OF MILK AND MICROBES: THE INTERPLAY OF MILK CELLS, MACRONUTRIENTS, MATERNAL DIET, AND VARIOUS MICROBIOMES OF THE MOTHER-INFANT DYAD

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by

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AUTHORIZATION TO SUBMIT DISSERTATION

This dissertation of Janet Elizabeth Williams, submitted for the degree of Doctor of Philosophy with a Major in Bioinformatics and Computational Biology and titled "OF MILK AND MICROBES: THE INTERPLAY OF MILK CELLS, MACRONUTRIENTS, MATERNAL DIET, AND VARIOUS MICROBIOMES OF THE MOTHER-INFANT DYAD," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

Human milk is generally considered to be the gold standard of nutrition for healthy human infants. It provides all of the essential nutrients and is rich in non-nutrients (e.g. indigestible carbohydrates), immune cells, and bacteria. To better understand these milk components, we conducted experiments examining the relationships and dynamics of milk-borne host cells, milk macronutrients, maternal diet, and the various microbiomes of the mother-infant dyad.

In Chapter 1, relationships among macronutrients, host cells, and bacterial communities in milk produced by 16 women over a 5-wk period are characterized. A wide variation of host cell types such as neutrophils, macrophages/secretory mammary epithelial cells, eosinophils, and lymphocytes was found. Distribution of these cell types varied greatly among women, but was relatively consistent over the sampling period within individual women. Myriad relationships existed between host cell profiles and the microbial community structure as well as relationships among several human milk oligosaccharides and the host cellular content.

In Chapter 2, the microbiome of milk produced by 21 healthy lactating women during the first 6 months postpartum is described, as well as associations between milk bacteria and other mediating factors such as maternal nutrient intake, delivery mode, and adiposity. Similar to the host cellular content, the microbial community structure of milk was variable among women but relatively constant over time within individual women. Relative abundances of several bacteria were associated with maternal adiposity, delivery mode, infant sex, and maternal diet.

In Chapter 3, relationships among microbial communities of milk, oral, and fecal samples from healthy lactating women, and oral and fecal samples from their infants over the first 6 months postpartum were explored. Microbial communities from each sample type were relatively unique. However, some similarities existed. For example, milk bacterial communities appeared to bridge the infant fecal and infant oral bacterial communities. Over time, however, milk bacterial communities became

more similar to the infant and maternal oral bacterial communities than that in infant feces.

In conclusion, these studies demonstrate that regulation of the components of human milk is likely a highly complex process, being individualized to a particular woman.

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DEDICATION

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INTRODUCTION

1.1 INTRODUCTION

Breastfeeding reduces the risk of diarrheal and respiratory infections (Horta and Victoria 2013), and this protection is conferred to the breastfed infant in both developed and developing countries. This is important as pneumonia and diarrheal disease together are responsible for nearly 30% of all of the deaths in children under 5 years of age (Liu et al. 2012). Indeed, each year, diarrhea is estimated to cause nearly 800,000 deaths globally in children under five (Horta and Victoria 2013; Liu et al. 2012). Finding ways to reduce these numbers is a priority worldwide and thus, gaining a better understanding of the mechanisms whereby breastfeeding reduces the risk of these infectious diseases is key.

The feeding of human milk to very low birth weight and preterm infants also reduces the incidence of necrotizing enterocolitis (Schanler et al. 1999; Herrmann and Carroll, 2014). Necrotizing enterocolitis is a devastating disease that afflicts up to 19% of VLBW infants and also can result in up 20-30% mortality in these infants (Torrazza et al. 2013). The disease is marked by severe inflammation of the intestinal tissue, which often becomes necrotic and deteriorates. Although it is still unclear as to the etiology of this disease, a dysbiosis of the microbial community in the infant's gastrointestinal (GI) tract is associated with development of this disease (Torrazza and Neu 2013). Since human milk is also known to influence the composition of the infant's GI bacterial community (O'Sullivan et al. 2015), understanding the interactions of milk macronutrients, milk host cells, and the milk bacterial community as well as the relationships between the milk and infant fecal bacterial communities may provide much needed insight into how human milk reduces the incidence of necrotizing enterocolitis.

Breastfeeding may also have long-term benefits for both the child and the mother (Chowdhury et al 2015; Horta et al. 2015). Studies have shown that infants that are exclusively fed human milk for up to 6 months are less likely to be obese at

age four. This trend continues even as these children reach their teen years. Additionally, increased total lifetime duration of breastfeeding is associated with reduced incidence of type 2 diabetes in mothers and any duration of breastfeeding is associated with a decreased risk of developing breast and ovarian cancer for the mother (Chowdhury et al. 2015).

This has led us to ask the question “What are the factors in human milk and/or the mammary gland that play a role in providing this protection?” Human milk is a complex fluid comprised of carbohydrates, lipids, proteins, and living cells (Jensen 1995). These living cells include immune cells and mammary secretory epithelial cells from the mother and also bacterial cells. And although it’s been long known that human milk contains bacteria, the bacteria were thought of as “contaminant” since bacterial growth was usually not observed during culture (Chiene and Ewart 1878). However, through improvement of culture methods, development of molecular methods, and the advent and use of methods such as high-throughput sequencing of the 16S rRNA gene, there is considerable evidence that healthy human milk contains a diverse community of bacteria (Martín et al. 2004; Hunt et al. 2011; Jost et al. 2013). But this has given rise to more questions. Where do the bacteria in milk originate? How does the bacterial community in milk change over time? What are the factors that affect the composition and membership of the milk bacterial community? And what, if any, are the functions of the milk microbiota and their relationships with the bacterial communities of the infant? The research provided in this dissertation attempts to provide insight to some of these questions by exploring the relationships among milk host cells, milk macronutrients, and the milk microbiome. Additionally, relationships among the various microbiomes of the mother-infant dyad are explored.

Characterizing the dynamics of the bacterial communities of the mother-infant dyad and identifying relationships among their microbial communities has significant potential to better infant and maternal health. By understanding the complexities of human milk and the role(s) the milk microbiota play, novel approaches can be developed to treat or reduce the incidence of diseases worldwide.

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CHAPTER 2

RELATIONSHIPS AMONG MICROBIAL COMMUNITIES, HOST CELL, OLIGOSACCHARIDE PROFILES, AND MACRONUTRIENT CONCENTRATIONS IN HUMAN MILK

2.1 ABSTRACT

Background. Human milk is generally considered to be the gold standard of nutrition for the healthy human infant, in that it provides all of the essential nutrients necessary for life. Human milk is also rich in non-nutrients (such as indigestible carbohydrates), immune cells, and bacteria. However, almost nothing is known about the interplay among these likely-related milk components. **Objectives.** The primary objective of this research was to characterize the relationships (if any) among milk macronutrients, milk-borne host cells, and milk-borne bacterial communities. **Methods.** Milk samples were collected 5 times each via complete breast expression from 16 healthy lactating women living in the inland northwest US. Milk protein, lipid, fatty acid, lactose, and human milk oligosaccharide (HMO) concentrations were analyzed using standard methods. Host cell concentrations and relative abundances were determined using microscopy. Milk microbial communities were determined using next-generation, culture-independent methods. **Results.** Host cell composition of milk varied greatly among women, but was relatively consistent over the sampling periods within individual women. Analyses suggested myriad relationships between host cell profiles and microbial community structure. For instance, relative abundances of *Staphylococcus* and *Corynebacterium* were associated with neutrophil concentration ($r = 0.27$, $P = 0.0128$ and $r = 0.24$, $P = 0.0356$); and concentration of Gram-positive bacteria in milk was positively associated with neutrophil concentration ($r = 0.35$, $P < 0.0016$). Concentrations of several HMO were also correlated with the host cellular content of milk. For instance, LNT and LNnT were negatively associated with somatic cell count ($r = -0.64$, $P = 0.0082$; $r = -0.52$, $P = 0.0387$, respectively). **Conclusion.** These data are the first, to our knowledge, to suggest that host- and environment-derived components in human milk (e.g.,

immune cells and nutrients) may be related to bacterial communities, thereof. **Key words:** human, lactation, milk, immune cells, human milk oligosaccharide, HMO, microbiome

2.2 INTRODUCTION

Researchers have long known that human milk contains myriad cells presumably involved in protecting both the mammary gland and recipient infant from infection. First described by Donné (1837), researchers have generally believed that the majority of cells in human milk are leukocytes (Smith and Goldman, 1968; Ho et al., 1979; Brooker, 1980). However, recent reports by Hartmann and colleagues (e.g., Cregan et al., 2007; Hassiotou et al., 2012) and others (Patki et al., 2010; Trend et al., 2015) suggest that the majority of cells found in milk produced by healthy women may actually be of mesenchymal origin, providing a rich source of multipotent stem cells to both mammary tissues and the recipient infant. Hassiotou et al. (2013b) have also published convincing evidence that both maternal and infant infection can stimulate a rapid increase in leukocyte abundance in milk. This finding combined with decades of research showing increased milk somatic cell count (SCC) is associated with mammary infection in both dairy cows and humans (Dufour et al., 2011; Kvist 2010) support a dynamic and responsive relationship among maternal health, infant health, and distribution/concentration of milk-borne cells. However, aside from maternal and infant infection, very little is known about factors related to variation in cell distribution found in mature human milk.

Cells of human origin, however, do not constitute all of the living cells found in human milk. Indeed, growing evidence from our laboratory and others (e.g., Hunt et al., 2011; Fernández et al., 2013; Jeurink et al., 2013; Jost et al., 2013a; Jost et al., 2013b) provides strong support for the paradigm-shifting concept that human milk is a probiotic fluid supplying a rich and diverse community of bacteria to the recipient infant (McGuire and McGuire, 2015). This paradigm shift has been spurred in large part by technological advances allowing the identification and quantification of difficult-to-culture bacteria via genomic sequencing rather than the use of more

biased and limited culture-dependent methods. In general, available literature suggests a high level of variation in microbial community structure among women and populations but a relatively consistent distribution of bacterial taxa within an individual woman (Hunt et al., 2011). However, almost nothing is known about factors related to variation in milk microbial community structure. It is likely, though, that the distribution of innate immune cells present in the milk and mammary gland may be associated with the relative abundance and presence of bacterial taxa.

We posit that a complex bidirectional relationship exists between immune cells and the bacteria in the healthy mammary gland. In support of this overarching hypothesis, several studies have shown that complex interactions exist between host immune cells and microbiota in the gastrointestinal (GI) tract and are necessary to maintain homeostasis in that physiological and anatomical niche (reviewed by Ivanov and Littman, 2011; Kabat et al., 2014; Walker and Iyengar, 2015). Although the innate immune system's response to bacteria in the mammary gland of the dairy cow has been examined (reviewed by Sordillo and Streicher, 2002; Rainard and Riollet, 2006; Alnakip et al., 2014), most of the work has focused on the interactions as they relate to mastitis. Almost nothing is known about interactions between microbiota in "healthy" milk and the mammary milieu in this regard. Additionally, only one recent study has described relationships between milk microbiota and total SCC in women (Boix-Amorós et al., 2016).

Interactions between host and bacterial cells in milk may also be influenced by the presence and/or concentration of various human milk oligosaccharides (HMO). HMO could influence both the bacterial community structure and the immune cell populations of milk by favoring the growth of specific bacteria (Hunt et al., 2012; Garrido et al., 2013), acting as "decoy" ligands to decrease attachment of certain bacteria (Morrow et al., 2005), and potentially changing the immune cell response by altering platelet-neutrophil-mediated inflammation (Bode et al., 2004a, Bode et al., 2004b, Bode, 2012). To our knowledge, however, the potential interactions among host immune cells, complex bacterial communities, and HMO in the mammary gland have not been reported.

Understanding the biological and environmental factors related to the cellular composition in the milk of healthy women is likely an important piece of the complex puzzle by which lactation and breastfeeding influence both maternal and infant health. In particular, to our knowledge, global relationships among human milk cell content and distribution, human milk microbial profiles, and milk macronutrient composition – especially HMO profiles – have not been investigated. As such, the main objective of this exploratory, hypothesis-generating study was to relate variation in milk immune cell populations to variations in milk microbial community membership and HMO profiles. In addition, we explored potential relationships with selected other milk components, such as fatty acids.

2.3 METHODS

2.3.1 *Subjects*

Breastfeeding women (*n* = 16) were recruited from Moscow, ID, Pullman, WA, and the surrounding area. To be eligible for participation, women had to be self-reported healthy, between the ages of 20-40 y, and breastfeeding or expressing milk at least 5 times each day. The Washington State University Institutional Review Board and the University of Idaho Human Assurances Committee approved all procedures, and written, informed consent was obtained from all participants. Information concerning general health and demographics of the mothers was collected during the week prior to the first sampling day. Mothers were weighed immediately prior to the first milk collection (Seca® Alpha, Model 770, Hamburg, Germany; ± 1.0 g); confirmation of health status occurred at each sampling period.

2.3.2 *Experimental Design and Milk Collection*

This research was conducted as a prospective, longitudinal, observational study during which 5 milk samples were collected from each woman at weekly intervals. Details of the sample collection have been described previously (Hunt et al., 2011). Briefly, milk samples were obtained from the same breast between 0700 and 1100 hr, and to assure adequate milk availability women were asked to have not fed or

expressed from the study breast for at least 2 hr prior to sample collection. All sample collections took place in the Human Metabolic Unit in the Department of Food Science and Human Nutrition at Washington State University. Before sample collection, the breast was cleaned with an iodine swab to reduce bacteria residing on the skin. Milk was collected using a single-use, sterile, Hygienikit® collection unit (Ameda, Cary, IL) attached to an electric breast pump (Model SMR-B-R, Ameda-Egnell, Inc., Cary, IL); milk was collected until milk flow ceased and placed on ice immediately following collection.

2.3.3. *Milk Microbiome Determination*

It is noteworthy that microbial community compositions of a subset of samples used in the present analysis were previously described at the genus level, and the methods used here are the same as those described in this earlier report (Hunt et al., 2011). Briefly, DNA was extracted from each milk sample, and PCR reactions with bar-coded primers were carried out to amplify the V1-V2 hypervariable region of the bacterial 16S rRNA gene. Pyrosequencing of the amplicons was performed with the Roche 454 FLX platform, and quality-control measures were employed to remove sequences of questionable quality. Sequences were then assigned a taxonomic name at the genus level using the Ribosomal Database Project (RDP) Bayesian Classifier (Wang et al., 2007).

2.3.4 *Differential Immune Cell Counting*

Somatic cell counts (SCC) were determined in triplicate on fresh milk using a DeLaval® DCC (Tumba, Sweden). Cytospin smears were prepared in triplicate with 1.6×10^5 cells/slide. Following enumeration of cells via SCC methodology, an appropriate amount of milk to provide the needed 1.6×10^5 cells was centrifuged at 500 x g for 20 min at 4 °C. The fat layer was removed and cells washed using 1 mL 0.1 % bovine serum albumin (BSA) in phosphate-buffered saline (PBS; Mediatec Inc., Herndon, VA). This suspension was then centrifuged at 500 x g for 10 min at 4 °C. The supernatant was aspirated, and the cell pellet was gently resuspended in 0.1 % BSA in PBS at a final concentration of 1.6×10^5 cells/50 µL. The loading chamber

of a cytofunnel was preloaded with 30 µL of 22% BSA in PBS and then followed with 50 µL of the resuspended cell pellet. Slides were centrifuged for 4 min at 1000 rpm using the medium setting on a cytocentrifuge (Cytopro® 7620, Wescor Inc., Logan, UT), air-dried, stained with Wright-Giemsa stain (VWG-032, Volu-Sol® Inc., Salt Lake City, UT) for 3 min, placed in stain-primed hematology buffer (VWB-032) for 5 min, rinsed with water, and allowed to dry completely.

*S*lides were evaluated using light microscopy; a total of 200 cells were counted on each slide. Cells were visually classified as macrophage/epithelial cells (excluding squamous epithelial cells), neutrophils, lymphocytes, eosinophils, basophils, and other (e.g., squamous epithelial cells and bare nuclei). It is noteworthy that, under the staining methods used, macrophages and secretory epithelial cells are visually indistinguishable and therefore, were grouped together as 'macrophage/secretory epithelia' (MSE).

2.3.5 *Biochemical Analyses*

Lipids were extracted from 1 mL of each milk sample using 2:1 chloroform:methanol (Clark et al., 1982), and percent lipid was determined in duplicate gravimetrically. Lipids were methylated using base-catalyzed transesterification (Christie, 1982) and fatty acid methyl esters were analyzed on a gas chromatograph (Hewlett-Packard 6890 series with auto injector) fitted with a flame-ionization detector and a 100 m x 0.25 mm (0.2 µm film) capillary column coated with CP-Sil 88 (Chrompack, Middelburg, the Netherlands). After sample injection, the oven temperature was 70 °C for 3 min and then increased to 175 °C at a rate of 3 °C/min and held for 3 min. Oven temperature was then increased to 185 °C at a rate of 1 °C/min and held for 20 min, increased to 215 °C at a rate of 3 °C/min, and then increased to 230 °C at a rate of 10 °C/min and held for 5 min.

Milk protein concentration was analyzed for each milk sample in duplicate using a modification of the spectrophotometric methods described by Lönnerdal et al. (1987) using the Bio-Rad protein assay kit II (Hercules, CA). Milk lactose concentrations were analyzed in duplicate using modified spectrophotometric methodologies described by Polberger and Lönnerdal (1993).

HMO concentrations and isoform distributions were determined *on a single sample* for each woman as previously described (Jantscher-Krenn et al., 2012). Lipids and proteins were removed from the samples by centrifugation and chloroform/methanol extraction. Lactose was removed by overnight incubation on lactase-immobilized beads (Invitrogen, Carlsbad, CA) at 37 °C. Residual peptides and salt were removed over Sep-Pak C18 cartridges followed by porous graphitized carbon (PGC) cartridges. The reducing ends of the dried oligosaccharides were labeled with the fluorescent tag 2-aminobenzamide (2AB) for 2 h at 65 °C. Free 2AB label was separated from the 2AB-labeled oligosaccharides using silica gel cartridges. 2AB-labeled oligosaccharides were analyzed by high performance liquid chromatography (HPLC) on an amide-80 column (4.6 mm ID x 25 cm, 5 µm, Tosoh Bioscience, Tokyo, Japan) with a 50-mM ammonium formate/acetonitrile buffer system. Separation was performed at 25 °C and monitored with a fluorescence detector at 360 nm excitation and 425 nm emission. Peak annotation was based on standard retention times and mass spectrometric (MS) analysis on a Thermo LCQ Duo Ion trap mass spectrometer equipped with a Nano-ESI-source. Total concentration of HMO was calculated as the sum of most common oligosaccharides including 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 3'-sialyllactose (3'SL), lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), lacto-N-fucopentaose I (LNFP I), lacto-N-fucopentaose II (LNFP II), and lacto-N-fucopentaose III (LNFP III), which collectively represent approximately 80% of all HMO in each sample.

2.3.6 *Statistical Analyses*

Spearman rank correlations were determined to evaluate associations among cellular content and other milk components such as relative abundances of microbiota, milk protein, milk lipid, milk fatty acids, and HMO. Significance for Spearman correlations was declared at $P < 0.01$; trends were noted at $P < 0.05$. Average linkage hierarchal clustering was performed on a Bray-Curtis dissimilarity matrix of the cell populations to explore the similarity of milk cellular composition within a subject across samples. Canonical correlation analyses (CCA) were conducted to investigate correlations between linear combinations of bacteria taxa and milk cellular composition. Principal

component analysis (PCA) and non-metric dimensional scaling (NMDS) analysis were conducted to determine if there were patterns or structure to the variation and similarity, respectively, of milk immune cell profiles among women. Generalized linear mixed models (GLMM) were used to investigate the effect of time on concentrations of immune cells and macronutrients. CCA, Spearman rank correlations, GLMM, PCA, and NMDS analyses were performed using SAS v9.4 (Cary, NC); hierarchal clustering was conducted using the stats, vegan, and gplots packages in R (http://www.r-project.org/) and Seed software (Beck et al., 2014).

2.4 RESULTS

2.4.1 *Description of Subjects and Disposition of Samples*

Descriptive statistics concerning demographic and anthropometric variables of participants are summarized in Table 2.1. In general, subjects were ~32 y of age, multiparous, and ~5 mo postpartum at enrollment. All subjects completed the study, although one woman did not provide one of the milk samples; this yielded a total of 79 milk samples.

2.4.2. *Somatic Cell Counts, Absolute Cell Concentrations, and Relative Cell Distributions*

Mean SCC, absolute host-cell concentrations (cells/mL), and relative host-cell distributions (% total) are provided in Table 2.2. In general, the most abundant type of cells (47.4%) was MSE, followed by neutrophils (32.3%), lymphocytes (14.1%), eosinophils (3.1%), and other (3.4%). Figure 2.1 shows stacked bar charts of the relative abundances of the various cell types for all women. The concentrations and relative abundances were relatively stable over time as evidenced by 1-way ANOVA. No effect of time was detected for the cell concentrations or relative abundances. Additionally, upon visually examining the slides made from milk collected from each woman, we also concluded that each subject appeared to present with her own distribution of cell types which remained relatively stable over the course of the study. In other words, each woman’s milk appeared to have a relatively consistent cellular

"fingerprint." A representative sample of slides prepared from milk collected from 4 of the women is shown in Figure 2.2. Qualitative analysis of these data suggests the distribution of cells in each milk sample could be categorized into one of four classifications as described here.

- **'Macrophage/Secretory Epithelium'** – cells predominantly and consistently macrophage or secretory epithelial cells (indistinguishable from each other)
- **'Neutrophil'** – cells predominantly and consistently neutrophils
- **'Macrophage/Neutrophil'** – consistently similar distribution of macrophage/secretory epithelial cells and neutrophils
- **'Unique'** – a unique distribution of cells that changed over time

Using this qualitatively-derived scheme, milk from 10 participants (1, 2, 3, 4, 10, 11, 12, 14, 15, 16) were generally categorized as 'Macrophage/Epithelium,' 2 participants (5, 8) as 'Neutrophil,' 2 participants (6, 9) as 'Macrophage/Neutrophil,' and 2 participants (7, 13) as 'Unique' (see Table 2.5 for cell counts of the various cell types by participant and sampling week). Some squamous epithelial cells were also observed, as were several binucleated cells (Figure 2.3).

Cellular composition data were also analyzed using hierarchical cluster analysis based on Bray-Curtis dissimilarities and the average linkage algorithm (Figure 2.4). The results from this analysis suggested that complex cellular profiles were relatively consistent within a particular woman over time.

2.4.3 *Milk Macronutrient Composition*

Mean milk protein, lipid, lactose, and fatty acid concentrations are provided in Table 2.3. Figure 2.5 shows the relative abundances of the major fatty acids identified for each woman at each sampling period. Values were well within what would be expected for milk produced by healthy women (Jensen et al., 1978; Lönnerdal et al., 1987; Nommsen et al., 1991; Mitoulas et al., 2003), and were consistent over time as assessed by 1-way ANOVA. HMO compositions are presented in Table 2.4, and are also typical of what would be expected in human milk (Chaturvedi et al., 2001; Smilowitz et al., 2013). Figure 2.6 shows the relative abundances of HMO for a single milk sample from each woman during the 5 week period.

2.4.4 *Milk Microbial Compositions*

The most abundant bacterial members (≥ 1% of identified bacteria) are provided in Table 2.6 and the relative abundances of the top 20 bacteria are depicted in Figure 2.7. At the genus level, *Streptococcus* (27%) and *Staphylococcus* (18%) were most common; this, too, is what would be expected given previously published data (Jost et al., 2013a; Boix-Amorós et al., 2016).

2.4.5 *Associations Among Absolute and Relative Proportions of Host Cells*

SCC was positively associated with the concentrations of neutrophils (r = 0.81, $P < 0.0001$), lymphocytes (r = 0.62, $P < 0.0001$), and MSE (r = 0.62, $P < 0.0001$). Lymphocyte concentration was also positively associated with concentrations of both neutrophils (r = 0.31, $P < 0.005$) and MSE (r = 0.67, $P < 0.0001$). Relative proportion of neutrophils was inversely associated with relative proportion of MSE (r = -0.73, $P < 0.0001$) and lymphocytes (r = -0.50, $P < 0.0001$).

2.4.6 *Associations Between Relative Bacterial Abundances and Relative Abundances and Concentrations of Host Cells*

Relative abundances of *Staphylococcus* and *Corynebacterium* tended to be associated with neutrophil concentration (r = 0.27, $P = 0.0128$ and r = 0.24, $P = 0.0356$, respectively). Relative abundances of *Serratia* and *Pseudomonas* were negatively associated with SCC (r = -0.47 and -0.49, respectively; $P < 0.0001$) and neutrophil concentration (r = -0.38 and -0.42, respectively; $P < 0.0006$). Concentration of Gram-positive bacteria in milk was positively associated with neutrophil concentration (r = 0.35, $P < 0.0016$); while the concentration of Gram-negative bacteria was negatively associated with SCC (r = -0.48, $P < 0.0001$).

2.4.7 *Associations Between SCC, Milk Cell Concentration, and Milk Macronutrient Content*

Milk protein concentration was positively correlated with SCC (r = 0.28, $P = 0.0119$) and neutrophil concentrations (r = 0.25, $P = 0.0239$), and inversely associated with eosinophil concentration (r = -0.41, $P = 0.0001$). Milk lactose concentration was

negatively associated with neutrophil concentration (r = -0.36, *P* = 0.0009), MSE concentration (r = -0.32, *P* = 0.0045), and SCC (r = -0.40, *P* = 0.0003). Milk lipid content (%) was strongly correlated with concentration of MSE (r = 0.42, *P* = 0.0001) and SCC (r = 0.33, *P* = 0.0033).

The concentrations of several fatty acids in milk were also related to immune cell profiles. For instance, behenic acid (C22:0) was positively correlated with relative abundance of neutrophils (r = 0.43; *P* < 0.0001) and tended to be negatively correlated with the MSE concentration (r = -0.28, *P* = 0.0127). Arachidonic acid (C20:4n6) was positively associated with eosinophil concentration (r = 0.39, *P* = 0.0004), and α-linolenic acid (C18:3n3) was negatively associated with lymphocyte concentration (r = -0.31, *P* = 0.0059).

Concentrations of several HMO were also correlated with the host cellular content of milk. For instance, LNT and LNnT were negatively associated with SCC (r = -0.64, *P* = 0.0082; r = -0.52, *P* = 0.0387, respectively) and MSE concentration (r = -0.68, *P* = 0.0038; and r = -0.65, P = 0.0067, respectively). In addition, 2'FL concentration tended to be positively associated with the relative abundance (%) of neutrophils (r = 0.52, *P* = 0.0387) and negatively associated with relative abundance (%) of MSE (r = -0.55, *P* = 0.0283). LNFP I concentration was also negatively associated with the MSE concentration (r = -0.54, *P* < 0.0293).

2.4.8 *Multivariate analysis of milk cellular composition*

PCA of the 5 main cell types identified in milk suggested that variation in the concentrations of MSE, neutrophils, and lymphocytes accounted for 75% of the total variation in milk cell composition. However, visual examination of both the PCA and the NMDS showed little to no clustering by subject (see Figure 2.9 for NMDS plot; PCA plot not shown).

CCA between linear combinations of proportions of milk cells (MSE, neutrophils, lymphocytes, and eosinophils) and linear combinations of the relative abundances of the six most-abundant bacterial genera (*Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Serratia*, *Pseudomonas*, and *Propionibacterium*) was 0.63 (*P* = 0.0002). The canonical correlation between linear combinations of

Gram negative and Gram positive bacteria and linear combinations had a weaker correlation of 0.45 (P = 0.0166). These results suggest a complex relationship exists among the host and bacterial cellular components in human milk (Figure 2.8).

2.5 DISCUSSION

The primary objective of this study was to characterize and investigate the relationships between immune cells and the bacterial communities in human milk. Because HMO can influence both bacterial growth and immune cell function, relationships between immune cell populations and HMO were also investigated, as were those between milk lipids and protein and the milk microbiome.

The subjects included in this study were generally representative of relatively healthy, typical breastfeeding US women as evidenced by the demographic and anthropometric data. Furthermore, SCC were within normal ranges reported previously for healthy lactating women (Goldman, 1993; Goldman and Goldblum, 1997); and milk lipid, protein, oligosaccharide, and lactose concentrations fell within normal ranges (Jensen, 1995). As such, we expect our results to apply to other healthy lactating women.

The presence of cells in milk has long been known. In 1956, Holmquist and Papanicolaou described the historical research of cells in milk and conducted cytologic examinations of exfoliated mammary gland cells during lactation. They observed a wide variation of cell types across different stages of lactation and also among individuals in the same stage of lactation. In agreement with this study and previous research (Järvinen and Suomalainen, 2002), we also found a wide variation of cell types and proportions of those cell types across the different individuals in the study.

Holmquist and Papanicolaou (1956) noted that many of the cell types were found in clusters and appeared to be of epithelial origin. Other researchers have also found that cells of epithelial origin are prevalent in mature human milk (Brooker, 1980; Hassiotou et al., 2013a). Our study would suggest that leukocytes also comprise a great proportion of the cell types in milk from some self-reported healthy

women. But it should be noted that one of the limitations in using cytological evaluation of the milk cells is that it is difficult to distinguish between macrophages and secretory epithelial cells. And thus differences in methodology may be one of the reasons why our results differ. However, even with the cytological evaluation, high relative numbers of neutrophils were still seen in several of the milk samples from mothers who self-reported themselves and their infants as being healthy. An overview of all the milk components examined in this study from 4 representative women is provided in Figure 2.10. Two of the profiles shown are from women who had high proportions of MSE in their milk across the 5 week sampling period, while the other two profiles are from women who had high proportions of neutrophils in their milk.

Interestingly, by using a cytological-based staining technique, we observed several large binucleated cells (Figure 2.3) as has been previously reported. Holmquist and Papanicolaou (1956) described the presence of large binucleated cells in many of the human milk samples they evaluated. Just recently, binucleated cells in the mouse have been shown to be present in milk and are formed through failed cytokinesis (Rios et al., 2016). This results in the cells having an increase in cytoplasmic volume. The researchers suggest that this increase in cell volume is needed to support more ribosomes, golgi, and endoplasmic reticula that in turn are needed to accommodate the production of milk proteins and lipids that occurs during secretory activation (Anderson et al., 2007). Rios and coworkers (2016) also suggest that since these binucleated cells are observed in lactating mammary glands across many species such as humans, cows, mice, seals, and wallabies, their function may be evolutionarily conserved. More research is warranted to investigate the origin and roles of these somewhat unique binucleated cells in human lactation.

Along with cells of epithelial origin, varying composition of leukocytes have also been observed by different researchers. The composition of the leukocyte populations appears to be influenced by a number of factors. One of the factors that has been shown to impact the immunomodulatory components of human milk is the health status of the infant. Recent evidence supports a bidirectional communication between infant and the immune response of the mammary gland. In a study

conducted by Riskin and coworkers (2012), there was a pronounced difference in CD45-positive cells in milk during times when the infant had an active illness. Additionally, a decrease in the number of macrophages was noted during the time between when the infant had an active illness and convalescence. This concurs with a similar finding by Hassiotou and coworkers (2013b) who also observed a small increase in the number of leukocytes in milk during the time that the infant had an infection. As leukocytes are central to the immunosurveillance of the mammary gland, the additional impact that these dynamic changes have on bacterial communities present in the mammary gland has yet to be investigated.

Recently, Trend and coworkers (2015) examined the leukocyte populations in human milk using flow cytometry. They reported that the median concentration of cells in milk was 313,500 cells/mL. Concentrations of CD45+ cells (leukocytes) were not different between transitional (median 27,500 cells/mL) and mature milk (median 23,650 cells/mL) but colostrum had a higher concentration (median 146,000 cells/mL) than both. These are similar concentrations to the values observed in the present study (Table 2.2), which focused on mature milk samples.

The immune cells in milk have long been thought to mitigate bacterial growth and/or keep surveillance on the bacteria encountered in the mammary gland such that infection does not develop (Robinson et al., 1978; Johnson et al., 1980). However, with the advent of molecular methods such as high-throughput sequencing and quantitative PCR, it is now generally accepted that healthy human milk contains a diverse community of bacteria that can range in concentration from 10^3 – 10^6 bacterial cells/mL (Heikkilä and Saris, 2003; Boix-Amorós et al., 2016). The relationships between the concentration and profile of immune cells and variation in this diverse bacterial community has not been well studied. Only recently Boix-Amorós and coworkers (2016) examined the correlation between total SCC and microbiota in human milk; they found a positive correlation between relative abundance of *Staphylococcus* and the SCC. In our study, we observed a weak positive correlation between *Staphylococcus* and neutrophils concentration, but not an association with SCC or with any of the concentrations of the cells identified. It should be noted, however, that relative abundance of *Staphylococcus* tended to be

weakly associated (r = 0.24, *P* = 0.035) with the total number of somatic cells estimated in the full milk expression volume (milk cells per mL multiplied by the milk expression volume). It is unclear as to why this discrepancy exists between studies.

Because Gram-negative and Gram-positive bacteria elicit different innate immune responses from the host (Bannerman, 2009; Skovbjerg et al., 2010), we wanted to investigate if the relative abundances of bacteria grouped by Gram status was associated with the host cell distribution in the milk. Indeed, the CCA indicated that combinations of Gram-negative and Gram-positive bacteria are moderately associated with linear combinations of host milk cells. Additionally, these broad categories revealed that relative abundance of Gram-positive bacteria was weakly associated with both relative proportions of neutrophils (r = 0.27, *P* = 0.0155) and MSE (r = -0.34, *P* = 0.0021). As to be expected with compositional data, relative proportion of Gram-negative bacteria was inversely correlated with neutrophils (r = -0.26, *P* = 0.0223) and MSE (r = 0.30, *P* = 0.0083). Unfortunately, cytokine/chemokine concentrations were not ascertained in this study to further assess the inflammatory state of the mammary gland related to the presence of the different classifications of bacteria. Our data suggest, however, that the presence of different types of bacteria in “healthy” human milk moderate the inflammatory response such that a balanced immune response is mounted in the mammary gland and homeostasis is maintained. When dysbiosis occurs in the mammary gland milieu or certain bacteria obtain an advantage and overgrowth occurs, diseases such as mastitis may be manifested. The mechanisms whereby these events are triggered are not well understood, and studies investigating the relationships between the milk bacterial community composition and milk cytokine/chemokine composition are warranted.

Because both immune cell and bacterial community composition of milk are likely linked, other downstream effects of individualized composition of bacteria, cells, and HMO on lactation could be important. For example, Gouon-Evans and coworkers (2002) demonstrated that macrophages play a role in the morphogenesis of the mammary gland during puberty (reviewed by Gjorevski and Nelson, 2011; Reed and Schwertfeger, 2010). Although hormones such as prolactin and progesterone are intimately involved in maintaining the proliferative phase of alveolar morphogenesis,

the impact of immune cells and/or bacteria in structuring the mammary architecture during lactation has not been studied and can only be speculated upon. It is intriguing to consider that just as signals such as colony-stimulating factor 1 from macrophages promote terminal bud synthesis in the developing gland (Gouon-Evans et al., 2002) signals from immune cell populations potentially shaped by the milk microbiome may mitigate continuing morphogenesis or maintenance of the secretory alveolar system during lactation. Further research is needed to investigate these potential actions of the immune cells on proliferating mammary cells.

