Growth, Tolerance and Stool Characteristics of Term Infants Consuming Short-chain Fructooligosaccharides in Infant Formula

ABSTRACT

Introduction: Human milk contains complex oligosaccharides that are associated with the *Bifidobacteria*-dominant microflora in the breastfed infant. The addition of short chain fructo-oligosaccharide (FOS) to infant formula may provide similar benefits to formula-fed infants. Two studies were conducted to assess the effects of FOS-supplemented formulas on infant growth, tolerance, stool patterns and stool microflora in healthy, term infants.

Methods: *Study 1:* 66 infants were fed Similac with Iron (SWI) for 2 weeks, then randomized to receive either a whey-enriched formula with 3.0 g/L (FOS3), 1.5 g/L (FOS1.5) or no added FOS (FOS0) for 2 weeks. *Study 2:* 102 infants were randomized to receive either SWI or SWI with 3 g/L added FOS from 8-112 days of age. Growth, intake, stool characteristics, and tolerance were monitored in both studies. Stool samples were collected in both studies and analyzed for selected anaerobes and aerobes; FOS, liver enzymes and cholesterol were measured in plasma samples, and FOS and ketones were measured in urine.

Results: Intake and tolerance were not affected by FOS in the studies. In Study 1, stools were looser in all infants while consuming the whey-enriched formulas. Mean microflora counts did not differ among groups, although infants receiving FOS-supplemented formula (FOS1.5 and FOS3 groups combined) experienced a decrease in *Clostridia* colonization compared to infants receiving the formula without FOS ($p = 0.047$). In Study 2, median rank stool consistency was softer for infants fed the FOS-supplemented formula than the control formula at day 28. At days 56, 84, and 112, the imputed logs of *Lactobacillus* counts were significantly higher in the FOS3 group (4.3-5.2) than in the FOS0 group (3.3-3.9), and more infants in the FOS3 group showed an increase in *Lactobacillus* status. FOS had no effects on other fecal anaerobes assessed, liver enzymes or cholesterol.

Conclusions: Infant formulas containing added FOS at the levels provided in these studies are well tolerated and support normal growth in term infants, and have minimal effects on fecal microflora.

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INTRODUCTION

Human milk is a complex substance that contains thousands of constituents, some of which are biologically active components (Picciano 2001). Included among the biologically active components in milk are oligosaccharides. Approximately 130 structurally distinct oligosaccharides have been identified in human milk (McVeagh and Miller 1997), and with concentrations in mature milk of nearly 13 g per liter (Coppa et al. 1993), oligosaccharides represent one of the largest solutes in human milk after lactose and lipids.

Oligosaccharides in human milk are largely resistant to digestion in the upper intestinal tract (Engfer 2000, Gnoth et al. 2000). The intact structure of the oligosaccharides in the colon provides a substrate for bacterial fermentation, resulting in the production of short-chain fatty acids, gases including hydrogen, carbon dioxide, and biomass. A small fraction of ingested human milk oligosaccharides are absorbed intact and excreted in urine (Rudloff et al. 1996).

Analysis of feces from infants using both classic culture as well as modern molecular techniques generally indicate a predominance of *Bifidobacteria* and *Lactobacilli* in breastfed infants, while formula fed infants tend to have a more diverse microflora with lower concentrations of *Bifidobacteria* and *Lactobacilli*, and a greater presence of species including *Clostridia* and *Bacteroides* (Stark and Lee 1982; Harmsen et al. 2000; Favier et al. 2002). The selective stimulation of *Bifidobacteria* and *Lactobacilli* in the infant microflora and concurrent inhibition of potentially pathogenic flora has been attributed primarily to the presence of oligosaccharides in human milk (Coppa et al. 2004). Cows' milk, which is frequently used in the production of infant formulas, contains only trace amounts of oligosaccharides and consequently formulas are a poor source of these nondigestible carbohydrates (Kunz et al. 1999).

The addition of oligosaccharides such as fructooligosaccharide (FOS) to infant formulas however, may provide formula-fed infants with some of the benefits attributed to

oligosaccharides in human milk. FOS, which belongs to the class of carbohydrates known as fructans, is a mixture of molecules containing one sucrose molecule to which one to three additional fructose units have been enzymatically added by a beta 2-1 linkage (average degree of polymerization (DP) of 3.5) (Carabin and Flamm 1999). FOS is structurally similar to oligofructose and inulin (average DP of 4-5 and >10, respectively), which are oligo- and polysaccharide fructans present in widely consumed foods such as wheat, onion, banana, and garlic (Moshfegh et al. 1999).

Similar to human milk oligosaccharides, FOS largely escapes digestion in the small intestine and is fermented by the colonic flora (Molis et al. 1996; Oku and Nakamura 2003). In a study of healthy adults, daily consumption of 5, 10 or 20 g FOS for one week significantly increased fecal *Bifidobacteria* counts above baseline levels, and fecal *Bifidobacteria* counts were significantly correlated to the ingested dose (Bouhnik et al. 1999). In a study of healthy term infants receiving 1.5 or 3.0 g FOS per liter of formula for a period of one week, FOS-supplemented formulas were found to have a minimal effect on fecal *Bifidobacteria* (Euler et al. 2005). Other studies in term infants, however, have shown that formulas containing 0.4 g/L FOS and 3.6 g/L galacto-oligosaccharide (GOS) or 0.8 g/L FOS and 7.2 g/L GOS stimulate the growth of colonic *Bifidobacteria* as measured in feces (Moro et al. 2002; Knol et al. 2005). A 90% GOS-10% FOS formula also produced higher fecal *Bifidobacteria* counts in preterm infants consuming formula containing a total of 1 g/L oligosaccharides (Boehm et al. 2002).

