Prebiotic Oligosaccharides in Premature Infants

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ABSTRACT

Objective: The aim of the study was to determine the impact of increasing doses of 2 prebiotic oligosaccharides and of an ''all-human diet'' on the intestinal microbiota of premature infants.

Methods: Twelve premature infants receiving formula feedings were randomly assigned to receive either galacto-oligosaccharide (F+GOS) or a pooled concentrated donor human milk product containing human milk oligosaccharides (F+HMO) in increasing doses during a 5-week period. A second group of 15 premature infants received their mother's own milk fortified with either a concentrated donor human milk product (H+H) or a bovine powdered fortifier (H+B). Serial stool specimens from each infant were analyzed by terminal restriction fragment length polymorphism and quantitative polymerase chain reaction for bacterial composition.

Results: All of the infants studied had relatively low levels of bifidobacteria and no measurable Lactobacilli. Infants from the F+GOS and F+HMO groups demonstrated an increase in relative numbers of Clostridia with increasing doses. Compared with the H+B group, the infants in the F+HMO and the H+H groups showed an unexpected trend toward an increase in γ-Proteobacteria over time/dose. Principal coordinate analyses and Shannon diversity scores were not significantly different among the 4 groups. Infants in the H+H group received more antibiotics during the study period than those in the other groups. Two of the infants receiving GOS developed feeding intolerance.

Conclusions: None of the prebiotic interventions resulted in significant increases in bifidobacteria compared with baseline specimens or the H+B group; however, many of the infants did not receive the highest doses of GOS and HMO, and antibiotic use in the H+H group was high.

Key Words: all-human diet, galacto-oligosaccharide, human milk oligosaccharide, late-onset sepsis, necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is a common and devastating disease of premature infants. The etiology of NEC is unclear and likely multifactorial; important risk factors include degree of prematurity, enteral feeding, and alterations in the intestinal microbiota (1). The possible roles of transfusions (2), maternal factors such as smoking (3), and genetic factors (4) in NEC are under investigation. The rate of advancement of feeding does not appear to be an important risk factor (5). To date, the most effective interventions to prevent NEC include mother's own milk (6), donor human milk (7), and probiotics (8). Fortification of mother's own milk or donor human milk with powdered or liquid bovine milk products is common. An ''all-human'' diet consisting of mother's own milk fortified with a pasteurized concentrated human milk product has been demonstrated to decrease the incidence of NEC (9) and to be cost-effective (10).

Prebiotics are nondigestible food ingredients that promote the growth of beneficial microbes; they differ from probiotics, which contain live organisms. Human milk oligosaccharides (HMOs) are the quintessential prebiotic, with dozens of complex structures that can only be digested by select species of bacteria (11). Commercial prebiotics are derived from plants or lactose and vary in composition, but lack the structural complexity and bacterial specificity of HMOs (12). Prebiotics are commonly added to a variety of foods including term infant formulas, and consist of fructose polymers (inulin and fructo-oligosaccharides [FOS]) and galactose polymers (galacto-oligosaccharides [GOS]) of varying length and acidity. The addition of prebiotic oligosaccharides to stimulate the growth of desirable commensal bacteria and bind pathogens within the intestinal lumen has been proposed as a safer method of prevention of NEC. A meta-analysis of 7 clinical trials in premature infants showed no improvement in NEC, sepsis, or time to full enteral feeding, but did demonstrate increased fecal bifidobacteria. The prebiotics used were predominantly short-chain GOS and/or long-chain FOS at doses from 0.4 to 1.0 g/dL of formula or human milk (13). No studies to date have analyzed different doses of prebiotics or compared commercial prebiotics with HMOs in premature infants. Dose escalation trials in this population are desirable but challenging. A comparison of several different doses of prebiotics with NEC as the primary outcome would require thousands of patients (8), given that differences that would be clinically significant are small relative to the within-group standard deviation of this outcome. Sample size requirements can be significantly reduced by selecting outcomes for which clinically significant effects are large relative to background variation (14). The fecal microbiota of the premature infant differs markedly from that of the term breast-fed infant, with the former dominated by Staphylococci, Streptococci, and Enterobacteriaceae and the latter by Bifidobacteria and Bacteroides. Given the association between

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an abnormal intestinal microbiota and NEC, we chose the composition of the fecal microbiota as our primary outcome, with secondary outcomes of safety and adverse effects. Herein we report the impact of prebiotic oligosaccharides on the fecal microbiota in 2 groups of premature infants. The first is a dose escalation trial of GOS or HMOs from a pooled donor human milk product in formula-fed premature infants. The second trial is a comparison among premature infants receiving mother's own milk plus either of 2 commercial milk fortifiers, one rich in HMOs and the other lacking HMOs. We hypothesized that both GOS and HMO would alter the fecal microbiota to become more like that of a term breast-fed infant in a dose-dependent manner and that an ''all-human diet'' (with the highest number and diversity of HMOs) would stimulate the growth of Bifidobacteria in the premature intestine to a greater extent than the other prebiotic interventions.

