JOBNAME: gas 40#2 2005 PAGE: 1 OUTPUT: Sat January 15 18:48:59 2005
lww/gas/91706/MPG158571
Prod #: MPG158571
MS #: 04-047

Journal of Pediatric Gastroenterology and Nutrition
40:157–164 © February 2005 Lippincott Williams & Wilkins, Philadelphia

Prebiotic Effect Of Fructo-Oligosaccharide Supplemented Term Infant Formula at Two Concentrations Compared with Unsupplemented Formula and Human Milk

*Arthur R. Euler, MD, †Douglas K. Mitchell, MD, *Randi Kline, RD, and ‡Larry K. Pickering, MD

**Wyeth Nutrition, Collegeville, Pennsylvania; †Center for Pediatric Research, Eastern Virginia Medical School, Children's Hospital of The King's Daughters, Norfolk, Virginia; ‡National Immunization Program, Centers for Disease Control and Prevention Atlanta, Georgia*

ABSTRACT

Background: Human milk components, including oligosaccharides, affect the gastrointestinal flora of infants. Previous studies in adults have demonstrated that fructo-oligosaccharides increase potentially beneficial fecal bacteria, including bifidobacteria. The purpose of this study was to determine the prebiotic effect of infant formula supplemented with fructo-oligosaccharides.

Methods: Healthy term infants 2 to 6 weeks of age were enrolled in a 5-week, prospective, randomized, crossover, single-site study with a nonrandomized human milk comparator group. Washout weeks preceded and followed a week of feeding with fructo-oligosaccharide-supplemented formula (1.5 or 3.0 g/L). Stool specimens were quantitatively cultured weekly for bacteroides, lactobacilli, bifidobacteria, clostridia and enterococci and were tested for *Clostridium difficile* toxin.

Results: Seventy-two of 87 infants completed the trial; 58 were formula fed and 14 were human milk fed. Mean counts of bifidobacteria and lactobacilli were similar in all groups at entry and no group experienced a significant change in counts with fructo-oligosaccharide supplementation. After 7 days of fructo-oligosaccharide supplementation the bifidobacteria counts were greater in the 1.5 g/L fructo-oligosaccharide formula group than in the human milk fed or 3.0 g/L fructo-oligosaccharide formula groups. Formula-fed infants had higher counts of enterococci and bacteroides before fructo-oligosaccharide supplementation, and these counts did not change after supplementation. *Clostridium* counts increased 7 days after supplementation in the 1.5 g/L fructo-oligosaccharide formula group ($P = 0.0356$). No human milk fed infants had *C. difficile* toxin in stools. Fructo-oligosaccharide (3.0 g/L) supplementation resulted in more frequent and significantly softer stools.

Conclusions: Infant formula supplemented with 1.5 or 3.0 g/L fructo-oligosaccharides was safe but had minimal effect on fecal flora and *C. difficile* toxin. ***JPGN 40:157–164, 2005***. © 2005 Lippincott Williams & Wilkins

INTRODUCTION

Human milk has some prebiotic function, thereby inducing the growth of a beneficial gut bacterial flora in infants (1–3). Human milk oligosaccharides are probably the major source of this prebiotic effect (4–6). Fructo-oligosaccharides (FOS) have been shown in adult humans and neonatal piglets to increase the gut populations of potentially beneficial bacteria such as bifidobacteria (7,8). The aim of this study was to determine if FOS at either of two concentrations in term infant formula had a beneficial prebiotic effect on the gastrointestinal tract flora of infants. Comparisons were made to an identical, non-FOS-supplemented term infant formula and to human milk-fed infants of the same age.

Received March 6, 2004; accepted October 7, 2004.

This study was supported by Wyeth Nutrition, Collegeville, Pennsylvania. Ms. Randi Kline is an employee of Wyeth Nutrition as was Dr. Euler at the time the study was designed and conducted. Dr. Pickering had no financial conflict of interest. Dr. Mitchell was the study's principal investigator. Wyeth Nutritional contracted with eastern Virginia Medical School (EVMS) for the conduct of the study. Dr. Mitchell was an EVMS employee during the conduct of the study. Dr. Euler was compensated by Wyeth Nutrition for his work in writing this manuscript.

Address correspondence and reprint requests to Arthur R. Euler, 2316 Stacey Hollow Place, Lafayette, IN 47905-8506. (e-mail: a.euler@insightbb.com).

MATERIALS AND METHODS

This study was approved by the Eastern Virginia Medical School Institutional Review Board. In all cases a parent/guardian signed and dated an informed consent form at enrollment.

