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Influence of Infant Feeding Type on Gut Microbiome Development in Hospitalized Preterm Infants

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Supplemental Digital Content 1. Figures illustrating variation boxplots for Lactobacillales and Bifidobacteriales. .doc

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Abstract

Background—Premature infants have a high risk for dysbiosis of the gut microbiome. Mother's own breastmilk (MOM) has been found to favorably alter gut microbiome composition in infants born at term. Evidence about the influence of feeding type on gut microbial colonization of preterm infants is limited.

Objective—The purpose of this study was to explore the effect of feeding types on gut microbial colonization of preterm infants in the neonatal intensive care unit (NICU).

Methods—Thirty-three stable preterm infants were recruited at birth and followed-up for the first 30 days of life. Daily feeding information was used to classify infants into six groups (mother's own milk [MOM], human donated milk [HDM], formula, MOM+HDM, MOM+Formula, and HDM+formula) during postnatal days 0–10, 11–20, and 21–30 after birth. Stool samples were collected daily. DNA extracted from stool was used to sequence the 16S rRNA gene. Exploratory data analysis was conducted with a focus on temporal changes of microbial patterns and diversities among infants from different feeding cohorts. Prediction of gut microbial diversity from feeding type was estimated using linear mixed models.

Results—Preterm infants fed MOM (at least 70% of the total diet) had highest abundance of *Clostridiales*, *Lactobacillales*, and *Bacillales* compared to infants in other feeding groups, whereas infants fed primarily human donor milk or formula had a high abundance of *Enterobacteriales* compared to infants fed MOM. After controlling for gender, postnatal age, weight and birth gestational age, the diversity of gut microbiome increased over time and was constantly higher in infants fed MOM relative to infants with other feeding types ($p < .01$).

Discussion—Mother's own breast milk benefits gut microbiome development of preterm infants, including balanced microbial community pattern and increased microbial diversity in early life.

Keywords

gut microbiome; preterm infant; human milk; 16S rRNA gene sequencing

The maintenance of human health and well-being relies on the interrelationship with a diverse gastrointestinal microbiome. Previous research demonstrates that the gastrointestinal microbiome is necessary for proper intestinal cell proliferation and migration, mucosal barrier protection, energy harvesting and storage, defense against pathogens, metabolism of food, regulation of blood pressure, and innate immunity (Leser & Mølbak, 2009; Moore, Munck, Sommer, & Dantas, 2011; Mshvildadze & Neu, 2010; Pluznick et al., 2013). An imbalance of species and communities of the gastrointestinal microbiome disrupts human health and leads to disease making microbiome research a priority (Groer et al., 2014; Manco, Putignani, & Bottazzo, 2010; Neu, 2013).

Environmental factors experienced during early development have been associated with phenotypic alterations through physiologic and epigenetic mechanisms with implications for

non-communicable disease (Hanson, Godfrey, Lillycrop, Burdge, & Gluckman, 2011). From this theoretical perspective of developmental plasticity, the intestinal microbiome influences many aspects of human health and it is necessary to understand what determines its composition and development. Microbiome is used to refer to the collective genomes of the microbes and to date, the gut microbiome also refers to a collection of microorganisms as well as the habitat they colonize in the gastrointestinal track (Weinstock, 2012).

Many factors early in the perinatal period influence the development of neonatal gut microbiome. Gut perfusion, stress, pharmacological interventions, hemodynamic stability, hospital environments and feeding practices during the perinatal period all potentially influence gut microbiome composition and proliferation (Cong, Xu, Romisher, et al., 2016; Groer et al., 2014; Khodayar-Pardo, Mira-Pascual, Collado, & Martinez-Costa, 2014; Putignani, Del Chierico, Petrucca, Vernocchi, & Dallapiccola, 2014). Thus, there is a research emphasis on identifying predictors of optimal microbiome development and associated health implications, especially within the preterm neonate population as preterm neonates are much more vulnerable to medical and developmental complications.