METHODS

The studies were performed in the neonatal intensive care unit of the University of California, Davis, Children's Hospital in Sacramento, beginning in June 2009 and ending in June 2011, were approved by the university's institutional review board. The inclusion criteria were birth weight <1500 g, gestational age <33 weeks, exclusive formula feeding during study 1 and exclusive feeding of mother's own milk during study 2, and enteral feeding volume of at least 100 mL · kg^{-1} · day^{-1}. The exclusion criteria were congenital gastrointestinal anomaly and a diet consisting of both formula and human milk. Enteral feeding was started as soon after birth as the infant was clinically stable and was advanced according to a written protocol with the goal of reaching full enteral feeding by 7 to 14 days of life. Informed consent was obtained from the parents of all infants before participation in the study.

In study 1, 12 premature infants were randomly assigned to receive either increasing doses of GOS (group F+GOS) or a pooled pasteurized donor human milk product (Prolacta Bioscience, Monrovia, CA, group F+HMO) added to each feeding for 5 weeks according to the dosage schedule in Table 1. Parents and caregivers were aware of the assigned study group (ie, the studies were not blinded). The GOS products contained short-chain GOS (<8 linkages). The human milk product for the F+HMO group differs from the commercial product Prolact+H^2MF in that it was skim milk and subjected to membrane filtration for addition to premature infant formula; the study product is not commercially available. This product was analyzed by mass spectrometry as described (15) and found to contain more of the high-molecular-weight HMOs (spectrum in supplemental Fig. 1, *http://links.lww.com/MPG/A266*; blue dots represent human milk oligosaccharides), but lower amounts of sIgA and lactoferrin (both of which are vulnerable to pasteurization) than unprocessed human milk. HMO concentration of this product was determined by gas chromatography as described (16). Total HMO concentration in the pooled donor human milk product averaged 10.3 g/L with a standard deviation of 1.5 for different batches (triplicate measurements). To avoid a conflict of interest, the investigators did not approach parents about participation in the formula-fed arms of this study until the mother had already decided not to provide her milk. Some of the enrolled infants received a mixture of mother's own milk and formula for a time before enrollment.

In study 2, an additional 15 premature infants had their mother's milk fortified with either a pasteurized concentrated donor human milk product (Prolact+4 or Prolact+6, Prolacta Bioscience, group H+H) or a powdered fortifier formulated from bovine milk (Similac Human Milk Fortifier, group H+B). The former contains HMOs, whereas the latter does not contain added prebiotic oligosaccharides. Fortification and feeding volume were adjusted by the attending neonatologist on an individual basis to achieve adequate growth. If mother's own milk was insufficient, infants in the H+H group were supplemented with pasteurized donor human milk (1 infant). One infant in the H+B group was removed from the study because of inadequate milk supply (patient 21). Figure 1 is a CONSORT (Consolidated Standards of Reporting Trials) chart summarizing enrollment in study 1 and study 2 and a concurrent trial of probiotics (17). The infants in study 2 were not randomly assigned to a group but were enrolled in blocks as described in the caption to Figure 1. During the study period, these infants were predominantly fed through a feeding tube or from a bottle.

Specimens of stool were collected from the formula-fed infants at baseline (before initiation of the supplement) and every week (just before the dose increase) for 5 weeks. Specimens from the human milk–fed infants were collected at baseline and every 2 weeks for 6 weeks. The stool specimens were collected from a soiled diaper by the bedside nurse into a sterile container and refrigerated at 4°C overnight. Stools were diluted 1:1 with phosphate-buffered solution, homogenized, then transported on dry ice and placed in an −80°C freezer until analysis of the microbiota. Specimens were labeled with a randomly generated specimen number to ensure blinded specimen analysis. Details regarding DNA extraction, qPCR, terminal restriction fragment length polymorphism (TRFLP) and Bacilli-specific TRFLP are contained in the supplementary information (*http://links.lww.com/MPG/A271*).

Statistical Analysis

QIIME software was used to generate principal coordinate analysis plots (18). Longitudinal trajectories of the patient's weekly 16S-TRFLP bacterial composition and diversity measures were modeled with mixed-effects extensions of generalized linear models, using PROC GLIMMIX in SAS/Stat software (19), adjusting for gestational age at first dose, recent antibiotic exposure, and small sample size (20). Taxon-specific relative abundances (eg, proportion bifidobacteria) were modeled using β regression (21). Weekly Shannon diversity scores (22) for the 6 taxon-specific relative abundances were modeled using β regression. Nonparametric methods were used for between-group comparisons for qPCR outcomes. For assessing the within-specimen agreement between relative abundance measures of bifidobacteria by qPCR and TRFLP, the coefficient of determination (R^2) was estimated using the linear regression procedure for clustered survey data (PROC SURVEYREG), designating specimens from the same infant as a separate cluster.

TABLE 1. Dosage schedule

Prebiotic	Week 1	Week 2	Week 3	Week 4	Week 5
HMO (mL added to 100 mL of formula, g/100 mL of the mixture)	20, 0.17	30, 0.24	40, 0.29	50, 0.34	50, 0.34
GOS (g/100 mL of formula)	0.25	0.5	1.0	1.5	2.0

GOS = galacto-oligosaccharide; HMO = human milk oligosaccharide.