Study Population

We enrolled 2- to 6-week-old healthy infants of gestational age between 37 and 42 weeks. At birth and at enrollment, weight for length ratios were required to be between the 10th and 90th percentiles according to the World Health Organization

growth charts. Infants were excluded if they had siblings with documented bovine milk protein allergy or a feeding problem that had necessitated a change from bovine milk-based formula to protein hydrolysate formula. Other exclusion criteria were birth by cesarean section, any diarrheal episode since birth, suspected or documented systemic or congenital infections and evidence of significant cardiac, respiratory, hematological, gastrointestinal or other systemic diseases. Infants were excluded or withdrawn from the study if they were exposed to smoking in the home or had received iron supplements, antimicrobial agents, antifungal medications (except topical preparations), suppositories, bismuth-containing medications or any medication that might neutralize or suppress gastric acid secretion.

Infants entering the formula-fed (FF) arms of the study consumed standard infant formula exclusively for 7 days before enrollment. Infants enrolled in the human milk (HM)-fed arm of the trial consumed HM exclusively for 7 days before enrollment. Mothers of HM-fed infants were required to be in good health. Mothers were not permitted to take antimicrobial agents or antifungal medications (except topical preparations), bismuth-containing medications, laxatives (except stool softeners) or medications that might suppress gastric acid secretion. After enrollment in this study, infants could not participate in any other clinical trial. All infants received free weekly medical examinations, and FF infants received free study formula and diapers.

Study Design

This study was a prospective, randomized, crossover (0.0 g and 1.5 g FOS/L or 0.0 g and 3.0 g FOS/L), outpatient, single-site study of FOS added to term infant formula (S-26® Gold; Wyeth Nutrition, Collegeville, PA) with a non-randomized comparison group of HM-fed infants conducted at the Center for Pediatric Research in Norfolk, VA.

The primary efficacy objective was to determine, by quantitative culture of stool, whether either FOS concentration was associated with a change in bifidogenic fecal flora or resulted in fecal flora similar to that of HM-fed infants. The secondary efficacy objective was to determine whether either FOS concentration resulted in a decreased colony count of enterococci, bacteroides or clostridia or was associated with an incidence of *Clostridium difficile* toxin-positive stools similar to that of HM-fed infants. The safety objective was to compare the tolerability and acceptability of the two FOS concentrations.

FF infants were randomized to one of four study groups receiving one of two experimental supplemented formulas. All infants received control formula during weeks 1, 3 and 5. In a blinded fashion, they received one of the supplemented formulas during either week 2 or week 4 with control formula during either week 4 or week 2. All formulas were identical except for the FOS concentration in the supplemented formulas. The FOS used in this study was inulin, a non-nutrient carbohydrate (Raftilose®P95; ORAFTI, Tienen, Belgium). Raftilose®P95 is produced by partial enzymatic hydrolysis of chicory inulin. It contains more than 93.2% of this oligofructose and less than 6.8% glucose, fructose and sucrose.

Formula was supplied as a powder providing 670 kcal (2804 kJ) per liter when reconstituted according to label directions. The three study formulas were as follows:

- Control formula: S-26® Gold (Wyeth Nutrition's commercially available term infant cow's milk based formula).
- Experimental formula #1: S-26® Gold with 1.5 g/L FOS.
- Experimental formula #2: S-26® Gold with 3.0 g/L FOS.

Feeding of non-study formula or HM was prohibited in the FF groups, and formula feeding was prohibited in the HM-fed group. Baby foods or solid foods were prohibited in all study groups.

Blinding was maintained as follows. After enrollment and signed informed consent, an infant was assigned a three-digit randomization number and one of the study formula sequences. The randomization number was used as the unique infant identifier throughout the study. Once a randomization number had been assigned, it was not assigned subsequently to any other infant. Infants who discontinued the study before the 28-day visit were replaced. The next infant to enroll after an infant discontinued received the same formula sequence as the infant who was removed, but the randomization number was different.

HM-fed and FF infants had the same schedule of visits and specimen collection. Weekly ($\pm$1 day) study visits included a history and physical examination with measurements of weight, length and occipitofrontal head circumference by standard methods (9,10). A diary recording formula intake, stool frequency, size, consistency and color was completed during the 24 hours before each weekly visit. Stool consistency was recorded using a descriptor corresponding to an assigned numerical value as follows: watery = 5, loose = 4, soft = 3, firm = 2 and hard = 1. Stool frequency was reported as number of stools in the previous 24-hour period. At each visit, the parent or guardian was questioned about possible adverse events. An adverse event was defined as any untoward, undesired, unplanned clinical event in the form of signs, symptoms, disease or laboratory or physiological observations occurring in a participating infant regardless of possible causal relationship to study formula ingestion.

All medications (including over-the-counter medicines) or therapies administered to infants and each corresponding duration of administration were recorded. Routine immunizations and use of vitamin/mineral supplements except iron were recorded.

Laboratory Methods

Immediately before all study visits, a stool specimen was collected in a pre-weighed collection tube containing 1% gelatin medium in phosphate buffered saline. The collection tube was refrigerated until use. A stool sample of approximately 2 to 5 g was placed in the collection tube and dispersed in the transport medium using a standard scoop. The tube was sealed and immediately agitated until stool and liquid were mixed thoroughly and the collection tube was then refrigerated. The sample was transported within 24 hours of collection to the Center for Pediatric Research laboratory where it was weighed and quantitatively cultured.

