Fecal Microbiota Composition of Breast-Fed Infants Is Correlated With Human Milk Oligosaccharides Consumed

*Mei Wang, *Min Li, †Shuai Wu, †Carlito B. Lebrilla, ‡Robert S. Chapkin, §Ivan Ivanov, and *Sharon M. Donovan

See "Should Infants Cry Over Spilled Milk? Fecal Glycomics as an Indicator of a Healthy Infant Gut Microbiome" by Frese and Mills on page 695.

ABSTRACT

Objectives: This study tested the hypothesis that the fecal bacterial genera of breast-fed (BF) and formula-fed (FF) infants differ and that human milk oligosaccharides (HMOs) modulate the microbiota of BF infants.

Methods: Fecal samples were obtained from BF ($n = 16$) or FF ($n = 6$) infants at 3-month postpartum. Human milk samples were collected on the same day when feces were collected. The microbiota was assessed by pyrosequencing of bacterial 16S ribosomal RNA genes. HMOs were measured by high-performance liquid chromatography–chip time-of-flight mass spectrometry.

Results: The overall microbiota of BF differed from that of FF ($P = 0.005$). Compared with FF, BF had higher relative abundances of *Bacteroides*, lower proportions of *Clostridium* XVIII, *Lachnospiraceae incertae sedis*, *Streptococcus*, *Enterococcus*, and *Veillonella* ($P < 0.05$). *Bifidobacterium* predominated in both BF and FF infants, with no difference in abundance between the 2 groups. The most abundant HMOs were lacto-N-tetraose + lacto-N-neotetraose (LNT + LNnT, 22.6%), followed by 2′-fucosyllactose (2′FL, 14.5%) and lacto-N-fucopentaose I (LNFP I, 9.5%). Partial least squares regression of HMO and microbiota showed several infant fecal bacterial genera could be predicted by their mothers' HMO profiles, and the important HMOs for the prediction of bacterial genera were identified by variable importance in the projection scores.

Conclusions: These results strengthen the established relation between HMO and the infant microbiota and identify statistical means whereby infant bacterial genera can be predicted by milk HMO. Future studies are needed to validate these findings and determine whether the supplementation of formula with defined HMO could selectively modify the gut microbiota.

Key Words: breast-fed, gut microbiota, human milk oligosaccharide, infants

(*JPGN* 2015;60: 825–833)

What Is Known

- HMOs are believed to serve as the primary substrate for the growth of beneficial bacteria.
- HMO consumption by gut bacteria has been studied in vitro.
- Little is known about the association between composition of the gut microbiota and HMO profiles in vivo.

What Is New

- Microbial composition of BF infants is positively and negatively correlated with the presence of a variety HMO in their mother's milk.
- Microbial composition of BF infants can be predicted by HMO consumed.
- If replicated in a larger population, the findings would support the supplementation of infant formula with defined HMO as a means to enrich the specific bacterial genera in the infant gut.

The development of the intestinal microbiota occurs primarily during infancy. Mutualistic interactions between the colonizing intestinal bacteria and the host are essential for healthy intestinal and immunological development (1). The stepwise microbial colonization process appears to have a long-lasting influence on the risk of not only gastrointestinal disease but also allergic, autoimmune, and metabolic diseases, in later life (2,3). Early microbial programming begins in utero and is substantially modulated by host and

Received August 29, 2014; accepted January 27, 2015.

From the *Department of Food Science and Human Nutrition, University of Illinois, Urbana, the †Department of Chemistry, University of California, Davis, the ‡Program in Integrative Nutrition and Complex Diseases and Center for Environmental and Translational Health Research, and the §Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station.

Address correspondence and reprint requests to Sharon M. Donovan, Department of Food Science and Human Nutrition, University of Illinois, 905 South Goodwin Ave, Urbana, IL 61801 (e-mail: sdonovan@illinois.edu).

Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (*www.jpgn.org*).

This study was supported by grants from National Institutes of Health (R01 HD061929 and 1P30ES023512-01), a Freedom to Discover Award from the Bristol-Myers Squibb Foundation to the Division of Nutritional Sciences at the University of Illinois, Urbana, and Hatch funds (Project ILLU-971-346) distributed through the Division of Nutritional Sciences Vision 20/20 program.

The authors report no conflicts of interest.

Copyright © 2015 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

DOI: 10.1097/MPG.0000000000000752

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

environmental factors, including the duration of gestation, mode of delivery, environmental microbes, antibiotic use, and diet (4,5).

Human milk is the optimal diet for infants, and exclusive breast-feeding is recommended for the first 6-months of life; however, a large proportion of US babies (~81%) are no longer exclusively breast-fed (BF) by 6 months of age (6). The influence of feeding type, breast or formula, on the composition of the microbiota is presently equivocal. Many studies have shown that the microbiota of BF infants is predominated by *Bifidobacterium*, whereas formula-fed (FF) infants are often colonized by more diverse microbiota, with a lower abundance of *Bifidobacterium* (7). In contrast, other studies reported that all of the infants were colonized by *Bifidobacterium*, with no differences in the prevalence or abundance between BF and FF infants (8,9).

Emerging evidence supports a role for HMOs in shaping the composition of the infant gut microbiota. HMOs are the third most abundant component of human milk after lactose and lipid, present at high concentrations (~20 g/L in colostrums, 5–10 g/L in mature milk) (10). In addition, HMOs exhibit great structural diversity, with >200 different structures being defined (11). HMOs are resistant to enzymatic hydrolysis in the upper gastrointestinal tract and the majority of HMOs (>90%) reach the colon (12), where they serve as the primary substrate for the growth of specific subsets of bacteria in the intestine of BF infants (13,14). Consumption of HMOs by gut bacteria has been studied in vitro by measuring the growth of pure bacterial isolates in culture media containing individual or mixtures of HMOs (14). Others have investigated the fermentation of individual HMO or a mixture of HMO ex vivo using fecal/intestinal microbiota from infants or piglets (15,16). Tracking HMO excretion can provide insight into the selective use by gut microbes in human infants in vivo (17,18). Little is, however, known about the association between composition of the gut microbiota and HMO profiles in vivo. Therefore, the goal of this study was to compare the fecal microbial composition between BF and FF infants and to examine the ability of milk HMOs to predict bacterial genera in BF infants.