In two separate studies reported herein, the effects of FOS-supplemented formulas on infant growth, tolerance, stool patterns, and stool microflora in healthy, term infants were investigated.

MATERIALS AND METHODS

The safety and efficacy of infant formulas containing FOS were evaluated in two separate experimental protocols. Both studies evaluated infant growth, formula intake, tolerance, and stool characteristics. Each study was placebo-controlled, randomized, blinded, and

parallel. In both studies, parents completed questionnaires on infant tolerance and data on fecal microflora and urine and plasma kestose (GF_2) and nystose (GF_3) response to treatment formulas were collected and evaluated.

Parental consent was obtained prior to infant enrollment. Both studies enrolled healthy full-term infants (38 to 42 weeks gestational age); not taking any medication or antibiotics for ≥ 3 wks prior to enrollment; having weight, length, and head circumference ≥ 5th percentile at birth; and without maternal history of diabetes, tuberculosis, or perinatal infection with proven adverse effects on the fetus. Formula-fed infants received only the assigned ready-to-feed (RTF) study formula for the study duration and no vitamin or mineral supplements with the exception of fluoride drops, when prescribed by physician. All formulas met or exceeded levels of nutrients as recommended by the Committee on Nutrition of the American Academy of Pediatrics, and all formulas were manufactured by Abbott Laboratories. The FOS used in all studies was obtained from GTC Nutrition Co. (Golden, CO) and consisted primarily of a single glucose unit linked with two or three fructose units by a beta 2-1 linkage (i.e., GF_2 and GF_3). The Institutional Review Boards of each study site approved the consent form and experimental protocols.

Subjects and Feeding Protocols

Study 1: FOS Tolerance and Efficacy

Healthy, formula-fed infants 4-10 weeks (±3 d) of age were recruited from several pediatric offices in the U.S. Infants were initially fed Similac with Iron (SWI; approximately 18% whey protein/82% casein). At a clinic visit two weeks later, infants were randomized to whey-enriched formulas (approximately 50% whey/ 50% casein) with 0 (FOS0), approximately 1.5 g (FOS1.5), or approximately 3 g (FOS3) of FOS per L for an additional 13 days. Formula FOS1.5 and FOS3 contained 1.5 and 3.1 g FOS per liter, respectively. By analysis, formula FOS1.5 contained 0.65 g GF_2 and 0.60 g GF_3 per liter (i.e., FOS with 2 or 3 fructose units comprised approximately 83% of the total FOS), and formula FOS3 contained 1.45 g GF_2 and 1.41 g GF_3 per liter (approximately 92% of

total FOS). The RTF formulas contained 20 (SWI and FOS0), 20.5 (FOS1.5), and 20.9 (FOS3) kcals per oz, respectively.

Study 2: FOS Growth Study

Healthy, formula-fed infants 1-8 (±3) days of age were recruited and randomized to receive either SWI (approximately 18% whey protein/82% casein) with no added FOS (FOS0), or SWI with approximately 3 g per L added FOS (FOS3) for approximately 16 weeks, until 112 days of age. By analysis, formula FOS0 contained 0 g per liter GF_2 and GF_3 and FOS3 contained 1.3 and 1.2 g GF_2 and GF_3 per liter, respectively (approximately 83% of total FOS). A third group of 25 infants was fed human milk exclusively for the duration of the study. The study was conducted at three sites across the U.S. The RTF formulas provided during the study period contained 20 (FOS0) and 20.9 (FOS3) kcals per oz.

Methods

Growth, Dietary Assessments, Tolerance and Stool Records: In Study 1, weight was recorded on day 1 (study entry), and during clinic visits on days 15 and 29 using standard procedures. In Study 2, length, weight, and head circumference measurements were taken at study entry (day 1) and during all study visits (days 28, 56, 84, and 112). In both studies, parents kept detailed records of formula intake, stool patterns (Study 1: frequency, consistency, gas, odor, straining, color; Study 2: frequency, consistency, straining, dryness), and incidence of spit-up and vomiting. Stool consistency was ranked as 1 = watery, 2 = loose, 3 = soft, 4 = formed, 5 = hard. The records were completed daily in Study 1, and during the 3 days prior to each visit after study entry in Study 2.

Urine, Plasma, and Stool Sample Collection: Urine was collected on days 15 and 29 (Study 1) or on days 8, 28, 56, 84, and 112 (Study 2) in a bag, transferred to a clean collection cup, refrigerated and brought to the clinic within 12 h. A 2 mL blood sample was collected by venipuncture from 4-5 infants per group on day 29 in Study 1, and by venipuncture or heel stick from all infants on days 28 and 112 in Study 2. Parents were

instructed to collect stool samples no longer than 8 h prior to the clinic visit at the end of the baseline period (day 15) and at the end of the study feeding period (day 29) in Study 1, and on days 8, 28, 56, 84 and 112 in Study 2. Parents transferred two marble-sized scoops of stool to a preweighed tube containing 15 mL Cary-Blair transport medium (Para-Pak C&S, Meridian Diagnostics, Cincinnati, Ohio).

