile* positive stools, the C. DIFF CHEK™ − 60 test was used (TECHLAB Inc, Blacksburg, VA). The test is an enzyme immunoassay designed to detect the *C difficile* antigen, glutamate dehydrogenase. A positive result confirms the presence of *C difficile* in a fecal specimen; the test does not distinguish between toxigenic and nontoxigenic strains of *C difficile*. The assay was performed at the Center for Pediatric Research (Norfolk, VA). Analysis of calprotectin was performed using the PhiCal™ ELISA test (Genova Diagnostics; Ashville, NC); the assay was performed at Genova Diagnostics. Data are reported as micrograms per gram (μg/g) feces.

Statistical Analyses

Sample size calculations were based on previous data collected by the sponsor, which indicated the mean (log-transformed) bifidobacteria count in the feces of HM-fed infants was 8.8 with a standard deviation of 1.9; equivalence margins for comparisons to the HM group were defined to be ±1.76 (20% of the mean), assuming 80% power and an α level of .05.

The primary objective of the study was to compare the intestinal microbiota (measured as fecal bacteria concentrations) among the feeding groups at 8 weeks (day 56). With the exception of safety outcomes, all analyses were performed in the per protocol (PP) population (n = 70), which excluded infants with major protocol violations. Infants were excluded from the PP population if they were not 5 to 14 days of age at baseline stool sample collection; were not exclusively fed either study formula or HM; were presently receiving or had received excluded medications or supplements; were a HM-fed infant whose mother was presently receiving or had received any postpartum antibiotics or antifungal medications (except topical); had received prohibited feeding during the study; had an inadequate stool sample at baseline or week 8; had the week 8 clinic visit outside of the accepted window (before day 49 or after day 63). Adverse events were evaluated in the safety population, which included all infants who received study formula or breast milk (n = 145).

Fecal microbiota data were compared in the study groups using 2-way analyses of covariance, with terms for baseline bacterial count, treatment, study site, and treatment by site interaction. Analyses were based on changes from baseline to week 8 in $\log_{10}$-transformed counts [$\log_{10}$(count)] per gram dry fecal weight. Sparse data prevented more than a descriptive summary of the number of infant stool samples positive for *C difficile*. Infants with missing baseline or week 8 stool data were not included in the summary. Statistical methods for analyzing fecal calprotectin concentrations were the same as described for fecal microbiota data; calprotectin concentrations were analyzed using models with terms for baseline, treatment, study site, and treatment by site interaction, and were $\log_{10}$ transformed prior to analysis. Stool frequency and consistency were analyzed for treatment difference at week 8 using a two-way analysis of variance with terms for treatment, site, and interactions. A similar analysis was completed for the number of wet diapers per day. Infant weights were analyzed using a 2-way analysis of covariance with terms for

Table 2. Fluorescent In Situ Hybridization Targets and Probes.

Probe	Target	Sequence (5′–3′) Cy5 Labeled at 5′ End	Reference
Bfra602	*Bacteroides*	GAGCCGCAAACTTTCACAA	Franks et al (1998)[23]
Bdis65f6	*Bacteroides*	CCGCCTGCCTCAAACATA	Franks et al (1998)[23]
Bif164	bifidobacteria	CATCCGGCATTACCACCC	Langendijk et al (1995)[24]
Chis150	clostridia	TTATGCGGTATTAATCTYCCTTT	Franks et al (1998)[23]
EBAC-1790	*Enterobacteriaceae*	CGTGTTTGCACAGTGCTG	Bohnert et al (2000)[25]
Lab 158	*Lactobacillus/Enterococcus*	GGTATTAGCAYCTGTTTCCA	Harmsen et al (1999)[26]
LGC354A	*Lactobacillus Bacillus* sub branch	TGGAAGATTCCCTACTGC	Meier et al (1999)[27]
STA	*Staphylococcus*	TCCTCCATATCTCTGCGC	Kempf et al (2000)[28]
ASUNI	Antisense UNI	CAGCAGCCGCGGTAATAC	Reverse complement of UNI519

treatment, site, and interactions, using the baseline measurement as the covariant.

Analyses were initially conducted comparing the 2 formula groups and excluding HM since it was not a randomized group; subsequently, separate analyses were done comparing each formula group to HM. Study sites that enrolled 20 or fewer subjects were pooled to form a single site for analysis purposes. Substitute values were not added if data were missing; infants with missing values for a particular outcome were not included in the analysis of that outcome.

Results

Subjects

Of 166 infants screened, a total of 145 infants between 5 and 15 days of age (mean ± SD = 11.2 ± 2.3 days) were enrolled in the study; 48 (33%) received CF, 47 (32%) received EF, and 50 (35%) continued to receive HM (Figure 1). Eighty-four infants completed the study. The number of discontinuations and the proportions of infants who discontinued were comparable across all 3 study groups (20 [42%] in CF, 22 [47%] in EF, and 19 [38%] in HM). Of the 84 infants who completed the study, 77 had stool samples at both baseline and week 8 and 70 met PP criteria. Infants in the PP subset were similar to those in the overall study population in age and weight, but had somewhat higher proportions of infants who were male or born by vaginal delivery, and slightly lower proportions of infants with maternal intrapartum antibiotic use. Characteristics of the PP population at the baseline study visit (study day 0) are shown in Table 3.