METHODS

Study Subjects and Design

Healthy, full-term, vaginally delivered, exclusively BF (n = 16) or FF (n = 6) (Enfamil LIPIL; Mead Johnson Nutrition, Evansville, IN) infants were eligible for the enrollment into the study. Details of the subject recruitment and inclusion and exclusion criteria have been previously described (19). Both mothers and infants were medically certified as healthy (asymptomatic and with no clinical indication of disease) during the study, and the mothers consumed their normal diet. Enrolled infants who subsequently received antibiotic treatment were excluded from the study. In brief, mothers of infants were recruited for the study between the third-trimester pregnancy and 1 month postpartum. We focused on recruiting second parity mothers who had either exclusively BF or FF their first infant to increase our likelihood of enrolling mothers who were secure in their child feeding decision. Enfamil LIPIL formulas were provided to mothers who decided to formula feed their infants before delivery so that the infant began on the formula immediately after birth. All of the infants were fed ad libitum. Freshly voided stool samples were collected from the infants' diaper by the parent at 3 months of age using a sterile spoon. Samples were placed into sterile 2-mL tubes (Corning Incorporated, Corning, NY). Human milk was collected on the same day that fecal samples were collected. Milk was collected by expressing the contents of one breast, while the infant nursed on the other breast. To ensure the "full" content of the breast had been expressed, participants continued to express the mammary gland until milk flow had subsided. The milk sample was mixed to obtain a homogenous sample, from which 30 mL was placed into a sterile 50-mL conical tube (Corning Incorporated) and remaining milk was retained by the parent. All of the stool and milk samples were stored at 4°C for 2 to 3 hours, before being transported on ice to the laboratory, where they were stored at −80°C. All of the study procedures were approved by the University of Illinois institutional review board and informed consent was obtained from parents before the participation in the study.

HMO Analysis

HMOs were extracted, reduced, and purified from milk samples according to previously described methods (20,21). HMO composition was profiled using an Agilent 6210 high-performance liquid chromatography–chip time-of-flight mass spectrometry system equipped with both a capillary pump for sample loading and a nanopump for sample separation (Agilent Technologies, Santa Clara, CA) as previously described (20,21). Data were collected in the positive mode and calibrated by a dual nebulizer electrospray source with a wide range of internal calibrant ions: *m/z* 118.086, 322.048, 622.029, 922.010, 1221.991, 1521.972, 1821.952, 2121.933, 2421.914, and 2721.895. HMO identification and quantitation was performed using Agilent Mass Hunter Qualitative Analysis software (version B.03.01) as described by Totten et al (22). The relative amount of each oligosaccharide species was calculated by normalizing the absolute abundance of the individual species to the total oligosaccharide ion abundance in each sample, yielding a relative abundance expressed as a percentage of the total.

DNA Isolation From Fecal Samples

DNA was extracted using a modification of the method of Yu and Morrison (23). The detailed protocol has been previously described (9). DNA quality was checked on a 1% agarose gel following ethidium bromide staining. DNAs from 3 to 4 extractions per sample were pooled and concentration was quantified on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Analysis of Fecal Microbiota by Pyrosequencing of 16S Ribosomal RNA Genes

Amplification of the V1-V3 regions of the bacterial 16S ribosomal RNA (rRNA) genes was performed with fusion primers. Each forward primer (from 5′ to 3′) included GS FLX Titanium Primer A (CCATCTCATCCCTGCGTGTCTCCGACTCAG), a Multiplex Identifier that was unique to each sample, and 27F-DegS (24). The reverse primer (from 5′ to 3′) contained Primer B (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG) and 534R (25). The FastStart High Fidelity Polymerase Chain Reaction (PCR) System, dNTPack (Roche Applied Science, Indianapolis, IN), was used for PCR amplification. The PCR reaction mixture contained 0.2 μmol/L of each primer, 10 ng of template DNA, 5 μL of 10× PCR reaction buffer, 200 μmol/L of each deoxyribonucleotide triphosphate, 2.5 μL bovine serum albumin (New England Biolabs, Ipswich, MA) at 1 mg/mL (final concentration 100 μg/mL), 1.8 mmol/L $MgCl_2$ and 1.25 U of FastStart Hi-Fi enzyme blend in a total volume of 25 μL. PCR was performed in a DNAEngine (Bio-Rad, Hercules, CA) under the following conditions: 94°C for 3 minutes followed by 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute, and a final elongation step at 72°C for 7 minutes. After PCR, the amplicons

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

from 3 separate reactions were pooled and purified using Agencourt AMPure XP according to manufacturer instructions (Beckman Coulter, Brea, CA). Before pyrosequencing, DNA concentration was measured with Quant-iT PicoGreen dsDNA Assay Kits (Life Technologies, Grand Island, NY), and DNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies). The amplicons were mixed in equimolar concentration and sequenced at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois using 454 Life Sciences Genome Sequencer FLX with GS FLX Titanium series reagents (Roche Applied Science).

Sequence Processing

The 16S rDNA sequences were processed and analyzed using the QIIME pipeline (version 1.6.0, *http://qiime.org*) (26). Sequences were removed from further analysis if their length was outside the range of 400 to 600 nt, or if they contained ambiguous bases, primer mismatches, homopolymer run >6 nucleotides, or uncorrectable barcodes. The remaining sequences were denoised with the Denoiser algorithm within QIIME and clustered into operational taxonomic units (OTUs) at 97% pairwise identity using the UCLUST algorithm within QIIME. The representative sequences from each OTU were picked, and the chimera sequences were identified via Chimera Slayer. After removal of chimeras, the remaining sequences were aligned to the Greengenes imputed core reference alignment (27) using PyNAST and the alignment were filtered to remove highly variable regions and columns composed of only gaps using a lane mask. The phylogenetic tree was constructed from filtered alignment using FastTree (28), and unweighted UnFrac distance matrix (29) was generated from phylogenetic tree. The representative sequence of each OTU was assigned to different taxonomic levels using Ribosomal Database Project (RDP release 11.1, *http://rdp.cme.msu.edu*) naive Bayesian rRNA Classifier at 80% confidence level (30). Alpha-diversity (observed OTUs, Chao1 and ACE estimators, Shannon and Simpson reciprocal indices) was calculated using QIIME after rarefying to an equal number of reads (7800) for all of the samples to control for unequal sampling effort.

Statistical Analysis

To detect whether the structure of the bacterial communities between BF and FF infants differed, principal coordinate analysis (PCoA) and distance-based redundancy analysis (dbRDA) were performed on unweighted UniFrac distance using QIIME and the capscale command of vegan package of R, respectively (31).

Univariate statistical analysis was performed using PROC MIXED procedure of SAS version 9.2 (SAS Institute, Cary, NC). When the data were not normally distributed, the Mann-Whitney U test was used. The Spearman rank-correlation test was applied to explore relation between bacterial genera. Statistical significance was set at $P < 0.05$.