Our recent study explored day-to-day gut microbiome patterns in preterm infants during their first 30 days of life in the neonatal intensive care unit (NICU) and investigated potential factors related to the development of the infant gut microbiome (Cong, Xu, Janton, et al., 2016). We found that *Proteobacteria* was the most abundant phylum in early life and factors including postnatal days, feeding types and gender were significant contributors to the dynamic development of the gut microbiome in preterm infants. Especially, infants fed mother's own breastmilk (MOM) had a higher microbial diversity and significantly higher abundance in *Clostridiales* and *Lactobacillales* than infants fed nonMOM (Cong et al., 2016).

Infant feeding may have a large effect on the gut microbiome early in development, as milk is one of the first external components to enter the neonatal intestinal tract (Khodayar-Pardo et al., 2014; Newburg & Morelli, 2015; Putignani et al., 2014; Rodríguez, 2014). The feeding of MOM is essential for preterm infants; however, this is not always a possibility due to insufficient supply of MOM leading to the necessity for the use of pasteurized human donor milk (HDM) and preterm infant formula. There is still little known about the influence of different feeding types on the development of gut microbiome in preterm infants. In term infants, MOM has been associated with favorable alteration of gut microbiome, such as *Bifidobacterium* and *Lactobacillus*, in addition to pathogenic inhibition (Jost, Lacroix, Braegger, & Chassard, 2015). Recent evidence also points to the milk glycobiome as an influential factor in gut microbiome development (Newburg & Morelli, 2015; Pacheco, Barile, Underwood, & Mills, 2015). The glycobiome is comprised of a complex array of carbohydrates commonly termed human milk oligosaccharides with varied structure and linkage (Pacheco et al., 2015). Human milk oligosaccharides have been reported to contribute to optimal microbiome development through the inhibition of potentially pathogenic microbes (Gonia et al., 2015; Manco et al., 2010; Neu, 2013; Putignani et al., 2014).

Additionally, preterm infants are provided with human milk fortifier to substitute and/or enhance the nutritional composition of breast milk to better meet the nutritional needs of infants born preterm. Collectively, reduced immunologic components including immunoglobulins, cytokines and lymphocytes coupled with altered nutritional and microbial composition associated HDM and other alternate feeding modalities may have implications for the microbial development and may result in altered short- and long-term health outcomes of these high-risk infants (Poroyko et al., 2011).

Purpose

The purpose of this study was twofold: (a) to explore the effect of different feeding types, including MOM, HDM, formula, and mixed feeding types on gut microbial patterns, and (b) to investigate the contribution of different feeding types on the development of gut microbial diversities over the first 30 days of life in preterm infants in the NICU.

Methods

Study Design and Participants

A secondary analysis of data used for Cong, Xu, Janton, et al. (2016) with additional subjects was performed. The primary study was a prospective exploratory study conducted on level IV NICUs of Connecticut Children's Medical Center, at two sites located in Hartford and Farmington, CT (Cong et al., 2016). Inclusion criteria were stable preterm infants who were: (a) 28 weeks 0 days – 32 weeks 6 days gestational age, (b) 0–7 days old, and (c) mothers were older than 18 years old to provide consent. Exclusion criteria were infants who had: (a) known congenital anomalies, (b) severe periventricular/ intraventricular hemorrhage (Grade III), (c) undergone minor or major surgical procedures, or (d) history of prenatal drug (illicit or other drugs) or other exposures. The implication of inclusion and exclusion criteria was to control for factors that could contribute to feeding types and gut microbial composition. All parents of infants meeting the criteria and hospitalized in the participating NICUs during the study period, from January, 2013 to March, 2015, were invited to participate. Research nurses in the NICUs then discussed study procedures with the parents and obtained informed consent from both of the parents of the eligible infant. After being enrolled in the study, preterm infants were followed over their first 30 days of life in the NICU.

The study protocol was approved by the institutional review boards (IRBs) of participating sites and authors' affiliated university. All study procedures including clinical data collection, stool sample collection, storage, sequencing, and data analysis were approved by the IRBs.

Outcome Measures and Data Collection

Infant and clinical data—Demographic information and infant health characteristics were abstracted from the medical record. Severity of illness was measured by the Score for Neonatal Acute Physiology – Perinatal Extension-II (SNAPPE-II; Richardson, Corcoran, Escobar, & Lee, 2001). Administration of antibiotics, such as days of antibiotic use during the NICU hospitalization was also collected.

