# Maternal Mineral Intake, Milk Minerals, and Milk Microbiome as Determinants of Infant Growth in a *Mam*-Mayan population in Guatemala

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*To my grandparents,*
*Irma, Ofelia, Adrian and Fernando, for their enduring legacy,*
*and to my nephew and niece, Nicolas and Isabella,*
*as a reminder that they can achieve anything they aspire to.*

# Table of Contents

## Abstract

**Introduction:** Impaired growth is frequently observed in Guatemalan infants under 6 months of age, despite breastfeeding. While nutrients in human milk are essential for infant development, research on the relationship between maternal mineral intake and milk mineral content, and how these interact with the milk microbiota, is limited, particularly in low- and middle-income settings. Although nutrients are crucial for the growth and survival of bacteria, studies on the human milk microbiome have predominantly focused on fatty acids and oligosaccharides and not minerals leaving a gap in our understanding of mineral-microbiome interactions and their potential influence on infant growth.

**Methods:** This research is divided into a cross sectional and a longitudinal study. In the cross-sectional study milk samples were collected from 77 *Mam*-Mayan mothers and classified into early [5 – 46 days (n=38)] and established lactation [4 – 6 months, (n= 39)]. The concentrations of 9 milk minerals (Na, Ca, Cu, Fe, Mg, Mn, K, Se, and Zn) were analyzed by ICP-MS and maternal dietary intake was obtained from 2 non-consecutive 24 hr recalls at early or established lactation. Milk microbiome diversity was obtained by 16S rRNA gene amplicon sequencing of the V5-V6 region. In the longitudinal study, 57 mothers were recruited and provided milk samples at early and established lactation for a total of 114 milk samples. Infant growth was calculated according to the World Health Organization (WHO) growth reference standards including weight-for-age Z-score (WAZ) and head circumference-for-age Z-score (HCAZ), which were subdivided into >-1 SD (normal growth) and <-1 SD (impaired growth) and length-for-age Z-score (LAZ), that was subdivided into >-2 SD (normal growth) and <-2 SD (impaired growth).

**Results:** In the cross-sectional study, milk mineral composition and not maternal dietary intake was strongly associated with the human milk microbiome. In early lactation, milk iron, manganese, copper, and selenium were correlated with the differentially abundant taxa whereas in established lactation, milk iron, manganese, selenium, calcium, and sodium were correlated with milk bacteria. Only iron, manganese and selenium had several associations with milk bacteria at both stages of lactation. These 3 minerals were correlated with members of the phyla Pseudomonadota (Fe and Mn) in early lactation, with Actinomycetota (Fe and Mn) in established lactation and with Bacillota in both stages of lactation (Mn and Se). In the longitudinal study,

infants with normal and impaired growth in WAZ and LAZ were exposed to different milk microbiomes in early lactation but not at established lactation. In established lactation, only infants with normal and impaired HCAZ were exposed to different milk microbiome. Manganese and magnesium emerged as important minerals in relation to milk microbiome and growth, with the greatest number of correlations with bacteria. *Cutibacterium acnes* was differentially abundant in the mild underweight and stunted groups and was negatively associated with manganese. In non-stunted and normal head circumference infants Phascolarctobacterium and *Streptococcus agalactiae* were positively associated with magnesium, and *Streptococcus pneumoniae* was positively associated with manganese.

**Conclusion:** Our findings reveal a complex and dynamic interplay between milk minerals and the milk microbiome, which seems to operate independently of maternal diet. The effect of milk microbiome on infant growth appears to be critical in early lactation, with magnesium and manganese showing significant associations with the milk microbiome that may influence growth outcomes. Specific bacteria seem to be linked to impaired or normal growth, *C. acnes* was differentially abundant in impaired weight and length and negatively correlated with manganese. *S. pneumoniae* was positively correlated with milk manganese, *S. aglactiae* and *Phascolarbacterium* were positively correlated with magnesium, *Janthinobacterium lividum* was negatively correlated with calcium and *Lactobacillus johnsonii* was negatively correlated with selenium and they were all differentially abundant species in normal length and head circumference. Our results highlight the importance of mineral-microbiome interactions in shaping early growth trajectories.

## Résumé

**Introduction :** Un retard de croissance est fréquent chez les nourrissons guatémaltèques de moins de 6 mois, malgré un fort taux d'allaitement national. Bien que les nutriments trouvés dans le lait maternel soient essentiels au développement des nourrissons, les liens entre l'apport en minéraux maternels, la teneur en minéraux du lait et leurs interactions avec le microbiote du lait maternel sont peu connus, surtout dans les pays à revenu faible et intermédiaire. Les études sur le microbiote du lait maternel se concentrent principalement sur les acides gras et les oligosaccharides, négligeant les minéraux et leurs interactions avec le microbiote du lait maternel, ainsi que leur potentielle influence sur la croissance infantile.

**Méthodes :** Ce travail de thèse comprend une étude transversale et une étude longitudinale. Dans l'étude transversale, des échantillons de lait maternel ont été collectés auprès de 77 mères *Mam*-Maya, pendant les périodes d'allaitement précoce [5 – 46 jours (n=38)] ou établi [4 – 6 mois (n=39)]. Neuf minéraux du lait maternel (Na, Ca, Cu, Fe, Mg, Mn, K, Se, Zn) ont été analysés par ICP-MS, et la composition du régime alimentaire maternel a été évalué par deux rappels alimentaires de 24 heures. La diversité du microbiome du lait maternel a été analysée via séquençage du gène 16S rRNA (région V5-V6). Dans l'étude longitudinale, 57 mères ont fourni des échantillons de lait maternel durant leur allaitement précoce et établi, pour un total de 114 échantillons. La croissance des nourrissons a été évaluée selon les critères standards de l'OMS : poids-pour-l'âge (P/EZ), périmètre crânien-pour-l'âge (PC/EZ) et taille-pour-l'âge (T/EZ), classés comme >-1 ou <-1 écart type (croissance normale/retardée), ou >-2 et <-2 SD pour T/EZ.

**Résultats :** Dans l'étude transversale, la composition minérale du lait maternel, et non l'alimentation maternelle, était fortement associée à la composition du microbiome du lait. Durant la période d'allaitement précoce, le Fe, Mn, Cu et Se étaient corrélés à des taxa bactériens spécifiques, tandis qu'en période d'allaitement établie, le Fe, Mn, Se, Ca et Na présentaient des corrélations. Le Fe, Mn et Se avaient des associations avec les bactéries aux deux stades d'allaitement: les Pseudomonadota (Fe, Mn) durant la période précoce, les Actinomycetota (Fe, Mn) durant la période établie, et les Bacillota (Mn, Se) durant les deux périodes. Dans l'étude longitudinale, les nourrissons avec une croissance normale ou retardée (P/EZ, T/EZ) étaient

exposés à des microbiomes du lait maternel distincts durant la période d'allaitement précoce, mais pas établie. Durant l'allaitement établi, le microbiome du lait maternel était différent entre les nourrissons avec un PC/EZ normal ou altéré. Mn et Mg se sont révélés être des minéraux clés, avec de nombreuses corrélations avec divers taxa bactériens. *Cutibacterium acnes* était plus abondant chez les nourrissons avec un retard de croissance et un faible poids, négativement associé au Mn. Chez les nourrissons sans retard de croissance, *Phascolarctobacterium* et *Streptococcus agalactiae* étaient positivement associés au Mg, tandis que *Streptococcus pneumoniae* était associé au Mn.

**Conclusion :** Nos résultats montrent des interactions complexes et dynamiques entre les minéraux du lait maternel et le microbiome du lait maternel, indépendantes du régime alimentaire maternel. L'effet du microbiome du lait maternel sur la croissance infantile semble crucial durant la phase d'allaitement précoce, où Mg et Mn montrent des associations significatives avec le microbiome. Des bactéries spécifiques semblent être liées à une croissance normale ou altérée. *C. acnes* était différentiellement abondante chez les enfants avec un poids et une longueur altérés et était négativement corrélée au Mn. *S. pneumoniae* était positivement corrélée au Mn du lait, *S. aglactiae* et Phascolarbacterium étaient positivement corrélés au Mg, *Janthinobacterium lividum* était négativement corrélé au calcium et *Lactobacillus johnsonii* était négativement corrélé au Se. Il s'agissait toutes d'espèces différentiellement abondantes chez les enfants présentant des valeurs de T/EZ et PC/EZ normales. Nos résultats soulignent l'importance des interactions minéraux-microbiome dans les trajectoires de croissance précoce.

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## Contribution to Original Knowledge

The results presented in this dissertation represent novel contributions to the field and are considered original scholarship. The original knowledge from the review paper and two original studies included in this dissertation are:

- To the best of our knowledge the current study is the first one to analyze collectively associations between maternal mineral intake, concentrations of milk minerals, human milk microbiome composition and infant growth in an Indigenous population of Guatemala

- We identified a novel usage of a set of primers in the regions V5 – V6 for 16s rRNA gene amplification in milk microbiome research, which is capable of capturing the expected genera in milk microbiome, including *Bifidobacterium* and *Cutibacterium.*

- We demonstrated the efficiency of amplifying the V5-V6 regions for identifying all expected genera in the milk microbiome, including the successful amplification of *Bifidobacterium* and *Cutibacterium*.

- We filled a knowledge gap by focusing this dissertation on minerals, including maternal mineral intake and milk minerals, which historically have been overlooked.

- By conducting this research in a *Mam*-Mayan population in Guatemala, we advanced our knowledge on indigenous populations and low- and middle-income countries.

- We provided more evidence on the lack of association between maternal mineral intake and milk mineral concentrations on a population with nutrient deficiencies.

- We advanced our understanding on the dynamic association between milk minerals and milk bacteria, and we identified that minerals associate differently with milk bacteria depending on the lactation stage.

- Through a longitudinal study, which was lacking in the available literature, we investigated the influence of milk minerals and the milk microbiome on infant growth at early and established lactation, identifying for the first time in a longitudinal study that these factors are critical for infant growth particularly during early lactation.

    - Our novel observations uncovered strong associations of milk mineral concentrations with the milk microbiome and a weak association with maternal diet.

    - In the cross-sectional study, we reported the associations between mineral and microbial composition of human milk, where iron, manganese and selenium stand out as minerals associated with the milk microbiome at both stages of lactation. These minerals were correlated with members of the phyla Pseudomonadota (Fe and Mn) in early lactation, with Actinomycetota (Fe and Mn) in established lactation and with Bacillota in both stages of lactation (Mn and Se)

    - When looking specifically to their collective association with infant growth, manganese continued to have an important role, while magnesium emerged also as a mineral with several correlations with milk bacteria and with an important association with infant growth.

    - We identified milk bacteria associated with impaired and normal growth.

        - In mildly underweight and stunted infants *Cutibacterium acnes* was differentially abundant and was negatively associated with milk manganese.

- In normal growth the bacteria differentially abundant were *Luteococcus peritonei* (in WAZ, LAZ and HCAZ)*, Bifidobacterium longum, Lancefieldella parvula,* Phascolarctobacterium*, Rothia mucilaginosa, Streptococcus agalactiae* and *Streptococcus pneumoniae* (in LAZ and HCAZ), *Janthinobacterium lividum* (in WAZ) and *Lactobacillus johnsonii* (in HCAZ).

- From the aforementioned bacteria, only some were correlated with milk minerals: *S. pneumoniae* was positively correlated with milk manganese, *S. aglactiae* and *Phascolarbacterium* were positively correlated with magnesium, *Janthinobacterium lividum* was negatively correlated with calcium and *Lactobacillus johnsonii* was negatively correlated with selenium.

## Contribution of Authors

This thesis is written in the manuscript format according to the Guidelines Concerning Thesis Preparation stipulated by McGill University. Lilian Lopez Leyva wrote this thesis. The first manuscript is published, the second manuscript has been accepted, and the third manuscript will be submitted during Summer 2025.

- **Chapter 1 - Thesis overview:** Written by Lilian Lopez Leyva. Edits were performed by Lilian Lopez Leyva, Dr. Kristine Koski and Dr. Corinne Maurice.

- **Chapter 2 - Manuscript 1 (Literature Review):**
  Adapted from:

  "Lopez Leyva, L., Brereton, N. J. B., & Koski, K. G. (2020). Emerging frontiers in human milk microbiome research and suggested primers for 16S rRNA gene analysis. *Computational and Structural Biotechnology Journal*, *19*, 121–133. https://doi.org/10.1016/j.csbj.2020.11.057"

  Lilian Lopez Leyva participated in the conceptualization and did the writing of the original draft. Dr. Nicholas Brereton participated in the conceptualization, wrote the majority of the section "Methods shape observed bacterial taxa in human milk" and reviewed & edited the original draft. Dr. Kristine Koski participated in the conceptualization and reviewed & edited the original draft.

- **Chapter 3 - Manuscript 2:** Lilian Lopez Leyva participated in the conceptualization and design of the study, did the DNA extractions of the milk samples, evaluated the maternal diet and milk mineral concentrations and wrote the manuscript. Dr. Emmanuel Gonzalez performed the statistical and bioinformatics analyses, created the figures of the manuscript, except for figures 1 and 2 which were done by Lilian Lopez Leyva and reviewed and edited the manuscript. Dr. Marieke Vossenaar and Dr. Anne Marie Chomat managed the field collection of dietary intake data and transformed it into nutrient intakes. Dr. Noel W. Solomons supervised the field data collection. Dr. Corinne F. Maurice

reviewed and edited the manuscript. Dr. Kristine Koski participated in the conceptualization and design of the study, reviewed and edited the manuscript.

- **Chapter 4 - Manuscript 3:** Lilian Lopez Leyva participated in the conceptualization and design of the study, did the DNA extractions of the milk samples and wrote the manuscript. Dr. Emmanuel Gonzalez performed the statistical and bioinformatics analyses, created the figures of the manuscript, except for figures 1, 2 and 3 that were done by Lilian Lopez Leyva and reviewed and edited the manuscript. Dr. Marieke Vossenaar, Dr. Anne Marie Chomat and Dr. Hilary Wren-Atiola took the infant anthropometric measurements and collected the milk samples. Dr. Noel W. Solomons and Dr. Marieke Vossenaar supervised the field data collection. Dr. Corinne F. Maurice reviewed and edited the manuscript. Dr. Kristine Koski participated in the conceptualization and design of the study, reviewed and edited the manuscript.

- **Chapter 5 - Thesis Discussion:** Discussion was written by Lilian Lopez Leyva. Edits were performed by Lilian Lopez Leyva, Dr. Kristine Koski and Dr. Corinne F. Maurice.

## List of Tables

# List of Figures

## List of Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| BLASTn | Basic Local Alignment Search Tool (nucleotide to nucleotide) |
| BMI | Body Mass Index |
| C3G | Canadian Centre for Computational Genomics |
| Ca | Calcium |
| CAP | Canonical Analysis of Principal Coordinates |
| CCA | Canonical Correspondence Analysis |
| CDC | Centers for Disease Control and Prevention |
| CeSSIAM | Center for Studies of Sensory Impairment, Aging and Metabolism |
| cm | centimeters |
| C-section | Caesarean section |
| Cu | Copper |
| DA | Differential Abundance/differentially abundant |
| DC | Dendritic cells |
| DNA | Deoxyribonucleic acid |
| DNAse | Deoxyribonuclease |
| ESVs | Exact sequence variants |
| FC | Fold change |
| FDR | False discovery rate |
| Fe | Iron |
| g | grams |
| HCAZ | head circumference-for-age Z-score |
| Hifi | High fidelity |
| HMOs | Human milk oligosaccharides |
| ICP-MS | Inductively coupled plasma mass spectrometry |
| INCAP | Institute of Nutrition of Central America and Panama |
| IRB | Institutional Review Board |
| K | Potassium |

| | |
|---|---|
| kcal | Kilocalories |
| kg | kilogram |
| L | Liter |
| LAZ | length-for-age Z-score |
| LOD | Limits of detection |
| m | meter |
| MFA | Multifactorial Analysis |
| Mg | Magnesium |
| mg | miligrams |
| MI4 | McGill Interdisciplinary Initiative in Infection and Immunity |
| min | minutes |
| ml | mililiters |
| mmol | millimole |
| Mn | Manganese |
| mo | months |
| MS | Multiple species |
| Na | Sodium |
| NCBI | National Center for Biotechnology Information |
| NEB | New England Biolabs |
| nr/nt | Nucleotide collection (non-redundant) |
| $O_2$ | Oxygen |
| PCA | Principal Component Analysis |
| PCoA | Principal Coordinate Analysis |
| PCR | Polymerase chain reaction |
| Rb | Rubidium |
| RDA | Redundancy Analysis |
| RefSeq | Reference Sequence |
| rlog | regularized log transformation |
| RNA | Ribonucleic acid |

| | |
|---|---|
| RNAse | Ribonuclease |
| rRNA | Ribosomal ribonucleic acid |
| s | seconds |
| SCM | Subclinical mastitis |
| SD | Standard deviation |
| Se | Selenium |
| spp | Several species |
| Sr | Stronium |
| stdev | Standard deviation |
| Taq | Thermus aquaticus |
| µg | micrograms |
| UNICEF | United Nations International Children's Emergency Fund |
| USA | United States of America |
| USAID | U.S. Agency for International Development |
| Vit | Vitamin |
| WAZ | weight-for-age Z-score |
| WHO | World Health Organization |
| Zn | Zinc |

# Chapter 1: Overview

## 1.1 Background

### 1.1.1 Impaired growth in breastfed infants in Guatemala

Stunting refers to the height-for-age more than two standard deviations below the WHO Child Growth Standards median and is the most prevalent form of child malnutrition (UNICEF, 2021). Stunted children suffer severe irreversible physical and cognitive damage that accompany stunted growth, such as muscular atrophy, slow bone growth, environmental enteric dysfunction, and increased risk of infections (Soliman et al., 2021). The effects of stunting can last a lifetime and are inter-generational (UNICEF, 2021). In 2022, stunting affected an estimated 22.3% children under 5 years old globally. That same year, stunting prevalence in Guatemala was estimated to be close to double of the global average, reaching 43.5% of children (UNICEF/WHO/World Bank Group, 2023). Guatemala has the sixth-highest rate of chronic malnutrition in the world resulting in stunting (or low height-for-age) and other conditions (USAID, 2018). The indigenous population in Guatemala is notably more affected with 58% of chronic undernutrition compared to non-indigenous population with 34%. The prevalence of stunting in Guatemala remains high despite several programs investing in affordable nutrition, primary healthcare, healthy motherhood and prenatal care, better sanitation, efforts to improve treatment of childhood diarrhea and food fortification. Breastfeeding has been identified as one of the key modifiable maternal factors protecting against impaired infant growth (Campos et al., 2020). Indeed, human milk has long been acknowledged as the ideal food for newborns and infants to meet their energy and nutrient needs, as well as to provide antibodies and immunological factors that protect them from infections. (*WHO | Breastfeeding*, 2018). Yet, in Guatemala there are increasing numbers of breastfed children younger than 6 months old that are stunted (Wren-Atilola et al., 2018). Is milk truly always the perfect food for infants or is it conditionally perfect, as it has recently been described? (Erick, 2018). Recent reports document for the first-time low concentrations of minerals and trace elements in human milk, suggesting that milk composition may not always be optimal for adequate infant growth, especially in settings of chronic and inter-generational malnutrition (Daniels et al., 2019; Donohue et al., 2020; Li et al., 2016, 2019; Reyes et al., 2023).

### 1.1.2 Human milk and factors that influence its composition.

Human milk is one of the most under-explored biological system in life sciences despite its recognized and multiple benefits to mothers' and infants' health (Christian et al., 2021). Breastfeeding mothers have reduced risk of breast and ovarian cancer, type 2 diabetes, and hypertension, while breastfed infants have a reduced risk of asthma, obesity, type 1 diabetes, severe lower respiratory disease, acute otitis media, sudden infant death syndrome, gastrointestinal infections and necrotizing enterocolitis for preterm infants (CDC, 2023). Human milk is a dynamic fluid that varies during the day, between breasts and between mothers (Samuel et al., 2020). Several maternal and infant factors can modify its composition such as maternal diet, nutritional status, weight and age, infant parity, mode of delivery, medical conditions, infant health, birthweight, infant sex, gestational age and stage of lactation (Samuel et al., 2020). Human milk is vital to the infant due to its nutritional components such as lactose, lipids, protein, minerals, and vitamins. It provides 65 – 70 kcal of energy per 100 ml, carbohydrates account 40% of the total calories, and lactose is the main carbohydrate. Lactose increases progressively throughout lactation, and it promotes the absorption and attachment of bioactive components, such as oligosaccharides and minerals, including calcium. Fat accounts for almost 50% of the total calories, it also increases progressively throughout lactation, and it is the most important macronutrient for the infant growth and development of central nervous system. Human milk protein is composed by whey, casein, and various peptides; its content is high in early lactation and slowly decreases throughout lactation (Yi & Kim, 2021).

Several other attributes have been credited to human milk beyond its nutritional compounds, such as bioactive factors like immunoglobulins, antibodies, growth factors, hormones and cytokines, and nonspecific compounds including oligosaccharides, bacteria and MicroRNAs (Erick, 2018; Eriksen et al., 2018; Gomez-Gallego et al., 2016; Słyk-Gulewska et al., 2023). These bioactive factors impact infant survival and health. For instance, lactoferrin is an antimicrobial compound with a high affinity for iron and with bacteriostatic activity against iron-requiring pathogens (Yi & Kim, 2021). Human milk oligosaccharides (HMOs) are important in the formation of neonate immunity. There are three major types of HMOs in human milk: Neutral (Fucosylated)

HMO, Neutral N-containing HMO, and Acid (sialylated) HMO (Wiciński et al., 2020) and they serve as prebiotics, that support the growth of beneficial gut bacteria and host defense (Moukarzel & Bode, 2017; Yi & Kim, 2021). Other milk components such as minerals and microbial communities, including their concentrations, diversity and interactions, have been poorly explored and require further research (Bravi et al., 2016; Daniels et al., 2019; Dror & Allen, 2018a; Petersohn et al., 2023).

### 1.1.3 Association of maternal diet with milk minerals

Human milk is the link between the mother and the infant. Therefore, if mothers have nutrient deficiencies, many of them will likely be reflected in the milk nutrient composition. The triad "mother – human milk – infant" has been described as a system in which variations in any of its components will have an effect on the infant's development and maternal health (Bode et al., 2020). For instance, there is evidence that maternal micronutrient deficiencies may continue during lactation and alter milk composition (Dror & Allen, 2018a). This is the case for selenium and iodine in human milk; while the concentrations of other minerals found in milk like calcium, iron, copper, and zinc remain consistent regardless of the mother's dietary intake (Allen, 1994, 2012; Erick, 2018). Yet, a study conducted in women of low socioeconomic status in Indonesia reported negative correlations of zinc in human milk with maternal intake (Gibson et al., 2020). The authors suggested that the low socioeconomic status could partially explain this effect by promoting maternal secretion of zinc, challenging the notion that zinc is unaffected by maternal diet or status intake (Gibson et al., 2020). As summarized in **Table 1.1**, the associations between maternal mineral intake and milk mineral concentrations are still unclear, as variations in milk mineral concentrations vary among different populations, depending on geographical location, maternal nutritional status, and socioeconomic status. This emphasizes the need for more studies in low- and middle-income countries and in vulnerable populations bearing the brunt of stunting.