The associations between HMO profiles and each bacterial genus were modeled by partial least squares (PLS) regression. The HMOs that contributed most to the relation were identified by calculating variable importance in the projection (VIP) scores (32). Data were log transformed and mean centered before the PLS regression. The predictive performance of PLS model was evaluated by 10-fold cross-validation. A variable with VIP ≥ 1.2 was considered influential. The direction of correlation (positive or negative) was determined according to PLS regression coefficients (Beta). Spearman correlation, PLS regression, and VIP analyses were performed under MATLAB R2011b environment (Mathworks, Natick, MA).

RESULTS

Demographics and Growth of Subjects

A total of 16 BF and 6 FF infants were recruited for the study, and most of the infants were white (Table 1). There was no difference in mean age between mothers of BF and FF infants. The sex distribution and birth length of infants were similar in both groups. Body length at birth, body weight at birth and 3 months of age did not differ between BF and FF infants (Table 1).

HMO Composition

In total, 141 types of oligosaccharides were detected, and the average number of HMO in all of the milk samples was 63.4 ± 1.4. Nonfucosylated neutral oligosaccharides accounted for $25.4 \pm 2.26\%$ of total HMO. Fucosylated, sialylated, or both fucosylated and sialylated oligosaccharides comprised $61.1\% \pm 2.46\%$, $11.0\% \pm 1.65\%$, and $2.53\% \pm 0.41\%$, respectively (Table 2). The predominant HMOs are shown in Table 2. Lacto-N-tetraose and lacto-N-neotetraose (LNT + LNnT, 22.6%) together were the most predominant HMOs, followed by 2′-fucosyllactose (2′FL, 14.5%), lacto-N-fucopentaose I (LNFP I, 9.48%), lacto-N-fucopentaose II (LNFP II, 8.17%), and lactodifucotetraose (LDFT, 6.61%). Other HMOs accounted for <5% of the total HMO.

Fecal Microbiota of BF and FF Infants by Pyrosequencing

Pyrosequencing of the V1-V3 regions of the 16S rRNA gene amplicons yielded 417,344 total reads with an average read length of 453 bp. After performing the quality control depletions as above, 321,822 sequences with a mean of 10,734 sequences (range 7863–13,410) per sample were used for further analysis. Unweighted UniFrac PCoA revealed that the fecal microbial structure of BF infants differed from that of FF infants (Fig. 1A). This was confirmed by dbRDA of unweighted UniFrac distances ($P = 0.005$, Fig. 1B).

To identify which bacteria differed between BF and FF infants, the sequences were classified using Ribosomal Database Project Classifier (version 2.6 trained on 16S rRNA training set 9). In total, 7 phyla and 62 genera were identified. Actinobacteria was the most abundant phylum (~53 %), with no difference between BF and FF infants (Fig. 2). Other bacterial phyla were Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Fusobacteria, and MT7. BF infants harbored greater relative abundance

TABLE 1. Demographics and growth of subjects

	BF, n = 16	FF, n = 6
Maternal age, y*	29.1 ± 4.7	30.7 ± 2.4
Infant sex	4 female, 12 male	2 female, 4 male
Infant ethnicity	13 white	6 white
	2 African white	
	1 African American	
Length at birth, cm*	53.5 ± 2.9	51.0 ± 2.5
Body weight, kg*		
Birth	3.73 ± 0.55	3.50 ± 0.20
3 mo	6.70 ± 0.58	6.45 ± 0.96

BF = breast-fed; FF = formula-fed; SEM = standard error of mean.
* Mean ± SEM.

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

TABLE 2. Composition of HMOs in breast milk

Name	Percentage of total HMO*	Name	Percentage of total HMO*
Nonfucosylated neutral	25.4 ± 2.26	FS-LNnH I	1.27 ± 0.91
Fucosylated	61.1 ± 2.46	*m/z* 855.3 @ 18 min	1.15 ± 0.65
Sialylated	11.0 ± 1.65	LNDFH II	1.09 ± 1.07
Fucosylated and sialylated	2.53 ± 0.41	*m/z* 636.3 @ 16 min	0.95 ± 0.78
LNT + LNnT	22.6 ± 7.36	3′SL	0.92 ± 0.47
2′FL	14.5 ± 12.1	*m/z* 1074.4 @ 21 min	0.88 ± 1.03
LNFP I	9.48 ± 5.62	3′FL	0.87 ± 0.69
LNFP II	8.17 ± 4.33	*m/z* 1439.5 @ 23 min	0.85 ± 0.36
LDFT	6.61 ± 3.66	LSTc	0.76 ± 0.14
MFLNH III	3.82 ± 3.38	IFLNH III	0.70 ± 1.01
LSTb	3.14 ± 3.48	*m/z* 709.3 @ 12 min	0.69 ± 0.34
DFLNHa	2.47 ± 1.90	MSLNnH	0.67 ± 0.55
LNnH	2.38 ± 1.60	LNDFH I	0.58 ± 0.19
DSLNT	1.95 ± 2.37	5130a	0.54 ± 0.33
LNH	1.93 ± 1.24	*m/z* 490.2 @ 14 min	0.53 ± 0.73
MFpLNH IV	1.91 ± 1.24	DFpLNH II	0.51 ± 0.42
DFLNHb	1.89 ± 2.30		

Only HMO with relative abundance >0.5% are shown. 2′FL = 2′-fucosyllactose; 3′FL = 3′-fucosyllactose; 3′SL = 3′-sialyllactose; DFLNHa = difucosyllacto-N-hexaose a; DFLNHb = difucosyllacto-N-hexaose b; DFpLNH II = difucosyl-para-lacto-N-hexaose II; DSLNT = disialyllacto-N-tetraose; FS-LNnH I = fucosyl-sialyl-lacto-N-neohexaose I; HMO = human milk oligosaccharide; IFLNH III = isomeric fucosylated lacto-N-hexaose III; LDFT = lactodifucotetraose; LNDFH I = lacto-N-difucohexaose I; LNDFH II = lacto-N-difucohexaose II; LNFP I = lacto-N-fucopentaose I; LNFP II = lacto-N-fucopentaose II; LNH = lacto-N-hexaose; LNnH = lacto-N-neohexaose; LNT + LNnT = lacto-N-tetraose + lacto-N-neotetraose; LSTb = sialyllacto-N-tetraose b; LSTc = sialyllacto-N-tetraose c; MFLNH III = monofucosyllacto-N-hexaose III; MFpLNH IV = monofucosyl-para-lacto-N-hexaose IV; MSLNnH = monosialyllacto-N-neohexaose I; SEM = standard error of mean.

* Mean ± SEM, n = 16.

of Bacteroidetes, whereas FF infants had higher Firmicutes ($P < 0.05$, Fig. 2).