Feeding—Infant daily feeding information was collected by trained research nurses in the NICUs, including the frequency of infant fed by mother’s own breastmilk (MOM), human donor milk (HDM), and/or formula over the first 30 postnatal days of life. The feeding type cohorts were defined based upon >70% of total frequency of feeding in 10-day intervals. The frequency and percentage of each feeding type (MOM, HDM, or formula) for each infant were calculated over three 10-day intervals. Since no standard cutoff point for feeding types has been reported in the literature and the majority of infants were fed with more than one feeding type, we categorized study infants in to 6 feeding cohort groups based on the 70% cutoff of the frequency of feeding types using 10-day intervals. These groups included MOM, MOM+HDM, MOM+Formula, HDM, Formula, and HDM+Formula groups. For example, an infant fed MOM for more than 70% of the total feeding time during the first 10-days, then he/she was assigned to the MOM group for the first 10-days. If during the second 10-day period, none of the individual feeding types exceeded 70%, but the infant consumed feedings of mixed MOM and formula together for 70% of total feedings, then the infant was grouped into MOM+Formula group for the second 10-days (please see examples in the results section). We then applied these six feeding groups to examine and visualize the microbiome patterns.

Stool samples—Infant stool samples were collected by trained bedside nurses daily, depending upon whether the infant produced a stool each day. Stool samples were collected using sterile, disposable spatulas during diaper changes and then placed into a sterile specimen container. Samples were immediately frozen upon collection at –80°C, then transferred on dry ice to the laboratory and stored at –80°C until processing.

Stool Sample DNA Extraction, Sequencing, and Data Processing

The DNA extraction and processing method and procedures were as follows (described previously in Cong, Xu, Janton, et al., 2016). DNA was extracted from 0.25g of fecal sample or negative controls using the MoBio Power Soil or PowerMag Soil DNA isolation kit (MoBio Laboratories, Inc) according to the manufacturer instruction for the Eppendorf epMotion 5076 Vac liquid handling robot or manually. DNA extracts were quantified using a Syngery HT (Biotek) with the Quant-iT PicoGreen kit (Invitrogen, ThermoFisher Scientific). Partial bacterial 16S rRNA genes (V4) were amplified using 30 ng extracted DNA as template. The V4 region was amplified using 515F and 806R with Illumina adapters and golay indices on the 3’ end (Caporaso et al., 2010). Samples were amplified in triplicate using Phusion High-Fidelity PCR master mix (New England BioLabs) with the addition of 10 µg BSA (New England BioLabs). The PCR reaction was incubated at 95°C for 3.5 minutes, the 30 cycles of 30 s at 95.0°C, 30 s at 50.0°C and 90 s at 72.0°C, followed by final extension as 72.0°C for 10 minutes. PCR products were quantified and visualized using the QIAxcel DNA Fast Analysis (Qiagen). PCR products were normalized based on the concentration of DNA in the 250–400 bp region and pooled using the QIAgility liquid handling robot (Qiagen). Pooled PCR products were cleaned using the Gene Read Size Selection kit (Qiagen) according to the manufacturer’s protocol. The cleaned pool was sequenced on the MiSeq using v2 2 × 250 base pair kit (Illumina, Inc).

The sequences were demultiplexed requiring 0 mismatches in the index sequences and Q25 minimum, merged using SeqPrep and filtered for length (maximum 300bp) using a custom script (https://github.com/mcnelsonphd/16S-RDS/blob/master/Qiime_Process) (Nelson, Morrison, Benjamino, Grim, & Graf, 2014). Using QIIME (Quantitative Insights Into Microbial Ecology) software, operational taxonomic units (OTUs) were determined by clustering reads to the Greengenes reference 16S reference dataset (2013–08 release) at a 97% identity, and then performing *de novo* OTU clustering on reads that failed to cluster to a reference (Caporaso et al., 2010; Nelson et al., 2014). The dataset was filtered to remove singleton and doubleton OTUs and then OTUs present at less than 0.0005% (Bokulich et al., 2013; Nelson et al., 2014). Potential chimeric sequences were removed with ChimeraSlayer (http://microbiomeutil.sourceforge.net/#A_CS). The data was rarified to 10,000 reads per sample. The validity of this approach was determined using a mock community (Nelson et al., 2014).