Fecal Microbiota and Intestinal Markers

Unadjusted mean $\log_{10}$-transformed bacteria counts per gram dry fecal weight at baseline and week 8 are shown in Table 4. After adjustment for baseline bacterial count, treatment, study site, and treatment by site interaction, infants in the EF group had a significantly greater increase in $\log_{10}$-transformed fecal bifidobacteria per gram dry fecal weight from baseline to week 8 compared with infants in the CF group (mean ± SE: EF group 0.70 ± 0.15 vs CF group 0.16 ± 0.12, $P = .008$). A model with adjustment for mode of delivery was also tested, with similar results (mean ± SE: EF group 0.69 ± 0.17 vs CF group 0.22 ± 0.13, $P = .030$). Since adjustment for mode of delivery did not change conclusions, this term was not included in the final statistical models. Furthermore, the increase in fecal bifidobacteria observed in the EF group was similar to the increase observed in the group fed HM (mean ± SE: HM group 0.72 ± 0.11 vs EF group, $P = .32$). Adjusted changes from baseline to week 8 for *Bacteroides*, clostridia, *Lactobacillus/Enterococcus*, and *Staphylococcus* were similar between the CF and EF groups and there were no differences between either formula group and HM for any of the bacterial targets (data not shown). The increase in adjusted *Enterobacteriaceae* $\log_{10}$-transformed counts per gram dry fecal weight from baseline to week 8 was significantly smaller in the CF group versus HM (mean ± SE: CF group 0.18 ± 0.12 vs HM group 0.58 ± 0.11, $P = .023$). The increase in *Enterobacteriaceae* in the EF group was not significantly different from the HM group but was numerically higher than in the CF group. This difference approached, but did not reach, the criterion for statistical significance (mean ± SE: EF group 0.33 ± 0.13 vs CF group, $P = .056$; EF group vs HM group, $P = .59$).

Among infants who had *C difficile* results available at both baseline and week 8 (n = 10-13 per group), none tested positive for *C difficile* at baseline and only 1 was positive for *C difficile* at week 8. The limited number of *C difficile*–positive stools precluded further statistical analysis. Fecal calprotectin concentrations at baseline (mean ± SE) were as follows: CF group 240.8 ± 45.9 μg/g feces; EF group 279.9 ± 45.4 μg/g feces;

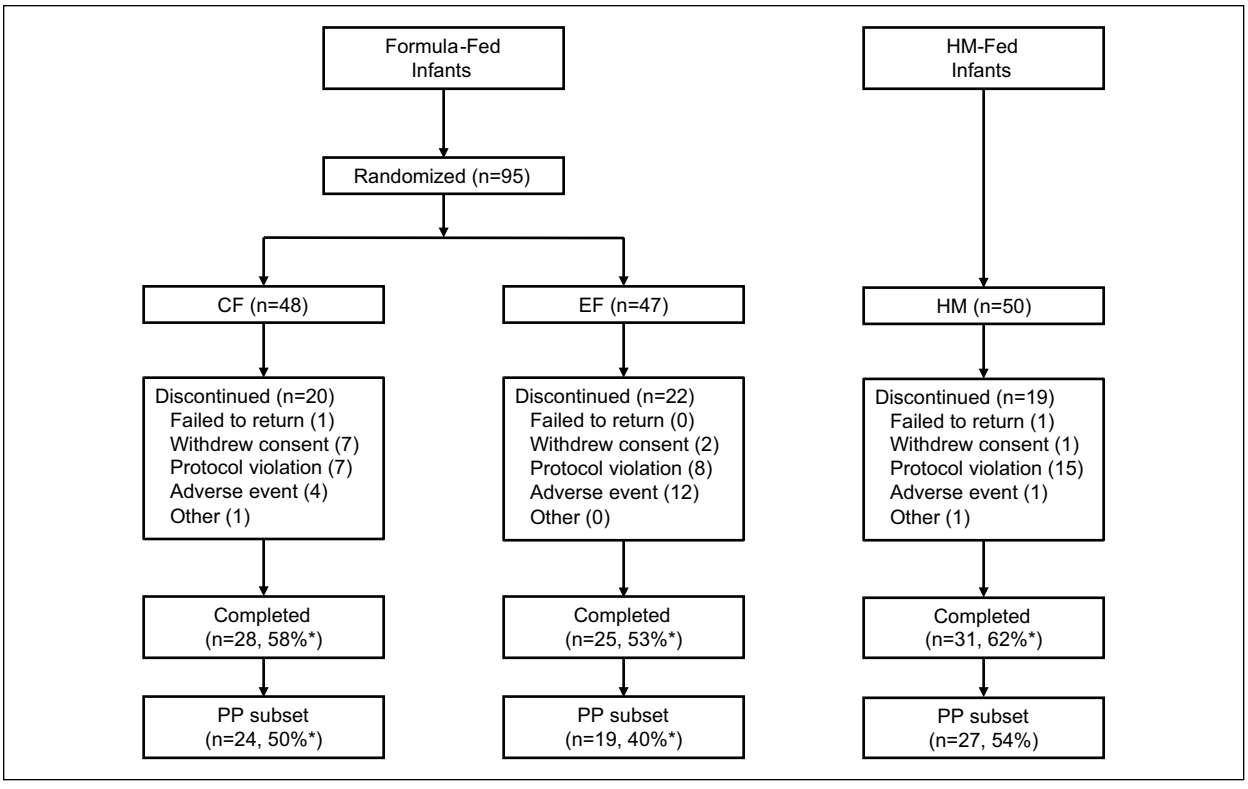

Figure 1. Study completion by feeding group. *Percentage reflects proportion of infants originally enrolled.
Abbreviations: CF, a whey-dominant infant formula enriched with α-lactalbumin; EF, CF supplemented with 3.0 g/L oligofructose; HM, human milk; PP, per protocol.