The relative abundances of predominating bacterial genera occurring in infant feces are shown in Table 3. *Bacteroides* were greater in BF than FF infants. In contrast, fecal *Clostridium* XVIII, *Lachnospiraceae incertae sedis*, *Streptococcus*, *Blautia*, *Clostridium* XI, *Clostridium sensu stricto*, *Eubacterium*, *Erysipelotrichaceae incertae sedis*, and *Haemophilus* were lower in BF than FF infants ($P < 0.05$). *Bifidobacterium* represented >50% of the total sequences in both BF and FF infants, with no difference between the 2 groups.

To compare diversity within samples, sequences were rarefied to an equal number of reads (7800) for all of the samples and observed OTUs, Chao1 and ACE estimators, Shannon and reciprocal Simpson indices were calculated (Table 4). The observed OTUs, Chao1 and ACE estimators did not differ between BF and FF infants. Similarly, no differences in the Shannon and reciprocal Simpson indices were detected between the 2 groups.

Relation Between Bacterial Genera

In order to assess the relation between the members of fecal microbiota, Spearman correlation analyses of relative abundances of bacterial genera were performed. In BF infants, the relative abundance of *Enterococcus* was positively correlated with abundances of *Bifidobacterium*, *Streptococcus*, and *Veillonella*, whereas negatively correlated with *Bacteroides* and *Clostridium* XVIII ($P < 0.05$, Table 5). Significant negative correlations were also detected between *Bifidobacterium* and *Bacteroides*, *Escherichia/Shigella*, and *Klebsiella* in BF infants. The only significant correlations found in FF infants were negative correlations between *Bifidobacterium* and *Clostridium* XVIII and between *Veillonella* and *Klebsiella* (Table 5).

Association Between HMO and Bacterial Genus

The associations between HMO profiles and each bacterial genus were modeled by PLS regression, and the influential HMOs for the prediction of bacterial genus were identified by VIP scores. PLS regression showed several bacterial genera detected in infant feces, including *Bifidobacterium*, *Bacteroides*, *Enterococcus*, *Veillonella*, and *Rothia*, could be predicted by their mothers' HMO profiles. As shown in Table 6 and supplemental Figure 1 (*http://links.lww.com/MPG/A436*), each bacterial genus revealed an association with multiple HMOs. For example, the relative abundance of fecal *Bifidobacterium* was positively linked with the presence of LNFP I, monofucosyllacto-N-hexaose III (MFLNH III), sialyllacto-N-tetraose b (LSTb) and disialyllacto-N-tetraose (DSLNT), and negatively linked with the presence of 2′FL and LDFT in human milk. Furthermore, most of HMOs were associated with multiple bacterial genera; for example, 2′FL was positively linked with *Bacteroides*, but negatively linked with *Bifidobacterium*, *Enterococcus*, *Veillonella*, and *Rothia*.

DISCUSSION

Feeding mode is one of the most important determinants of gut microbial diversity in neonates; however, its impact on the composition of the infant microbiota is often contradictory (3). These inconsistencies in the results may arise from the different analytical approaches used to enumerate the microbiota, geographically distinct infant groups studied or the variability in the composition of infant formula. To reduce those variations, we applied a high-throughput sequencing approach, enrolled infants from same geographic region and fed all of the FF infants with same formula throughout the experimental period. In agreement with several previous studies (7,9), our PCoA and dbRDA showed that the fecal microbiota composition of BF differed from that of FF infants. BF

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

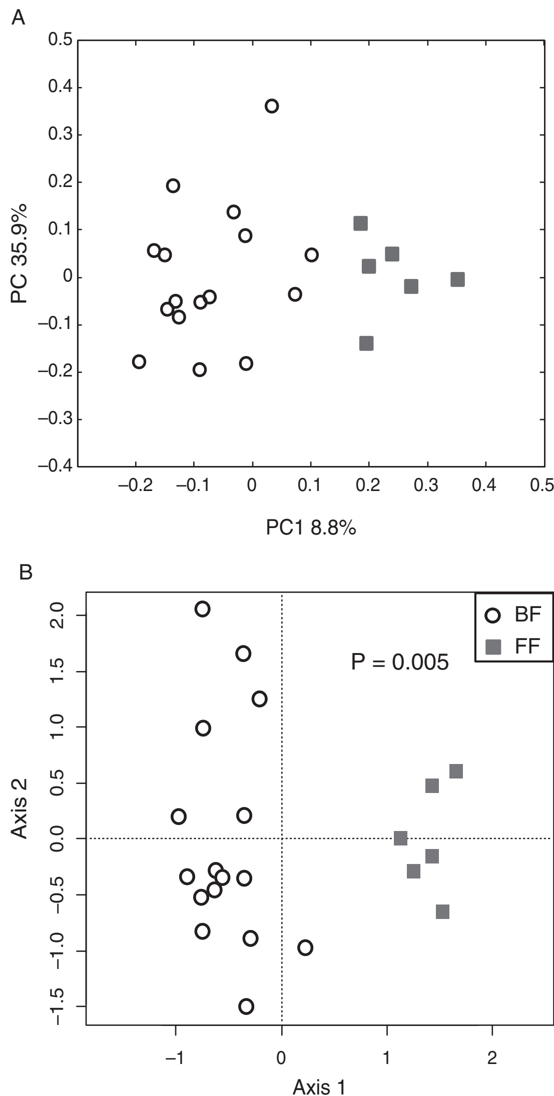

FIGURE 1. PCoA (A) and dbRDA (B) based on unweighted UniFrac distances generated from fecal samples of 3-month-old BF and FF infants. n = 16 (BF), n = 6 (FF). BF = breast-fed; dbRDA = distance-based redundancy analysis; FF = formula-fed; PCoA = principal coordinate analysis; PC = principal coordinate.

infants harbored greater relative abundance of *Bacteroides*, which belongs to Bacteroidetes, whereas FF infants had higher abundances of bacterial genera classified as Firmicutes, such as *Clostridium* XVIII, *Lachnospiraceae incertae sedis*, *Streptococcus*, *Enterococcus*, and *Veillonella*.

Bifidobacterium constitute a significant portion of the intestinal microbiota and are frequently used as probiotics to provide health-promoting benefits on their host (33). Colonization of *Bifidobacterium* in the neonatal intestine tract has been extensively studied; however, the results are often contradictory. Many studies showed that that *Bifidobacterium* rapidly dominated the microbiota in BF infants (34), whereas others reported that *Bifidobacterium* occurred in relatively low frequency and abundance in the fecal microbiota of BF infants (8). It is now generally accepted that *Bifidobacterium* are usually highly abundant in BF infants and that studies that deviated from this experienced methodological or sampling errors (35). For example, forward primer used in the study of Palmer et al (8) has a 3 base pair mismatch against *B longum*, and *Bifidobacterium* genus in general do not have 100% sequence identity to the forward primer.