The following organisms were quantitated on selective media to the genus level: *Bacteroides* – Bacteroides Bile Esculin agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD); *Lactobacillus* - LBS agar with tomato juice (BBL, Becton Dickinson Microbiology Systems); *Bifidobacterium* – Reinforced Clostridial agar plus nalidixic acid (20 mg/L), polymixin B (8.5 mg/mL), kanamycin (12.5 mg/mL), iodoacetic Acid

JOBNAME: gas 40#2 2005 PAGE: 3 OUTPUT: Sat January 15 18:49:00 2005
lww/gas/91706/MPG158571

(12 mg/L) and 2,3,5 triphenyl tetrazolium chloride (25 mg/mL) (BBL, Becton Dickinson Microbiology Systems); *Clostridium* - Clostrisel agar (BBL, Becton Dickinson Microbiology Systems); *Enterococcus* – KF Streptococcal agar (BBL, Becton Dickinson Microbiology Systems) (11). Quantitative culture was performed by plating 100 microliters of diluted stool onto each agar medium using an automated spiral plater system (Autoplate® 4000; Spiral Biotech, Inc., Norwood, MA). Plate surfaces were allowed to dry for 15 minutes, then were inverted and placed in an appropriate environment. Bacteroides, lactobacilli, bifidobacteria and clostridia plates were incubated in an anaerobic chamber at 37°C for a minimum of 4 days. Enterococci plates were incubated for 48 hours in 5% carbon dioxide at 37°C.

Total colony counts were performed using the CASBA™ 4 system (Spiral Biotech, Inc.) and were reported as less than detectable ($<10^2$) or detectable ($>10^2$). This methodology detected at least 10^2 colony forming units/g stool. Colony counts are reported as log (base 10) throughout this report. *Clostridium difficile* toxin was detected by an enzyme immunoassay (Premier Cytoclone A + B EIA; Meridian Diagnostics, Cincinnati, OH).

If an infant developed diarrhea during the study a stool sample was delivered to the laboratory within 2 to 3 hours of collection. The specimen was tested for occult blood (Hemoccult SENSA; SmithKline Diagnostics, Inc., San Jose, CA), fecal leukocytes by microscopy and for reducing substances (Ames Clinitest tablets; Bayer Corporation, Tarrytown, NY) (12). Diarrhea samples also were tested for rotavirus (Rotaclone EIA; Meridian Diagnostics), enteric adenovirus (Premier Adenoclone-Type 40/41 EIA; Meridian Diagnostics), *Giardia lamblia*, Cryptosporidium (ProSpecT *Giardia*/Cryptosporidium Microplate Assay; Alexon-Trend, Inc., Ramsey, MN) and *C. difficile* toxin (Premier Cytoclone A+B EIA, Meridian Diagnostics).

Statistical Methods

Sixty-five infants (52 FF and 13 exclusively HM-fed) were needed to complete the study until at least day 28 to detect a three-fold increase (from 30% to 90%) in the percentage of infants with a clinically relevant increase in fecal organism counts. The study design included 13 infants in each of the four formula study groups and 13 HM-fed infants (n = 65). A significance level of $P = 0.05$ with a power of 0.80 was used to make decisions regarding significant mean differences.

The first analysis was a comparison of organism counts (lactobacilli and bifidobacteria) in stools of the 1.5 g/L and 3.0 g/L FOS formula groups obtained before and after the week of FOS supplementation. The Wilcoxon signed-rank test was used to confirm that the median counts of the groups receiving supplementation at the second or fourth study week did not differ. The relative change in organism counts for each infant was computed from the time before FOS supplementation to the end of 1 week of FOS supplementation. The proportion of infants in the 1.5 g/L and 3.0 g/L FOS groups who attained relative increases in organism counts of 10%, 15% and 25% was compared.

The second analysis was performed to detect an association between the dosage of FOS and the quantitation of the five organisms. The log-transformed values of organism counts for each infant were used in this analysis. The distribution of counts was expected to significantly deviate from normality. Therefore, the method of generalized estimating equations (GEE) was used to analyze the data because this approach does not force one to assume a normal distribution for the outcome variable and allows for adjustments for the intra-infant correlation in measurements. The model included two time variables, one for each of the experimental formulas. One variable was assigned a value of 1 if the infant was fed 1.5 g/L FOS formula during the previous period and assigned a value of 0 otherwise. The other variable was assigned a value of 1 if the infant was fed 3.0 g/L FOS formula during the previous period and a value of 0 otherwise. The model also included an indicator variable that assigned a value of 1 if an infant was fed HM throughout the study period and a value of 0 otherwise. Time (7, 14, 21 and 28 days) also was included as a covariant. All organism counts measured for each infant were used in this analysis.