Table 3. Baseline Characteristics of Infants by Feeding Group, Per Protocol Population.[a]

	CF (n = 24)	EF (n = 19)	HM (n = 27)
Age, days, mean (SD)	11.6 (1.9)	10.8 (2.6)	11.5 (2.8)
Weight, kg, mean (SD)	3.4 (0.4)	3.5 (0.6)	3.6 (0.5)
Male gender, n (%)	14 (58.3)	12 (63.2)	10 (37.0)
Vaginal delivery, n (%)	14 (58.3)	16 (84.2)	19 (70.4)
Maternal use of intrapartum antibiotics, n (%)	6 (25.0)	5 (26.3)	1 (3.7)

Abbreviations: CF, a whey-dominant infant formula enriched with α-lactalbumin; EF, CF supplemented with 3.0 g/L oligofructose; HM, human milk.
[a]Continuous variables are reported as mean (standard deviation), categorical variables are reported as number (%).

and HM group 530.8 ± 126.9 μg/g feces; concentrations at week 8 (mean ± SE) were similar—CF group 243.2 ± 27.5 μg/g feces; EF group 220.9 ± 43.5 μg/g feces; and HM group 520.2 ± 109.5 μg/g feces. Among infants who had calprotectin results at both baseline and week 8 (10-12 infants per feeding group), there were no significant differences in adjusted change from baseline in the EF versus CF groups. Furthermore, there were no significant differences in adjusted change from baseline in either formula feeding group compared with the HM group.

Stool Frequency and Consistency

At week 8, there was no significant difference in the mean number of stools passed in a 24-hour period by infants in the EF and CF groups (mean ± SE: EF group 1.59 ± 0.43 stools/d vs CF group 2.18 ± 0.32 stools/d, $P = .28$). However, infants in the formula groups passed, on average, fewer stools per day than HM-fed infants (mean ± SE: HM group 3.71 ± 0.42 vs EF group, $P = .004$; HM group vs CF group, $P = .007$). Mean stool consistency scores at baseline and week 8 are shown in

Table 4. Intestinal Microbiota at Baseline and Week 8 by Feeding Group.[a]

	Time Point	CF (n = 20)	EF (n = 19)	HM (n = 23)
bifidobacteria[b]	Baseline	12.11 ± 0.14	12.08 ± 0.12	11.62 ± 0.17
	Week 8	12.33 ± 0.14	12.68 ± 0.08	12.45 ± 0.13
Bacteroides	Baseline	11.32 ± 0.11	11.34 ± 0.17	10.73 ± 0.13
	Week 8	11.57 ± 0.09	11.62 ± 0.10	11.53 ± 0.14
clostridia	Baseline	11.44 ± 0.16	11.47 ± 0.14	10.89 ± 0.17
	Week 8	11.76 ± 0.08	11.68 ± 0.09	11.61 ± 0.11
Enterobacteriaceae[c]	Baseline	11.45 ± 0.13	11.56 ± 0.09	11.23 ± 0.12
	Week 8	11.54 ± 0.10	11.81 ± 0.09	11.81 ± 0.11
Lactobacillus/Enterococcus	Baseline	11.75 ± 0.12	11.59 ± 0.10	11.37 ± 0.12
	Week 8	11.76 ± 0.10	11.74 ± 0.09	11.76 ± 0.08
Staphylococcus	Baseline	11.79 ± 0.14	11.60 ± 0.13	11.34 ± 0.13
	Week 8	11.97 ± 0.08	11.89 ± 0.07	11.77 ± 0.12

Abbreviations: CF, a whey-dominant infant formula enriched with α-lactalbumin; EF, CF supplemented with 3.0 g/L oligofructose; HM, human milk.
[a]Data presented as unadjusted baseline and week 8 values ± standard error (SE) for mean bacteria $\log_{10}$ transformed counts [$\log_{10}$(count)] expressed per gram dry fecal weight.
[b]In the adjusted model presented in the text, the increase in bifidobacteria from baseline to week 8 was significantly greater in the EF group versus CF (mean ± SE: EF group 0.70 ± 0.15 vs CF group 0.16 ± 0.12, $P = .008$).
[c]In the adjusted model presented in the text, the increase in *Enterobacteriaceae* from baseline to week 8 was significantly smaller in the CF group versus HM (mean ± SE: CF group 0.18 ± 0.12 vs HM group 0.58 ± 0.11, $P = .023$).

Figure 2. HM-fed infants passed softer stools at week 8 than either formula group ($P < .001$ vs CF group; $P = .021$ vs EF group). The average stool consistency score for infants in the EF group was intermediate between the CF and HM-fed groups at week 8, and infants in the EF group passed significantly softer stools than infants in the CF group ($P = .015$). Similarly, at week 8 the distribution pattern of stool consistency categories (hard, firm, soft, loose, or watery) in the EF group was intermediate between the CF and HM-fed groups (Figure 3). Adjusting stool frequency and consistency for baseline values made little difference to formula group comparisons thus these terms were not included in final models.