Urine, Plasma, and Stool Analytical Methods: Urine was analyzed for ketones using a Keto stix Strip [Miles Inc., Diagnostic Division]) and then frozen at -70ºC. Blood samples were centrifuged and frozen immediately at -70ºC or -20ºC. Urine and plasma samples were shipped on dry ice to the analytical laboratory (Abbott Laboratories). In Study 2, aliquots of urine from day 84 were analyzed for indican and creatinine using a method derived from the Abbott standard procedure for total plasma tryptophan (indican), and Abbott assays using the Abbott Spectrum EPx Clinical Chemistry Analyzer (creatinine). Plasma samples were analyzed for FOS as GF_2 and GF_3 via ion chromatography using a Carbo Pack column with a base resistant latex anionic exchange resin (Dionex Corp, Sunnyvale, CA). In both studies, Abbott assays using the Abbott Spectrum EPx Clinical Chemistry Analyzer were used to analyze the plasma samples for alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and a cholesterol analysis (Abbott Spectrum EPx) was completed in Study 2.

The stool samples were mixed with Cary-Blair transport medium and the tubes were held at room temperature until frozen at -70ºC (maximum time until frozen was 24 h). Specimens were transported on dry ice to the central microbiological laboratory for processing (Children's Hospital, Columbus, OH), which occurred within 2 months of collection. Analysis of stool samples in Study 1 included identification and enumeration of the anaerobic species *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Clostridium*, *Peptostreptococcus*, and the aerobic species *Escherichia*, enteric gram-negative rods (GNR) other than *Escherichia*, *Pseudomonas*, *Enterococcus*, *Staphylococcus*, *Streptococcus*, and *Candida*. In Study 2, stool samples were evaluated for the presence of *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Clostridium*, and *Clostridium difficile*.

Stool samples were incubated for the appropriate time period specified for each organism. Isolated colonies were subcultured to ensure purity, followed by Gram staining. Isolates were then identified for the genus or species level using standard methods. Colonies with the following characteristics were considered *Lactobacillus spp.*: large creamy white to yellowish colonies on LBS agar, anaerobic or microaerophilic Gram positive rods, in chains when grown in thioglycollate broth, and vancomycin resistant (or producing lactic acid as the sole end product of glucose fermentation by GLC determination). Colonies with the following characteristics were considered *Bifidobacterium spp*.: medium to large creamy white colonies, some with pink centers on B1M-25 agar, anaerobic Gram positive rods, many with Y and V shapes (bifurcated), vancomycin sensitive, and producing acetic acid and lactic acid in a 3-2 molar ratio by GLC when the above criteria were not definitive. The concentration of each species was based on a stool wet weight basis using dilution plates with counts between 30 and 300 when possible and expressed as number of organisms per g of stool. Concentrations of *Bifidobacterium spp.* were based on the mean number of organisms from duplicate plates. *Clostridium spp.* were isolated using the alcohol treatment method (Balows 1991). When dilution plates did not permit accurate colony counts, data were expressed as $< 10^4$ or $\geq 10^{10}$.

Statistical Analyses

Sample Size. Study 1. Sample size was estimated using a standard deviation of 0.6 of the primary variable, mean rank stool consistency (MRSC), from similar study populations. A total sample size of 51 subjects (17 per group) will have 80% power to detect differences in means of 0.6 using a two-sided 0.05 level t-test. Based on an estimated 20% attrition rate, enrollment of 66 infants is planned (22 per group). Study 2. Sample size was estimated using the standard deviation, 5.0 g/d, of the primary variable, weight gain per day from 14 to 112 days of age from similar study populations. A sample size of 70 (35 per group) infants will have 80% power to detect a difference in means of 3 grams per day using a two group t-test with a 0.05 two-sided significance level. Based on an estimated 30% attrition rate, enrollment of 100 infants is planned (50 per group).

Continuous variables were analyzed using ANOVA. Continuous variables which are rarely normally distributed were ranked prior to analysis by ANOVA (e.g. anthropometric data, formula intake, number of feedings, number of stools, stool characteristics, tolerance outcomes). Categorical variables (e.g. sex, race, predominant stool color) were analyzed with Chi-square, or Fisher's exact tests given a cell frequency less than 5. Proportions were analyzed by ANOVA on transformed data. Pairwise differences were determined with Tukey's multiple comparisons. In Study 1, analyses of intake and stool data were performed on infants who completed the study, and tolerance outcomes were analyzed on all non-protocol failures. In Study 2, intake, tolerance and stooling patterns were analyzed on all available data.