Several older studies reported that FF infants harbor a lower abundance of *Bifidobacterium* compared with BF infants (34), whereas a review, which summarized the studies performed after 1980, concluded that in most studies *Bifidobacterium* are found equally often and in similar counts in BF and FF infants (36). In the present study, *Bifidobacterium* were predominant (>50% of sequences) in the feces of both BF and FF infants with no difference in the relative abundances between the 2 groups. The high abundance of *Bifidobacterium* detected in FF infants in the present study suggests that the formula was able to support the growth of *Bifidobacterium*. In recent years, several approaches have been used to improve infant formulas to induce a microbiota profile more similar to that in BF infants. These approaches, including providing an optimal ratio of casein and whey protein, and adding prebiotics or probiotics to infant formula, have been successful in increasing bifidobacteria in FF infants (37,38). The formula used in this study did not contain added prebiotics, although presently marketed Enfamil does contain 4 g/L of a 1:1 mixture of polydextrose and galactooligosaccharides. In terms of protein composition, the formula was whey predominant and studies have shown whey-predominant formula induce a fecal microbiota generally closer to that of BF babies than did a casein-predominant formula (38). Although similar proportions of *Bifidobacterium* genera were detected in BF and FF infants in our study, the *Bifidobacterium* composition at species level could differ between the 2 groups, as previously demonstrated. For example, Haarman and Knol (39) showed that *B longum* subsp *infantis*, *B longum* subsp *longum*, and *B breve* were the predominant species found in BF infants, whereas the microbiota of FF infants contained relatively more *B catenulatum* and *B adolescentis*, 2 species that are commonly found in adults.

Bacteroides are predominant in the gut of human adults and several studies have confirmed that *Bacteroides* also dominate the intestinal microbiota of some infants (40,41). In our study, *Bacteroides* represented the second most predominant bacterial genus in BF infants, after *Bifidobacterium*, and a significantly greater proportion of *Bacteroides* was detected in BF compared with FF infants (13.4% vs 0.03%). The presence of higher levels of *Bacteroides* may be beneficial for the BF infant, as members of *Bacteroides* have been shown to exert immunomodulatory proprieties on the host (42). For example, polysaccharide A produced by *Bacteroides fragilis* directs the cellular and physical maturation of the host immune system, specifically promoting the functional development of $CD4^+$ T cells (41). In addition, *Bacteroides* spp have extensive machinery to metabolize complex polysaccharides (such as starch, pectin, and host-derived glycan) (43). Thus, the presence of *Bacteroides* in the intestine of BF infants may confer stability and adaptability to microbiota during the transition from human milk to solid foods (44). Furthermore, degradation of polysaccharides by *Bacteroides* produces short-chain fatty acids, which contribute significantly to host nutrition and overall health of the colon (45).

Differences in the ratio of Firmicutes-to-Bacteroidetes were detected between lean and obese mice and human adults (46). Compared with lean mice, the cecal microbiota of obese mice had 50% fewer Bacteroidetes, and correspondingly more Firmicutes (46). Human adult studies have shown that the proportion of Bacteroidetes is decreased in obese individuals by comparison with lean people and that increases in Bacteroidetes and reductions in Firmicutes has been documented with weight loss (47). In our study, a higher Firmicutes-to-Bacteroidetes ratio was detected in FF than BF infants; however, no differences in body weight between the 2 groups were observed during the study period. Previous studies

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

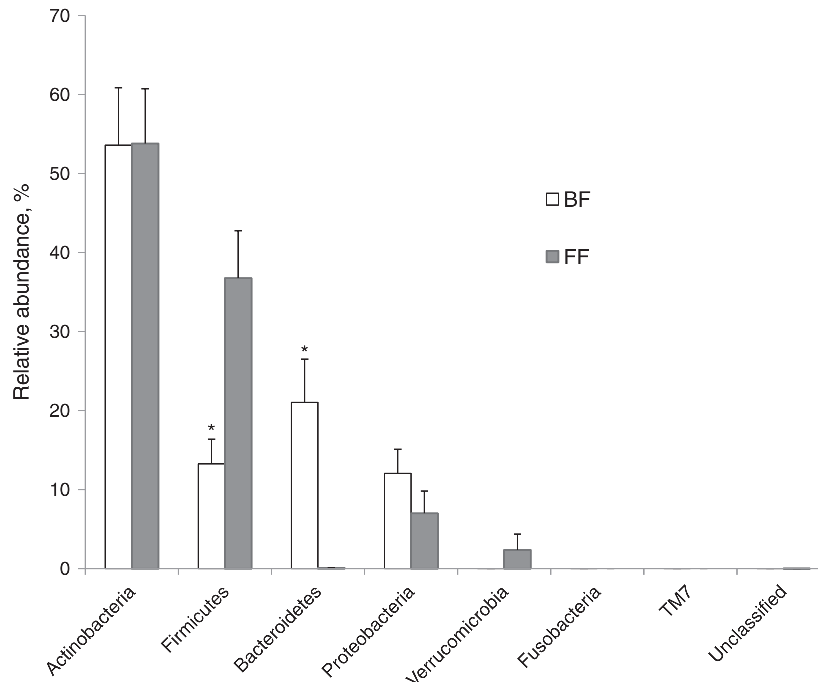

FIGURE 2. Relative abundances of bacterial phyla within fecal microbiota of BF and FF infants at 3 months of age. n = 16 (BF), n = 6 (FF). *FF differed from BF infants, Mann-Whitney *U* test, $P < 0.05$. BF = breast-fed; FF = formula-fed.

TABLE 3. Relative abundances of bacterial genera within fecal microbiota of BF and FF infants, median (25th–75th percentiles)