The generalized estimating equations approach allowed evaluation of any dose-response relationship between the level of supplementation with FOS and the organism counts for each infant. It also allowed determination of whether supplementation with FOS modified microbial counts to levels comparable to those in HM-fed infants and the age on organism counts. Because each infant fed FOS supplemented formula also was fed control formula feedings for four study periods, these data are analogous to data collected from a crossover clinical trial.

A secondary study outcome was the presence of fecal *C. difficile* toxin. The generalized estimating equations approach was used to make comparisons between the stool specimens collected before and after 7 days of FOS supplementation and between the two formula groups and the HM group.

RESULTS

Study Population

Of eighty-seven infants enrolled, 72 (58 FF and 14 HM fed) were considered evaluable for the per-protocol population. The reasons for discontinuing participation in the study included adverse events (three infants), failure to return (1), ingestion of non-study feeding (4), prohibited medications (5) and other (2). Data were analyzed for 87 infants in the intent-to-treat population (all randomized infants who received one feeding of control formula or who began the HM regimen). The intent-to-treat population was used for safety analyses.

At enrollment the infants in all three study groups had similar demographic and birth characteristics including gender, ethnicity, birth weight and length, birth weight for length and gestational age. The head circumference of the HM-fed infants at birth (34.8 ± 1.4 cm) was significantly larger than the FF groups (1.5 g/L group, 34.4 + 1.3 cm; 3.0 g/L group, 33.7 + 1.3 cm) ($P < 0.05$). The larger head circumference of HM-fed infants persisted from birth to study entry to study completion (data not shown) with normal growth rates in all three groups. At the end of the study, there were no other significant differences in anthropometrics between groups, including weight.

JOBNAME: gas 40#2 2005 PAGE: 4 OUTPUT: Sat January 15 18:49:01 2005
lww/gas/91706/MPG158571

Microbiology

There were no differences in bacterial counts between males and females at baseline or after FOS supplementation. The results for males and females were therefore combined for further analysis. Before FOS supplementation, the HM-fed and FF groups had similar fecal lactobacillus and bifidobacterium colony counts (Table 1). Mean values for lactobacilli were similar in all groups immediately after FOS supplementation. The mean bifidobacteria counts, however, were statistically greater ($P < 0.0450$) in the 1.5 g/L FOS formula group than in either the HM fed or 3.0 g/L FOS formula groups (Table 2) after supplementation. Seven days after the conclusion of FOS supplementation, there were no significant differences in lactobacillus or bifidobacterium counts among the treatment groups (Table 3).

All FF group had approximately 100-fold greater colony counts of enterococci and bacteroides than the HM-fed group before FOS supplementation (Table 1). Immediately after FOS supplementation, changes in enterococcus and bacteroides counts were comparable in all groups (Table 2). Seven days after conclusion of FOS supplementation, the FF groups continued to have approximately 100-fold greater counts of bacteroides ($P = 0.016$) and enterococcus ($P = 0.0001$) than did the HM-fed group (Table 3).

Clostridia colony counts were similar in HM-fed and FF groups before FOS supplementation ($P = 0.1448$) (Table 1). Immediately after FOS supplementation the counts in the two supplemented groups were similar and both were higher than counts in the HM-fed group ($P = 0.0054$) (Table 2). Seven days after the conclusion of FOS supplementation clostridia colony counts were highest for the 1.5 g/L FOS formula group and were similar in the HM-fed and 3.0 g/L FOS formula group.

TABLE 1. *Summary of log (base 10) organism counts before fructo-oligosaccharide supplementation*

Organism Type	HM (n = 14)	FOS 1.5 (n = 28)	FOS 3.0 (n = 30)	*P* value
		Bifidobacterium		
Mean	8.2	8.8	8.6	0.3386
Median	8.4	9.4	8.5	
SD	1.45	1.51	1.14	
		Lactobacillus		
Mean	4.6	4.7	4.6	0.9954
Median	4.5	5.0	4.2	
SD	2.43	2.41	2.34	
		Enterococcus		
Mean	5.6*	7.8†	7.7†	0.0001
Median	5.8	7.9	7.8	
SD	1.80	1.52	1.17	
		Bacteroides		
Mean	3.4*	5.5†	5.5†	0.0182
Median	2.0	5.9	6.0	
SD	1.91	2.32	2.79	
		Clostridium		
Mean	6.5	7.1	7.2	0.1448
Median	6.5	7.0	7.3	
SD	1.26	1.43	1.69	

HM = human milk; FOS-fructo-oligosaccharides.
*†Significantly different ($P < 0.05$).