Statistical Analysis

Clinical data, as well as OTU tables and the Gini–Simpson α -diversity index that were calculated from QIIME process were imported to R 3.3.1 and SAS version 9.4 (Cary, NC) for statistical analysis. Exploratory data analyses including scatter plot, and taxonomy graph techniques were conducted to display the composition of organisms in the preterm infants' gut-microbiome community, the α -diversity (Gini–Simpson diversity index), and β -diversity. To further examine the effect of feeding types, demographic and clinical characteristics on gut microbiome, generalized linear mixed models (GLMM) were used to analyze the association between these variables with Gini–Simpson α -diversity index. β -diversity was analyzed using both Bray–Curtis and Jaccard in Vegan package (<https://cran.r-project.org/web/packages/vegan/index.html>) and visualized using multidimensional scaling (MDS). To further examine the effect of demographic and clinical characteristics and feeding on β -diversity of gut microbiome, the permutational multivariate analysis of variance using distance matrices (PERMANOVA) were conducted.

Results

Participants

A total of 38 preterm infants were invited to participate and 33 preterm infants were enrolled in the study and completed without attrition. The majority of the infants were female (51.5%), white (78.8%), non-Hispanic (66.7%), and delivered by cesarean-section (60.6%). Average gestation age at birth was 31.1 weeks ($SD = 1.8$); average birth weight was 1444 g ($SD = 442.7$) (Table 1).

Feeding Types

Enteral feedings were introduced between postnatal Day 1 to Day 6 ($M = 2.3$; $SD = 1.2$), depending on infant condition and tolerance to enteral nutrition. During the study period, 63.5% of the total feedings among the 33 infants used MOM and the days of using MOM for each infant varied from 0 to 28 days ($M = 17.7$, $SD = 7.9$). Nothing by mouth (NPO) status during the first 30 days of life ranged from 0 to 5 days ($M = 1.4$; $SD = 1.3$). As described in the Methods, infants were classified into six major feeding types during the

first, second and third 10-day intervals during the investigational period. Table 2 presents numbers and percentages of infants in each of feeding type groupings in the three 10-day intervals.

Evaluation of infant feedings included information regarding exclusive, mixed major feeding types and formula composition types. Among the infants who were fed formula during the study period, standard formula types were used in 45.4% of the cases, followed by elemental formula types used in 32.1% of the cases. Given the standard NICU practice of adding fortification to breastmilk (MOM or HDM), bovine-based human milk fortifier was added to the feedings of 24 (72.2%) infants in the second 10 days while 19 infants received the same additive during the third 10 days of the investigational period and the contribution of this additive was considered in all analyses.

Gut Microbial Patterns with Feeding Types

A total of 419 stool samples were collected from 33 preterm infants during their first 30 days of life. The average number of stool collections for each infant was 12.7 ($SD = 5.1$; range: 2–21). The mean time interval of sample collection was 1.5 days ($SD = 1.0$; $Mdn = 1$). Thirteen out of the 419 samples including nine meconium stools yielded DNA concentration less than 1 ng/ml and were therefore excluded from the sequencing. In total, 25.4 million high quality and chimera free reads were produced. Sixteen samples yielded less than 10,000 reads and were excluded for statistical analysis. One sample with a community that was significantly different than the rest of the samples were removed from the analysis as an outlier. A total of 389 samples were included in the data analysis.

To evaluate the association of infant feeding type with microbiome development, microbial taxonomic order-level quantifications (% composition) obtained from infant stool samples were compared across the six feeding groups, as well as at 10-day intervals within each group (Figure 1). Among the 6 groups, the MOM group had the highest abundance of *Clostridiales*, *Lactobacillales*, and *Bacillales* and the lowest abundance of *Enterobacteriales*. The groups of MOM+HDM as well as MOM+Formula also had higher abundance of *Clostridiales*, *Lactobacillales*, *Bacillales* and *Bacteroidales* compared to the groups of HDM, formula and HDM+Formula. Stool samples of the infants in the HDM, formula and HDM+formula groups had a high abundance of *Enterobacteriales* at all time points. Additionally, abundance variation boxplots of *Lactobacillales* (Supplemental Digital Content 1, see Figure A) and *Bifidobacteriales* (Supplemental Digital Content 1, see Figure B) showed that the MOM feeding group had the highest abundance of *Lactobacillales*, while the MOM+Formula group had the highest abundance of *Bifidobacteriales* during the first 30 days of life.