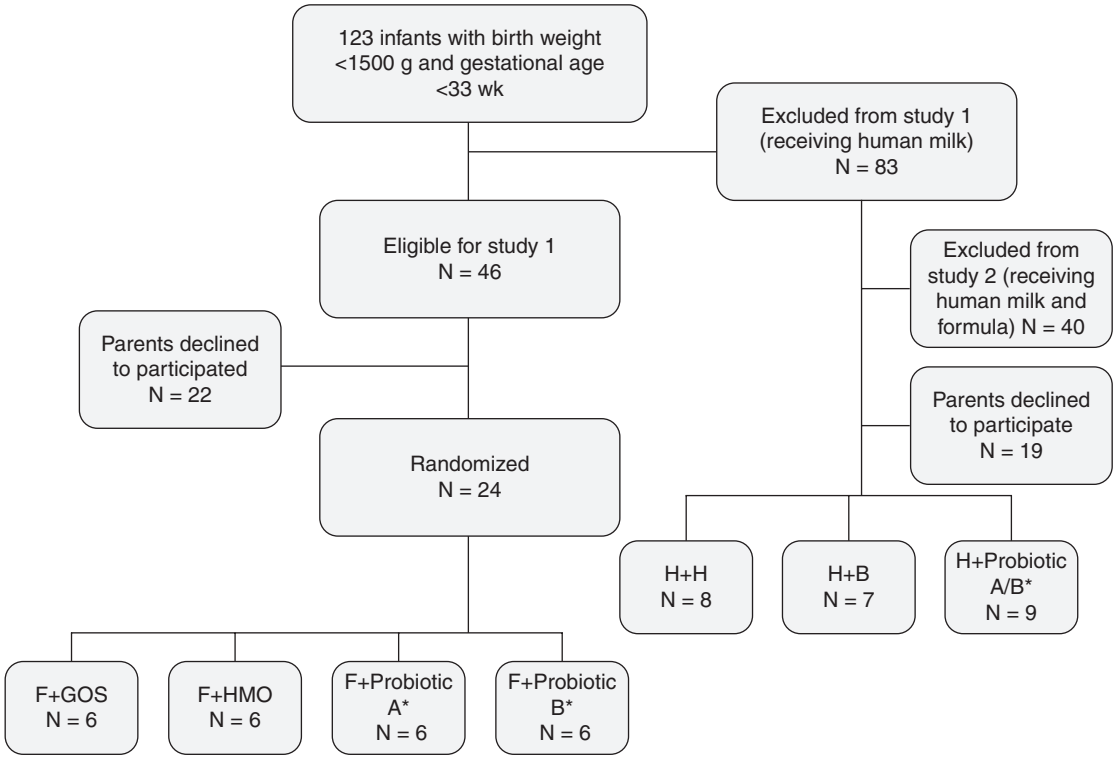

FIGURE 1. CONSORT (Consolidated Standards of Reporting Trials) diagram. The 24 infants eligible for study 1 (exclusive formula feeding) were randomly assigned to 1 of 4 groups. Infants receiving probiotics are noted with an asterisk (*) and were reported in a separate publication (17). The 24 infants eligible for study 2 (receiving exclusively human milk) were not randomly assigned to a group. The H+H group was enrolled first and then when additional funding was obtained, the H+B group and 1 additional patient in group H+H were enrolled. The H+probiotic group (also noted with an asterisk [*]) was enrolled last and these infants were randomly assigned to begin with probiotic A or B in a crossover design as reported in a separate publication (17). GOS = galacto-oligosaccharide; HMO = human milk oligosaccharide.

RESULTS

Safety and Clinical Outcomes

Descriptive statistics for the 4 groups are summarized in Table 2. The first 2 infants in the F+GOS group developed blood-streaked stools with mild abdominal distension, but without diarrhea, vomiting, or abnormal findings on screening radiographs or blood tests. The bloody stools resolved quickly after stopping the GOS syrup supplementation (both infants were removed from the study at that time). The prebiotic product administered in previous studies (23) was not available to us; therefore, we switched to a powdered GOS product for the remaining 4 infants in this group. There were no infants with vomiting, abdominal distension, constipation, diarrhea, bloody stools, or NEC during or following study enrollment among the 4 infants receiving the powdered GOS or the 6 infants in the F+HMO group. In the F+GOS group, 2 infants (patients 19 and 24) were discharged before study completion and 1 infant (patient 38) was removed from the study at the parents' request. In the F+HMO group, 2 infants were discharged before study completion (patients 2, 33) and 1 infant (patient 39) was removed from the study for poor growth (this was a 22-week-old infant who had stage 3 NEC requiring resection of a large portion of small bowel and primary anastamosis several weeks before the enrollment in the study at 4 months of age). In the H+H group, 1 infant (patient 3) developed coagulase-negative staphylococcal pneumonia, 1 infant (patient 13) developed stage 3 NEC shortly after a transfusion and died, 1 infant (patient 10) developed coagulase-negative staphylococcal sepsis, and 1 infant (patient 30) developed *Staphylococcus aureus* sepsis and endocarditis. In the H+B group, 1 infant (patient 14) developed stage 2 NEC and was treated with antibiotics for 7 days, and 1 infant (patient 26) was treated with antibiotics for 7 days for feeding intolerance and suspicion of sepsis (blood culture negative).