Several studies have demonstrated that the mammary gland has several complex mechanisms that work in concert together to protect it from going into a state of microbial dysbiosis (Sordillo et al.,1997). Presence of immune cells within the mammary gland provides one such mechanism. But perhaps a commensal community of bacteria in the mammary gland regulates the functionality of immune cells to elicit an appropriate immune response. Vong and colleagues (2014) demonstrated that bacteria can directly impact the function of neutrophils. Co-culture of the probiotic *Lactobacillus rhamnosus* strain GG with neutrophils resulted in decreased production of reactive oxygen species and the formation of neutrophil extracellular traps. The researchers suggest that this mechanism may change the local innate immune response and help protect against adverse intestinal inflammation. We posit that a similar relationship exists between the milk microbiota and the immune cells in the mammary gland to maintain a homeostatic, health-promoting environment. Other components in milk such as HMO impact innate and/or adaptive immune responses to bacteria and thus may influence the composition of immune cells present in milk. HMO can directly block pathogen binding to host cell surface glycans and receptors (reviewed by Bode, 2012) and also alter the immunomodulatory response of various cell types such as peripheral blood mononuclear cells (Comstock et al., 2014; Duska-McEwen et al., 2014) and intestinal epithelial cells (Lane et al., 2013; He et al., 2016). In our study, we found few associations between the cellular components of milk and HMO. However, it should be noted that there were only 16 samples (one from each woman) that were analyzed in these correlations. Future studies are necessary to investigate more

thoroughly the repercussions of various HMO on cytokine production and activation and other immunomodulatory factors of cells in the mammary gland.

As bacteria, immune cells, and HMO are subsequently passed on to the nursing young, it is important to consider how these milk components impact infant health. One such example in regards to the cellular content of human milk, is the well-described phenomena in many species in which colostral leukocytes traffic into the neonatal circulation. Reber and coworkers (2005, 2008a, 2008b) describe how this translocation of maternal immune cells may promote the development of the neonatal immune system and in particular, enhance the ability to stimulate a mixed leukocyte response. Stem cells from human milk have also been shown to translocate and integrate into many different tissues of the offspring (Hassiotou et al. 2014a; Hassiotou et al. 2014b). The implications of maternal cells on infant development and health need further study.

Understanding the complex interplay between the bacteria, immune cells and other milk components such as HMO is critical so that we can better understand how these factors maintain and promote health of the mother and of the infant or how imbalances occur and result in diseases such as mastitis for the mother or diseases such as necrotizing enterocolitis for the infant.

2.6 CONCLUSIONS

Our results suggest a complex interaction exist among the host's immune cells, milk bacteria, and milk nutrients – including HMO. We propose that this interaction may help maintain homeostasis in the "healthy" lactating mammary gland. However, it is unknown whether the different bacterial populations in milk are responsible for driving the variation in immune cell profile in the mammary gland or vice-versa. Indeed, it is likely a complex interplay that ultimately regulates the cell profiles of both. More studies are needed to discern the mechanisms by which both bacterial communities and immune cell populations are regulated within the mammary gland during lactation.

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Table 2.1 *Maternal Demographic and Anthropometric Variables at Enrollment*[1]

Age (y)	31.6 ± 0.5
Weight (kg)	73.4 ± 1.5
Height (cm)	167.5 ± 0.7
BMI (kg/m^2)	26.0 ± 0.4
Time Postpartum (days)	161.3 ± 15.1
Parity (#)	1.6 ± 0.1

[1]Values represent means ± SEM; n = 16.

Table 2.2 *Somatic Cell Counts* (10^3 cells/mL), *Relative Cell Distributions (%), and Absolute Cell Concentrations* (10^3 cells/mL)[1]

Variable	Mean ± SEM
SCC	62.6 ± 21.8
Macrophage/epithelial	
Relative	47.4 ± 5.2
Absolute	18.9 ± 3.7
Neutrophils	
Relative	32.3 ± 6.8
Absolute	35.7 ± 21.3
Lymphocytes	
Relative	14.1 ± 2.2
Absolute	5.1 ± 0.9
Eosinophils	
Relative	3.1 ± 2.6
Absolute	1.2 ± 0.8
Other cells	
Relative	3.4 ± 0.6
Absolute	2.2 ± 0.7

[1]Values represent means ± SEM*; n* = 79.
[2]Relative values represent the average of the relative percentages across samples.
[3]Absolute values represent the average of the concentrations across all samples.

Table 2.3 *Macronutrient and Fatty Acid Contents of Milk Samples* [1,2]

Variable	Mean ± SEM
Protein (mg/mL)	10.1 ± 0.2
Lactose (mg/mL)	64.1 ± 0.4
Lipids (mg/mL)	4.6 ± 0.2
Caproic acid (C6:0)	0.09 ± 0.00
Caprylic acid (C8:0)	0.19 ± 0.01
Capric acid (C10:0)	1.31 ± 0.02
Lauric acid (C12:0)	4.91 ± 0.16
Myristic acid (C14:0)	5.45 ± 0.17
Myristoleic acid (C14:1n5)	0.20 ± 0.01
Pentadecanoic acid (C15:0)	0.31 ± 0.01
Palmitic acid (C16:0)	20.25 ± 0.24
Palmitoleic acid (C16:1n7)	1.96 ± 0.07
Margaric acid (C17:0)	0.33 ± 0.01
Hexadecadienoic acid (C16:2n4)	0.19 ± 0.01
Stearic acid (C18:0)	7.24 ± 0.14
Vaccenic acid (C18:1n7)	0.65 ± 0.04
Oleic acid (C18:1n9)	31.79 ± 0.39
Linoleic acid (C18:2n6)	16.29 ± 0.37
Aracidic acid (C20:0)	0.21 ± 0.00
α-Linolenic acid (C18:3n3)	1.53 ± 0.06
γ-Linolenic acid (C18:3n6)	0.38 ± 0.01
Eicosadienoic acid (C20:2n6)	0.20 ± 0.01
Behenic acid (C22:0)	0.34 ± 0.01
Arachidonic acid (C20:4n6)	0.49 ± 0.01
Eicosapentaenoic acid (C20:5n3)	0.06 ± 0.00
Docosahexaenoic acid (C22:6n3)	0.20 ± 0.01

[1]Values represent means ± SEM; $n = 79$.
[2]Values represent % of total fatty acid methyl esters identified.

Table 2.4 *Mean Human Milk Oligosaccharide (HMO) Concentrations*[1]

Variable	nmol/mL	µg/mL
Total HMO	3356 ± 327	1919 ± 177
2'-fucosyllactose (2'FL)	1961 ± 296	958 ± 145
3-fucosyllactose (3FL)	202 ± 38	99 ± 19
3'-sialyllactose (3'SL)	338 ± 31	214 ± 19
Lacto-N-tetraose (LNT)	452 ± 63	320 ± 44
Lacto-N-fucopentaose I (LNFP I)	154 ± 23	131 ± 20
Lacto-N-fucopentaose II (LNFP II)	127 ± 15	108 ± 13
Lacto-N-neotetraose (LNnT)	127 ± 19	90 ± 14

[1]Values represent means ± SEM; n = 16.

Table 2.5 *Milk cell distribution (%) by subject ID and sampling week.*

ID-Week	MEC	Neutrophils	Lymphocytes	Eosinophils	Other cells
1-1	65	16	13	0	6
1-2	65	19	13	0	3
1-3	77	9	9	0	5
1-4	47	13	29	9	2
1-5	49	11	25	7	8
2-1	56	21	12	0	11
2-2	66	11	16	0	7
2-3	40	29	13	0	18
2-4	67	5	11	0	17
2-5	61	21	7	1	10
3-1	64	27	9	0	0
3-2	44	4	19	0	33
3-3	73	9	13	0	5
3-4	77	10	13	0	0
3-5	52	3	34	0	11
4-1	67	12	15	0	6
4-2	62	32	4	0	2
4-3	46	17	32	0	5
4-4	45	19	31	0	5
4-5	66	19	13	0	2
5-1	12	87	1	0	0
5-2	12	85	3	0	0
5-3	34	38	21	0	7
5-4	9	82	7	0	2
5-5	25	63	8	0	4
6-1	52	38	9	0	1
6-2	49	36	7	0	8
6-3	37	52	10	0	1
6-4	25	56	16	0	3
6-5	33	55	9	0	3
7-1	39	8	12	41	0
7-2	50	10	21	18	1
7-3	58	11	11	20	0
7-4	20	18	11	51	0
7-5	22	27	15	34	2
8-1	20	69	10	0	1
8-2	6	91	3	0	0
8-3	7	89	4	0	0
8-4	10	82	7	0	1
8-5	4	95	1	0	0
9-1	35	44	16	5	0
9-2	31	49	15	2	3
9-3	39	40	20	1	0
9-4	33	46	19	2	0
9-5	48	29	14	7	2
10-1	48	24	27	0	1
10-2	63	15	21	1	0
10-3	42	24	9	25	0
10-4	72	15	13	0	0
10-5	77	4	14	4	1

11-1	73	11	14	0	2
11-2	50	27	20	0	3
11-3	55	17	26	0	2
11-5	58	11	25	0	6
12-1	48	16	35	1	0
12-2	58	28	13	0	1
12-3	67	21	5	7	0
12-4	50	4	41	5	0
12-5	72	11	17	0	0
13-1	4	93	2	0	1
13-2	18	64	18	0	0
13-3	34	32	31	3	0
13-4	35	9	31	25	0
13-5	3	93	4	0	0
14-1	59	11	27	0	3
14-2	66	12	15	7	0
14-3	67	15	15	1	2
14-4	57	10	29	3	1
14-5	62	16	20	0	2
15-1	49	24	8	0	19
15-2	59	25	13	0	3
15-3	71	8	16	0	5
15-4	72	1	15	0	12
15-5	81	8	10	0	1
16-1	68	15	15	2	0
16-2	21	40	38	0	1
16-3	58	10	28	3	1
16-4	56	7	34	3	0
16-5	57	6	33	3	1

Table 2.6 *Relative Abundance of Bacterial Groups Comprising ≥ 1% of Total Sequence Reads*

Genus	%
Streptococcus	26.7 ± 2.3
Staphylococcus	18.1 ± 2.2
Corynebacterium	6.7 ± 1.1
Serratia	5.9 ± 0.6
Pseudomonas	4.1 ± 0.4
Propionibacterium	3.6 ± 0.5
Ralstonia	3.1 ± 0.5
Novosphingobium	2.9 ± 0.5
Rothia	2.7 ± 0.4
Sphingomonas	1.9 ± 0.3
Veillonella	1.8 ± 0.3
Bradyrhizobium	1.6 ± 0.2
Gemella	1.2 ± 0.2
Actinomyces	1.1 ± 0.2

[1]Values represent means ± SEM; $n = 79$.

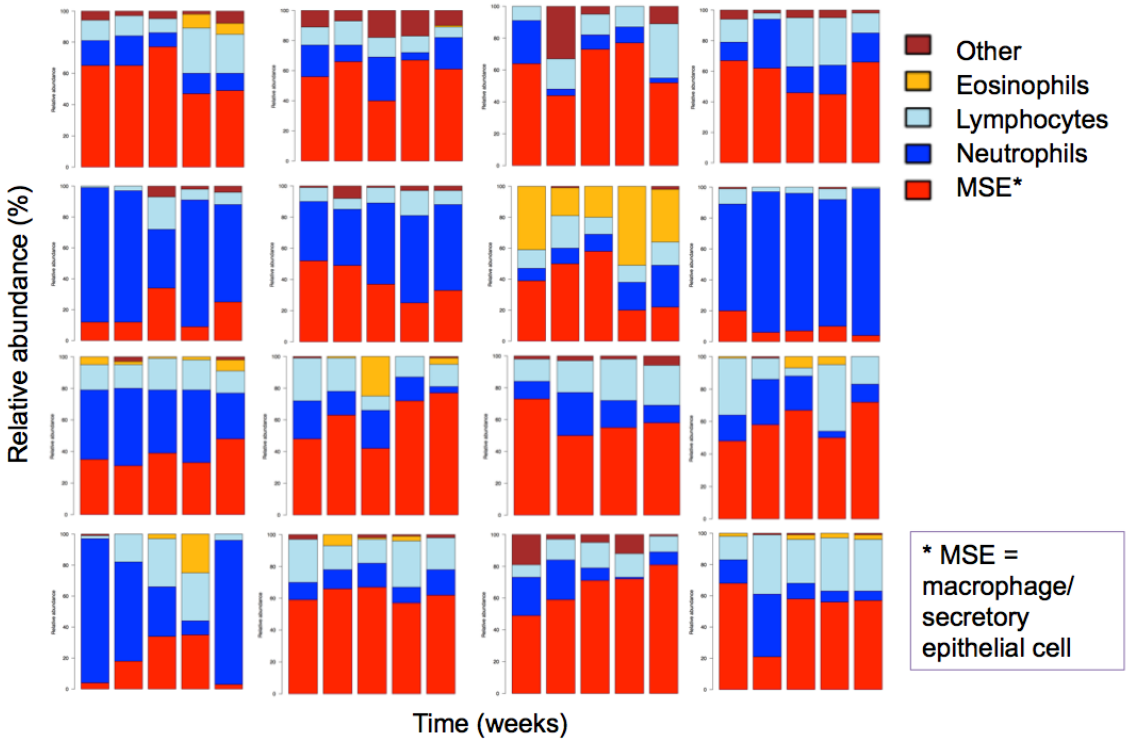

Figure 2.1 Stacked barcharts of the relative abundances of milk host cells from each subject across the 5 week sampling period.

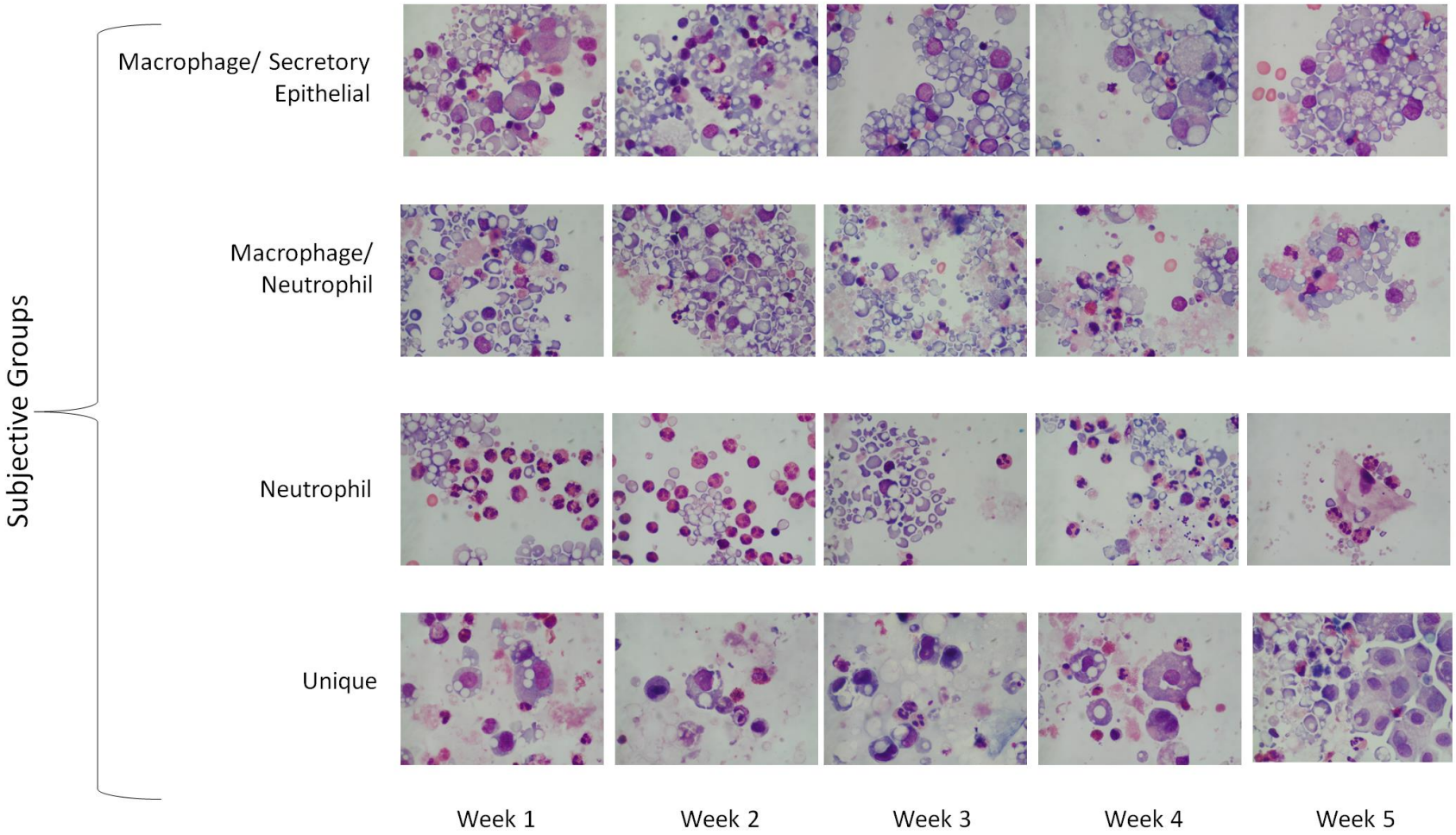

Figure 2.2 Representative micrograph series of milk immune cell type groups.
Each example of a group includes 5 micrographs made from milk produced by single woman. Of the 16 women, 9 were classified as having milk dominated by macrophages and secretory epithelial cells, 3 by similar percentages of macrophages and neutrophils, 1 dominated by neutrophils, and 3 had an inconsistent combination of cell types at each sampling period (unique).

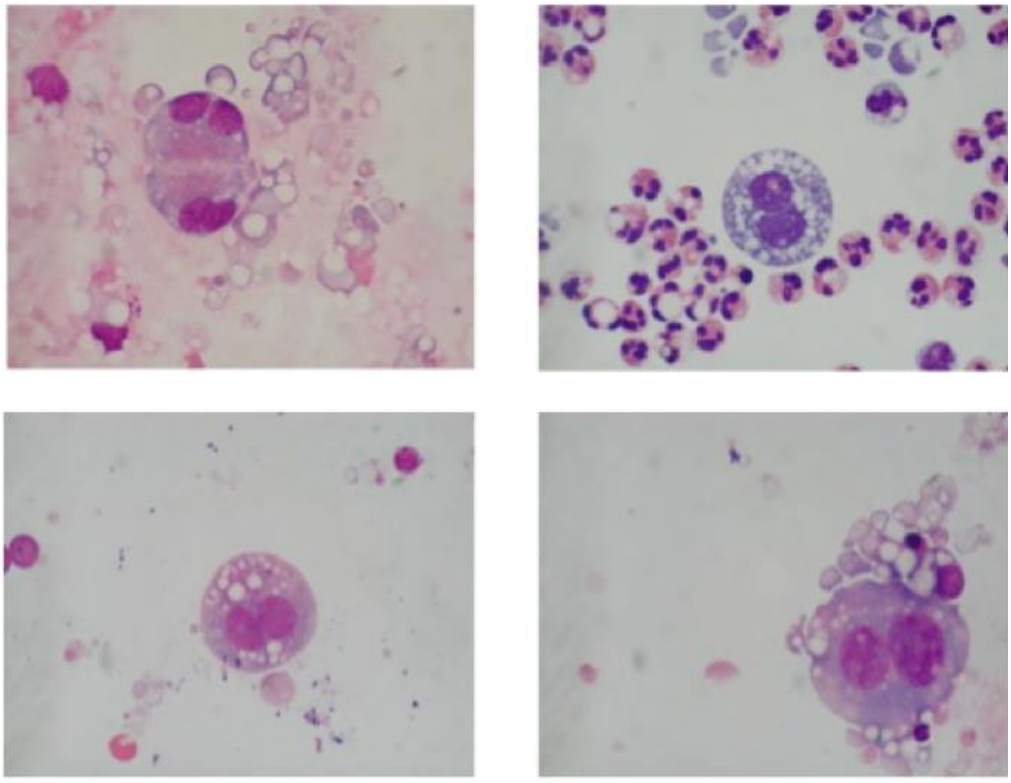

Figure 2.3 Micrographs showing 4 examples of binucleatedcells in human milk.

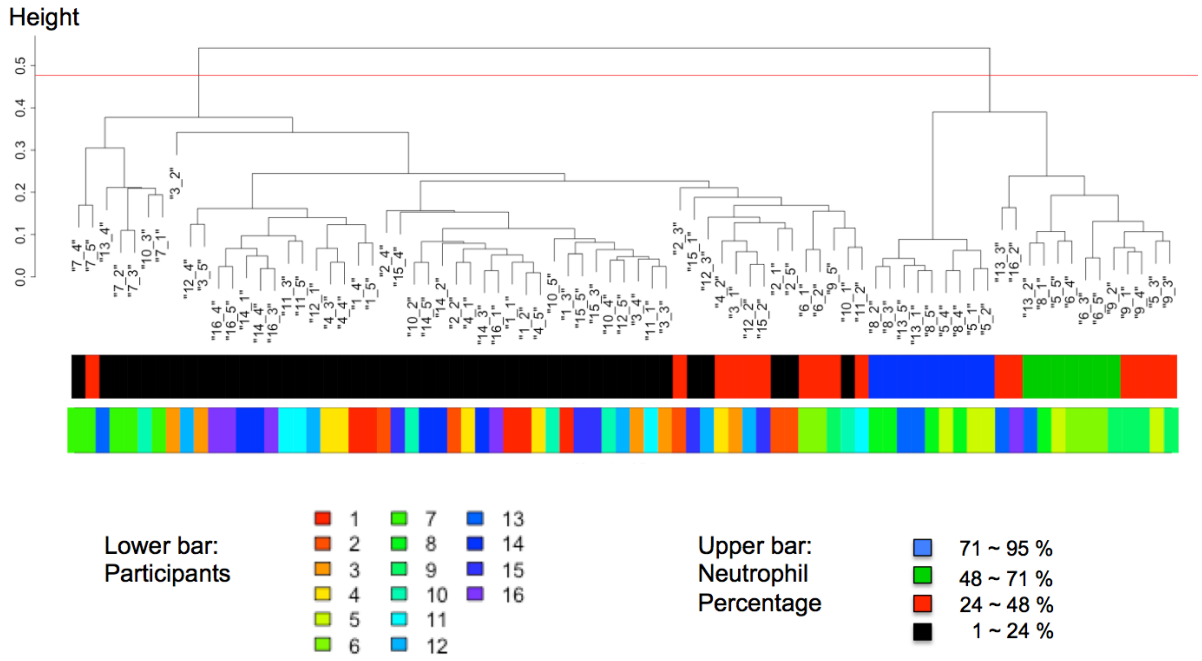

Figure 2.4 Hierarchical clustering of the immune cell populations. Colors in the lower bar represent different participants. Colors in the upper bar represent percentages of neutrophils in milk.

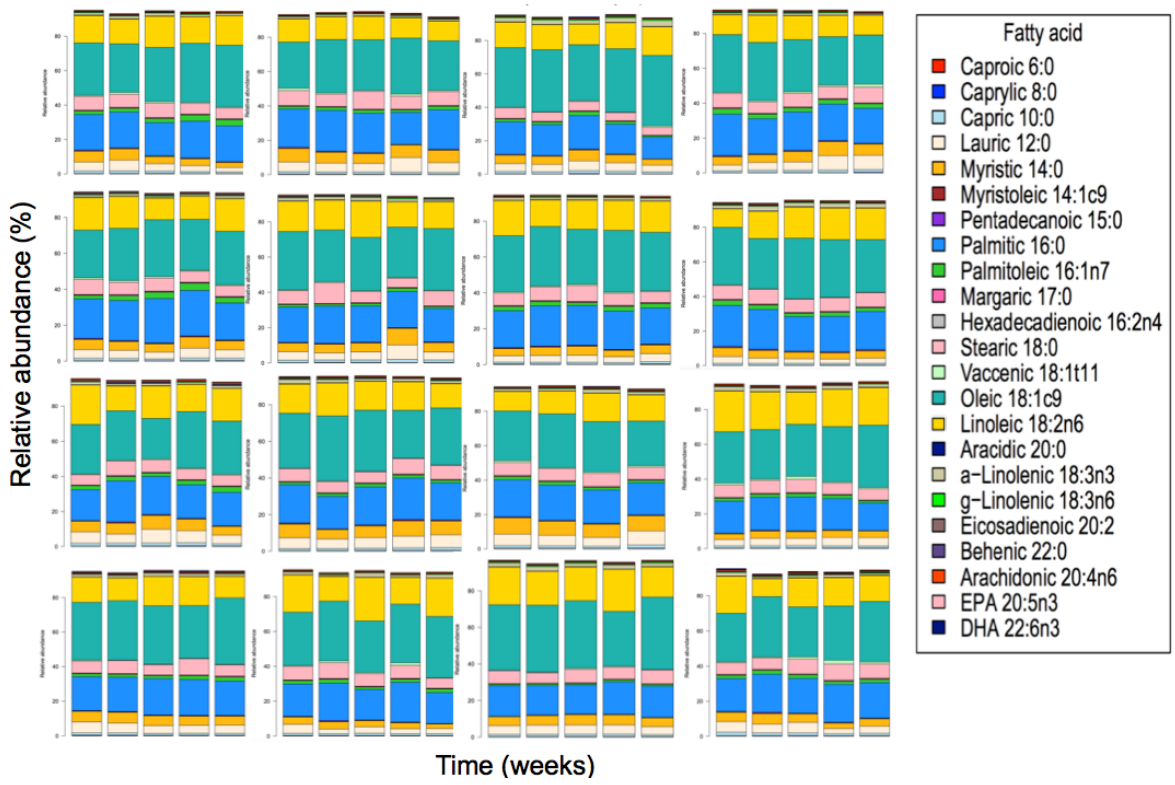

Figure 2.5 Stacked barcharts of the relative abundances of the milk fatty acids from each subject across the 5 week sampling period.

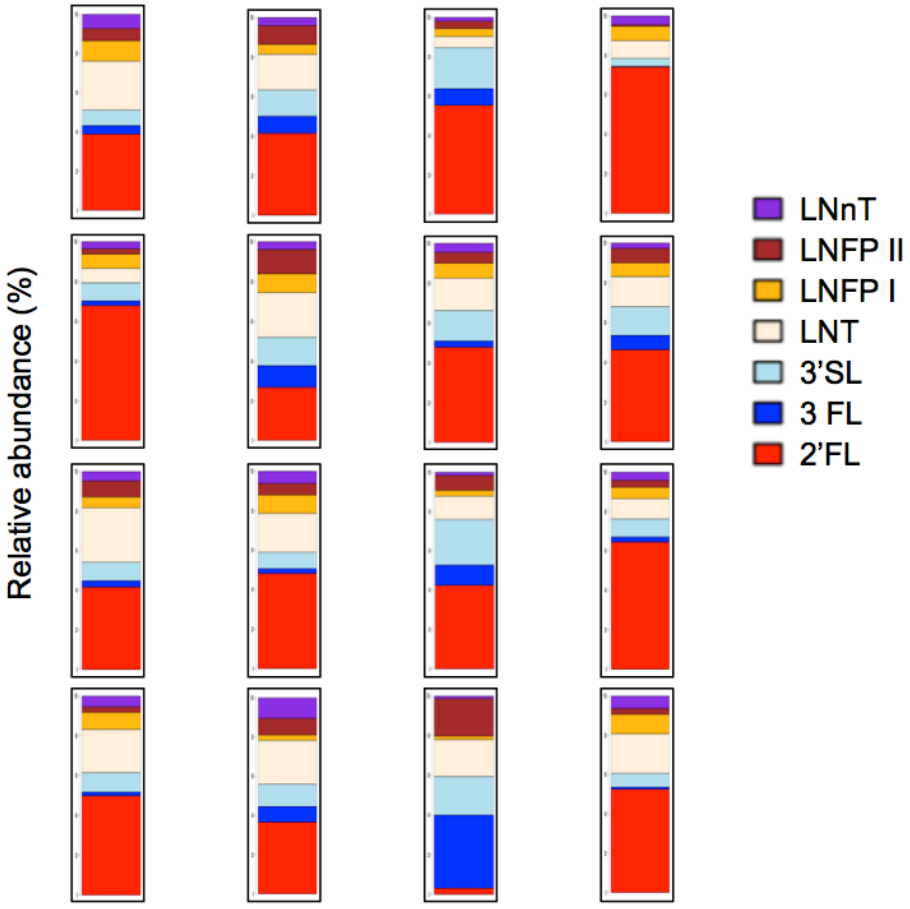

Figure 2.6 Stacked barcharts of the relative abundances of HMO in milk from each subject at one of the sampling periods

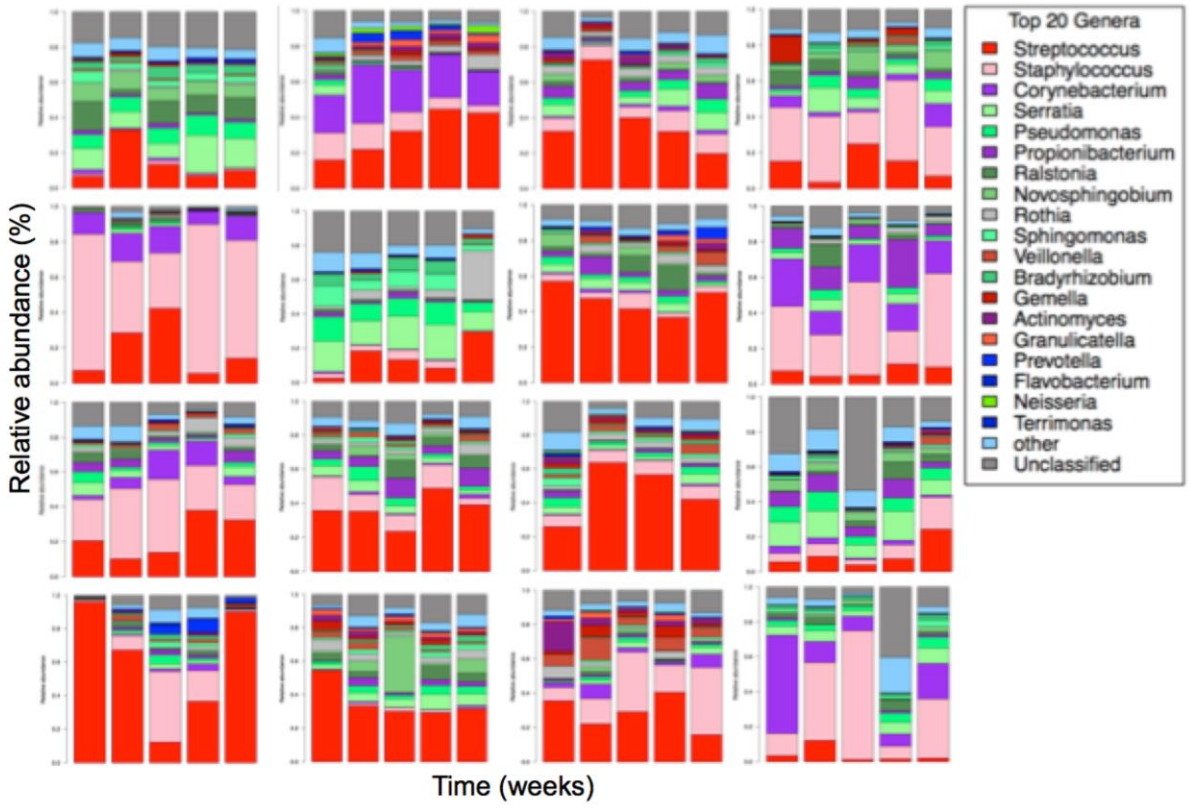

Figure 2.7 Stacked barcharts of the relative abundances of the 20 most abundant genera in milk from each subject across the 5 week sampling period.

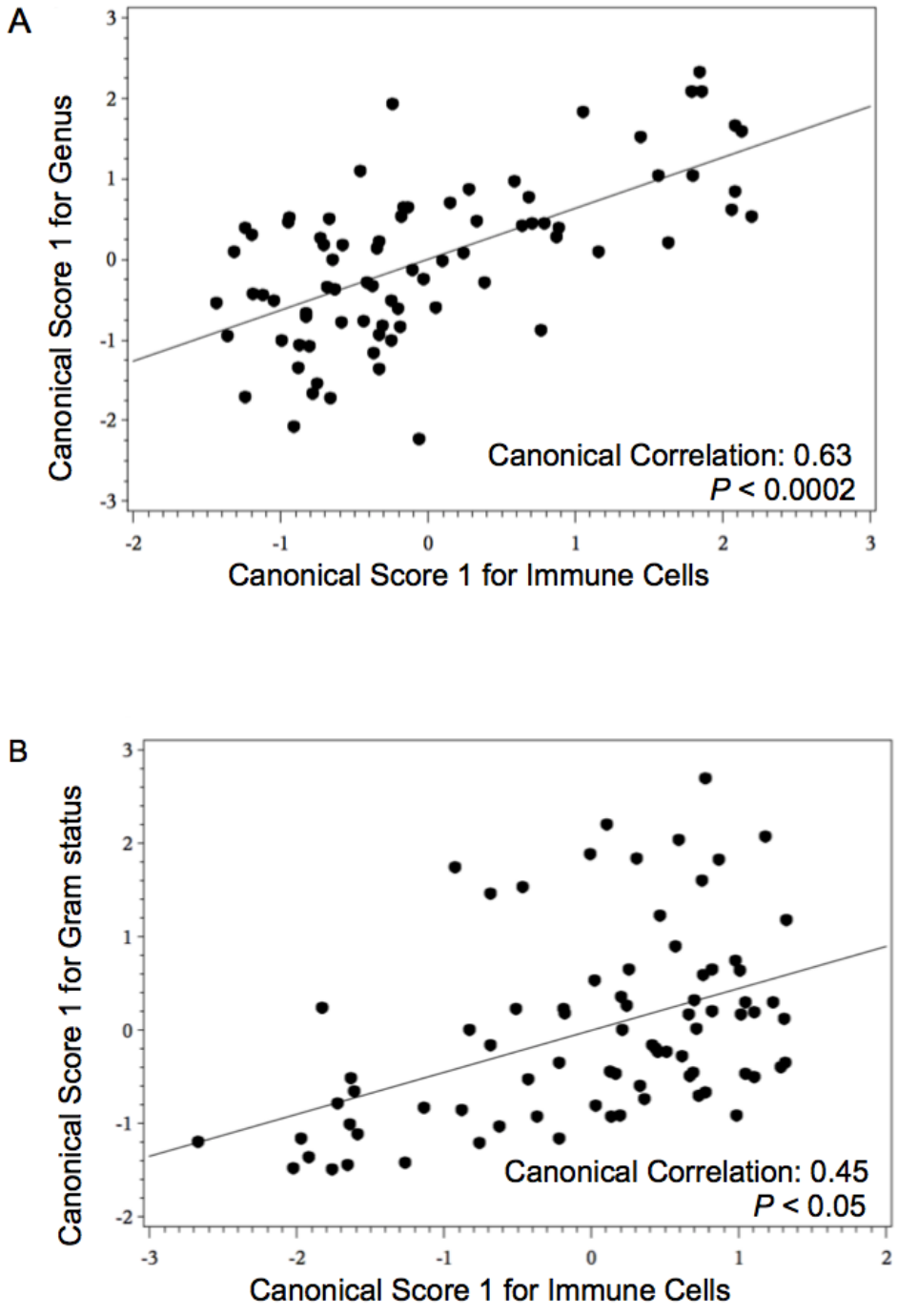

Figure 2.8 Canonical correlations between linear combinations of (A) milk-borne host cells and relative abundances of most-abundant genera in milk and (B) milk-borne host cells and relative abundances of gram-positive and gram-negative bacteria.