Mean log counts of fecal flora were used for the ANOVA of mean values of stool microorganisms. Only infants with quantified numbers of organisms ($10^4 – 10^{10}$) were used to calculate the mean log value. The Cochran-Mantel-Haentzel technique was used to compare the proportion of subjects with improved microorganism populations stratified on initial status (detectable or non-detectable). Improvement was defined as an increase in beneficial bacteria or a decrease in pathogenic bacteria. Subjects who were beyond detectable at baseline for beneficial bacteria were excluded from the analysis. A significant improvement in *Bifidobacteria* or *Lactobacillus* was defined as increasing from undetectable ($<10^4$) to detectable ($10^4 – 10^9$), changing from detectable to beyond detectable ($\geq 10^{10}$), or increasing at least 2 on the log scale compared to entry count. A significant improvement in *Clostridia* was defined as changing from beyond detectable ($\geq 10^{10}$) to detectable ($10^4 – 10^9$), from detectable to undetectable ($<10^4$), or decreasing by at by at least 2 on the log scale compared to entry count. For all analyses, the level of significance was set at $p < 0.05$. In Study 1, an analysis of *Bifidobacteria* and *Clostridia* was performed comparing all three groups and separate analyses were made comparing each FOS group to the control group, and the FOS groups combined compared to the control group.

In Study 2, the effect of site was not addressed since the majority of infants were from one site (101 of 127, 80%). For fecal microflora analysis, at each visit an ANOVA was

performed on the ranks of imputed logs of enumerated bacteria: a log of 3 was assigned to bacterial counts that were less than detectable ($<10^4$), a log of 9 was assigned to bacterial counts that were greater than detectable ($\geq 10^{10}$), and the log of the recorded reading was assigned to the detectable values (10^4-10^9). Additionally, at each visit ordinal chi-square tests were performed comparing the distribution of the categories (less than detectable, detectable, beyond detectable) among the feeding groups.

RESULTS

A summary of entry and exit information for subjects enrolled in both studies is presented in Table 1. Baseline demographic characteristics among groups within each study were similar. In Study 1, infants in the FOS3 group were older than infants in the FOS0 and FOS1.5 groups ($p<0.05$), and in Study 2, infants in the formula groups were older than infants in the human milk group ($p<0.05$).

Exit Data & Growth, Dietary Assessments, Tolerance and Stool Characteristics

Study 1: A total of 66 infants were enrolled and 63 infants participated in the study (Table 1). Three infants (FOS0 = 2, FOS3 = 1) exited the study prior to initiation of the test formulas due to treatment or protocol failures; these infants were excluded from all analyses. A total of 7 (11%) treatment failures were reported (FOS0 = 1, FOS1.5 = 2, FOS3 = 4). With the exception of one infant in the FOS3 group who rejected the study formula, all other treatment failures were determined to be due to intolerance. The reported events resulting in treatment failure included vomiting or spit up, diarrhea, watery stools, fussiness, increased stool frequency, and weight loss. During the study, there were no protocol failures for the FOS0 or FOS3 groups and 3 protocol failures for the FOS1.5 group. The protocol failures included: 1 infant removed by parents because of fussiness/gassiness, 1 for failure to complete intake and tolerance records, and 1 because the infant did not have study stool samples. Excluding the study protocol failures, the proportion of treatment failures between the FOS1.5 and FOS0 groups (10.5% [2 of 19] vs. 4.8% [1 of 21]) and the FOS3 and FOS0 groups (20.0% [4 of 20] vs. 4.8% [1 of 21]) was not significantly different ($p = 0.462$ and 0.156, respectively). All intolerances resolved when infants returned to the SWI formula.

There were no differences among groups in average weight gain per day (Table 1) or NCHS weight z-scores (data not shown). The mean number of feedings per day and average formula intake (mL) per day were similar among the groups (Table 1).

At baseline, the median percent of feedings with spit up in the FOS0, FOS1.5 and FOS3 groups was 8, 6, and 17%, respectively; at the end of the study, the corresponding values were 13, 6, and 25% (Table 2). There were no statistically significant differences among the groups in percent change from baseline in number of feedings with spit up and/or vomit, when analyzed on ranked data (Table 2).

Stool characteristics including number per day, ranked consistency, and predominant consistency did not differ among the groups (Table 2). In all groups, stools were looser (i.e., lower rank) during the feeding period as compared to the baseline period, with median stool consistency rankings changing from 2.6 to 1.6, 2.7 to 1.6, and 2.8 to 1.8 in the FOS0, FOS1.5, FOS3 groups, respectively. The predominant stool color and percent of stools with straining, odor, or gas did not differ among the groups (data not shown).

Study 2: A total of 127 infants were enrolled and 93 infants completed the study. A total of 14 (15%) treatment failures were reported (FOS0 = 6, FOS3 = 8) (Table 1), with adverse events including symptoms of milk intolerance (FOS0 = 2, FOS3 = 4), colic (FOS3 = 1), diarrhea or watery stools (FOS0 = 2, FOS3 = 1), constipation (FOS0 = 2, FOS3 = 1), and gassiness (FOS3 = 1). There was no statistical difference in the number of treatment failures between the formula groups. A total of 20 (21%) protocol failures were reported (FOS0 = 12, FOS3 = 6, HM = 2). Reasons for protocol failure included: missed visits (FOS0 = 7, FOS3 = 4, HM = 1), removed by investigator before study formula was started (FOS0 = 3, FOS3 = 1), removed by investigator because of fever (FOS0 = 1), removed by parents due to failure to grow normally in absence of any organic symptom or other condition (FOS3 = 1), consumed other foods (FOS0 = 1), unable to collect fecal specimens (HM = 1).