Composition and Diversity of the Intestinal Microbiota

Figure 2 presents the composition of the fecal microbiota at the class/genus level from each group over time as determined by TRFLP. Supplemental Figure 2 (*http://links.lww.com/MPG/A267*; B = baseline specimen) shows the same data at the phylum level. Supplemental Figure 3A and B (*http://links.lww.com/MPG/A268* and *http://links.lww.com/MPG/A269*) shows the individual TRFLP data for each patient and specimen. The bacterial classes γ-Proteobacteria (phylum Proteobacteria) and Bacilli (phylum Firmicutes) were dominant in all of the specimens. Bacteroidetes were absent, and Bifidobacteria (phylum Actinobacteria) were absent or minimally present in most specimens, as is typical of premature infants (24). In the formula-fed infants (study 1), relative abundances of Clostridia increased with increasing doses of HMO (adjusted per-week odds ratio [AOR] 2.0, 95% [confidence interval] CI 1.2–3.3, $P = 0.01$) and of GOS (AOR 2.7, 95% CI 1.1–6.8, $P = 0.03$). There were no differences between these groups (F+HMO vs F+GOS) in weekly changes for γ-Proteobacteria (relative adjusted per-week odds ratio [RAOR] 1.2, 95% CI 0.8–1.8, $P = 0.60$), Bacilli (RAOR 0.9, 95% CI 0.4–2.1,

TABLE 2. Demographics

	F+HMO (n = 6)	F+GOS (n = 6)	H+H (n = 8)	H+B (n = 7)
Gender, number F	4	1	3	5
Birth weight (SD)	936 (456)	933 (312)	943 (312)	952 (151)
Gestational age at birth, mean (SD)	27 (4)	28 (3)	27 (2)	27 (2)
Corrected gestational age at study enrollment, mean (SD)	33 (5)	31 (2)	30 (1)	30 (2)
Cesarean section	5	4	5	6
Apgar score at 1 min (range)	2 (1–8)	4 (2–8)	6	5 (2–8)
Apgar score at 5 min (range)	5 (2–9)	7 (4–9)	8	6 (2–9)
Age, days at study onset (SD)	39 (46)	25 (14)	22 (12)	18 (7)
Hispanic	1	3	2	2
Black	1	1	1	1
Multiples	2	0	0	1 surviving twin
Days on antibiotics (at or before onset of study), mean (SD)	14 (16)	6 (8)	6 (4)	9 (8)
Days on antibiotics (during study period), mean (SD)	1 (1)	2 (3)	8 (12)	5 (5)

F+HMO = formula with added human milk oligosaccharides; F+GOS = formula with added galacto-oligosaccharide; H+H = all-human diet; H+B = human milk fortified with bovine powdered fortifier; SD = standard deviation.

$P = 0.77$), Bifidobacteria (RAOR 0.8, 95% CI 0.4–2.0, $P = 0.69$), or Clostridia (RAOR 0.7, 95% CI 0.3–2.1, $P = 0.54$). Some of the infants in the F+GOS group appear to have a response to increasing doses of GOS with increases in Bifidobacteria (patients 24 and 38), and 1 infant demonstrated a significant decrease in γ-Proteobacteria with increasing GOS dose (patient 38); however, neither of these trends was significant for the entire group (for Bifidobacteria, AOR 1.1, 95% CI 0.6–2.1, $P = 0.83$; for γ-Proteobacteria, AOR 0.9, 95% CI 0.6–1.4, $P = 0.75$).

In the human milk-fed infants (study 2), the H+B infants showed a significant decrease over time in the relative abundance of bifidobacteria (AOR 0.76, 95% CI 0.61–0.95, $P = 0.02$); this was not seen in the H+H group. Four of the H+B infants showed a decrease in γ-Proteobacteria over time (patients 16, 21, 26, and 27), but this did not reach statistical significance for the entire group (RAOR 0.88, 95% CI 0.69–1.1, $P = 0.30$). An unexpected increase in relative abundance of γ-Proteobacteria over time/dose was seen in the H+H group compared to the H+B group (ROAR 1.5, 95% CI 1.01–2.0, $P = 0.04$) and a decrease for Bacilli (0.59, 95% CI 0.35–0.999, $P < 0.05$). In the principal coordinate analysis of TRFLP data from all specimens, there were no obvious differences between groups (data not shown).

Shannon diversity scores for the 6 taxa were moderately low, as is typical of premature infants (mean score [cluster-adjusted 95%

