Abstract

Background: Premature infants have a great risk for dysbiosis of the gut microbiome. Mother’s own breastmilk (MOM) has been found to increase the diversity of gut microbiota in term infant, however, the influence of feeding type on gut microbial colonization of preterm infants is still unknown.

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. Feeding types and 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. Predictions for gut microbial diversity was estimates using linear mixed models.

Results: Preterm infants fed MOM at least 70% total diet had highest abundance of Clostridiales, Lactobacillales, and Bacillales compared to infants in other feeding groups, while 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 microbiota increased over time and was constantly higher in infants fed MOM relative to infants with other feeding types (p<0.01).

Discussion: Mother’s 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
Introduction

The maintenance of human health and well-being relies on the interrelationship with a diverse gastrointestinal microbiota. Previous research demonstrates that the gastrointestinal microbiota is necessary for proper intestinal cell proliferation and migration, mucosal barrier protection, growth of blood vessels, energy harvesting and storage, defense against pathogens, metabolism of food, regulation of blood pressure, and innate immunity (Hooper et al., 2001; Leser & Mølbak, 2009; Moore, Munck, Sommer, & Dantas, 2011; Mshvildadze & Neu, 2010; Pluznick et al., 2013; Turnbaugh et al., 2006). An imbalance of species and community of the gastrointestinal microbiota disrupts human health and leads to disease making microbiome research a priority (Groer et al., 2014; Manco, Putignani, & Bottazzo, 2010; Neu, 2013).

Given the intestinal microbiota influences many aspects of human health, it is necessary to understand what determines its composition and development. Some research suggest that intestinal microbiota colonization begins at birth (Dominguez-Bello et al., 2010; Neu, 2013; Putignani, Del Chierico, Petrucca, Vernocchi, & Dallapiccola, 2014). Many factors early in the perinatal period influence the development of neonatal gut microbiota (Khodayar-Pardo, Mira-Pascual, Collado, & Martinez-Costa, 2014). Medical interventions and exposures as well diet and individual genetic components are all factors influencing the complex process of early inoculation of the gut (Groer et al., 2014; Khodayar-Pardo et al., 2014). Thus, there is a research emphasis on determining these predictors and health implications, especially within the preterm neonate population as preterm neonates are much more vulnerable to health and development complications. Gut perfusion, stress, pharmacological interventions, hemodynamic stability and feeding practices during the perinatal period all potentially influence gut microbiota composition and proliferation (Dominguez-Bello et al., 2010; Khodayar-Pardo et al., 2014; Putignani et al., 2014). Thus, there is a research emphasis on identifying these predictors and health implications,
especially within the preterm neonate population as preterm neonates are much more vulnerable to health and development complications.

The feeding mode has a large effect on the gut microbiome early in development as milk is one of the first external components to enter the preterm neonatal intestinal tract (Cabrera-Rubio et al., 2012; Khodayar-Pardo et al., 2014; Newburg & Morelli, 2015; Putignani et al., 2014; Rodriguez, 2014). Human breast milk is fed to preterm neonates in the form of raw breast milk or pasteurized human donor milk. Recent evidence points to the milk glycobiome as an influential factor in gut microbiome development (Newburg & Morelli, 2015; Pacheco, Barile, Underwood, & Mills, 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. There are significant compositional differences between raw and pasteurized human milk and alteration in immunological, nutritional and bacterial components of human milk can have short term and long term effects on preterm neonatal microbiome development and health (Poroyko et al., 2011). Collectively, reduced immunologic components including immunoglobulins, cytokines and lymphocytes coupled with altered nutritional and microbial composition associated pasteurized human banked milk and other alternate feeding modalities have implications for the microbial community development in preterm infants. The purpose of this study was to explore the effect of different feeding types on gut microbiome development of preterm infants in the neonatal intensive care unit (NICU).
Methods

Study Design and Participants

A prospective exploratory study was conducted in the level IV NICUs of Connecticut Children’s Medical Center, at two sites, Hartford and Farmington, CT. Inclusion criteria were stable preterm infants who were: 1) 28 0/7 – 32 6/7 weeks gestational age, 2) 0 - 7 days old, and 3) mothers were older than 18 years old to provide consent. Exclusion criteria were infants who had: 1) known congenital anomalies, 2) severe periventricular/ intraventricular hemorrhage (≥ Grade III), 3) undergone minor or major surgery procedures, or 4) positive drug exposure history. Parents of infants meeting the criteria and present in the participating NICUs during the study period 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 up over their first 30 days of life in the NICU.

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

Outcome Measures and Data Collection

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 stay was also collected.
Infant daily feeding information was collected by 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 days of life. The feeding cohort types were defined based upon >70% of total frequency of feeding in 10-day intervals (see the Statistical Analysis section).