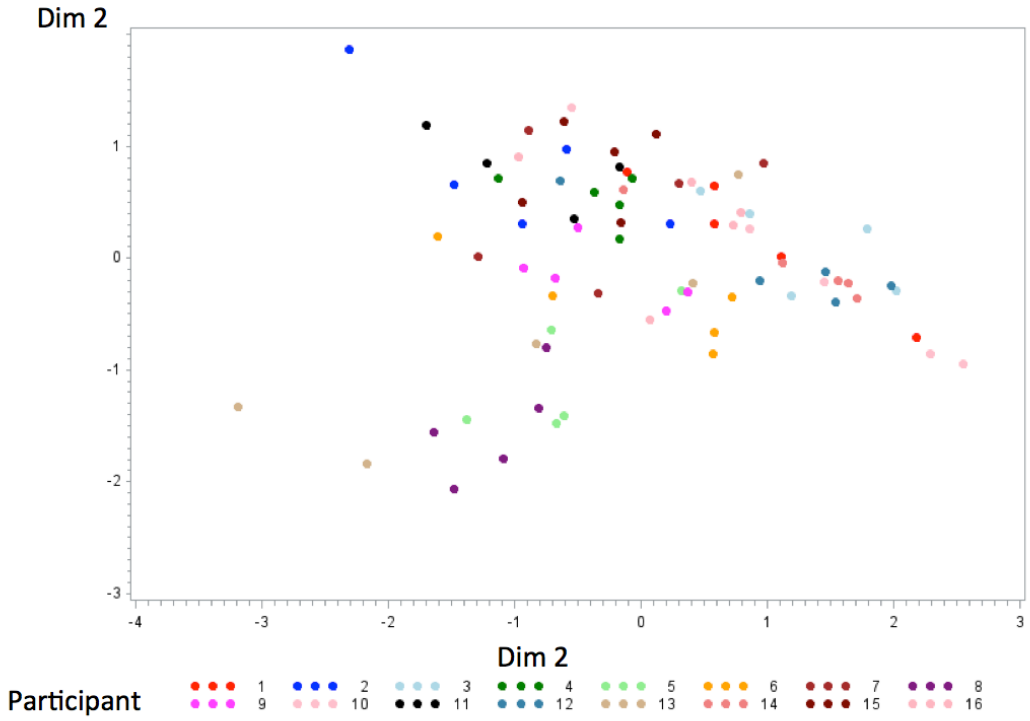

Figure 2.9 NMDS plot of the relative abundances of milk cells. Different colors represent milk samples from different participants

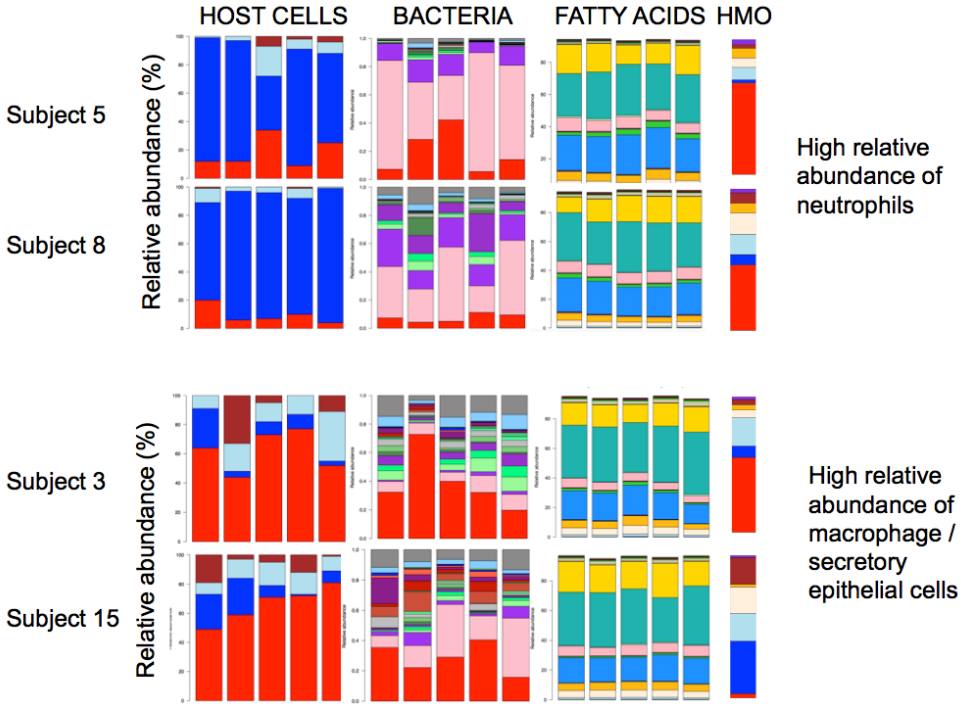

Figure 2.10 Overview of stacked barcharts of various components in human milk from 4 subjects. Each row represents (from left to right) relative abundances of host cells, bacteria, fattty acids, and HMO in milk from a different subject. The first two rows represent milk components from two women (subjects 5 and 8) whose milk had high percentages of neutrophils. The second two rows represent milk components from two women (subjects 3 and 15) whose milk had high percentages of MSE.

CHAPTER 3

MILK MICROBIAL COMMUNITY STRUCTURE IS RELATIVELY STABLE OVER TIME AND RELATED TO VARIATION IN MACRONUTRIENT AND MICRONUTRIENT INTAKES IN LACTATING WOMEN

3.1 ABSTRACT

Background. The human milk microbiome has been characterized in some populations, but little is known about changes over time and relationships with maternal factors such as nutrient intake. **Objective.** Our objectives were to characterize the microbiome of milk produced by healthy lactating women during the first 6 mo postpartum and determine associations between milk bacteria and possible mediating factors such as maternal nutrient intake, delivery mode, and adiposity. **Methods.** Milk samples were collected at 9 time points from 21 lactating women from d 2 to 6 mo postpartum; bacterial taxa were characterized using high-throughput sequencing of the 16S rRNA gene. **Results.** The microbial community structure of milk was relatively constant over time within a woman, although the relative abundances of several bacterial taxa were associated with maternal adiposity, delivery mode, infant sex, and chronic maternal dietary intake. For instance, current BMI was positively correlated with *Lactobacillus* (r = 0.32, *P* = 0.005), and higher energy consumption was associated with higher relatively abundance of *Gemella* (r = 0.58, *P* = 0.007*)*. Overall consumption of saturated and monounsaturated fatty acids was related to higher relative abundance of members of the Proteobacteria phylum*,* and total carbohydrates, as well as lactose and total disaccharides, were inversely associated with relative abundance of Firmicutes (r = -0.54, -0.51, -0.47; *P* = 0.008, 0.004, 0.001, respectively). Total protein intake was positively correlated with relative abundance of *Gemella* (r = 0.46; *P* = 0.005). We also observed multiple relationships between chronic micronutrient consumption and milk microbiome patterns. **Conclusions.** Factors mitigating the composition of the bacterial community in milk are complex, and may include chronic maternal nutrient,

maternal adiposity, delivery mode, and infant sex. Future studies designed to investigate these relationships should strive to analyze milk samples for their nutrient composition. **Keywords:** milk, human, maternal, microbiome, microbiota, nutrients

3.2 INTRODUCTION

Milk, a complex matrix that supports the development of the nursing young, contains lipids, simple sugars (mainly in the form of lactose), oligosaccharides, proteins, and many other biologically-active factors such as immune cells, stem cells, hormones, and immunoglobulins. The mammary gland and milk also provide a unique niche for a diverse community of bacteria (reviewed by McGuire and McGuire, 2015). Culture-dependent methods have long shown the presence of various bacterial populations, such as *Staphylococcus* spp. (Eidelman and Szilagyi, 1979) particularly in milk produced by women with mastitis, and *Lactobacillus* spp. (Martín et al. 2003; Heikkilä and Saris, 2003), but culture-independent methods now suggest that a much more complex bacterial community exists, even in milk produced by healthy women (Collado et al. 2009; Hunt et al. 2011; Jost et al. 2013). Differences in the proportions of various bacteria have been reported among studies, but at the phylum level, Firmicutes have usually been shown to be predominant followed by Proteobacteria (Cabrera-Rubio et al. 2015). At the genus level, *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Pseudomonas*, *Propionibacterium*, and *Bifidobacterium* are often reported as being present in greater relative abundances than other bacterial genera (Hunt et al. 2011; Jost et al. 2013).

Composition of human milk bacterial communities may be influenced by a variety of factors including introduction of bacteria from the infant's mouth (Ramsey et al. 2004; Geddes 2009), skin bacteria, and potentially the maternal gastrointestinal (GI) system. Results from several studies (Perez et al. 2007; Donnett-Hughes et al. 2010; Jiménez et al. 2008; Arroyo et al. 2010; Jost et al. 2014) have provided evidence for the transfer of bacteria from the maternal GI

tract to the mammary gland through an entero-mammary pathway, first hypothesized by Martín and coworkers in 2004. Both *in vivo* and *in vitro* studies provide support that dendritic cells may translocate non-invasive bacteria from the GI epithelium to the mammary gland via the lymphatic system, and thus maternal GI bacteria may become part of the milk microbiome. Consequently, factors such as maternal nutrient intake thought to directly influence the maternal GI bacterial community may also indirectly impact the milk microbiome.

The relationship between maternal nutrient intake and maternal GI bacterial communities was investigated recently by Carrothers et al. (2015), who provided initial evidence for statistical associations between myriad macro- and micronutrients and maternal fecal microbial community structure. Additionally, since maternal diet influences concentration of some substances (e.g., fatty acids) in milk (reviewed by Lönnerdal 1986; Jensen 1995; Ballard and Morrow 2013; Innis 2014), maternal nutrient intake may indirectly help shape the bacterial community membership and structure in milk simply due to its impact on milk nutrient composition. To our knowledge, however, there exists no literature exploring the link between maternal nutrient intake (particularly micronutrients) and the milk microbiome.

Therefore, this study was designed primarily to investigate the potential association between maternal nutrient intake and milk microbial communities during the first 6 mo postpartum in healthy breastfeeding women. We hypothesized that we would find correlations similar to those (between maternal dietary intake and maternal fecal microbiome) found previously by Carrothers et al. (2015), although we expected the relationships to be less consistent and/or strong. It is noteworthy that the milk samples analyzed in the present study were collected in conjunction with the fecal samples collected from the women studied previously by Carrothers and colleagues (2015).

3.3 METHODS

3.3.1 *Subjects and study design*

This was a prospective, longitudinal investigation of 21 self-reported healthy, breastfeeding women who, in their third trimester of pregnancy, were recruited from the Pullman, WA/Moscow, ID area. Written informed consent was obtained in accordance with procedures approved by the Washington State University and the University of Idaho Institutional Review Boards. Samples and data were collected on 2, 5 and 10 d (± 1 d), and 1, 2, 3, 4, 5, and 6 mo (± 1 d) postpartum. Body mass index (BMI) was considered in two ways: pre-pregnancy BMI (as reported by each subject at enrollment) and current BMI (as measured at each sampling period). Each woman was classified as either normal weight (< 25 kg/m^2) or overweight/obese (≥ 25 kg/m^2).

3.3.2 *Sample collection*

Milk samples were collected at each of the 9 time points either at the subject's home, a local hospital, Washington State University, or the University of Idaho. Women were asked to provide a full expression from one breast using an Ameda-Egnell Elite™ pump and single-use sterile collection kit (Ameda HygieniKit®). Fresh milk samples were aliquoted and stored immediately at -80 °C (when collected at a university site) or kept on ice until transported to the university (when collected at a home or hospital site) and then frozen at -80 °C.

3.3.3 *Maternal diet records*

With the assistance of trained study personnel, a comprehensive, quantitative 24-hr dietary recall was completed for each subject at each time point. All foods and beverages (but not dietary supplements) were recorded and included in the analysis. Diet records were entered into Genesis R&D (version 7.6, ESHA Research, Salem, OR) and energy, macronutrient intakes, and selected micronutrient intakes estimated.

3.3.4 *Extraction and amplification of bacterial DNA*

Milk samples (0.5 to 10 mL, depending on the volume available) were thawed on ice and then centrifuged at 13,000 x g for 10 min at 4 °C. The fat layer was carefully removed and the supernatant discarded. The remaining cell pellet was resuspended in 0.5 mL TE50 (10 mM Tris-HCl, 50 mM EDTA, pH 8). Samples were subjected to enzymatic lysis by adding 100 µL of a mixture containing 50 µL lysozyme (10 mg/mL in nuclease-free water; Sigma-Aldrich, St. Louis, MO), 6 µL mutanolysin (25 KU/mL in nuclease-free water; Sigma-Aldrich), 3 µL lysostaphin (4000 U/mL in 20 mM sodium acetate; Sigma-Aldrich), and 41 µL TE50 for one hr at 37 °C on a dry-heat block, and then physical disruption by bead-beating with 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) for 1 min on setting 5 using a FastPrep FP120 (Qbiogene, Carlsbad, CA). DNA was subsequently extracted using the QIAamp DNA Mini Kit (Qiagen Cat. 51304, Valencia, CA) following the manufacturer's protocol. TE50 (0.5 mL) was used as a negative control. Extracted DNA was eluted in 25 µL nuclease-free water and stored at -80 °C until further analysis. A dual-barcoded two-step PCR was conducted to amplify the V1-V3 hypervariable region of the bacterial 16S rRNA gene; a 7-fold degenerate forward primer targeting position 27 and a reverse primer targeting position 534 (positions numbered according to the *Escherichia coli* rRNA) were used. Primer sequences are provided in Appendix B.

DNA was amplified in a dedicated PCR hood. The first PCR mixture contained 5-10 µL extracted DNA, 0.05 µM target-specific primers (Integrated DNA Technologies, Coralville, IA), 5 µL 10X PCR buffer (Life Technologies, Carlsbad, CA), 3 mM $MgCl_2$ (Life Technologies), 0.24 mg/mL bovine serum albumin (Sigma, St. Louis, MO), 0.2 mM dNTP (Life Technologies), 0.25% DMSO, and 0.05 U/µL AmpliTaq® DNA 360 polymerase (Life Technologies) in a total volume of 50 µL. PCR were conducted using either an Applied Biosystems 2720, Veriti, or ProFlex model thermocycler under the following conditions: 95 °C for 2 min, then 95 °C for 20 sec, 60 °C for 30 sec, and 72 °C for 1 min for 20 cycles with a 0.5 °C step-down in the annealing temp each cycle, then 95 °C for 20 sec, 50 °C for 30 sec, and 72 °C for 1 min for 20 cycles, and then a final

extension step of 72 °C for 5 min. Samples were held at 4 °C in the thermocycler until being stored at -20 °C.

Products from the first PCR were electrophoresed on 1% agarose gels made with tris-acetate-ethylenediamine tetraacetic acid (TAE; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer and containing ethidium bromide (0.0007 mg/mL). Gels were allowed to run for 30 min at 80 V, and bands viewed using the BioRad UltraCam Digital Imaging System (Hercules, CA). Samples with high-quality amplicons (relatively bright band of interest at ~534 bp), low primer-dimers, and absence of unwanted bands or smears were deemed acceptable for the second PCR reaction. PCR products were diluted (1:14) with nuclease-free water and 2-4 µL were subjected to a second round of PCR in a reaction mix containing 75 nM primers with dual-index barcodes and Illumina sequencing adapters (University of Idaho IBEST Genomics Resources Core Facility), 2 µL 10X PCR buffer (Life Technologies, Carlsbad, CA), 4.5 mM $MgCl_2$ (Life Technologies), 0.6 mg/mL bovine serum albumin (Sigma, St. Louis, MO), 0.2 mM dNTP (Life Technologies), and 0.05 U/µL AmpliTaq DNA 360 polymerase (Life Technologies) in a total volume of 20 µL. PCR was conducted using an Applied Biosystems 2720 thermocycler under the following conditions: 94 °C for 5 min, then 94 °C for 30 sec, 60 °C for 45 sec, and 72 °C for 1.5 min for 20 cycles with a 0.5 °C step-down in the annealing temp each cycle, then 94 °C for 30 sec, 50 °C for 45 sec, and 72 °C for 1.5 min for 10 cycles, and then a final extension step of 72 °C for 5 min. Samples were held at 4 °C in the thermocycler until being stored at -20 °C. Quality of 2nd PCR amplicons was evaluated by diluting the 2nd PCR 1:14 with QX DNA Dilution Buffer (Qiagen) and using a QIAxcel DNA screening cartridge (Qiagen Cat. 929004), and DNA quantified using picogreen (Life Technologies).

An appropriate volume of each amplicon (containing 50 ng DNA) was pooled to create a composite sample for high-throughput sequencing. Amplicon pools were size-selected using AMPure beads (Beckman Coulter, Indianapolis, IN). The cleaned amplicon pool was quantified using the KAPA Illumina library quantification kit (KAPA Biosciences, Wilmington, MA) and the Applied

Biosystems StepOne Plus real-time PCR system. Sequences were obtained using an Illumina MiSeq v3 (San Diego, CA) paired-end 300-bp protocol for 600 cycles.

3.3.5 *Sequence analysis*

Raw DNA sequence reads from the Illumina MiSeq were demultiplexed and classified in the following manner. The custom python application dbcAmplicons (https://github.com/msettles/dbcAmplicons) was used to identify and assign reads by both expected barcode and primer sequences. Specifically, the application performs processing of raw reads (preprocessing), joining of overlapping paired reads into longer single reads, classification of reads to the genus level, and generation of abundance tables. During preprocessing, barcodes were allowed to have at most 1 mismatch (hamming distance), and primers were allowed to have at most 4 mismatches (Levenshtein distance) as long as the final 4 bases of the primer perfectly matched the target sequence. Reads identified as not having a corresponding barcode and primer sequence were discarded. Reads were then trimmed of their primer sequence and merged into a single amplicon sequence using a modified version of FLASH (Magoč and Salzberg 2011) and the Ribosomal Database Project Bayesian classifier (Wang et al. 2007) was used to assign sequences to phylotypes. Reads were assigned to the first RDP taxonomic level with a bootstrap score ≥ 50.

3.3.6 *Longitudinal characterization of bacterial community composition*

Sequence counts were converted to relative abundance values and visualized using area graphs. A generalized linear mixed model assuming a beta distribution and logit link was used to separately assess the effect of time on the relative proportion of each of the 10 most abundant bacterial populations at each taxa level (Stroup 2012). Non-metric dimensional scaling (NMDS) and principal component (PCA) analysis of relative bacterial proportions were conducted to examine patterns among the similarity and variation, respectively, among

complex bacterial community structures and other factors such as time, dietary intake of various nutrients, and birth mode.

3.3.7 *Spearman rank correlation analysis*

To characterize and visualize associations present between bacterial taxa proportions and adiposity (BMI) or nutrient intakes, heat maps of Spearman rank correlation coefficients were constructed using the vegan and gplots packages in R 3.2.2 (R Core Team 2015). To help control for multiple comparisons, associations were deemed significant in this discovery phase of the analysis if $P \leq 0.01$ and the Spearman rho was ≤ -0.3 or ≥ 0.3; however, due to the exploratory nature of this analysis we also denote weaker "trends" at $P \leq 0.05$ and similar Spearman rho. Correlations between dietary intake and bacterial abundances were examined using both 1) nutrient intake and bacterial abundance variables at each sampling time point (acute affects) and 2) averages of the nutrient intake and bacterial abundance variables across all time points (chronic affects).

3.3.8 *Additional inferential statistics*

Additional analyses to relate selected metadata (e.g., birth mode, dietary variables) to variation in complex microbial community structure were assessed using GLMM in SAS as appropriate. The dependent variables included in these models were the proportions of the four most abundant phyla (Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria) and the 10 most abundant genera; independent variables were categorical metadata such as subject, parity, and birth mode. All statistical analyses, other than the generation of heat maps as mentioned above, were carried out using SAS 9.4 (SAS Institute Inc., Cary, NC).

3.4 RESULTS

3.4.1 *Subject description and sample disposition*

Information related to basic anthropometrics and reproductive history for all 21 women at enrollment has been described previously (Carrothers et al. 2015). Briefly, women were 30 ± 4 y old, weighed 64 ± 7 kg prior to pregnancy, and had a mean parity of 1.8 ± 1 children. Although milk samples were collected from most women at each time point, some women did not provide milk samples at certain time points, and some milk samples did not yield sufficient PCR amplicon products. In total, this resulted in 165 milk samples for which we could obtain sequencing data. In addition, because our goal was to characterize bacterial communities of milk produced by "healthy" women and their infants, data from 22 of these samples were excluded from the data analysis because the mother reported that she or her infant had taken antibiotics during the time since the previous sample was collected.

3.4.2 *Dietary intake*

Mean dietary energy, macronutrient, and selected micronutrient intakes have also been described previously (Carrothers et al., 2015). In general, women consumed energy and nutrients at levels that would be expected for well-nourished, lactating women (Institute of Medicine, 2006). It is noteworthy that there was an effect of time on carbohydrate (g/d) and energy (kcal/d) intakes such that these dietary components generally decreased as time postpartum advanced; there was, however, no effect of time on intake of any of the other nutrients that we assessed.

3.4.3 *Overall sequencing depth summary and final disposition of samples*

A total of 1,807,857 sequences were obtained following cleaning and quality checking with a range of 19 to 30,900 sequences per sample. Of the 165 samples sequenced, 133 samples from 21 women yielded > 5000 sequences (range 5180-30,900; mean ± SEM = 12,897 ± 505). After removing samples due to low sequencing read counts, i.e. < 5000 reads, or antibiotic use by mother

and/or infants, a total of 104 milk samples were utilized for this analysis: 5, 12, 9, 17, 15, 14, 10, 10, and 12 samples at 2, 5, and 10 d and 1, 2, 3, 4, 5, and 6 mo, respectively.

To assess sampling and sequencing depth, individual-based rarefaction and accumulation curves were generated. Individual-based rarefaction curves (see Figure 3.1) suggested that we could confidently analyze data classified at the order, class, and phylum levels from samples with at least 5000 sequences. Consequently, analysis of data at the genus and family levels was completed to obtain preliminary results and generate hypotheses for future investigations at greater sequencing depth. Accumulation curves (see Figure 3.2) suggested that sampling depth (n = 165) was sufficient at the family, order, class, and phylum level but more samples would be needed to have sufficient sampling at the genus level. As such, analysis of data at the genus level was completed to obtain preliminary results and generate hypotheses for future investigations.

3.4.4 *Overall milk bacterial community structure*

Relative proportions of the 4 most-abundant phyla and 10 most-abundant genera over all time points are found in Table 3.1. Area charts showing the 10 most-abundant bacterial groups at each time point for phylum and genus levels are shown in Figure 3.3 with an enlarged view of the taxa without the dominant phyla and genera shown in Figure 3.1C and 3.1D, respectively. In general, members of the Firmicutes phylum were predominant across all time points, followed by lesser amounts of Actinobacteria, Proteobacteria, and Bacteroidetes. At the genus level, bacterial communities were dominated by *Streptococcus* and *Staphylococcus.* Three genera (*Streptococcus*, *Staphylococcus*, *Propionibacterium*) were found in all samples; while 7 other genera (*Pseudomonas*, *Veillonella*, *Pilibacter*, *Gemella*, *Bacteroides*, *Prevotella*, and *Corynebacterium*) were found in at least 90% of the samples.

3.4.5 *Effects of time, adiposity, parity, infant sex, and maternal age on milk microbiome*

Effect of time on relative abundance of the most-abundant bacterial genera in milk is shown in Table 3.2. Analysis using GLMM indicated only relative abundance of *Gemella* consistently decreased over time ($P < 0.03$); this decrease occurred in the first 10 d postpartum, after which time relative abundance of *Gemella* remained constant. There was also an effect of subject ($P < 0.05$) on the 10 most abundant bacterial groups across the taxonomic levels, suggesting likely important inter-woman differences.

Because of the relative stability of the milk bacterial communities over time, additional analyses designed to mediate investigative factors considered all of the samples together, regardless of when they were collected. Overall and utilizing the GLIMMIX analysis, there was no effect of mother's age or parity on the relative abundances of the overall 10 most-abundant genera, although pre-pregnancy BMI and delivery mode were both related ($P < 0.05$) to the relative abundance of *Veillonella*. Milk produced by overweight/obese mothers (pre-pregnancy BMI ≥ 25 kg/m^2) had higher relative abundance of *Veillonella* compared to that produced by women with a BMI < 25 kg/m^2 (3.8 ± 1.0 vs. 1.9 ± 0.5%, respectively; $P < 0.05$). Milk produced by mothers who underwent a cesarean-section had a lower relative abundance of *Veillonella* than milk produced by women who delivered vaginally (1.1 ± 0.5 vs. 3.0 ± 0.6%, respectively; $P < 0.05$). Additionally, milk from mothers of male infants had a higher relative abundance of *Streptococcus* compared to milk from mothers of female infants (53.5 ± 4.6 vs. 35.6 ± 5.6%; $P < 0.05$).

To investigate factors related to complex bacterial community composition (rather than single genera), PCA of the relative abundances of the overall 10 most-abundant genera in milk found that the first component axis accounted for 77.8% of the variation and that *Staphylococcus* and *Streptococcus* were the primary contributors to this axis. Visual examination of the PCA ordination plots (plots not shown) suggested no clustering patterns by time, pre-pregnancy BMI, infant sex, delivery mode, subject, or parity. NMDS plots (plots not shown) also showed no clustering associated with the variables listed above.

3.4.6 *Associations between diet and relative abundance of single bacterial taxa*

As mentioned previously, we evaluated the relationship between nutrient intake and milk microbiome in two ways: using nutrient intake and milk microbiome data at each time point (acute effect) and assessing relationships between mean dietary and microbiome values across all time points (chronic effect). When investigating the acute effect, only total ash (minerals) met both the *P* value threshold ($P \leq 0.05$ for trend or ≤ 0.01 for significance) and a Spearman correlation coefficient (≤ -0.3 or ≥ 0.3) at several different taxonomic levels [i.e. *Prevotellaceae* (family), *Bacteroidale*s (order), *Bacteroidia* (class), *Bacteroidetes* (phylum)]. However, we found substantially more relationships between diet and milk microbiome when we looked at them on a more chronic basis. Indeed, heat maps illustrating correlations between chronic dietary variables (BMI, and average energy and nutrient intake values over the study) and mean relative abundance of the top 10 bacterial phyla and genera (Figure 3.4) revealed numerous correlations. Some of these associations are highlighted and summarized in Table 3.3. Heat maps illustrating correlations at the class, order, and family level are depicted in Figure 3.5.

For instance, current BMI was positively correlated with relative abundance of *Lactobacillus* in milk ($r = 0.32$, $P = 0.005$), and higher energy consumption was associated with higher relatively abundance of *Gemella* ($r = 0.58$, $P = 0.007$); both of these genera are members of the Firmicutes phylum. In general, overall consumption of saturated and monounsaturated fatty acids was related to higher relative abundance of members of the Proteobacteria phylum. Consumption of total carbohydrates, as well as lactose and total disaccharides, were found to be inversely associated with relative abundance of Firmicutes ($r = -0.54, -0.51, -0.47$; $P = 0.008, 0.004, 0.001$, respectively). *Rothia* (a member of the Actinobacteria phylum) was highest in milk produced by women consuming the most insoluble fiber ($r = 0.48$, $P = 0.011$). Total protein intake was positively correlated with relative abundance of *Gemella* ($r = 0.46$; $P = 0.005$), although in general, greater consumption of the essential amino acids was related to increased abundance of members of the Proteobacteria and Fusobacteria phyla.

We also observed multiple relationships between chronic micronutrient consumption and milk microbiome patterns. For instance, consumption of pantothenic acid was positively related to relative abundance of *Staphylococcus* (r = 0.38; *P* = 0.047) and negatively correlated with that of *Streptococcus* (r = -0.44; *P* = 0.018). Riboflavin and vitamin D consumption were both positively associated with *Veillonella* (r = 0.52, 0.31; *P* = 0.031, 0.004, respectively); consumption of the latter was inversely correlated with percent abundance of member of the Firmicutes phylum (r = -0.50; *P* = 0.007). *Veillonella* was positively associated with consumption of total ash (r = 0.32; *P* = 0.005) and several essential minerals such as calcium, chloride, iodine, molybdenum, and phosphorus (r = 0.32 to 0.58; *P* = 0.0001 to 0.012).

3.5 DISCUSSION

Results from this study reveal relatively stable microbial communities within the milk produced by a woman, although differences clearly exist among women – supporting data previously published by our research group (Hunt et al. 2011). Only the relative abundance of *Gemella* showed an effect of time. However, it is worth noting that the overall 10 most abundant genera did not always represent the top 10 most abundant genera at each time point (see Figure 3.3 and Table 3.3), and inspection of the relative abundances of these individual genera did indicate some minor changes over time. It is also possible that future studies that more closely inspect the relative abundances of the rarer taxa may indicate additional changes in overall community composition across early lactation.

Nonetheless, our data concerning microbial composition of human milk are generally similar to that previously published by others (Jost et al. 2015). For example, microbial composition of milk samples (*n* = 5) collected by us on d 2 postpartum were similar in composition to those described by Obermajer and coworkers (2015) with *Gemella*, *Streptococcus*, and *Staphylococcus* being present in all samples. *Pseudomonas*, *Prevotella*, *Propionibacterium*, *Pilibacter*, *Veillonella*, *Corynebacterium*, *Clostridium*, and *Bacteroides* were also present in

all d 2 samples, and *Lactobacillus* and *Lactococcus* were present in 3 of the 5 samples. Unlike what was reported by Cabrera-Rubio and coworkers (2012), however, *Weisella* and *Leuconostoc* were not present in our d 2 milk samples. Additionally, *Bifidobacterium*, *Enterococcus*, and *Enterobacter* were not identified in any of the samples collected at d 2 postpartum.

Importantly, this study is the first, to our knowledge, that describes the relationships between the mother's diet and the bacterial communities in her milk over the first 6 mo of lactation. These data, combined with those supporting an endogenous cellular route for the translocation of bacteria from the maternal GI tract to the mammary gland (Rodríguez 2014) suggest that maternal diet (particularly when considered on a chronic basis) may play a key role in determining the bacterial community in milk. Maternal diet may be exerting this influence through a variety of modes such as alteration of the composition of milk components and/or by dictating the representation of specific bacterial groups in the maternal GI tract.

Whereas there is almost no information about the relationship between maternal diet and milk microbiome, several studies have linked diet to alterations in the GI microbiota (reviewed by Albenberg and Wu, 2014). Recently, we have also provided evidence of associations of particular bacteria in the maternal GI tract with dietary intake of several nutrients. For example, data from our group (Carrothers et al. 2015) suggest that increased maternal consumption of pantothenic acid, riboflavin, vitamin B-6, and vitamin B-12 are related to increased relative abundance of *Prevotella* ($r = 0.45$, 0.39, 0.34, and 0.24, respectively; $P \leq 0.01$) in maternal feces during lactation. However, *Prevotella* was not one of the most abundant bacterial genera in the milk produced by these women. However, we uncovered myriad other relationships that are noteworthy. For instance, maternal consumption of copper, magnesium, manganese, and molybdenum appear to be positively associated with relative abundance of Firmicutes ($r = 0.33$, 0.38, 0.44, and 0.51, respectively; $P \leq 0.01$) in milk; and consumption of several minerals was associated with abundance of *Veillonella* in the milk. Essential amino acid-rich diets were correlated with increased

abundance of Proteobacteria and Fusobacteria, and saturated and monounsaturated fatty acid intakes were correlated with abundance of Proteobacteria.

We posit that one mechanism whereby maternal diet can play a role in the composition of the milk bacteria is through the following pathway. Since diet influences the composition of the GI microbiota, the types of bacteria sampled, harbored, and transported by dendritic cells as demonstrated by Macpherson and Uhr (2004) could also be altered. The increased trafficking of these cells and/or PBMC to the mammary gland during pregnancy and lactation (Donnet-Hughes et al. 2010) would then be directing different bacteria to the mammary gland and result in a varied bacterial composition. However, more research is needed to investigate this potential mechanism and better understand the migration of cells in the mucosal immune system during pregnancy and lactation.

Nonetheless, many previously published studies have demonstrated that maternal diet can influence the concentrations of some of the components in milk, many of which mitigate bacterial growth (Hoppu et al. 2012; Thum et al. 2012). For example, maternal diet plays a key role in determination of the lipid composition in milk (Finley et al. 1985; Hachey et al. 1989). Triglycerides comprise at least 98% of the lipids (Jensen 1995) and, upon *in vivo* hydrolysis by lipases, fatty acids are released. Both *in vivo* (Isaacs et al. 1990) and *in vitro* (Kelsey et al. 2006) studies have demonstrated that these resultant fatty acids can have bacteriostatic or bactericidal properties, with some being more potent than others. Alternatively, some fatty acids have been shown to promote the growth of particular bacterial populations such as *Lactobacillus* spp. (Lhuillery et al. 1981; Williams and Fieger 1946; Partanen et al. 2001). In a recent study, Nejrup and coworkers (2015) tested various combinations of non-esterified fatty acids, monoacylglycerols, and sphingosine that mimicked products from hydrolysis of human milk fat in an *in vitro* fermentation system with infant fecal bacteria as the inoculum. Relative abundances of both *Bifidobacterium* and *Lactobacillus* as determined by quantitative PCR were found to be different in the varying mixtures. Using 16S rRNA sequencing of the fermentation samples,

relative abundance of Proteobacteria decreased with the addition of long-chain fatty acids and sphingosine. Interestingly, in our study relative abundance of Proteobacteria was positively related to maternal intake of several fatty acids, including unsaturated fatty acids [e.g., pentadecanoic (15:0), palmitic (C16:0), and stearic (C18:0) acids]; monounsaturated fatty acids (e.g., myristoleic (C14:1), palmitoleic (C16:1), C17:1, and oleic (C18:1*c*9) acids); and the polyunsaturated fatty acid rumenic acid (C18:2*c*9*t*11). Unfortunately, fatty acid analysis was not completed on these milk samples, so we cannot evaluate whether maternal intake of these fatty acids was associated with milk fatty acid concentrations. More work is needed to ascertain relationships between specific milk components, such as fatty acids, and the microbial community profile of human milk. Results from our study, however, suggest it is plausible that maternal diet influences bacterial community structure in milk. Additional studies will need to be conducted to ascertain whether dietary intake of various nutrients induce changes in the milk composition which can then be associated with variation in milk bacterial communities.

Maternal diet may also be one of the factors driving the observed differences in milk microbial communities among women with different body weights (Cabrera-Rubio et al. 2012) and also among women with different pregnancy weight gains (Collado et al. 2012). Collado and coworkers (2012) found higher levels of both *Staphylococcus* and *Akkermansia* and lower levels of *Bifidobacterium* in milk produced by overweight mothers as compared with normal-weight women at 1 and 6 mo postpartum. We also found higher relative abundance of *Staphylococcus* in milk produced by overweight/obese women. Collado and colleagues (2012) also found that weight gain during pregnancy was associated with composition of milk microbiota during the first 6 mo postpartum. Specifically, when compared to milk produced by women with healthy weight gain (11.5 – 16.0 and 7.0 – 11.5 kg for normal weight and overweight women, respectively; defined in accordance with the recommendations by the Institute of Medicine, 1990) during pregnancy, excessive pregnancy weight gain (> 16 and > 11.5 kg for normal-weight and overweight mothers, respectively) was associated

with higher relative levels of *Staphylococcus* in colostrum and lower relative abundance of *Bifidobacterium* in milk produced at 1 mo postpartum.

Cabrera-Rubio and coworkers (2012) also reported differences in bacterial diversity produced by overweight mothers (lower estimates of richness in colostrum and 1 mo samples) as compared to milk from normal-weight mothers. In concurrence with the results from Collado and coworkers, they also detected higher numbers of *Staphylococcus* and lower numbers of *Bifidobacterium* in the milk from obese as compared with normal-weight over the first 6 mo postpartum. Both of these studies investigating body weight composition and weight gain over 6 mo could be a reflection of the chronic dietary intake by individuals. Indeed, our data suggest that use of chronic maternal dietary intake levels (averaged over the 6 mo of our study) yielded stronger correlations with average milk microbial communities than relating acute dietary intake data with acute milk microbiome data.

Of particular interest is a general relationship between consumption of a nutrient-rich diet and presence of relatively high abundances of Proteobacteria in milk. These results differ from those we observed in the maternal fecal bacterial community in which a nutrient-rich diet was associated with a higher relative abundance of Firmicutes and lower relative abundances of *Bacteroidetes*. Proteobacteria are known to be remarkably versatile in using various carbon sources such as carbohydrates, amino acids, and lipids for ATP and energy production (Kazakov et al. 2009). In our study we also noted positive associations with intakes of several amino acids and Fusobacteria. It is interesting to note that most species of Fusobacteria are proteolytic (Gillespie and Hawkey, 2006). Perhaps both Proteobacteria and Fusobacteria are able to take advantage of possible changes in milk composition that occur with a more nutrient-dense diet. However, milk composition was not determined in this study, and thus the direct relationships among dietary nutrient density, milk nutrient composition, and milk microbiome structure could not be evaluated. Since these data are compositional in nature and no directionality or causality can be inferred from correlations such as these, future studies are warranted to elucidate

whether one or both populations of bacteria can be impacted by variation in nutrient intake. Understanding these relationships is key to providing important insight as to ways that maternal factors influence infant health and development, and potentially mammary health.