Examples of gut microbial developmental patterns of individual infants with different feeding types are shown in Figure 2. For instance, infant A fed MOM and infant C were fed HDM constantly over the 30-day investigational period. Infant B fed MOM+HDM during the first 10-day interval, then the feeding type grouping shifted to MOM+HDM+Formula in the second 10-days, and then to HDM+Formula in the third 10-days of life. Infant D was fed HDM in the first 10-days and then grouped as HDM+Formula at second, and third 10 days.

Gut Microbial α - and β -Diversity with Feeding Types

Among the six feeding type groups, α -diversity of gut microbial community was constantly higher in the MOM group compared to other groups over the three 10-day intervals, while the HDM, formula and HDM+Formula groups had lower α -diversity (Figure 3). To determine the contributing factors to the microbial α diversity, a general linear mixed effects model was conducted and the arcsine transformation was applied to Gini-Simpson diversity to obtain the normal distribution. The analysis of the mixed effects model showed that higher α -diversity of the microbial community was associated with older day of life ($p < .001$), fed MOM ($p < .01$) and gender of female ($p < .05$). Factors including days of antibiotics use, PROM, daily weight, human milk fortifier and birth gestational age did not significantly contribute to the α -diversity of microbial profiles (Table 3). Multicollinearity among the factors was evaluated using variance inflation factors (VIF) and results of all the factors were less than 2.3.

Figure 4 shows β -diversity using nonmetric multidimensional scaling (NMDS) of the samples based on Bray-Curtis dissimilarity of the OTUs. This demonstrates the similarity or dissimilarity of the communities within each feeding grouping. The effect of the demographic factors including feeding type on community structure (β -diversity) were evaluated using PERMANOVA by controlling for subject effects. Feeding type explained the greatest variance in the community structure of the factors tested (11%; $p < .001$). Gender was the next most influential factor (6%; $p < .001$). The interaction between gender and feeding explained an additional 8% ($p < .05$) of the variation in the communities even after the effect of the single factors were removed. Infant gestational age and postnatal age, antibiotic use, and PROM also significantly explained 2–3% ($p < .001$) of the variability each with very little variance explained by their interaction terms.

Discussion

This investigation provides a basic understanding of the patterns and diversity of the intestinal microbial community in preterm neonates based upon feeding type. Microbial diversity is significant as nutritional exposures in the early postnatal period can induce permanent alterations in microbiome and these changes can impose later disease risk (Collado et al., 2015). Detecting a greater α -diversity in infants being fed MOM is consistent with our hypothesized associations. In considering specific microbes, the infants receiving MOM had a higher abundance of *Clostridiales*, *Lactobacillales*, and *Bacillales* compared to both the HDM and formula groups (Figure 1). Furthermore, compared to infants who received no MOM, *Clostridiales*, *Lactobacillales*, and *Bacillales* contributed to greater microbial diversity in infants fed alternate milks (HDM or formula) with a small quantity of MOM combined with the feeding (Figure 1). Hence, a small quantity of MOM was associated with a more favorable microbial community. Additionally, we report higher abundance of potential pathogenic *Enterobacteriales* at all time points in the infants fed HDM and formula compared to the MOM. We have expanded our previous findings (Cong, Xu, Janton, et al., 2016) by reporting effects of MOM, HDM, formula and mixed feeding types on the development of gut microbial community. These findings were anticipated as fresh MOM contains a complex combination of immunological, nutritional and

antimicrobial properties (Civardi et al., 2013). Different bacteria genera have been reported in breast milk of mothers given birth to full-term infants, including *Bifidobacterium*, *Lactobacillus*, *Staphylococcus*, *Bacteroides*, and *Clostridium*, and these microbes are present both in the MOM and the infant's stool, indicating a vertical microbial transmission (Bezirtoglou, Tsiotsias, & Welling, 2011; Cabrera-Rubio et al., 2012; Collado, Delgado, Maldonado, & Rodríguez, 2009; Jost, Lacroix, Braegger, & Chassard, 2012; Khodayar-Pardo et al., 2014; Moles et al., 2015; Solís, de Los Reyes-Gavilan, Fernández, Margolles, & Gueimonde, 2010).