### 1.1.4 The human milk microbiome composition

The term microbiome refers to the collection of all genes contained within a community of microorganisms; it refers to the entire habitat including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (i.e., coding and non-coding DNA), and

the surrounding environmental conditions. The methods used to study microbiomes have changed and developed through time. The initial studies used bacterial culture techniques. However, anaerobic bacteria would not survive experimental procedures, resulting in biased results favouring aerobic bacteria. To overcome these limitations, molecular approaches were developed, in which bacterial species are identified based on the sequence of their 16S ribosomal RNA genes (Sarangi et al., 2019).

Research has aimed to determine the origins of the milk microbiome through different pathways. The retrograde flow and the entero- mammary pathway are sources of skin, saliva and maternal gut bacteria, which together constitute the milk microbiome. In detail, the retrograde flow refers to the infant-to-mother transfer in which microorganisms are transmitted via the skin and saliva from the infant's oral cavity into the duct during suckling; the entero-mammary route consists in the translocation of bacteria through the intestinal epithelial barrier, where the dendritic cells can cross the paracellular space of the intestinal epithelium to take up bacteria directly from the intestine lumen. Then, circulation of lymphocytes within the mucosal associated lymphoid tissue allows the maternal gastrointestinal tract microbiota to reach distant mucosal surfaces such as the lactating mammary gland (Martín et al., 2004; Perez et al., 2007; Rescigno et al., 2001; Thum et al., 2012).

Some studies have attempted to define a "core" microbiome of human milk, with contrasting results. The dominant bacterial taxa are *Staphylococcus* and *Streptococcus* (Lackey et al., 2019), followed by other genera such as *Corynebacteria, Lactobacillales, Propionibacteria*, and *Bifidobacteria* (Fernández et al., 2013). The composition of the human milk microbiome may be influenced by diverse factors such as environment, lactation stage, breastfeeding practices, maternal BMI, maternal age, maternal diet, mode of delivery, parity and use of antibiotics (Cabrera-Rubio et al., 2012, 2016; Khodayar-Pardo et al., 2014; Lopez Leyva et al., 2020; Moossavi, Sepehri, et al., 2019; Notarbartolo et al., 2022).

### 1.1.5 Association of milk minerals with human milk microbiota composition

Milk components and their effects on infant growth and mothers' health have typically been studied individually rather than as an ecosystem. For instance, most studies focus only on milk nutrient composition (Bzikowska-Jura et al., 2018; Daniels et al., 2019; Dror & Allen, 2018b; Lockyer et al., 2021) or only on milk microbiota composition (Ajeeb et al., 2022b; Robertson et al., 2019; Selma-Royo et al., 2022), without considering the interactions between these two components. Such interactions might explain the variations in milk composition and the resulting different outcomes on infant health, including infant growth.

Knowledge about the composition and diversity of the milk microbiome, as well as the factors altering it, lags behind other human-associated microbial communities such as the gut microbiome (Lewandowska-Pietruszka et al., 2022; Lopez Leyva et al., 2020). In addition, the human milk microbiome is less characterized than the bovine milk microbiome composition (Ruegg, 2022). A recent review on the gut microbiome with studies in animals and humans i reported that bacterial strains from the genera *Streptococcus, Lactobacillus,* and *Bifidobacteria* improve the bioaccessibility and bioavailability of iron, zinc, calcium, selenium, and magnesium (Bielik & Kolisek, 2021). Likewise, another review suggested a prebiotic effect of lactose on the gut microbiota, increasing *Bifidobacterium adolescentis* and lactic acid bacteria, while Bacteroides and Clostridia decreased (Grenov et al., 2016). This could facilitate host energy utilization and a better absorption of growth-promoting minerals, including calcium, magnesium, manganese, and zinc (Grenov et al., 2016). These studies highlight existing interactions between gut bacterial taxa and minerals typically found in human milk, yet direct evidence of such interactions is lacking in the human milk. One of the few studies that analysed milk mineral composition and milk microbiome diversity reported an association between calcium, magnesium, and selenium with the human milk microbiome diversity in 99 Spanish mothers who breastfed for <6 months or >6 months (Sanjulián et al., 2021). Calcium was negatively correlated with *Streptococcus, Prevotella, Actinobacteria*, and *Bacteroidetes* (Sanjulián et al., 2021). Magnesium showed a positive correlation with *Streptococcus*, while Selenium was negatively correlated with *Staphylococcus*, in accordance with the documented inhibitory effects of selenium against *S. aureus* (K. Liu et al., 2020; Malbe et al., 2006; Tran & Webster, 2011). Based

on this prior work, one can hypothesize that the milk microbiome could also alter the milk mineral content and therefore impact infant growth, yet such evidence is still needed.

### 1.1.6 Association of infant growth with human milk microbiota composition

Infant growth for its part has been associated with several human milk factors including minerals and cytokines concentrations, as well as subclinical mastitis (SCM) in our Guatemala study population and globally (Li et al., 2016, 2019; Lockyer et al., 2021; Wren et al., 2015; Wren-Atilola et al., 2018, 2021). In contrast, the association between infant growth and the milk microbiome remains underexplored, with only cross-sectional studies performed to date (Ajeeb et al., 2022a, 2022b, 2024; Cheema et al., 2022). While important, such studies don't provide insight into possible causal relationships between milk microbiome composition and infant growth.

It is currently hypothesized that human milk is the immediate next source of microbes seeding the infant gut after birth. This statement is supported by the fact that gut microbiota composition from breastfed and formula-fed infants is different, with breastfed infants dominated by species involved in the metabolism of human milk oligosaccharides such as *Streptococcus*, *Veillonella*, and *Rothia* (Pannaraj et al., 2017), whereas formula-fed infants have Ruminococcaceae and Bacteroidaceae. In addition, primarily breastfed infants share 28% of their stool microorganisms with their mother's milk microorganisms, consisting mainly of *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Staphylococcus*; and the frequency of shared microorganisms between human milk and infant stools increases with the proportion of daily human milk intake (Le Doare et al., 2018). However, it could be because these bacteria are the ones metabolically equipped to metabolize human milk and they are actually acquired from the environment rather than from the milk itself, therefore further investigation is needed. Another study in preterm infants reported that breastfeeding promoted microbial succession in early life, suggesting an essential role of human milk in the assembly of the infant gut microbiota (Korpela et al., 2018). Considering the recognized influence of breastfeeding on infant growth, the relationship between milk composition and gut microbiome may be a primary target for addressing impaired infant growth (Robertson et al., 2019). Indeed, there is increasing evidence that the infant gut microbiome is associated with infant growth, as alterations in gut microbiota establishment in the first two years of life have been shown to impact infant growth (Robertson et al., 2019; Ronan et al., 2021). For

instance, a study in Malawi conducted on twins discordant for kwashiorkor, a form of severe protein malnutrition, reported that the twin suffering from malnutrition had an immature gut microbiome that produced severe weight loss and perturbations in the carbohydrate and amino acid metabolism (M. I. Smith et al., 2013). Similarly, a study in mice showed that germ-free mice colonized with the gut microbiota of severely stunted and underweight children had impaired growth patterns (Blanton et al., 2016). The study showed that the gut microbiota immaturity, defined in the study as low to absent relative abundance of age-discriminatory bacterial taxa in relation to infant age, is related to undernutrition (Blanton et al., 2016). However, these studies focused on infant growth after the breastfeeding period, and the links between human milk microbiome composition and infant growth are therefore currently unknown.

### 1.2 Thesis Rationale

Currently, most studies done on the human milk microbiome consider well-nourished mothers from developed countries with diverse characteristics regarding mode of delivery, antibiotics usage, maternal weight, and duration of breastfeeding. Our study population solely consists of mothers from a *Mam*-Mayan population from eight rural *Mam*-speaking communities in Guatemala. This is a unique population with homogenous characteristics, such as vaginal deliveries, no antibiotics usage, normal weight, and importantly for us, most of them exclusively breastfed for 6 months, which aligns with current WHO recommendations (WHO, 2020b). Collectively, this makes an ideal population for studying the composition of the milk microbiome, as many factors known to affect the milk microbiota are not relevant in this population such as C-section, high maternal weight, antibiotic usage, or a westernized diet (Cabrera-Rubio et al., 2012; Hermansson et al., 2019; Lopez Leyva et al., 2020).

Advancing our understanding of the human milk microbiome composition is important since human milk is the first food the neonate consumes, it accounts for the initial bacterial colonization of the neonatal gut, and it is central to the maintenance of the microbiome of healthy infants throughout lactation.

Importantly, this PhD thesis is divided into two investigations, a cross-sectional and a longitudinal study. To date, there are very limited longitudinal studies on milk microbiota. For instance, a prospective cohort study followed 110 Chinese women for 3 months and analyzed the variations of human milk oligosaccharides, milk microbiome and infant gut microbiome composition, and the associations with maternal characteristics. In relation to the milk microbiota, they reported that alpha-diversity, the diversity within samples, increased over time, with 20 genera (mainly *Acinetobacter, Bacteroides, Microbacterium, Ochrobactrum*, and *Strenotrophormonas*) constituting up to 50% of the microbiota and the relative abundance of *Bifidobacterium* and *Lactobacillus* were both less than 1% (F. Liu et al., 2022). Another study followed 107 American mothers for 12 months to determine the association between the maternal human milk and areolar skin and infant gut bacterial communities. They reported that breastfed infants received 27.7% of their gut bacteria from breast milk and 10.4% from areolar skin during the first month of life (Pannaraj et al., 2017). Last, a longitudinal cohort study that followed 210 Swedish infants for 5 years, aimed to characterize the human milk microbiota and define associations with saliva and fecal microbiota and selected diseases in preschool children. They concluded that distinct microbiotas exist for the milk, saliva and feces, though resemblance between milk and oral swab microbiota increased by age (Lif Holgerson et al., 2023). Despite their valuable results, none of them have focused on the correlation of milk composition and infant growth. Therefore, the need for longitudinal studies regarding these associations remains.

To summarise, maternal mineral intake, milk minerals and milk microbiome composition have been associated with infant growth separately and with contrasting results. It is however becoming increasingly clear that all human milk components are interacting and contributing to maternal and infant health during the lactation period. This PhD work aims to fill the knowledge gap regarding the impact of maternal mineral intake on milk mineral concentrations and the milk microbiome as an ecosystem, that is collectively associated with infant growth. Findings will enable us to identify microorganisms that promote neonatal growth and to determine if certain microorganisms underscore the paradoxical growth faltering even in women exclusively breastfeeding, which has for long time been an unresolved issue in this population.

### 1.2.1 Conceptual Framework

The conceptual framework of this dissertation is based on two main concepts: (1) the triad "Mother – Human Milk – Infant" and the (2) "Lactocrine Programming". The first concept highlights the human milk as the link between the mother and the infant (Bode et al., 2020; Verduci et al., 2021). The Lactocrine programming concept is the process by which non-nutrient bioactives, such as the milk microbiota, affect the developmental program of cells, tissues, and organs in nursing offspring. The term "programming" refers to the lasting effect on form, function, and/or health in adulthood (Bartol et al., 2013; de Weerth et al., 2023). These two concepts justify the main interconnections in this framework of mother, infant, and human milk. Specifically, this dissertation will look into the maternal mineral intake as the maternal element of this triad, as maternal micronutrient deficiencies may affect milk composition (Dror & Allen, 2018a). In relation to human milk, our work will focus on the interactions between milk minerals and milk microbiota composition. These interactions might affect the absorption and functions of both the minerals and microorganisms (Bielik & Kolisek, 2021). Finally, the infant element considered will be infant growth, as it is impacted by milk composition, including milk nutrients and milk microbiome (Ma et al., 2022) **(Figure 1.1).**

### 1.2.2 Study objectives and hypotheses

This thesis is divided in two investigations. The first is a cross-sectional study (N = 77 milk samples from 77 mothers) looking at the association of milk minerals with maternal mineral intake and human milk microbiome diversity. The second investigation is a longitudinal study (N= 114 milk samples from 57 mothers) that explores the association of milk minerals with milk microbiome diversity and their impact on infant growth. The advantage of the second study lays in the fact that we have a bigger sample size and that milk samples and growth measurements come from the same dyads mother-infant at early (<1 month) and established (4-6 months) lactation. This

will enable us to validate the known variations in milk microbiome composition at early and established lactation (Gonzalez et al., 2021)

The two hypotheses of my work are that: (1) maternal mineral intake is associated with milk mineral concentrations; and (2) the milk minerals interact in a bidirectional way with the milk microbiome and collectively impact infant growth.

The objectives were to:

1) Explore the history and sources of milk microbiome, as well as the factors that affect milk microbiome and the evolution of the methods used for studying it (Chapter 2)

2) Associate maternal mineral dietary intake, the human milk mineral concentrations and the human milk microbiome diversity at early and established lactation in a cross-sectional study (Chapter 3)

3) Determine if human milk minerals are associated with human milk microbiome diversity at early and established lactation in a longitudinal study and associate human milk microbiome diversity with adequate or impaired infant growth through the Growth Reference Standards [weight-for-age (WAZ), length-for-age (LAZ), and head circumference-for-age (HCAZ) in a longitudinal study (Chapter 4).

### 1.3 Tables

**Table 1.1 – Association between maternal mineral intake and milk mineral concentrations**

| Author | Type of study | Population and Site | Diet Measurement | Stage of lactation | Minerals affected by maternal diet | Minerals not affected by maternal diet |
|---|---|---|---|---|---|---|
| (Allen, 1994; Erick, 2018) | Systematic Review | N/A | N/A | N/A | I, Se (Group 1 nutrients) | Folate, Ca, Fe, Cu, Zn (Group 2 nutrients) |
| (Dror & Allen, 2018a) | Systematic Review | N/A | N/A | N/A | Ca (habitual low intake), I, Se, | Fe, Cu, Zn,P, Mg |
| (Gibson et al., 2020) | Longitudinal | Indonesia (n=207) | In-home weighed food records on 3 nonconsecutive days and 24h recall | 2 mo and 5 mo | Ca (+ weak), Fe (+), Zn (-) | K |
| (Daniels et al., 2019) | Longitudinal | Indonesia (n = 110) | In-home weighed food records on 3 nonconsecutive days and 24h recall | 2 mo and 5 mo | | Ca, Fe, K, Zn |
| (Butts et al., 2018) | Cross-sectional | New Zealand (n= 72) | three-day food diary | 6 - 8 weeks postpartum | Fe (+), K (+), Mg (+), Zn (+) (All weak) | Ca |
| (Finley et al., 1985) | Longitudinal (Vegetarian vs Nonvegetarian) | North America (n = 52) | N/A | 2 - 20 mo of lactation | | Ca, Fe, K, Mg, |
| (Vuori et al., 1980) | Longitudinal | Finland (n = 15) | two 7-day food records | 6 -8 weeks and 17 - 22 weeks | Mn (+) | Cu, Fe, Zn |

## 1.4 Figures

**Figure 1.1 – Thesis Conceptual Framework: Mothers and infants are linked by the human milk**

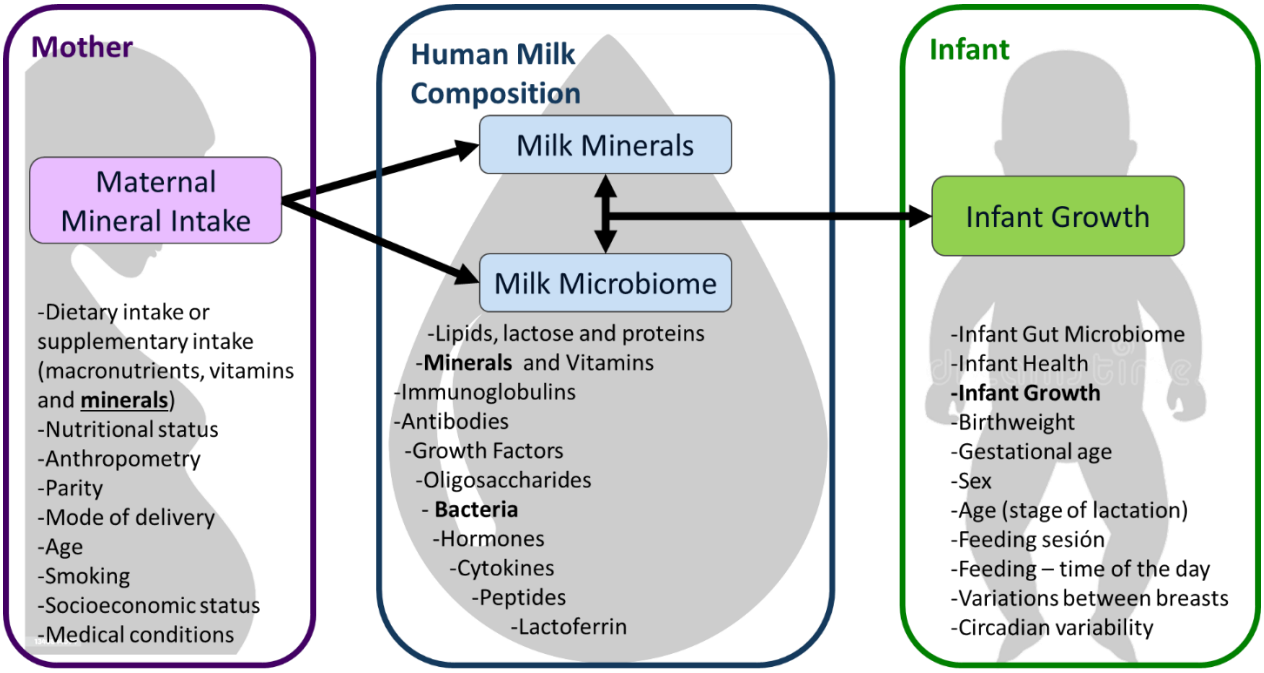


**Figure 1.1. Mothers and infants are linked by the human milk.** This research is focusing on the impact of the maternal mineral intake on the milk mineral composition, which is closely associated with the milk microbiome. We will also analyze the association between the milk microbiome with the infant growth. Maternal and infant factors known to affect milk composition are mentioned as well as milk components (Samuel et al., 2020). The factors that will be studied in this research are bolded.

# Chapter 2: Manuscript 1 (Literature Review)

## Emerging frontiers in human milk microbiome research and suggested primers for 16S rRNA gene analysis

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## 2.1 Abstract

Human milk is the ideal food for infants due to its unique nutritional and immune properties, and more recently human milk has also been recognized as an important source of bacteria for infants. However, a substantial amount of fundamental human milk microbiome information remains unclear, such as the origin, composition and function of the community and its members. There is emerging evidence to suggest that the diversity and composition of the milk microbiome might differ between lactation stages, due to maternal factors and diet, agrarian and urban lifestyles, and geographical location. The evolution of the methods used for studying milk microbiota, transitioning from culture dependent-approaches to include culture-independent approaches, has had an impact on findings and, in particular, primer selection within 16S rRNA gene barcoding studies have led to discrepancies in observed microbiota communities. Here, the current state-of-the-art is reviewed and emerging frontiers essential to improving our understanding of the human milk microbiome are considered.

**Key Words:**

Microbiome; Microbiota; 16S rRNA gene; Human Milk; Microbial barcoding; PCR primers

## 2.2 History of milk microbiome

Although many of the nutritional and immune system benefits of human milk have been established (Eriksen et al., 2018; Mosca & Giannì, 2017; Triantis et al., 2018; WHO, 2020a), the influence of the milk microbiome on health is still in the early days of exploration. A recent review (van den Elsen et al., 2019), while stressing further evidence is needed, has explored the idea that human milk could represent a reservoir of bacteria which influences infant gut microbiome diversity and aspects of health such as allergy prevention. The existence of the human microbiome is well-established (Savage, 1977) and was studied as far back as Antonie van Leeuwenhoek (1632–1723); however, microbiome study in human milk is very recent due to a long-standing belief that human milk was sterile (McGuire & McGuire, 2017). In the late ‘60s, the presence of bacteria in human milk was related to the low levels of personal hygiene and environmental sanitation in women from Guatemala (Wyatt & Mata, 1969) **(Table 1)**. Later, diverse methods were employed in order to make milk ‘bacteriologically safe’, such as heating and freezing (Carroll et al., 1979; El-Mohandes et al., 1993). By the late ‘80s it had been

recognized that human milk contained growth-promoting substances postulated to be involved in the development of microbiota (Hill & Marsh, 1990). In the early 2000s, interest and study of the microbiome significantly increased and promptly led to important discoveries, including the existence of commensal bacteria in human milk (Heikkilä & Saris, 2003). Bacteria were recognized as components of the natural microbiota rather than contaminants (Martín et al., 2004) resulting in notable differences among human body sites that included highly diverse communities in skin, less variable communities within the oral cavity compared to other habitats and highly variable gut communities between individuals but with low variability over time (Costello et al., 2009).

One of the most important advances in microbiome research began in 2007 with the Human Microbiome Project conducted by the National Institutes of Health, the first large-scale effort to characterize the healthy human microbiome (Knight et al., 2017). The study included a population of 242 healthy adults and a description of the microbiomes of up to 18 body sites. A total of 5,298 samples were collected within the human airways, skin, oral cavity, gut and vagina. The result was the collection of 11,174 primary biological specimens representing the human microbiome that formed the backdrop for further microbiome research. However, at that time, sites like the mammary glands or human milk were not analysed.

**2.3 Sources of the milk microbiome**

Research has aimed to explain the sources of the milk microbiome **(Figure 1)**. Two main pathways have been recognized as sources that might potentially contribute to the human milk microbiome: retrograde flow and the entero-mammary pathway.