Bacterial genus	BF, n = 16	FF, n = 6	*P*
Actinobacteria			
Bifidobacterium	59.8 (36.1–76.9)	54.2 (41.3–65.3)	0.796
Rothia	0.11 (0.01–0.36)	0.03 (0.01–0.04)	0.318
Bacteroidetes			
Bacteroides	13.4 (2.35–41.0)	0.03 (0.01–0.06)	0.009
Parabacteroides	0 (0–0.21)	0 (0–0)	0.068
Firmicutes			
Clostridium XVIII	0 (0–1.39)	4.64 (2.23–8.40)	0.006
Lachnospiraceae incertae sedis	0 (0–0.18)	0.88 (0.86–0.94)	0.001
Streptococcus	0.34 (0.06–1.08)	4.87 (2.69–5.95)	0.001
Enterococcus	0.14 (0.01–0.41)	0.50 (0.09–0.93)	0.112
Veillonella	0.60 (0.04–1.37)	0.22 (0.11–1.10)	0.912
Blautia	0 (0–0)	0.01 (0–4.04)	0.021
Flavonifractor	0 (0–0.05)	0.01 (0–0.03)	0.432
Clostridium XI	0 (0–0.02)	0.23 (0.19–0.47)	0.003
Clostridium sensu stricto	0 (0–0.02)	0.56 (0.28–0.75)	0.004
Clostridium XIVa	0 (0–0.07)	0.01 (0–0.02)	0.932
Eubacterium	0 (0–0)	0.04 (0–0.39)	0.004
Erysipelotrichaceae incertae sedis	0 (0–0)	0.17 (0.04–0.55)	0.005
Lactobacillus	0 (0–0.02)	0 (0–0)	0.146
Anaerostipes	0 (0–0)	0 (0–0.09)	0.123
Staphylococcus	0.02 (0–0.13)	0 (0–0.01)	0.056
Proteobacteria			
Escherichia/Shigella	6.11 (2.13–14.1)	4.49 (1.49–6.57)	0.631
Klebsiella	0.03 (0–0.86)	0.27 (0.11–0.70)	0.279
Sutterella	0 (0–0.18)	0 (0–0)	0.581
Haemophilus	0.06 (0–0.17)	0 (0–0)	0.03
Morganella	0 (0–0)	0 (0–0)	0.61
Verrucomicrobia			
Akkermansia	0 (0–0)	0 (0–1.89)	0.096

Only bacterial genera with mean relative abundance >0.05% were analyzed. *P* values were obtained by Mann-Whitney *U* test. BF = breast-fed; FF = formula-fed.

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

TABLE 4. Diversity measures obtained from fecal samples of BF and FF infants, mean ± SEM

	Observed OTUs, n	Shannon	Reciprocal Simpson	ACE	Chao1
BF, n = 16	48.0 ± 2.4	2.75 ± 0.11	4.29 ± 0.35	83.4 ± 5.37	92.7 ± 10.6
FF, n = 6	60.0 ± 3.3	3.10 ± 0.23	4.83 ± 0.73	87.8 ± 7.57	89.9 ± 7.03
P	0.0894	0.1299	0.4645	0.6605	0.855

All samples were rarefied to 7800 sequences. *P* values were obtained by one-way ANOVA. ACE = abundance-base coverage estimator; ANOVA = analysis of variance; BF = breast-fed; FF = formula-fed; OTU = operational taxonomic unit; SEM = standard error of mean.

have shown breast-feeding decreases children's risk of obesity (48); therefore, the impact of the higher ratio of Firmicutes-to-Bacteroidetes in FF infants on the development of overweight and obesity later in life deserves further investigation.

Similar to the findings of a study by Jost et al (40), the proportion of *Bifidobacterium* was inversely correlated with the proportion of *Bacteriodes*; however, such relation was detected only in BF, not in FF infants. This may be partly owing to the presence of HMO in human milk. Human milk contains large quantity of structurally diverse oligosaccharides (10), whereas bovine milk, the basis for most infant formula, contains only trace amounts of predominantly siallylated oligosaccharides (49). In vitro fermentation studies have shown that some species of *Bifidobacterium* (eg, *B longum* subsp *infantis* and *B bifidus*) and *Bacteroides* (eg, *B thetaiotaomicron*, *B vulgatus*, and *B fragilis*) can grow efficiently in minimal medium containing HMO as the sole carbon source (50,51). Moreover, whole genome transcriptional profiling has revealed that some members of *Bifidobacterium* and *Bacteroides* express glycoside hydrolase and intestinal membrane transporters that are essential for the degradation of HMO (51,52). Thus, the inverse correlation between the 2 genera may result from competition for HMO as metabolic substrates when both *Bifidobacterium* and *Bacteroides* are present in the gut of BF infants. In addition, interindividual differences in HMO composition, such as the structural complexity of the HMO, may be an important selective force because previous studies have demonstrated that *Bifidobacterium* (eg, *B longum* subsp *infantis*) preferentially consume short HMOs (53), whereas *Bacteroides* have the capacity to use a broad range of HMOs, with a slight preference for larger ones (50). For example, a short HMO, LNnT, selectively expanded the abundance of *B longum* subsp *infantis* relative to *B thetaiotaomicron* in bi-associated gnotobiotic mice (51).

To date, most studies of HMO use by gut microbes have been performed in vitro by assessing the growth of single bacterial species in culture media containing HMO (14), or through ex vivo fermentation of HMO using fecal/intestinal microbiota from infants or animals (15,16). Few studies investigate associations between the composition of the gut microbiota and consumed HMO profiles in vivo. A study by De Leoz et al (18) investigated the relation between fecal bacterial populations and HMO excreted in the stool of 2 BF infants. Samples were collected at birth, 1, 2, and 13 to 14 weeks of age. Their final time point is consistent with the 3-month sample in the present study. In both infants, they observed a shift in the fecal bacterial population from non-HMO users, such as *Enterobacteriaceae* and *Staphylococcaceae* to the HMO consumers, *Bacteroidaceae*, and *Bifidobacteriaceae* (18). The relations between fecal HMO isomers and the relative abundances of order-level bacterial taxa were determined by Pearson product-moment correlation coefficients. Consistent with our findings, both positive and negative correlations were detected between the specific HMO and bacterial taxa. As an example, the relative abundance of Lactobacillales was positively correlated with the abundance of fecal MFLNH I, LnNH, pLNH, and an HMO with a mass of 5130a, and not negatively correlated with any of the HMO reported. Because these HMOs were excreted and not used, this indicates that none of these HMOs stimulate the growth of Lactobacillales. The relative abundance of Bifidobacteriales was negatively correlated with the abundance of MFLNH I, IFLNH I, LNT, LnNH and an HMO with a mass of 5230b in feces and not positively correlated with any of the HMO reported (18). Thus, MFLNH I and LnNH had opposite effects on the relative abundance of Lactobacillales and Bifidobacteriales.

Herein, PLS regression was applied to investigate the relation between milk HMO profiles and infant gut bacterial genus, and influential HMOs were identified by VIP scores. PLS regression is a supervised method that allows for the modeling of complex biological events by considering different factors at the same time (54) and is not affected by data collinearity. PLS regression coupled with VIP scores has been shown to be an excellent tool in identifying influencing variables (32,55). Our results indicate that relative abundances of some bacterial genera detected in infant feces can be predicted by the HMO consumed.

TABLE 5. Correlation between relative abundances of bacterial genera detected in feces of BF or FF infants

Bacterial genus	Bacterial genus	BF, n = 16	FF, n = 6
Bifidobacterium	*Bacteroides*	−0.75*	0.14
Bifidobacterium	*Clostridium XVIII*	−0.45	−0.89*
Bifidobacterium	*Enterococcus*	0.53*	−0.54
Bacteroides	*Enterococcus*	−0.53*	0.66
Escherichia/Shigella	*Klebsiella*	−0.51*	0.49
Clostridium XVIII	*Enterococcus*	−0.51*	0.66
Streptococcus	*Enterococcus*	0.68*	0.26
Enterococcus	*Veillonella*	0.52*	−0.66
Veillonella	*Klebsiella*	−0.04	−0.89*

Values were Spearman correlation coefficients. Only bacterial genera present in >50% infants and with mean relative abundances >0.5% were analyzed. BF = breast-fed; FF = formula-fed.