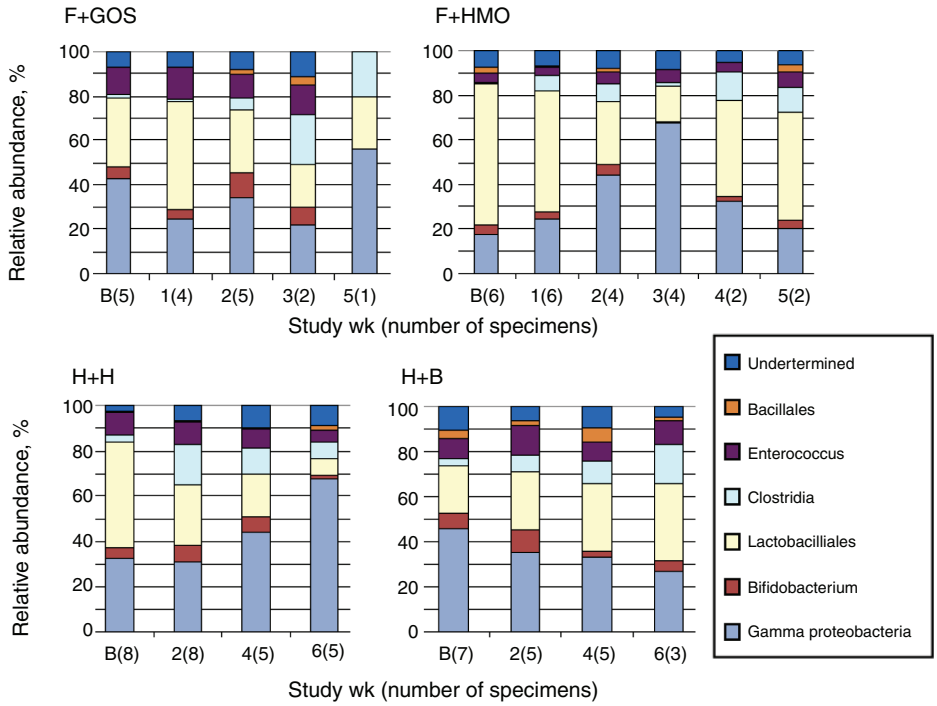

FIGURE 2. Universal Bacteria 16S-terminal restriction fragment length polymorphism analysis. Each color represents the mean percentage of the noted bacterial class present in the specimen by group with x-axis representing time/dose for the formula-fed infants and time for the human milk–fed infants. B = baseline specimen; GOS = galacto-oligosaccharide; HMO = human milk oligosaccharide.

CI]): F+HMO 2.5 (2.1–3.0), F+GOS 3.3 (2.7–3.9), H+H 3.0 (2.6–3.4), H+B 3.6 (3.2–3.9). In regression analyses, we found no differences within or between groups in changes over time/dose.

The bacteria of the class Bacilli were further characterized by focused TRFLP of baseline and final specimens in 3 infants in groups F+GOS, F+HMO, and H+B as summarized in supplemental Figure 4 (*http://links.lww.com/MPG/A270*; each color represents the percentage of all of the bacteria of the class Bacilli (orders Bacillales and Lactobacillales) of the noted bacterial species or genus in selected specimens). No differences between the groups were apparent, but all of the groups showed an early predominance of *S aureus*, which resolved over time as described (24). Lactobacilli were not detected.

Figure 3 presents more quantitative data based on qPCR analysis of mean total bacteria and mean number and percentage of Bifidobacteria (with the individual qPCR data for each specimen in Fig. 4). For percentage Bifidobacteria in this study, the correlation between TRFLP and qPCR was modest ($R^2 = 28\%$, $P < 0.001$). Infants in all 4 groups demonstrated low percentages of Bifidobacteria, as is common in this population. In the infants receiving increasing doses of HMO, there was no obvious impact on the numbers of total bacteria or numbers of Bifidobacteria. Three of the infants receiving GOS demonstrated increases in Bifidobacteria >0.1%. Only 1 of the infants assigned to the GOS group (patient 34) was able to complete all doses of the study; this baby appeared to have a dose response with the first 3 GOS doses and persistent increased percentages of Bifidobacteria with doses 4 and 5.

The infants in the F+HMO group received more antibiotics before study enrollment than the other groups, and the infants in the H+H group received more antibiotics during the study period than the other groups. Figure 4 also includes the gestational age at birth, the age at enrollment, and details regarding antibiotic administration for each infant.

DISCUSSION

Prebiotics are dietary supplements that alter the intestinal microbiota through differential consumption by desirable commensal bacteria rather than potentially pathogenic microbes. Commercial prebiotics have been shown to be safe in premature infants without significant adverse effects (13). The feeding intolerance seen in the 2 infants receiving the GOS syrup may be coincidental or may be related to the osmolality or composition of the syrup itself (60% GOS, 40% lactose, glucose, and galactose). The number of infants in the H+H group with nosocomial infections was higher than expected. This may also be coincidental and is inconsistent with previous reports (9,25).

Human milk contains complex oligosaccharides that are highly abundant, but not digestible by the infant (15). The production of these HMOs requires a significant energy investment by the mother. The functions of HMOs appear to be 3-fold: prebiotic activity (26), pathogen binding within the gut lumen (27), and perhaps neural development (28). The evidence for HMO prebiotic activity is compelling. Most women produce >100 different HMO structures, many of which stimulate the growth of specific strains of Bifidobacteria and Bacteroides in vitro (11). In the term infant, HMOs and other human milk components shape the microbiota of the infant until the introduction of solid foods (29). This process is disrupted in the premature infant in part because of the neonatal intensive care unit (NICU) environment (feeding tubes, antibiotics, delays in feeding, acid suppression, and hospital-acquired organisms), but also in part as a result of the immaturity of the premature infant gut and differences in ''premature'' human milk (30,31). For these reasons, the diet of the premature infant (unlike that of the term infant) does not appear to have a significant influence on the intestinal microbiota (32,33).