TABLE 2. *Summary of log (base 10) organism counts at the visit after fructo-oligosaccharide supplementation*

Organism Type	HM (n = 14)	FOS 1.5 (n = 28)	FOS 3.0 (n = 30)	*P* value
		Bifidobacterium		
Mean	8.0*	9.1†	8.6*†	0.0450
Median	7.9	9.0	8.4	
SD	1.37	1.33	1.17	
		Lactobacillus		
Mean	5.3	5.2	4.8	0.7115
Median	6.5	5.3	5.3	
SD	2.58	2.69	2.60	
		Enterococcus		
Mean	5.7*	7.8†	7.6†	0.0001
Median	5.9	8.0	7.7	
SD	1.50	1.23	1.05	
		Bacteroides		
Mean	3.9	5.7	5.5	0.0870
Median	2.0	6.2	6.1	
SD	2.50	2.78	2.71	
		Clostridium		
Mean	6.0*	7.8†	6.9†	0.0054
Median	5.8	7.6	7.3	
SD	1.34	1.75	1.69	

HM = human milk; FOS-fructo-oligosaccharide.
*†Significantly different ($P < 0.05$).

TABLE 3. *Summary of log (base 10) organism counts at the visit 7 days after termination of fructo-oligosaccharides supplementation*

Organism Type	HM (n = 14)	FOS 1.5 (n = 28)	FOS 3.0 (n = 30)	*P* value
		Bifidobacterium		
Mean	8.6	9.3	8.5	0.0886
Median	8.4	9.2	8.4	
SD	1.65	1.28	1.14	
		Lactobacillus		
Mean	4.5	4.3	4.1	0.8924
Median	4.6	4.5	4.3	
SD	2.54	2.35	1.91	
		Enterococcus		
Mean	5.8*	8.0†	7.4†	0.0001
Median	6.2	7.9	7.6	
SD	1.62	1.10	1.17	
		Bacteroides		
Mean	3.5*	5.9†	5.9†	0.0158
Median	2.0	6.1	6.6	
SD	2.28	2.60	2.27	
		Clostridium		
Mean	6.6*†	7.4*	6.5†	0.0356
Median	6.1	7.4	6.5	
SD	1.81	1.60	1.54	

HM = human milk; FOS-fructo-oligosaccharides.
*†Significantly different ($P < 0.05$).

Clostridium difficile Toxin

No HM-fed infants had detectable *C. difficile* toxin at any time during the study. From the visit before FOS supplementation to the visit after supplementation, groups fed 1.5 g/L FOS and 3.0 g/L FOS experienced similar declines in the proportions of infants with *C. difficile* toxin (from 14% to 4% and from 23% to 17%, respectively) (Table 4). When comparisons were made between the two supplemented groups and the HM-fed group, no significant differences in the proportion of positive stools were noted before or after FOS supplementation ($P = 0.1320$ and $P = 0.0893$, respectively) (Table 4). Combining the two FOS supplemented groups, a post hoc analysis of the change in *C. difficile* toxin incidence comparing the visits before and after FOS supplementation revealed a decrease in the toxin incidence (Table 5) that did not reach statistical significance. Before supplementation 11 infants from both supplemented groups (19%) were *C. difficile* toxin positive. After supplementation six infants (10%) were toxin positive ($P = 0.0588$). Seven days after FOS supplementation ended, the incidence of toxin-positive stools increased similarly in the two FF groups, but the differences among the three groups were not statistically significant ($P = 0.0939$) (Table 4).

TABLE 5. *CLOSTRIDIUM DIFFICILE toxin change from the visit before fructo-oligosaccharides supplementation to the visit after fructo-oligosaccharides supplementation*

Stool EIA	HM (n = 14)	FOS 1.5/3.0 (n = 28)
	Visit before FOS supplementation	
Positive	0 (0.0%)	11 (19.0%)
Negative	14 (100.0%)	47 (81.0%)
	Visit after FOS supplementation	
Positive	0 (0.0%)*	6 (10.3%)†
Negative	14 (100.0%)	52 (89.7%)

Values are n (%).
EIA = enzyme immunoassay.
*A P value for the HM group could not be calculated as the result of a lack of change;
† $P = 0.0588$.

Adverse Events, Tolerability, and Acceptability

Ten infants (59%) taking HM, 30 infants (83%) taking 1.5 g/L FOS and 33 infants (97%) taking 3.0 g/L FOS experienced adverse events. Adverse events included all events occurring during the 5 weeks of study including only 1 week on experimental formula. The digestive system adverse events reported for the 1.5 g/L and 3.0 g/L FOS groups were increased flatulence (39% and 44%, respectively), increased spit-ups (39% and 44%), loose stools (14% and 32%), thrush (11% and 9%), emesis (8% and 3%) and decreased appetite (3% and 6%). One infant in the 3.0 g/L FOS regimen 2 group developed diarrhea in the first week of the study before FOS supplementation and was discontinued from the study.

During the week of FOS supplementation, FF infants experienced increased flatulence ($P = 0.0344$), increased spit-ups ($P = 0.0355$), and looser stools ($P = 0.0242$). HM-fed infants experienced no change in these events during the comparable period. The incidence of adverse events was lower in the 1.5 g/L FOS group than in the 3.0 g/L FOS group: seven infants (21%) versus 10 (31%) with flatulence, four infants (12%) versus nine (28%) with spit-ups and five infants (15%) versus 10 (31%) with looser stools.