Hydration Status

Physician-assessed hydration status assessed at baseline, week 4, and week 8 was normal for all infants. Baseline-adjusted infant weights at week 8 did not differ by formula feeding group (mean ± SE: EF group 5.6 ± 0.1 kg vs CF group 5.4 ± 0.1 kg, $P = .38$); nor was there a difference in mean body weight between either of the 2 formula groups and the HM group (mean ± SE: HM group 5.6 ± 0.1 kg vs EF group, $P = .30$; HM group vs CF group, $P = .73$). Results were similar for the safety population at week 8 (mean ± SE: CF group 5.4 ± 0.1 kg, EF group 5.6 ± 0.1 kg, HM group 5.5 ± 0.1 kg). There was no significant difference in the adjusted mean number of wet diapers per day at week 8 between the EF and CF groups (mean ± SE: EF group 9.9 ± 0.9 wet diapers/d vs CF group 8.2 ± 0.7 wet diapers/d, $P = .13$); nor was there a difference between either of the 2 formula groups and the HM group (mean ± SE: HM group 8.4 ± 0.6 wet diapers/d vs EF group, $P = .13$; HM group vs CF group, $P = .83$). Results were similar for the safety population at week 8 (mean ± SE: CF group 8.1 ± 0.5 wet diapers/d, EF group 8.9 ± 0.7 wet diapers/d, HM group 7.9 ± 0.4 wet diapers/d).

Safety

A total of 28 (19%) infants experienced an adverse event considered by the physician to be possibly, probably or definitely related to feeding; 13 (27%) in the CF group, 15 (32%) in the EF group and none in the HM group. Feeding-related gastrointestinal adverse events were evenly distributed between the 2 formula groups (13 in each group) and none were considered serious. There were 7 withdrawals because of feeding-related gastrointestinal adverse events (vomiting, spitting up, and abdominal pain) in the EF group and 2 in the CF group because of vomiting and flatulence.

Discussion

The present study demonstrates that infants fed the OF-supplemented formula had a greater increase in fecal bifidobacteria concentrations over 8 weeks than infants fed control formula without OF. HM-fed infants are known to have a fecal microbiota dominated by bifidobacteria[2-4]; here, the increase in bifidobacteria in the

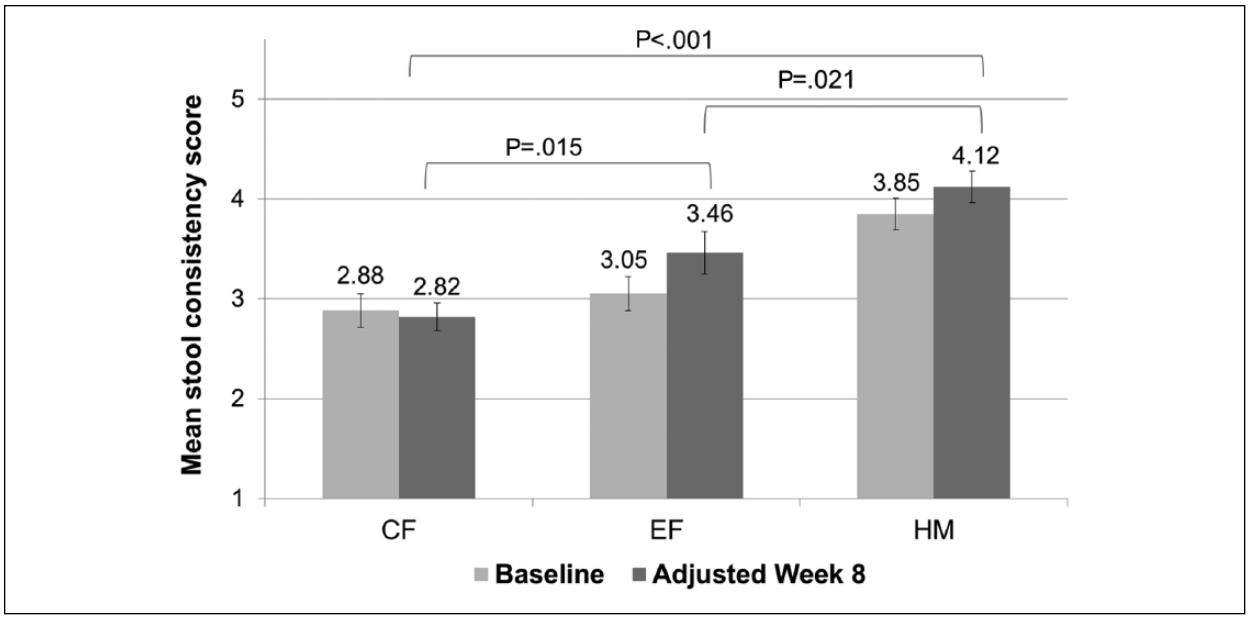

Figure 2. Mean stool consistency scores at baseline and week 8 by feeding group.
Abbreviations: CF, a whey-dominant infant formula enriched with α-lactalbumin; EF, CF supplemented with 3.0 g/L oligofructose; HM, human milk. Mean stool consistency scores at baseline and week 8 are shown as mean ± standard error (SE). Week 8 stool consistency scores were adjusted for treatment, study site, and treatment by site interaction. Results based on a 5-point scale: 1 = hard; 2 = firm; 3 = soft; 4 = loose; 5 = watery.