* $P < 0.05$.

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

TABLE 6. HMO showing influential effects for the prediction of bacterial genera in BF infants

Bacterial genus	MSE	Influential HMO (VIP ≥ 1.2)	
		Positive	Negative
Bifidobacterium	0.258	MFLNH III, LSTb, LNFP I, DSLNT	2′FL, LDFT
Bacteroides	0.686	2′FL, LNFP I, LDFT	LSTb, DFLNHa, DSLNT
Veillonella	0.142	LNFP I, DSLNT, LNFP II	2′FL
Enterococcus	0.068	DSLNT	2′FL, LDFT
Rothia	0.014	LDFT, LSTb	2′FL, MFLNH III, DSLNT

Data were log transformed and mean centered before PLS regression. The predictive performance was evaluated by 10-fold cross-validation. Only bacterial genera presented in >50% BF infants were analyzed. The direction of correlation (positive or negative) was determined by calculating PLS regression coefficients. 2′FL = 2′-fucosyllactose; BF = breast-fed; DFLNHa = difucosyllacto-N-hexaose a; DSLNT = disialyllacto-N-tetraose; HMO = human milk oligosaccharides; LDFT = lactodifucotetraose; LNFP I = lacto-N-fucopentaose I; LNFP II = lacto-N-fucopentaose II; LSTb = sialyllacto-N-tetraose b; MFLNH III = monofucosyllacto-N-hexaose III; MSE = mean-squared errors for PLS model; PLS = partial least square; VIP = variable importance in the projection.

For example, the relative abundance of *Bifidobacterium* and *Bacteroides* in infant stool were correlated with the HMO of their mothers' milk, which is consistent with previous culture studies showing that some strains of *Bifidobacterium* and *Bacteroides* are able to use HMO with high efficiency (14). In agreement with previous in vitro studies (14), the present results suggest that the relative abundances of *Escherichia*/*Shigella*, *Streptococcus*, and *Staphylococcus* in infants' feces are not associated with the HMO consumed. However, differences from previous reports were also observed in the present study. For example, in vitro culture studies have shown *Enterococcus* and *Veillonella* strains grew little or not at all when HMO was used as the carbon source (14). The results of this study demonstrate that the proportion of *Enterococcus* and *Veillonella* in infant fecal samples were associated with HMO profiles of mothers' milk.

Those seemingly contradictory observations may be partly because of the metabolic cross-feeding between members of gut bacteria. Cross-feeding is the phenomenon that metabolic products produced from one bacterial species provide substrates to support the growth of other species. Cross-feeding can result in metabolic consequences that would not be predicted simply from the substrate use of isolated bacteria (56). Cross-feeding has been found between strains of *B adolescentis* and butyrate-producing bacteria isolated from the human gut (56). Thus, *Enterococcus* and *Veillonella* may not themselves degrade HMO, but they may be able to use partial breakdown products or the fermentation end products produced by other gut bacteria, such as *Bifidobacterium* and *Bacteroides*.

In conclusion, we have compared the composition of fecal microbiota between BF and FF infants by pyrosequencing of bacterial 16S rRNA genes and correlated the microbiota of BF infants with the HMO profiles consumed. Our results indicate that the fecal microbial composition of BF infants differ from that of FF infants with a higher proportion of Bacteroidetes and lower abundance of Firmicutes. Moreover, we also demonstrate that the microbial composition of BF infants is correlated with the presence of HMO in their mother's milk. The ability of specific HMO to predict bacterial genera colonizing the infant gut should be validated in a larger cohort. If replicated, the findings would support investigating whether supplementation of infant formula with defined HMO would provide a means to selectively enrich specific bacterial genera in the infant gut (57).

Acknowledgments: The authors are grateful to all of the study subjects for participating in the study. The authors also thank the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois for performing pyrosequencing, and Mead Johnson Nutrition for providing infant formula.

REFERENCES

1. Rautava S, Luoto R, Salminen S, et al. Microbial contact during pregnancy, intestinal colonization and human disease. *Nat Rev Gastroenterol Hepatol* 2012;9:565–76.
2. Kull I, Melen E, Alm J, et al. Breast-feeding in relation to asthma, lung function, and sensitization in young schoolchildren. *J Allergy Clin Immunol* 2010;125:1013–9.
3. Li M, Wang M, Donovan SM. Early development of the gut microbiome and immune-mediated childhood disorders. *Semin Reprod Med* 2014;32:74–86.
4. Dominguez-Bello MG, Blaser MJ, Ley RE, et al. Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. *Gastroenterology* 2011;140:1713–9.
5. Collado MC, Cernada M, Baüerl C, et al. Microbial ecology and host-microbiota interactions during early life stages. *Gut Microbes* 2012; 3:352–65.
6. Centers for Disease Control and Prevention. Breastfeeding Report Card—United States; 2014. *http://www.cdc.gov/breastfeeding/data/reportcard.htm.* Accessed November 4, 2014.
7. Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. *Nature* 2012;486:222–7.
8. Palmer C, Bik EM, DiGiulio DB, et al. Development of the human infant intestinal microbiota. *PLoS Biol* 2007;5:e177.
9. Schwartz S, Friedberg I, Ivanov I, et al. A metagenomic study of diet-dependent interaction between gut microbiota and host in infants reveals differences in developmental and immune responses. *Genome Biol* 2012;13:R32.
10. Kunz C, Rudloff S, Baier W, et al. Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu Rev Nutr* 2000;20: 699–722.
11. Ninonuevo MR, Park Y, Yin H, et al. A strategy for annotating the human milk glycome. *J Agric Food Chem* 2006;54:7471–80.
12. Gnoth MJ, Kunz C, Kinne-Saffran E, et al. Human milk oligosaccharides are minimally digested in vitro. *J Nutr* 2000;130:3014–20.
13. Ward RE, Niñonuevo M, Mills DA, et al. In vitro fermentation of breast milk oligosaccharides by *Bifidobacterium infantis* and *Lactobacillus gasseri*. *Appl Environ Microbiol* 2006;72:4497–9.
14. Marcobal A, Barboza M, Froehlich JW, et al. Consumption of human milk oligosaccharides by gut-related microbes. *J Agric Food Chem* 2010;58:5334–40.
15. Li M, Bauer LL, Chen X, et al. Microbial composition and in vitro fermentation patterns of human milk oligosaccharides and prebiotics differ between formula-fed and sow-reared piglets. *J Nutr* 2012; 142:681–9.
16. Vester Boler BM, Rossoni Serao MC, Faber TA, et al. In vitro fermentation characteristics of select nondigestible oligosaccharides by infant fecal inocula. *J Agric Food Chem* 2013;61:2109–19.