In addition to these relationships between maternal diet and milk microbial community structure, we found a difference in relative abundance of *Veillonella* between women delivering via cesarean section and those delivering via vaginal birth: milk produced by the former had a lower relative abundance of *Veillonella* then milk produced by the latter (1.1 ± 0.5 vs. 3.0 ± 0.6%, respectively; $P < 0.05$). In addition, milk produced by mothers of male infants had a higher relative abundance of *Streptococcus* compared to milk from mothers of female infants (53.5 ± 4.6 vs. 35.6 ± 5.6%; $P < 0.05$). The significance of these findings (if any) is currently unknown, and others should verify these relationships prior to suggesting such.

Understanding and manipulating the genesis and dynamics of the bacteria in milk potentially likely have tremendous health implications for both mothers and infants. Maternal susceptibility or duration of mastitis could be directly related to the proportions and types of bacteria that are present in the mammary gland (Jiménez et al. 2008; Fernández et al. 2016). Alteration to the milk bacterial community through maternal supplementation of probiotics provides evidence for the importance of various bacterial populations in regulating mammary health. Jiménez and coworkers (2008) demonstrated that women who ingested *Lactobacillus gasseri* CECT5714 and *Lactobacillus salivarius* CECT5713 had a shorter duration of mastitis and lower staphylococcal counts in their milk. Additionally, in both this study and that by Arroyo and coworkers (2010), orally administered probiotic strains were isolated in the milk samples collected from the women who had taken the probiotics. Once again, this provides evidence that maternal intake of probiotics or nutrients (i.e. the maternal diet) may indeed alter the microbial dynamics in the mammary gland.

For the infant, bacteria in milk clearly represent some of the first bacteria to which the infant is exposed. As such, bacteria in milk may serve as or interact

with some of the first colonizers of the breastfed infant's GI tract, likely stimulating the infant's immune system and setting in motion the development of immune response, not only for the immediate period of time but also perhaps for a lifetime. Additional controlled, clinical intervention studies related to the impact of environmental choices (e.g., maternal nutrient intake, probiotic consumption, exercise) are, therefore, warranted to understand the basic factors regulating human milk microbial composition, and variability therein. These studies should utilize methods appropriate for estimating chronic dietary intake (e.g., repeated 24-hr recalls or 3-day food records) and include milk composition analyses.

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Table 3.1 Overall mean relative abundances (%) of top bacterial phyla and genera in human milk samples (n = 104) produced by healthy women (n = 21) from day 2 to 6 months postpartum.

Taxonomic level	Mean ± SEM	Range (min – max)
Phylum		
Firmicutes	85.1 ± 1.2	22.7 – 97.5
Actinobacteria	5.9 ± 0.9	0.1 – 71.2
Proteobacteria	2.3 ± 0.3	0.1– 21.3
Bacteroidetes	1.3 ± 0.3	0.1 – 26.7
Genus		
Streptococcus	45.2 ± 2.6	0.2 – 88.4
Staphylococcus	25.3 ± 2.6	0.1 – 89.1
Gemella	3.6 ± 0.8	0.0 – 51.6
Veillonella	2.5 ± 0.4	0.0 – 17.4
Rothia	2.4 ± 0.4	0.0 – 23.1
Lactobacillus	1.4 ± 0.6	0.0 – 40.7
Propionibacterium	1.0 ± 0.2	0.0 – 11.9
Corynebacterium	1.0 ± 0.3	0.0 – 18.2
Pseudomonas	0.6 ± 0.1	0.0 – 4.8

Table 3.2 Mean (± SEM) relative abundances of top identified and unclassified genera in milk produced by healthy women (n = 21) from day 2 to 6 months postpartum.

Genus	d 2	d 5	d 10	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo
Streptococcus	31.4 ± 5.3	36.0 ± 6.8	40.3 ± 7.3	43.2 ± 7.4	50.6 ± 6.5	56.0 ± 8.3	42.6 ± 10.7	54.7 ± 8.4	41.3 ± 6.9
Staphylococcus	33.1 ± 10.7	35.7 ± 6.4	23.1 ± 8.4	28.9 ± 6.7	17.9 ± 5.8	22.6 ± 7.6	37.1 ± 12.3	13.3 ± 6.8	20.6 ± 7.1
Gemella	12.6 ± 6.2	11.0 ± 4.4	5.0 ± 2.3	4.3 ± 1.9	1.3 ± 0.7	1.0 ± 0.3	0.7 ± 0.3	0.9 ± 0.3	0.7 ± 0.3
Veillonella	0.4 ± 0.2	1.8 ± 0.7	2.4 ± 1.5	1.9 ± 0.6	2.0 ± 1.1	1.8 ± 0.8	1.9 ± 1.1	3.7 ± 1.4	6.1 ± 1.6
Rothia	1.1 ± 1.1	1.3 ± 1.1	3.6 ± 2.1	1.6 ± 1.3	2.3 ± 1.1	2.1 ± 1.1	3.4 ± 1.9	3.5 ± 1.4	2.6 ± 0.6
Lactobacillus	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.5 ± 2.6	3.5 ± 2.1	1.7 ± 1.1	0.6 ± 0.6	0.3 ± 0.2	0.0 ± 0.0
Propionibacterium	2.0 ± 1.4	0.2 ± 0.1	2.7 ± 0.8	0.8 ± 0.4	1.3 ± 0.6	1.2 ± 0.8	0.3 ± 0.2	0.4 ± 0.2	0.6 ± 0.4
Corynebacterium	0.5 ± 0.1	1.5 ± 0.9	1.7 ± 1.2	1.5 ± 1.1	0.3 ± 0.1	0.7 ± 0.3	0.2 ± 0.1	0.7 ± 0.5	2.0 ± 1.3
Granulicatella	0.3 ± 0.3	0.2 ± 0.1	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.8 ± 0.7	1.6 ± 1.0	2.3 ± 0.8
Pseudomonas	0.2 ± 0.1	0.3 ± 0.1	0.9 ± 0.4	0.4 ± 0.2	1.0 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	1.0 ± 0.5	0.2 ± 0.1

Prevotella	1.6 ± 1.6	0.1 ± 0.0	0.1 ± 0.1	1.0 ± 0.9	0.4 ± 0.2	0.4 ± 0.3	0.2 ± 0.1	0.5 ± 0.3	0.4 ± 0.1
Actinomyces	0.0 ± 0.0	0.1 ± 0.1	1.5 ± 1.5	0.6 ± 0.5	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.5 ± 0.2	1.4 ± 0.5
Clostridium sensu stricto	0.1 ± 0.0	0.5 ± 0.2	0.9 ± 0.4	0.6 ± 0.3	1.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
Neisseria	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 1.6
Bifidobacterium	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 1.6	0.0 ± 0.0	0.1 ± 0.1	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Haemophilus	0.3 ± 0.3	0.5 ± 0.3	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Other identified	18.5 ± 3.3	12.2 ± 0.9	20.2 ± 5.0	13.7 ± 2.4	19.6 ± 2.3	12.5 ± 1.4	12.5 ± 1.8	21.3 ± 4.7	25.8 ± 5.0
Unclassified	14.5 ± 3.1	9.5 ± 0.7	13.2 ± 3.3	9.1 ± 0.9	13.6 ± 1.8	9.7 ± 1.1	9.6 ± 1.4	15.2 ± 4.6	14.2 ± 2.3

Table 3.3 Selected significant relationships (Spearman rho and *p* values) between chronic energy and nutrient intakes and mean percent abundance of bacterial taxa in milk produced by healthy women (*n* = 21).

Diet-related variable	Bacterial taxa	Spearman Rho	*P* value
Energy and BMI			
Current BMI (kg/m^2)	*Lactobacillus*	0.32	0.005
Energy, kcal/d	*Gemella*	0.58	0.007
Lipids and fatty acids			
Total lipids, % of energy	*Verrucomicrobia*	-0.34	0.0008
MUFA, g/d	*Gemella*	0.38	0.040
12:0, g/d	*Streptococcus*	-0.40	0.030
15:0, g/d	Proteobacteria	0.35	0.009
16:0, g/d	Proteobacteria	0.40	0.0007
14:1, g/d	Proteobacteria	0.41	0.003
16:1, g/d	Proteobacteria	0.43	0.010
17:1, g/d	Proteobacteria	0.41	0.0001
18:1, g/d	Proteobacteria	0.37	0.007
18:2c9t11, g/d	Proteobacteria Fusobacteria	0.48 0.40	0.000 0.003
20:1(n-9), g/d	*Streptococcus*	0.33	0.033
Carbohydrates			
Total carbohydrates, g/d	Firmicutes *Gemella* *Veillonella*	-0.54 0.50 0.38	0.008 0.018 0.047
Disaccharides, g/d	Firmicutes Fusobacteria	-0.47 0.40	0.001 0.000003

Lactose, g/d	Firmicutes Fusobacteria *Veillonella*	-0.51 0.46 0.43	0.004 0.0000001 0.003
Maltose, g/d	*Streptococcus* *Bacteroides*	-0.33 0.58	0.039 0.034
Total fiber, g/d	*Bacteroides*	0.35	0.027
Insoluble fiber, g/d	*Rothia*	0.48	0.011
Protein and amino acids			
Total protein, g/d	*Gemella*	0.46	0.005
Cysteine, g/d	Proteobacteria Fusobacteria	0.48 0.51	0.00002 0.00009
Cysteine, g/d	Fusobacteria	0.51	0.00009
Glutamic acid, g/d	Proteobacteria	0.51	0.002
Isoleucine, g/d	Proteobacteria	0.42	0.006
Leucine, g/d	Proteobacteria Fusobacteria	0.43 0.37	0.002 0.007
Phenylalanine, g/d	Proteobacteria	0.41	0.008
Proline, g/d	Proteobacteria Fusobacteria	0.44 0.33	0.00009 0.0002
Serine, g/d	Proteobacteria Fusobacteria	0.41 0.34	0.004 0.008
Tryptophan, g/d	Proteobacteria	0.46	0.008
Tyrosine, g/d	Proteobacteria	0.40	0.005
Valine, g/d	*Proteobacteria*	0.41	0.006
Vitamins			
Pantothenic acid, mg/d	*Streptococcus* *Staphylococcus*	-0.44 0.38	0.018 0.047

Riboflavin, mg/d	*Veillonella*	0.52	0.031
Thiamin, mg/d	*Gemella*	0.50	0.001
Vitamin D, IU/d	*Veillonella*	0.31	0.004
Choline, mg/d	*Veillonella*	0.41	0.029
Vitamin D, IU/d	Firmicutes	-0.50	0.007
Mineral			
Total minerals, g/d	*Veillonella*	0.32	0.005
Calcium, mg/d	*Veillonella* *Fusobacteria*	0.58 0.37	0.005 0.0006
Chloride, mg/d	*Veillonella*	0.57	0.0001
Iodine, µg/d	*Veillonella*	0.52	0.002
Iron, mg/d	*OD1*	0.58	0.00001
Molybdenum, µg/d	*Veillonella*	0.49	0.012
Chromium, µg/d	*Gemella*	0.37	0.016
Phosphorus, mg/d	*Veillonella*	0.32	0.006

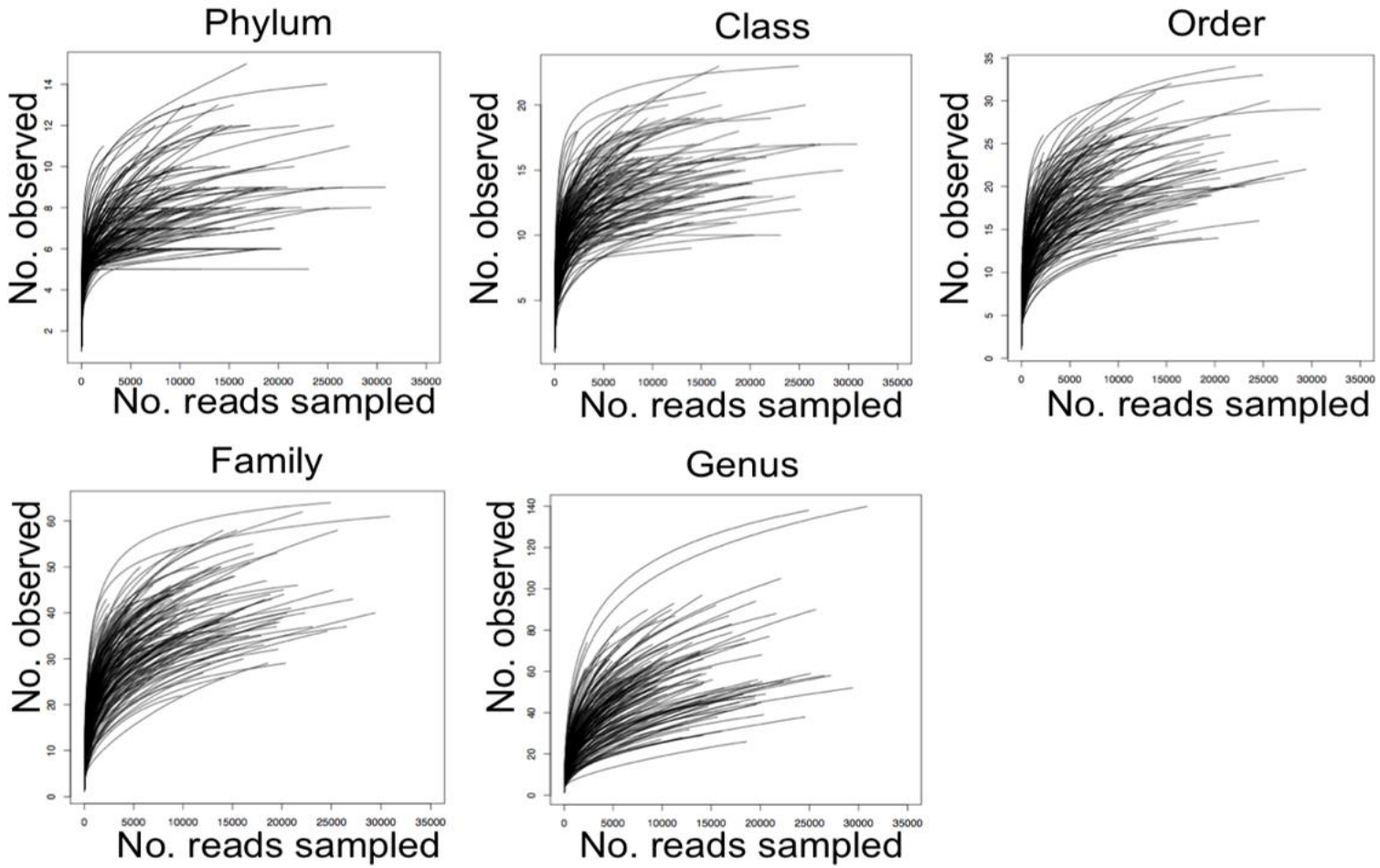


Figure 3.1 Individual-based rarefaction curves at each taxonomic level. Rarefaction curves depict estimated number of taxa observed given increased sequencing depth.

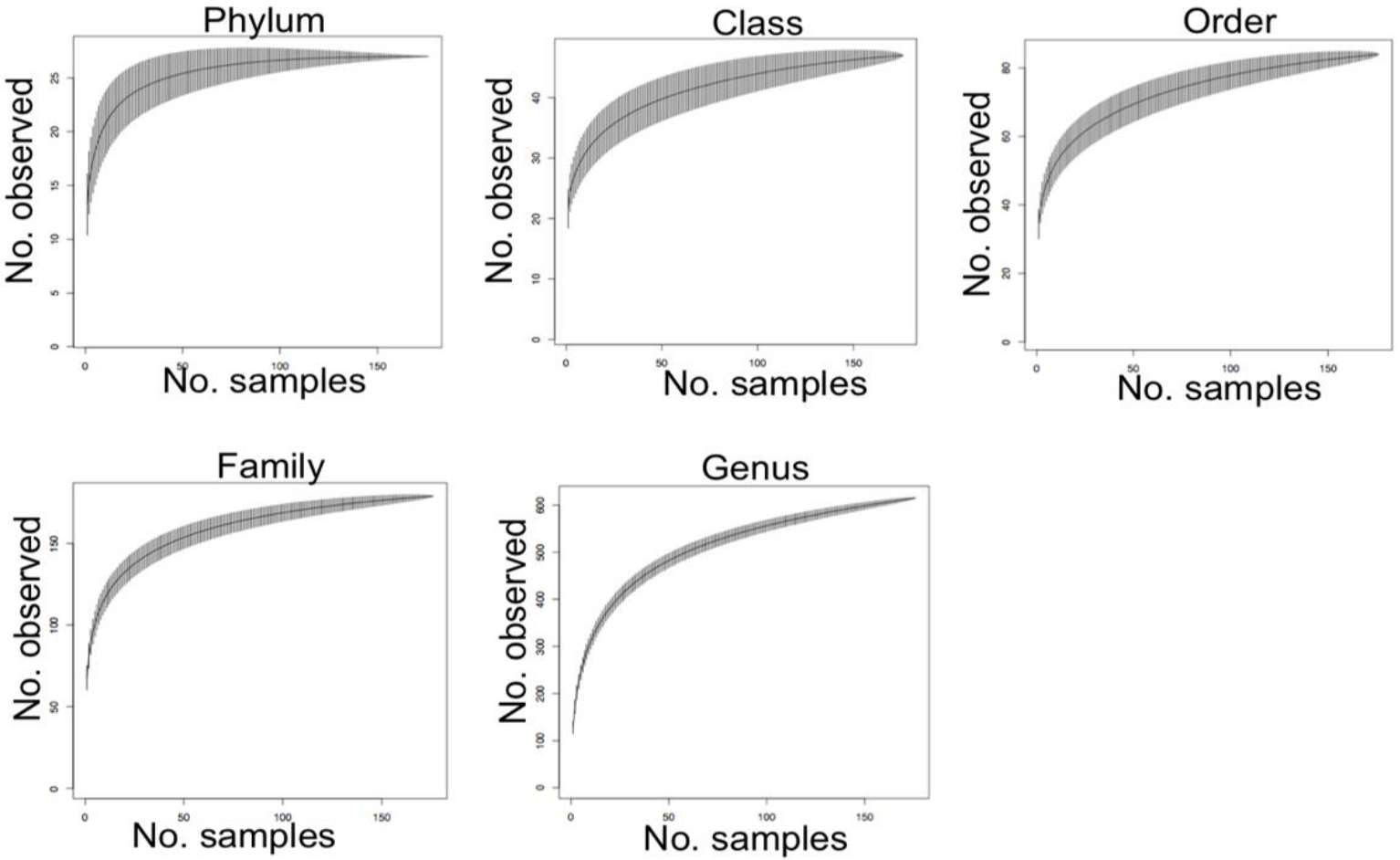


Figure 3.2 Accumulation curves at each taxonomic level. Accumulation curves depict number of estimated taxa observed given increased sampling.

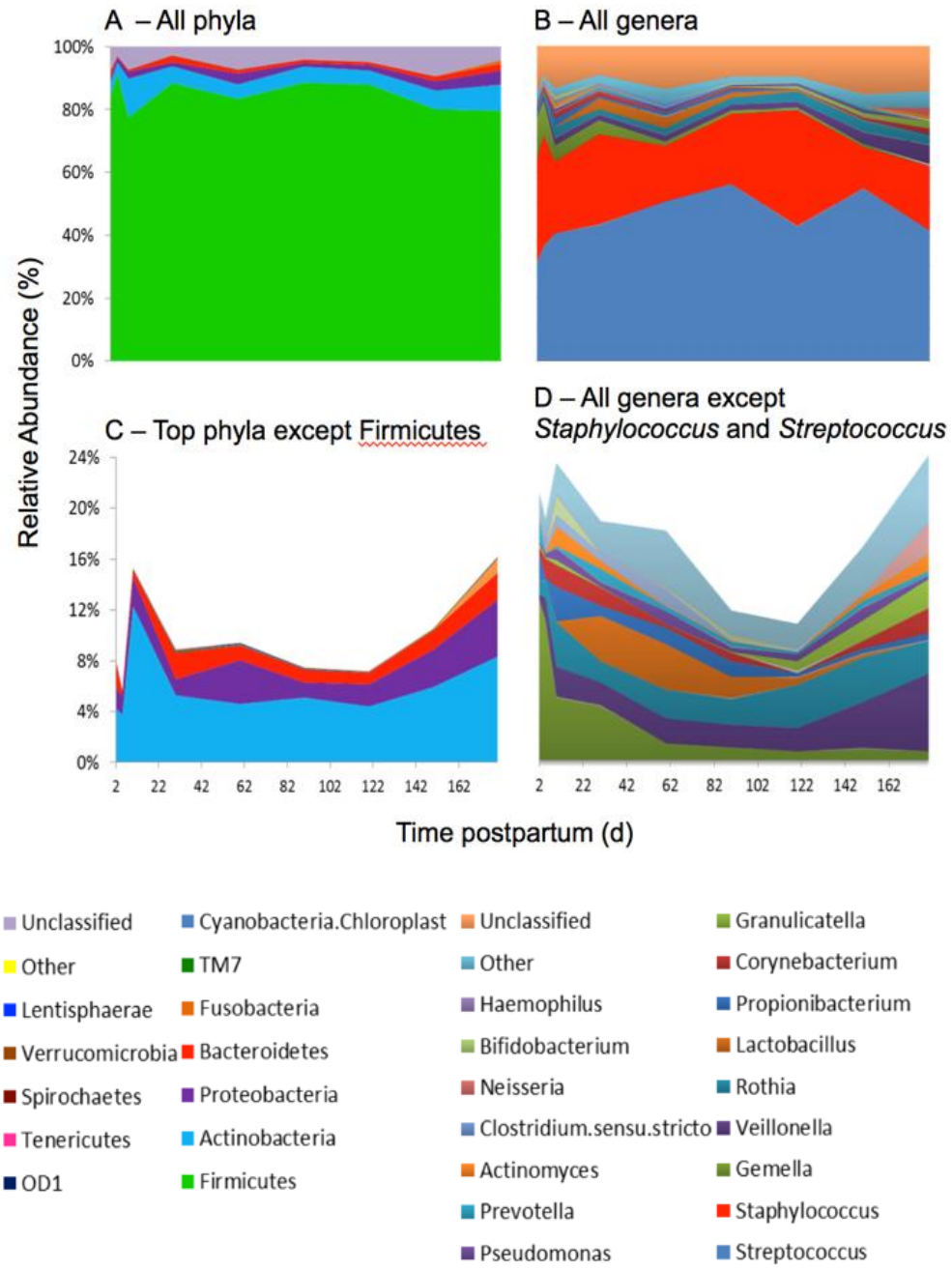


Figure 3.3 Relative abundance of 10 most abundant bacterial taxa across each time point at the phylum and genus levels (A and B); enlarged view of the most-abundant phyla with relative abundance of Firmicutes omitted (C); and the most-abundant genera with *Streptococcus* and *Staphylococcus* omitted (D).

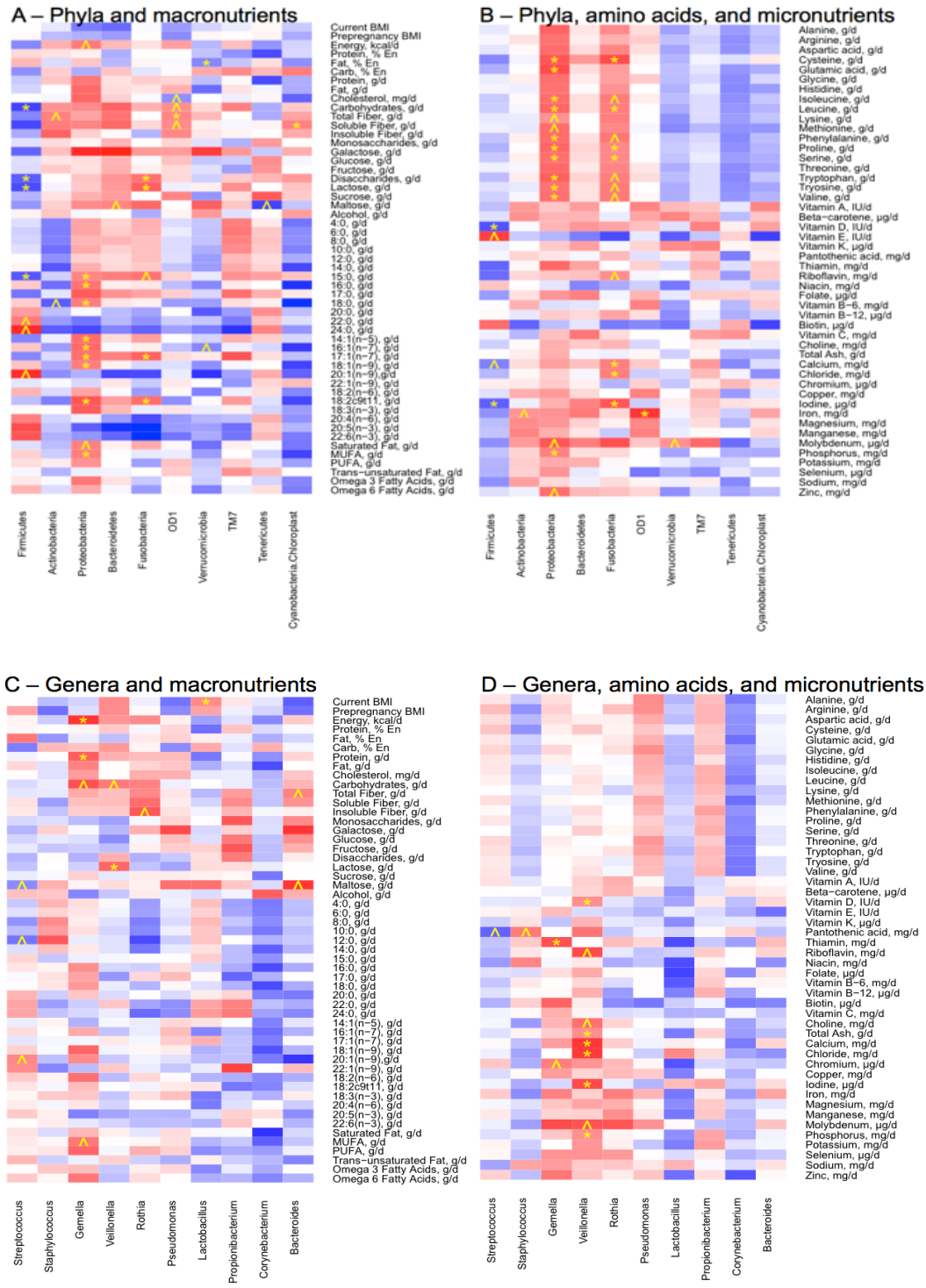
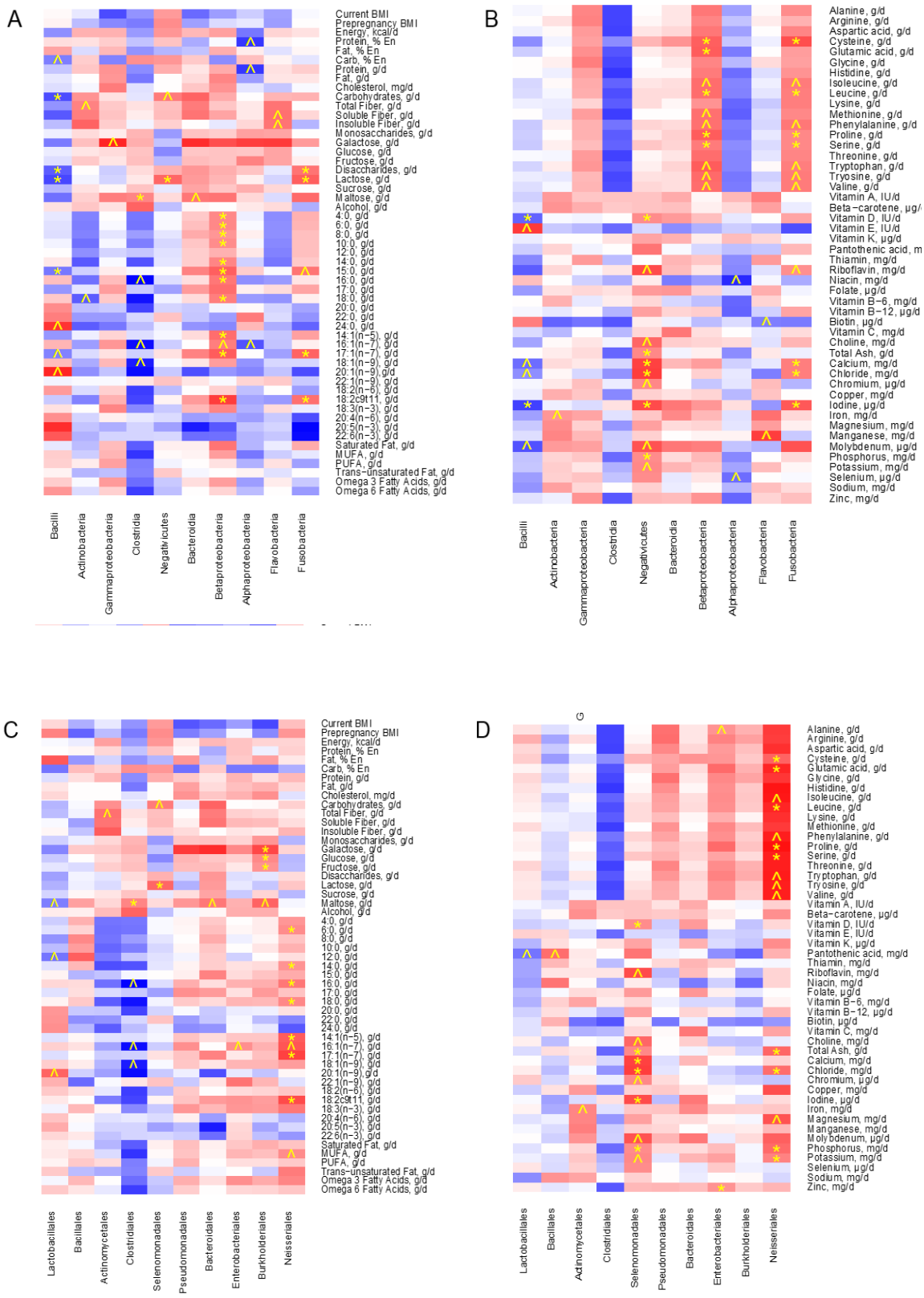


Figure 3.4 Heat maps of Spearman rank correlations between BMI, energy intake, and nutrient intake and relative abundance of the 10 most abundant bacterial phyla (A and B) and genera (C and D) in milk samples. ^ Trend ($P \leq 0.05$); *Significant ($P \leq 0.01$). Variables for each woman were averaged across all time points. % En, percentage of energy.


A
Current BMI
Prepregnancy BMI
Energy, kcal/d
Protein, % En
Fat, % En
Carb, % En
Protein, g/d
Fat, g/d
Cholesterol, mg/d
Carbohydrates, g/d
Total Fiber, g/d
Soluble Fiber, g/d
Insoluble Fiber, g/d
Monosaccharides, g/d
Galactose, g/d
Glucose, g/d
Fructose, g/d
Disaccharides, g/d
Lactose, g/d
Sucrose, g/d
Maltose, g/d
Alcohol, g/d
4:0, g/d
6:0, g/d
8:0, g/d
10:0, g/d
12:0, g/d
14:0, g/d
15:0, g/d
16:0, g/d
17:0, g/d
18:0, g/d
20:0, g/d
22:0, g/d
24:0, g/d
14:1(n−5), g/d
16:1(n−7), g/d
17:1(n−7), g/d
18:1(n−9), g/d
20:1(n−9), g/d
22:1(n−9), g/d
18:2(n−6), g/d
18:2c9t11, g/d
18:3(n−3), g/d
20:4(n−6), g/d
20:5(n−3), g/d
22:6(n−3), g/d
Saturated Fat, g/d
MUFA, g/d
PUFA, g/d
Trans−unsaturated Fat, g/d
Omega 3 Fatty Acids, g/d
Omega 6 Fatty Acids, g/d
Bacilli
Actinobacteria
Gammaproteobacteria
Clostridia
Negativicutes
Bacteroidia
Betaproteobacteria
Alphaproteobacteria
Flavobacteria
Fusobacteria
B
Alanine, g/d
Arginine, g/d
Aspartic acid, g/d
Cysteine, g/d
Glutamic acid, g/d
Glycine, g/d
Histidine, g/d
Isoleucine, g/d
Leucine, g/d
Lysine, g/d
Methionine, g/d
Phenylalanine, g/d
Proline, g/d
Serine, g/d
Threonine, g/d
Tryptophan, g/d
Tryosine, g/d
Valine, g/d
Vitamin A, IU/d
Beta−carotene, µg/
Vitamin D, IU/d
Vitamin E, IU/d
Vitamin K, µg/d
Pantothenic acid, m
Thiamin, mg/d
Riboflavin, mg/d
Niacin, mg/d
Folate, µg/d
Vitamin B−6, mg/d
Vitamin B−12, µg/d
Biotin, µg/d
Vitamin C, mg/d
Choline, mg/d
Total Ash, g/d
Calcium, mg/d
Chloride, mg/d
Chromium, µg/d
Copper, mg/d
Iodine, µg/d
Iron, mg/d
Magnesium, mg/d
Manganese, mg/d
Molybdenum, µg/d
Phosphorus, mg/d
Potassium, mg/d
Selenium, µg/d
Sodium, mg/d
Zinc, mg/d
Bacilli
Actinobacteria
Gammaproteobacteria
Clostridia
Negativicutes
Bacteroidia
Betaproteobacteria
Alphaproteobacteria
Flavobacteria
Fusobacteria
C
Current BMI
Prepregnancy BMI
Energy, kcal/d
Protein, % En
Fat, % En
Carb, % En
Protein, g/d
Fat, g/d
Cholesterol, mg/d
Carbohydrates, g/d
Total Fiber, g/d
Soluble Fiber, g/d
Insoluble Fiber, g/d
Monosaccharides, g/d
Galactose, g/d
Glucose, g/d
Fructose, g/d
Disaccharides, g/d
Lactose, g/d
Sucrose, g/d
Maltose, g/d
Alcohol, g/d
4:0, g/d
6:0, g/d
8:0, g/d
10:0, g/d
12:0, g/d
14:0, g/d
15:0, g/d
16:0, g/d
17:0, g/d
18:0, g/d
20:0, g/d
22:0, g/d
24:0, g/d
14:1(n−5), g/d
16:1(n−7), g/d
17:1(n−7), g/d
18:1(n−9), g/d
20:1(n−9), g/d
22:1(n−9), g/d
18:2(n−6), g/d
18:2c9t11, g/d
18:3(n−3), g/d
20:4(n−6), g/d
20:5(n−3), g/d
22:6(n−3), g/d
Saturated Fat, g/d
MUFA, g/d
PUFA, g/d
Trans−unsaturated Fat, g/d
Omega 3 Fatty Acids, g/d
Omega 6 Fatty Acids, g/d
Lactobacillales
Bacillales
Actinomycetales
Clostridiales
Selenomonadales
Pseudomonadales
Bacteroidales
Enterobacteriales
Burkholderiales
Neisseriales
D
Alanine, g/d
Arginine, g/d
Aspartic acid, g/d
Cysteine, g/d
Glutamic acid, g/d
Glycine, g/d
Histidine, g/d
Isoleucine, g/d
Leucine, g/d
Lysine, g/d
Methionine, g/d
Phenylalanine, g/d
Proline, g/d
Serine, g/d
Threonine, g/d
Tryptophan, g/d
Tryosine, g/d
Valine, g/d
Vitamin A, IU/d
Beta−carotene, µg/d
Vitamin D, IU/d
Vitamin E, IU/d
Vitamin K, µg/d
Pantothenic acid, mg/d
Thiamin, mg/d
Riboflavin, mg/d
Niacin, mg/d
Folate, µg/d
Vitamin B−6, mg/d
Vitamin B−12, µg/d
Biotin, µg/d
Vitamin C, mg/d
Choline, mg/d
Total Ash, g/d
Calcium, mg/d
Chloride, mg/d
Chromium, µg/d
Copper, mg/d
Iodine, µg/d
Iron, mg/d
Magnesium, mg/d
Manganese, mg/d
Molybdenum, µg/d
Phosphorus, mg/d
Potassium, mg/d
Selenium, µg/d
Sodium, mg/d
Zinc, mg/d
Lactobacillales
Bacillales
Actinomycetales
Clostridiales
Selenomonadales
Pseudomonadales
Bacteroidales
Enterobacteriales
Burkholderiales
Neisseriales

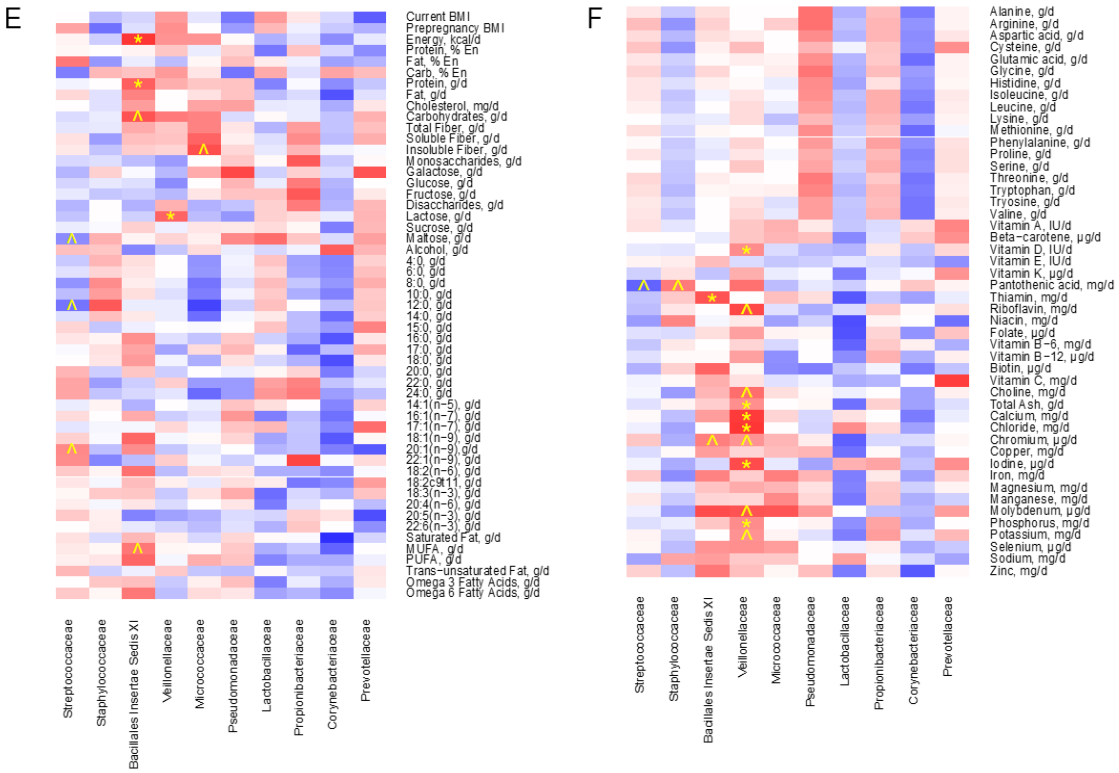


Figure 3.5 Heat maps of Spearman rank correlations between BMI, energy intake, and nutrient intake and relative abundance of the 10 most abundant bacterial classes (A and B), orders (C and D) and families (E and F) in milk samples. ^ Trend ($P \leq 0.05$); *Significant ($P \leq 0.01$). Variables for each woman were averaged across all time points. % En, percentage of energy.