Retrograde flow is an infant-to-mother transfer in which microbes are transmitted via the skin and saliva from the infant's oral cavity into the duct during suckling (Ramsay et al., 2004). This could explain how bacteria commonly found in the infant oral cavity, such as those within the genera *Veillonella, Leptotrichia* and *Prevotella* (Cabrera-Rubio et al., 2012), or bacteria commonly found in the vagina, such as *Lactobacillus* (Ravel et al., 2011) are sometimes found in human milk. The vaginal bacteria could be acquired by the infant through a vaginal delivery and then transferred to the human milk through retrograde flow. Recent evidence has suggested that the mode of delivery might influence human milk composition (Cabrera-Rubio et al., 2012, 2016;

Hermansson et al., 2019; Khodayar-Pardo et al., 2014; Toscano et al., 2017). Some studies show higher bacterial diversity and richness in the milk from vaginal deliveries (Cabrera-Rubio et al., 2012, 2016; Hermansson et al., 2019; Toscano et al., 2017). Human milk from vaginal deliveries has been reported to have higher *Bifidobacterium* (Khodayar-Pardo et al., 2014), *Streptococcus* and *Haemophilus*, and lower abundance of *Finegoldia* spp., *Halomonas* spp., *Prevotella* spp., *Pseudomonas* spp. and *Staphylococcus* spp. (Toscano et al., 2017). However, despite these differences, findings are not consistent as other studies did not report differences (Moossavi, Sepehri, et al., 2019; Pannaraj et al., 2017; Sakwinska & Bosco, 2019; Soto et al., 2014).

The entero-mammary route has emerged as another potential route for how maternal gut microbiota might enter and enrich the human milk microbiome. The route proposes that maternal gut bacteria are translocated through the intestinal epithelial barrier via dendritic cells which cross the paracellular space of the intestinal epithelium directly into the intestine lumen (Martín et al., 2004; Perez et al., 2007; Rescigno et al., 2001; Thum et al., 2012). Circulation of lymph within the mucosal associated lymphoid tissue could then allow the maternal gastrointestinal tract microbiota to reach distant mucosal surfaces, such as the lactating mammary gland.

### 2.4 Major factors that influence the human milk microbiome

Milk microbiome composition is influenced by diverse factors such as stage of lactation, maternal BMI, diet and use of antibiotics (Arroyo et al., 2010; Cabrera-Rubio et al., 2012; Davé et al., 2016a; Hermansson et al., 2019; Khodayar-Pardo et al., 2014; Kumar et al., 2016; S.-W. Li et al., 2017; Moossavi, Sepehri, et al., 2019; Soto et al., 2014; Williams et al., 2017; Zimmermann & Curtis, 2020). However, there are other factors that remain unexplored and might have an important influence in human milk composition including age, parity and geographical location, interactions with the environment.

#### 2.4.1 Stage of lactation

There is evidence of substantial shifts in microbiota composition at different stages of lactation. Chen et al. (P.-W. Chen et al., 2018) found that colostrum and transitional milk share 48.9% of bacterial genera and 42% of bacterial species, so there are common and unique bacteria between

the two stages of milk. Cabrera et al. (Cabrera-Rubio et al., 2012) reported initially that the microbiota of colostrum was dominated by *Weissella, Leuconostoc, Staphylococcus* spp*., Streptococcus* spp*.,* and *Lactococcus* spp*.* Later, milk samples collected 1–6 months post-partum had higher levels of *Veillonella, Prevotella, Leptotrichia, Lactobacillus* spp*, Streptococcus* spp., and increasing levels of *Bifidobacterium* and *Enterococcus* spp*,* which the authors speculated was related to the frequent interaction with the infant's oral microbiota. Similar results were reported by (Khodayar-Pardo et al., 2014), in which *Bifidobacterium* and *Enterococcus* spp*.* counts increased throughout the lactation period. Another study reported that colostrum had a higher diversity typical of skin and maternal gut bacteria, and as lactation progressed, mature milk became less diverse but increased in infant oral and skin associated bacteria (LaTuga et al., 2014). While improved technologies will help elucidate the current discrepancies in microbiota across lactation stage, a consistent finding is that the microbiome is dynamic and remodels as lactation progresses (Collado et al., 2012; Gueimonde et al., 2007). Given this, other factors known to change throughout lactation, such as the nutritional and immunological composition of milk, will need to be explored as modifiers of human milk microbiome.

### 2.4.2 Maternal BMI and diet

The relationship between BMI and human milk microbiota is not clear. Some studies did not find any significant associations (S.-W. Li et al., 2017; Williams et al., 2017) but others have reported that maternal BMI and weight gain during pregnancy do impact the diversity of bacterial community in human milk (Cabrera-Rubio et al., 2012; Collado et al., 2008). Those studies that found an association reported that obesity and excessive weight gain reduced diversity of the milk microbiome (Cabrera-Rubio et al., 2012; Collado et al., 2008). Milk samples of mothers with higher BMI had higher abundance of *Lactobacillus* in colostrum and higher abundance of *Staphylococcus* and *Akkermansia* in mature milk (Cabrera-Rubio et al., 2012) as well as higher *Granulicatella* (Williams et al., 2017). On the other hand, higher BMI has been related to reductions in the genera *Bifidobacterium* in milk produced at 6 months (Cabrera-Rubio et al., 2012) and *Bacteroides* (Williams et al., 2017) and, more broadly, reductions in the phyla Proteobacteria (Moossavi, Sepehri, et al., 2019) and Firmicutes (Kumar et al., 2016).

Maternal diet, a factor often associated with BMI, could influence microbial composition of human milk directly or as a secondary effect of influencing human milk nutritional content. Some studies have reported that maternal diet influences the nutrient concentration of milk (Ballard & Morrow, 2013; Innis, 2014; Lönnerdal, 1986) and likely shape its bacterial community (Williams et al., 2017). With regards to macronutrients, maternal intake of saturated fatty acids and monosaturated fatty acids were inversely associated with *Corynebacterium*, and protein consumption was positively correlated with the relative abundance of *Gemella* (Williams et al., 2017). In relation to micronutrients, a negative correlation was observed between pantothenic acid intake and *Streptococcus* and between *Lactobacillus* with thiamin, niacin, vitamin B-6 and chromium and a positive correlation was found between riboflavin and calcium with *Veillonella* (Williams et al., 2017). Since the relationship between diet and gut microbiota has been extensively explored (David et al., 2014; Filippo et al., 2010; Ley et al., 2008; G. D. Wu et al., 2011), the diet induced alterations of maternal gut microbiota might also influence human milk microbes, and therefore vertical transfer to the infant (Wang et al., 2019). Nonetheless, there is limited evidence about the direct relationship of maternal diet on the human milk microbiome, or the interactions between maternal diet, maternal microbiota and infant microbiota. Despite the limited evidence, it is valuable that research suggests that maternal diet can influence human milk microbial composition, as maternal diet is one of the most modifiable factors by which interventions could be explored for modulating the human milk microbiome.

### 2.4.3 Use of antibiotics and probiotics

Results regarding the use of antibiotics are contradictory. For instance, in a study of women living in Germany and Austria abundance of lactobacilli or bifidobacteria was lower in women who had received antibiotics during pregnancy or lactation (Soto et al., 2014). However, another study reported that the effect of antibiotic exposure on human milk microbiota at 1 month postpartum appeared to be an increase in bacterial richness and diversity (Hermansson et al., 2019). Similarly, it has also been reported that mean bacterial counts in milk produced by women receiving antibiotics were higher than in milk from women taking probiotics (Arroyo et al., 2010). Although probiotics are largely considered to support microbiome diversity, a gut microbiome study found a similar pattern and reported that after the use of antibiotics, the introduction of probiotics

actually delayed and impaired mucosal microbiome reconstitution (Suez et al., 2018). However, the impact of the consumption of probiotics during pregnancy and lactation on the milk microbiome and infant health following antibiotic use has not been widely explored.

## 2.5 Inconsistencies in dominant milk microbiome bacteria persist

Although lactation stage, maternal BMI, diet and use of antibiotics are thought to drastically influence the microbiota present in human milk, a number of taxa are consistently observed across studies. The genera often found include *Staphylococcus, Streptococcus*, *Lactobacillus, Bifidobacterium Pseudomonas, Corynebacteria, Acinetobacter, Sphingomonas, Serratia, Ralstonia, Bradyrhizobiaceae and Cutibacterium* (Boix-Amorós et al., 2016; Fernández et al., 2013; Hunt et al., 2011; Jiménez et al., 2015; S.-W. Li et al., 2017; Murphy et al., 2017; Ojo-Okunola et al., 2018; Urbaniak et al., 2014). According to the latest systematic review 590 different genera have been detected via sequencing human milk, from which the 10 most frequently found were: *Staphylococcus* (genera found in 97% of studies; range of relative abundance 5–83%), *Streptococcus* (95%; < 1 to 74%), *Lactobacillus* (63%; < 1 to 5%), *Pseudomonas* (50%; < 1 to 17%)*, Bifidobacterium* (42%; < 1 to 5%), *Corynebacterium* (42%; < 1 to 6%), *Enterococcus* (42%; 1%), *Acinetobacter* (39%; 2 to 4%), *Rothia* (34%; 1 to 6%) and *Cutibacterium* (29%; < 1 to 3%) (Zimmermann & Curtis, 2020).

Some studies have attempted to define a “core” microbiome of human milk (Fernández et al., 2013; Hunt et al., 2011), but the presence of some bacteria in human milk remains controversial as different studies have considered specific genera as either contaminants or as part of the “core” microbiome. Salter *et al.* (Salter et al., 2014) found that laboratory reagents were commonly contaminated with species from genera that are repeatedly reported as present in human milk, such as *Acinetobacter, Cutibacterium, Novosphingobium*, *Pseudomonas*, *Ralstonia*, *Sphingomonas* and *Streptococcus*, and which can become spurious signals in samples with low bacterial load during 16S rRNA gene amplification. Jiménez et al., speculated that while species within genera such as *Cutibacterium* and *Streptococcus* have been isolated using culture-dependent techniques, species from genera such as *Sphingomonas* and *Pseudomonas* (containing many readily culturable species) had not, suggesting these genera could commonly be contamination from laboratory reagents. More recently, similar warnings that reagent

contamination can substantially influence low bacterial load human milk microbiome samples were raised within Douglas *et al*. (Douglas et al., 2020), which provided evidence that species within *Pseudomonas* are common contaminants and also likely genuinely present in human milk (highlighting the current challenges within the field). Similarly, *Acinetobacter,* a bacteria commonly found in soil, has been reported previously in human milk (Boix-Amorós et al., 2016; Kumar et al., 2016; Sakwinska et al., 2016; Urbaniak et al., 2014). Sakwinska et al., and Urbaniak et al., both detected *Acinetobacter* in their milk samples where milk collection was done without aseptic cleansing of the breast and also rejection of foremilk. However, Sakwinska et al. found it unlikely that the predominance of *Acinetobacter* was due to the collection protocol and argued that *Acinetobacter* might be a specific feature of breastfeeding associated microbiota. Other studies that have reported bacteria commonly associated with soil in their samples correlated it to the maternal diet based in legumes (Drago et al., 2017) or to the proximity to a soil environment (Blum et al., 2019).

Care should be taken to clearly report the use of PCR controls in microbiome studies as the presence of species from certain bacterial genera in human milk remains controversial. Because of this, interpretation of observed genera should also include their potential as contamination while not precluding novel discovery within this understudied field. Few studies analysing human milk microbiome have been conducted at species level **(Table 2)**, despite the advantages improved resolution would have by providing more information about functionality and in facilitating biological interpretation. However, research at species level is increasing as 16S rRNA gene barcoding and shotgun metagenomics technologies improve.

**2.6 Methods shape observed bacterial taxa in human milk**

The methods used to study the human milk microbiome continue to advance. The initial studies used bacterial culture techniques followed by phenotyping of isolated strains using morphological and biochemical characteristics. These studies isolated only a limited number of genera, predominantly facultative anaerobes such as members of the *Staphylococcus* spp., *Streptococcus* spp., *Propionibacterium* spp. (now *Cutibacterium* in the case of *C. acnes*), *Rothia* spp., *Enterococcus* spp. and *Lactobacillus* spp. (Albesharat et al., 2011; Jiménez et al., 2008; Martín et al., 2003). Studies that have used culturing techniques have generally reported that human milk

harbours relatively low mean viable bacterial counts often < log 3 cfu/ml (Heikkilä & Saris, 2003; Jost et al., 2013; Perez et al., 2007). While still a powerful tool for assessing viability of specific bacterial strains, culture-dependent analyses are limited in revealing only taxa capable of surviving sampling procedures and growth under laboratory conditions (Sakwinska & Bosco, 2019). This selection can potentially reduce the observed microbiota community in complex habitats to below 1% of the diversity currently estimated by culture-independent approaches (Handelsman, 2004), although research suggests 50% of bacteria may a more reasonable rough estimate in the human gut (Lagkouvardos et al., 2017). This difficulty to culture can be due to the lack of specific required nutrients in the culture medium, toxicity of the culture medium, inhibition by other bacteria or a dependence on association with other species (such as present in bacterial consortia or eukaryote host interactions) (Wade, 2002). Therefore, although culture-based techniques are vital for the study of specific bacteria of clinical importance or functional interest (H. P. Browne et al., 2016; Sarangi et al., 2019), they can be heavily biased and drastically underestimate the diversity when used to assess a microbiome community.

To overcome these challenges, culture-independent metagenomic approaches have been developed in which high-throughput sequencing is harnessed to identify bacteria within microbiome samples using by shotgun metagenomics or the 16S ribosomal RNA gene (Sarangi et al., 2019). The major advantage of these approaches is the possibility to detect difficult or yet-to-be-cultured bacteria in addition to improved sample throughput without a requirement of viable cells (allowing the use of frozen samples) (Sakwinska & Bosco, 2019). Shotgun metagenomics attempts to sequence DNA directly from DNA fragments derived from all the genomic material present within a microbiome sample and attempts *de novo* assembly of as many entire genomes or large contiguous sequences as possible in order to infer taxonomic and functional information (Almeida et al., 2019; Sarangi et al., 2019). As this approach does not target a specific gene for PCR, it does not include the same amplification biases associated to 16S rRNA gene barcoding and is widely considered the gold standard of microbiome research. However, in addition to the high expense currently associated to high depth shotgun metagenomics (which is rapidly dropping (van Nimwegen et al., 2016)), the approach generates large amounts of reads from complex samples which are challenging to assemble *de novo* (Sczyrba et al., 2017; Vollmers et al.,

2017). While *de novo* shotgun metagenomics has yet to become common for the study of the human milk microbiota, recent research has used shotgun metagenomics sequence reads as markers for mapping to reference libraries (Asnicar et al., 2017; Jiménez et al., 2015; Pärnänen et al., 2018; Ward et al., 2013). While read mapping approaches are still improving in terms of accuracy for quantitative analysis, made difficult by database limitations, very low mapping rates and extensive sequence ambiguity, these rapid improvements in sequencing and bioinformatics methodologies suggest an exciting future for high-resolution identification of metagenomes which includes distinct inventories of genes between human milk microbiome communities. Alternatively, the 16S ribosomal RNA (rRNA) gene has been the most popular approach used for microbial community assessment over recent decades (Caporaso et al., 2010; FOX et al., 1977; Muyzer et al., 1993; Pace et al., 1986; Schloss et al., 2009). The value of the 16S rRNA gene as a 'barcode' for the identification and phylogenetic classification of bacterial species lies in the very highly conserved function of 16S rRNA leading to regions of hyper-conservation within the gene (Martinez-Porchas et al., 2017; Woese et al., 1983). These regions of conservation can then be targeted by primers in order to amplify proximal hyper-variable sequence regions (an amplicon) used as a potentially unique barcode of life (Klindworth et al., 2012).

In human milk research, 16S rRNA is still the most popular tools for profiling microbiome samples for quantitative comparison of groups or treatments **(Table 2)**. While some limitations of the 16S rRNA gene barcoding approach are generally well recognised in the field, including the impact of low bacterial load in human milk (Salter et al., 2014) and poor utility for inference of biological functions in the community (Sarangi et al., 2019), primer specificity has been less well addressed. Certain primers have, in the past, been considered "universal" to prokaryotes and thought to amplify hypervariable regions from all bacteria. Research including that conducted by Klindworth et al. (Klindworth et al., 2012) has now demonstrated that no known primer pair is universal in amplifying 16S rRNA gene regions across all currently known and well-characterised bacterial species, although many have coverage of over 90% of known bacteria. Given that the observed absence or presence of certain bacteria can be determined by primer pair selection and the extensive range of primers used in human milk microbiome research **(Table 2)**, it is perhaps not

unsurprising that the “core” human milk microbiome genera or species have been reported as inconsistent in systematic literature reviews (Fitzstevens et al., 2017; Jeurink et al., 2013).

Using the TestPrime tools in the Silva rRNA database (Quast et al., 2013) and the research by Klindworth et al. (Klindworth et al., 2012), it is possible to assess the specific utility of primers commonly used in human milk microbiome research to amplify the putative “core” milk genera *in silico*. Interestingly, the primers 27F/533R (V1 region targeting), which are often used in human milk microbiome studies (Cabrera-Rubio et al., 2012; Hunt et al., 2011; Lackey et al., 2019; Mediano et al., 2017; Williams et al., 2017), have high coverage for amplification of the genus *Cutibacterium* but not species within the genus *Bifidobacterium* (with the most common 27F variant, **Figure 2,** both of which are considered putative “core” genera (Hunt et al., 2011; Jiménez et al., 2015; Jost et al., 2013) **(Table 2)**. This shortfall in the coverage of *Bifidobacterium* of the commonly used 27F primer was reported by Frank et al. (Frank et al., 2008), who designed a high degeneracy variant of seven primers (27F YM+3) with improved coverage in the genera which has been used in human milk microbiome research (Lackey et al., 2019; Meehan et al., 2018). Conversely, the other most commonly used primers 515F/806R primer pair (V4 region targeting) will likely amplify species within the genus *Bifidobacterium* but have very low coverage and do not amplify species from the genera *Cutibacterium*. To overcome these discrepancies, the 27F YM+3 high degeneracy variant (within 27F/533R, V1-V3 targeting) or the 784F/1061R primer pair (V5-V6 targeting) should be considered more suitable for human milk research due to high coverage within all of the genera currently considered “core” in human milk **(Figure 2)**. It is important to recognize that these primers might still fail to amplify yet-to-be-identified species but will allow for the quantitative assessment of relative changes in most species within these important genera when experimentally comparing groups of mothers. While there is a desperate need to increase research into the human milk microbiome using tools such as 16S rRNA gene barcoding, care needs to be taken with all culture-independent techniques to not assume a perfect snapshot of the community, similar to the lessons previously learned with culture-based community assessment (Jost et al., 2013; Martín et al., 2003).

## 2.7 Emerging frontiers in human milk microbiome research

### 2.7.1 Overlooked maternal health issues: Age and Parity

Two overlooked factors that could be impacting the human milk microbiome include maternal age and parity. To date, only one study has analysed the relationship with maternal age, finding no association with human milk microbiota at family level resolution (Moossavi, Sepehri, et al., 2019). However, as there are established differences in the development of breast immunity (Bartlett et al., 1998), nutritional requirements and human milk nutrient composition (Dror & Allen, 2018a) throughout a women's lifetime, these factors could also affect the human milk microbiota and should be assessed at higher taxonomic resolution. Parity has also been reported only in one study finding a sex-dependent association with milk microbiome diversity (Moossavi, Sepehri, et al., 2019). This study reported an association of human milk microbiota composition with multiparity in female infants but did not characterize the microbiome. Parity could be a factor influencing milk microbiome if we consider the interactions of milk microbiome such as the retrograde flow from the infant (Ramsay et al., 2004). Assuming retrograde flow, it could be expected that a multiparous mother will have a mammary gland with more diverse microbiota since it has been previously inoculated by the bacteria transferred by her previous infant(s) during earlier pregnancies (Nasioudis et al., 2017).

### 2.7.2 Geographical location

Another factor to consider when identifying dominant bacteria in human milk is geographical location, lifestyle, and community. Multi-country studies confirm that human milk microbiome composition differs between and within countries as well as the presence of unique bacteria exclusively related to some study sites (Gómez-Gallego et al., 2018; Kumar et al., 2016; Lackey et al., 2019).

Some human milk microbiome studies have characterized their study population based on their lifestyle and community, such as rural (Meehan et al., 2018), urban (Corona-Cervantes et al., 2020; Gomez-Gallego et al., 2016; Kumar et al., 2016; Sakwinska et al., 2016), rural and urban (Lackey et al., 2019; Vaidya et al., 2017) or low socioeconomic (Carvalho-Ramos et al., 2018; Davé et al., 2016a). These population characteristics have been proposed as factors that might potentially affect the human milk microbiota composition (Gomez-Gallego et al., 2016; Kumar et

al., 2016; Lackey et al., 2019; Meehan et al., 2018; Sakwinska et al., 2016). A recent study by Lackey et al. (Lackey et al., 2019) aimed to elucidate if a "core" microbiome in human milk exists in mothers of different countries. The sample included 413 mothers and their infants from 8 countries: Ethiopia, Gambia, United States, Ghana, Kenya, Peru, Spain and Sweden. In addition, Ethiopia and Gambia populations were subdivided in rural and urban. The researchers used homogenous methodology in the selected countries in order to understand if the differences in milk microbiota previously reported in other studies (Cabrera-Rubio et al., 2012; Hunt et al., 2011; Moossavi, Sepehri, et al., 2019) were due to diverse methodologies, genuine differences among populations, or if they were due to differences in the collection, storage, processing and analysis of milk. This study reported differences in milk microbiota at phyla and genus levels. From the 15 phyla identified, Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes represented 97.7% of the microbiota diversity. At the genus level, there was more variation between countries than within countries. On the other hand, some countries showed unique bacteria. For example, only milk collected in rural Ethiopia contained *Acidothermus, Demequina, Flaviflexus* and *Pediococcus,* milk from urban Gambia uniquely contained *Chroococcidiopsis* and *Isoptericolar* and milk from urban Ghana uniquely contained *Akkermansia* and *Butyricicoccus*. Importantly, none of the developed countries included in the study reported unique bacteria. The authors interpreted these results as the confirmation of the existence of a small "core" microbiome among all countries consisting of *Staphylococcus* and *Streptococcus* since they were found in 98.7 and 97.7% of all samples respectively, which aligns to previous conclusions (Fitzstevens et al., 2017). However, while reporting that a "core" microbiome might exist across different populations, Lackey et al. (Lackey et al., 2019) concluded that geographical location was not the only factor influencing the structure and diversity of microbiota in human milk. They emphasized that the existence of additional factors such as antibiotic use, infant age, parity, infant sex and exclusive breastfeeding status may also play an understudied role in milk microbiome.