Two infants (6%) in the 3.0 g/L FOS group experienced serious adverse events. One developed a urinary tract infection requiring antibiotic therapy and one developed pyloric stenosis requiring surgical intervention. Three infants in the 1.5 g/L FOS group (8%) and one infant (3%) in the 3.0 g/L FOS group experienced adverse events that led to discontinuation of study formula. There was no statistical significance in serious adverse events between the human milk fed group and either of the FF groups. All other adverse events resolved without intervention.

Satisfaction ratings for formula acceptability and tolerability declined at the visit after FOS supplementation and were slightly higher for the 1.5 g/L FOS group

TABLE 4. *CLOSTRIDUM DIFFICILE toxin detection*

Stool EIA	HM (n = 14)	FOS 1.5 (n = 28)	FOS 3.0 (n = 30)	P value
Visit before FOS supplementation				0.1320
Positive	0 (0.0%)	4 (14.3%)	7 (23.3%)	
Negative	14 (100.0%)	24 (85.7%)	23 (76.7%)	
Visit after FOS supplementation				0.0893
Positive	0 (0.0%)	1 (3.6%)	5 (16.7%)	
Negative	14 (100.0%)	27 (96.4%)	25 (83.3%)	
Visit 7 days after FOS supplementation				0.0939
Positive	0 (0.0%)	3 (10.7%)	7 (23.3%)	
Negative	14 (100.0%)	25 (89.3%)	23 (76.6%)	

Values are n (%).
EIA = enzyme immunoassay.

than for the 3.0 g/L FOS group: 67% (24 infants) versus 59% (20 infants).

Comparisons of stool evaluations were made between the visits before FOS supplementation and the visit after FOS supplementation. The frequency of bowel movements was greater and stool consistency was looser for HM-fed infants than for FF infants ($P < 0.05$ for both). In HM-fed infants mean stool frequency was 4.6 per day and mean stool consistency rating was 3.9. During FOS supplementation in the 1.5 g/L group, stool frequency decreased from 2.1 per day to 1.5 per day and stool consistency rating changed minimally from 2.6 to 2.7. During FOS supplementation in the 3.0 g/L group, stool frequency increased from 1.5 to 2.0 per day and the stool consistency rating changed from 2.7 to 3.2. There was a significant difference ($P < 0.01$) in the change in stool consistency between the two FOS treatments. FOS 3.0 had a significantly greater change in stool consistency (mean, 0.6 $\pm$ 0.77; median, 1.0) when compared with FOS 1.5 (mean, 0.07 $\pm$ 0.81; median, 0.0).

DISCUSSION

Factors in HM, such as lactoferrin, glycoconjugates, iron, nucleotides and sialic acid may function as anti-infective agents in the infant gastrointestinal tract (13,14). These factors also may contribute to the differences in the gastrointestinal flora of HM-fed and FF infants observed in some studies (15,16). In both HM-fed and FF infants the gut initially is colonized by enterobacteria. By 6 days of life HM-fed infants have a bifidobacteria predominant flora with a ratio of bifidobacteria to enterobacteria of more than 1000:1. In FF infants, the ratio is less than 10:1 (17). A bifidobacteria-predominant gut flora may suppress the growth of coliforms and other potential pathogens and may be a factor that decreases the occurrence of infectious diseases in HM-fed infants (18,19).

The gut flora of FF infants may be altered by the addition of probiotics or prebiotics to formula. Probiotics change flora by adding organisms that may temporarily or chronically populate the gastrointestinal tract. (18). Prebiotics are substances that promote a luminal environment conducive to the growth of desirable bacteria such as bifidobacteria and lactobacilli. A number of reports have shown that probiotics can establish a flora closer to that of HM-fed infants. In a study in which *Streptococcus thermophilus* and *Lactobacillus helveticus* were given as probiotic agents, term infants grew well and the prevalence of bifidobacteria after 1 month was similar to that of HM-fed controls (19). Some trials have shown that probiotic-supplemented term infant formula is more efficacious than placebo in preventing some types of gastroenteritis. Most studies have found probiotic supplementation less effective than HM in preventing such disease (20–23).

The composition and fermentative activity of the colon microflora of human infants may be influenced by the type of feeding (24). HM oligosaccharides and the FOS content in solid foods seem to be important factors of impacting microflora (5,24,25). A study in adults showed that 2.5 g of FOS per day was well tolerated and was associated with an increase in colon bifidobacteria (7). We therefore decided to evaluate two concentrations of FOS (1.5 and 3.0 g/L) and found that both were safe and well tolerated by study infants.

The adverse events reported in our patients were mainly gastrointestinal, as would be predicted in subjects ingesting a non-absorbable carbohydrate. As expected, adverse events were more common in the higher concentration group. The infants being fed the 3.0 g/L FOS formula experienced an increased incidence of spitting up. A possible explanation from studies in adults receiving a lower daily FOS dose than our patients is an increased number of transient lower esophageal sphincter relaxations in subjects taking FOS (26).