CHAPTER 4

DIVERSITY AND RELATIONSHIPS AMONG BACTERIAL COMMUNITIES IN VARIOUS MATERNAL AND INFANT NICHES DURING THE FIRST SIX MONTHS POSTPARTUM

4.1 ABSTRACT

Background. Gastrointestinal bacterial community structure is largely established early in life, and may be related to bacterial communities of the mother – including that of her milk. Currently, however, very little is known about how the diversity and dynamics of and relationships among complex bacterial communities in mother/infant dyads. **Objective.** Our objective was to characterize the dynamics of microbial communities of milk, oral, and fecal samples from healthy lactating women and oral and fecal samples from their infants over the first six months postpartum and to explore relationships among the different sample types. **Methods.** Samples were collected 9 times from d 2 to 6 mo postpartum from 21 lactating women and their infants, and bacterial taxa were characterized using high-throughput sequencing of the 16S rRNA gene. Profiles of the bacterial communities in each sample type were determined and relationships among sampling sites were visualized and analyzed using principal coordinates analysis, and multivariate analyses such as non-metric multidimensional scaling. **Results.** Multivariate analyses revealed that bacterial community structures of the various sites studied here are relatively unique, and that the milk microbiome is more similar to those of infant fecal and oral samples than to maternal oral and fecal samples during early postpartum. But during later postpartum, milk becomes more similar to infant and maternal oral samples. **Conclusions.** We conclude that, although there are slight (and perhaps important) shifts in maternal and infant microbial communities during the first 6 mo after birth, there is also considerable stability in milk, feces, and saliva of healthy breastfeeding mothers and their infants in this regard. **Keywords:** microbiota, milk, feces, saliva, maternal, infant

4.2 INTRODUCTION

High-throughput sequencing has unveiled complex consortia of microbial communities that exist in and on the human body (Costello et al. 2009; Human Microbiome Project Consortium 2012; Costello et al. 2013; Zhou et al. 2013), and their compositions are likely associated with various health and disease states, such as obesity, diabetes, and inflammatory intestinal diseases (reviewed by DeGruttola et al. 2016). In the mother-infant dyad these communities likely interact to influence health and disease of both the mother and infant (Neu 2015; Soderborg et al. 2016). For example, we and other researchers have postulated that the bacteria in milk play a role in structuring the bacterial communities in the infant gastrointestinal (GI) tract either directly through serving as “founder” species in the GI tract or indirectly through stimulating the innate or adaptive immune system of the infant. These interactions may not only extend to milk and GI bacterial communities but also to others niches such as the oral cavity, skin, and respiratory tract. Therefore, understanding these bacterial communities in healthy individuals is critical to understanding the role of the microbiome in disease and during critical stages of physiologic development. As such, the first step is to better understand what bacteria make up these communities in healthy mother and infant pairs, how the communities are related (or not related) to each other, and how they interact and change during lactation and the postpartum period.

Here, we performed longitudinal sampling and 16S rRNA gene sequencing of samples from mother – infant dyads across the first 6 months of lactation. Samples included milk, oral swabs, and feces from the mothers and oral swabs and feces from their infants. Our goals were to (1) profile and define the dynamics of the microbiomes in the respective samples over time, and (2) assess the dissimilarity/similarity of the bacterial communities among the sample types, particularly as this relates to the potential origin of the bacteria in milk. Our overarching hypothesis was that the milk microbial community structure would be

more similar to the infant fecal microbial community than other sites investigated here.

4.3 METHODS

4.3.1 *Subjects and study design*

This study was carried out as a prospective, longitudinal investigation of 21 self-reported healthy, breastfeeding women in their third trimester of pregnancy recruited from the Pullman, WA-Moscow, ID area. Written informed consent was obtained in accordance with procedures approved by the Washington State University and the University of Idaho Institutional Review Boards. Samples and data were collected on 2, 5 and 10 d (± 1 d), and 1, 2, 3, 4, 5, and 6 mo (± 1 d) postpartum.

4.3.2 *Sample collection*

Methods used to collect maternal feces and milk have been described in detail previously (Carrothers et al. 2015; see Chapter 2). Oral samples from the mothers and their infants were collected at each time point either at the subject's home, the hospital, Washington State University, or the University of Idaho. Oral samples were obtained by swabbing the dorsum of the tongue and the interior cheek surfaces of the women and their infants using a sterile viscose-tipped swab (Sarstedt, Germany; #80.625). Infant fecal samples were collected from a freshly soiled diaper in the home by the participants within 24 hours of the sampling day with a sterile viscose-tipped transport swab (Sarstedt, Germany; #80.625) and stored in their home freezer. Samples were stored immediately at -80 °C (when collected at a university site) or kept on ice until transported to the university (when collected at a home site or hospital) and then frozen at -80 °C.

4.3.3 *Extraction and amplification of bacterial DNA*

Extraction of DNA from maternal feces and milk has been described in detail previously (Carrothers et al. 2015; see Chapter 2). For the oral samples, the ends of the swabs were carefully cut and placed in sterile 1.5 mL microcentrifuge

tubes with 0.5 mL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8). Samples were vortexed for 30 sec and the liquid then transferred to a new sterile 1.5 mL microcentrifuge tube. Samples were centrifuged at 13,000xg for 10 min at 4 °C. The supernatant was then decanted and the remaining pellet resuspended in 0.5 mL TE50 (10 mM Tris-HCl, 50 mM EDTA, pH 8.0). Samples were then subjected to enzymatic lysis by adding 100 µL of a mixture containing 50 µL lysozyme (10 mg/mL in nuclease-free water; Sigma-Aldrich, St. Louis, MO), 6 µL mutanolysin (25 KU/mL in nuclease-free water; Sigma-Aldrich), 3 µL lysostaphin (4000 U/mL in 20 mM sodium acetate; Sigma-Aldrich), and 41 µL TE50 for 1 hr at 37 °C on a dry-heat block and then physical disruption by bead-beating with 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) for 1 min on setting 5 using a FastPrep FP120 (Qbiogene, Carlsbad, CA). DNA was subsequently extracted using the QIAamp DNA Mini Kit (Qiagen Cat. 51304, Valencia, CA) following the manufacturer's protocol. TE50 (0.5 mL) was used as a negative control. Extracted DNA was eluted in 50 µL nuclease-free water and stored at -80 °C until further analysis.

DNA was extracted from the infant fecal samples by first cutting the tip of the swab into a sterile 1.5 mL microcentrifuge tube with 0.5 mL TE 50. Samples were vigorously vortexed and then the liquid transferred to a new tube. The samples were then processed using the QIAamp DNA Stool Mini Kit (Qiagen Cat. 51504), following the manufacturer's protocol.

A dual-barcoded two-step PCR was conducted to amplify the V1-V3 hypervariable region of the bacterial 16S rRNA gene; a 7-fold degenerate forward primer targeting position 27 and a reverse primer targeting position 534 (positions numbered according to the *Escherichia coli* rRNA) were used. Primer sequences are provided in Appendix B.

Bacterial DNA from maternal and infant feces were amplified as described previously (Carrothers et al. 2015). Bacterial DNA from milk was amplified as described previously in Chapter 2. All of the PCR were done in a dedicated PCR hood.

Bacterial DNA from maternal and infant saliva were amplified with the following reaction mixture and thermocycling conditions. The first PCR mixture contained 10 µL extracted DNA, 0.05 µM target-specific primers (Integrated DNA Technologies, Coralville, IA), 5 µL 10X PCR buffer (Life Technologies, Carlsbad, CA), 3 mM $MgCl_2$ (Life Technologies), 0.24 mg/mL bovine serum albumin (Sigma, St. Louis, MO), 0.2 mM dNTP (Life Technologies), 0.25% DMSO, and 0.05 U/µL AmpliTaq® DNA 360 polymerase (Life Technologies) in a total volume of 50 µL. PCR were conducted using either an Applied Biosystems 2720, Veriti, or ProFlex model thermocycler under the following conditions: 95 °C for 2 min, then 95 °C for 20 sec, 60 °C for 30 sec, and 72 °C for 1 min for 20 cycles with a 0.5 °C step-down in the annealing temp each cycle, then 95 °C for 20 sec, 50 °C for 30 sec, and 72 °C for 1 min for 20 cycles, and then a final extension step of 72 °C for 5 min. Samples were held at 4 °C in the thermocycler until being stored at -20 °C.

Products from the first PCR were electrophoresed on 1% agarose gels made with tris-acetate-ethylenediamine tetraacetic acid (TAE; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer and containing ethidium bromide (0.0007 mg/mL). Gels were allowed to run for 30 min at 80 V, and bands viewed using the BioRad UltraCam Digital Imaging System (Hercules, CA). Samples with high-quality amplicons (relatively bright band of interest at ~534 bp), low primer-dimers, and absence of unwanted bands or smears were deemed acceptable for the second PCR reaction. PCR products were diluted (1:14) with nuclease-free water and 2-4 µL were subjected to a second round of PCR in a reaction mix containing 75 nM primers with dual-index barcodes and Illumina sequencing adapters (University of Idaho IBEST Genomics Resources Core Facility), 2 µL 10X PCR buffer (Life Technologies, Carlsbad, CA), 4.5 mM $MgCl_2$ (Life Technologies), 0.6 mg/mL bovine serum albumin (Sigma, St. Louis, MO), 0.2 mM dNTP (Life Technologies), and 0.05 U/µL AmpliTaq DNA 360 polymerase (Life Technologies) in a total volume of 20 µL. PCR was conducted using an Applied Biosystems 2720 thermocycler under the following conditions: 95 °C for 10 min, then 95 °C for 15 sec, 51 °C for 30 sec, and 72 °C for 1 min for 13 cycles, and

then a final extension step of 72 °C for 3 min. Samples were held at 4 °C in the thermocycler until being stored at -20 °C. Quality of 2nd PCR amplicons was evaluated using a QIAxcel DNA screening cartridge (Qiagen), and DNA quantified using picogreen (Life Technologies).

An appropriate volume of each amplicon (containing 50 ng DNA) was pooled to create a composite sample for high-throughput sequencing. Amplicon pools were size-selected using AMPure beads (Beckman Coulter, Indianapolis, IN). The cleaned amplicon pool was quantified using the KAPA Illumina library quantification kit (KAPA Biosciences, Wilmington, MA) and the Applied Biosystems StepOne Plus real-time PCR system. Sequences were obtained using an Illumina MiSeq v3 (San Diego, CA) paired-end 300-bp protocol for 600 cycles.

4.3.4 *Sequence analysis*

Raw DNA sequence reads from the Illumina MiSeq were demultiplexed and classified in the following manner. The custom python application dbcAmplicons (https://github.com/msettles/dbcAmplicons) was used to identify and assign reads by both expected barcode and primer sequences. Specifically, the application performs processing of raw reads (preprocessing), joining of overlapping paired reads into longer single reads, classification of reads to the genus level, and generation of abundance tables. During preprocessing, barcodes were allowed to have at most 1 mismatch (hamming distance), and primers were allowed to have at most 4 mismatches (Levenshtein distance) as long as the final 4 bases of the primer perfectly matched the target sequence. Reads identified as not having a corresponding barcode and primer sequence were discarded. Reads were then trimmed of their primer sequence and merged into a single amplicon sequence using a modified version of FLASH (Magoč and Salzberg, 2011) and the Ribosomal Database Project (RDP) Bayesian classifier (Wang et al., 2007) was used to assign sequences to phylotypes. Reads were assigned to the first RDP taxonomic level with a bootstrap score ≥ 50.

Individual-based rarefaction and accumulation curves were generated to visualize whether sequencing and sampling depth, respectively, was sufficient across taxonomic levels.

4.3.5 *Longitudinal characterization and statistical analyses of bacterial community composition*

To assess sampling and sequencing depth, individual-based rarefaction and accumulation curves were generated for each sample type. The individual-based rarefaction curves and accumulation curves (not shown) suggested that the threshold for sequencing depth and sampling depth varied across sample types and taxonomic levels but that we could confidently analyze data classified at the phylum level with at least 2,000 sequences and ~ 50 samples. However, because we were interested in comparing our data to others previously published (at the genus level) and generating hypotheses for future investigations with more sequencing depth, we also examined bacterial community structures at the genus level. As there was considerable variation in the total sequencing read count for each sample and across sample types, sample read counts were rarefied at 2,000 reads per sample prior to calculation of the various indices and multivariate analyses.

The presence and distribution (relative abundance) of each bacterial taxon in each sample were used to assess and compare bacterial diversity and community structure within and among the sampling sites. Sequence read counts were converted to relative abundance values and summarized as overall relative abundance by time PP and sampling site.

To compare the structure of the bacterial communities among the sample types, alpha diversity indices were calculated at the genus taxonomic level. Alpha diversity (Whittaker 1972), which describes the “within” sample diversity, was assessed using Shannon’s diversity number, Inverse Simpson, Pielou’s J evenness metric and richness at the genus level. Statistical analyses of diversity indices were performed in SAS 9.4 using a generalized linear mixed model and specifying a Poisson distribution. Beta diversity, which describes the “across”

sample diversity, was examined using principal coordinate analysis (PCoA) using Hellinger-transformed data and non-metric dimensional scaling (NMDS) using the Bray-Curtis dissimilarity matrix. Analysis of similarity (ANOSIM; Clarke 1993) and non-parametric multivariate ANOVA (ADONIS; Anderson 2001) functions in the vegan package in the R environment, respectively, were utilized to test for differences in microbial community composition among sample types. Bray-Curtis dissimilarity distance matrices were utilized in both tests. ANOSIM tests whether groups (e.g., sample types) are significantly different by comparing the ranks of distances between the groups and within the groups. An ANOSIM R value can range from -1 to 1, where a “1” indicates dissimilarity between groups, “0” indicates an even distribution of high and low ranks within and between groups, and “-1” indicates dissimiliarity within groups is greater than between groups. ADONIS tests the differentiation between the means or centroids of the distances within groups in order to explain the percentage of the variation by computing the effective size (R^2) and a p-value. In both cases, analyses were conducted with 999 permutations.

To further investigate the relationships among the bacterial communities of the different sample types, we conducted canonical correlation analyses (CCA) among the sample types using the relative abundances of the most abundant taxa in each sample type using the rarefied count data. Bacterial taxa for each sample type included in the CCA were chosen based on their presence in the top 10 taxa for at least one time point in that particular sample type. A complete listing of the taxa included for each sample type is listed in the supplementary information. The CCA was done using PROC CANCORR in SAS v9.4 (Cary, NC).

4.4 RESULTS

4.4.1 *Subject description and sample disposition*

Information related to basic anthropometrics and reproductive history for all 21 women at enrollment has been described previously (Carrothers et al. 2015). On

average, women were 30 ± 4 years old, weighed 64 ± 7 kg prior to pregnancy, and had a mean parity of 1.8 ± 1 children. Most samples were collected at each time point, but we were unable to obtain all of the samples due to either subject unavailability during the sampling period, the subject did not provide a sample, or sample was unaccounted for at time of processing. Ultimately, 181, 184, 167, 183, and 168) infant fecal, infant oral, maternal fecal, maternal oral, and milk samples, respectively, were obtained.

4.4.2 *Sequencing summary*

In total, 911 samples (with some being duplicates) were sequenced yielding a total of 18,534,383 sequencing reads (range 2 – 154,386; mean ± SEM = 20,345 ± 590) following cleaning and quality checking. Sequencing results for each sample type are summarized in Table 4.1. After removing duplicates and samples with read counts < 2000 reads, 818 samples (Infant feces, n = 165; infant oral, n = 152; maternal feces, n = 163; maternal oral, n = 182; milk, n = 156) were used in the analyses.

4.4.3 *Alpha and beta diversity of bacterial communities*

There was no interaction between time and sample type on Simpson evenness, Pielou's J evenness, and Shannon diversity. There was no effect of time or sample type on Simpson evenness. However, there was an effect of sample type on Pielou's J evenness index (P = 0.0169) with maternal feces having more evenness (0.58 ± 0.06; Bonferroni adjusted P < 0.0171) than infant saliva (0.34 ± 0.06). Although there was no effect of time on Shannon diversity, there was an effect of sample type (P < 0.0001); maternal fecal samples were more diverse (2.3 ± 0.1; Bonferroni adjusted P < 0.001) and infant saliva was less diverse (infant saliva, 0.9 ± 0.1; Bonferroni adjusted P < 0.01) than all of the other sample types (maternal saliva, 1.7 ± 0.1; milk, 1.3 ± 0.1; infant feces, 1.4 ± 0.1). A sample type by time interaction was evident (Bonferroni adjusted P < 0.0001) with respect to richness. A SLICE procedure was performed to determine the effect of time on richness within each sample type, and all sample types except

maternal feces showed an effect of time ($P < 0.0001$). Although an effect of time was detected for these other sample types, only infant feces showed a consistent decrease from day 2 to day 10 and then showed a gradual increase in richness as time progressed. See Figure 4.1 for plots of the various alpha diversity metrics.

Both PCoA and NMDS analyses of the genus-level data revealed structure to the variation in the community composition and in the structure of the (dis)similarity of samples by sample site, respectively. Samples clearly clustered by sampling site despite some overlap (see Figures 4.2 and 4.3 for ordination plots for PCoA at genus and phylum levels, respectively, and Figures 4.4 and 4.5 for NMDS ordination plots at genus and phylum levels, respectively), suggesting that each niche hosts a unique bacterial community. Both ANOSIM ($r = 0.6773$; $P = 0.001$) and ADONIS ($r^2 = 0.42$; $P = 0.001$) tests at the genus level indicated a difference among sample types supporting the clustering observed in the PCoA and NMDS ordination plots.

Although each sample type had a diverse and somewhat unique community structure, there were also several noteworthy similarities in membership among sample types. For ease, the average relative abundances for the 20 most abundant taxa at the genus and phylum levels in each sample type are presented in Tables 4.2 - 4.5. Tables 4.7 - 4.36 list the relative abundances of the 20 most abundant taxa at the genus and phylum level in each sample type for each time point. Similarities among the sample types can be noted by examining the relative abundances of the members in each community. For example, maternal and infant oral samples were both dominated by *Streptococcus* (53.6 ± 1.3% and 68.5 ± 1.8%, respectively); whereas the most abundant bacterial taxon in maternal and infant feces was *Bacteroides* (22.2 ± 1.3% and 21.0 ± 2.2%, respectively). And although maternal and infant fecal samples shared similar relative abundances of *Bacteroides*, only four other taxa were shared in the top 20 most abundant bacteria from these sites; these were *Lachnospiracea incertae sedis* (7.6 ± 0.4% and 2.6 ± 0.6% in maternal and infant fecal samples, respectively), *Lachnospiraceae* (5.4 ± 0.2% and 1.3 ± 0.3% in

maternal and infant fecal samples, respectively), *Clostridium XIVa* (1.2 ± 0.2% and 1.8 ± 0.5% in maternal and infant fecal samples, respectively) and *Parabacteroides* (2.3 ± 0.2% and 4.3 ± 1.0% in maternal and infant fecal samples, respectively) (See Table 4.3). The top 5 most abundant genera in both milk and the infant oral samples were *Streptococcus*, *Staphylococcus*, *Gemella*, *Rothia*, and *Veillonella*. It is interesting to note that on d 2, milk and infant feces shared 12 of the 20 most abundant taxa at the genus level (*Clostridium sensu stricto*, *Comamonas*, *Gemella*, *Haemophilus*, *Lactococcus*, *Leuconostoc*, *Prevotella*, *Propionibacterium*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Weissella*); whereas, on d 30 only 4 (*Clostridium sensu stricto*, *Pseudomonas*, *Streptococcus*, *Veillonella*) of the 20 most abundant taxa in each group were shared between milk and infant feces (See Tables 4.7, 4.8, 4.16, 4.17).

The relationships among bacterial communities in the various sample types were further characterized using CCA. See Table 4.6 and Figure 4.6. Three of the canonical correlations (infant feces:infant oral, infant oral:maternal oral, and milk:maternal oral) were not significant. The other canonical correlations had a minimum of 1 significant canonical component (range 1-6), and all of the canonical correlations were moderate to strong (range 0.63 ~ 1.00). Milk and infant saliva had the greatest number of significant canonical components (number of significant components, 6; canonical correlations ranged from 0.92 – 0.69; $P < 0.01$). These six canonical components together accounted for ~ 85% of the data variability with the first canonical component accounting for 31% of the variation in the data. Milk and infant fecal were highly correlated as evidenced by the canonical correlation coefficient being very close to “1” in the first canonical component. The first axis accounted for ~ 96% of the variation. Relative abundance of both *Lactococcus* and *Weisella* in infant feces had a strong correlation with the first milk canonical variable while relative abundance of *Gemella* in milk was strongly associated with the first infant fecal canonical variable. Milk and maternal feces also had a strong canonical correlation of 0.73 ($P = 0.0012$).

4.5 DISCUSSION

This study is the first, to our knowledge that provides a relatively comprehensive cross-biome view of the maternal-infant dyad during the first six months postpartum. Bacterial communities from maternal feces, mouth, milk, and infant feces and mouth were assessed using high throughput sequencing of the V1-V3 region of the16S rRNA gene. To our surprise, our data did not support our *a priori* hypothesis that the milk microbiome would most closely resemble the infant fecal microbiome. In fact, the bacterial community structure of milk was more similar to both the infant oral and as time progressed to the maternal oral microbiomes. But the canonical correlations suggested that these two microbial communities are somehow related.

Bacterial communities from each site reflected similar bacterial composition as previously reported in maternal oral (Davé et al. 2016), fecal (Koren et al. 2012; Jost et al. 2014), and milk (Hunt et al. 2011) samples. Additionally, the bacterial composition of infant fecal and oral samples we observed concurred with previous reports (Jost et al. 2012; Bäckhed et al 2015; oral bacterial communities reviewed by Zaura et al. 2014).

Samples from the different sampling sites showed distinct clustering at the genus level as has been shown previously for smaller subsets of mother-infant pairs or shorter time periods in different populations (Bisanz et al. 2015) and in other studies that have investigated the bacterial communities across the human body (Ding and Schloss 2014). Indeed we note a similar finding in the US population that Bisanz and coworkers noted in the Tanzanian cohort that bacterial communities of infant feces and oral samples seemed to be more closely related to human milk. They also noted that at both 0 to 10 days after birth and 10 to 25 days after birth there was more similarity between the infant's oral microbiota and their mother's milk microbiota than between the infant's feces and their mother's milk. Samples from our study also support this finding and provide additional indication that this trend continues with communities of the oral microbiota and milk microbiota becoming more similar to each other and less similar to infant feces at 180 days after birth. Indeed, the complex bacterial

composition of human milk bridges those of infant saliva and infant feces early in the infant's life (see Figure 4.2).

Over time the composition of the milk bacterial community and the infant oral bacterial community become more similar to each other and also to the maternal oral bacterial community. This is evident as we observed that the most common genera across all time points in all three sample types were *Streptococcus*, *Gemella*, *Rothia*, *Veillonella*. Infant saliva and milk both had a greater relative abundance of *Staphylococcus* than was found in maternal saliva. Indeed, our results concur with Boustedt and coworkers (2015) who investigated the salivary microbiomes from 149 infants within 2 days after birth and then monthly thereafter and then also the salivary microbiome of their mothers at 6 mo. They too found that that the salivary microbiomes of both mother and infant were dominated by *Streptococcus* in most of the subjects. We observed a similar finding in that *Streptococcus* was the dominant bacterial genus in both maternal and infant oral samples at each time point (d 2 – 180). Additionally, in infant oral samples from d 2 – 30, *Staphylococcus* had a high relative abundance (range 2 – 11.6%) and then decreased to less than 1% by d 60. The high relative abundance of *Staphylococcus* in the infant's mouth is likely due to the high proportions of *Staphylococcus* present in milk and the frequent bi-directional interaction between the bacteria in the mammary gland and the infant's mouth. This interaction between saliva and milk has been shown to result in changes in the reactive oxygen species in the infant's saliva and thus, may play important roles in structuring the bacterial communities in both milk and the infant's mouth (Al-Shehri et al. 2015). More research is needed to understand the oral and mammary milieu and how this intertwined system inhibits and / or supports the presence and growth of various bacterial populations in both environments.

Additionally, results from the CCA provide more evidence that the bacterial community in the milk is linked to both the infant oral and fecal bacterial communities during the first six months postpartum. The strong canonical correlation between the bacterial communities of the milk and infant feces suggest though that although these bacterial communities may not be similar in

membership composition later in lactation, the microbial communities continue to be intimately linked. Similarly, although the community composition of the maternal fecal community was distinct from the milk bacterial community as evidenced in the NMDS plot (see Figure 4.4), there was a strong canonical correlation (0.73, $P = 0.0012$) between these two microbial communities. This is interesting in that if the bacterial community of maternal feces is a proxy for the bacterial community in the maternal GI tract, then maternal diet, which likely plays a role in the membership of the GI microbiota, may indeed influence the milk microbiota. More research is needed to better understand these relationships.

Dominguez-Bello and coworkers (2010) also found distinct bacterial community structure when examining the microbial communities of maternal oral, vaginal, and skin microbiota on the first day after giving birth. However, they did not observe separate clustering of the infant oral, skin, and rectal microbiota. Instead, infant samples differentiated into groups by delivery mode. In the current study although we observed distinct groupings by sample type at the genus level, the distinction between groups was less obvious at the phylum level in both the PCoA and especially in the NMDS plots. It is interesting to note that Dominguez-Bello and coworkers utilized the UniFrac metric, which is based on the fraction of branch length shared between two communities within a phylogenetic tree constructed from the 16S rRNA gene sequences, to compare the bacterial communities. In our study we utilized the Bray-Curtis distance to compare sample types. Therefore, the comparison of sample types at the phylum level in our study may be more similar to the relationships seen by Dominguez-Bello and coworkers.

In the present study the predominant bacterial genera in maternal feces at each time point was *Bacteroides*, followed by lower relative abundances of *Faecalibacterium*, *Prevotella*, and *Lachnospiracea incertae sedis*. Although these taxa are similar to what others have reported as being present in maternal feces (Koren et al. 2012; Jost et al. 2014), we observed a greater relative abundance of *Bacteroides* in our population than in the previous studies. In another

comparison, the maternal fecal bacterial community in Tanzania was reported to be dominated by *Prevotella* (Bisanz et al. 2015). These differences in distributions of the bacterial community membership may be due to differences in diet among populations but host genetic differences may also be a factor. Recently several researchers have examined the heritability of the microbiome (Goodrich et al. 2014; Blekhman et al. 2015; Davenport et al. 2015; Davenport 2016) and found that individual taxa in the GI microbiome appeared to be heritable. Davenport (2016) suggested that when looking at these studies together that most of the heritable bacteria seemed to be part of either the Proteobacteria or Firmicute phyla. These phyla, however, comprise a large variety of bacterial species in the GI system and thus more work is needed to identify the specific bacteria that are heritable and the implications this may have for GI health and disease. These genetic associations also extend not only to the GI microbiome but to bacterial community structures of other body sites (Blekhman et al. 2015) and many of these associations, not unexpectedly, are related to immunity-related genes. Knights and coworkers (2014) also demonstrated that complex host genetics are involved in risk and development of diseases such as inflammatory bowel disease. Other factors that may influence the microbial community structure such as trans-generational epigenetic modification may also explain these findings. Being able to tease apart and understand the connection between host genetics and the microbiomes of the mother-infant dyad may help untangle complex diseases which have been long-time enigmas, such as necrotizing enterocolitis in infants.

Interestingly, in both infant feces > 1 mo and maternal feces, we identified *Lachnospiracea* as one of the most abundant taxa, but it was not prevalent in the milk or saliva samples. Recently, Sagheddu and coworkers (2016) described this bacterial family and concurred that *Lachnospiracea* is found in high frequency in the early infant GI. They posit that the abundance and diversity of genera within this family may influence the growth of other bacteria. Bacteria within this family have genes that are involved in metabolic pathways involved in complex sugar degradation (Cervera-Tison et al. 2012; Biddle et al. 2013) and in iso-bile

detoxification pathways (Devlin and Fischbach 2015) and thus could potentially influence the presence and abundance of other bacteria. Additional studies are needed to look more in depth at the role these bacteria play in evolvement of the bacterial community structure in both maternal and infant GI environments and especially during the dynamic succession of bacterial colonization during the first two months in the infant's GI system.

There was a time by sample type interaction that was evident in the infant fecal bacterial communities. The richness of the bacterial community was initially high at d 2 and then decreased quickly through d 10. Following this initial decrease however, richness then gradually increased. Favier and coworkers (2002) also noted an increase in richness of the bacterial community of the infant gastrointestinal tract that occurred with time. Although they noted that during the first few days of life the bacterial communities appeared simple, the complexity increased over time.

There are several strengths to this study with one of the major ones being that this is the first to our knowledge of longitudinal sampling of maternal feces, maternal saliva, milk, infant feces, and infant saliva from mother-infant pairs during the first six months postpartum. Information regarding the microbial community composition was then obtained at various taxonomic levels through the use of high-throughput sequencing, which allowed the detection of hard-to-culture bacteria and bacteria found in low abundance, and examine the complex nature and relationships of the diverse bacterial communities found in these samples.

Several limitations, however, are also noteworthy in the present study. For instance, because bacterial communities in feces do not necessarily reflect the bacterial composition of the GI bacterial communities, only limited inferences concerning the latter should be made from our data. Indeed, Romano-Keeler and coworkers (2014) have demonstrated that distinct biogeography exists in the GI tract and question how reliable fecal communities represent the developing GI microbial communities. Further studies are needed to ascertain whether fecal community structure or particular bacterial populations can serve as accurate

markers or indicators of intestinal bacterial communities. In addition, the women and babies participating in our study represent a relatively homogenous population, both in terms of environmental exposures and genetic backgrounds. Future studies should be designed to include a more diverse cohort of maternal/infant dyad. Additionally, other factors such as maternal intrapartum antibiotic prophylaxis and probiotic usage need to be examined in light of recent work that indicates these factors influence both infant GI (Azad et al. 2016; Corvaglia et al. 2016) and milk (Fernández et al. 2014; Bisanz et al. 2015) microbial compositions. Finally, although our findings suggest that maternal and infant salivary microbiomes are interconnected with the milk microbiome, intervention studies will be needed to confirm the directionality of these relationships.

Nonetheless, a growing literature suggests that microbial communities of the mother/infant dyad represent complex microcosms that are likely linked and interact to maintain health in both the mother and the infant (Dogra et al. 2015a; Dogra et al. 2015b; Garcia-Mantrana and Collado 2016; Soderborg et al. 2016). The close proximity and the interchange that goes on between mother and infant likely influence bacterial communities of the mother as well as her infant. Understanding these complex cross-biome interactions is important as establishment of these different microbiota in various body habitats likely has consequences for future health of both the mother and infant.

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Table 4.1 *Sequencing summary for the various sample types*

Sample Type	Total number of reads	Average reads (mean ± SEM)
Infant feces (*n* = 201)*	2,426,600	12,073 ± 711
Infant oral (*n* = 184)	2,319,632	12,607 ± 1,470
Maternal feces (*n* = 167)	5,897,388	35,313 ± 1,548
Maternal oral (*n* = 183)	6,064,070	33,137 ± 565
Milk (*n* = 176)*	1,826,693	10,379 ± 520

* Some samples were duplicated in order to increase likelihood of obtaining a good quality sample.