The variance observed between countries could be related to disparities between rural and urban populations or socioeconomical status, characteristics previously proposed as factors that might potentially affect the microbiota composition (Filippo et al., 2010; Vaidya et al., 2017). However, evidence is limited because the majority of published studies characterising microbial

communities have been conducted in developed countries and urban settings **(Figure 3).** The few studies available in rural communities have reported that human milk composition differs in these populations and suggested this could be due to their agrarian lifestyles' activities. Meehan et al. (Meehan et al., 2018) compared foragers with horticulturalist women in the Central African Republic, and observed that even though both groups spent considerable time in proximity to each other, milk microbial communities varied significantly within populations and between ethnic groups. Vaidya et al. (Vaidya et al., 2017) concluded that human milk microbiota of rural Indian women was more diverse than the milk microbiota of urban women; at phyla level women from rural communities had more species from Firmicutes while human milk from urban women had more Proteobacteria. Lackey et al. (Lackey et al., 2019) noted differences in the milk microbiota of mothers from developed countries and two developing countries (Ethiopia and Gambia), which were also sub-divided into rural and urban populations. Milk from rural-Ethiopia differed from the other populations and was characterized by a relatively high abundance of *Rhizobium* and *Achromobacter;* intermediate abundances of *Streptococcus* and *Staphylococcus;* and very little *Cutibacterium*/*Propionibacterium, Dyella*, and *Rothia.* However, the methodology used for collection and processing of the milk in Ethiopia differed from other populations, which calls into question that the uniqueness of the Ethiopian results relates to the rural aspect of the community.

There is only one study that has described its population as a low socioeconomic community, Dave et al. (Davé et al., 2016a) reported an uncommonly high abundance of *Streptococcus* and therefore a reduced diversity when compared to other populations, suggesting that differences might be related either to ethnicity, socioeconomic status or other factors (Cabrera-Rubio et al., 2012) related to culture including dietary patterns, rituals and customs particular to certain region or geographical location that could affect the interaction of the mother and infant with their environment.

### 2.7.3 Environmental Factors

The potential for the environment to modify the human milk microbiome has not been widely researched. Few studies have discussed the source of the bacteria in human milk and have commonly focused on identifying taxa potentially originating from oral, skin or gut habitats

(Cabrera-Rubio et al., 2012; LaTuga et al., 2014). Togo et al. (Togo et al., 2019) analysed where each species identified in human milk studies (820 species from 242 articles, 38 countries, 11,124 women and 15,489 samples) were originally isolated. The study found that only 40% of breast milk bacteria were first isolated from human tissue (including gut, respiratory tract, oral cavity, urinary tract, skin, vagina, milk) while the other 60% was first observed in association with the environment, plants, animals and food. From this 60%, environment associated bacteria were the most prevalent with 34% (Togo et al., 2019). This same study recognized that only one species was initially associated with milk, suggesting extensive interaction between the microbiome of humans and their environment. These observations highlight the potential influence of the environment in shaping the breast milk microbiome, which to-date has most often been perceived as contaminants rather than being normally present in the breast milk (P. D. Browne et al., 2019; Salter et al., 2014).

The presence of soil and water associated bacteria has consistently been observed in breast milk studies. These include *Acinetobacter* (Drell et al., 2017; Kumar et al., 2016; Patel et al., 2017; Sakwinska et al., 2016; Urbaniak et al., 2016), Bradyrhizobiaceae (Hunt et al., 2011), *Novosphingobium* (Jiménez et al., 2012), *Pseudomonas* (Hunt et al., 2011; Jiménez et al., 2015; Moossavi, Sepehri, et al., 2019; Pannaraj et al., 2017; Patel et al., 2017; Sakwinska et al., 2016), *Ralstonia* (Hunt et al., 2011; Kumar et al., 2016; Moossavi, Sepehri, et al., 2019; Vaidya et al., 2017; Williams et al., 2017), *Sphingobium* (Jiménez et al., 2012; Mueller et al., 2015), *Sphingomonas* (Davé et al., 2016a; Ding et al., 2019; Hermansson et al., 2019; Hunt et al., 2011; Jiménez et al., 2015; Kumar et al., 2016; S.-W. Li et al., 2017; Urbaniak et al., 2014), *Stenotrophomonas* (Urbaniak et al., 2014), and *Xanthomonadaceae* (Davé et al., 2016a; Urbaniak et al., 2014). Bacteria commonly found in the environment and also found in breast milk samples have the potential to simply be contamination, particularly as contaminants from PCR reagents as suggested by Ruiz et al. (Ruiz et al., 2019). Douglas et al. (Douglas et al., 2020) has provided evidence that, for at least some of these species, their presence in human milk may be genuine.

Soil bacteria observed in breast milk microbiota have been related to the maternal diet, seasonality, the environment and occupation, such as horticulturalists (Meehan et al., 2018) or hunter-gatherers (Blum et al., 2019; Clemente et al., 2015; Yatsunenko et al., 2012), rather than

as a product of cross-contamination during the analysis (Sakwinska et al., 2016; Salter et al., 2014; Urbaniak et al., 2014). On the other hand, soil microbes can differ enormously from region to region. There are only a few species that can be found in all soils, while there are numerous rare species that only occur in particular soils or geographical areas (Wall et al., 2015). Therefore, generalisations among soil bacteria are difficult to do and further research in milk microbiome that aims to study the source of bacteria should include soil analysis.

Soil is considered to harbour one of the most diverse microbial populations, with several thousand of species often observed in samples. This large microbial diversity in soil results in diverse functional ecology, which includes primary productivity and nutrient cycling such as increased nutrient use efficiency and uptake, which may improve plant resilience and resistance against stressors (Blum et al., 2019). For instance, bacteria identified in soil can produce more than 50 antibiotics to protect plants from pathogenic bacteria and rhizobia can associate with plant hosts like alfalfa, soybeans and clover to help provide the plants with nutrients (Brereton et al., 2020). As soil is part of the habitat of humans providing space for living, recreation and food production (Brevik et al., 2020) it is probable that the soil contributes to the human microbiome due to its close contact as opposed to similar provision of functional diversity. It has been observed that hunter-gatherers have a gut microbiome with a higher species richness than that of humans consuming westernized food or from an urbanized society (Blum et al., 2019; Clemente et al., 2015; Schnorr et al., 2014; Vaidya et al., 2017; Yatsunenko et al., 2012). While these observations could be related to genetics, diet and their unique environments (Tasnim et al., 2017) the findings highlight the potential for largely unexplored links between agricultural practices, soil transmitted parasites and protozoa and human health (Scott & Koski, In press).

### 2.7.4 Benefits of environmental bacteria

A milk microbiome enriched in diversity and associated functionality, in contrast to assuming certain observed species are *de facto* contamination, could be explored as potentially conferring some benefit to mother or infant. For instance, Chan *et al.* (Chan et al., 2016) have speculated that *Sphingobium yanoikuyae* found in nipple aspirate fluid might activate a pathway that could inhibit cancer progression. A human microbiome enriched by soil bacteria could be beneficial through functions well established in soil communities, such as suppression of soil-borne

pathogens, exposure to immunoregulation-inducing soil microorganisms, immune tolerance and increase of microbiota diversity (Tasnim et al., 2017). The influence of bacteria associated with soils and water-types have been somewhat explored in the gut microbiome but research in the breast milk microbiome is still scarce. Blum et al. (59) linked the soil microbiome and the human intestinal microbiome as “superorganisms” which, by close contact (soil, faeces and food), can replenish each other as inoculants and provide beneficial microorganisms which could positively impact human health. It is also speculated that urbanization and industrialization of agriculture may have decreased the richness of an overlapping of soil and human microbiota (Blum et al., 2019). Additionally, there is evidence that microbes from diverse habitats like soil can colonize the germ-free gut (Seedorf et al., 2014) and contribute commensal microbes which enrich gut microbiota diversity, which can reduce inflammatory disease risk, reduce asthma and improve child health (Tasnim et al., 2017).

Although the previous studies linked the benefits of environmental bacteria specifically to the gut microbiome, it is plausible that similar relationships exist with the breast milk microbiome. Further research is essential to explore the relationship of breastfeeding practices, such as the introduction of water, beverages and food, as a factor for introducing soil and water bacteria into breast milk microbiome as microbial diversity might influence neonatal gut colonisation, impact the maturation of the immune system, supress pathogenic bacteria such as *Staphylococcus aureus* and therefore prevent maternal and neonatal infections as well as increase breast milk production.

**2.8 Conclusion**

Human milk is the first source of nutrients and immunity that the infant receives, supplying microbes to the newborn infant during a critical period of growth and development. Despite this important role for human development and health, there is scarce evidence of how some factors, such as maternal age and diet, area and environment, might influence milk microbiota composition. Contemporary high-resolution 16S rRNA gene amplicon and shotgun metagenomics are powerful approaches capable of more accurate genus and species-level microbiome assessment. These tools should be used alongside controls for contamination to study the source of environmental bacteria in breast milk and whether they originate from breastfeeding practices,

maternal diet or environment proximity. Additionally, expansion of breast milk research to developing countries and rural areas represents low-hanging fruit for important discovery, as the vast majority of available evidence is in developed countries and urban areas. While the study of the breast milk microbiome has faced diverse challenges, there are extensive new strategies and opportunities to advance our understanding and promote future interventions in maternal and infant health.

### 2.9 CRediT authorship contribution statement

**Lilian Lopez Leyva:** Conceptualization, Writing - original draft. **Nicholas Brereton:** Conceptualization, Writing - review & editing. **Kristine. Koski:** Conceptualization, Writing - review & editing.

### 2.10 Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.


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## 2.12 Tables

**Table 2.1 Historical Study of Milk Microbiome**

| Time | Microbiome Concept | Historical Human Milk Microbiome Findings |
|---|---|---|
| '60s | *Enterobacteriaceae* detected | The presence of *Enterobacteriaceae* in human breast milk is related to the low levels of personal hygiene and environmental sanitation in women from Guatemala. It is concluded that an interchange of breast milk bacteria between the mother's breast and the infant's mouth is possible (Wyatt & Mata, 1969). |
| '70s | Heat sterilization | Bacteria in milk were detected. Breast milk was heated in order to be "bacteriologically safe" (Carroll et al., 1979). |
| '80s | Microbial growth promotion suggested | Growth-promoting substances in human milk postulated to be involved in the development of microbiota. Components like lactoferrin and a saccharide containing N-acetyl glucosamine could provide an adequate environment for bacterial growth (Hill & Marsh, 1990). |
| '90s | Bacteria considered solely contamination | Presence of bacteria considered to be due to contamination in frozen milk. Contamination levels of human milk compared to pasteurized cow's milk to develop guidelines for the acceptable microbial quality of human milk (El-Mohandes et al., 1993). |
| '00s | Commensal bacteria | A study looks for commensal bacteria inhibiting *Staphylococcus aureus* as published reports at that time had only focused on pathogenic bacteria (Heikkilä & Saris, 2003). |
| | Entero-mammary pathway | Bacteria are recognized as components of natural microbiota rather than contaminants. The hypothesis of an Entero-Mammary route is proposed (Martín et al., 2004). |
| | Retrograde flow | Description of the "retrograde infant-to-mother transfer" in which microbes are transmitted via the skin and via retrograde-flow of milk into the duct during suckling (Ramsay et al., 2004). |
| | Human Microbiome Project (HMP) | The Human Microbiome Project started in 2007 by the National Institute of Health. The first large-scale effort to characterize the |

| | | |
|---|---|---|
| | | healthy human microbiome across 5 body sites: the digestive tract, mouth, skin, nasal cavity and vagina (NIH HMP Working Group et al., 2009). |
| | Anaerobic species | Alternative route for microbial transfer due to the presence of the anaerobic genus *Bifidobacterium* in breast milk samples (Gueimonde et al., 2007). Although this led to important new theories about the origin of milk bacteria, *Bifidobacterium* is now recognised as containing extensive strain variance in $O_2$ tolerance and sensitivity (Shimamura et al., 1992; P. J. Simpson et al., 2005). |
| | Gut and milk link to lymphatic system | Evidence of an internal microbial transfer pathway due to the presence of maternal gut and breast milk bacteria in the lymphatic system is strengthen, although more evidence is necessary due to the characteristics of the study. (Perez et al., 2007). |
| | Tissue specific microbiome patterning | Identification of distinct patterns of the microbiome among human body sites (13). |
| '10s | Vertical transfer widely accepted | The vertical transfer to the neonate via breastfeeding was strengthened when it was found that the same bacteria is shared by the breast milk, maternal and infant faeces (63). |

**Table 2.2 - Summary of most abundant bacteria, lifestyle or community considered and methods employed in breast milk microbiome studies**

| Most Abundant Taxa | Cohort location | Method | Region | Primer[a] | Primer sequences (including degeneracy variants) | Template free PCR controls[b] | Reference |
|---|---|---|---|---|---|---|---|
| *Staphylococcus, Streptococcus, Serratia, Pseudomonas, Corynebacterium, Ralstonia, Cutibacterium/Propionibacterium, Sphingomonas* and *Bradyrhizobiaceae* | USA | 16S rRNA gene sequencing | V1-V2 | 27F[C] and 338R | 27F: `5-AGAGTTTGATCCTGGCTCAG-3'<br>338R: `5-TGCTGCCTCCCGTAGGAGT-3' | Yes | (Hunt et al., 2011) |
| *Weisella, Leuconostoc, Staphylococcus, Streptococcus, Lactococcus, Veillonella*, *Leptotrichia*, and *Prevotella* | Finland | 16S rRNA gene sequencing | V1-V3 | 27F and 533R | N/A | Yes | (Cabrera-Rubio et al., 2012) |
| *Cutibacterium acnes, Staphylococcus epidermis, Streptococcus (S. salivarius,S. thermophilus, S.vestibularis, S. mitis, S. pneumoniae, Staphylococcus (S. lugdunensis, . aureus, S. haemolyticus, S. hominis, S. pasteuri, S. wameri) Veillonella (V. atypical, V. dispar, V. parvula), , Rothia mucilaginosa, Propionibacterium granulosum, Bifidobacterium breve, Klebsiella pneumoniae,* | Switzerland | 16S rRNA gene sequencing | V5 – V6 | 784F and 1061R | 784F: `5-AGGATTAGATACCCTGGTA-3'<br>1061R: `5-C**RR**CACGAGCTGACGAC-3' | No | (Jost et al., 2013) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Escherichia/Shigella, Lactobacillus (L. gasseri, L, brevis), Enterococcus (E. faecalis, E. gallinarum)* | | | | | | | |
| *Staphylococcus* spp., *Streptococcus* spp., *Veillonella* spp., *Corynebacterium* spp., *Rothia* spp., *Enterococcus* spp., *Lactobacillus* spp., *Escherichia/Shigella* spp., *Klebsiella* spp. | Switzerland | 16S rRNA gene sequencing | V5 – V6 | 784F and 1061R | 784F: `5-AGGATTAGATACCCTGGTA-3’<br>1061R: `5-C**RR**CACGAGCTGACGAC-3’ | No | (Jost et al., 2014) |
| *Eubacterium, Lactobacillus, Acinetobacter, Xanthomonadaceae, Stenotrophomonas* | Canada | 16S rRNA gene sequencing | V6 | Fw`5-CWACGCGARGAACCTTACC-3’ Rv: `5-ACRACACGAGCTGACGAC-3’ ’ | Fw: `5-C**W**ACGCGA**R**GAACCTTACC-3’<br>Rv: `5-AC**R**ACACGAGCTGACGAC-3’ | Yes | (Urbaniak et al., 2014) |
| *Staphylococcus, Streptococcus, Bacteroides, Faecalibacterium, Ruminococcus, Lactobacillus*, *Cutibacterium/Propionibacterium, Staphylococcus aureus* and *Staphylococcus epidermis* | Spain | Shotgun metagenomic sequencing | - | - | - | - | (Jiménez et al., 2015) |
| *Streptococcus, Staphylococcus,* and *Neisseria* | USA | 16S rRNA gene sequencing | V4 | 515F and 806R | 515F: GTGCCAGC**M**GCCGCGGTAA<br>806R: GGACTAC**HV**GGGT**W**TCTAAT | No | (Davé et al., 2016a) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Staphylococcus, Pseudomonas, Streptococcus* and *Acinetobacter* | Spain | 16S rRNA gene sequencing | V1-V3 | 27F and 533R | 27F: 5'-AGAGTTTGATC**M**TGGCTCAG-3') 533R: 5'-GCCTTGCCAGCCCGCTCAGGC-3' | No | (Boix-Amorós et al., 2016) |
| *Staphylococcus, Pseudomonas, Enterobacteriaceae, Streptococcus* and *Lactobacillus* | Canada | 16S rRNA gene sequencing | V6 | Fw`5-CWACGCGARGAACCTTACC-3' Rv: `5-ACRACACGAGCTGACGAC-3' | Fw: 5'-C**W**ACGCGA**R**GAACCTTACC-3'<br>Rv: 5'-AC**R**ACACGAGCTGACGAC -3' | Yes | (Urbaniak et al., 2016) |
| *Staphylococcus, Streptococcus, Pseudomonas* and *Acinetobacter* | Spain, Finland, South Africa and China | 16S rRNA gene sequencing | V4 | 515F and 806R | N/A | Yes | (Kumar et al., 2016) |
| *Streptococcus Staphylococcus* and *Acinetobacter* | China | 16S rRNA gene sequencing | V4 | 515F and 806R | 515F: 5'-GTGCCAGC**M**GCCGCGGTAA<br>806R: 5'-GGACTAC**HV**GGGT**W**TCTAAT-3' | No | (Sakwinska et al., 2016) |
| *Streptococcus, Pseudomonas, Staphylococcus, Lactobacillus, Propionibacterium, Herbaspirillum, Rothia, Stenotrophomonas, Acinetobacter, Bacteroides,* | Taiwan and China | 16S rRNA gene sequencing | V1 – V2 | 27F and 338R | 8F/27F-mod: 5'-AG**R**GTTTGAT**YM**TGGCTCAG-3'<br>338R: 5'-TGCTGCCTCCCGTAGGAGT-3' | No | (S.-W. Li et al., 2017) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Halomonas, Veillonella, Sphingomonas, Delftia,* and *Corynebacterium* | | | | | | | |
| *Pseudomonas*, *Staphylococcus* and *Streptococcus* | Ireland | 16S rRNA gene sequencing | V3 – V4 | Bakt314F: 5'-CCTACGGG**N**GGC**W**GCAG-3' Bakt805R: 5'-GACTAC**HV**GGGTATCTAATCC-3' | Bakt314F: 5'-CCTACGGG**N**GGC**W**GCAG-3' Bakt805R: 5'-GACTAC**HV**GGGTATCTAATCC-3' | No | (Murphy et al., 2017) |
| *Streptococcaceae, Staphylococcaceae, Gamellaceae, Alicyclobacillaceae, Enterobacteriaceae, Pseudomonadaceae, Moraxellaceae, Xanthomonadaceae, Bradyrhizobiaceae, Caulobacteraceae, Neisseriaceae* and *Weeksellaceae* | USA | 16S rRNA gene sequencing | V4 | 515F and 806R | 515F: GTGCCAGC**M**GCCGCGGTAA 806R: GGACTAC**HV**GGGT**W**TCTAAT | Yes | (Pannaraj et al., 2017) |
| *Pseudomonas,* unclassified *Enterobacteriaceae, Enterobacter,* | India | 16S rRNA gene sequencing | V2 – V3 | 101F and 518R | 101F:5'-ACTGGCGGACGGGTGAGTAA 3' | No | (Vaidya et al., 2017) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| unclassified *Pseudomonadaceae, Klebsiella, Ralstonia, Acinetobacter* and *Serratia*, *Bacillaceae, Staphylococcus, Enterococcus, Bicullus,* unclassified *Lachnospiraceae, Streptococcus* | | | | | 518R: 5'-CGTATTACCGCGGCTGCTGG-3' | | |
| *Streptococcus, Staphylococcus, Veillonella, Corynebacterium, Rhodococcus, Dyella, Lactobacillus, Prevotella, Micrococcus* and *Hafnia.* | Central African Republic | 16S rRNA gene sequencing | V1 – V3 | 27F-YM+3 and 534R | 27F-YM+3: 5'-AG**M**GTT**Y**GAT**YM**TGGCTCAG-3'<br>534R: 5'-ATTACCGCGGCTGCTGGC-3' | Yes | (Meehan et al., 2018) |
| *Streptococcus, Staphylococcus, Ralstonia, Acidovorax, Acinetobacter, Aquabacterium, Massilia, Agrobacterium, Rheinheimera, Veillonella, Vogesella, Nocardioides* and *Pseudomonas.* | Canada | 16S rRNA gene sequencing | V4 | 515F and 806R | 515F: 5'-GTGCCAGC**M**GCCGCGGTAA-3'<br>806R: 5'-GGACTAC**HV**GGGT**W**TCTAAT-3' | Yes | (Moossavi, Sepehri, et al., 2019) |
| *Streptococcus, Staphylococcus, Corynebacterium, Cutibacterium/Propionibacterium, Rhizobium, Lactobacillus, Dyella, Rothia, Kocuria, Veillonella, Bifidobacterium, Acinetobacter, Klebsiella, Gemella, Achromobacter, Escherichia/Shigella, Bacillus, Stenotrophomonas, Enterococcus,* | Ethiopia, Kenya, Ghana, Gambia, Peru, Spain, Sweden and USA | 16S rRNA gene sequencing | V1 – V3 | 27F-YM+3 and 534R | 27F-YM+3: 5'-AG**M**GTT**Y**GAT**YM**TGGCTCAG-3'<br>534R: 5'-ATTACCGCGGCTGCTGGC-3' | Yes | (Lackey et al., 2019) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Janthinobacterium, Anaerococcus, Acidocella, Enterobacter, Bacteroides, Pseudomonas, Chryseobacterium, Tatumella, Psychrobacter, Clostridium sensu stricto 18.* | | | | | | | |
| *Staphylococcus, Kaistobacter, Paracoccus, Pseudomonas, Bradyrhizobium, Methylobacterium, Acinetobacter, Cutibacterium/Propionibacterium, Corynebacterium,* and *Microbacterium*; three families *Phyllobacteriaceae, Sphingomonadaceae, Gemellaceae* | Mexico | 16S rRNA gene sequencing | V3 | 341F and 518R | 341F: CCTACGGGAGGCAGCAG 518R: ATTACCGCGGCTGCTGG | Yes | (Corona-Cervantes et al., 2020) |