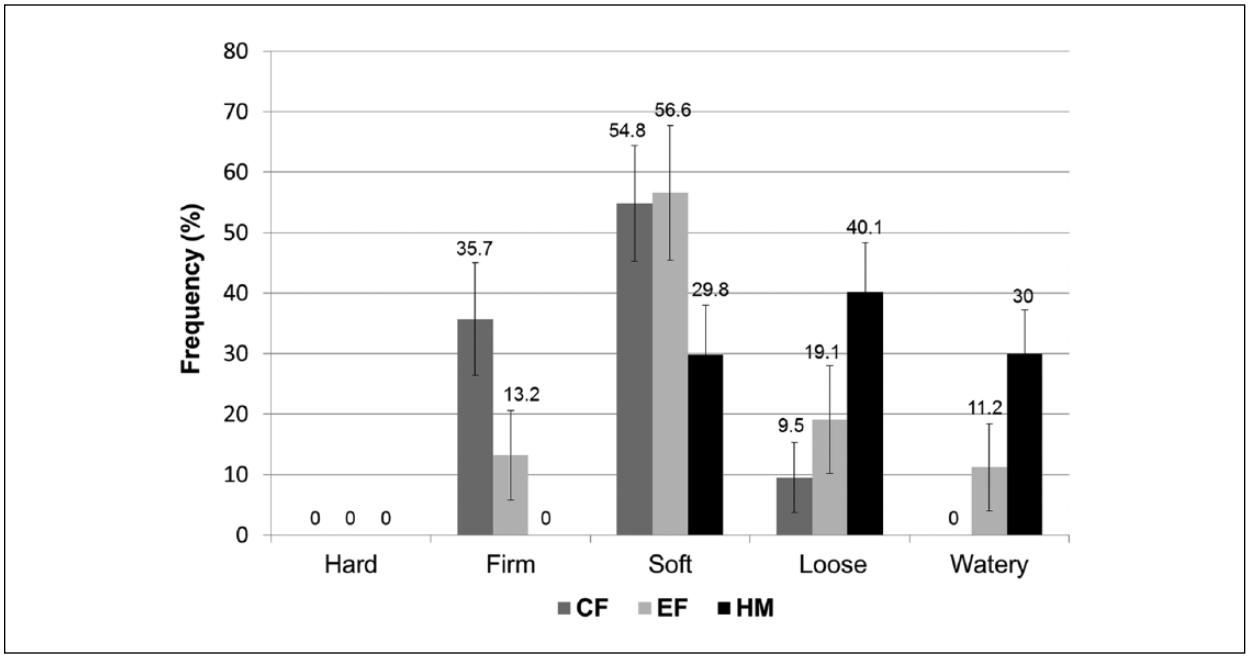

Figure 3. Stool consistency distribution at week 8 by feeding group.
Abbreviations: CF, a whey-dominant infant formula enriched with α-lactalbumin; EF, CF supplemented with 3.0 g/L oligofructose; HM, human milk. Stool category frequencies are shown as mean ± standard error (SE).

OF-supplemented group was similar to the increase observed among infants who received HM. Two previous studies of OF-supplemented formula in exclusively FF infants have demonstrated little or no

change in fecal microbiota after OF supplementation; however, the duration of supplementation in these studies was short, only 1 to 2 weeks.[18,20] Furthermore, at least one of these studies measured fecal microbiota with quantitative culture, a detection method with relatively low sensitivity.[20] A study by Yao et al[21] evaluated the effects of a formula supplemented with OF and a high *sn*-2 palmitate fat blend on fecal microbiota using FISH. After 8 weeks, infants who received the formula with 3.0 g/L OF and high *sn*-2 palmitate had significantly higher fecal bifidobacteria counts compared with infants who received a standard control formula, and counts in the OF and *sn*-2 supplemented infants did not differ from HM-fed infants. However, the changes in fecal bifidobacteria reported in this comparison represent the combined effects of both *sn*-2 palmitate and OF. Other studies have demonstrated increases in fecal bifidobacteria following OF supplementation in preterm infants and adults.[16,29]

In the present study, both the mode of delivery and administration of intrapartum antibiotics differed among the study groups. Since mode of delivery has been shown to be associated with differences in neonatal fecal microbiota,[30] an additional statistical evaluation was performed which adjusted for this variable; however, adjustment for mode of delivery made little difference to the results of the statistical analysis or study conclusions. Intrapartum antibiotic use was similar between the formula groups and lower in the HM-fed group. Although intrapartum antibiotic administration can alter the bacterial colonization of infants, these alterations are not dramatic[31] and would not be expected to substantially influence the results of this study.

Compared with HM-fed infants, FF infants have previously been reported to have higher proportions of fecal bacteria from groups that include potentially pathogenic bacterial species, such as *Bacteroides* and *Clostridium*.[2-4] However, in contrast to the findings for bifidobacteria, significant differences in change from baseline between the 2 formula groups were not observed for counts of *Bacteroides*, clostridia, *Enterobacteriaceae, Lactobacillus/Enterococcus*, or *Staphylococcus*. Consistent with the results for clostridia counts, changes in the number of *C difficile*–positive stools were not observed among infants receiving the EF compared to infants receiving the formula without OF although power for this endpoint was low since only 1 infant tested positive for *C difficile*. Similar to these findings, a previous study demonstrated little or no benefit of 1 week of OF-supplementation at 3.0 g/L on counts of enterococci, *Bacteroides*, or *Clostridium*, although reductions in the proportion positive for *C difficile* toxin were seen in the OF-supplemented groups.[20] However, other studies have demonstrated reduced counts of known or potential pathogens in response to OF supplementation; in adults, supplementation of OF at 15 g/d for 2 weeks reduced counts of clostridia, bacteroides, and fusobacteria.[16] Studies in infants that have demonstrated changes in potentially pathogenic fecal bacteria have generally used higher prebiotic doses than the present study. For example, 2 weeks of OF supplementation at 4 g/L in preterm infants reduced fecal counts of *Escherichia coli* and enterococci[29] while healthy term infants who received infant formula with 8 g/L of a 50:50 combination of OF and inulin had lower counts of *Bacteroides* and *Enterobacteriaceae* than infants who received the control formula.[32] Together with data from the present study, these results suggest that, although OF at 3.0 g/L is sufficient to increase fecal bifidobacteria, a dose higher than 3.0 g/L OF may be required to produce changes in potentially pathogenic intestinal bacteria.