Table 4.2 *Overall relative abundances of most abundant genera identified in milk, infant saliva, and maternal saliva*

Milk	Mean ± SEM	Infant saliva	Mean ± SEM	Maternal saliva	Mean ± SEM
Streptococcus	44.9 ± 2.1	Streptococcus	68.5 ± 1.8	Streptococcus	53.6 ± 1.3
Staphylococcus	22.4 ± 2.1	Gemella	9.7 ± 1.3	Rothia	6.3 ± 0.5
Bacteria	4.9 ± 0.5	Rothia	5.7 ± 0.9	Gemella	6.2 ± 0.5
Gemella	3.5 ± 0.8	Staphylococcus	4.0 ± 0.9	Prevotella	5.8 ± 0.5
Rothia	2.3 ± 0.4	Veillonella	2.5 ± 0.3	Veillonella	5.2 ± 0.3
Veillonella	2.3 ± 0.3	Lactobacillales	1.2 ± 0.0	Neisseria	4.5 ± 0.4
Lactobacillales	1.6 ± 0.1	Granulicatella	1.1 ± 0.2	Haemophilus	2.8 ± 0.2
Pseudomonas	1.5 ± 0.6	Haemophilus	0.8 ± 0.3	Granulicatella	2.7 ± 0.2
Lactobacillus	1.5 ± 0.6	Bacteria	0.6 ± 0.1	Actinomyces	1.2 ± 0.1
Propionibacterium	1.3 ± 0.2	Soonwooa	0.5 ± 0.3	Fusobacterium	1.2 ± 0.1
Unknown	1.2 ± 0.2	Prevotella	0.5 ± 0.3	Porphyromonas	0.9 ± 0.2
Corynebacterium	1.0 ± 0.2	Porphyromonas	0.5 ± 0.2	Lactobacillales	0.8 ± 0.0
Bacillales	0.8 ± 0.0	Actinomyces	0.4 ± 0.1	Prevotellaceae	0.8 ± 0.1
Bacilli	0.6 ± 0.0	Lactobacillus	0.4 ± 0.2	Leptotrichia	0.7 ± 0.1
Granulicatella	0.5 ± 0.1	Neisseria	0.4 ± 0.2	Schlegelella	0.6 ± 0.1
Prevotella	0.5 ± 0.1	Bacilli	0.3 ± 0.0	Neisseriaceae	0.5 ± 0.0
Actinomyces	0.5 ± 0.1	Bacillales	0.2 ± 0.0	Bacteria	0.4 ± 0.0
Staphylococcaceae	0.4 ± 0.0	Pasteurellaceae	0.2 ± 0.1	Pasteurellaceae	0.3 ± 0.0
Firmicutes	0.4 ± 0.0	Pilibacter	0.2 ± 0.0	Abiotrophia	0.3 ± 0.1
Actinomycetales	0.4 ± 0.0	Actinomycetales	0.2 ± 0.0	Actinomycetales	0.3 ± 0.0

Table 4.3 *Overall relative abundances of most abundant genera identified in maternal and infant feces*

Maternal feces	Mean ± SEM	Infant feces	Mean ± SEM
Bacteroides	22.2 ± 1.3	Bacteroides	21.0 ± 2.2
Faecalibacterium	8.6 ± 0.6	Escherichia/Shigella	14.5 ± 1.8
Prevotella	7.9 ± 1.0	Veillonella	10.2 ± 1.3
Lachnospiracea_incertae_sedis	7.6 ± 0.4	Clostridium sensu stricto	9.7 ± 1.6
Lachnospiraceae	5.4 ± 0.3	Bifidobacterium	4.8 ± 0.8
Porphyromonas	4.1 ± 0.6	Parabacteroides	4.3 ± 1.0
Ruminococcaceae	3.3 ± 0.2	Streptococcus	4.2 ± 1.1
Oscillibacter	3.1 ± 0.3	Pseudomonas	3.0 ± 0.6
Clostridiales	2.8 ± 0.2	Lachnospiracea_incertae_sedis	2.6 ± 0.6
Parabacteroides	2.3 ± 0.2	Klebsiella	2.4 ± 0.8
Dialister	1.7 ± 0.3	Clostridium XlVa	1.8 ± 0.5
Roseburia	1.5 ± 0.1	Clostridium XVIII	1.6 ± 0.4
Bacteria	1.3 ± 0.2	Enterobacter	1.6 ± 0.5
Blautia	1.2 ± 0.2	Enterobacteriaceae	1.5 ± 0.3
Clostridium XlVa	1.2 ± 0.2	Haemophilus	1.3 ± 0.5
Peptoniphilus	1.1 ± 0.2	Lachnospiraceae	1.3 ± 0.3
Campylobacter	1.1 ± 0.3	Lactococcus	1.0 ± 0.3
Anaerosphaera	1.0 ± 0.2	Megasphaera	0.8 ± 0.4
Anaerococcus	1.0 ± 0.2	Comamonas	0.8 ± 0.4
Alistipes	1.0 ± 0.1	Clostridiaceae 1	0.6 ± 0.1

Table 4.4 *Overall relative abundances of most abundant phyla identified in milk, infant saliva, and maternal saliva*

Milk	Mean ± SEM	Infant saliva	Mean ± SEM	Maternal saliva	Mean ± SEM
Firmicutes	82.0 ± 1.2	Firmicutes	88.9 ± 1.3	Firmicutes	70.9 ± 1.1
Actinobacteria	6.1 ± 0.7	Actinobacteria	6.5 ± 1.0	Proteobacteria	9.8 ± 0.6
Bacteria	5.0 ± 0.5	Bacteroidetes	2.1 ± 0.5	Bacteroidetes	8.3 ± 0.6
Proteobacteria	3.8 ± 0.7	Proteobacteria	1.7 ± 0.6	Actinobacteria	7.9 ± 0.5
Bacteroidetes	1.6 ± 0.2	Bacteria	0.6 ± 0.1	Fusobacteria	2.3 ± 0.2
Unknown	1.2 ± 0.2	Fusobacteria	0.1 ± 0.1	Bacteria	0.4 ± 0.0
Fusobacteria	0.1 ± 0.1	TM7	0.1 ± 0.0	TM7	0.2 ± 0.0
TM7	0.0 ± 0.0	Unknown	0.1 ± 0.0	Cyanobacteria/Chloroplast	0.1 ± 0.0
Tenericutes	0.0 ± 0.0	Cyanobacteria/Chloroplast	0.0 ± 0.0	Spirochaetes	0.0 ± 0.0
Cyanobacteria/Chloroplast	0.0 ± 0.0	Tenericutes	0.0 ± 0.0	SR1	0.0 ± 0.0

Table 4.5 *Overall relative abundances of most abundant phyla identified in maternal and infant feces*

Maternal feces	Mean ± SEM	Infant feces	Mean ± SEM
Firmicutes	51.0 ± 1.1	Firmicutes	39.5 ± 2.4
Bacteroidetes	41.1 ± 1.1	Proteobacteria	27.8 ± 2.0
Proteobacteria	3.3 ± 0.3	Bacteroidetes	26.3 ± 2.6
Bacteria	1.3 ± 0.2	Actinobacteria	5.5 ± 0.9
Actinobacteria	1.2 ± 0.2	Verrucomicrobia	0.5 ± 0.5
Verrucomicrobia	0.9 ± 0.2	Bacteria	0.3 ± 0.0
Unknown	0.7 ± 0.2	Unknown	0.0 ± 0.0
Fusobacteria	0.3 ± 0.1	Fusobacteria	0.0 ± 0.0
Synergistetes	0.1 ± 0.0	TM7	0.0 ± 0.0
Cyanobacteria/Chloroplast	0.0 ± 0.0	Cyanobacteria/Chloroplast	0.0 ± 0.0

Table 4.6 *Canonical correlation results for the pairwise comparisons between sample types*

Pairwise Canonical Correlations	Axes	Canonical Correlation	Proportion of variability explained	Cumulative variability explained	P value
Infant feces – infant oral	1	0.86	0.29	0.29	0.0975
Infant feces – maternal feces	1	0.83	0.21	0.21	< 0.0001
	2	0.80	0.17	0.38	< 0.0001
	3	0.79	0.15	0.53	0.0018
Infant feces – maternal oral	1	0.72	0.22	0.22	0.0004
	2	0.64	0.14	0.36	0.0306
Infant oral – maternal feces	1	0.82	0.31	0.31	0.0002
Infant oral – maternal oral	1	0.66	0.23	0.23	0.0660
Maternal feces – maternal oral	1	0.70	0.25	0.25	< 0.0001
	2	0.65	0.19	0.44	< 0.0001
	3	0.58	0.13	0.57	0.0014
	4	0.55	0.11	0.68	0.0325
Milk – infant feces	1	1.00	0.96	0.96	< 0.0001
	2	0.81	0.01	0.97	0.0002
	3	0.75	0.01	0.98	0.0446
Milk – infant oral	1	0.92	0.31	0.31	< 0.0001
	2	0.88	0.19	0.50	< 0.0001
	3	0.84	0.14	0.64	< 0.0001
	4	0.80	0.10	0.74	< 0.0001
	5	0.69	0.05	0.80	0.0002
	6	0.69	0.05	0.85	0.0074
Milk – maternal feces	1	0.73	0.23	0.23	0.0012
Milk – maternal oral	1	0.63	0.27	0.27	0.2545

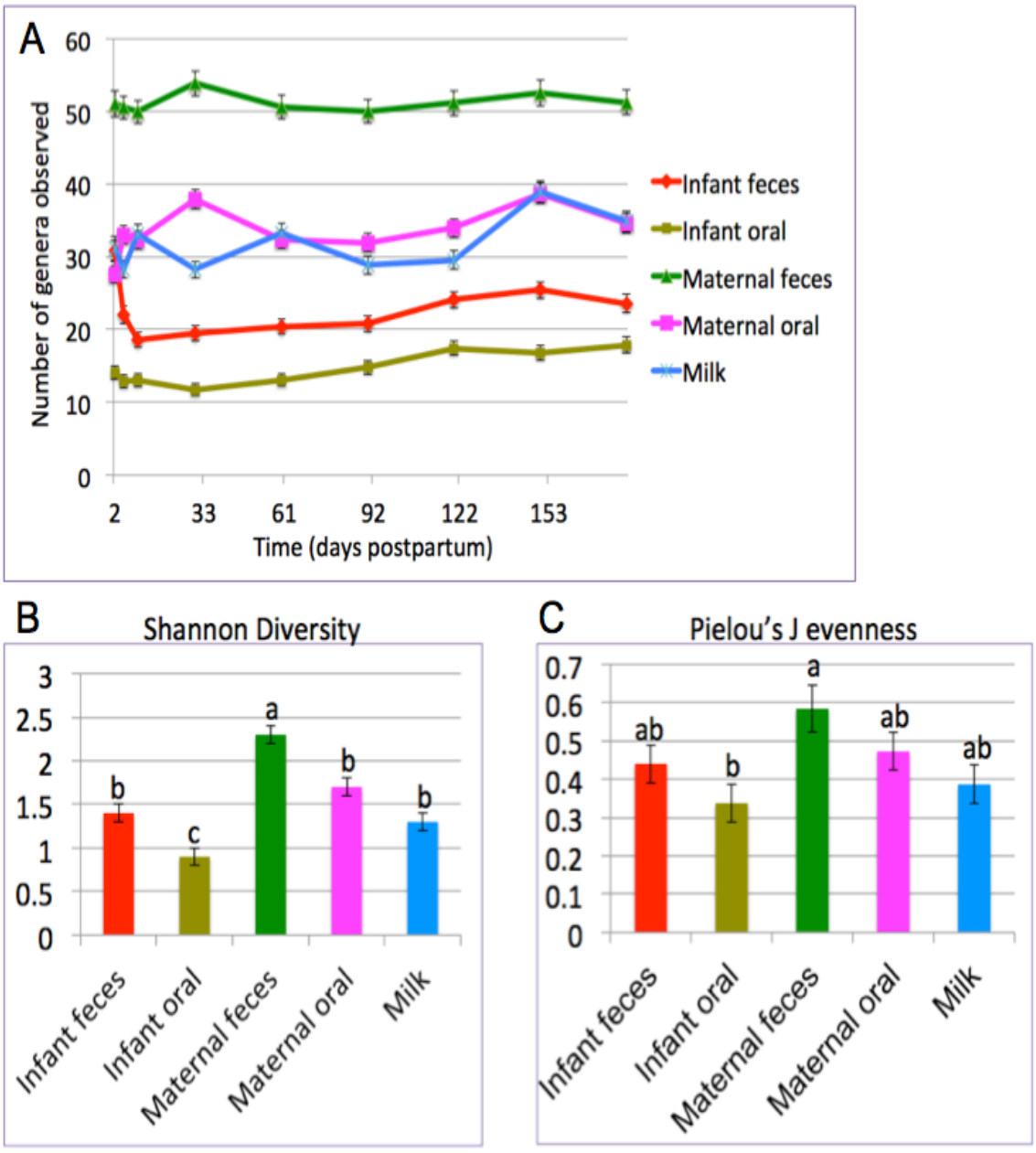


Figure 4.1 *Diversity indices at genus level A) Interaction plot of richness by sample type and time; B) Shannon indices by sample type; C) Pielou's J evenness indices by sample type. Columns within a plot not sharing a common letter differ ($P < 0.05$).*

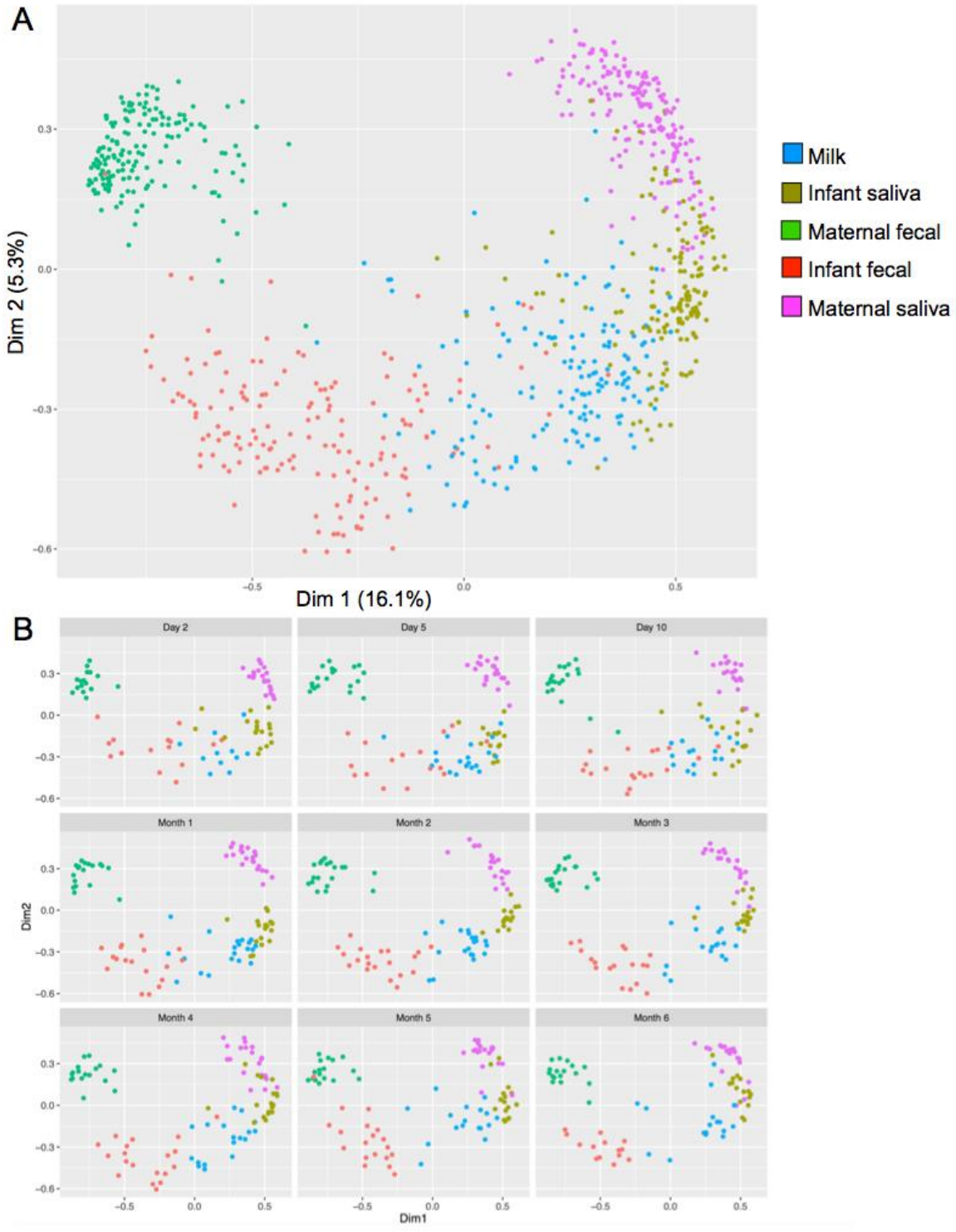


Figure 4.2 *PCoA plot of genus level rarefied sequence read count data from each sample type. A) PCoA plot with all time points; B) PCoA plots by time postpartum*

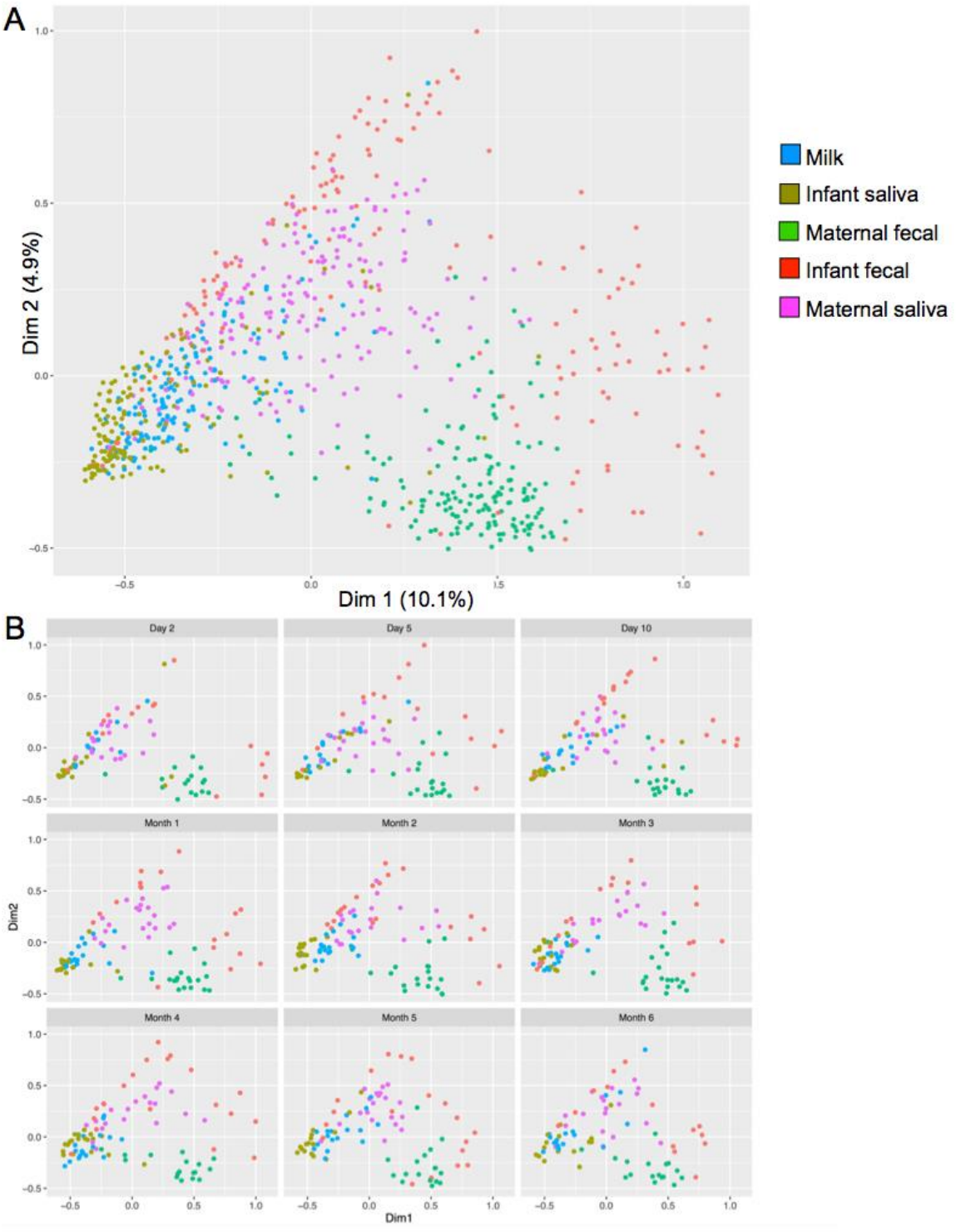


Figure 4.3 *PCoA plot of phylum level rarefied sequence read count data from each sample type. A) PCoA plot with all time points; B) PCoA plots by time postpartum*

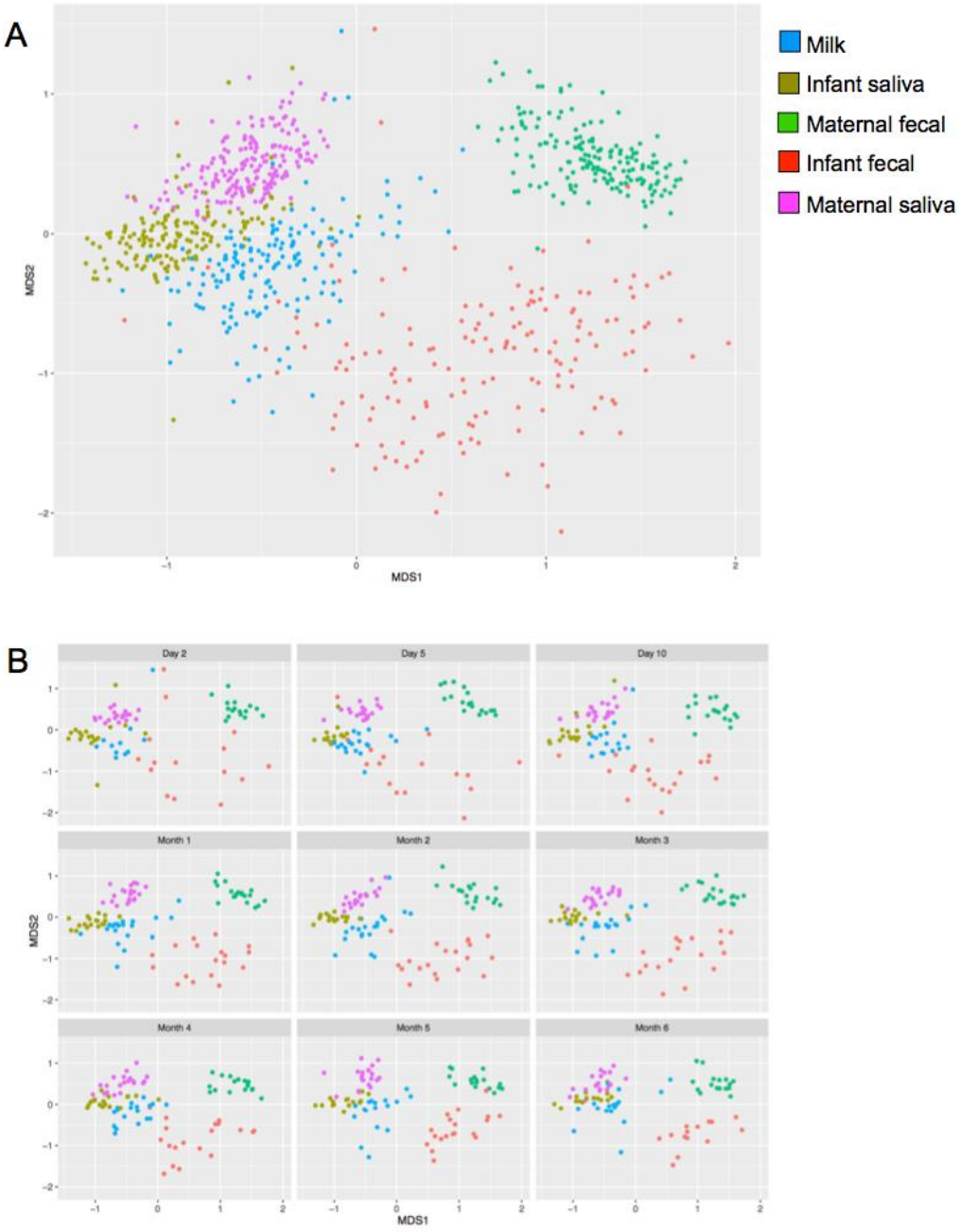


Figure 4.4 *NMDS plot of genus level rarefied sequence read count data from each sample type. A) NMDS plot with all time points; B) NMDS plots by time postpartum*

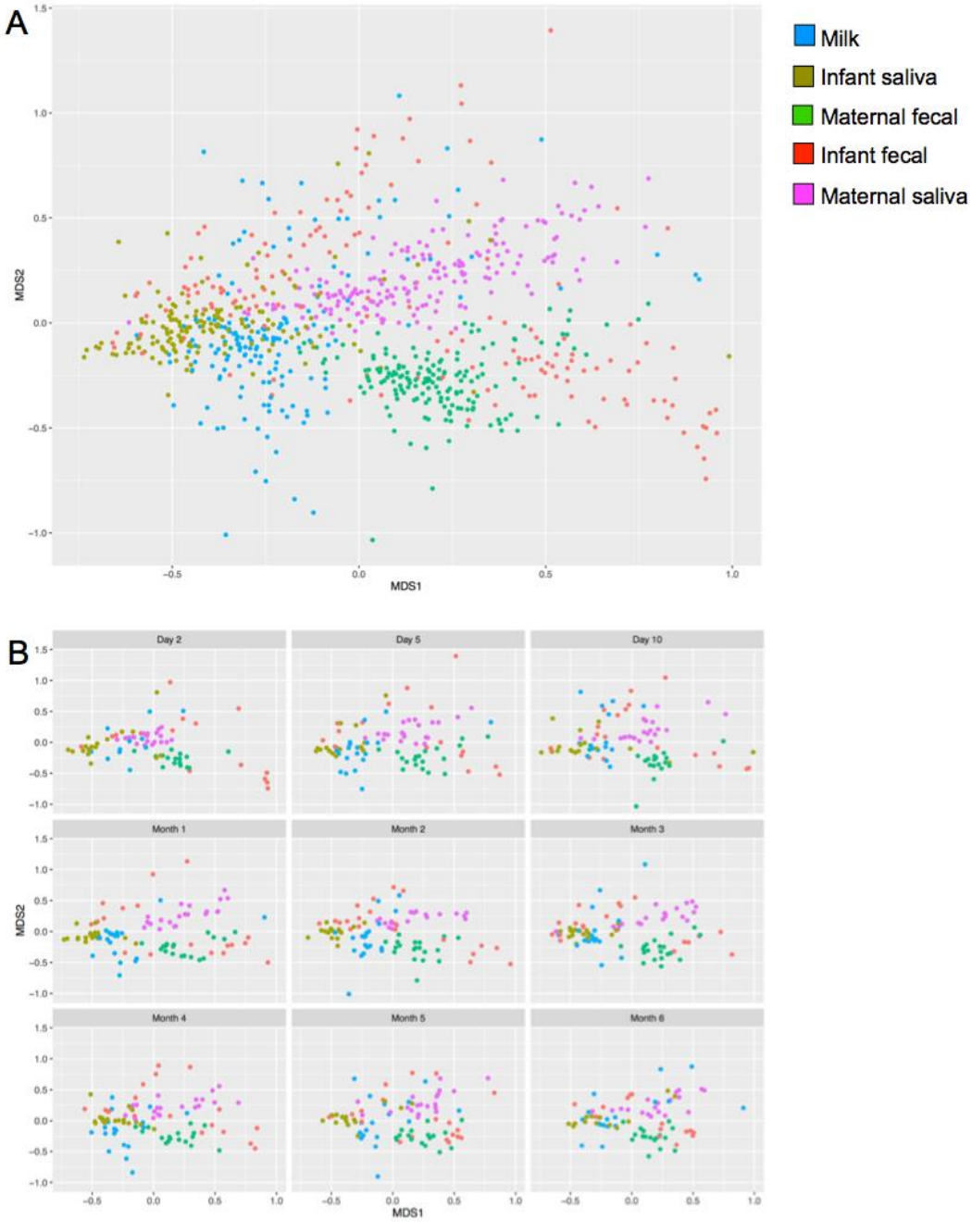
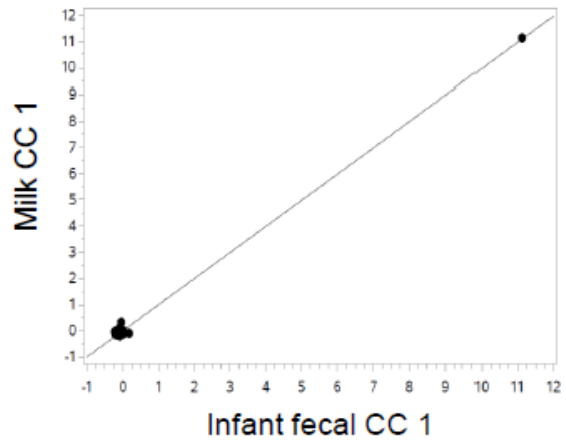
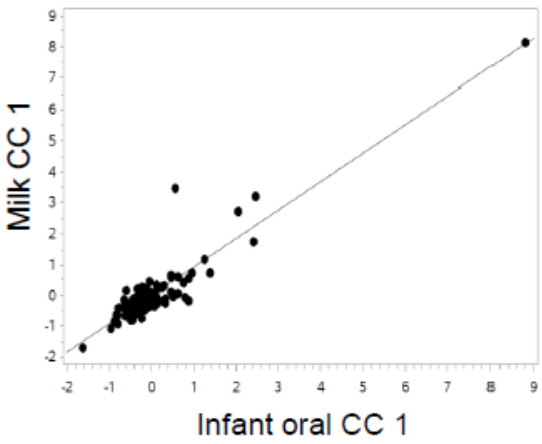
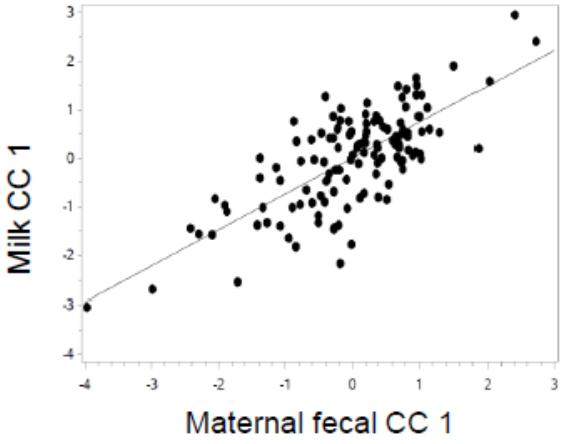
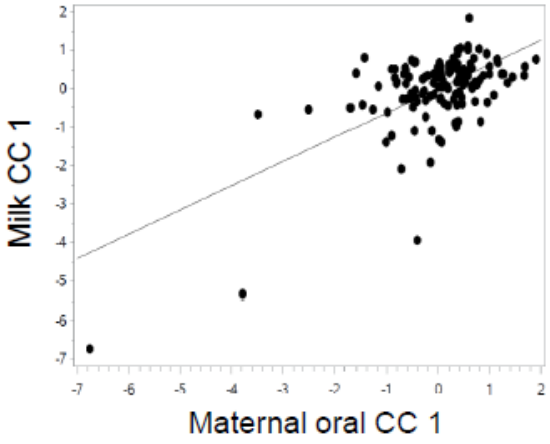


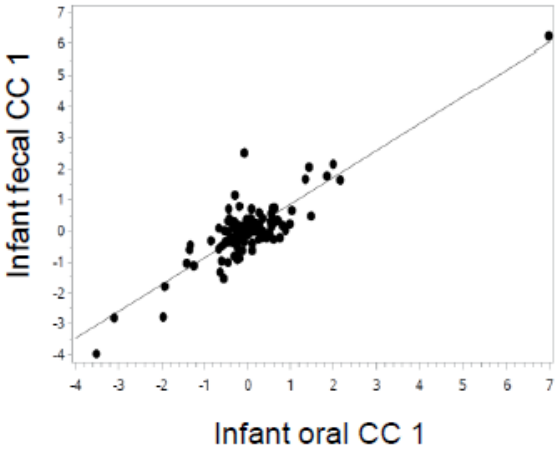
Figure 4.5 *NMDS plot of phylum level rarefied sequence read count data from each sample type. A) NMDS plot with all time points; B) NMDS plots by time postpartum*

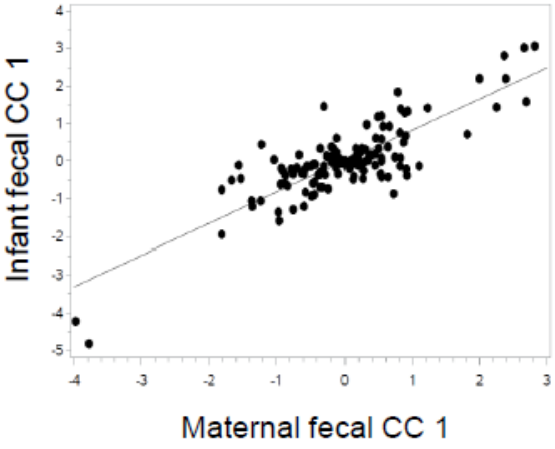

Milk CC 1
Infant fecal CC 1


Milk CC 1
Infant oral CC 1


Milk CC 1
Maternal fecal CC 1


Milk CC 1
Maternal oral CC 1


Infant fecal CC 1
Infant oral CC 1


Infant fecal CC 1
Maternal fecal CC 1

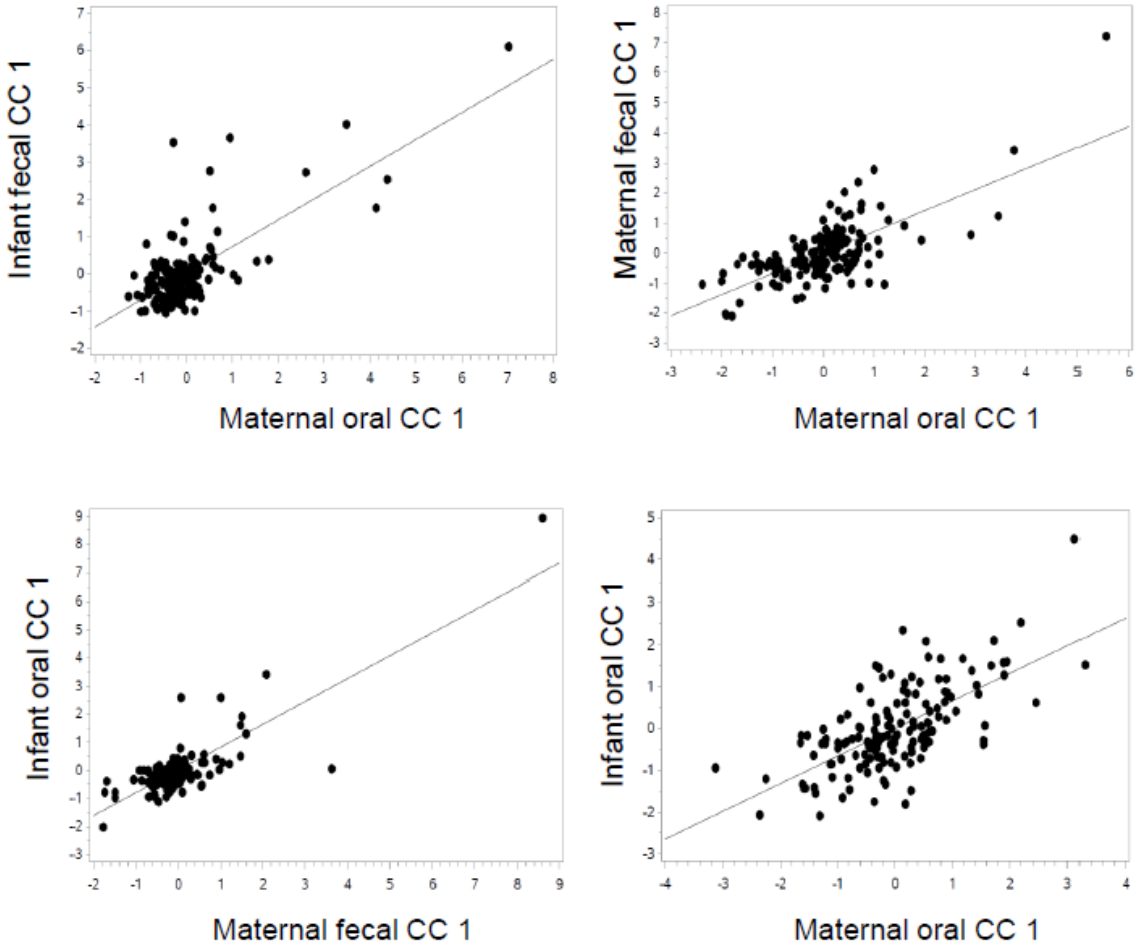


Figure 4.6 *Plots of the first canonical components in each pairwise CCA between sample types. Pairwise canonical correlation between (A) milk and infant feces, (B) milk and infant oral, (C) milk and maternal feces, (D) milk and maternal oral, (E) infant feces and infant oral, (F) infant feces and maternal feces, (G) infant feces and maternal oral, (H) maternal feces and maternal oral, (I) infant oral and maternal feces, and (J) infant oral and maternal oral.*