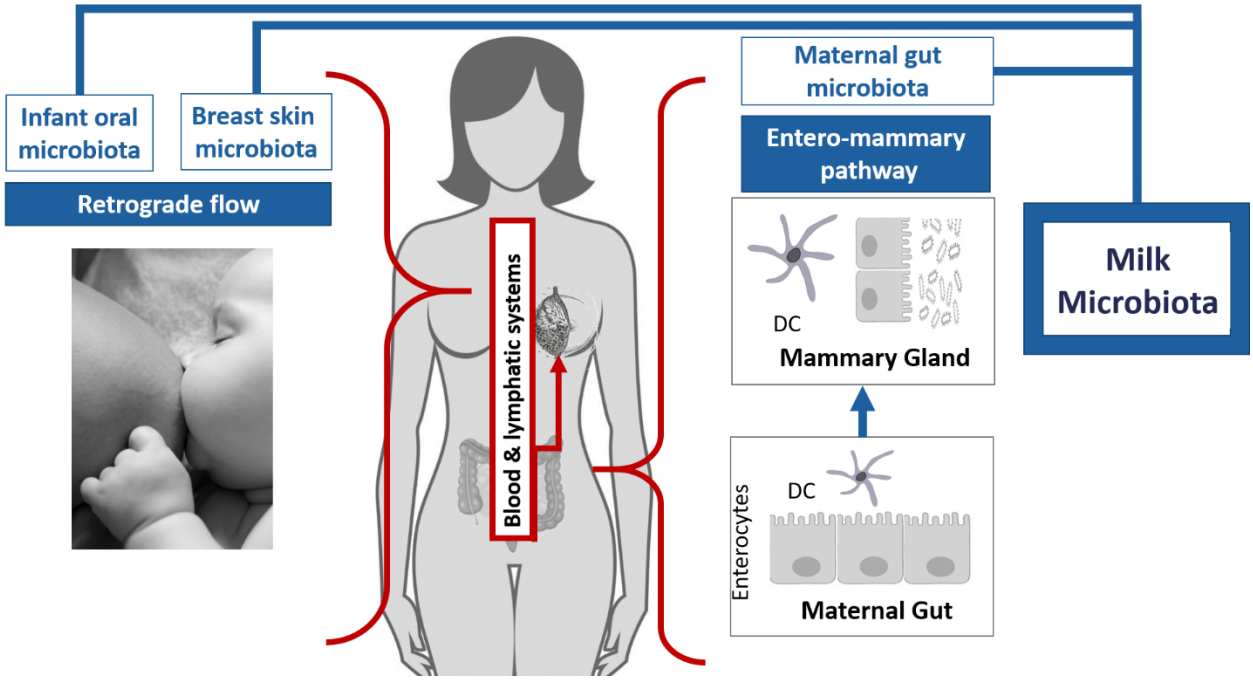
[a] Primer labels are often inconsistent in the literature and so exact sequences are provided where reported

[b] The use of no template PCR controls does not preclude the possibility that any observed sequences were contamination

[c] "27F" with no degeneracy is sometimes called 8F

## 2.13 Figures

**Figure 2.1 - Sources of milk microbiota**

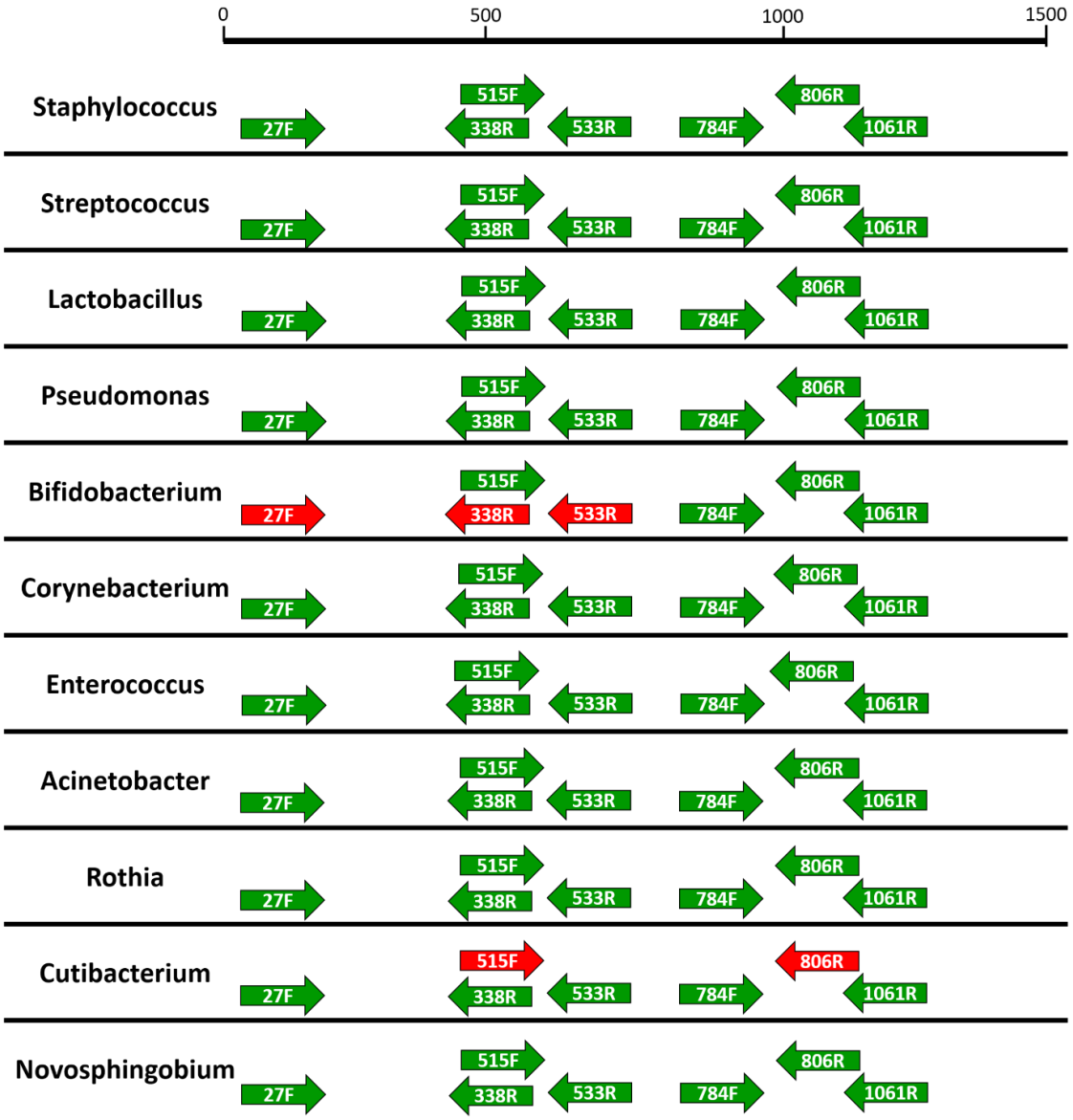



Two potential sources of bacteria which could contribute to the breast milk microbiome: retrograde flow (Ramsay et al., 2004) and the entero-mammary pathway (Martín et al., 2004; Perez et al., 2007; Thum et al., 2012). The retrograde flow pathway suggests that the during suckling, infant oral microbiota and breast skin microbiota can reach the breast milk microbiota. The entero-mammary pathway suggests that dendritic cells in the maternal gut that cross the intestinal epithelium can take up bacteria from the intestinal lumen which are then taken to the mammary gland through the blood and lymphatic systems.

**Figure 2.2 - 16S rRNA gene primers pair coverage of major breast milk microbiome genera**

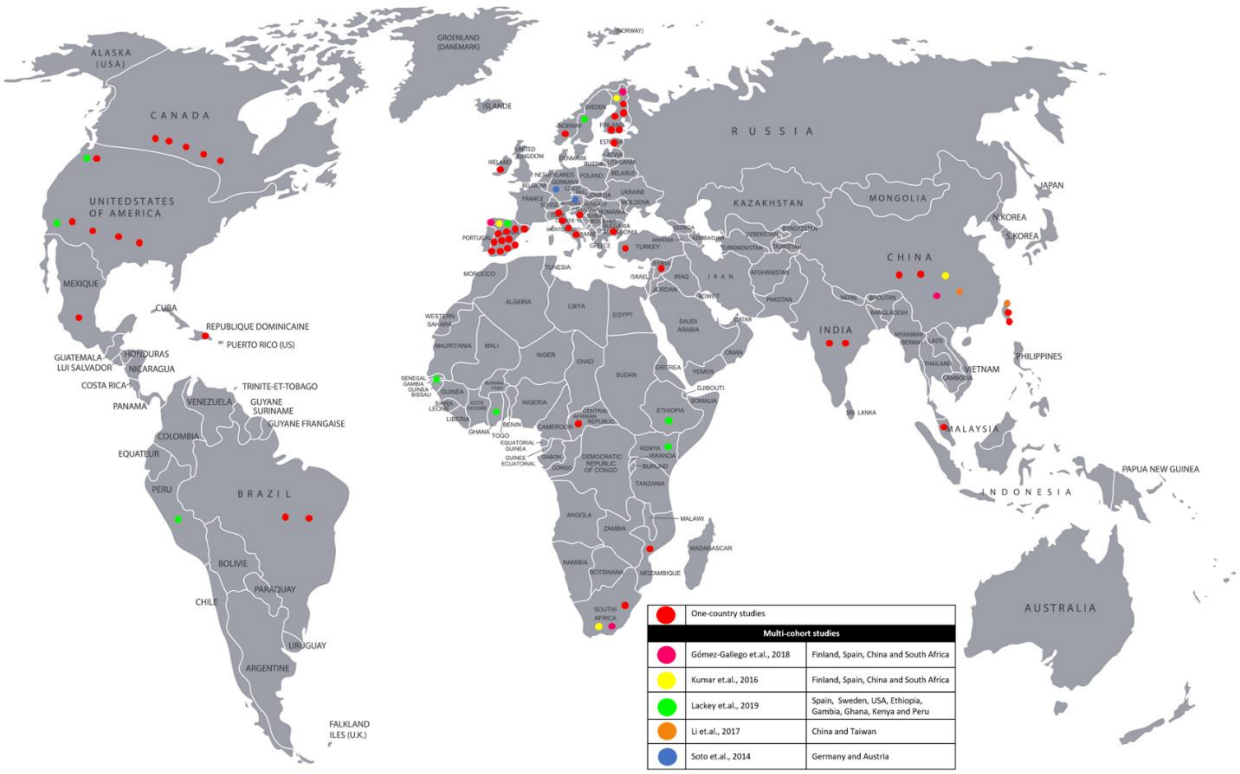
Primer pair coverage (% sequences theoretically amplified within the genus, 1 mismatch in 5` 5nt only)

| 16S region targeted | Common | Common | F Primer | R Primer | F seq | R seq | Staphylococcus | Streptococcus | Lactobacillus | Pseudomonas | Bifidobacterium | Corynebacterium | Enterococcus | Acinetobacter | Rothia | Cutibacterium | Novosphingobium |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| V4 | U515F | 806R | S-*-Univ-0515-a-S-19 | S-D-Bact-0787-b-A-20 | GTGCCAGCMGCCGCGGTAA | GGACTACHVGGGTATCTAAT | 72.7% | 84.1% | 90.4% | 88.1% | 92.9% | 81.4% | 90.1% | 89.9% | 88.0% | 0.1% | 93.5% |
| V1-V2 | 27F | 338R | S-D-Bact-0008-d-S-20 | S-D-Bact-0338-a-A-19 | AGAGTTTGATCMTGGCTCAG | TGCTGCCTCCCGTAGGAGT | 90.0% | 88.0% | 85.0% | 83.2% | 14.1% | 87.0% | 88.2% | 92.3% | 73.8% | 88.9% | 95.8% |
| V1-V3 | 27F | 533R | S-D-Bact-0008-d-S-20 | S-D-Bact-0515-a-A-19 | AGAGTTTGATCMTGGCTCAG | TTACCGCGGCTGCTGGCAC | 90.0% | 88.0% | 84.1% | 83.6% | 13.5% | 89.7% | 87.9% | 91.8% | 71.4% | 87.3% | 95.8% |
| V5-V6 | 784F | 1061R | S-D-Bact-0784-a-S-19 | S-D-Bact-1061-a-A-17 | AGGATTAGATACCCTGGTA | CRRCACGAGCTGACGAC | 90.9% | 92.4% | 88.3% | 88.6% | 91.3% | 95.2% | 87.5% | 88.6% | 95.7% | 80.8% | 92.6% |
| | | | | | Number of sequences in Silva | | 4274 | 4924 | 3877 | 7435 | 449 | 2941 | 1551 | 2460 | 582 | 1643 | 552 |



Common 16S rRNA gene primer pairs 515F-806R, 27F-338R, 27F-533R and 784F-1061R (Zimmermann et.al. 2020) were tested in silico against all sequences within major breast milk microbiome genera in the SILVA database using the TestPrime tool (set to allow 1 mismatch outside the 30 first 5 nucleotides; https://www.arb-silva.de/search/testprime/) (Zimmermann et.al. 2020). Percentage of sequences (coverage) within each genus is reported. High and low coverage primer pairs are illustrated in green and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Figure 2.3 - Geographical distribution of breast milk microbiome studies**




**Fifty-seven studies into the breast milk microbiome and factors that influence its composition grouped by geographical region. Thirty-two studies were conducted in Europe** (Aakko et al., 2017; Biagi et al., 2017, 2018; Boix-Amorós et al., 2016, 2019; Cabrera-Rubio et al., 2012, 2016; Collado et al., 2009, 2012; Drago et al., 2017; Drell et al., 2017; Gomez-Gallego et al., 2016; Heikkilä & Saris, 2003; Hermansson et al., 2019; Jiménez et al., 2008, 2015; Jost et al., 2014; Khodayar-Pardo et al., 2014; Kumar et al., 2016; Lackey et al., 2019; Martín et al., 2007; Moles et al., 2015; Murphy et al.,

2017; Obermajer et al., 2015; Olivares et al., 2015; Pärnänen et al., 2018; M. R. Simpson et al., 2018; Soto et al., 2014; Toscano et al., 2017; Tuominen et al., 2018; Tuzun et al., 2013), **11 studies in Asia** (Albesharat et al., 2011; P.-W. Chen et al., 2018; Dahaban et al., 2013; Ding et al., 2019; Gomez-Gallego et al., 2016; Huang et al., 2019; Kumar et al., 2016; S.-W. Li et al., 2017; Patel et al., 2017; Sakwinska et al., 2016; Vaidya et al., 2017), **11 studies in North America** (Cacho et al., 2017; Davé et al., 2016b; Hunt et al., 2011; Lackey et al., 2019; Moossavi, Atakora, et al., 2019; Moossavi, Sepehri, et al., 2019; Pannaraj et al., 2017; Urbaniak et al., 2014, 2016; Ward et al., 2013; Williams et al., 2017), **6 study in Africa** (Gomez-Gallego et al., 2016; González et al., 2013; Kumar et al., 2016; Lackey et al., 2019; Meehan et al., 2018; Ojo-Okunola et al., 2018) **and 5 studies in Latin- America** (Bender et al., 2016; Carvalho-Ramos et al., 2018; Corona-Cervantes et al., 2020; Damaceno et al., 2017; Lackey et al., 2019). **Five multi-country studies included: Germany and Austria** (Soto et al., 2014)**, China, Finland, South Africa and Spain** (Gomez-Gallego et al., 2016; Kumar et al., 2016)**, China and Taiwan** (S.-W. Li et al., 2017) **, and Ethiopia, Gambia, Ghana, Kenya, Peru, US and Sweden** (Lackey et al., 2019)**. Studies have most frequently been conducted in Spain (15 studies)** (Boix-Amorós et al., 2016, 2017; Cabrera-Rubio et al., 2016; Collado et al., 2009, 2012; Gomez-Gallego et al., 2016; Jiménez et al., 2008, 2015; Khodayar-Pardo et al., 2014; Kumar et al., 2016; Lackey et al., 2019; Martín et al., 2003, 2007; Moles et al., 2015; Olivares et al., 2015).

### 2.14 References


Aakko, J., Kumar, H., Rautava, S., Wise, A., Autran, C., Bode, L., Isolauri, E., & Salminen, S. (2017). Human milk oligosaccharide categories define the microbiota composition in human colostrum. *Beneficial Microbes*, *8*(4), 563–567. Scopus. https://doi.org/10.3920/BM2016.0185

Albesharat, R., Ehrmann, M. A., Korakli, M., Yazaji, S., & Vogel, R. F. (2011). Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. *Systematic and Applied Microbiology*, *34*(2), 148–155. https://doi.org/10.1016/j.syapm.2010.12.001

Almeida, A., Mitchell, A. L., Boland, M., Forster, S. C., Gloor, G. B., Tarkowska, A., Lawley, T. D., & Finn, R. D. (2019). A new genomic blueprint of the human gut microbiota. *Nature*, *568*(7753), Article 7753. https://doi.org/10.1038/s41586-019-0965-1

Arroyo, R., Martín, V., Maldonado, A., Jiménez, E., Fernández, L., & Rodríguez, J. M. (2010). Treatment of Infectious Mastitis during Lactation: Antibiotics versus Oral Administration of Lactobacilli Isolated from Breast Milk. *Clinical Infectious Diseases*, *50*(12), 1551–1558. https://doi.org/10.1086/652763

Asnicar, F., Manara, S., Zolfo, M., Truong, D. T., Scholz, M., Armanini, F., Ferretti, P., Gorfer, V., Pedrotti, A., Tett, A., & Segata, N. (2017). Studying Vertical Microbiome Transmission from Mothers to Infants by Strain-Level Metagenomic Profiling. *mSystems*, *2*(1). https://doi.org/10.1128/mSystems.00164-16

Ballard, O., & Morrow, A. L. (2013). Human Milk Composition. *Pediatric Clinics of North America*, *60*(1), 49–74. https://doi.org/10.1016/j.pcl.2012.10.002

Bartlett, J. A., Schleifer, S. J., Demetrikopoulos, M. K., Delaney, B. R., Shiflett, S. C., & Keller, S. E. (1998). Immune Function in Healthy Adolescents. *Clinical and Diagnostic Laboratory Immunology*, *5*(1), 105–113. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC121401/

Bender, J. M., Li, F., Martelly, S., Byrt, E., Rouzier, V., Leo, M., Tobin, N., Pannaraj, P. S., Adisetiyo, H., Rollie, A., Santiskulvong, C., Wang, S., Autran, C., Bode, L., Fitzgerald, D., Kuhn, L., & Aldrovandi, G. M. (2016). Maternal HIV infection influences the microbiome of HIV-uninfected infants. *Science Translational Medicine*, *8*(349), 349ra100-349ra100. https://doi.org/10.1126/scitranslmed.aaf5103

Biagi, E., Aceti, A., Quercia, S., Beghetti, I., Rampelli, S., Turroni, S., Soverini, M., Zambrini, A. V., Faldella, G., Candela, M., Corvaglia, L., & Brigidi, P. (2018). Microbial Community Dynamics in Mother's Milk and Infant's Mouth and Gut in Moderately Preterm Infants. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.02512

Biagi, E., Quercia, S., Aceti, A., Beghetti, I., Rampelli, S., Turroni, S., Faldella, G., Candela, M., Brigidi, P., & Corvaglia, L. (2017). The Bacterial Ecosystem of Mother's Milk and Infant's Mouth and Gut. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01214

Blum, W. E. H., Zechmeister-Boltenstern, S., & Keiblinger, K. M. (2019). Does Soil Contribute to the Human Gut Microbiome? *Microorganisms*, *7*(9). https://doi.org/10.3390/microorganisms7090287

Boix-Amorós, A., Collado, M. C., & Mira, A. (2016). Relationship between Milk Microbiota, Bacterial Load, Macronutrients, and Human Cells during Lactation. *Frontiers in Microbiology*, *7*, 492. https://doi.org/10.3389/fmicb.2016.00492

Boix-Amorós, A., Martinez-Costa, C., Querol, A., Collado, M. C., & Mira, A. (2017). Multiple Approaches Detect the Presence of Fungi in Human Breastmilk Samples from Healthy Mothers. *Scientific Reports*, *7*. https://doi.org/10.1038/s41598-017-13270-x

Boix-Amorós, A., Puente-Sánchez, F., du Toit, E., Linderborg, K. M., Zhang, Y., Yang, B., Salminen, S., Isolauri, E., Tamames, J., Mira, A., & Collado, M. C. (2019). Mycobiome Profiles in Breast Milk from Healthy Women Depend on Mode of Delivery, Geographic Location, and Interaction with Bacteria. *Applied and Environmental Microbiology*, *85*(9). https://doi.org/10.1128/AEM.02994-18

Brereton, N. J. B., Gonzalez, E., Desjardins, D., Labrecque, M., & Pitre, F. E. (2020). Co-cropping with three phytoremediation crops influences rhizosphere microbiome community in contaminated soil. *The Science of the Total Environment*, *711*, 135067. https://doi.org/10.1016/j.scitotenv.2019.135067

Brevik, E. C., Slaughter, L., Singh, B. R., Steffan, J. J., Collier, D., Barnhart, P., & Pereira, P. (2020). Soil and Human Health: Current Status and Future Needs. *Air, Soil and Water Research*, *13*, 1178622120934441. https://doi.org/10.1177/1178622120934441

Browne, H. P., Forster, S. C., Anonye, B. O., Kumar, N., Neville, B. A., Stares, M. D., Goulding, D., & Lawley, T. D. (2016). Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature*, *533*(7604), Article 7604. https://doi.org/10.1038/nature17645

Browne, P. D., Aparicio, M., Alba, C., Hechler, C., Beijers, R., Rodríguez, J. M., Fernández, L., & de Weerth, C. (2019). Human Milk Microbiome and Maternal Postnatal Psychosocial Distress. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02333

Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., & Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *The American Journal of Clinical Nutrition*, *96*(3), 544–551. https://doi.org/10.3945/ajcn.112.037382

Cabrera-Rubio, R., Mira-Pascual, L., Mira, A., & Collado, M. C. (2016). Impact of mode of delivery on the milk microbiota composition of healthy women. *Journal of Developmental Origins of Health and Disease*, *7*(1), 54–60. https://doi.org/10.1017/S2040174415001397

Cacho, N. T., Harrison, N. A., Parker, L. A., Padgett, K. A., Lemas, D. J., Marcial, G. E., Li, N., Carr, L. E., Neu, J., & Lorca, G. L. (2017). Personalization of the Microbiota of Donor Human Milk with Mother's Own Milk. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01470

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), Article 5. https://doi.org/10.1038/nmeth.f.303

Carroll, L., Osman, M., Davies, D. P., & Mcneish, A. S. (1979). BACTERIOLOGY OF RAW BREAST MILK. *The Lancet*, *314*(8153), 1186. https://doi.org/10.1016/S0140-6736(79)92409-7

Carvalho-Ramos, I. I., Duarte, R. T. D., Brandt, K. G., Martinez, M. B., & Taddei, C. R. (2018). Breastfeeding increases microbial community resilience. *Jornal de Pediatria*, *94*(3), 258–267. https://doi.org/10.1016/j.jped.2017.05.013

Chan, A. A., Bashir, M., Rivas, M. N., Duvall, K., Sieling, P. A., Pieber, T. R., Vaishampayan, P. A., Love, S. M., & Lee, D. J. (2016). Characterization of the microbiome of nipple aspirate fluid of breast cancer survivors. *Scientific Reports*, *6*(1), Article 1. https://doi.org/10.1038/srep28061