The composition of the intestinal microbiota appears to be an important risk factor for the development of NEC in premature infants. The intestinal microbiota of premature infants differs significantly from that of term infants (24,34). The risk of developing NEC in premature infants increases with the number of days the infant receives empiric antibiotics in the NICU (35–37). Alterations in the intestinal microbiota of premature infants appear to precede the onset of NEC with a bloom of γ-Proteobacteria (mostly Gram-negative Enterobacteriaceae) (38,39). Although no human studies have demonstrated a decrease in NEC related to prebiotic administration, in a rat NEC model an isomer of an HMO (disialyllacto-*N*-tetraose) improved survival and decreased NEC pathology scores (40). This is in marked contrast to probiotics in which changes in the fecal microbiota (17) and decreased risk of NEC (8) have been demonstrated in premature infants.

Reports of the impact of commercial prebiotics on the fecal microbiota in premature infants have been mixed with some studies

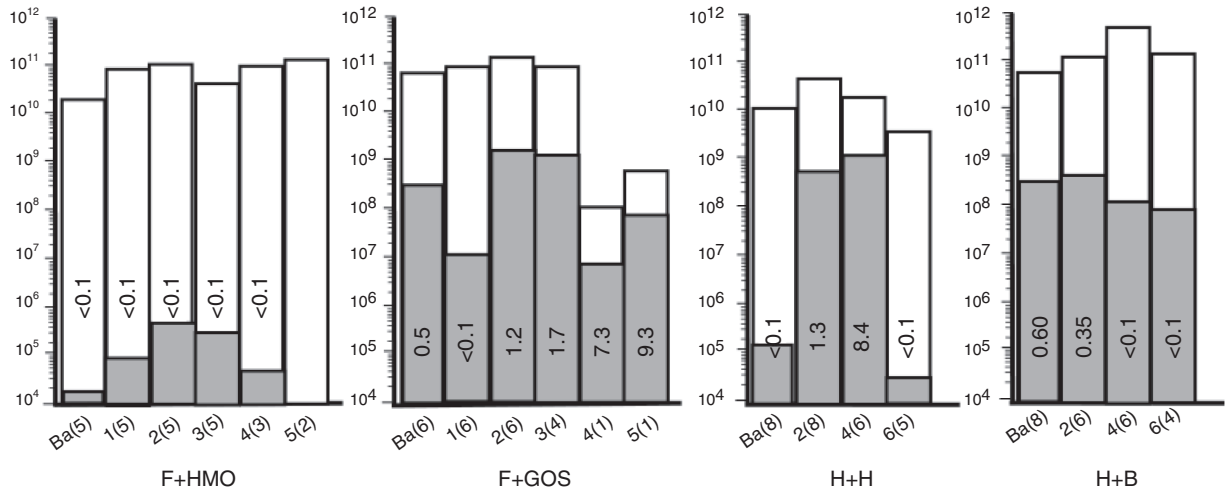

FIGURE 3. Quantitative polymerase chain reaction analysis of fecal specimens. For each group, mean total bacteria (white bars), mean total Bifidobacteria (gray bars), and mean percentage of Bifidobacteria (number within each bar) are presented. Note that the y-axis is a logarithmic scale and represents copies of 16S rRNA gene per gram of stool.