Table 4.7 *Relative abundances of most abundant genera identified in infant feces from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Bacteroides	23.9 ± 9.2	Bacteroides	20.4 ± 7.1	Escherichia/Shigella	18.7 ± 7.1
Parabacteroides	11.2 ± 6.7	Escherichia/Shigella	16.5 ± 6.9	Bacteroides	18.5 ± 6.7
Streptococcus	7.9 ± 4.8	Streptococcus	13.3 ± 6.2	Streptococcus	11.2 ± 5.8
Veillonella	7.8 ± 4.8	Veillonella	10.3 ± 6.2	Clostridium sensu stricto	10.5 ± 5.2
Escherichia/Shigella	7.4 ± 6.2	Haemophilus	7.2 ± 3.8	Veillonella	9.7 ± 3.6
Lactococcus	7.4 ± 3.1	Clostridium sensu stricto	4.9 ± 3.3	Enterobacter	7.1 ± 3.4
Clostridium sensu stricto	6.3 ± 6.2	Parabacteroides	4.5 ± 2.3	Enterobacteriaceae	3.2 ± 1.8
Weissella	3.4 ± 1.4	Klebsiella	4.2 ± 4.2	Haemophilus	2.6 ± 2.0
Leuconostoc	3.1 ± 1.4	Pseudomonas	2.2 ± 0.8	Pseudomonas	2.3 ± 0.9
Haemophilus	2.1 ± 1.1	Enterobacter	2.0 ± 1.2	Clostridium XVIII	2.2 ± 1.2
Pseudomonas	1.9 ± 0.7	Propionibacterium	1.5 ± 1.5	Parabacteroides	1.7 ± 0.9
Staphylococcus	1.5 ± 0.8	Sutterella	1.3 ± 1.1	Clostridium XlVa	1.4 ± 1.1
Comamonas	1.2 ± 0.9	Lactococcus	1.1 ± 1.0	Bifidobacterium	1.1 ± 0.6
Prevotella	0.9 ± 0.4	Enterobacteriaceae	0.9 ± 0.2	Klebsiella	1.0 ± 0.7
Enterobacteriaceae	0.8 ± 0.3	Enterococcus	0.9 ± 0.9	Sutterella	0.9 ± 0.9
Gemella	0.7 ± 0.6	Weissella	0.7 ± 0.6	Sarcina	0.9 ± 0.5
Enterobacter	0.7 ± 0.5	Staphylococcus	0.6 ± 0.2	Megasphaera	0.9 ± 0.7
Propionibacterium	0.7 ± 0.4	Leuconostoc	0.6 ± 0.6	Staphylococcus	0.7 ± 0.3
Acinetobacter	0.6 ± 0.2	Phascolarctobacterium	0.6 ± 0.6	Yokenella	0.6 ± 0.2
Roseburia	0.5 ± 0.5	Megasphaera	0.5 ± 0.5	Cellulosilyticum	0.5 ± 0.5

Table 4.8 *Relative abundances of most abundant genera identified in infant feces from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Bacteroides	24.4 ± 6.8	Bacteroides	19.1 ± 7.0	Clostridium sensu stricto	24.2 ± 7.6
Escherichia/Shigella	17.1 ± 6.3	Clostridium sensu stricto	12.3 ± 5.2	Bacteroides	16.8 ± 6.2
Veillonella	10.2 ± 4.7	Veillonella	12.3 ± 3.4	Escherichia/Shigella	13.6 ± 4.3
Clostridium sensu stricto	9.0 ± 4.2	Escherichia/Shigella	12.1 ± 4.5	Veillonella	12.1 ± 4.0
Klebsiella	5.5 ± 4.2	Bifidobacterium	8.9 ± 3.3	Pseudomonas	7.2 ± 3.3
Parabacteroides	5.1 ± 2.8	Klebsiella	6.7 ± 4.0	Bifidobacterium	4.4 ± 1.4
Bifidobacterium	4.8 ± 2.5	Clostridium XlVa	4.2 ± 2.7	Clostridium XlVa	3.7 ± 2.7
Clostridium XVIII	2.7 ± 1.4	Lachnospiracea incertae sedis	4.1 ± 2.8	Lachnospiracea incertae sedis	2.9 ± 1.8
Citrobacter	2.3 ± 2.3	Enterobacteriaceae	2.9 ± 1.3	Parabacteroides	2.0 ± 1.1
Lachnospiracea incertae sedis	2.2 ± 1.6	Lachnospiraceae	2.7 ± 1.7	Clostridiaceae 1	1.4 ± 0.4
Enterobacteriaceae	1.9 ± 0.7	Clostridium XVIII	2.7 ± 1.8	Anaerobacter	1.3 ± 0.4
Megasphaera	1.7 ± 1.7	Enterobacter	1.2 ± 0.5	Lachnospiraceae	1.0 ± 0.4
Enterobacter	1.5 ± 0.8	Streptococcus	1.1 ± 0.6	Clostridium XlVb	1.0 ± 1.0
Streptococcus	1.2 ± 0.4	Parabacteroides	0.9 ± 0.5	Clostridium XVIII	1.0 ± 0.8
Lachnospiraceae	1.2 ± 0.8	Anaerobacter	0.9 ± 0.4	Streptococcus	0.8 ± 0.3
Pseudomonas	1.1 ± 0.3	Citrobacter	0.8 ± 0.8	Enterobacteriaceae	0.6 ± 0.1
Sutterella	0.9 ± 0.6	Pseudomonas	0.5 ± 0.2	Lactobacillus	0.6 ± 0.6
Clostridium XlVa	0.7 ± 0.4	Clostridiaceae 1	0.5 ± 0.2	Blautia	0.5 ± 0.4
Olsenella	0.5 ± 0.5	Haemophilus	0.5 ± 0.5	Bacteria	0.5 ± 0.3
Clostridium XI	0.5 ± 0.3	Sutterella	0.4 ± 0.3	Pseudomonadaceae	0.5 ± 0.2

Table 4.9 *Relative abundances of most abundant genera identified in infant feces from 120 d through 180 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Escherichia/Shigella	22.7 ± 6.2	Bacteroides	20.4 ± 5.5	Bacteroides	27.9 ± 7.7
Bacteroides	17.9 ± 6.1	Escherichia/Shigella	16.2 ± 5.4	Veillonella	10.6 ± 3.2
Veillonella	12.7 ± 3.4	Veillonella	9.4 ± 2.7	Escherichia/Shigella	9.1 ± 2.7
Clostridium sensu stricto	10.9 ± 4.9	Bifidobacterium	7.8 ± 3.6	Clostridium sensu stricto	8.2 ± 3.8
Bifidobacterium	10.0 ± 3.1	Lachnospiracea incertae sedis	6.4 ± 2.8	Bifidobacterium	5.6 ± 2.1
Pseudomonas	3.7 ± 1.4	Parabacteroides	5.7 ± 3.4	Klebsiella	4.0 ± 2.3
Streptococcus	3.2 ± 2.3	Akkermansia	4.3 ± 4.3	Lachnospiracea incertae sedis	3.9 ± 1.5
Lachnospiracea incertae sedis	2.8 ± 1.6	Clostridium sensu stricto	3.5 ± 2.0	Clostridium XVIII	3.6 ± 2.8
Parabacteroides	2.6 ± 1.5	Prevotella	3.0 ± 2.2	Clostridium XlVa	3.4 ± 2.0
Megasphaera	1.9 ± 1.9	Clostridium XlVa	2.4 ± 1.0	Faecalibacterium	3.3 ± 3.3
Enterobacteriaceae	1.0 ± 0.3	Lachnospiraceae	2.1 ± 0.6	Lachnospiraceae	3.2 ± 0.9
Clostridiaceae 1	1.0 ± 0.4	Megasphaera	1.9 ± 1.8	Parabacteroides	2.6 ± 2.6
Clostridium XVIII	0.7 ± 0.4	Clostridium XVIII	1.8 ± 0.9	Pseudomonas	1.7 ± 1.2
Anaerobacter	0.7 ± 0.3	Pseudomonas	1.4 ± 0.5	Enterobacteriaceae	1.5 ± 0.4
Blautia	0.7 ± 0.4	Streptococcus	0.9 ± 0.4	Enterobacter	1.2 ± 1.0
Lachnospiraceae	0.6 ± 0.2	Enterobacteriaceae	0.8 ± 0.2	Clostridiaceae 1	1.0 ± 0.5
Citrobacter	0.5 ± 0.5	Ruminococcaceae	0.7 ± 0.3	Citrobacter	0.8 ± 0.5
Leuconostoc	0.5 ± 0.4	Bacteria	0.7 ± 0.3	Anaerobacter	0.8 ± 0.4
Lactobacillus	0.4 ± 0.3	Cellulosilyticum	0.6 ± 0.6	Akkermansia	0.6 ± 0.5
Clostridium XlVa	0.3 ± 0.2	Megamonas	0.5 ± 0.5	Flavonifractor	0.6 ± 0.3

Table 4.10 *Relative abundances of most abundant genera identified in infant saliva from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Streptococcus	54.2 ± 6.3	Streptococcus	46.6 ± 5.5	Streptococcus	53.4 ± 6.8
Gemella	27.4 ± 5.8	Gemella	21.4 ± 6.1	Gemella	12.5 ± 4.1
Haemophilus	3.6 ± 2.6	Rothia	12.4 ± 6.7	Staphylococcus	11.7 ± 4.5
Soonwooa	3.3 ± 2.2	Staphylococcus	11.1 ± 3.8	Rothia	10.2 ± 4.9
Staphylococcus	3.1 ± 1.4	Lactobacillales	1.3 ± 0.2	Prevotella	2.1 ± 2.1
Neisseria	1.8 ± 1.8	Bacteria	1.0 ± 0.2	Porphyromonas	2.0 ± 1.5
Lactobacillales	0.9 ± 0.1	Veillonella	1.0 ± 0.4	Lactobacillales	1.1 ± 0.1
Veillonella	0.7 ± 0.5	Bacilli	0.7 ± 0.1	Pasteurellaceae	0.9 ± 0.9
Bacteria	0.7 ± 0.2	Bacillales	0.6 ± 0.1	Haemophilus	0.9 ± 0.8
Rothia	0.6 ± 0.3	Haemophilus	0.6 ± 0.4	Bifidobacterium	0.7 ± 0.7
Pasteurellaceae	0.5 ± 0.2	Unknown	0.4 ± 0.3	Bacteria	0.6 ± 0.1
Bacilli	0.4 ± 0.1	Soonwooa	0.4 ± 0.1	Veillonella	0.5 ± 0.2
Bacillales	0.4 ± 0.1	Neisseria	0.2 ± 0.2	Bacilli	0.5 ± 0.1
Granulicatella	0.4 ± 0.3	Flavobacteriaceae	0.2 ± 0.1	Bacillales	0.5 ± 0.1
Flavobacteriaceae	0.3 ± 0.1	Pasteurellaceae	0.2 ± 0.1	Actinomyces	0.3 ± 0.2
Firmicutes	0.1 ± 0.0	Actinomyces	0.2 ± 0.2	Actinomycetales	0.2 ± 0.1
Pilibacter	0.1 ± 0.0	Staphylococcaceae	0.2 ± 0.1	Staphylococcaceae	0.2 ± 0.1
Neisseriaceae	0.1 ± 0.1	Pilibacter	0.2 ± 0.0	Granulicatella	0.1 ± 0.1
Prevotella	0.1 ± 0.1	Actinomycetales	0.2 ± 0.1	Flavobacteriaceae	0.1 ± 0.0
Streptococcaceae	0.1 ± 0.0	Firmicutes	0.1 ± 0.0	Firmicutes	0.1 ± 0.0

Table 4.11 *Relative abundances of most abundant genera identified in infant saliva from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Streptococcus	73.0 ± 5.9	Streptococcus	80.2 ± 2.9	Streptococcus	79.5 ± 2.3
Staphylococcus	9.6 ± 4.3	Gemella	5.1 ± 1.7	Gemella	5.6 ± 1.4
Gemella	6.9 ± 2.4	Rothia	4.7 ± 1.1	Rothia	5.0 ± 1.0
Rothia	2.1 ± 1.6	Veillonella	3.2 ± 1.2	Veillonella	3.1 ± 0.9
Veillonella	2.0 ± 0.8	Lactobacillus	1.3 ± 0.9	Lactobacillales	1.1 ± 0.1
Lactobacillus	1.5 ± 1.4	Lactobacillales	1.2 ± 0.2	Granulicatella	1.0 ± 0.5
Lactobacillales	1.1 ± 0.2	Staphylococcus	0.7 ± 0.6	Bacteria	0.9 ± 0.6
Bacteria	0.4 ± 0.1	Granulicatella	0.4 ± 0.2	Soonwooa	0.3 ± 0.2
Prevotella	0.4 ± 0.4	Bacteria	0.3 ± 0.1	Flavobacteriaceae	0.3 ± 0.1
Bacilli	0.3 ± 0.1	Neisseria	0.3 ± 0.3	Pilibacter	0.3 ± 0.0
Bacillales	0.3 ± 0.1	Pilibacter	0.3 ± 0.1	Actinomycetales	0.2 ± 0.1
Porphyromonas	0.3 ± 0.2	Bacilli	0.2 ± 0.1	Porphyromonas	0.2 ± 0.2
Actinomycetales	0.2 ± 0.1	Firmicutes	0.2 ± 0.1	Bacilli	0.2 ± 0.1
Flavobacteriaceae	0.2 ± 0.1	Actinomyces	0.2 ± 0.1	Firmicutes	0.2 ± 0.0
Actinomyces	0.2 ± 0.1	Actinomycetales	0.2 ± 0.1	Prevotellaceae	0.2 ± 0.2
Pilibacter	0.2 ± 0.0	Atopobium	0.2 ± 0.1	Lactobacillus	0.1 ± 0.1
Prevotellaceae	0.2 ± 0.2	Haemophilus	0.2 ± 0.1	Staphylococcus	0.1 ± 0.0
Granulicatella	0.1 ± 0.1	Bacillales	0.1 ± 0.1	Pseudomonas	0.1 ± 0.1
Firmicutes	0.1 ± 0.0	Flavobacteriaceae	0.1 ± 0.0	Neisseria	0.1 ± 0.1
Staphylococcaceae	0.1 ± 0.0	Streptococcaceae	0.1 ± 0.0	Haemophilus	0.1 ± 0.1

Table 4.12 *Relative abundances of most abundant genera identified in infant saliva from 120 d through 180 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Streptococcus	76.4 ± 2.2	Streptococcus	78.5 ± 3.0	Streptococcus	72.6 ± 4.1
Rothia	7.4 ± 1.3	Rothia	5.3 ± 1.2	Veillonella	5.1 ± 1.6
Veillonella	3.1 ± 0.7	Veillonella	4.1 ± 0.9	Rothia	4.1 ± 1.0
Gemella	2.7 ± 0.7	Granulicatella	2.1 ± 0.7	Gemella	4.1 ± 1.0
Granulicatella	2.2 ± 0.7	Gemella	1.9 ± 0.4	Granulicatella	3.3 ± 0.7
Lactobacillales	1.4 ± 0.2	Lactobacillales	1.2 ± 0.1	Lactobacillales	1.3 ± 0.2
Prevotella	0.7 ± 0.5	Haemophilus	1.1 ± 0.9	Porphyromonas	1.1 ± 0.5
Prevotellaceae	0.7 ± 0.5	Actinomyces	1.0 ± 0.4	Actinomyces	1.1 ± 0.5
Bacteria	0.5 ± 0.1	Bacteria	0.5 ± 0.1	Prevotella	1.0 ± 0.5
Porphyromonas	0.4 ± 0.2	Porphyromonas	0.3 ± 0.2	Haemophilus	0.7 ± 0.3
Actinomyces	0.4 ± 0.1	Prevotella	0.3 ± 0.1	Bacteria	0.5 ± 0.1
Actinomycetales	0.3 ± 0.1	Actinomycetales	0.3 ± 0.1	Leptotrichia	0.5 ± 0.5
Pilibacter	0.3 ± 0.0	Pilibacter	0.3 ± 0.0	Neisseria	0.4 ± 0.3
Firmicutes	0.3 ± 0.0	Fusobacterium	0.2 ± 0.2	Prevotellaceae	0.4 ± 0.2
Soonwooa	0.3 ± 0.1	Firmicutes	0.2 ± 0.0	Firmicutes	0.3 ± 0.1
Pseudomonas	0.3 ± 0.2	Neisseria	0.2 ± 0.2	TM7_genera_incertae_sedis	0.2 ± 0.2
Bacilli	0.2 ± 0.0	TM7_genera_incertae_sedis	0.2 ± 0.2	Actinomycetales	0.2 ± 0.1
Haemophilus	0.2 ± 0.1	Bacilli	0.2 ± 0.0	Pilibacter	0.2 ± 0.0
Hallella	0.1 ± 0.1	Staphylococcus	0.1 ± 0.1	Pasteurellaceae	0.2 ± 0.1
Lactobacillus	0.1 ± 0.1	Pseudomonas	0.1 ± 0.1	Bacilli	0.2 ± 0.0

Table 4.13 *Relative abundances of most abundant genera identified in maternal feces from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Bacteroides	23.2 ± 3.6	Bacteroides	20.4 ± 7.2	Bacteroides	22.7 ± 3.7
Lachnospiracea_incertae_sedis	8.2 ± 1.6	Escherichia/Shigella	16.5 ± 6.9	Prevotella	10.1 ± 3.9
Faecalibacterium	7.9 ± 1.9	Streptococcus	13.3 ± 6.2	Faecalibacterium	9.3 ± 1.6
Prevotella	7.9 ± 3.02	Veillonella	10.3 ± 6.2	Lachnospiracea_incertae_sedis	8.1 ± 1.2
Lachnospiraceae	5.1 ± 0.7	Haemophilus	7.2 ± 3.8	Lachnospiraceae	5.8 ± 0.8
Porphyromonas	3.9 ± 1.8	Clostridium sensu stricto	4.9 ± 3.3	Porphyromonas	3.3 ± 1.5
Ruminococcaceae	3.5 ± 0.7	Parabacteroides	4.5 ± 2.3	Ruminococcaceae	2.7 ± 0.5
Clostridiales	3.2 ± 0.6	Klebsiella	4.2 ± 4.2	Bacteria	2.4 ± 1.3
Oscillibacter	3.1 ± 1.0	Pseudomonas	2.2 ± 0.8	Parabacteroides	2.4 ± 0.8
Dialister	2.3 ± 1.1	Enterobacter	2.0 ± 1.2	Roseburia	2.1 ± 0.5
Parabacteroides	2.0 ± 0.6	Propionibacterium	1.5 ± 1.5	Oscillibacter	2.1 ± 0.6
Akkermansia	1.8 ± 1.1	Sutterella	1.3 ± 1.1	Clostridiales	2.0 ± 0.3
Anaerosphaera	1.6 ± 0.8	Lactococcus	1.1 ± 1.0	Unknown	1.8 ± 1.1
Bacteria	1.5 ± 0.3	Enterobacteriaceae	0.9 ± 0.2	Clostridium XVIII	1.7 ± 1.6
Subdoligranulum	1.5 ± 0.5	Enterococcus	0.9 ± 0.9	Clostridium XlVa	1.7 ± 1.1
Roseburia	1.5 ± 0.3	Weissella	0.7 ± 0.6	Dialister	1.4 ± 0.7
Ruminococcus	1.4 ± 0.6	Staphylococcus	0.6 ± 0.2	Prevotellaceae	1.2 ± 0.7
Alistipes	1.3 ± 0.5	Leuconostoc	0.6 ± 0.6	Blautia	1.1 ± 0.4
Clostridium IV	1.1 ± 0.6	Phascolarctobacterium	0.6 ± 0.6	Finegoldia	0.8 ± 0.5
Clostridium XlVa	0.8 ± 0.2	Megasphaera	0.5 ± 0.5	Anaerococcus	0.8 ± 0.4

Table 4.14 *Relative abundances of most abundant genera identified in maternal feces from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Bacteroides	23.3 ± 4.1	Bacteroides	21.8 ± 3.6	Bacteroides	23.4 ± 4.0
Faecalibacterium	8.6 ± 1.3	Lachnospiracea_incertae_sedis	6.9 ± 1.0	Prevotella	10.0 ± 3.4
Prevotella	8.4 ± 2.9	Faecalibacterium	5.8 ± 1.2	Faecalibacterium	8.8 ± 1.6
Lachnospiracea_incertae_sedis	7.1 ± 0.8	Porphyromonas	5.4 ± 1.9	Lachnospiracea_incertae_sedis	7.5 ± 1.1
Lachnospiraceae	6.1 ± 0.9	Lachnospiraceae	4.8 ± 0.6	Lachnospiraceae	5.4 ± 0.6
Ruminococcaceae	3.9 ± 0.7	Oscillibacter	4.5 ± 1.8	Ruminococcaceae	3.4 ± 0.5
Porphyromonas	3.6 ± 1.5	Prevotella	4.2 ± 1.1	Oscillibacter	3.3 ± 0.9
Oscillibacter	3.5 ± 0.8	Ruminococcaceae	3.8 ± 0.7	Porphyromonas	3.3 ± 1.5
Clostridiales	2.8 ± 0.5	Clostridiales	3.6 ± 0.9	Clostridiales	2.9 ± 0.5
Parabacteroides	2.5 ± 0.7	Lactobacillus	3.2 ± 3.2	Campylobacter	2.2 ± 1.3
Roseburia	1.5 ± 0.3	Dialister	2.7 ± 1.4	Dialister	1.7 ± 0.6
Dialister	1.3 ± 0.4	Parabacteroides	2.3 ± 0.9	Roseburia	1.5 ± 0.3
Clostridium XlVa	1.3 ± 0.5	Campylobacter	1.8 ± 1.0	Parabacteroides	1.5 ± 0.4
Bacteria	1.3 ± 0.3	Unknown	1.7 ± 1.6	Bacteria	1.2 ± 0.3
Anaerococcus	1.1 ± 0.7	Roseburia	1.5 ± 0.4	Finegoldia	1.1 ± 0.5
Peptoniphilus	1.0 ± 0.5	Akkermansia	1.5 ± 1.0	Peptoniphilus	1.1 ± 0.4
Anaerosphaera	1.0 ± 0.5	Bacteria	1.4 ± 0.4	Blautia	1.0 ± 0.4
Finegoldia	1.0 ± 0.4	Anaerosphaera	1.1 ± 0.5	Anaerococcus	1.0 ± 0.6
Porphyromonadaceae	0.9 ± 0.4	Peptoniphilus	1.1 ± 0.4	Unknown	0.9 ± 0.6
Subdoligranulum	0.9 ± 0.2	Ruminococcus	1.0 ± 0.3	Ruminococcus	0.9 ± 0.2

Table 4.15 *Relative abundances of most abundant genera identified in maternal feces from 120 d through 180 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Bacteroides	24.7 ± 4.2	Bacteroides	21.7 ± 3.5	Bacteroides	20.7 ± 3.6
Faecalibacterium	11.5 ± 2.4	Faecalibacterium	8.5 ± 1.9	Faecalibacterium	10.5 ± 3.2
Lachnospiracea_incertae_sedis	9.0 ± 1.5	Prevotella	8.1 ± 3.2	Lachnospiracea_incertae_sedis	7.7 ± 1.0
Lachnospiraceae	5.3 ± 0.8	Lachnospiracea_incertae_sedis	7.6 ± 1.7	Prevotella	6.7 ± 3.2
Parabacteroides	4.2 ± 1.4	Lachnospiraceae	4.9 ± 0.6	Lachnospiraceae	6.3 ± 1.0
Prevotella	3.9 ± 1.8	Porphyromonas	4.2 ± 1.7	Ruminococcaceae	4.0 ± 0.9
Ruminococcaceae	3.3 ± 0.5	Oscillibacter	3.6 ± 1.2	Oscillibacter	3.8 ± 1.2
Oscillibacter	2.9 ± 0.8	Ruminococcaceae	3.4 ± 0.7	Dialister	2.8 ± 1.3
Blautia	2.1 ± 1.3	Clostridiales	2.8 ± 0.4	Porphyromonas	2.7 ± 1.6
Lactobacillus	2.1 ± 1.4	Parabacteroides	2.7 ± 0.7	Clostridiales	2.4 ± 0.3
Clostridium XlVa	2.0 ± 1.1	Blautia	1.7 ± 0.7	Clostridium XlVa	2.0 ± 1.3
Clostridiales	1.9 ± 0.2	Campylobacter	1.4 ± 1.0	Alistipes	1.9 ± 1.0
Dialister	1.8 ± 0.9	Peptoniphilus	1.4 ± 0.6	Blautia	1.8 ± 0.8
Porphyromonas	1.6 ± 0.5	Roseburia	1.3 ± 0.4	Roseburia	1.6 ± 0.5
Roseburia	1.3 ± 0.3	Bacteria	1.3 ± 0.3	Parabacteroides	1.6 ± 0.3
Ruminococcus	1.2 ± 0.3	Alistipes	1.2 ± 0.2	Anaerococcus	1.3 ± 1.0
Bacteria	1.1 ± 0.2	Subdoligranulum	1.1 ± 0.3	Clostridium IV	1.2 ± 0.6
Alistipes	1.1 ± 0.3	Akkermansia	1.0 ± 0.5	Peptoniphilus	1.1 ± 0.6
Subdoligranulum	1.0 ± 0.3	Anaerosphaera	1.0 ± 0.4	Bacteria	0.9 ± 0.1
Coprococcus	1.0 ± 0.3	Clostridium IV	0.9 ± 0.4	Subdoligranulum	0.9 ± 0.2

Table 4.16 *Relative abundances of most abundant genera identified in milk from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Streptococcus	30.2 ± 6.5	Staphylococcus	33.0 ± 4.8	Streptococcus	35.3 ± 5.5
Staphylococcus	26.2 ± 8.7	Streptococcus	31.1 ± 4.9	Staphylococcus	23.4 ± 6.4
Gemella	13.7 ± 7.8	Gemella	7.4 ± 3.0	Bacteria	5.7 ± 1.7
Bacteria	4.3 ± 1.4	Bacteria	5.3 ± 1.4	Gemella	5.7 ± 2.2
Propionibacterium	3.5 ± 1.7	Rothia	4.3 ± 2.0	Lactobacillus	4.9 ± 4.9
Lactococcus	1.8 ± 1.8	Corynebacterium	2.0 ± 1.0	Rothia	3.3 ± 1.7
Comamonas	1.8 ± 1.7	Propionibacterium	1.6 ± 1.0	Veillonella	2.7 ± 1.2
Leuconostoc	1.7 ± 1.7	Lactobacillales	1.5 ± 0.2	Corynebacterium	2.1 ± 1.2
Lactobacillales	1.6 ± 0.3	Unknown	1.5 ± 0.8	Propionibacterium	2.1 ± 0.6
Pseudomonas	1.0 ± 0.5	Bacillales	1.3 ± 0.1	Lactobacillales	1.5 ± 0.2
Prevotella	0.9 ± 0.7	Veillonella	1.1 ± 0.5	Actinomyces	1.2 ± 0.9
Bacillales	0.9 ± 0.2	Bacilli	0.9 ± 0.1	Unknown	1.1 ± 0.3
Weissella	0.8 ± 0.8	Staphylococcaceae	0.8 ± 0.1	Bacillales	0.9 ± 0.1
Unknown	0.8 ± 0.3	Pseudomonas	0.7 ± 0.3	Pseudomonas	0.9 ± 0.3
Haemophilus	0.8 ± 0.5	Clostridium sensu stricto	0.7 ± 0.2	Bifidobacterium	0.9 ± 0.9
Bacilli	0.8 ± 0.1	Actinomycetales	0.5 ± 0.2	Clostridium sensu stricto	0.8 ± 0.4
Rothia	0.7 ± 0.5	Hafnia	0.4 ± 0.3	Bacilli	0.7 ± 0.1
Clostridium sensu stricto	0.6 ± 0.4	Bacteroides	0.4 ± 0.3	Prevotella	0.5 ± 0.2
Corynebacterium	0.5 ± 0.3	Firmicutes	0.4 ± 0.1	Actinomycetales	0.5 ± 0.2
Staphylococcaceae	0.5 ± 0.1	Haemophilus	0.4 ± 0.2	Staphylococcaceae	0.4 ± 0.1

Table 4.17 *Relative abundances of most abundant genera identified in milk from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Streptococcus	43.5 ± 6.7	Streptococcus	53.6 ± 5.3	Streptococcus	59.0 ± 6.8
Staphylococcus	30.1 ± 6.3	Staphylococcus	14.8 ± 4.4	Staphylococcus	19.0 ± 6.2
Gemella	3.6 ± 1.6	Bacteria	7.9 ± 1.9	Bacteria	3.7 ± 0.7
Lactobacillus	3.2 ± 2.2	Lactobacillus	2.8 ± 1.6	Veillonella	1.9 ± 0.7
Bacteria	2.1 ± 0.3	Rothia	2.0 ± 0.9	Rothia	1.8 ± 0.9
Lactobacillales	1.6 ± 0.3	Veillonella	1.8 ± 0.7	Lactobacillales	1.7 ± 0.3
Veillonella	1.6 ± 0.5	Unknown	1.7 ± 0.6	Lactobacillus	1.3 ± 0.8
Rothia	1.4 ± 1.2	Lactobacillales	1.4 ± 0.2	Gemella	1.0 ± 0.2
Corynebacterium	1.3 ± 0.9	Gemella	1.2 ± 0.6	Propionibacterium	0.9 ± 0.7
Bacillales	1.1 ± 0.1	Propionibacterium	1.2 ± 0.5	Bacillales	0.8 ± 0.2
Prevotella	1.0 ± 0.8	Clostridium sensu stricto	0.9 ± 0.3	Unknown	0.6 ± 0.1
Bacilli	0.7 ± 0.1	Pseudomonas	0.9 ± 0.2	Bacilli	0.6 ± 0.1
Propionibacterium	0.7 ± 0.3	Firmicutes	0.8 ± 0.2	Corynebacterium	0.6 ± 0.2
Actinomyces	0.5 ± 0.4	Bacillales	0.6 ± 0.1	Pseudomonas	0.5 ± 0.2
Unknown	0.5 ± 0.1	Finegoldia	0.5 ± 0.5	Firmicutes	0.4 ± 0.1
Pseudomonas	0.5 ± 0.2	Bacilli	0.5 ± 0.1	Bifidobacterium	0.4 ± 0.4
Clostridium sensu stricto	0.5 ± 0.2	Hafnia	0.4 ± 0.3	Prevotella	0.4 ± 0.3
Staphylococcaceae	0.4 ± 0.1	Prevotella	0.4 ± 0.1	Staphylococcaceae	0.4 ± 0.1
Actinomycetales	0.4 ± 0.1	Bacteroides	0.3 ± 0.1	Actinomycetales	0.3 ± 0.1
Firmicutes	0.4 ± 0.1	Staphylococcaceae	0.3 ± 0.1	Pilibacter	0.3 ± 0.1

Table 4.18 *Relative abundances of most abundant genera identified in milk from 120 d through 180 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Streptococcus	47.7 ± 6.9	Streptococcus	54.5 ± 5.7	Streptococcus	54.5 ± 5.7
Staphylococcus	29.0 ± 8.2	Staphylococcus	10.7 ± 4.4	Staphylococcus	10.7 ± 4.4
Bacteria	4.6 ± 1.2	Bacteria	5.9 ± 1.8	Bacteria	5.9 ± 1.8
Veillonella	2.2 ± 0.8	Veillonella	3.3 ± 0.9	Veillonella	3.3 ± 0.9
Rothia	2.1 ± 1.0	Pseudomonas	3.2 ± 1.7	Pseudomonas	3.2 ± 1.7
Unknown	1.6 ± 0.7	Unknown	2.5 ± 1.1	Unknown	2.5 ± 1.1
Lactobacillales	1.5 ± 0.2	Rothia	2.3 ± 0.8	Rothia	2.3 ± 0.8
Propionibacterium	0.8 ± 0.5	Lactobacillales	1.5 ± 0.2	Lactobacillales	1.5 ± 0.2
Gemella	0.7 ± 0.2	Granulicatella	1.4 ± 0.6	Granulicatella	1.4 ± 0.6
Bacillales	0.7 ± 0.1	Gemella	1.2 ± 0.3	Gemella	1.2 ± 0.3
Pseudomonas	0.7 ± 0.2	Prevotella	0.8 ± 0.3	Prevotella	0.8 ± 0.3
Granulicatella	0.6 ± 0.4	Propionibacterium	0.8 ± 0.5	Propionibacterium	0.8 ± 0.5
Bacilli	0.5 ± 0.1	Actinomyces	0.7 ± 0.3	Actinomyces	0.7 ± 0.3
Staphylococcaceae	0.4 ± 0.1	Corynebacterium	0.7 ± 0.3	Corynebacterium	0.7 ± 0.3
Bacteroides	0.4 ± 0.2	Bacillales	0.5 ± 0.1	Bacillales	0.5 ± 0.1
Actinomyces	0.4 ± 0.3	Firmicutes	0.5 ± 0.1	Firmicutes	0.5 ± 0.1
Corynebacterium	0.4 ± 0.2	Pseudomonadaceae	0.4 ± 0.1	Pseudomonadaceae	0.4 ± 0.1
Lactobacillus	0.4 ± 0.3	Bacilli	0.4 ± 0.1	Bacilli	0.4 ± 0.1
Firmicutes	0.3 ± 0.0	Ruminococcaceae	0.4 ± 0.2	Ruminococcaceae	0.4 ± 0.2
Actinomycetales	0.3 ± 0.1	Bacteroides	0.4 ± 0.2	Bacteroides	0.4 ± 0.2

Table 4.19 *Relative abundances of most abundant genera identified in maternal saliva from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Streptococcus	66.2 ± 2.5	Streptococcus	55.0 ± 3.4	Streptococcus	62.7 ± 3.1
Gemella	6.7 ± 1.2	Veillonella	7.2 ± 1.3	Rothia	4.5 ± 1.0
Rothia	4.4 ± 1.2	Rothia	7.1 ± 1.6	Prevotella	4.5 ± 1.0
Veillonella	4.1 ± 1.0	Prevotella	6.6 ± 1.4	Veillonella	4.4 ± 0.8
Prevotella	3.9 ± 1.1	Gemella	4.4 ± 0.9	Gemella	4.2 ± 1.0
Haemophilus	2.4 ± 0.6	Granulicatella	2.7 ± 0.7	Neisseria	2.4 ± 0.9
Granulicatella	2.2 ± 0.7	Haemophilus	2.1 ± 0.6	Haemophilus	1.9 ± 0.4
Neisseria	2.0 ± 0.6	Neisseria	1.7 ± 0.6	Granulicatella	1.5 ± 0.3
Lactobacillales	0.9 ± 0.1	Porphyromonas	1.5 ± 1.0	Schlegelella	1.4 ± 0.8
Actinomyces	0.8 ± 0.3	Actinomyces	1.4 ± 0.2	Actinomyces	1.3 ± 0.5
Porphyromonas	0.6 ± 0.3	Lactobacillales	1.0 ± 0.1	Fusobacterium	1.1 ± 0.4
Prevotellaceae	0.5 ± 0.2	Prevotellaceae	0.8 ± 0.3	Porphyromonas	1.1 ± 0.4
Abiotrophia	0.4 ± 0.2	Lactobacillus	0.8 ± 0.7	Lactobacillales	0.9 ± 0.1
Neisseriaceae	0.3 ± 0.1	Fusobacterium	0.8 ± 0.3	Prevotellaceae	0.8 ± 0.4
Soonwooa	0.3 ± 0.2	Abiotrophia	0.5 ± 0.3	Abiotrophia	0.4 ± 0.2
Pasteurellaceae	0.3 ± 0.1	Leptotrichia	0.4 ± 0.2	Soonwooa	0.4 ± 0.2
Schlegelella	0.2 ± 0.1	Schlegelella	0.4 ± 0.1	Bacteria	0.4 ± 0.1
Bacteria	0.2 ± 0.1	Bacteria	0.4 ± 0.0	Neisseriaceae	0.4 ± 0.1
Fusobacterium	0.2 ± 0.1	Actinomycetales	0.3 ± 0.1	Leptotrichia	0.3 ± 0.1
Pilibacter	0.2 ± 0.0	Hallella	0.2 ± 0.1	Pasteurellaceae	0.3 ± 0.1