The MOM also contains oligosaccharides, the abundant component in human milk, function as a prebiotic that promotes the growth and activity of beneficial gut microorganisms including *Bifidobacterium spp.* and *Lactobacillus* (Coppa, Zampini, Galeazzi, & Gabrielli, 2006). The human milk oligosaccharides interfere with the adhesion of potentially microbial pathogens like *Escherichia coli*, *Helicobacter jejuni*, *Shigella*, *Vibrio cholerae*, and *Salmonella* species to the neonate's intestinal epithelial surface thus reducing the neonate's risk of infections. Knowledge about MOM from mothers given preterm birth is still limited. Their breast milk may have higher protein, fat, carbohydrate and immunological compositions, but less diverse and mature human milk oligosaccharides compared to term milk (Bauer & Gerss, 2011; Castellote et al., 2011; De Leoz et al., 2012; Moltó-Puigmartí, Castellote, Carbonell-Estrany, & López-Sabater, 2011). Thus, the "premature" MOM may affect the preterm infant's gut through different mechanisms (Gabrielli et al., 2011; Underwood et al., 2015). In comparison to the gut microbial composition of full-term breast-fed infants who have the *Bifidobacteria* as one of the most predominant intestinal microorganisms (Arbolea et al., 2013), our study reports that the MOM feeding group had the highest abundance of *Lactobacillales* (Supplemental Digital Content 1, see Figure A), while the MOM+Formula group had the highest abundance of *Bifidobacteriales* (Supplemental Digital Content 1, see Figure B) during early life. Our finding related to *Bifidobacteria* in preterm infants fed MOM diet necessitates further investigation.

In HDM, the human milk oligosaccharides may not be significantly affected by the pasteurization process, however, because of the lack of live bacteria in HDM, this processing may result in loss of the early interaction of the gut microbiome and oligosaccharides, which may be beneficial in early life (Neu, 2015). Potential impact of HDM on bioactive components in association with microbiome development would contribute to the existing literature and warrants further research (Li, Hosseinian, Tsopmo, Friel, & Beta, 2009; Montjaux-Régis et al., 2011).

Furthermore, fatty acids and monoglycerides rendered from the hydrolysis of milk triglycerides also have antimicrobial properties that support the infant's innate immunity against *Giardia lamblia*, *H. influenzae*, group B *streptococci*, *Staphylococcus epidermidis*, respiratory syncytial virus, and herpes simplex virus type 1 (Hamosh, 1998; Pacheco et al., 2015). Bifidus factor and other bioactive components, prevent the growth of pathogenic microorganisms indirectly by promoting the growth of probiotic bacteria like *Lactobacillus bifidus* (Koenig, de Albuquerque Diniz, Barbosa, & Vaz, 2005; Landers & Hartmann, 2013; McPherson & Wagner, 2001; Silvestre, Ruiz, Martínez-Costa, Plaza, & López, 2008; Untalan, Keeney, Palkowetz, Rivera, & Goldman, 2009).

Antibiotics are used routinely with premature infants and markedly influence gut bacterial colonization with implications for microbiome development during this vulnerable period when colonization of the naïve gut is occurring for the first time. With limited number and types of bacteria initially, then compounded by the elimination or reduction of one or a few species through antibiotic use has the potential to influence colonization. In this investigation antibiotic type, dose and duration of use was similar across our cohort. Thus, our findings provide evidence of real-time microbiome development of hospitalized preterm infants receiving routine antibiotic therapy and are not generalizable to healthy term infants. Given our statistical control and routine use of antibiotics across the cohort, variations in microbial composition we report are not attributable to variations in antibiotic therapy across feeding groups.

Feeding type changes and modular additives are routine for preterm infants in the neonatal intensive care setting based upon progress and protocols aimed to promote optimal growth. From this perspective, we grouped infants according to categories of total feedings consumed thus limiting our ability to account for the influence of feeding change on microbiome development at different timepoints. The only way to discern different feeding types is by comparing clean groups. However, in real life, depending on the availability of MOM, HDM, and preterm infant formula, this can be controlled only to a limited extent by the caregiver.