To assure that fecal flora were as equivalent as possible before FOS supplementation, the two FF groups were fed the same unsupplemented cow's milk protein based term infant formula, with a whey/casein ratio of 60:40 for 7 days. Microbiologic analyses obtained after this 7-day period revealed similar lactobacilli and bifidobacteria colony counts in HM and FF infants. This is in contrast to previous studies that have generally demonstrated different fecal flora in HM-fed and FF infants, particularly the counts of lactobacilli and bifidobacteria. (7,15–18) The lack of a difference between our study groups and HM-fed controls before FOS supplementation was unexpected and may indicate that the control formula in our study also promotes lactobacilli and bifidobacteria. Our study formula contained predominantly whey proteins. In one study in which a whey-predominant formula was compared with a casein-predominant formula, the whey-predominant formula promoted a pattern of bifidobacteria and other fecal microflora closer to that of HM-fed infants (27,28). In another study in which a whey-predominant formula was compared with a casein-predominant formula, the casein-predominant formula supported an increased growth of lactobacilli. The reason that our patients and controls had similar bifidobacteria and lactobacilli counts after 1 week of control formula feeding remains to be determined.

After 1 week of FOS supplementation, regardless of FOS concentration or whether the dose was given during week 2 (regimen 1) or week 4 (regimen 2), the counts of lactobacilli and bifidobacteria did not increase significantly above baseline. Directly after the week of supplementation, however, the 1.5 g/L FOS formula group had a statistically greater number of bifidobacteria than the HM-fed and 3.0 g/L FOS formula groups. Other trials in which oligosaccharide supplementation has been evaluated have generally shown an increase in counts of lactobacilli and bifidobacteria with oligosaccharide

supplementation (29–32). These trials differed from ours in design and study population. In none of these trials was an assessment of fecal flora performed before oligosaccharide supplementation, and a HM comparator group was included in only one trial (33). In two of the trials a mixture of galacto-oligosaccharides and fructo-oligosaccharides was fed. In one of the studies only preterm infants were enrolled (30,31). The duration of supplementation sometimes differed from that of our trial, with one trial supplementing for 4 days and two others for 28 days (29–31). Given these differences in study design and subjects, it is difficult to use them to shed light on the absence of an increase in bifidobacteria and lactobacilli after 1 week of FOS supplementation in our study.

During FOS supplementation, infants experienced looser and more frequent stools, especially in the 3.0 g/L group. No parents reported overt diarrhea during FOS supplementation. Parents, however, did report that stools became more frequent and significantly softer, but not loose, during 3.0 g/L FOS supplementation. This is an important finding because hard stools are a common problem in FF infants and softer or loose stools are more typical of the breast-fed infant (34).

In conclusion, we found that infants fed a whey-predominant term infant formula during the neonatal period had counts of fecal lactobacilli and bifidobacteria similar to those seen in HM-fed infants and that these counts did not change after feeding FOS-supplemented formula. We found higher colony counts of enterococci, bacteroides and clostridia in fecal samples from FF compared with HM infants. Counts of these organisms in the FF groups did change after feeding FOS at either concentration. *C. difficile* toxin was never detected in the stools of HM-fed infants. There was a trend towards decreased proportion of FF infants with toxin-positive stools after FOS-supplemented formula. Both FOS concentrations were safe and well tolerated. It is possible that an increase in the lactobacilli and bifidobacteria counts may occur with a longer feeding period.

Acknowledegement: The authors gratefully appreciate the professionalism and dedication of Teresa Hedrick, MT, ASCP for her contribution to the laboratory portion of this study.