Table 4.20 *Relative abundances of most abundant genera identified in maternal saliva from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Streptococcus	48.8 ± 3.5	Streptococcus	51.2 ± 4.2	Streptococcus	49.4 ± 4.6
Rothia	8.3 ± 2.1	Gemella	7.3 ± 1.7	Gemella	8.8 ± 2.0
Veillonella	6.0 ± 1.0	Prevotella	6.9 ± 2.0	Neisseria	7.5 ± 1.8
Prevotella	6.0 ± 1.2	Rothia	6.5 ± 2.1	Veillonella	5.8 ± 1.2
Gemella	4.9 ± 0.9	Neisseria	5.6 ± 1.6	Rothia	5.7 ± 1.4
Neisseria	4.9 ± 1.3	Veillonella	4.9 ± 1.2	Prevotella	5.6 ± 1.4
Granulicatella	3.2 ± 0.5	Haemophilus	3.1 ± 0.6	Granulicatella	2.9 ± 0.5
Haemophilus	3.1 ± 0.6	Granulicatella	2.5 ± 0.4	Haemophilus	2.6 ± 0.6
Fusobacterium	1.5 ± 0.3	Porphyromonas	1.3 ± 0.7	Fusobacterium	1.7 ± 0.5
Actinomyces	1.2 ± 0.2	Prevotellaceae	1.1 ± 0.4	Actinomyces	0.9 ± 0.2
Leptotrichia	1.1 ± 0.5	Fusobacterium	0.9 ± 0.3	Prevotellaceae	0.8 ± 0.2
Porphyromonas	0.9 ± 0.3	Actinomyces	0.9 ± 0.2	Porphyromonas	0.8 ± 0.3
Schlegelella	0.8 ± 0.3	Lactobacillales	0.8 ± 0.1	Lactobacillales	0.8 ± 0.1
Lactobacillales	0.8 ± 0.1	Leptotrichia	0.7 ± 0.2	Leptotrichia	0.7 ± 0.2
Prevotellaceae	0.7 ± 0.2	Schlegelella	0.7 ± 0.2	Neisseriaceae	0.7 ± 0.1
Neisseriaceae	0.5 ± 0.1	Neisseriaceae	0.4 ± 0.1	Pasteurellaceae	0.3 ± 0.1
Bacteria	0.4 ± 0.1	Bacteria	0.3 ± 0.0	Bacteria	0.3 ± 0.0
Pasteurellaceae	0.4 ± 0.1	Pasteurellaceae	0.3 ± 0.1	Abiotrophia	0.3 ± 0.1
Soonwooa	0.4 ± 0.1	Abiotrophia	0.2 ± 0.1	Schlegelella	0.2 ± 0.1
Actinomycetales	0.3 ± 0.1	TM7_genera_incertae_sedis	0.2 ± 0.1	Actinomycetales	0.2 ± 0.0

Table 4.21 *Relative abundances of most abundant genera identified in maternal saliva from 120 d through 180 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Streptococcus	51.2 ± 4.6	Streptococcus	46.4 ± 3.1	Streptococcus	50.6 ± 3.8
Gemella	7.2 ± 1.8	Rothia	7.1 ± 1.5	Gemella	6.8 ± 1.5
Prevotella	6.2 ± 1.9	Prevotella	6.1 ± 1.2	Rothia	6.6 ± 1.5
Rothia	6.1 ± 1.1	Veillonella	5.9 ± 1.1	Prevotella	6.1 ± 2.0
Neisseria	5.9 ± 1.4	Gemella	5.4 ± 1.0	Neisseria	5.3 ± 1.1
Veillonella	4.0 ± 0.7	Neisseria	5.1 ± 1.0	Veillonella	4.5 ± 0.8
Haemophilus	3.2 ± 0.8	Granulicatella	3.5 ± 0.5	Granulicatella	3.6 ± 0.6
Granulicatella	2.5 ± 0.3	Haemophilus	3.5 ± 1.0	Haemophilus	3.0 ± 0.9
Actinomyces	1.4 ± 0.5	Fusobacterium	2.2 ± 0.6	Fusobacterium	1.5 ± 0.5
Fusobacterium	1.1 ± 0.3	Actinomyces	1.8 ± 0.4	Actinomyces	1.3 ± 0.3
Lactobacillales	0.8 ± 0.1	Leptotrichia	1.1 ± 0.3	Leptotrichia	0.9 ± 0.3
Prevotellaceae	0.8 ± 0.3	Porphyromonas	0.9 ± 0.2	Lactobacillales	0.8 ± 0.1
Neisseriaceae	0.7 ± 0.2	Schlegelella	0.8 ± 0.6	Prevotellaceae	0.8 ± 0.2
Porphyromonas	0.7 ± 0.2	Prevotellaceae	0.8 ± 0.2	Porphyromonas	0.7 ± 0.2
Leptotrichia	0.6 ± 0.2	Lactobacillales	0.7 ± 0.1	Neisseriaceae	0.7 ± 0.1
Schlegelella	0.5 ± 0.2	Actinobacillus	0.7 ± 0.6	Moraxella	0.5 ± 0.5
Bacteria	0.4 ± 0.1	Pasteurellaceae	0.5 ± 0.3	Bacteria	0.4 ± 0.1
Pasteurellaceae	0.3 ± 0.1	Neisseriaceae	0.5 ± 0.1	Pasteurellaceae	0.3 ± 0.1
Streptophyta	0.3 ± 0.2	Bacteria	0.4 ± 0.1	Schlegelella	0.3 ± 0.1
Abiotrophia	0.3 ± 0.1	Abiotrophia	0.3 ± 0.1	Actinomycetales	0.3 ± 0.1

Table 4.22 *Relative abundances of most abundant genera identified in infant feces from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Firmicutes	41.8 ± 9.6	Proteobacteria	36.2 ± 7.9	Firmicutes	40.7 ± 7.5
Bacteroidetes	37.0 ± 11.2	Firmicutes	36.2 ± 8.3	Proteobacteria	36.9 ± 6.5
Proteobacteria	19.1 ± 6.0	Bacteroidetes	25.5 ± 8.5	Bacteroidetes	20.7 ± 7.5
Actinobacteria	1.4 ± 0.6	Actinobacteria	1.8 ± 1.4	Actinobacteria	1.5 ± 0.7
Bacteria	0.4 ± 0.1	Bacteria	0.2 ± 0.0	Bacteria	0.2 ± 0.1
TM7	0.2 ± 0.1	Cyanobacteria/Chloroplast	0.0 ± 0.0	Unknown	0.1 ± 0.0
Fusobacteria	0.1 ± 0.1	Spirochaetes	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0
Spirochaetes	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0	Cyanobacteria/Chloroplast	0.0 ± 0.0
Fibrobacteres	0.0 ± 0.0	Unknown	0.0 ± 0.0	OP11	0.0 ± 0.0
Unknown	0.0 ± 0.0	TM7	0.0 ± 0.0	Acidobacteria	0.0 ± 0.0

Table 4.23 *Relative abundances of most abundant phyla identified in infant feces from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Firmicutes	32.8 ± 6.6	Firmicutes	43.3 ± 7.2	Firmicutes	52.3 ± 8.5
Proteobacteria	31.5 ± 6.9	Proteobacteria	25.9 ± 5.3	Proteobacteria	23.4 ± 5.5
Bacteroidetes	29.7 ± 7.9	Bacteroidetes	20.4 ± 7.3	Bacteroidetes	19.1 ± 7.0
Actinobacteria	5.7 ± 3.0	Actinobacteria	10.0 ± 3.7	Actinobacteria	4.7 ± 1.4
Bacteria	0.3 ± 0.1	Bacteria	0.3 ± 0.1	Bacteria	0.5 ± 0.3
Unknown	0.0 ± 0.0	Unknown	0.0 ± 0.0	Unknown	0.0 ± 0.0
Cyanobacteria/Chloroplast	0.0 ± 0.0	Fibrobacteres	0.0 ± 0.0	OD1	0.0 ± 0.0
Deinococcus-Thermus	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0	Tenericutes	0.0 ± 0.0
Fusobacteria	0.0 ± 0.0	TM7	0.0 ± 0.0	Verrucomicrobia	0.0 ± 0.0
Verrucomicrobia	0.0 ± 0.0	Verrucomicrobia	0.0 ± 0.0	Acidobacteria	0.0 ± 0.0

Table 4.24 *Relative abundances of most abundant phyla identified in infant feces from 30 d through 90 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Firmicutes	38.4 ± 7.6	Firmicutes	36.2 ± 6.7	Firmicutes	42.4 ± 6.3
Proteobacteria	29.7 ± 6.1	Bacteroidetes	29.8 ± 7.5	Bacteroidetes	31.0 ± 8.4
Bacteroidetes	20.7 ± 7.0	Proteobacteria	21.0 ± 5.6	Proteobacteria	19.7 ± 4.1
Actinobacteria	10.9 ± 3.3	Actinobacteria	8.0 ± 3.6	Actinobacteria	6.0 ± 2.3
Bacteria	0.2 ± 0.0	Verrucomicrobia	4.2 ± 4.2	Verrucomicrobia	0.6 ± 0.6
Cyanobacteria/Chloroplast	0.0 ± 0.0	Bacteria	0.6 ± 0.2	Bacteria	0.3 ± 0.1
Unknown	0.0 ± 0.0	Unknown	0.1 ± 0.0	Unknown	0.0 ± 0.0
Fusobacteria	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0
Archaea	0.0 ± 0.0	TM7	0.0 ± 0.0	Spirochaetes	0.0 ± 0.0
Tenericutes	0.0 ± 0.0	Acidobacteria	0.0 ± 0.0	Acidobacteria	0.0 ± 0.0

Table 4.25 *Relative abundances of most abundant phyla identified in infant saliva from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Firmicutes	88.2 ± 5.5	Firmicutes	83.6 ± 7.1	Firmicutes	81.0 ± 7.3
Proteobacteria	6.4 ± 4.7	Actinobacteria	13.0 ± 7.0	Actinobacteria	11.6 ± 5.5
Bacteroidetes	3.8 ± 2.4	Proteobacteria	1.4 ± 0.8	Bacteroidetes	4.5 ± 2.6
Actinobacteria	0.9 ± 0.4	Bacteria	1.0 ± 0.2	Proteobacteria	2.2 ± 1.9
Bacteria	0.7 ± 0.2	Bacteroidetes	0.7 ± 0.2	Bacteria	0.6 ± 0.1
Unknown	0.1 ± 0.1	Unknown	0.4 ± 0.3	TM7	0.0 ± 0.0
TM7	0.0 ± 0.0	TM7	0.0 ± 0.0	Unknown	0.0 ± 0.0
Fibrobacteres	0.0 ± 0.0	Tenericutes	0.0 ± 0.0	Acidobacteria	0.0 ± 0.0
Planctomycetes	0.0 ± 0.0	Acidobacteria	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0
Tenericutes	0.0 ± 0.0	Archaea	0.0 ± 0.0	OD1	0.0 ± 0.0

Table 4.26 *Relative abundances of most abundant phyla identified in infant saliva from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Firmicutes	95.7 ± 1.9	Firmicutes	93.5 ± 1.0	Firmicutes	92.0 ± 1.3
Actinobacteria	2.6 ± 1.8	Actinobacteria	5.2 ± 1.1	Actinobacteria	5.4 ± 1.1
Bacteroidetes	1.2 ± 0.8	Proteobacteria	0.6 ± 0.5	Bacteroidetes	1.1 ± 0.3
Bacteria	0.4 ± 0.1	Bacteroidetes	0.3 ± 0.1	Bacteria	0.8 ± 0.5
Proteobacteria	0.2 ± 0.1	Bacteria	0.3 ± 0.0	Proteobacteria	0.6 ± 0.2
Fusobacteria	0.0 ± 0.0	Unknown	0.0 ± 0.0	TM7	0.0 ± 0.0
Cyanobacteria/Chloroplast	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0
Deinococcus-Thermus	0.0 ± 0.0	Spirochaetes	0.0 ± 0.0	Unknown	0.0 ± 0.0
Acidobacteria	0.0 ± 0.0	TM7	0.0 ± 0.0	Spirochaetes	0.0 ± 0.0
Archaea	0.0 ± 0.0	Tenericutes	0.0 ± 0.0	Tenericutes	0.0 ± 0.0

Table 4.27 *Relative abundances of most abundant phyla identified in infant saliva from 120 d through 180 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Firmicutes	87.8 ± 1.4	Firmicutes	89.3 ± 2.0	Firmicutes	87.7 ± 2.3
Actinobacteria	8.2 ± 1.3	Actinobacteria	6.8 ± 1.3	Actinobacteria	5.7 ± 1.3
Bacteroidetes	2.6 ± 1.2	Proteobacteria	1.8 ± 1.1	Bacteroidetes	3.2 ± 0.9
Proteobacteria	0.7 ± 0.3	Bacteroidetes	1.0 ± 0.3	Proteobacteria	1.7 ± 0.7
Bacteria	0.5 ± 0.1	Bacteria	0.5 ± 0.1	Fusobacteria	0.8 ± 0.7
TM7	0.1 ± 0.0	Fusobacteria	0.4 ± 0.3	Bacteria	0.5 ± 0.1
Unknown	0.0 ± 0.0	TM7	0.2 ± 0.2	TM7	0.3 ± 0.3
Fusobacteria	0.0 ± 0.0	Unknown	0.0 ± 0.0	Unknown	0 ± 0.0
Cyanobacteria/Chloroplast	0.0 ± 0.0	Cyanobacteria/Chloroplast	0.0 ± 0	Cyanobacteria/Chloroplast	0 ± 0.0
Verrucomicrobia	0.0 ± 0.0	OD1	0 ± 0.0	Fibrobacteres	0 ± 0

Table 4.28 *Relative abundances of most abundant phyla identified in maternal feces from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Firmicutes	51.0 ± 3.5	Firmicutes	46.7 ± 3.2	Firmicutes	48.4 ± 3.6
Bacteroidetes	41.4 ± 3.4	Bacteroidetes	44.8 ± 3.3	Bacteroidetes	43.1 ± 3.9
Proteobacteria	3.2 ± 0.7	Proteobacteria	3.4 ± 1.0	Proteobacteria	2.4 ± 0.5
Verrucomicrobia	2.0 ± 1.2	Actinobacteria	1.8 ± 0.8	Bacteria	2.3 ± 1.2
Bacteria	1.4 ± 0.3	Verrucomicrobia	1.0 ± 0.6	Unknown	1.7 ± 1.1
Actinobacteria	0.8 ± 0.4	Bacteria	0.9 ± 0.1	Actinobacteria	1.0 ± 0.4
Unknown	0.1 ± 0.0	Fusobacteria	0.6 ± 0.3	Verrucomicrobia	0.7 ± 0.3
Fusobacteria	0.1 ± 0.0	Synergistetes	0.4 ± 0.2	Fusobacteria	0.2 ± 0.2
Synergistetes	0.0 ± 0.0	Unknown	0.4 ± 0.3	Synergistetes	0.1 ± 0.1
Lentisphaerae	0.0 ± 0.0	Cyanobacteria/Chloroplast	0.0 ± 0.0	Tenericutes	0.0 ± 0.0

Table 4.29 *Relative abundances of most abundant phyla identified in maternal feces from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Firmicutes	51.5 ± 2.9	Firmicutes	51.9 ± 3.7	Firmicutes	49.6 ± 2.9
Bacteroidetes	42.2 ± 3.0	Bacteroidetes	39.0 ± 3.4	Bacteroidetes	41.9 ± 3.0
Proteobacteria	2.3 ± 0.5	Proteobacteria	3.5 ± 1.1	Proteobacteria	4.7 ± 1.4
Bacteria	1.3 ± 0.3	Unknown	1.7 ± 1.6	Bacteria	1.2 ± 0.2
Actinobacteria	0.9 ± 0.2	Verrucomicrobia	1.4 ± 0.9	Actinobacteria	1.1 ± 0.5
Fusobacteria	0.7 ± 0.4	Bacteria	1.4 ± 0.4	Unknown	0.9 ± 0.6
Verrucomicrobia	0.7 ± 0.3	Actinobacteria	0.8 ± 0.3	Verrucomicrobia	0.5 ± 0.2
Unknown	0.3 ± 0.2	Fusobacteria	0.3 ± 0.2	Fusobacteria	0.1 ± 0.0
Synergistetes	0.0 ± 0.0	Synergistetes	0.0 ± 0.0	Cyanobacteria/Chloroplast	0.0 ± 0.0
Lentisphaerae	0.0 ± 0.0	Tenericutes	0.0 ± 0.0	Tenericutes	0.0 ± 0.0

Table 4.30 *Relative abundances of most abundant phyla identified in maternal feces from 120 d through 180 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Firmicutes	55.2 ± 4.3	Firmicutes	49.1 ± 2.8	Firmicutes	56.9 ± 3.5
Bacteroidetes	38.4 ± 4.4	Bacteroidetes	41.5 ± 2.8	Bacteroidetes	37.4 ± 3.6
Proteobacteria	3.0 ± 0.4	Proteobacteria	4.0 ± 1.2	Proteobacteria	2.8 ± 0.5
Actinobacteria	1.1 ± 0.3	Actinobacteria	2.1 ± 0.9	Actinobacteria	1.3 ± 0.7
Bacteria	1.1 ± 0.2	Bacteria	1.2 ± 0.3	Bacteria	0.8 ± 0.1
Verrucomicrobia	0.5 ± 0.2	Verrucomicrobia	1.0 ± 0.5	Verrucomicrobia	0.6 ± 0.3
Fusobacteria	0.4 ± 0.3	Fusobacteria	0.6 ± 0.5	Unknown	0.1 ± 0.1
Unknown	0.3 ± 0.2	Unknown	0.4 ± 0.2	Synergistetes	0.1 ± 0.1
Cyanobacteria/Chloroplast	0.0 ± 0.0	Synergistetes	0.1 ± 0.1	Fusobacteria	0.0 ± 0.0
Synergistetes	0.0 ± 0.0	Tenericutes	0.0 ± 0.0	TM7	0.0 ± 0.0

Table 4.31 *Relative abundances of most abundant phyla identified in maternal milk from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Firmicutes	80.7 ± 3.9	Firmicutes	80.2 ± 4.9	Firmicutes	78.6 ± 5.1
Proteobacteria	6.7 ± 3.5	Actinobacteria	9.1 ± 3.3	Actinobacteria	10.8 ± 4.5
Actinobacteria	5.7 ± 1.9	Bacteria	5.3 ± 1.4	Bacteria	6.0 ± 1.7
Bacteria	4.3 ± 1.3	Proteobacteria	2.9 ± 1.1	Proteobacteria	2.3 ± 0.5
Bacteroidetes	1.6 ± 0.8	Unknown	1.5 ± 0.8	Bacteroidetes	1.3 ± 0.3
Unknown	0.8 ± 0.3	Bacteroidetes	0.9 ± 0.4	Unknown	0.9 ± 0.3
Fusobacteria	0.1 ± 0.0	OD1	0.0 ± 0.0	Verrucomicrobia	0.0 ± 0.0
Fibrobacteres	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0	Cyanobacteria/Chloroplast	0.0 ± 0.0
Verrucomicrobia	0.0 ± 0.0	TM7	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0
Chloroflexi	0.0 ± 0.0	Verrucomicrobia	0.0 ± 0.0	OD1	0.0 ± 0.0

Table 4.32 *Relative abundances of most abundant phyla identified in maternal milk from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Firmicutes	89.0 ± 2.2	Firmicutes	81.4 ± 2.5	Firmicutes	88.2 ± 1.8
Actinobacteria	4.5 ± 1.6	Bacteria	8.3 ± 1.9	Actinobacteria	4.3 ± 1.4
Bacteria	2.2 ± 0.3	Actinobacteria	3.9 ± 1.0	Bacteria	3.7 ± 0.8
Bacteroidetes	2.1 ± 1.3	Proteobacteria	3.2 ± 0.6	Proteobacteria	1.8 ± 0.4
Proteobacteria	1.5 ± 0.3	Unknown	1.7 ± 0.6	Bacteroidetes	1.2 ± 0.5
Unknown	0.5 ± 0.1	Bacteroidetes	1.3 ± 0.4	Unknown	0.6 ± 0.1
TM7	0.1 ± 0.1	Cyanobacteria/Chloroplast	0.1 ± 0.0	Cyanobacteria/Chloroplast	0.1 ± 0.1
Tenericutes	0.0 ± 0.0	OD1	0.0 ± 0.0	Tenericutes	0.0 ± 0.0
OD1	0.0 ± 0.0	TM7	0.0 ± 0.0	OD1	0.0 ± 0.0
Spirochaetes	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0	Spirochaetes	0.0 ± 0.0

Table 4.33 *Relative abundances of most abundant phyla identified in maternal milk from 120 d through 180 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Firmicutes	85.5 ± 2.2	Firmicutes	77.4 ± 3.2	Firmicutes	75.2 ± 5.2
Bacteria	4.7 ± 1.2	Proteobacteria	6.2 ± 2.2	Proteobacteria	9.2 ± 5.2
Actinobacteria	4.4 ± 1.4	Bacteria	5.9 ± 1.8	Actinobacteria	7.3 ± 1.3
Proteobacteria	2.4 ± 0.6	Actinobacteria	5.4 ± 1.2	Bacteria	4.0 ± 0.7
Unknown	1.6 ± 0.7	Unknown	2.5 ± 1.1	Bacteroidetes	2.4 ± 0.9
Bacteroidetes	1.2 ± 0.4	Bacteroidetes	2.3 ± 0.6	Fusobacteria	0.7 ± 0.6
Fusobacteria	0.1 ± 0.0	TM7	0.1 ± 0.0	Unknown	0.7 ± 0.2
OD1	0.0 ± 0.0	Fusobacteria	0.0 ± 0.0	Tenericutes	0.2 ± 0.1
Cyanobacteria/Chloroplast	0.0 ± 0.0	Tenericutes	0.0 ± 0.0	TM7	0.1 ± 0.0
Spirochaetes	0.0 ± 0.0	OD1	0.0 ± 0.0	OD1	0.0 ± 0.0

Table 4.34 *Relative abundances of most abundant phyla identified in maternal saliva from 2 d through 10 d*

Day 2	Mean ± SEM	Day 5	Mean ± SEM	Day 10	Mean ± SEM
Firmicutes	82 ± 2.1	Firmicutes	73.5 ± 2.8	Firmicutes	76.2 ± 2.6
Proteobacteria	5.9 ± 1.0	Bacteroidetes	9.8 ± 2.0	Bacteroidetes	7.4 ± 1.5
Actinobacteria	5.7 ± 1.3	Actinobacteria	9.1 ± 1.8	Proteobacteria	7.3 ± 1.4
Bacteroidetes	5.6 ± 1.2	Proteobacteria	5.3 ± 1.1	Actinobacteria	6.4 ± 1.2
Fusobacteria	0.5 ± 0.2	Fusobacteria	1.5 ± 0.4	Fusobacteria	1.7 ± 0.5
Bacteria	0.3 ± 0.0	Bacteria	0.4 ± 0.1	Bacteria	0.4 ± 0.1
TM7	0.1 ± 0.0	Cyanobacteria/Chloroplast	0.2 ± 0.2	Cyanobacteria/Chloroplast	0.3 ± 0.2
Cyanobacteria/Chloroplast	0.0 ± 0.0	TM7	0.2 ± 0.0	TM7	0.2 ± 0.1
Unknown	0.0 ± 0.0	Spirochaetes	0.0 ± 0.0	Spirochaetes	0.1 ± 0.1
Verrucomicrobia	0.0 ± 0.0	Tenericutes	0.0 ± 0.0	Synergistetes	0.0 ± 0.0

Table 4.35 *Relative abundances of most abundant phyla identified in maternal saliva from 30 d through 90 d*

Day 30	Mean ± SEM	Day 60	Mean ± SEM	Day 90	Mean ± SEM
Firmicutes	66.3 ± 3.3	Firmicutes	68.7 ± 3.9	Firmicutes	69.3 ± 4.1
Proteobacteria	10.7 ± 1.7	Proteobacteria	11.1 ± 1.8	Proteobacteria	12.1 ± 2.1
Actinobacteria	10.2 ± 2.2	Bacteroidetes	9.8 ± 2.6	Bacteroidetes	8.1 ± 1.5
Bacteroidetes	8.7 ± 1.4	Actinobacteria	7.7 ± 2.0	Actinobacteria	6.9 ± 1.4
Fusobacteria	3.2 ± 0.7	Fusobacteria	2.1 ± 0.6	Fusobacteria	2.9 ± 0.7
Bacteria	0.4 ± 0.1	Bacteria	0.3 ± 0.0	Bacteria	0.3 ± 0.1
TM7	0.3 ± 0.1	TM7	0.3 ± 0.1	TM7	0.2 ± 0.0
Cyanobacteria/Chloroplast	0.1 ± 0.0	Cyanobacteria/Chloroplast	0.0 ± 0.0	Cyanobacteria/Chloroplast	0.1 ± 0.0
Spirochaetes	0.0 ± 0.0	SR1	0.0 ± 0.0	SR1	0.0 ± 0.0
Synergistetes	0.0 ± 0.0	Unknown	0.0 ± 0.0	Verrucomicrobia	0.0 ± 0.0

Table 4.36 *Relative abundances of most abundant phyla identified in maternal saliva from 120 d through 180 d*

Day 120	Mean ± SEM	Day 150	Mean ± SEM	Day 180	Mean ± SEM
Firmicutes	68.2 ± 4.1	Firmicutes	65.0 ± 2.6	Firmicutes	68.5 ± 3.7
Proteobacteria	12.3 ± 1.9	Proteobacteria	13.0 ± 2.1	Proteobacteria	11.0 ± 1.8
Bacteroidetes	8.6 ± 2.3	Actinobacteria	9.1 ± 1.7	Actinobacteria	8.6 ± 1.8
Actinobacteria	7.7 ± 1.3	Bacteroidetes	8.4 ± 1.4	Bacteroidetes	8.5 ± 2.2
Fusobacteria	2.2 ± 0.5	Fusobacteria	3.7 ± 0.8	Fusobacteria	2.8 ± 0.6
Bacteria	0.4 ± 0.1	Bacteria	0.4 ± 0.0	Bacteria	0.3 ± 0.1
Cyanobacteria/Chloroplast	0.3 ± 0.2	TM7	0.3 ± 0.1	TM7	0.2 ± 0.1
TM7	0.2 ± 0.1	Cyanobacteria/Chloroplast	0.1 ± 0.0	Cyanobacteria/Chloroplast	0.0 ± 0.0
Unknown	0.0 ± 0.0	Tenericutes	0.0 ± 0.0	SR1	0.0 ± 0.0
Spirochaetes	0.0 ± 0.0	Spirochaetes	0.0 ± 0.0	Synergistetes	0.0 ± 0.0

CHAPTER 5
CONCLUSIONS

5.1 CONCLUSIONS

The microbial communities of the mother-infant dyad are indeed complex, and the results presented in this dissertation suggest they are inexplicably linked. Factors mitigating the composition of these bacterial communities are likely many and include maternal diet, interaction with other microbiomes of the maternal-infant dyad, genetics, breastfeeding practices, delivery mode, maternal and infant health status, and other environmental exposures to name a few. For example, we observed that the milk microbiome is dynamic over the first 6 months postpartum and is associated with the dietary intake of several nutrients. Variation in host cells (e.g., maternal immune cells) in the milk is also associated with the structure of the bacterial community. Different milk bacterial profiles appear to be present when the distribution of host milk cells are either high in macrophages / secretory epithelial cells or when there is a high relative abundance of neutrophils. In Chapter 4, we present evidence that the bacterial communities of the maternal mouth, feces, and milk are strongly associated with the bacterial communities of the infant mouth and feces.

However, the data in this dissertation have only been examined through associations and correlations. More research is needed to tease apart whether these associations are causal in nature and, if so, their directionality, if any. Experiments specifically designed to test the causality of various factors are needed to discern how and whether indeed, for example, maternal diet regulates the presence and growth of various members of the milk microbial community. Further interrogation of the current data using statistical and ecological modeling strategies will also likely lead to a better understanding of the complex interactions that exist among bacterial communities, milk micro- and macronutrients, and host cells over time.

Another potential area of further investigation is to further understand the origins of bacteria in human milk. Although studies demonstrate a bidirectional flow of fluid between the mouth of the infant and the mammary gland, this does not explain presence of bacteria in the mammary gland of women who have not undergone lactation and nursed a child.

In conclusion, the work presented in this dissertation provides initial insight into the relationships among many of the components in human milk. Importantly, these results provide a framework to continue to investigate how breastfeeding, lactation, and the infant's consumption of human milk reduces the risk of developing disease and how the components within human milk may provide the necessary factors that promote the health and well-being of both mother and child.

APPENDIX A: APPROVAL OF RESEARCH PROTOCOLS

University of Idaho
Office of Research Assurances
Institutional Review Board
PO Box 443010
Moscow ID 83844-3010

Phone: 208-885-6162
Fax: 208-885-5752
irb@uidaho.edu

To: Mark McGuire
Cc: Susan Buttram & Katherine Hunt

From: Traci Craig PhD
Chair, University of Idaho Institutional Review Board
University Research Office
Moscow Idaho 83844-3010

IRB No.: IRB00000843

FWA: FWA00005639

Date: March 15, 2011

Project: "Relationships among time postpartum, related factors and human milk microbiome"
IRB Number: 09-246
Approved: June 17, 2011
Expires: June 16, 2012

On behalf of the Institutional Review Board at the University of Idaho, I am pleased to inform you that the first-year extension of your proposal is approved as offering no significant risk to human subjects as no changes in protocol have been made on this project.

This extension of approval is valid until the date stated above at which time a second extension will need to be requested if you are still working on this project. If not, please advise the IRB committee when the project is completed.

Should there be any significant changes in your proposal within the year, it will be necessary for you to resubmit it for review.

Thank you for submitting your extension request.

Traci Craig

University of Idaho
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Institutional Review Board
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Moscow, Idaho 83844-3043
208-885-6324
Fax: 208-885-7710

Federalwide Assurance: FWA00005639
Federal Assigned IRB #: 00000843
UI Assigned Number: 06-029

MEMORANDUM

TO: Mark McGuire
Animal and Veterinary Sciences Department - 2330

FROM: Steve Meier, Chair
Human Assurances Committee

DATE: April 26, 2006

SUBJECT: Approval of "Development of Procedures for Identification and Characterization of Cells from Human Milk"

On behalf of the Human Assurances Committee at the University of Idaho, I am pleased to inform you that the above-named proposal is approved as offering no significant risk to human subjects. This approval is valid for **one year** from the date of this memo. Should there be a significant change in your proposal, it will be necessary for you to resubmit it for review. Thank you for submitting your proposal to the Human Assurances Committee.

Steve E. Meier
SEM/ed

University of Idaho
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Federalwide Assurance: FWA00005639
Federal Assigned IRB #: 00000843
UI Assigned Number: 06-029

MEMORANDUM

TO: Mark McGuire
Animal and Veterinary Sciences Department - 2330

FROM: Steve E. Meier, Chair
Human Assurances Committee

DATE: May 20, 2006

SUBJECT: Approval of Revisions to "Development of Procedures for Identification and Characterization of Cells from Human Milk"

On behalf of the Human Assurances Committee at the University of Idaho, I am pleased to inform you that the above-named proposal revisions are approved as offering no significant risk to human subjects. This approval is valid for **one year** from the date of the original proposal approval date of April 26, 2006. Should there be a significant change in your proposal, it will be necessary for you to resubmit it for review. Thank you for submitting your proposal to the Human Assurances Committee.

Steve E. Meier
SEM/ed

APPENDIX B: PRIMER SEQUENCES

Illumina V1-V3 regions of 16S rRNA genes

Illumina sequencing analysis was done for each of the samples. We amplified the bacterial 16S rRNA gene using primers that flanked the variable regions 1 and 3 (Escherichia coli positions 27F-534R). Generations of sample amplicons were performed using a double round of PCR and dual indexing on the Illumina MiSeq. The first round of PCR extracts the targeted 16S V1 to V3 region. The second round of PCR attaches the sample barcode and sequencing adapters. The 1st PCR primer sequences are below.

27F Primer Primer Sequence

27F-YM1 5' - ACACTGACGACATGGTTCTACAGT**AGAGTTTGATCCTGGCTCAG** – 3'
27F-YM2 5' - ACACTGACGACATGGTTCTACA***C***GT**AGAGTTTGATCATGGCTCAG** – 3'
27F-YM3 5' – ACACTGACGACATGGTTCTACA***AC***GT**AGAGTTTGATTCTGGCTCAG** – 3'
27F-YM4 5' – ACACTGACGACATGGTTCTACA***TAC***GT**AGAGTTTGATTATGGCTCAG** – 3'
27F-Bif 5' - ACACTGACGACATGGTTCTACA***GTAC***GT**AGGGTTCGATTCTGGCTCAG** – 3'
27F-Bor 5' -ACACTGACGACATGGTTCTACA***CGTAC***GT**AGAGTTTGATCCTGGCTTAG** – 3'
27F-Chl 5' - ACACTGACGACATGGTTCTACA***ACGTAC***GT**AGAATTTGATCTTGGTTCAG** – 3'

534R Primer Primer Sequence

534R_15' - TACGGTAGCAGAGACTTGGTCTCC**ATTACCGCGGCTGCTGG** - 3'
534R_25' - TACGGTAGCAGAGACTTGGTCT***G***CC**ATTACCGCGGCTGCTGG** - 3'
534R_35' - TACGGTAGCAGAGACTTGGTCT***TG***CC**ATTACCGCGGCTGCTGG** - 3'
534R_45' - TACGGTAGCAGAGACTTGGTCT***ATG***CC**ATTACCGCGGCTGCTGG** - 3'
534R_55' - TACGGTAGCAGAGACTTGGTCT***CATG***CC**ATTACCGCGGCTGCTGG** - 3'
534R_65' - TACGGTAGCAGAGACTTGGTCT***TCATG***CC**ATTACCGCGGCTGCTGG** - 3'
534R_75' - TACGGTAGCAGAGACTTGGTCT***ATCATG***CC**ATTACCGCGGCTGCTGG** - 3'

Adapter Primers Primer Sequence

P7 – CS2 5' -
CAAGCAGAAGACGGCATACGAGAT*NNNNNNNN*TACGGTAGCAGAGACTTGGTCT- 3
P5 – CS1 5' -
AATGATACGGCGACCACCGAGATCTACAC*NNNNNNNN*ACACTGACGACATGGTTCTACA- 3

, where the underlined sequences are universal sequences CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGGTCT). Bold Sequences denote the universal 16S rRNA primers 27F and 534R, we used seven different 27F primer sequences in order to capture the greatest number of taxa.

The bold, italized bases are added to the template specific primers in order to introduce variability of base calls during Illumina sequencing. The adapter primers included the illumina specific sequences P7 and P5 for dual indexing where the unique sequence barcodes are denoted by the 8 italicized N's. This allowed us to sequence the amplicons from many samples simultaneously using relatively few barcoded adapter primers, and afterwards assign each sequence to the sample they were obtained from (40 P7-CS2 primers and 40 P5-CS1 primers enable 800 unique samples identifiers).