Our finding of significantly lower α -diversity and a differential microbial community in the stool samples of infants receiving HDM compared to infants receiving MOM or formula raises questions about the surrounding processing practices of HDM. We attribute our finding of lower infant microbiome α -diversity in HDM fed infants to the current regulatory standards for processing HDM prior to use. All milk donors of the Human Milk Banking Association of North America (HMBANA) are screened for potential pathogenicity or toxicity. In addition to the initial screening, HDM is pasteurized to eradicate potential viral or bacterial pathogens (Reeves, Johnson, Vasquez, Maheshwari, & Blanco, 2013). The pasteurization process alters composition of the human donor milk. Although HDM is a preferable alternative to cow's milk based infant formulas, many immunological, nutritional, and microbial factors are lost during pasteurization (Landers & Hartmann, 2013; Li et al., 2009; Reeves et al., 2013). Multiple research groups have reported that pasteurization reduces the innate immunological properties of breast milk. More specifically, lactoferrin and key inflammatory cytokines are reduced which reduces the immune benefits attributed to human milk feeding (McPherson & Wagner, 2001). Concentrations of IgA, IgG, IgM, total protein, lysosomes, glutathione, and interleukin-10 are all reduced in pasteurized human milk (Bertino et al., 2013; McPherson & Wagner, 2001; Untalan et al., 2009). Pasteurization also destroys the B- and T-cell components of milk (Tully, Jones, & Tully, 2001). Detecting a higher abundance of potential pathogenic *Enterobacteriales* at all time points in the infants fed HDM compared to the MOM and formula fed groups suggests an impact of pasteurization on the immunologic quality and ultimate composition of the fecal microbiome. Unlike raw MOM, HDM is considered a partial sterile product after pasteurization, impacting the presence of good bacteria present in raw breast milk, such as *Bifidobacterium breve* and several *Clostridium* species (Jost, Lacroix, Braegger, Rochat, & Chassard, 2014). Our finding of a higher abundance of *Clostridiales*, *Lactobacillales*,

Bacillales and *Pasteurellales* taxa in the MOM cohort is a pattern reported by Jost and colleagues in a cohort evaluating vertical transmission fed MOM (Jost et al., 2014). Ultimately, understanding the developmental process of the neonatal gut microbiome is important in learning the role of the gut microbiome in preterm neonatal health outcomes.

Both inter- and intra-participant variability within naturally occurring microbial communities is anticipated and our results show the same pattern. However, given our large sampling scheme, we were able to detect significant drivers of community structure in our demographic factors. Feeding type, gender, and the interaction of feeding type and gender explains a substantial 34% of the variability of the communities. The strength of the interaction effect of gender and feeding type as well as other factors is interesting and could be a fruitful path of future inquiry (Cong, Xu, Janton, et al., 2016).

Although it is still relatively unclear how different neonatal feeding types influence the gut microbiome over time, this investigation made it evident that there are significant differences in gut microbial composition of preterm neonates based upon feeding type. While it is well known that raw MOM is the most optimal nutritional source for preterm neonates, further evidence is needed regarding dose-effect and timing relationships for combined feeding of MOM with HDM or formula for preterm neonatal feeding when raw MOM supplies are insufficient. Our evidence supports need for increased lactation support for mothers of hospitalized preterm infants for sustained breastmilk production during hospitalization. Although HDM is a preferable alternative when MOM is not available, further work is necessary in processing safe human milk for donation while maintaining compositional integrity toward fostering more diverse microbiome development (Peila et al., 2016). Given our finding of higher potentially pathogenic microbiota in infants fed non MOM, further research is also necessary in identifying methods preventing colonization due to pathogenic bacteria before severe infections and neonatal compromise occur. Knowledge on how preterm neonatal feeding contributes to intestinal microbial composition may ultimately influence the development of standard preterm neonatal feeding practices in neonatal intensive units nationally to subsequently optimize short- and long- term neonatal health outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Distribution of mean relative abundance of taxa among six feeding types and temporal development of taxa over three 10-day intervals across the first 30 days of life. Each of the above stacked bar plots illustrates the average relative abundance (y -axis) of the most abundant gut microbiota at the order level. The x -axis shows six feeding types over first, second and third 10-days intervals. HDM = human donor milk; MOM = mother's own milk.