Chen, P.-W., Lin, Y.-L., & Huang, M.-S. (2018). Profiles of commensal and opportunistic bacteria in human milk from healthy donors in Taiwan. *Journal of Food and Drug Analysis*, *26*(4), 1235–1244. https://doi.org/10.1016/j.jfda.2018.03.004

Clemente, J. C., Pehrsson, E. C., Blaser, M. J., Sandhu, K., Gao, Z., Wang, B., Magris, M., Hidalgo, G., Contreras, M., Noya-Alarcón, Ó., Lander, O., McDonald, J., Cox, M., Walter, J., Oh, P. L., Ruiz, J. F., Rodriguez, S., Shen, N., Song, S. J., … Dominguez-Bello, M. G. (2015). The microbiome of uncontacted Amerindians. *Science Advances*, *1*(3), e1500183. https://doi.org/10.1126/sciadv.1500183

Collado, M. C., Cernada, M., Baüerl, C., Vento, M., & Pérez-Martínez, G. (2012). Microbial ecology and host-microbiota interactions during early life stages. *Gut Microbes*, *3*(4), 352–365. https://doi.org/10.4161/gmic.21215

Collado, M. C., Delgado, S., Maldonado, A., & Rodríguez, J. M. (2009). Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. *Letters in Applied Microbiology*, *48*(5), 523–528. https://doi.org/10.1111/j.1472-765X.2009.02567.x

Collado, M. C., Isolauri, E., Laitinen, K., & Salminen, S. (2008). Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. *The American Journal of Clinical Nutrition*, *88*(4), 894–899. https://doi.org/10.1093/ajcn/88.4.894

Corona-Cervantes, K., García-González, I., Villalobos-Flores, L. E., Hernández-Quiroz, F., Piña-Escobedo, A., Hoyo-Vadillo, C., Rangel-Calvillo, M. N., & García-Mena, J. (2020). Human milk microbiota associated with early colonization of the neonatal gut in Mexican newborns. *PeerJ*, *8*, e9205. https://doi.org/10.7717/peerj.9205

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., & Knight, R. (2009). Bacterial Community Variation in Human Body Habitats Across Space and Time. *Science*, *326*(5960), 1694–1697. https://doi.org/10.1126/science.1177486

Dahaban, N. M., Romli, M. F., Roslan, N. R., Kong, S. S.-S., & Cheah, F.-C. (2013). Bacteria in Expressed Breastmilk from Mothers of Premature Infants and Maternal Hygienic Status. *Breastfeeding Medicine*, *8*(4), 422–423. https://doi.org/10.1089/bfm.2012.0109

Damaceno, Q. S., Souza, J. P., Nicoli, J. R., Paula, R. L., Assis, G. B., Figueiredo, H. C., Azevedo, V., & Martins, F. S. (2017). Evaluation of Potential Probiotics Isolated from Human Milk and

Colostrum. *Probiotics and Antimicrobial Proteins*, *9*(4), 371–379. https://doi.org/10.1007/s12602-017-9270-1

Davé, V., Street, K., Francis, S., Bradman, A., Riley, L., Eskenazi, B., & Holland, N. (2016a). Bacterial microbiome of breast milk and child saliva from low-income Mexican-American women and children. *Pediatric Research*, *79*(6), 846–854. https://doi.org/10.1038/pr.2016.9

Davé, V., Street, K., Francis, S., Bradman, A., Riley, L., Eskenazi, B., & Holland, N. (2016b). Bacterial microbiome of breast milk and child saliva from low-income Mexican-American women and children. *Pediatric Research*, *79*(6), 846–854. https://doi.org/10.1038/pr.2016.9

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563. https://doi.org/10.1038/nature12820

Ding, M., Qi, C., Yang, Z., Jiang, S., Bi, Y., Lai, J., & Sun, J. (2019). Geographical location specific composition of cultured microbiota and Lactobacillus occurrence in human breast milk in China. *Food & Function*, *10*(2), 554–564. https://doi.org/10.1039/C8FO02182A

Douglas, C. A., Ivey, K. L., Papanicolas, L. E., Best, K. P., Muhlhausler, B. S., & Rogers, G. B. (2020). DNA extraction approaches substantially influence the assessment of the human breast milk microbiome. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-019-55568-y

Drago, L., Toscano, M., De Grandi, R., Grossi, E., Padovani, E. M., & Peroni, D. G. (2017). Microbiota network and mathematic microbe mutualism in colostrum and mature milk collected in two different geographic areas: Italy versus Burundi. *The ISME Journal*, *11*(4), Article 4. https://doi.org/10.1038/ismej.2016.183

Drell, T., Štšepetova, J., Simm, J., Rull, K., Aleksejeva, A., Antson, A., Tillmann, V., Metsis, M., Sepp, E., Salumets, A., & Mändar, R. (2017). The Influence of Different Maternal Microbial Communities on the Development of Infant Gut and Oral Microbiota. *Scientific Reports*, *7*(1), 9940. https://doi.org/10.1038/s41598-017-09278-y

Dror, D. K., & Allen, L. H. (2018). Overview of Nutrients in Human Milk. *Advances in Nutrition*, *9*(suppl_1), 278S-294S. https://doi.org/10.1093/advances/nmy022

El-Mohandes, A. E., Schatz, V., Keiser, J. F., & Jackson, B. J. (1993). Bacterial contaminants of collected and frozen human milk used in an intensive care nursery. *American Journal of Infection Control*, *21*(5), 226–230. https://doi.org/10.1016/0196-6553(93)90413-X

Eriksen, K. G., Christensen, S. H., Lind, M. V., & Michaelsen, K. F. (2018). Human milk composition and infant growth. *Current Opinion in Clinical Nutrition and Metabolic Care*, *21*(3), 200–206. https://doi.org/10.1097/MCO.0000000000000466

Fernández, L., Langa, S., Martín, V., Maldonado, A., Jiménez, E., Martín, R., & Rodríguez, J. M. (2013). The human milk microbiota: Origin and potential roles in health and disease. *Pharmacological Research*, *69*(1), 1–10. https://doi.org/10.1016/j.phrs.2012.09.001

Filippo, C. D., Cavalieri, D., Paola, M. D., Ramazzotti, M., Poullet, J. B., Massart, S., Collini, S., Pieraccini, G., & Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences*, *107*(33), 14691–14696. https://doi.org/10.1073/pnas.1005963107

Fitzstevens, J. L., Smith, K. C., Hagadorn, J. I., Caimano, M. J., Matson, A. P., & Brownell, E. A. (2017). Systematic Review of the Human Milk Microbiota. *Nutrition in Clinical Practice*, *32*(3), 354–364. https://doi.org/10.1177/0884533616670150

FOX, G. E., PECHMAN, K. R., & WOESE, C. R. (1977). Comparative Cataloging of 16S Ribosomal Ribonucleic Acid: Molecular Approach to Procaryotic Systematics. *International Journal of Systematic and Evolutionary Microbiology, 27*(1), 44–57. https://doi.org/10.1099/00207713-27-1-44

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., & Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and Environmental Microbiology*, *74*(8), 2461–2470. https://doi.org/10.1128/AEM.02272-07

Gomez-Gallego, C., Garcia-Mantrana, I., Salminen, S., & Collado, M. C. (2016). The human milk microbiome and factors influencing its composition and activity. *Seminars in Fetal and Neonatal Medicine*, *21*(6), 400–405. https://doi.org/10.1016/j.siny.2016.05.003

Gómez-Gallego, C., Morales, J. M., Monleón, D., du Toit, E., Kumar, H., Linderborg, K. M., Zhang, Y., Yang, B., Isolauri, E., Salminen, S., & Collado, M. C. (2018). Human Breast Milk NMR Metabolomic Profile across Specific Geographical Locations and Its Association with the Milk Microbiota. *Nutrients*, *10*(10). https://doi.org/10.3390/nu10101355

González, R., Mandomando, I., Fumadó, V., Sacoor, C., Macete, E., Alonso, P. L., & Menendez, C. (2013). Breast Milk and Gut Microbiota in African Mothers and Infants from an Area of High HIV Prevalence. *PLOS ONE*, *8*(11), e80299. https://doi.org/10.1371/journal.pone.0080299

Gueimonde, M., Laitinen, K., Salminen, S., & Isolauri, E. (2007). Breast Milk: A Source of Bifidobacteria for Infant Gut Development and Maturation? *Neonatology*, *92*(1), 64–66. https://doi.org/10.1159/000100088

Handelsman, J. (2004). Metagenomics: Application of Genomics to Uncultured Microorganisms. *Microbiology and Molecular Biology Reviews*, *68*(4), 669–685. https://doi.org/10.1128/MMBR.68.4.669-685.2004

Heikkilä, M. P., & Saris, P. E. J. (2003). Inhibition of Staphylococcus aureus by the commensal bacteria of human milk. *Journal of Applied Microbiology*, *95*(3), 471–478. https://doi.org/10.1046/j.1365-2672.2003.02002.x

Hermansson, H., Kumar, H., Collado, M. C., Salminen, S., Isolauri, E., & Rautava, S. (2019). Breast Milk Microbiota Is Shaped by Mode of Delivery and Intrapartum Antibiotic Exposure. *Frontiers in Nutrition*, *6*. https://doi.org/10.3389/fnut.2019.00004

Hill, M. J., & Marsh, P. D. (1990). *Human Microbial Ecology*. CRC Press.

Huang, M.-S., Cheng, C.-C., Tseng, S.-Y., Lin, Y.-L., Lo, H., & Chen, P.-W. (2019). Most commensally bacterial strains in human milk of healthy mothers display multiple antibiotic resistance. *MicrobiologyOpen*, *8*(1), e00618. https://doi.org/10.1002/mbo3.618

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M. E., Beck, D. L., Abdo, Z., Fox, L. K., Williams, J. E., McGuire, M. K., & McGuire, M. A. (2011). Characterization of the Diversity and Temporal Stability of Bacterial Communities in Human Milk. *PLoS ONE*, *6*(6), e21313. https://doi.org/10.1371/journal.pone.0021313

Innis, S. M. (2014). Impact of maternal diet on human milk composition and neurological development of infants. *The American Journal of Clinical Nutrition*, *99*(3), 734S-741S. https://doi.org/10.3945/ajcn.113.072595

Jeurink, P. v., van Bergenhenegouwen, J., Jiménez, E., Knippels, L. m. j., Fernández, L., Garssen, J., Knol, J., Rodríguez, J. m., & Martín, R. (2013). Human milk: A source of more life than we imagine. *Beneficial Microbes*, *4*(1), 17–30. https://doi.org/10.3920/BM2012.0040

Jiménez, E., de Andrés, J., Manrique, M., Pareja-Tobes, P., Tobes, R., Martínez-Blanch, J. F., Codoñer, F. M., Ramón, D., Fernández, L., & Rodríguez, J. M. (2015). Metagenomic Analysis of Milk of Healthy and Mastitis-Suffering Women. *Journal of Human Lactation*, *31*(3), 406–415. https://doi.org/10.1177/0890334415585078

Jiménez, E., Delgado, S., Maldonado, A., Arroyo, R., Albújar, M., García, N., Jariod, M., Fernández, L., Gómez, A., & Rodríguez, J. M. (2008). Staphylococcus epidermidis: A differential trait of the fecal microbiota of breast-fed infants. *BMC Microbiology*, *8*, 143. https://doi.org/10.1186/1471-2180-8-143

Jiménez, E., Espinosa, I., Arroyo, R., Fernández, L., & Rodríguez, J. M. (2012). *Book of abstracts, 3rd TNO Beneficial Microbes Conferences, 26-28 March 2012*.

Jost, T., Lacroix, C., Braegger, C., & Chassard, C. (2013). Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *British Journal of Nutrition*, *110*(7), 1253–1262. https://doi.org/10.1017/S0007114513000597

Jost, T., Lacroix, C., Braegger, C. P., Rochat, F., & Chassard, C. (2014). Vertical mother-neonate transfer of maternal gut bacteria via breastfeeding: Mother-neonate bacterial transfer. *Environmental Microbiology*, *16*(9), 2891–2904. https://doi.org/10.1111/1462-2920.12238

Khodayar-Pardo, P., Mira-Pascual, L., Collado, M. C., & Martínez-Costa, C. (2014). Impact of lactation stage, gestational age and mode of delivery on breast milk microbiota. *Journal of Perinatology*, *34*(8), 599–605. https://doi.org/10.1038/jp.2014.47

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2012). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), e1. https://doi.org/10.1093/nar/gks808

Knight, R., Callewaert, C., Marotz, C., Hyde, E. R., Debelius, J. W., McDonald, D., & Sogin, M. L. (2017). The Microbiome and Human Biology. *Annual Review of Genomics and Human Genetics*, *18*(1), 65–86. https://doi.org/10.1146/annurev-genom-083115-022438

Kumar, H., du Toit, E., Kulkarni, A., Aakko, J., Linderborg, K. M., Zhang, Y., Nicol, M. P., Isolauri, E., Yang, B., Collado, M. C., & Salminen, S. (2016). Distinct Patterns in Human Milk Microbiota and Fatty Acid Profiles Across Specific Geographic Locations. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01619

Lackey, K. A., Williams, J. E., Meehan, C. L., Zachek, J. A., Benda, E. D., Price, W. J., Foster, J. A., Sellen, D. W., Kamau-Mbuthia, E. W., Kamundia, E. W., Mbugua, S., Moore, S. E., Prentice, A. M., K., D. G., Kvist, L. J., Otoo, G. E., García-Carral, C., Jiménez, E., Ruiz, L., … McGuire, M. K. (2019). What's Normal? Microbiomes in Human Milk and Infant Feces Are Related to Each Other but Vary Geographically: The INSPIRE Study. *Frontiers in Nutrition*, *6*, 45. https://doi.org/10.3389/fnut.2019.00045

Lagkouvardos, I., Overmann, J., & Clavel, T. (2017). Cultured microbes represent a substantial fraction of the human and mouse gut microbiota. *Gut Microbes*, *8*(5), 493–503. https://doi.org/10.1080/19490976.2017.1320468

LaTuga, M., Stuebe, A., & Seed, P. (2014). A Review of the Source and Function of Microbiota in Breast Milk. *Seminars in Reproductive Medicine*, *32*(01), 068–073. https://doi.org/10.1055/s-0033-1361824

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., Schlegel, M. L., Tucker, T. A., Schrenzel, M. D., Knight, R., & Gordon, J. I. (2008). Evolution of mammals and their gut microbes. *Science (New York, N.Y.)*, *320*(5883), 1647–1651. https://doi.org/10.1126/science.1155725

Li, S.-W., Watanabe, K., Hsu, C.-C., Chao, S.-H., Yang, Z.-H., Lin, Y.-J., Chen, C.-C., Cao, Y.-M., Huang, H.-C., Chang, C.-H., & Tsai, Y.-C. (2017). Bacterial Composition and Diversity in Breast Milk Samples from Mothers Living in Taiwan and Mainland China. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.00965

Lönnerdal, B. (1986). Effects of Maternal Dietary Intake on Human Milk Composition. *The Journal of Nutrition*, *116*(4), 499–513. https://doi.org/10.1093/jn/116.4.499

Martín, R., Heilig, H. G. H. J., Zoetendal, Erwin. G., Jiménez, E., Fernández, L., Smidt, H., & Rodríguez, J. M. (2007). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. *Research in Microbiology*, *158*(1), 31–37. https://doi.org/10.1016/j.resmic.2006.11.004

Martín, R., Langa, S., Reviriego, C., Jimínez, E., Marín, M. L., Xaus, J., Fernández, L., & Rodríguez, J. M. (2003). Human milk is a source of lactic acid bacteria for the infant gut. *The Journal of Pediatrics*, *143*(6), 754–758. https://doi.org/10.1016/j.jpeds.2003.09.028

Martinez-Porchas, M., Villalpando-Canchola, E., Ortiz Suarez, L. E., & Vargas-Albores, F. (2017). How conserved are the conserved 16S-rRNA regions? *PeerJ*, *5*. https://doi.org/10.7717/peerj.3036

Martín, R., Langa, S., Reviriego, C., Jiménez, E., Marín, M. L., Olivares, M., Boza, J., Jiménez, J., Fernández, L., Xaus, J., & Rodríguez, J. M. (2004). The commensal microflora of human milk: New perspectives for food bacteriotherapy and probiotics. *Trends in Food Science & Technology*, *15*(3–4), 121–127. https://doi.org/10.1016/j.tifs.2003.09.010

McGuire, M. K., & McGuire, M. A. (2017). Got bacteria? The astounding, yet not-so-surprising, microbiome of human milk. *Current Opinion in Biotechnology*, *44*, 63–68. https://doi.org/10.1016/j.copbio.2016.11.013

Mediano, P., Fernández, L., Jiménez, E., Arroyo, R., Espinosa-Martos, I., Rodríguez, J. M., & Marín, M. (2017). Microbial Diversity in Milk of Women With Mastitis: Potential Role of Coagulase-Negative Staphylococci, Viridans Group Streptococci, and Corynebacteria. *Journal of Human Lactation: Official Journal of International Lactation Consultant Association*, *33*(2), 309–318. https://doi.org/10.1177/0890334417692968

Meehan, C. L., Lackey, K. A., Hagen, E. H., Williams, J. E., Roulette, J., Helfrecht, C., McGuire, M. A., & McGuire, M. K. (2018). Social networks, cooperative breeding, and the human milk microbiome. *American Journal of Human Biology*, *30*(4), e23131. https://doi.org/10.1002/ajhb.23131

Moles, L., Escribano, E., de Andrés, J., Montes, M. T., Rodríguez, J. M., Jiménez, E., Sáenz de Pipaón, M., & Espinosa-Martos, I. (2015, February 22). *Administration of Bifidobacterium breve PS12929 and Lactobacillus salivarius PS12934, Two Strains Isolated from Human Milk, to Very Low and Extremely Low Birth Weight Preterm Infants: A Pilot Study* [Research Article]. Journal of Immunology Research; Hindawi. https://doi.org/10.1155/2015/538171

Moossavi, S., Atakora, F., Miliku, K., Sepehri, S., Robertson, B., Duan, Q. L., Becker, A. B., Mandhane, P. J., Turvey, S. E., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Subbarao, P., Field, C. J., Bode, L., Khafipour, E., & Azad, M. B. (2019). Integrated Analysis of Human Milk Microbiota With Oligosaccharides and Fatty Acids in the CHILD Cohort. *Frontiers in Nutrition*, *6*, 58. https://doi.org/10.3389/fnut.2019.00058

Moossavi, S., Sepehri, S., Robertson, B., Bode, L., Goruk, S., Field, C. J., Lix, L. M., de Souza, R. J., Becker, A. B., Mandhane, P. J., Turvey, S. E., Subbarao, P., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Khafipour, E., & Azad, M. B. (2019). Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors. *Cell Host & Microbe*, *25*(2), 324-335.e4. https://doi.org/10.1016/j.chom.2019.01.011

Mosca, F., & Giannì, M. L. (2017). Human milk: Composition and health benefits. *La Pediatria Medica E Chirurgica: Medical and Surgical Pediatrics*, *39*(2), 155. https://doi.org/10.4081/pmc.2017.155

Mueller, N. T., Bakacs, E., Combellick, J., Grigoryan, Z., & Dominguez-Bello, M. G. (2015). The infant microbiome development: Mom matters. *Trends in Molecular Medicine*, *21*(2), 109–117. https://doi.org/10.1016/j.molmed.2014.12.002

Murphy, K., Curley, D., O'Callaghan, T. F., O'Shea, C.-A., Dempsey, E. M., O'Toole, P. W., Ross, R. P., Ryan, C. A., & Stanton, C. (2017). The Composition of Human Milk and Infant Faecal Microbiota Over the First Three Months of Life: A Pilot Study. *Scientific Reports*, *7*. https://doi.org/10.1038/srep40597

Muyzer, G., de Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, *59*(3), 695–700. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC202176/

Nasioudis, D., Forney, L. J., Schneider, G. M., Gliniewicz, K., France, M., Boester, A., Sawai, M., Scholl, J., & Witkin, S. S. (2017). Influence of Pregnancy History on the Vaginal Microbiome of Pregnant Women in their First Trimester. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/s41598-017-09857-z

NIH HMP Working Group, Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., Schloss, J. A., Bonazzi, V., McEwen, J. E., Wetterstrand, K. A., Deal, C., Baker, C. C., Di Francesco, V., Howcroft, T. K., Karp, R. W., Lunsford, R. D., Wellington, C. R., Belachew, T., Wright, M., … Guyer, M. (2009). The NIH Human Microbiome Project. *Genome Research*, *19*(12), 2317–2323. https://doi.org/10.1101/gr.096651.109

Obermajer, T., Lipoglavšek, L., Tompa, G., Treven, P., Lorbeg, P. M., Matijašić, B. B., & Rogelj, I. (2015). Colostrum of Healthy Slovenian Mothers: Microbiota Composition and Bacteriocin Gene Prevalence. *PLOS ONE*, *10*(4), e0123324. https://doi.org/10.1371/journal.pone.0123324

Ojo-Okunola, A., Nicol, M., & du Toit, E. (2018). Human Breast Milk Bacteriome in Health and Disease. *Nutrients*, *10*(11). https://doi.org/10.3390/nu10111643

Olivares, M., Albrecht, S., De Palma, G., Ferrer, M. D., Castillejo, G., Schols, H. A., & Sanz, Y. (2015). Human milk composition differs in healthy mothers and mothers with celiac disease. *European Journal of Nutrition*, *54*(1), 119–128. https://doi.org/10.1007/s00394-014-0692-1

Pace, N. R., Stahl, D. A., Lane, D. J., & Olsen, G. J. (1986). The Analysis of Natural Microbial Populations by Ribosomal RNA Sequences. In K. C. Marshall (Ed.), *Advances in Microbial Ecology* (pp. 1–55). Springer US. https://doi.org/10.1007/978-1-4757-0611-6_1

Pannaraj, P. S., Li, F., Cerini, C., Bender, J. M., Yang, S., Rollie, A., Adisetiyo, H., Zabih, S., Lincez, P. J., Bittinger, K., Bailey, A., Bushman, F. D., Sleasman, J. W., & Aldrovandi, G. M. (2017). Association Between Breast Milk Bacterial Communities and Establishment and Development of the Infant Gut Microbiome. *JAMA Pediatrics*, *171*(7), 647. https://doi.org/10.1001/jamapediatrics.2017.0378

Pärnänen, K., Karkman, A., Hultman, J., Lyra, C., Bengtsson-Palme, J., Larsson, D. G. J., Rautava, S., Isolauri, E., Salminen, S., Kumar, H., Satokari, R., & Virta, M. (2018). Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. *Nature Communications*, *9*(1), Article 1. https://doi.org/10.1038/s41467-018-06393-w