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

17. Albrecht S, Schols HA, van den Heuvel EGHM, et al. Occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk. *Carbohydr Res* 2011;346:2540–50.
18. De Leoz ML, Kalanetra KM, Bokulich NA, et al. Human milk glycomics and gut microbial genomics in infant feces show a correlation between human milk oligosaccharides and gut microbiota: a proof-of-concept study. *J Proteome Res* 2015;14:491–502.
19. Chapkin RS, Zhao C, Ivanov I, et al. Stool-based detection of infant gastrointestinal development using gene expression profiles from exfoliated epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2010;298:G582–9.
20. Wu S, Tao N, German JB, et al. Development of an annotated library of neutral human milk oligosaccharides. *J Proteome Res* 2010;9:4138–51.
21. Wu S, Grimm R, German JB, et al. Annotation and structural analysis of sialylated human milk oligosaccharides. *J Proteome Res* 2011;10:856–68.
22. Totten SM, Zivkovic AM, Wu S, et al. Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. *J Proteome Res* 2012;11:6124–33.
23. Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* 2004;36:808–12.
24. van den Bogert B, de Vos WM, Zoetendal EG, et al. Microarray analysis and barcoded pyrosequencing provide consistent microbial profiles depending on the source of human intestinal samples. *Appl Environ Microbiol* 2011;77:2071–80.
25. Yildirim S, Yeoman CJ, Sipos M, et al. Characterization of the fecal microbiome from non-human wild primates reveals species specific microbial communities. *PLoS One* 2010;12:e13963.
26. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;7:335–6.
27. DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 2006;72:5069–72.
28. Price MN, Dehal PS, Arkin AP. FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS One* 2010;5:e9490.
29. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 2005;71:8228–35.
30. Wang Q, Garrity GM, Tiedje JM, et al. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;73:5261–7.
31. Oksanen J, Blanchet FG, Kindt R, et al. *Vegan: Community Ecology Package*. R package version 2.0-10; 2013. *http://CRAN.R-project.org/package=vegan*. Accessed December 16, 2013.
32. Chong IG, Jun CH. Performance of some variable selection methods when multicollinearity is present. *Chemom Intell Lab Syst* 2005;78:103–12.
33. Vaughan EE, Schut F, Heilig HG, et al. A molecular view of the intestinal ecosystem. *Curr Issues Intest Microbiol* 2000;1:1–12.
34. 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.
35. Sela DA. Bifidobacterial utilization of human milk oligosaccharides. *Int J Food Microbiol* 2011;149:58–64.
36. Adlerberth I, Wold AE. Establishment of the gut microbiota in Western infants. *Acta Paediatr* 2009;98:229–38.
37. Veereman-Wauters G, Staelens S, Van de Broek H, et al. Physiological and bifidogenic effects of prebiotic supplements in infant formulae. *J Pediatr Gastroenterol Nutr* 2011;52:764–72.
38. Hascoët JM, Hubert C, Rochat F, et al. Effect of formula composition on the development of infant gut microbiota. *J Pediatr Gastroenterol Nutr* 2011;52:756–62.
39. Haarman M, Knol J. Quantitative real-time PCR assays to identify and quantify fecal Bifidobacterium species in infants receiving a prebiotic infant formula. *Appl Environ Microbiol* 2005;71:2318–24.
40. Jost T, Lacroix C, Braegger CP, et al. New insights in gut microbiota establishment in healthy breast fed neonates. *PLoS One* 2012;7:e44595.
41. Vaishampayan PA, Kuehl JV, Froula JL, et al. Comparative metagenomics and population dynamics of the gut microbiota in mother and infant. *Genome Biol Evol* 2010;2:53–66.
42. Mazmanian SK, Liu CH, Tzianabos AO, et al. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 2005;122:107–18.
43. Comstock LE. Importance of glycans to the host-bacteroides mutualism in the mammalian intestine. *Cell Host Microbe* 2009;5:522–6.
44. Marcobal A, Sonnenburg JL. Human milk oligosaccharide consumption by intestinal microbiota. *Clin Microbiol Infect* 2012;18 (suppl 4):12–5.
45. Hamer HM, Jonkers D, Venema K, et al. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* 2008;27:104–19.
46. Ley RE, Turnbaugh PJ, Klein S, et al. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006;444:1022–3.
47. Nadal I, Santacruz A, Marcos A, et al. Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents. *Int J Obes (Lond)* 2009;33:758–67.
48. Grummer-Strawn LM, Mei Z. Centers for Disease Control and Prevention Pediatric Nutrition Surveillance System. Does breastfeeding protect against pediatric overweight? Analysis of longitudinal data from the Centers for Disease Control and Prevention Pediatric Nutrition Surveillance System. *Pediatrics* 2004;113:e81–6.
49. Martín-Sosa S, Martín MJ, García-Pardo LA, et al. Sialyloligosaccharides in human and bovine milk and in infant formulas: variations with the progression of lactation. *J Dairy Sci* 2003;86:52–9.
50. Asakuma S, Hatakeyama E, Urashima T, et al. Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. *J Biol Chem* 2011;286:34583–92.
51. Marcobal A, Barboza M, Sonnenburg ED, et al. Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. *Cell Host Microbe* 2011;10:507–14.
52. Sela DA, Chapman J, Adeuya A, et al. The genome sequence of *Bifidobacterium longum* subsp. infantis reveals adaptations for milk utilization within the infant microbiome. *Proc Natl Acad Sci U S A* 2008;105:18964–9.
53. LoCascio RG, Ninonuevo MR, Freeman SL, et al. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *J Agric Food Chem* 2007;55:8914–9.
54. Palermo G, Piraino P, Zucht HD. Performance of PLS regression coefficients in selecting variables for each response of a multivariate PLS for omics-type data. *Adv Appl Bioinforma Chem* 2009;2:57–70.
55. Mehmood T, Martens H, Sæbø S, et al. A partial least squares based algorithm for parsimonious variable selection. *Algorithms Mol Biol* 2011;6:27.
56. Belenguer A, Duncan SH, Calder AG, et al. Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. *Appl Environ Microbiol* 2006;72:3593–9.
57. Chichlowski M, German JB, Lebrilla CB, et al. The influence of milk oligosaccharides on microbiota of infants: opportunities for formulas. *Annu Rev Food Sci Technol* 2011;2:331–51.

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.