Throughout the study measures of weight, length and head circumference, and weight gains did not differ among the groups (Table 1). Weight z-scores also were similar among the groups (data not shown). Number of feedings per day, daily formula intake, and daily caloric intake also did not differ between the formula groups (Table 1).

The percentage of feedings with spit up and/or vomit did not differ among the three groups (Table 2). Daily stool frequency did not differ among the groups (0.8 – 2.0) except at d 28 when infants fed human milk had a greater number of stools per day than infants fed either of the study formulas (3.7 vs 1.7 – 2.0). At d 28, 56, and 84, median stool consistency was softer for infants fed human milk as compared to infants fed formulas (2.0 vs 2.4 – 3.0), and at d 28, median rank stool consistency was significantly less for infants in the FOS3 formula group (2.8) than in the FOS0 group (3.0). Percent of stools with straining or dryness did not differ based on type of feeding.

Plasma and Urine Analyses

Study 1: No GF_2 or GF_3 was detected in plasma samples from any infants, and mean plasma AST and ALT values were normal and did not differ among groups (Table 3). Following the feeding period, GF_2 was detected in urine samples from 4 of 11 (36%) infants consuming FOS1.5, and GF_3 was detected in urine from 5 of the infants (45%) (Table 4). Among the 11 infants consuming FOS3 who were tested for urinary FOS following the feeding period, quantifiable amounts of GF_2 and GF_3 were detected in samples from 8 and 9 infants, respectively. GF_2 and GF_3 were not detected in urine samples collected from infants prior to consumption of the FOS-containing formulas, or in infants fed FOS0 before or after the feeding period. Ketones were not detected in urine from any infants before or after the feeding period.

Study 2: The plasma concentrations of AST and ALT were normal and means values did not differ between the formula groups, although values were generally lower but within normal ranges, in the formula groups as compared to infants consuming human milk

(Table 3). Mean cholesterol levels were higher in the HM group on both sampling days (28 and 112) compared to the formula groups, but did not differ between the formula groups (Table 3). GF_2 was detected in 38 to 100% of the urine samples at each visit for infants fed FOS3, while GF_3 was detected in 0 to 83% of the samples. With the exception of one infant in the FOS0 group found to have 4.7 ppm GF_2 in urine collected on day 56 and one infant in the HM group with 186.9 ppm GF_2 and 183.9 ppm GF_3 on day 8, GF_2 and GF_3 were not detected in urine samples from infants consuming FOS0 or human milk. GF_2 and GF_3 were not detected in any plasma samples. Ketones were not detected in any urine samples collected from infants during the study visits. The indican/creatinine values were not different among groups as measured on day 84 (median ratios – FOS0: 0.10, FOS3 = 0.10, HM = 0.11).

Fecal Microflora Analysis

Study 1: There were no significant differences in fecal bifidobacteria counts among the formula groups after the 2-week feeding period. Fecal clostridia counts decreased in the population of infants fed formula containing 1.5 g scFOS/L as compared to infants fed the scFOS-free formula.

Study 2: Stool microflora population counts were obtained at the end of the baseline feeding period (day 8) and at study days 28, 56, 84, and 112. ANOVA on ranks of imputed logs revealed no differences between formula groups at any time points in fecal *Bifidobacteria*, *Clostridia*, and *C. difficile* counts (data not shown). At days 56, 84 and 112, the mean imputed logs of *Lactobacillus* counts were significantly higher in the FOS3 group (4.4-5.2) than in the FOS0 group (3.3-3.6).

When changes in anaerobic bacteria were evaluated from study day 8-112 in response to the test feedings or human milk, no differences were found between the formula groups for *Bifidobacteria*, *Bacteroides*, *Clostridia*, and *C. difficile*. At days 56, 84 and 112, the FOS3 group showed a significantly larger increase in *Lactobacillus* colonization than the FOS0 group ($p<0.05$).

DISCUSSION

In both studies, the safety and efficacy of infant formulas containing added FOS, a source of oligosaccharides, were assessed during feeding periods of approximately 2 weeks or 4 months.

The safety of infant formulas containing added FOS was assessed by examining measures of infant growth, formula intake, and tolerance. In both the 2-week and 4-month feeding trials, infants consuming formulas containing up to 3 g/L FOS showed similar growth to infants fed control formulas as assessed by anthropometric measures of weight, length and head circumference and daily gains over the study period. Infants consuming the supplemented and control formulas also consumed comparable volumes of total formula per day and consumed formula with equivalent daily frequency, though formula-fed infants tended to have fewer feedings per day than breast-fed infants.

In both studies, tolerance reasons were cited for a small number of infants who did not complete the study feeding. The percents of treatment failures in the 3 and 1.5 g/L FOS groups were approximately 18-20% and 11% of enrolled infants excluding protocol failures, respectively, while treatment failures in the control groups ranged from 5-15% of enrolled infants. The data suggest mildly greater intolerance at the highest FOS intake (3 g/L), though no statistically significant differences in treatment failures between the FOS and control groups were found within either study.