Patel, S. H., Vaidya, Y. H., Patel, R. J., Pandit, R. J., Joshi, C. G., & Kunjadiya, A. P. (2017). Culture independent assessment of human milk microbial community in lactational mastitis. *Scientific Reports*, *7*. https://doi.org/10.1038/s41598-017-08451-7

Perez, P. F., Dore, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., Segura-Roggero, I., Schiffrin, E. J., & Donnet-Hughes, A. (2007). Bacterial Imprinting of the Neonatal Immune System: Lessons From Maternal Cells? *PEDIATRICS*, *119*(3), e724–e732. https://doi.org/10.1542/peds.2006-1649

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(Database issue), D590–D596. https://doi.org/10.1093/nar/gks1219

Ramsay, D. T., Kent, J. C., Owens, R. A., & Hartmann, P. E. (2004). Ultrasound Imaging of Milk Ejection in the Breast of Lactating Women. *PEDIATRICS*, *113*(2), 361–367. https://doi.org/10.1542/peds.113.2.361

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S. K., McCulle, S. L., Karlebach, S., Gorle, R., Russell, J., Tacket, C. O., Brotman, R. M., Davis, C. C., Ault, K., Peralta, L., & Forney, L. J. (2011). Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4680–4687. https://doi.org/10.1073/pnas.1002611107

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.-P., & Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology*, *2*(4), 361–367. https://doi.org/10.1038/86373

Ruiz, L., García-Carral, C., & Rodriguez, J. M. (2019). Unfolding the Human Milk Microbiome Landscape in the Omics Era. *Frontiers in Microbiology*, *10*, 1378. https://doi.org/10.3389/fmicb.2019.01378

Sakwinska, O., & Bosco, N. (2019). Host Microbe Interactions in the Lactating Mammary Gland. *Frontiers in Microbiology*, *10*, 1863. https://doi.org/10.3389/fmicb.2019.01863

Sakwinska, O., Moine, D., Delley, M., Combremont, S., Rezzonico, E., Descombes, P., Vinyes-Pares, G., Zhang, Y., Wang, P., & Thakkar, S. K. (2016). Microbiota in Breast Milk of Chinese Lactating Mothers. *PLOS ONE*, *11*(8), e0160856. https://doi.org/10.1371/journal.pone.0160856

Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., Turner, P., Parkhill, J., Loman, N. J., & Walker, A. W. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology*, *12*(1), 87. https://doi.org/10.1186/s12915-014-0087-z

Sarangi, A. N., Goel, A., & Aggarwal, R. (2019). Methods for Studying Gut Microbiota: A Primer for Physicians. *Journal of Clinical and Experimental Hepatology*, *9*(1), 62–73. https://doi.org/10.1016/j.jceh.2018.04.016

Savage, D. C. (1977). Microbial ecology of the gastrointestinal tract. *Annual Review of Microbiology*, *31*, 107–133. https://doi.org/10.1146/annurev.mi.31.100177.000543

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Horn, D. J. V., & Weber, C. F. (2009). Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541. https://doi.org/10.1128/AEM.01541-09

Schnorr, S. L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., Turroni, S., Biagi, E., Peano, C., Severgnini, M., Fiori, J., Gotti, R., De Bellis, G., Luiselli, D., Brigidi, P., Mabulla, A., Marlowe, F., Henry, A. G., & Crittenden, A. N. (2014). Gut microbiome of the Hadza hunter-gatherers. *Nature Communications*, *5*(1), Article 1. https://doi.org/10.1038/ncomms4654

Scott, M., & Koski, K. (In press). Soil-Transmitted Helminths. In *Does Nutrition Make a Difference? In: Nutrition and Infectious Diseases: Shifting the Clinical Paradigm*. Springer.

Sczyrba, A., Hofmann, P., Belmann, P., Koslicki, D., Janssen, S., Dröge, J., Gregor, I., Majda, S., Fiedler, J., Dahms, E., Bremges, A., Fritz, A., Garrido-Oter, R., Jørgensen, T. S., Shapiro, N., Blood, P. D., Gurevich, A., Bai, Y., Turaev, D., … McHardy, A. C. (2017). Critical Assessment of Metagenome Interpretation—A benchmark of metagenomics software. *Nature Methods*, *14*(11), Article 11. https://doi.org/10.1038/nmeth.4458

Seedorf, H., Griffin, N. W., Ridaura, V. K., Reyes, A., Cheng, J., Rey, F. E., Smith, M. I., Simon, G. M., Scheffrahn, R. H., Woebken, D., Spormann, A. M., Van Treuren, W., Ursell, L. K., Pirrung, M., Robbins-Pianka, A., Cantarel, B. L., Lombard, V., Henrissat, B., Knight, R., & Gordon, J. I. (2014). Bacteria from Diverse Habitats Colonize and Compete in the Mouse Gut. *Cell*, *159*(2), 253–266. https://doi.org/10.1016/j.cell.2014.09.008

Shimamura, S., Abe, F., Ishibashi, N., Miyakawa, H., Yaeshima, T., Araya, T., & Tomita, M. (1992). Relationship between oxygen sensitivity and oxygen metabolism of Bifidobacterium species. *Journal of Dairy Science*, *75*(12), 3296–3306. https://doi.org/10.3168/jds.S0022-0302(92)78105-3

Simpson, M. R., Avershina, E., Storrø, O., Johnsen, R., Rudi, K., & Øien, T. (2018). Breastfeeding-associated microbiota in human milk following supplementation with Lactobacillus rhamnosus GG, Lactobacillus acidophilus La-5, and Bifidobacterium animalis ssp. Lactis Bb-12. *Journal of Dairy Science*, *101*(2), 889–899. https://doi.org/10.3168/jds.2017-13411

Simpson, P. J., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2005). Intrinsic tolerance of Bifidobacterium species to heat and oxygen and survival following spray drying and storage. *Journal of Applied Microbiology*, *99*(3), 493–501. https://doi.org/10.1111/j.1365-2672.2005.02648.x

Soto, A., Martín, V., Jiménez, E., Mader, I., Rodríguez, J. M., & Fernández, L. (2014). Lactobacilli and Bifidobacteria in Human Breast Milk: Influence of Antibiotherapy and Other Host and Clinical Factors. *Journal of Pediatric Gastroenterology and Nutrition*, *59*(1), 78–88. https://doi.org/10.1097/MPG.0000000000000347

Suez, J., Zmora, N., Zilberman-Schapira, G., Mor, U., Dori-Bachash, M., Bashiardes, S., Zur, M., Regev-Lehavi, D., Ben-Zeev Brik, R., Federici, S., Horn, M., Cohen, Y., Moor, A. E., Zeevi, D., Korem, T., Kotler, E., Harmelin, A., Itzkovitz, S., Maharshak, N., … Elinav, E. (2018). Post-Antibiotic Gut Mucosal Microbiome Reconstitution Is Impaired by Probiotics and Improved by Autologous FMT. *Cell*, *174*(6), 1406-1423.e16. https://doi.org/10.1016/j.cell.2018.08.047

Tasnim, N., Abulizi, N., Pither, J., Hart, M. M., & Gibson, D. L. (2017). Linking the Gut Microbial Ecosystem with the Environment: Does Gut Health Depend on Where We Live? *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01935

Thum, C., Cookson, A. L., Otter, D. E., McNabb, W. C., Hodgkinson, A. J., Dyer, J., & Roy, N. C. (2012). Can Nutritional Modulation of Maternal Intestinal Microbiota Influence the Development of the Infant Gastrointestinal Tract? *The Journal of Nutrition*, *142*(11), 1921–1928. https://doi.org/10.3945/jn.112.166231

Togo, A., Dufour, J.-C., Lagier, J.-C., Dubourg, G., Raoult, D., & Million, M. (2019). Repertoire of human breast and milk microbiota: A systematic review. *Future Microbiology*, *14*, 623–641. https://doi.org/10.2217/fmb-2018-0317

Toscano, M., De Grandi, R., Peroni, D. G., Grossi, E., Facchin, V., Comberiati, P., & Drago, L. (2017). Impact of delivery mode on the colostrum microbiota composition. *BMC Microbiology*, *17*(1), 205. https://doi.org/10.1186/s12866-017-1109-0

Triantis, V., Bode, L., & van Neerven, R. J. J. (2018). Immunological Effects of Human Milk Oligosaccharides. *Frontiers in Pediatrics*, *6*, 190. https://doi.org/10.3389/fped.2018.00190

Tuominen, H., Rautava, S., Collado, M. C., Syrjänen, S., & Rautava, J. (2018). HPV infection and bacterial microbiota in breast milk and infant oral mucosa. *PLOS ONE*, *13*(11), e0207016. https://doi.org/10.1371/journal.pone.0207016

Tuzun, F., Kumral, A., Duman, N., & Ozkan, H. (2013). Breast milk jaundice: Effect of bacteria present in breast milk and infant feces. *Journal of Pediatric Gastroenterology and Nutrition*, *56*(3), 328–332. https://doi.org/10.1097/MPG.0b013e31827a964b

Urbaniak, C., Angelini, M., Gloor, G. B., & Reid, G. (2016). Human milk microbiota profiles in relation to birthing method, gestation and infant gender. *Microbiome*, *4*(1), 1. https://doi.org/10.1186/s40168-015-0145-y

Urbaniak, C., McMillan, A., Angelini, M., Gloor, G. B., Sumarah, M., Burton, J. P., & Reid, G. (2014). Effect of chemotherapy on the microbiota and metabolome of human milk, a case report. *Microbiome*, *2*(1), 24. https://doi.org/10.1186/2049-2618-2-24

Vaidya, Y. H., Patel, S. H., Patel, R. J., Pandit, R. J., Joshi, C. G., & Kunjadia, A. P. (2017). Human milk microbiome in urban and rural populations of India. *Meta Gene*, *13*, 13–22. https://doi.org/10.1016/j.mgene.2017.04.001

van den Elsen, L. W. J., Garssen, J., Burcelin, R., & Verhasselt, V. (2019). Shaping the Gut Microbiota by Breastfeeding: The Gateway to Allergy Prevention? *Frontiers in Pediatrics*, *7*. https://doi.org/10.3389/fped.2019.00047

van Nimwegen, K. J. M., van Soest, R. A., Veltman, J. A., Nelen, M. R., van der Wilt, G. J., Vissers, L. E. L. M., & Grutters, J. P. C. (2016). Is the $1000 Genome as Near as We Think? A Cost Analysis of

Next-Generation Sequencing. *Clinical Chemistry*, *62*(11), 1458–1464. https://doi.org/10.1373/clinchem.2016.258632

Vollmers, J., Wiegand, S., & Kaster, A.-K. (2017). Comparing and Evaluating Metagenome Assembly Tools from a Microbiologist's Perspective—Not Only Size Matters! *PLOS ONE*, *12*(1), e0169662. https://doi.org/10.1371/journal.pone.0169662

Wade, W. (2002). Unculturable bacteria—The uncharacterized organisms that cause oral infections. *Journal of the Royal Society of Medicine*, *95*(2), 81–83. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1279316/

Wall, D. H., Nielsen, U. N., & Six, J. (2015). Soil biodiversity and human health. *Nature*, *528*(7580), Article 7580. https://doi.org/10.1038/nature15744

Wang, S., Ryan, C. A., Boyaval, P., Dempsey, E. M., Ross, R. P., & Stanton, C. (2019). Maternal Vertical Transmission Affecting Early-life Microbiota Development. *Trends in Microbiology*, S0966842X19302082. https://doi.org/10.1016/j.tim.2019.07.010

Ward, T. L., Hosid, S., Ioshikhes, I., & Altosaar, I. (2013). Human milk metagenome: A functional capacity analysis. *BMC Microbiology*, *13*, 116. https://doi.org/10.1186/1471-2180-13-116

WHO. (2020). *Breastfeeding*. https://www.who.int/westernpacific/health-topics/breastfeeding

Williams, J. E., Carrothers, J. M., Lackey, K. A., Beatty, N. F., York, M. A., Brooker, S. L., Shafii, B., Price, W. J., Settles, M. L., McGuire, M. A., & McGuire, M. K. (2017). *Human Milk Microbial Community Structure Is Relatively Stable and Related to Variations in Macronutrient and Micronutrient Intakes in Healthy Lactating Women*. *147*(9), 1739–1748.

Woese, C. R., Gutell, R., Gupta, R., & Noller, H. F. (1983). Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. *Microbiological Reviews*, *47*(4), 621–669.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. D., & Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial

enterotypes. *Science (New York, N.Y.)*, *334*(6052), 105–108. https://doi.org/10.1126/science.1208344

Wyatt, R. G., & Mata, L. J. (1969). BACTERIA IN COLOSTRUM AND MILK OF GUATEMALAN INDIAN WOMEN *†. *Journal of Tropical Pediatrics*, *15*(4), 159–162. https://doi.org/10.1093/tropej/15.4.159

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227. https://doi.org/10.1038/nature11053

Zimmermann, P., & Curtis, N. (2020). Breast milk microbiota: A complex microbiome with multiple impacts and conditioning factors. *The Journal of Infection*, *81*(1), 17–47. https://doi.org/10.1016/j.jinf.2020.01.023

## Connecting Statement 1

The previous publication allowed us to refine the objectives and study design of the following two manuscripts that constitute Chapters 3 and 4. By expanding our knowledge on the factors that affect milk microbiome composition, we identified a knowledge gap with regards to maternal mineral intake, milk mineral composition, and infant growth. We also noted that most of the studies on milk microbiome had been conducted in developed countries, emphasizing the need for more studies in low- and middle-income countries for a broader understanding of these interactions.

Most importantly, this first publication highlighted that the methods used to analyze the milk microbiome composition shape the observed results and that there had been no comprehensive review on the matter for the milk microbiome. Therefore, it was crucial to identify a set of primers capable of capture all the expected genera in milk microbiome, including *Bifidobacterium* and *Cutibacterium*. We concluded that the 784F/1061R primer pair, targeting the V5-V6 variable regions, or the 27F YM + 3 high degeneracy variant of the typically used 27F/533R primer pair targeting the V1-V3 variable region are more suitable for human milk research.

In the next chapter, we explored the associations between maternal mineral intake, milk mineral composition, and milk microbiome diversity in an indigenous population of Guatemala. We also considered the main drivers of milk microbiome composition identified in this chapter, such as lactation stage, to better design our study for this upcoming chapter. For the milk microbiome diversity analysis, we sequenced the variable regions V5-V6 with primers P609F/P699R, that share the same characteristics and advantages as the primer pair identified in Chapter 2, as those were not available at the sequencing facility we used.

# Chapter 3: Manuscript 2

## Milk mineral composition
## is strongly associated with the human milk microbiome.

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**3.1 Abstract**

**Introduction:** Associations between maternal mineral intake, human milk mineral concentrations, and their interactions with the milk microbiota remain understudied, especially in low- and middle-income countries. To understand potential interactions and gain insight into milk composition dynamics, we explored associations of milk mineral concentrations with maternal mineral intakes and the human milk microbiome in an indigenous Guatemalan community.

**Methods:** In this cross-sectional study, milk samples were collected from 77 Mam-Mayan mothers and classified into early and established lactation. Concentrations of 9 milk minerals were analyzed, and maternal dietary intake was obtained from two 24-hour recalls. Microbiome diversity was assessed by 16S rRNA gene sequencing (V5–V6 region). DESeq2 was used for differential abundance analysis. PCA and Spearman's rank correlation explored relationships among milk minerals, maternal mineral intake, and differentially abundant microbial taxa; results with FDR-adjusted p-values < 0.1 were retained.

**Results:** Our multifactorial analysis revealed strong associations between milk minerals and the milk microbiome and weak associations with maternal intake. Several maternal intakes (Ca, Se, K, Fe, Mn) and milk mineral concentrations (Ca, Se, K, Mg, Na) were below reference values. In early lactation, milk Fe, Mn, Se, and Cu correlated with differentially abundant taxa, while in established lactation, Fe, Mn, Se, Ca, and Na were correlated. Fe and Mn accounted for 64% of bacterial associations in early lactation and 75% in established lactation. These minerals were correlated with Pseudomonadota (early), Actinomycetota (established), and Bacillota (both), but all species were unique to each stage.

**Conclusion:** Our findings reveal a complex interplay between milk minerals and the microbiome. Iron, manganese, and selenium were consistently associated with milk bacteria across lactation stages. These correlations may reflect microbial responses to mineral availability. Further longitudinal studies with larger samples are needed to clarify how this interaction influences mineral bioavailability and infant growth.

### 3.2 Introduction

Human milk is widely recognized as a complete and optimal source of nutrients for infants (Keikha et al., 2017); however, these assumptions are being challenged, especially for micronutrients, including vitamins and minerals (Erick, 2018). Indeed, recent reports document low concentrations of minerals in human milk that are insufficient to meet the full requirements of the developing infant (Daniels et al., 2019; Donohue et al., 2020; Li et al., 2016).

Prior research had focused on the links between maternal intake of vitamins and fatty acids with milk micronutrient composition (Bravi et al., 2016; Daniels et al., 2019; Dror & Allen, 2018a; Petersohn et al., 2023) resulting in the classification of milk nutrients into 2 groups, depending on their association or not with maternal diet (Allen, 1994, 2012; Erick, 2018). Group I nutrients such as thiamin, riboflavin, vitamin B-6, vitamin B-12, choline, retinol, vitamin A, vitamin D, selenium, and iodine were dependent on maternal diet. Supplementing mothers with these nutrients boosted concentrations in human milk and enhanced infant health (Donohue et al., 2020). On the other hand, Group II nutrients such as folate, calcium, iron, copper, and zinc remained largely unchanged in human milk regardless of maternal intake or status (Allen, 2012; Christian et al., 2021; Dror & Allen, 2018a). In this scenario, maternal supplementation primarily benefited the mother and was not associated with milk composition or infant outcomes (Allen, 2012; Erick, 2018). The lack of association between maternal mineral intake and milk mineral concentrations might be due to well-regulated homeostatic processes that some minerals like zinc, iron, and copper have, such as active transport mechanisms in the mammary gland (Domellöf et al., 2004). However, the available evidence does not report if the lack of association differs according to the adequacy of maternal mineral intake (Bravi et al., 2016; Dror & Allen, 2018a; Mandiá et al., 2021). It is therefore possible that the underlying milk mineral homeostatic processes would be distinct between lactating mothers experiencing chronic nutrient deficiencies relative to those with adequate nutrient intakes. The lack of association between maternal mineral intake and milk mineral concentrations supports that other determinants could influence mineral bioavailability in human milk (Barone et al., 2022).

The collection of microorganisms naturally found in human milk, the milk microbiota, could be interacting with milk minerals and bidirectionally affecting their concentration and bioavailability. Indeed, microorganisms rely on micronutrients for their growth and metabolism (Hood & Skaar, 2012; Lopez & Skaar, 2018; Murdoch & Skaar, 2022) and several studies have explored these links in the gut

(Barone et al., 2022; Fan et al., 2023). For instance, calcium intake has been associated with a higher proportion of *Clostridium* cluster XVIII in the gut microbiota (Trautvetter et al., 2018), whereas iron supplementation induced a depletion of *Bifidobacterium* and *Lactobacillus* levels in children's guts (Simonyté Sjödin et al., 2019). However, evidence of such interactions in human milk is scarce. One of the few studies exploring the association between milk minerals and milk microbiota composition reported that calcium was negatively correlated with *Streptococcus, Prevotella*, Actinomycetota, and Bacteroidota; magnesium was positively correlated with *Streptococcus* abundance; and selenium was negatively correlated with *Staphylococcus* (Sanjulián et al., 2021).

One of the recognized paths of milk microbiome colonization is the entero-mammary route (Perez et al., 2007; Rodríguez, 2014; Thum et al., 2012). Data from animal studies suggest that modulation of the maternal gut microbiota, via diet or probiotics, may influence the milk microbiota composition (Costello et al., 2009, 2012; Sindi et al., 2021; Sprockett et al., 2018). Considering the importance of human milk for infant development, a better understanding of the relationship between milk microbiome composition, milk mineral concentrations, and maternal mineral intake could guide nutritional and microbiome-based interventions in pregnant women and breastfed infants.

Considering that 92% of infants born per minute globally (Population Growth in Low vs. High Income Countries | Wilson Center, 2023) come from developing countries where nutrient deficiencies are common, both for the mother and the infant, it is essential to increase our understanding in these populations. To fill this knowledge gap, we analyzed the associations among milk mineral concentrations, maternal mineral intakes, and human milk microbiome in mothers from eight rural *Mam*-speaking indigenous communities in Guatemala. Our specific objectives were to (1) analyze the association of the maternal mineral intake with milk mineral concentrations, (2) characterize the milk microbiome composition of a group of indigenous mothers from Guatemala, and (3) analyze the associations of milk mineral concentrations and maternal mineral intake with milk microbiome composition.

### 3.3 Materials and Methods

**Study site and participants.** This cross-sectional study was part of a collaboration between McGill University and the Center for Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM) in the Republic of Guatemala. Field studies were conducted from June 2012 through January 2013 in eight rural *Mam*-speaking communities of the San Juan Ostuncalco region in Guatemala (Chomat et

al. 2015). The inclusion criteria were indigenous lactating women with infants at 1) early stage of lactation (5 - 46 days) or established stage of lactation (109 – 184 days) and who 2) had a vaginal delivery and 3) did not report any health issue (Chomat et al., 2015). The exclusion criteria were: 1) mothers with colostrum (milk < 4 days postpartum) and 2) mothers treated with antibiotics during postpartum period. We did not exclude mothers with subclinical mastitis (SCM) in order to maintain sample size; 21% (n = 16) of the total sample size had subclinical mastitis, 31.5% (n = 12) had SCM in the group of early lactation and 10% (n= 4) in the group of established lactation. We did not control for breastfeeding practices, as 77% of mothers exclusively or predominantly breastfed, from which 97% breastfed at early lactation and 56% at established lactation. Maternal age, parity and infant sex were controlled for in the analyses. The participants had low diet diversity (3.4 + 1.3), low food security (38%), and urinary and gastrointestinal infections were rare (5%). This population has been described in detail previously (Chomat et al., 2015). Ethical approval was obtained from the Institutional Review Boards of both institutions and permission was obtained from indigenous community leaders and the local authorities of the Ministry of Health. All mothers provided written informed consent for participation in the study.

**Study Design.** In this cross-sectional study, milk samples were manually collected from 77 mothers in 2012-2013, with each lactating mother contributing a single milk sample in either early or established lactation. Mothers and their milk were classified into early lactation (5-46 days postpartum) (n = 38) and established lactation (109 – 184 days postpartum) (n =39). These ranges were chosen to be consistent with our previous studies that measured infant growth (Li et al., 2016, 2019; Wren-Atilola et al., 2018). The power analysis, based on a previous study (Ajeeb et al., 2022), indicated that a minimum of 20 samples was necessary to reach a power of 0.95 (using a p-value correction of 0.001, a minimum fold change of 2, and considering a 12% loss to follow-up). This higher power threshold was selected to offset known limitations in false positive control when using DESeq2 in high-dimensional microbiome datasets and to increase the reliability of observed associations.