Infant stool samples were collected by trained bedside nurses on a daily basis, depending upon whether the infant had a stool. 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, extraction and processing method and procedures were used as described previously (Cong et al., 2016). DNA was extracted from 0.25g of fecal sample 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 quantification
and visualization 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 x 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) (M. C. 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; M. C. 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; M. C. Nelson et al.,
2014). The data was rarified to 10,000 reads per sample.

**Statistical Analysis**

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 for every 10-day periods. These groups include MOM,
MOM+HDM, MOM+formula, HDM, Formula, and HDM+formula groups. For instance, an
infant was 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; during the second 10-days period, none of the individual feeding types exceeds 70%, but the mixed feeding of MOM and formula together ≥ 70%, 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.

Clinical data, OTU tables and the Gini–Simpson α-diversity index calculated from QIIME process were imported to R 3.2.0 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 organism in the preterm infants’ gut-microbiome community, the a-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

A total of 33 preterm infants were enrolled in the study from January, 2014 to March 2015. The majority of the infants were female (51.5%), white (78.8%), non-Hispanic (66.7%), and delivered by cesarean-section (60.6%). Infants were born at 31.1 ± 1.8 weeks of gestation and with 1444 ± 442.7 g of birth weight (Table 1).

Feeding Types
Enteral feedings were introduced between postnatal day 1 to day 6 (2.3 ± 1.2 days), depending on the infants’ 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 varies from 0 to 28 days (17.7 ± 7.9 days). Nothing by mouth (NPO) status during the first 30 days of life ranges from 0 to 17.7 ± 7.9 days. As described in the methods, infants were classified into 6 major feeding types during the first, second and third 10-days of life during the investigational period. Table 2 presents numbers and percentages of infants in each of feeding types in the three 10-day intervals.

Feeding composition includes information regarding exclusive or mixed major feeding types as well as formula types. Among the infants who were fed formula during the study period, standards formula types were used in 45.4% of the cases, followed by elemental formula types used in 32.1% of the formula feeding. Given the standard NICU practice of adding fortification to breastmilk, bovine based human milk fortifier was added to the nutrition of 24 (72.2%) infants in the second 10 days while 19 infants received the same additive during the third 10 days of the investigation period.

**Gut Microbial Patterns with Feeding Types**

A total of 424 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 12.9 ± 5.2 with a median frequency of 21 collections per infant and minimum of 2 sample per infant. The mean time interval of sample collection was 1.4 ± 1.0 days with a median of 1. Fourteen out of the 424 samples including 9 meconium stools yielded DNA concentration less than 1 ng/ml and were therefore excluded from the sequencing. In total, 25.5million high quality and chimera free reads
were produced. Eighteen samples yielded less than 10,000 reads and were excluded for statistical analysis.

To evaluate the association of infant feeding type with microbiome development, microbial taxonomic order-level species quantifications (% composition) obtained from infant stool samples were compared across the 6 feeding groups, as well as at 10-day intervals within each group (Fig. 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.

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 fed HDM constantly over 30-days of life. Infant B fed MOM+HDM in the first 10-days and then, feeding type shifted to MOM+HDM+formula in the second 10-days, and to HDM+formula in the third 10-days of life. Infant D fed HDM in the first 10-days and then shifted to HDM+formula at second, and third 10 days. The progression of microbial development seems different among these infants.

**Gut Microbial α and β Diversities with Feeding Types**

Among the 6 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 levels of α diversity (Fig 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 \( \alpha \)-diversity of the microbial community was related to older day of life \( (p<0.001) \), fed MBM \( (p<0.01) \) and gender of female \( (p<0.05) \). Factors including birth gestational age, PROM, and daily weight, days of NPO, human milk fortifier and days of antibiotics use were not significantly contributing to the \( \alpha \)-diversity of microbiota (Table 3). Multicollinearity among the factors was evaluated using Variance inflation factors (VIF) and results of all the factors are less than 2.3.

Figure 4 shows \( \beta \) 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 cohort groups. The effect of the demographic factors including feeding type on community structure, \( \beta \) diversity, was evaluated by PERMANOVA (Table 4). Feed type explains the greatest variance in the community structure of the factors tested, 9%. Gender was the next most influential factor, 6%. The interaction between gender and feeding explains an additional 8% of the variation in the communities even after the effect of the single factors were removed. Day of life, gestational age, and PROM also significantly explained 2 - 3% of the variability each with very little variance explained by their interaction terms.

**Discussion**

This investigation provides a basic understanding of the diversity of the intestinal microbiota in preterm neonates based upon feeding type. This microbial diversity is significant as according to the “early programming” theory, nutritional exposures in the early postnatal period can induce permanent alterations in some biological processes and cause diseases.
(Collado et al., 2015). Thus, it is essential that the preterm neonatal diet be perfected to positively support preterm intestinal development and associated biological processes.