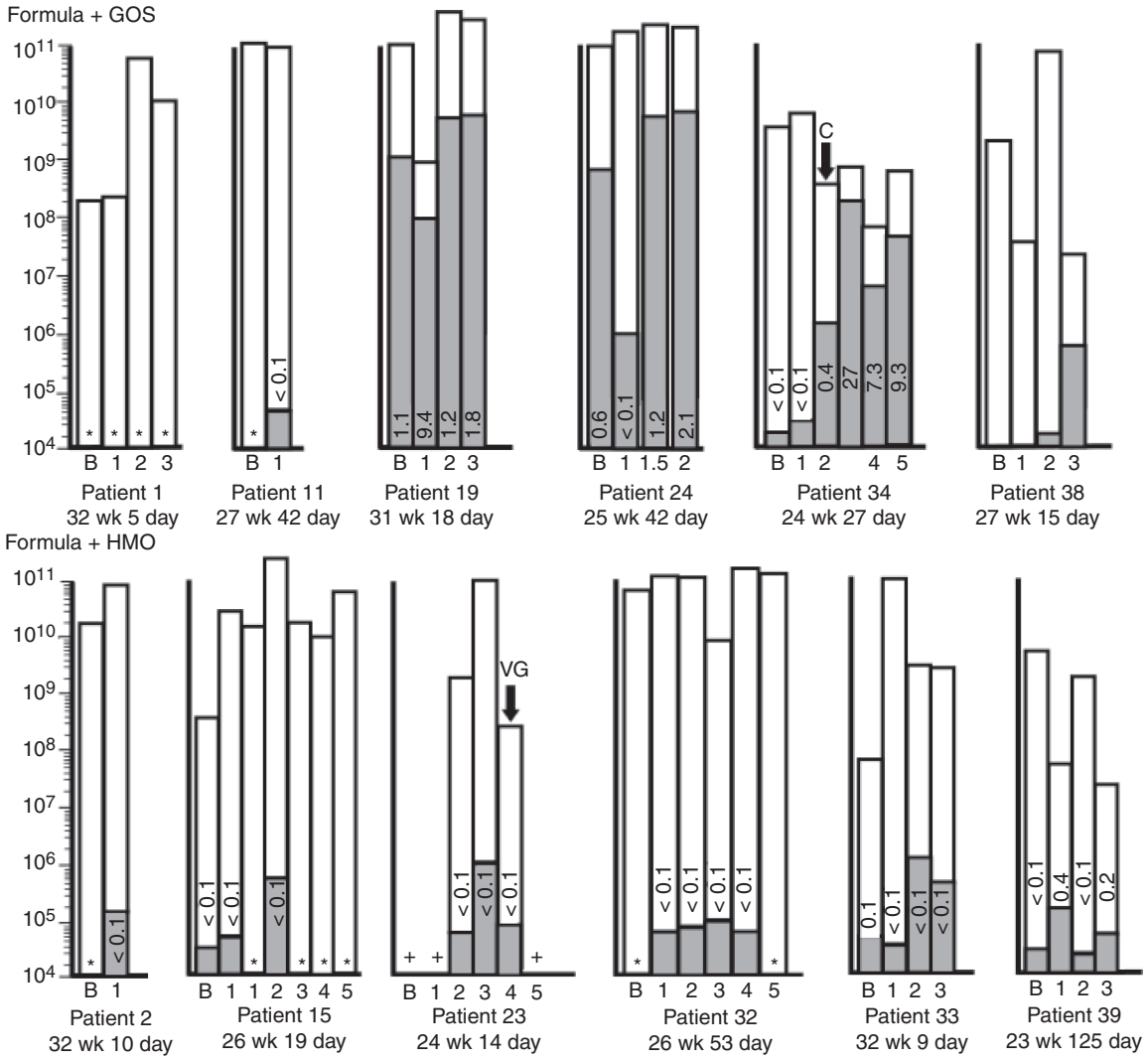

FIGURE 4. Quantitative polymerase chain reaction data for each individual specimen and each individual infant with total bacteria (white bars), total Bifidobacteria (gray bars), and percentage of Bifidobacteria (number within each bar). $*$ = no Bifidobacteria detectable (limit of detection 1×10^4 copies of 16S rRNA gene per gram of stool). $+$ = DNA not available for this specimen. B = baseline specimen. Arrows represent antibiotics given during the week before the specimen collection (V = vancomycin, G = gentamicin, A = ampicillin, C = cephalosporin, N = nafcillin). The y-axis is a logarithmic scale and represents copies of 16S rRNA gene per gram of stool. The numbers below each graph are the gestational age at birth (weeks) and the age at enrollment (days).

showing an increase in fecal Bifidobacteria (41,42), others showing no significant differences in fecal Bifidobacteria (43), and some showing a decrease in clinically relevant fecal pathogens (44). Possible explanations for the lack of increase in Bifidobacteria in the F+GOS group include the small sample size (although this is less likely given the multiple samples from each infant), differences in the GOS structure (although the GOS products administered in this study are readily consumed by several bifidobacterial strains (45)), a selection bias (although the parents of all eligible infants were invited to participate), few infants receiving the higher doses of GOS, and the antibiotics administered before or during the study. The low numbers of Bifidobacteria in the F+HMO, H+H, and H+B groups (all of whom received HMO) suggest that, unlike term infants, HMO alone is not sufficient to stimulate significant growth of Bifidobacteria in premature infants. Milk from women delivering preterm is more variable in HMO composition than milk from women delivering at term (31). The hypothesis that ''preterm milk'' does not provide an adequate composition of HMOs and therefore is a less effective prebiotic is not supported by these observations because both the F+HMO and the H+H groups received HMOs from donors that mostly delivered at term. The hypothesis that the relative lack of sIgA, lactoferrin, or other bioactive molecules diminished by pasteurization is responsible for the low levels of Bifidobacteria is also not supported by the data because the infants in groups H+H and H+B received unpasteurized mother's own milk. It is possible that environmental factors unique to the NICU and/or factors unique to the premature immune system preclude an initial inoculation with commensal organisms so that the provision of HMO is ineffective, that is, that the preterm infant requires both probiotics and prebiotics for significant colonization with Bifidobacteria. We have demonstrated that an HMO-consuming strain of *Bifidobacterium longum* ssp *infantis* colonizes the premature infant