Formula-fed infants have also been reported to have higher rates of gastrointestinal infections compared with HM-fed infants[33]; however, alterations in the fecal microbiota in response to OF supplementation suggest the possibility that OF may contribute to a reduced risk and/or severity of common intestinal diseases among infants. Indeed, among older infants approximately 1 year of age attending daycare centers, OF supplementation provided in weaning foods or drinks reduced fever episodes and antibiotic use[34,35] and severity of diarrheal disease.[36] In these studies, OF supplementation was provided for 21 days[34] or 6 months,[35,36] and data on fecal microbiota were not collected. The present study collected data on levels of fecal calprotectin, which has been recognized as a noninvasive indicator of intestinal disease and inflammatory activity in children and adults, and proposed for use as a marker of intestinal distress in neonates.[37] However, changes in calprotectin concentrations were not different in the OF-supplemented group compared with control. This finding may reflect a low rate of intestinal distress among the healthy, term, U.S.-based population of infants included in the present study, a possibility supported by the mean calprotectin concentrations in the FF groups at baseline and week 8, which fell below a recently proposed screening threshold for digestive disease among neonates (350 μg/g feces).[37] In contrast, relatively high fecal calprotectin concentrations have been reported in populations with overt or subclinical intestinal disease.[38-40] Fecal calprotectin concentrations in higher risk populations may be more amenable to nutritional interventions.

Formula-fed infants typically have harder stools than HM-fed infants[5,6] and parents are often concerned about the stool consistency of FF infants.[41] Considered the

gold standard in infant nutrition, HM-fed infants typically have soft, loose stools.[5] Previously, a 12-week study of infants receiving formula supplemented with 3.0 g/L OF demonstrated that constipation was less frequent among infants receiving 3.0 g/L OF compared with control, with no increase in the frequency of loose stools.[19] Similarly, there was a significantly greater change in stool consistency in infants who received formula supplemented with 3.0 g/L OF versus 1.5 g/L OF for 1 week; infants receiving formula with 3.0 g/L OF also had softer and more frequent stools than infants receiving the formula with the lower OF dose. Loose stools were also significantly increased during OF supplementation, with a greater frequency of looser stool adverse events among infants receiving 3.0 g/L OF compared with 1.5 g/L OF.[20] The present study found softer stools among infants fed EF compared with CF and mean stool consistency in the EF group was intermediate between the stool consistencies of infants receiving CF and infants receiving HM. This increase in stool softness was not accompanied by an increase in stool frequency. The European Food Safety Authority has recognized that changes in bowel function such as softer stools (without diarrhea) may be considered beneficial physiological effects.[42]

Concern has been expressed related to water balance in infants receiving OF-supplemented formula due to looser stools than in infants receiving unsupplemented formulas.[14] To assess hydration status the current study employed a consistent definition of hydration status across all study sites, based on common clinical measures of hydration including assessments of infant weight, mucous membranes, skin turgor, formula intake, and number of wet diapers per day.[43-45] The α-lactalbumin-enriched formula supplemented with OF did not adversely affect hydration status compared to control; physician-assessed hydration status, evaluated at 3 time points during the study, was normal for all infants. Of the 4 studies that have evaluated OF-supplemented infant formula in exclusively FF healthy term infants,[18-21] none employed a detailed evaluation of infant hydration status. However, normal hydration status has been reported in infants receiving formula containing a 50:50 combination of OF and inulin at 4 g/L[46] and 8 g/L.[32,46]

In the present study, both the total proportion of infants who discontinued the study for any reason and the overall incidence of adverse events were similar among the three groups. Although the number of feeding-related gastrointestinal adverse events was evenly distributed between the 2 formula groups, the EF group had a greater number of withdrawals due to feeding-related gastrointestinal adverse events than the CF group. However, none of these events was considered serious and in both groups, the events leading to the withdrawals represented common occurrences that are typical among infants of this age (vomiting, spitting up, flatulence, and abdominal pain). Previous studies of OF-supplemented infant formulas (at levels ranging from 1.5 to 5.0 g/L OF) provided to exclusively FF term and preterm infants consistently reported that these formulas were well tolerated.[18-21,29] In addition, formulas containing a 50:50 combination of OF and inulin evaluated in healthy term infants at doses up to 8 g/L[32,46] were also found to be safe and well tolerated.

Strengths of the present study include its randomized, multicenter design comparing formulas with and without added OF. By testing a formula with a single prebiotic (OF), which was not used in combination with other prebiotics such as galactooligosaccharides or inulin, the study was able to directly evaluate the effect of OF on fecal microbiota, stool consistency and frequency, and hydration. In addition, infants were excluded who consumed medications that could influence study endpoints. The possibility that mode of delivery confounded the effect of the formulas on fecal microbiota counts was excluded in the statistical analysis. Limitations included the study discontinuation and data exclusion rates, which affected the proportion of infants who remained in the study and met PP criteria, and could thus be included in the analysis, although based on the study discontinuation/data exclusion rates reported in other published infant prebiotic studies, this is not uncommon.[32,46,47] Where the events leading to study discontinuation are published, they typically represent common symptoms in infant populations, such as gastroesophageal reflux, regurgitation, and hunger[46]; as is the case here, rates of study discontinuation are typically similar between the formula groups.[32,46,47]