FIGURE 2.

Four examples of daily gut microbiome development from individual infants with different feeding types. Infant A was fed MOM; *Clostridiales* and *Lactobacillales* dominated. Infant B was fed MOM+HDM on days 0–10; then, feeding type shifted to MOM+HDM+Formula for days 11–20, and to HDM+Formula for days 21–30, with increased *Enterobacteriales* and decreased *Clostridiales*. Infant C was fed HDM; *Enterobacteriales* dominated. Infant D was fed HDM during days 0–10; then then shifted to HDM+Formula for days 11–20 and days 21–30; *Enterobacteriales* dominated. HDM = human donor milk. MOM = mother's own milk.

FIGURE 3.

Gini-Simpson α -diversity index over three 10-days intervals and among six feeding types.

HDM = human donor milk; MOM = mother's own milk feeding.

FIGURE 4.

Nonmetric multidimensional scaling (NMDS) of the samples based on Bray-Curtis dissimilarity of the operational taxonomic units (OTUs). All samples belonging to the same feeding group have the same color and shape combination. This demonstrates the similarity of the communities within each feeding cohort. HDM = human donor milk; MOM = mother's own milk.

TABLE 1**Infant Characteristics**

Characteristic	<i>M</i>	<i>(SD)</i>	Range
Gestational age (weeks)	31.1	(1.8)	28.1–33.4
Birth weight (g)	1444.2	(442.7)	703–2640
Birth length (cm)	39.8	(4.2)	32.5–50.5
Birth head circumference (cm)	28.0	(2.4)	24.0–34.5
SNAPPEII	9.2	(11.0)	0–31
Mother age (years)	28.8	(7.1)	15–37
	<i>n</i>	<i>(%)</i>	
Gender (male)	16	(48.5)	
Race			
White	26	(78.8)	
African American	5	(15.2)	
Asian	1	(3.0)	
Not known	1	(3.0)	
Ethnicity (Hispanic)	11	(33.3)	
Delivery type (vaginal)	13	(39.4)	
Premature rupture of membranes (yes)	15	(45.5)	
Singleton (yes)	19	(57.6)	
Resuscitation at birth (yes)	21	(63.6)	
Antibiotic use first 48–72 hours (yes)	32	(97.0)	

Note. *N* = 33. SNAPPEII = Score for Neonatal Acute Physiology–Perinatal Extension-II.

TABLE 2

Feeding Types Over 30 Days

Type	Days 0–10		Days 11–20		Days 21–30	
	n	(%)	n	(%)	n	(%)
MOM	13	(39.4)	15	(45.6)	13	(39.4)
MOM+HDM	12	(36.4)	6	(18.2)	3	(9.1)
MOM+Formula	6	(18.2)	8	(24.2)	9	(27.3)
HDM	2	(6.1)	2	(6.1)	3	(9.1)
Formula	0		1	(3.0)	3	(9.1)
HDM+Formula	0		1	(3.0)	2	(6.1)

Note. N = 33. HDM = human donor milk; MOM = Mother's own milk. This table represents the percentages of infants being fed with either MOM, HDM or formula over the first 30 days after birth at each of the intervals. A feeding type was attributed to an infant when he/she received that particular feeding type for more than 70% of the time in a 10 days period.

TABLE 3Mixed Effects Model: Factors Contributing to Gini-Simpson Diversity Index (α -Diversity)^a

Effect	Estimate	(SE)	p
Time interval			
0–10 days	–0.24	(0.07)	<.001
11–20 days	–0.14	(0.03)	<.0001
21–30 days ^b			
Covariates			
MOM	0.23	(0.07)	<.001
Gender (female)	0.14	(0.05)	.01
Antibiotic use (days)	–0.13	(0.07)	.07
PROM	0.05	(0.05)	.38
Weight (g)	0.00	(0.00)	.63
HMF	0.00	(0.06)	.98
Birth GA (weeks)	–0.01	(0.02)	.76

Note. *N* = 33. GA = gestational age; HMF = human milk fortifier; MOM = mother's own breastmilk feeding; PROM = premature rupture of membranes.

^a Arcsine transformed.

^b Referent category.