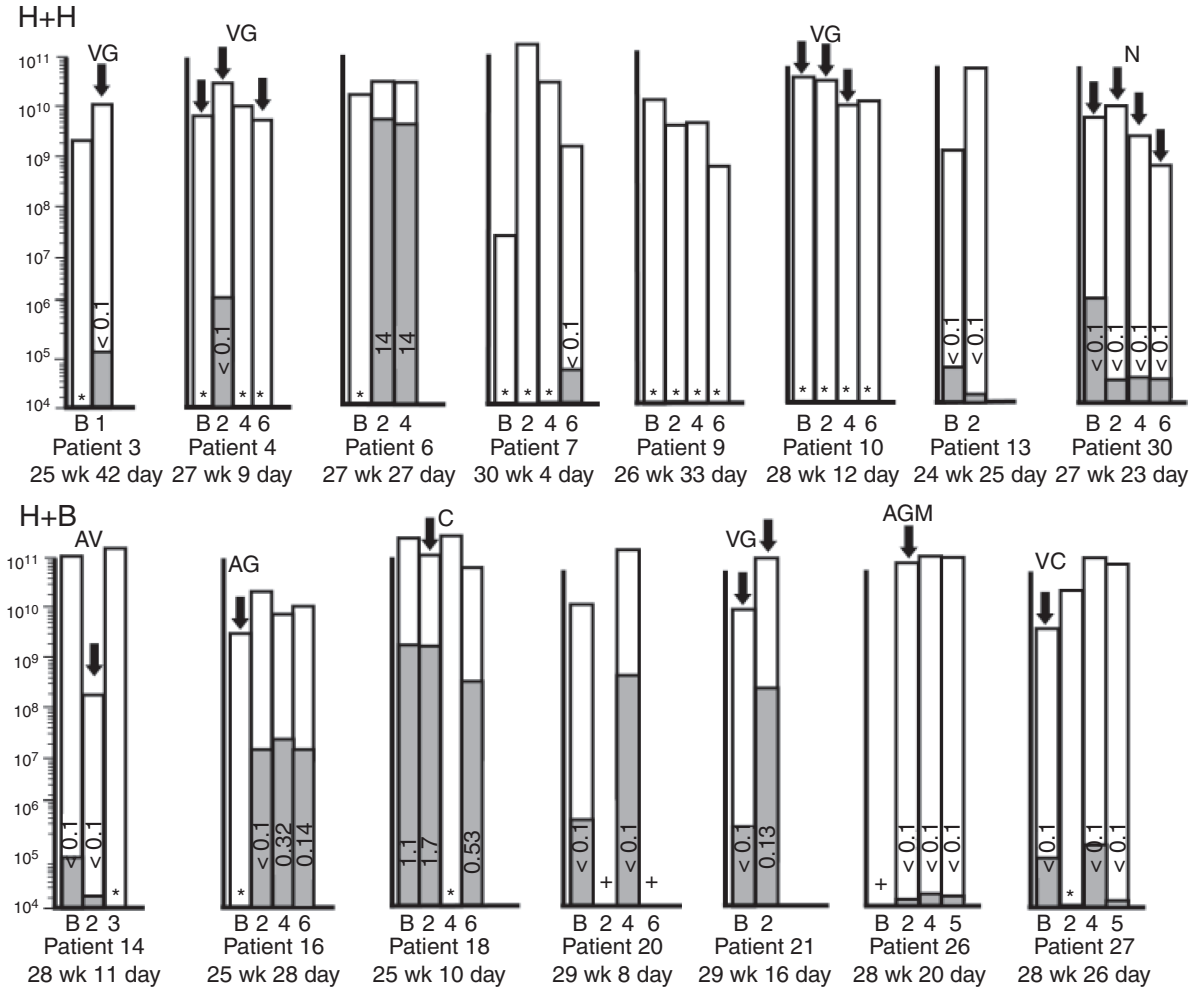

FIGURE 4. (*Continued*).

gut more effectively in the human milk–fed infant than in the formula-fed infant (17).

We hypothesized that administration of an all-human diet (H+H) would increase fecal Bifidobacteria because of higher numbers and a broader range of HMOs and that this change is an important mechanism by which this diet appears to be protective against NEC. This hypothesis was not supported by the data (no differences in fecal Bifidobacteria between the H+B group and the H+H group). The single published study comparing an all-human diet to standard fortification with bovine milk products is limited by its small sample size and the introduction of premature infant formula for the comparison group (ie, it is unclear whether the benefit seen is because of differences in fortification or differences in amount of formula received or both) (9). In our study, a decrease in γ-Proteobacteria over time was seen in the group receiving standard therapy (H+B), but not in the other groups; the significance of this observation is unclear.

The significance of the observed increase in relative abundance of Clostridia with increasing doses of both GOS and HMO in the formula-fed infants (study 1) is unclear. The class Clostridia includes a wide variety of genera including Clostridium species associated with degradation of antigen-specific immunoglobulin A (46), increased expression of proinflammatory cytokines (47), and increased irritability in premature infants (48), as well as the abundant commensal *Faecalibacterium prausnitzii* associated with decreased inflammation in patients with inflammatory bowel disease (49). *Clostridium perfringens* has been associated with outbreaks of NEC (50) and has been demonstrated to thrive in the presence of GOS (51).

This study has many limitations. The sample size is small, there are significant differences between groups (eg, corrected gestational age, days of antibiotics), many of the infants were born by cesarean section, there is a large degree of variability within each group, only infants who were receiving only formula (study 1) or only human milk (study 2) were eligible, which may limit generalizability, lack of completion of the study period by several infants (mostly as a result of being discharged), and the infants in study 2 were enrolled in blocks rather than randomized to 1 fortifier or the other. In addition, antibiotic administration has a significant impact on the intestinal microbiota and represents a major confounder. Among the human milk–fed infants (study 2), some of the infants received occasional feedings at the breast (unfortified), which may also represent a confounder.

CONCLUSIONS

Prebiotic administration to premature infants is not the standard of care in the United States. We compared 2 prebiotic

oligosaccharides in a dose escalation study. The addition of a pooled concentrated human donor product or GOS to infant formula in increasing doses did not increase fecal Bifidobacteria. No optimum dose was identified. Furthermore, an all-human diet did not increase fecal Bifidobacteria. The trend toward increased γ-Proteobacteria in both the F+HMO and H+H groups is puzzling and may represent antibiotic effect. In spite of the limitations outlined in the Discussion, the lack of development of significant numbers of commensal Bifidobacteria in any of the groups underscores the challenges of altering the microbiota of the premature infant.

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