Measures of formula tolerance and stool characteristics collected throughout the feeding periods provide additional information suggesting comparable acceptability of the FOS and control formulas. In both studies, the percent of feedings with spit up or vomit did not significantly differ among the feeding groups. The median percent of feedings with spit up in the 4-month study (study 2) tended to be lower in the FOS group compared to the control formula and human milk groups. In contrast, the occurrence of intolerance appeared to increase in both the control and FOS3 groups in the 2-week FOS tolerance study (study 1). At the end of the feeding period, spit up was reported at a median of 25% of feedings in the FOS3 group and 13% of feedings in the control group compared

to17% and 8% of feedings at baseline, respectively. The occurrence of spit up was considerably lower in the FOS1.5 group as measured both before and after the feeding period (6%), suggesting the FOS3 group may have been comprised of fussier infants, which was supported by parental comments during the study. Given the absence of a dose-response to FOS concentrations in the study formulas, it is unclear that the intolerance experienced in this group can be attributed solely to the FOS.

Stool characteristics were assessed during the studies to monitor tolerance of the formulas. In study 1, stools became significantly looser for all groups of infants fed the whey-enriched formula regardless of the addition of FOS, though daily frequency of stools was unaffected. The stool softening effect is likely attributed to the formula consumed during the feeding period, as other investigators have reported looser stools during consumption of whey-predominant formulas (Malacaman et al. 1985; Harrison et al. 1987). Euler and colleagues (2005) recently reported that consumption of a whey-predominant formula supplemented with FOS resulted in softer stools after one week, and infants consuming 3 g/L FOS had a greater change in consistency (more soft) than infants consuming 1.5 g/L FOS.

In study 2, the addition of FOS resulted in softer stools compared to the control formula, though the difference was significant only after one month of feeding. The addition of 3g FOS per liter formula had no effect on daily stool frequency in our study, though breastfed infants tended to have more frequent stools than formula fed infants. Across both studies, FOS had no consistent effect on stool frequency. In another study, up to 3 g/d FOS consumed in cereal by infants 16-46 weeks of age was reported to be well tolerated, and infants consuming the FOS cereal were more likely to have soft or loose stools than the control group, and also more frequent stools (Moore et al. 2003).

Infants consuming FOS-supplemented formulas had small quantifiable amounts of GF_2 and GF_3 in their urine though no levels were detected in blood. These findings are consistent with a study in adults in which approximately 0.12% of the ingested amount of

FOS was recovered unchanged in urine after 24 hours (Molis et al, 1996). Human milk oligosaccharides also are found in the urine of breastfeeding infants (Rudloff et al. 1996).

In both the 2-week tolerance study and the 4-month growth study, measurements of liver enzymes including ALT and AST in plasma indicated no abnormal levels or any effects of FOS. Plasma cholesterol also was not affected by the addition of 3 g/L to infant formula, as levels were comparable between the FOS and control group. Plasma cholesterol levels measured in breastfeeding infants where higher than levels in formula-fed infants, a finding consistent with other research (Owen et al. 2002).

Oligosaccharides represent one of the largest solutes in human milk, and infants may be exposed to concentrations as high as approximately 13 g/L through the consumption of mature human milk (Coppa et al. 1993). Human milk oligosaccharides are associated with the selective stimulation of these bacteria in the infant microflora (Coppa et al 2004). The oligosaccharides are not hydrolyzed in the upper small intestine and reach the large intestine intact where they serve as substrates for bacterial metabolism. Indirectly, the growth of beneficial microorganisms, such as *Bifidobacteria*, inhibit growth of pathogenic organisms by production of short chain fatty acids, thereby acidifying the colonic contents and producing an environment that inhibits the growth of the less acid tolerant pathogenic microorganisms.

The addition of the indigestible carbohydrate FOS to an infant formula provides a substrate for fermentation by intestinal bacteria. Several studies in adults indicate that consumption of as little as 4 g FOS results in significant increases in fecal *Bifidobacteria* (Bouhnik et al. 2004; Bouhnik et al. 1999; Buddington et al. 1996; Williams et al. 1994). Studies in infants, however, have not confirmed this bifidogenic effect. In the current series of studies, FOS-supplemented formulas had no consistent, significant effects on fecal counts of anaerobic bacteria including *Bifidobacteria*, *Lactobacilli*, *Clostridium*, *C. difficile*, or *Bacteroides* and selected aerobes. A decrease in *Clostridia* colonization was observed in the 2-week tolerance study, though the effect was not detected in the 4-month

study. In the 4-month study, an increase in *Lactobacillus* colonization was detected. Overall, the FOS-supplemented formulas had minimal effects on the fecal flora.

Euler and colleagues (2005) also reported that the FOS-supplemented formulas had no significant effects on fecal *Bifidobacteria*, *Lactobacilli*, *Enterococcus*, *Bacteroides*, or *Clostridium* following one week of intake. Results from studies in which term and preterm infants consumed a combination of GOS (90%) and FOS (10%) in formula for 28 days or longer, however, indicate a favorable, significant and dose-dependent shift in fecal *Bifidobacteria* (Boehm et al. 2002; Moro et al. 2002; Rinne et al. 2005; Knol et al. 2005). Moro and colleagues (2002) also reported a significant increase in fecal *Lactobacilli* with the addition of GOS/FOS to infant formula, though the addition of GOS/FOS was not found to have an effect on *Lactobacilli* in another study (Boehm et al. 2002). No changes in potentially pathogenic species (*Bacteroides*, *Clostridium* species, *E coli*, *Enterobacter*, *Citrobactei*, *Proteus*, *Klebsiella*, and *Candida*) were detected in infants consuming the GOS/FOS-supplemented formulas (Moro et al. 2002; Boehm et al. 2002).