REFERENCES

1. Wold AE, Adlerberth I. Breast feeding and the intestinal microflora of the infant: implications for protection against infectious diseases. *Adv Exp Biol* 2000;478:77–93.
2. Oddy WH. Breastfeeding protects against illness and infection in infants and children: a review of the evidence. *Breastfeed Rev* 2001; 9:11–8.
3. Hanson LA. Human milk and host defence: immediate and long-term effects. *Acta Paediatr Suppl* 1999;88:42–6.
4. Newburg DS. Do the binding properties of oligosaccharides in human milk protect human infants from gastrointestinal bacteria? *J Nutr* 1997;127:S980–4.
5. Gudiel-Urbano M, Goni I. Human milk oligosaccharides. The rule in the health and development of the infants. *Arch Latinoam Nutr* 2001;51:332–9.
6. Kunz C, Rudloff S. Biological function of oligosaccharides in human milk. *Acta Paediatr* 1993;82:903–12.
7. Bouhnik Y, Flourie B, Riottot M, et al. Effects of fructo-oligosaccharides ingestion on fecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. *Nutr Cancer* 1996;26:21–9.
8. Howard MD, Gordon DT, Pace LW, et al. Effects of dietary supplementation with fructo-oligosaccharides on colonic microbiota populations and epithelial cell proliferation in neonatal pigs. *J Pediatr Gastroenterol Nutr* 1995;21:297–303.
9. Fomon SJ, Nelson SE. Size and growth. In: Fomon SJ, ed. *Nutrition of Normal Infants*. St. Louis: Mosby; 1993:36–84.
10. Johnson TS, Engstrom JL, Gelhar DK. Intra- and interexaminer reliability of anthropometric measurements of term infants. *J Pediatr Gastroenterol Nutr* 1997;24:497–505.
11. Ben C, Martins J, Zoya I, et al. The magnitude of the global problem of diarrheal disease: a ten-year update. *Bull World Health* Organ 1992;70:705–14.
12. Lonnerdal B, Adkins Y. Developmental changes in breast milk protein composition during lactation. In: Sanderson IR, Walker WA, eds. *Development of the Gastrointestinal Tract*. Hamilton: BC Decker; 2000:227–44.
13. Kohler H, Donarski S, Stocks B, et al. Antibacterial characteristics in the feces of breast-fed and formula-fed infants during the first year of life. *J Pediatr Gastroenterol Nutr* 2002;34:188–93.
14. Balmer SE, Wharton BA. Diet and faecal flora in the newborn: breast milk and infant formula. *Arch Dis Child* 1989;64:1672–7.
15. Balmer SE, Wharton BA, Scott PH. Sorrento studies of diet and fecal flora in the newborn. *Acta Paediatr Jpn* 1994;36:579–84.
16. Yoshioka H, Iseki K, Fujita K. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics* 1983;72:317–21.
17. Iseki K. Development of intestinal flora in neonates. *Hokkaido Igaku Zasshi* 1987;62:895–906.
18. Langhendries JP, Detry J, Van Hees J, et al. Effect of a fermented infant formula containing viable bifidobacteria on the fecal flora composition and pH of healthy full-term infants. *J Pediatr Gastroenterol Nutr* 1995;21:177–81.
19. Saavedra JM, Bauman NA, Oung I, et al. Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *Lancet* 1994; 344:1046–9.
20. Vanderhoof JA, Whitney DB, Antonson DL, et al. Lactobacillus GG in the prevention of antibiotic-associated diarrhea in children. *J Pediatr* 1999;135:564–8.
21. Szajewska H, Mrukowicz JZ. Probiotics in the treatment and prevention of acute infectious diarrhea in infants and children: a systematic review of published randomized, double-blind, placebo-controlled trials. *J Pediatr Gastroenterol Nutr* 2001;33:S17–25.
22. Mastretta E, Longo P, Laccisaglia A, et al. Effect of *Lactobacillus* GG and breast-feeding in the prevention of rotavirus nosocomial infection. *J Pediatr Gastroenterol Nutr* 2002;35:527–31.
23. Flickinger EA, Hatch TF, Wofford RC, et al. In vitro fermentation properties of selected fructooligosacccharide-containing vegetables and in vivo colonic microbial populations are affected by the diets of healthy human infants. *J Nutr* 2002;132:2188–94.
24. Vandenplas Y. Oligosaccharides in infant formula. *Br J Nutr* 2002; 87:S293–6.
25. Piche T, des Varannes SB, Sacher-Huvelin S, et al. Colonic fermentation influences lower esophageal sphincter function in gastroesophageal reflux disease. *Gastroenterology* 2003;124:894–902.
26. Balmer SE, Scott PH, Wharton BA. Diet and faecal flora in the newborn: casein and whey proteins. *Arch Dis Child* 1989;64:1678–84.
27. Kleessen B, Bunke H, Tovar K, et al. Influence of two infant formulas and human milk on the development of the faecal flora in newborn infants. *Acta Paediatr* 1995;84:1347–56.

28. Rubaltelli FF, Biadaioli R, Pecile P, et al. Intestinal flora in breast- and bottle-fed infants. *J Perinat Med* 1998;26:186–91.
29. Boehm G, Lidestri M, Casetta P, et al. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Arch Dis Child Fetal Neonatal Ed* 2002;86:F178–81.
30. Moro G, Minoli I, Mosca M, et al. Dosage-related bifidogenic effects of galacto- and fructo-oligosaccharides in formula-fed term infants. *J Pediatr Gastroenterol Nutr* 2002;34:291–5.
31. Hopkins MJ, Macfarlane GT. Nondigestible oligosaccharides enhance bacterial colonization resistance against *Clostridium difficile* in vitro. *Appl Environ Microbiol* 2003;69:1920–97.
32. Oguz F, Uysal G, Dasdemir S, et al. The role of *Clostridium difficile* in childhood nosocomial diarrhea. *Scand J Infect Dis* 2001;33:731–3.
33. Kyne L, Farrell RJ, Kelly CP. *Clostridium difficile*. *Gastroenterol Clin North Am* 2001;30:753–77.
34. Weaver LT, Ewing G, Taylor LC. The bowel habits of milk-fed infants. *J Pediatr Gastoenterol Nutr* 1988;7:568–71.