In conclusion, it is well recognized that breast-feeding is the optimal means of providing nutrition to the healthy term infant. Improvements to infant formula may match some, but not all, of the benefits of HM feeding. The addition of 3.0 g/L OF to an α-lactalbumin enriched term infant formula resulted in increases in fecal bifidobacteria over an 8-week period similar to increases in HM-fed infants, and greater than increases in infants receiving a control formula without OF. Hard stools and constipation are common concerns among parents of FF infants[41]; here, FF infants receiving OF had stools that were softer than those in infants receiving control formula and more similar to those in HM-fed infants without increases in stool frequency or adverse impacts on hydration status. Overall, the present study supports the safety and tolerability of an OF-containing infant formula, including hydration status. Future studies should

measure species-level changes in the fecal microbiota caused by OF supplementation; these may or may not parallel changes in broad bacterial classes, such as those detected by the FISH method. In addition, future studies should evaluate whether the benefits of OF supplementation persist after supplementation ends, and seek to better understand any long term benefits associated with improvements in fecal microbiota and stool consistency resulting from OF supplementation.

Acknowledgments

The authors are grateful to the investigators and their study staff for participating in this multicenter trial: Jack Seidel, Dallas, Texas; Stan Block, Bardstown, Kentucky; Frank Hughes, Bossier City, Louisiana; Laura Ann Sass, Norfolk, Virginia; John Spitzer, Kalamazoo, Michigan; Frances Rushton, Beaufort, South Carolina; Lisa Lowe, Murfreesboro, Tennessee; Richard Schwartz, Vienna, Virginia; Linda Garrison, Portage, Michigan; and Michael Warren, Lancaster, Pennsylvania.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Susan Wernimont, Robert Northington, Manjiang Yao, and Jodi Bettler are employees of Nestlé Nutrition. Martin Kullen was an employee of Wyeth Nutrition at the time of the study and is currently employed by DuPont Nutrition & Health, Wilmington, Delaware.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was sponsored by Wyeth Nutrition, a Nestlé business, King of Prussia, Pennsylvania. The infant formula evaluated in this study was a Wyeth Nutrition product.

References

1. Donovan SM. Human milk: nutritional properties. In: Duggan C, Watkins JB, Walker WA, eds. *Nutrition in Pediatrics: Basic Science and Clinical Applications*. 4th ed. Hamilton, Ontario, Canada: BC Decker; 2008:341S-353S.
2. Fallani M, Young D, Scott J, et al. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr*. 2010;51:77-84.
3. Fanaro S, Chierici R, Guerrini P, Vigi V. Intestinal microflora in early infancy: composition and develop-ment. *Acta Paediatr*. 2003;91:48-55.
4. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr*. 2000;30:61-67.
5. Weaver LT, Ewing G, Taylor LC. The bowel habit of milk-fed infants. *J Pediatr Gastroenterol Nutr*. 1988;7:568-571.
6. Quinlan PT, Lockton S, Irwin J, Lucas AL. The relationship between stool hardness and stool composi-tion in breast- and formula-fed infants. *J Pediatr Gastroenterol Nutr*. 1995;20:81-90.
7. Lonnerdal B. Bioactive proteins in human milk: mechanisms of action. *J Pediatr*. 2010;156(2 suppl):S26-S30.
8. Petschow BW, Talbott RD. Response of bifidobacterium species to growth promoters in human and cow milk. *Pediatr Res*. 1991;29:208-213.
9. Frantz D, Kullen MJ. Effects of bovine α-lactalbumin on the growth of bifidobacteria. Paper presented at: The 107th General Meeting of the American Society for Microbiology; May 21-25, 2007; Toronto, Ontario, Canada.
10. Kullen MJ, Lambers T, Venema K, Bettler J. Characterization of peptides derived from α-lactalbumin digestion and demonstration of their prebiotic effect through stimulation of bifidobacteria in a model of the gastrointestinal tract. *J Pediatr Gastroenterol Nutr*. 2009;46:e197.
11. Brück WM, Graverholt G, Gibson GR. A two-stage continuous culture system to study the effect of supplemental α-lactalbumin and glycomacropeptide on mixed cultures of human gut bacteria with entero-pathogenic *Escherichia coli* and *Salmonella* serotype Typhimurium. *J Appl Microbiol*. 2003;95:44-53.
12. Brück WM, Graverholt G, Gibson GR. Use of batch culture and a two-stage continuous culture system to study the effect of supplemental α-lactalbumin and glycomacropeptide on mixed populations of human gut bacteria. *FEMS Microbiol Ecol*. 2002;41:231-237.
13. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology*. 2012;22:1147-1162.
14. Scientific Panel on Dietetic Products, Nutrition and Allergies. Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission relating to the safety and suitability for particular nutritional use by infants of fructooligosaccharides in infant formulae and follow-on formulae. *EFSA J*. 2004;31:1-11.
15. Bouhnik Y, Vahedi K, Achour L, et al. Short-chain fructo-oligosaccharide administration dose-dependently increases fecal bifidobacteria in healthy humans. *J Nutr*. 1999;129:113-116.
16. Gibson GR, Beatty ER, Wang X, Cummings JH. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. *Gastroenterology*. 1995;108:975-982.
17. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr*. 1995;125:1401-1412.
18. Guesry PR, Bodanski H, Tomsit E, Aeschlimann JM. Effect of 3 doses of fructo-oligosaccharides in infants. *J Pediatr Gastroenterol Nutr*. 2000;31:S252.
19. Bettler J, Euler A. An evaluation of the growth of term infants fed formula supplemented with fructo-oligosaccharide *Int J Probiot Prebiot*. 2006;1:19-26.