It is unclear why the addition of FOS did not cause a more significant shift in the fecal microflora populations assessed in our studies. Roberfroid and colleagues (1998) studied the relationship between FOS intake (from chicory oligofructose and synthetic FOS) and the log increase in fecal *Bifidobacteria* populations and found that an increase in the number of *Bifidobacteria* is correlated with the initial *Bifidobacteria* level, and not the ingested dose. It is possible that the healthy infants enrolled in these studies had relatively high *Bifidobacteria* levels that could not easily be perturbed with the doses provided. As seen in the 4-month study, the percent of infants with detectable fecal *Bifidobacteria* at approximately day 8 of life was nearly identical in one of the formula groups (FOS0, 52%) and the human milk group (59%), though the percent was slightly lower in the other formula group (FOS3, 36%). After one month of feeding, *Bifidobacteria* levels in all feeding groups were comparable and remained so over the

duration of our observations. The use of newer molecular techniques to study fecal microflora populations also may enable smaller changes to be detected (Tannock 2001).

In conclusion, results from the two studies indicates that infant formula containing added FOS at the levels provided in these studies (up to 3 g/L) are well tolerated and support normal growth in term infants. The addition of the fermentable fiber at these levels, however, has only small effects on fecal microflora.

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TABLE 1. *Characteristics of infants enrolled in studies evaluating safety and efficacy of fructooligosaccharide added to infant formulas*[1]

	Study 1[2]			Study 2[3]		
	FOS0	FOS1.5	FOS3	FOS0	FOS3	HM
Enrolled (M/F) [completed]	23 (18/5) [20][4]	22 (13/9) [17]	21 (11/10) [16][4]	52 (25/27) [34]	50 (23/27) [36]	25 (13/12) [23]
Treatment failures	1	2	4	6	8	0
Protocol failures	0	3	0	12	6	2
Gestational age (wk)	40 ± 0.2	40 ± 0.2	40 ± 0.2	39.2 ± 0.2	39.5 ± 0.2	39.8 ± 0.2
Age at study entry (d)	39 ± 2 [a]	41 ± 3 [a]	49 ± 3 [b]	7 (1-18) [a]		0 (0 – 9) [b]
Weight at study entry (g)	4737 ± 206 (23)	4692 ± 157 (22)	4957 ±156 (21)	3334 ± 66 (47)	3474 ± 66 (49)	3545 ± 98 (25)
Head circumference at study entry (cm)	NR	NR	NR	35 ± 0.0 (42)	36 ± 0.0 (42)	36 ± 0.0 (25)
Length at study entry (cm)	NR	NR	NR	50 ± 0.0 (47)	50 ± 0.0 (49)	51 ± 0.0 (25)
Weight at study end (g)	5615 ± 180 (21)	5477 ± 164 (18)	5801 ± 169 (18)	6426 ± 113 (33)	6629 ± 122 (36)	6471 ± 192 (23)
Weight gain (g/d)	28.2 ± 4.0 (21)	29.2 ± 2.3 (18)	25.4 ± 2.0 (18)	24.6 ± 1.0 (33)	23.1 ± 1.0 (36)	20.8 ± 2.0 (23)
Head circumference gain (mm/d)	NR	NR	NR	0.4 ± 0.0 (29)	0.3 ± 0.0 (29)	0.3 ± 0.0 (23)
Length gain (mm/d)	NR	NR	NR	1.0 ± 0.1 (33)	0.8 ± 0.1 (36)	0.9 ± 0.1 (23)
Feeding volume (mL/d)	830 ± 32 (21)	826 ± 30 (19)	862 ± 35 (19)	1040 ± 49 (34)	974 ± 45 (36)	NR
Feeding frequency (No./d)	5.9 ± 0.2 (21)	6.6 ± 0.4 (19)	6.1 ± 0.3 (19)	6.2 ± 0.4 (29)[a]	6.0 ± 0.3 (29)[a]	7.2 ± 0.5 (23)[b,5]

[1] Study outcome means are based on data collected over the entire study period except when noted. All values are mean ± SEM (n). Within studies, values with different superscripts are significantly different ($p<0.05$). All study formulas manufactured by Abbott.
[2] Study 1: FOS0 = 0 g/L added FOS; FOS1.5 = 1.5 g/L added FOS; FOS3 = 3.0 g/L added FOS. A whey-enriched formula was used in all groups during the feeding period.
[3] Study 2: SWI = 0 g/L added FOS; FOS3 = 3 g/L added FOS; HM = human milk. Similac with Iron was used in both formula groups.
[4] 2 infants in the FOS0 group and 1 in the FOS3 group exited the study prior to initiation of the test formulas due to treatment or protocol failures; these infants were excluded from all analyses.
[5] Breast-feeding infants had significantly more feedings per day compared to formula groups on days 28, 56, and 84 (ANOVA on ranked data).
NR: Not reported or measured in the corresponding study.