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. Furthermore, compared to infants who received no MOM, Clostridiales, Lactobacillales, and Bacillales contributed to greater microbiota diversity in infants fed alternate milks (HDM or formula) combined with MOM. These findings were anticipated as unpasteurized MOM contains a complex combination of immunological, nutritional and antimicrobial properties (Civardi et al., 2013). In contrast to HDM that has been pasteurized, raw MOM contains many nutritional and immunological components, including immunoglobulins (IgA, IgG, IgM), cytokines and lymphocytes (Li, Hosseinian, Tsopmo, Friel, & Beta, 2009; Montjaux-Regis et al., 2011). MOM also contains oligosaccharides that 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. Additionally, fatty acids and monoglycerides rendered from the hydrolysis of milk triglycerides 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). In addition to lipids, hormones and growth factors found in raw breast milk also play a role in neonatal immunity. Hormones and growth factors help mature the preterm neonates’ gastrointestinal lining making them less susceptible to pathogenic microorganisms. One growth factor found in raw breast milk, bifidus factor, prevents the growth of pathogenic
microorganisms indirectly by promoting the growth of probiotic bacteria like *Lactobacillus bifidus*. Other immunological components found in raw breast milk include lactoferrin, antioxidants, cytokines, B-lymphocytes, and T-lymphocytes (Koenig, de Albuquerque Diniz, Barbosa, & Vaz, 2005; Landers & Hartmann, 2013; McPherson & Wagner, 2001; M. M. Nelson, 2013; Silvestre, Ruiz, Martinez-Costa, Plaza, & Lopez, 2008; Untalan, Keeney, Palkowetz, Rivera, & Goldman, 2009).

Given the profound importance of breastmilk to infant health and microbiome development, the WHO has recommended a standard practice of providing hospitalized preterm infants with human donor milk in situations where mothers own milk is absent or insufficient (World Health Organization, 2011). Our finding of significantly lower α-diversity in the stool samples of infants receiving HDM compared to infants receiving MOM or formula raises questions about the consensus surrounding the benefits of HDM in this investigation. 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. Under FDA and CDC regulations, 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, donor milk 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 donor milk 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; M. M. Nelson, 2013; 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 (McPherson & Wagner, 2001; M. M. Nelson, 2013; Untalan et al., 2009). Pasteurization also destroys the B- and T-cell components of milk. 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 composition of the fecal microbiota. 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 are destroyed (Jost, Lacroix, Braegger, Rochat, & Chassard, 2014). Our finding of a higher abundance of Clostridiales, Lactobacillales, Bacillales and Pasteurellales taxa in the MOM cohort corroborates with the earlier work by Jost and colleagues (Jost et al., 2014). Ultimately, understanding the developmental process of the neonatal gut microbiome is important in learning the role of the gut microbiota in preterm neonatal health.

Variability within naturally occurring microbial communities is often strongest between samples, our results show the same pattern. However, given our large sampling scheme, we were still 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 23% of the variability of the communities. The strength of the interaction effect of gender and feeding type is interesting and could be a fruitful path of future inquiry because it is not clear why this is the case (Cong et al., 2016).
Although it is still relatively unclear how different neonatal feeding types influence the gut microbiome, this investigation made it evident that there are significant differences between the gut microbiota 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 lactogenesis 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 microbiota development (Tully, Jones, & Tully, 2001). Given our finding of higher potentially pathogenic microbiota in infants fed non MOM, further research is also necessary in identifying methods to inhibit proliferation of potentially pathogenic gut microbiota before severe infections and neonatal compromise occur. Knowledge on how preterm neonatal feeding contributes to intestinal microbiota composition will ultimately influence the development of standard preterm neonatal feeding practices in neonatal intensive units nationally and will promote the most optimal short and long term neonatal health outcomes.
References


Figure Captions:

**Figure 1.** Distribution of mean relative abundance of taxa among 6 feeding types and temporal (over three 10-days intervals) development of taxa during 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. X-axis is 6 feeding types over first, second and third 10-days intervals. MOM=mother’s own milk feeding; HDM=Human donor milk.

**Figure 2.** Examples of daily gut microbiome development from individual infants with different feeding types. Infant A fed MOM with Clostridiales and Lactobacillales dominated; Infant C fed HDM with Enterobacteriales dominated; Infant B fed MOM+HDM in the first 10-days and then, feeding type shifted to MOM+HDM+formula in the second 10-days, and to HDM+formula in the third 10-days of life; Infant D fed HDM in the first 10-days and then shifted to HDM+formula at second, and third 10 days.

**Figure 3.** Gini-Simpson α-diversity index over three 10-days intervals and among six feeding types. MOM=mother’s own milk feeding; HDM=Human donor milk.

**Figure 4.** Nonmetric multidimensional scaling (NMDS) of the samples based on Bray-Curtis dissimilarity of the 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.