20. Euler AR, Mitchell DK, Kline R, Pickering LK. Prebiotic effect of fructo-oligosaccharide supplemented term infant formula at two concentrations compared with unsupplemented formula and human milk. *J Pediatr Gastroenterol Nutr*. 2005;40:157-164.
21. Yao M, Lien EL, Capeding MR, et al. Effects of term infant formulas containing high sn-2 palmitate with and without oligofructose on stool composition, stool characteristics, and bifidogenicity: a randomized, double-blind, controlled trial [published online May 30, 2014]. *J Pediatr Gastroenterol Nutr*. doi:10.1097/MPG.0000000000000443.
22. Bettler J, Kullen MJ. Infant formula enriched with α-lactalbumin has a prebiotic effect in healthy term infants *J Pediatr Gastroenterol Nutr*. 2007;44(supplement 1):e197.
23. Franks AH, Harmsen HJ, Raangs GC, Jansen GJ, Schut F, Welling GW. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microb*. 1998;64:3336-3345.
24. Langendijk PS, Schut F, Jansen GJ, et al. Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl Environ Microb*. 1995;61:3069-3075.
25. Bohnert J, Hübner B, Botzenhart K. Rapid identification of Enterobacteriaceae using a novel 23S rRNA-targeted oligonucleotide probe. *Int J Hyg Environ Health*. 2000;203:77-82.
26. Harmsen H, Elfferich P, Schut F, Welling G. A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent in situ hybridization. *Microb Ecol Health Dis*. 1999;11:3-12.
27. Meier H, Amann R, Ludwig W, Schleifer KH. Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G + C content. *Syst Appl Microbiol*. 1999;22:186-196.
28. Kempf VA, Trebesius K, Autenrieth IB. Fluorescent In situ hybridization allows rapid identification of microorganisms in blood cultures. *J Clin Microbiol*. 2000;38:830-838.
29. Kapiki A, Costalos C, Oikonomidou C, Triantafyllidou A, Loukatou E, Pertrohilou V. The effect of a fructo-oligosaccharide supplemented formula on gut flora of preterm infants. *Early Hum Dev*. 2007;83:335-339.
30. Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C. Mode of delivery affects the bacterial community in the newborn gut. *Early Hum Dev*. 2010;86(suppl 1):13-15.
31. Jaureguy F, Carton M, Panel P, Foucaud P, Butel MJ, Doucet-Populaire F. Effects of intrapartum penicillin prophylaxis on intestinal bacterial colonization in infants. *J Clin Microbiol*. 2004;42:5184-5188.
32. Closa-Monasterolo R, Gispert-Llaurado M, Luque V, et al. Safety and efficacy of inulin and oligofructose supplementation in infant formula: results from a randomized clinical trial. *Clin Nutr*. 2013;32:918-927.
33. Agostoni C, Braegger C, Decsi T, et al. Breast-feeding: a commentary by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr*. 2009;49:112-125.
34. Waligora-Dupriet AJ, Campeotto F, Nicolis I, et al. Effect of oligofructose supplementation on gut microflora and well-being in young children attending a day care centre. *Int J Food Microbiol*. 2007;113:108-113.
35. Tschernia A, Moore N, Abi-Hanna A, et al. Effects of long-term consumption of a weaning food supplemented with oligofructose, a prebiotic, on general infant health status. *J Pediatr Gastroenterol Nutr*. 1999;29:503.
36. Saavedra J, Tschernia A, Moore N, et al. Gastrointestinal function in infants consuming a weaning food supplemented with oligofructose, a prebiotic. *J Pediatr Gastroenterol Nutr*. 1999;29:513.
37. Kapel N, Campeotto F, Kalach N, Baldassare M, Butel MJ, Dupont C. Faecal calprotectin in term and preterm neonates. *J Pediatr Gastroenterol Nutr*. 2010;51:542-547.
38. Josefsson S, Bunn SK, Domellof M. Fecal calprotectin in very low birth weight infants. *J Pediatr Gastroenterol Nutr*. 2007;44:407-413.
39. Aomatsu T, Yoden A, Matsumoto K, et al. Fecal calprotectin is a useful marker for disease activity in pediatric patients with inflammatory bowel disease. *Dig Dis Sci*. 2011;56:2372-2377.
40. Liu JR, Sheng XY, Hu YQ, et al. Fecal calprotectin levels are higher in rural than in urban Chinese infants and negatively associated with growth. *BMC Pediatr*. 2012;12:129.
41. Morley R, Abbott RA, Lucas A. Infant feeding and maternal concerns about stool hardness. *Child Care Health Dev*. 1997;23:475-478.
42. EFSA Panel on Dietetic Products, Nutrition and Allergies. Guidance on the scientific requirements for health claims related to gut and immune function. *EFSA J*. 2011;9:1984.
43. Kleinman RE, ed. *Pediatric Nutrition Handbook*. Elk Grove Village, IL: American Academy of Pediatrics; 2009.
44. Gorelick MH, Shaw KN, Murphy KO. Validity and reliability of clinical signs in the diagnosis of dehydration in children. *Pediatrics*. 1997;99:E6.
45. Steiner MJ, DeWalt DA, Byerley JS. Is this child dehydrated? *JAMA*. 2004;291:2746-2754.
46. Veereman-Wauters G, Staelens S, Van de Broek H, et al. Physiological and bifidogenic effects of prebiotic supplements in infant formulae. *J Pediatr Gastroenterol Nutr*. 2011;52:763-771.
47. Bruzzese E, Volpicelli M, Squeglia V, et al. A formula containing galacto- and fructo-oligosaccharides prevents intestinal and extra-intestinal infections: an observational study. *Clin Nutr*. 2009;28:156-161.