TABLE 2. *Tolerance and stool characteristics of infants consuming formula with added FOS*[1]

	Study 1[2]						Study 2[3]											
	FOS0		FOS1.5		FOS3		FOS0				FOS3				HM			
	week 2	week 4	week 2	week 4	week 2	week 4	day 28	day 56	day 84	day 112	day 28	day 56	day 84	day 112	day 28	day 56	day 84	day 112
Tolerance - feedings with (%, median)																		
Spit up	8	13	6	6	17	25	15	23	13	25	17	9	12	15	22	27	20	24
Vomit	1	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0
Stool characteristics																		
Number of stools/day (median)	1.3	1.2	1.2	1.1	1.3	1.3	1.7[a]	1.0	1.3	1.0	2.0[a]	1.0	1.0	1.0	3.7[b]	2.0	1.3	0.8
Consistency (median rank)[4]	2.6	1.6	2.7	1.6	2.8	1.8	3.0[a]	2.6[a]	2.4[a]	2.7	2.8[b]	2.5[a]	2.7[a]	2.0	2.0[c]	2.0[b]	2.0[b]	2.0
Predominant consistency (%)																		
Watery	5	48	5	53	5	42	0	5	8	6	10	11	11	23	13	23	10	10
Loose	36	48	38	42	15	42	17	38	36	21	21	26	24	14	61	64	71	48
Soft	41	5	48	0	65	16	48	41	36	44	48	39	39	37	22	14	10	33
Formed	14	0	10	0	10	0	24	3	0	6	7	0	8	3	0	0	0	0
Hard[5]	--	--	--	--	--	--	5	0	0	0	2	0	0	0	0	0	0	0
Mixed or Missing[6]	5	0	0	5	5	0	7	14	19	24	12	24	19	23	4	0	10	10

[1] Values in Study 1 based on all non-protocol failures; values in Study 2 based on all available data. Within studies, values for parameters with different superscripts are significantly different (ANOVA on ranked data; $p<0.05$).

[2] Study 1: FOS0 = 0 g/L added FOS; FOS1.5 = 1.5 g/L added FOS; FOS3 = 3.0 g/L added FOS. A whey-enriched formula was used in all groups during the feeding period.

[3] Study 2: SWI = 0 g/L added FOS; FOS3 = 3 g/L added FOS; HM = human milk. Similac with Iron was used in both formula groups.

[4] Ranked on a 5-point scale: 1 = watery, 2 = loose/mushy, 3 = soft, 4 = formed, and 5 = hard.

[5] Category not included in results for Study 1.

[6] Predominant stool consistency result was a tie between two categories (mixed) or was missing for a subject.

TABLE 3. *Liver enzyme and cholesterol analysis in plasma of infants consuming FOS-supplemented formulas*

	Study 1			Study 2					
	FOS0	FOS1.5	FOS3	FOS0		FOS3		HM	
	day 29	day 29	day 29	day 28	day 112	day 28	day 112	day 28	day 112
	Mean ± SEM (n) [1]								
ALT (U/L)[3]	32 ± 5 (7)	30 ± 10 (5)	37 ± 19 (3)	30 ± 1 (39)	32 ± 2[a] (32)	34 ± 2 (38)	37 ± 3[a] (33)	38 ± 4 (22)	49 ± 6[b] (21)
AST (U/L)[4]	46 ± 6 (7)	47 ± 11 (5)	46 ± 15 (3)	42 ± 2[a] (39)	46 ± 2[a] (32)	43 ± 2[a] (38)	49 ± 3[a] (33)	56 ± 4[b] (22)	65 ± 6[b] (21)
Cholesterol (mg/dL)[5]	- - -[2]	- - -	- - -	102 ± 3[a] (39)	117 ± 3[a] (31)	110 ± 4[a] (38)	119 ± 4[a] (33)	139 ± 5[b] (22)	148 ± 5[b] (21)

[1] Values at the same time point with unlike superscripts are different at $p<0.05$.
[2] Not measured.
[3]Reference range for infants (newborn – 12 months of age) is 13-45 U/L (Tietz 1995).
[4]Reference range for infants (newborn – 12 months of age) is 15-60 U/L (Tietz 1995).
[5]Reference range for infants newborn – 1 month of age is 45-198 mg/dL and for infants 2 months – 6 months of age is 60-218 (Soldin et al 1999).

TABLE 4. *Urinary FOS as GF_2 and GF_3 following consumption of FOS-supplemented formulas*

	Study 1		Study 2				
	FOS1.5 day 29	FOS3 day 29	FOS3 day 8	FOS3 day 28	FOS3 day 56	FOS3 day 84	FOS3 day 112
	ppm (range) (quantifiable samples/total samples)						
GF_2	39.6 – 56.6 (4/6)	59.2 – 114.0[1] (8/9)	2.3 – 217.6 (9/24)	26.6 – 102.9 (5/6)	2.1 – 34.6 (7/9)	33.9 – 108.4 (4/6)	26.4 – 55.5 (2/2)
GF_3	48.5 – 91.3 (5/6)	63.6 – 140.2 (9/9)	5.2 – 130.3 (8/24)[2]	12.6 – 110.6 (5/6)	1.5 – 26.7 (5/9)[3]	7.1 – 64.2 (3/6)[3]	- - - (0/2)[3]

[1] GF_2 was detected in one sample but not quantifiable.
[2] GF_3 was detected in one sample but not quantifiable.
[3] GF_3 was detected in two samples but not quantifiable.