**Human milk sample collection.** Prior to collection, the nipple and areola of the breast were cleaned with 70% ethyl alcohol. Human milk samples were collected during a 3-hour time window in the morning from the breast not recently used for breastfeeding via full manual expression by a trained midwife, who used hand sanitizer before and after collection. This is important since other studies have shown differences in milk microbiome diversity with the use of breast pumps (Fehr et al., 2020; Marín et al., 2009; Moossavi, Sepehri, et al., 2019). Milk was collected into 60 ml plastic vials

and immediately placed on ice. Samples were partitioned into 15 ml tubes at the field laboratory, stored at -30°C prior to transfer on dry ice to McGill University where they were stored at -80°C, which is known to preserve the milk microbiome integrity (Lyons et al., 2021), until DNA extraction for microbiome analysis was performed.

**Milk mineral concentrations.** Milk concentrations of 9 minerals (Na, Ca, Cu, Fe, Mg, Mn, K, Se, and Zn) were measured as described previously (Li et al., 2016, 2018). Milk samples were thawed and thoroughly homogenized. Overnight digestion of the samples was completed in plastic DigiTUBEs (SCP Science) through use of trace metal–grade concentrated nitric acid followed by a 3-h reaction with trace metal–grade hydrogen peroxide (30%, Ultrex II, JT Baker), followed by heating at 90°C for 3 h, as previously described (Li et al., 2016). Minerals were quantified by inductively coupled mass spectrometry (ICP-MS) using a Varian ICP-820MS (Analytik Jena, Germany) equipped with a Collision Reaction Interface using PlasmaCAL Calibration Standards (SCP Science, Ref #141-110-015). Limits of detection for each of the 9 elements, measured on 8 replicates of the lowest calibration standard were: calcium 1.505 µg/L, copper 0.396 µg/L, iron 1.34 µg/L, magnesium 0.232 µg/L, manganese 0.005 µg/L, potassium 4.887 µg/L, selenium 0.007 µg/L, sodium 1.816 µg/L, and zinc 0.116 µg/L (Li et al., 2016, 2018). Milk mineral concentrations were compared to the average mineral content in human milk considered by the INCAP (Institute of Nutrition of Central America and Panama) for the Guatemalan population (Menchú et al., 2012).

**Maternal Diet Assessment**. Maternal dietary intake was based on 2 non-consecutive 24 hr recalls at either early or established lactation, administered by trained nutritionists as previously described (Chomat et al., 2015). Despite recall bias, underreporting, and difficulty in capturing complex dietary patterns (Brown, 2006), the validity of 2 non-consecutive food frequency questionnaires is similar to the validity of 1 – 3 days of recall (Resnicow et al., 2000). In addition, this approach has been used in previous research projects by CESSIAM (Chomat et al., 2015). Briefly, staff nutritionists at CESSIAM conducted 2 comprehensive quantitative non-consecutive days of 24-h recalls in Spanish or Mam in both early and late lactation. All foods and beverages were recorded and included in the analysis. We acknowledge that the source of dietary minerals (i.e., animal or plants) can affect the bioavailability of the minerals, but the source was not considered in our analysis. This population did not consume any type of supplements, but consumed fortified foods such as “Incaparina”, “Bienestarina”, and “Protemás”. Incaparina is fortified with iron, zinc, and calcium, and also contains added vitamins like vitamin A and B-complex. Bienestarina is fortified with iron, zinc,

and calcium, along with added vitamins like vitamin A and folic acid; while Protemás is fortified with iron and zinc, plus other nutrients such as vitamin A and B-complex vitamins. These fortified foods were considered in the evaluation of the maternal mineral intake. The diet data were converted into nutrients for analysis. The nutrients collected were water, energy, lipids, carbohydrates, protein, and 9 minerals (calcium, copper, iron, magnesium, manganese, potassium, selenium, sodium and zinc). The maternal mineral intakes were compared to the daily dietary recommendations by the INCAP for the Guatemalan population (Menchú et al., 2012).

**DNA extraction.** DNA extraction was done using 1ml of milk with the Quick-DNA Fungal/Bacterial Miniprep kit from Zymo Research according to the manufacturer's protocol. DNA was sent to the McGill Genome Centre for quality control, library preparation, and sequencing. Upon reception of the samples, a Picogreen test was done to measure the DNA concentration. As the concentration of the samples was very low, a speed vac was used to concentrate the DNA before resuspension in 12uL DNAse/RNAse free water. The V5-V6 region was amplified in two steps using the P609F and P699R primers. PCR amplification was performed in triplicate with controls using the NEB Phusion Taq DNA Polymerase with the following cycle conditions: initial denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 58°C for 30 s, 72°C for 30 s, and concluded by a final extension at 72°C for 5 min. The barcoding was done using the Kapa Hifi 2X ready mix with the following conditions: initial denaturation at 95°C for 3 min, followed by 12 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30s, and concluded by a final extension at 72°C for 5 min. Following amplicon amplifications, the samples that amplified were pooled and normalized. The normalization was followed by two 1X AMPure clean-up to remove primer-dimers before quantification using qPCR and LabChip. The resulting amplicons were sequenced using an Illumina MiSeq platform at the McGill Genome Centre and assembled from 300 paired-end reads; reagent controls were below the detection limit.

**Microbial Community Characterization.** The sequencing data were processed using the ANCHOR pipeline for microbial analysis (Gonzalez et al., 2019). Reads were aligned and dereplicated using Mothur (Schloss et al., 2009). Exact sequence variants (ESVs) were selected based on a count threshold of 36 across all samples. Taxonomic annotations were performed using BLASTn against multiple databases, including NCBI 16S RefSeq and NCBI nr/nt, applying criteria of >99% identity and coverage (Altschul et al., 1990; Callahan et al., 2016). In cases of multiple hits with 100% identity, NCBI 16S rRNA RefSeq was prioritized due to the high standard of curation. All annotations, particularly species calls, should be considered putative even when sharing 100% sequence identity to a single

species due to potential errors in databases (Gonzalez et al., 2019). Given the low biomass nature of these samples, contamination was controlled for at all stages: experimentally with the use of PCR controls and reagent controls (Gonzalez et al., 2019).

**Microbial Community Assessment** To visualize the microbial community composition at the phylum level, the relative abundances of exact sequence variants (ESVs) were computed from the normalized data. A custom JavaScript script utilizing the D3.js library (Bostock et al., 2011) was used to create the flower diagram representing cumulative microbial abundance. Alpha and beta diversity analyses were conducted to compare microbial diversity between early and established lactation stages. Alpha diversity was calculated using the Shannon and Simpson indices, while beta diversity was evaluated using Bray-Curtis dissimilarity. The vegan (Oksanen et al., 2020), phyloseq (McMurdie & Holmes, 2013), and ggplot2 (Wickham, 2016) packages in R were used for these calculations and visualizations. Canonical Analysis of Principal Coordinates (CAP) was employed to assess beta diversity and to visualize the separation between the two lactation stages. The significance of group separation was tested using ANOVA-like permutation tests, with p-values corrected using the Benjamini-Hochberg procedure.

**Differential Abundance Analysis.** Differential abundance of microbial taxa between early and established lactation stages was assessed using DESeq2 (Love et al., 2014). Raw read counts were imported into DESeq2, and regularized log transformation (rlog) was applied as the normalization method to stabilize variance across samples. Differentially abundant exact sequence variants (ESVs) were identified with a significance threshold set at a false discovery rate (FDR) of 0.1. This threshold was chosen due to the multiple testing context of microbiome research and in consideration of the sample size. An FDR <0.1 allows more leniency while maintaining acceptable control over false positives. For visualization of the results, the phyloseq (McMurdie & Holmes, 2013) and ggplot2 (Wickham, 2016) R packages were used. Differentially abundant taxa were represented by grouping species by phylum and ordering them according to their log fold change. A dashed red line was used to denote “infinite” log fold changes, indicating that some ESVs were detected in samples from only one group (either early or established lactation) and absent in the other.

**Multifactorial Analysis.** Principal Component Analysis (PCA) was performed to explore the relationships between milk mineral concentrations, maternal mineral intake, and the differentially abundant microbial taxa. Correlation circles (Abdi & Williams, 2010) are visual tools derived from PCA

to illustrate the relationships between different factors (milk minerals, maternal intake, and microbial taxa) by representing their correlations as angles and their importance to the principal components as their distance from the center and the edge of the circle. The edge of the correlation circle represents a perfect correlation of 1. The closer a variable is to the center of the correlation circle, the weaker its correlation with the principal components being displayed (usually PC1 and PC2), and thus, the less important it is for interpreting these components. On the other hand, the closer a variable is to the edge of the correlation circle, the more of its variance is explained by these components. The angle between the vectors of two variables on the correlation circle reflect their correlation with each other. An acute angle (< 90°) indicates a positive correlation between the two variables. An obtuse angle (> 90°) indicates a negative correlation. A right angle (90°) suggests no correlation between the two variables. Variables that are diametrically opposite on the circle are strongly negatively correlated.

The PCA was conducted using the FactoMineR package (Lê et al., 2008) in R, with the first five principal components capturing the primary sources of variation in the data. Active variables included the normalized abundances of differentially abundant exact sequence variants (ESVs). Supplementary variables were categorized as either qualitative (anthropologic information) or quantitative (milk nutrients and maternal dietary minerals). The fviz_pca_var function from the factoextra package was used to generate a correlation circle plot, visualizing the relationships and contributions of variables to the principal components.

**Correlation Analysis.** Spearman's rank correlation was used to examine the relationships among maternal dietary factors, milk mineral concentrations and differentially abundant bacterial species (ESVs) in early and established lactation samples. The Kendall rank correlation coefficient was calculated using the psych (Revelle, 2017) and corrplot (Wei & Simko, 2021) R packages. Milk nutrients and dietary minerals were standardized prior to analysis. Bacterial abundance data consisted of ESVs identified as differentially abundant. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method, and results with an adjusted p-value (FDR) < 0.1 were retained. ESVs labeled as "Unknown" were excluded from the final heatmap. The correlation matrix was visualized using the corrplot package, where the intensity of color represented the strength of the correlation, and statistical significance was indicated with asterisks.

### 3.4 Results

**Population Characteristics**

This cross-sectional study included 77 lactating mothers: 38 (49.4%) were recruited at early stage of lactation (5 – 46 days) and 39 (50.6%) at established stage (4 – 6 mo). The average maternal age was 24 yrs; BMI was 23.5 kg/m$^2$ and 70.1% of participants had a normal BMI (18.5 - 24.9 kg/m$^2$) **(Supplementary Table 3.1)**. Differences in maternal dietary intakes and milk mineral concentrations between early and established lactation are also summarized in **Supplementary Table 3.1**.

Higher maternal intakes of water, energy, macronutrients, calcium, selenium, sodium, and lower intakes of zinc were observed during early compared to established lactation. No differences were observed for dietary intake of fiber, copper, iron, magnesium, manganese, and potassium. Milk concentrations of calcium, copper, iron, potassium, selenium, sodium, and zinc were higher during early lactation, whereas magnesium and manganese concentrations in milk did not differ between early and established lactation. Few correlations emerged between maternal mineral intake and milk mineral concentrations that met the criteria for a FDR < 0.1. The few significant correlations were all negative and weak, indicating that maternal mineral intake in our study population did not strongly correlate with milk mineral concentrations (**Supplementary Figures 3.1 and 3.2).**

**Comparison of maternal mineral intakes and milk mineral concentrations with INCAP reference standards**

A comparison of maternal mineral intake with the daily dietary recommendations of INCAP for the Guatemalan population during early and established lactation is summarized in **Figure 3.1**. Considering the interquartile ranges, more than 75% of mothers had intakes of calcium, iron, manganese, and potassium during early and established lactation below INCAP recommendations whereas the majority of mothers exceeded recommendations for magnesium and zinc at both stages of lactation. We also observed that mothers during established lactation had intakes of copper, selenium, and sodium below the INCAP recommendation.

The comparison of milk mineral concentrations with the recommended INCAP mineral concentrations in human milk also uncovered differences between early and established lactation **(Figure 3.2)**. Considering the interquartile ranges, we observed that >75% of mothers had milk concentrations below INCAP references during early lactation for calcium, magnesium, and selenium, whereas during

established lactation > 75% of mothers had milk concentrations of calcium, selenium, and sodium below the INCAP references. In contrast, milk concentrations of copper, manganese, and zinc at both stages of lactation exceeded the INCAP references (**Figure 3.2)**.

**Milk microbiome composition during lactation**

A total of 2,121 ESVs were assembled and captured 6,539,504 sequence reads across all 77 human milk samples**.** From those 2,121 ESVs we were able to annotate 1,419 ESVs within 402 genera and 165 bacterial families. The remaining 702 ESVs could not be recognized as 99% similar (in both identity and coverage) to any known taxa and were labeled as "unknown". However, these ESVs were retained in the statistical analyses to ensure a comprehensive assessment of microbiome composition. Notably, a subset of these 'unknown' taxa that were found to be significant in our differential abundance analyses were subsequently identified (100% identity) using BLASTn against a broader reference database. To ensure robust and reliable microbiome comparisons, we used regularized log (RLOG) transformation for normalization. This approach was chosen for its ability to stabilize variance across different abundance levels. To validate the normalization process, we generated abundance vs. variance plots, which confirmed the absence of stochastic effects and ensured appropriate variance stabilization. As contamination is a known challenge in microbiome studies, we included negative (blank) controls during sample processing to monitor potential contaminants.

Of the 1,419 ESVs annotated as putative species, the average BLASTn return identity was 99.9%, including 1,147 perfect hits (100% identity). The most abundant phyla were Pseudomonadota*,* Bacillota*,* Actinomycetota*,* and Bacteroidota **(Figure 3.3)**. The most abundant ESVs across all samples were *Pseudomonas spp; Staphylococcus hominis_2;* and *Streptococcus_MS_12* (which could represent one or more of the species *S. oralis, S. mitis,* or *S. tigurinus)* making up 25.91%, 12.37%, and 8.06% of the microbial community, respectively.

The estimation of within (alpha) and between (beta) sample diversity in these milk samples is shown in **Figure 3.4** and **Figure 3.5**, respectively**.** Microbial diversity indices, such as Shannon (FDR = 0.01), and Simpson (FDR = 0.008) identified significant differences between early and established lactation **(Figure 3.4)**; the indices Observed and Chao1 were not significant. Beta-diversity analyses segregated both lactation stages, along the Canonical Correspondence Analysis (CCA) ($p$ = 1.0E-04), Redundancy

Analysis (RDA) ($p$ = 2.0E-04) and Canonical Analysis of Principal Coordinates (CAP) ($p$ = 1.0E-04) ordinations **(Figure 3.5 and Supplementary Figure 3.3**).

**Microbial Differential Abundance Analyses (DA) between Early and Established Lactation**

Differential abundance analysis using DESeq2 identified taxa which were significantly different in relative abundance between early and established lactation. In total, 120 ESVs were identified as significantly differentially abundant, including 50 which were in higher relative abundance in early lactation and the remaining 70 higher in established lactation **(Figure 3.6).** These could be annotated at various taxonomic levels, spanning 37 species, 26 genera, 26 families, as well as 61 which could not be determined (labelled as "Unknown"). Bacteria from the Bacillota phylum, a common abundant gut bacterial phylum, were similarly present in early (8 ESVs) and established lactation (13 ESVs). Early lactation was dominated by members of the Pseudomonadota phylum (17 ESVs in early lactation vs 6 ESVs in established lactation). In contrast, taxa from the Actinomycetota (3 ESVs vs 1 ESVs), Bacteroidota (9 ESVs vs 2 ESVs), and Fusobacteriota (1 ESVs vs 0 ESVs) phyla dominated at established lactation.

The ESVs identified as having significantly higher relative abundance in early lactation and that had the highest fold change (FC) were *Acinetobacter spp.* (FC = -6.1)*,* and *Chryseobacterium spp.* (FC = -10.3), while at established lactation were *Granulicatella elegans* (FC = 7.5) and *Leptotrichia_8* (FC = 7). The other ESVs shown in the figure were also significant but had smaller fold changes **(Figure 3.6)**.

**Multifactorial Associations Among Maternal Diet, Milk Mineral Composition, and the Milk Microbiome**

The multifactorial analysis between milk mineral concentrations, maternal mineral intake, and milk microbiome composition shows weak associations between maternal mineral intake and milk mineral concentrations, whereas milk mineral concentrations were negatively associated with milk bacterial diversity **(Figure 3.7)**. Milk manganese and milk magnesium are in the same direction and close to the ESVs vectors, meaning that these milk minerals are positively correlated with the milk microbiome.

Given the mineral and microbial differences between early and established lactation (**Figure 3.5** and **Figure 3.6)**, we proceeded to explore associations between maternal diet, milk mineral composition,

and milk microbial diversity within each stage of lactation in **Figures 3.8 and 3.9**. The heatmap in **Figure 3.8** shows the bivariate correlations between the differentially abundant ESVs with milk minerals and maternal mineral intake at early lactation. The milk minerals with more correlations in descending order were iron (4), manganese (3), copper (3), and selenium (1). Iron and manganese accounted for 64% of all the associations. Iron in the milk was negatively correlated with *Acinetobacter johnsonii, Acinetobacter spp., Pseudomonas fluorescens,* and *Pseudomonas spp.* Milk manganese was negatively correlated with *Ewingella americana* and positively correlated with *Staphylococcus hominis* and *Staphylococcus spp.* Milk copper was also negatively correlated with *Acinetobacter spp.* and *Pseudomonas spp,* while it was positively correlated with *Bacillus pumilus.* Finally, milk selenium was positively correlated with *Staphylococcus caprae.* In relation to maternal mineral intake, only dietary selenium was positively correlated with *E. americana,* and dietary zinc was positively correlated with *S. hominis.*

The heatmap in **Figure 3.9** shows the correlations between the differentially abundant ESVs with milk minerals and maternal mineral intake at established lactation. The milk minerals with more correlations in descending order were manganese (8), iron (7), calcium (2), sodium (2), and selenium (1). All these correlations were positive. Iron and manganese accounted for 75% of all the associations. Some taxa were correlated with more than one mineral. For instance, *Amaricoccus spp.* and *Garnderella spp.* were correlated with milk calcium, iron, and sodium; *Luteococcus peritonei, Planococcus chinesis, Pseudarthrobacter polychromogenes,* and *Streptococcus spp.* were correlated with milk iron and manganese. Finally, *Turicibacter bilis* was correlated with milk manganese and selenium. Regarding the maternal mineral intakes, dietary calcium was positively correlated with *Rothia terrae* and *Veillonella spp.*, dietary sodium was positively correlated with *Enterobacter spp*, dietary magnesium and copper were positively correlated with *Enterobacter asburiae* and dietary iron, magnesium, potassium, copper, and manganese were negatively correlated with *Gardnerella spp.* Moreover, *Bifidobacteria* and *Lactobacillus* were not significantly associated with any milk mineral, nor maternal mineral intake at both lactation stages.

### 3.5 Discussion

To the best of our knowledge the current study is the first to analyze collectively associations between concentrations of milk minerals and maternal mineral intakes with the human milk microbiome in an Indigenous population of Guatemala. The primer selection (V5-V6) used in this study allowed us to

capture a wide range of bacteria, including *Bifidobacteria,* which are typically not captured when using other primers and regions such as V3-V4 (Lopez Leyva et al., 2020). Our population also provided a unique opportunity to analyze the milk microbiome, as many of the factors known to affect its composition are homogeneous in the *Mam*-Mayan mothers, including exclusive or partial breastfeeding for >6 months (Lopez Leyva et al., 2022), the absence of therapeutic use of antibiotics, and manual milk extraction rather than the use of a breast pump (Fehr et al., 2020; Marín et al., 2009; Moossavi, Sepehri, et al., 2019).

Our multifactorial analysis among maternal diet, milk mineral concentrations, and the milk microbiome identified strong associations of milk mineral concentrations with the milk microbiome. All associations reported are exploratory in nature and do not imply causation. Early lactation was dominated by Pseudomonadota taxa, whereas Actinomycetota, Bacteriodota, and Fusobacteriota dominated at established lactation. The Pseudomonadota phylum is known to contain several pathogens and putative pathobionts (Rizzatti et al., 2017) and Actinomycetota, Bacteriodota, and Fusobacteriota contribute to various physiological functions, including digestion and immune modulation (Yersin & Vonaesch, 2024). In early lactation, milk iron, manganese, copper, and selenium were correlated with the differentially abundant taxa while in established lactation, milk iron, manganese, selenium, calcium, and sodium were correlated with milk bacteria. Only iron, manganese and selenium were associated with milk bacteria at both stages of lactation, and iron and manganese had the greatest number of associations with differentially abundant ESVs. These minerals were correlated with members of the phyla Pseudomonadota (Fe and Mn) in early lactation, with Actinomycetota (Fe and Mn) in established lactation and with Bacillota in both stages of lactation (Mn and Se).

**Associations of maternal diet with milk mineral concentrations**

Previous research had focused on the maternal intake of fatty acids and vitamins and their association with human milk composition (Bravi et al., 2016; Petersohn et al., 2023), but evidence regarding milk minerals is limited and generally considered less related to maternal diet (Bravi et al., 2016; Samuel et al., 2020). Previous studies had reported correlations between maternal intake and milk concentrations for selenium (Dror & Allen, 2018a), zinc (Ortega et al., 1997), calcium (Gibson et al., 2020), and iron (Daniels et al., 2019; Gibson et al., 2020). In contrast, other studies had not observed significant associations between maternal mineral intakes and milk concentrations for zinc

(Aumeistere et al., 2018; Daniels et al., 2019), calcium (Daniels et al., 2019), and potassium (Daniels et al., 2019; Gibson et al., 2020), which aligns with our data. In our study, the observation that all significant correlations between maternal mineral intakes and milk mineral concentrations were negative and weak is intriguing. Other studies in low-socioeconomic communities have also observed inverse associations between milk concentrations and maternal diet for zinc (Bates & Tsuchiya, 1990; Domellöf et al., 2004; Gibson et al., 2020; Sian et al., 2002) suggesting that during maternal deficiencies there is mobilization and transport of the mineral to the milk (Geissler et al., 1978; Gibson et al., 2020; Krebs, 1998) Importantly, mothers in our study had inadequate intakes of several minerals based on dietary INCAP requirements. With regards to INCAP's suggested milk mineral concentrations, there was evidence of both inadequate and adequate milk concentrations in mothers' milk during both early and established lactation, suggesting weak correlations between intakes and milk concentration. Our correlation circle and heat maps further supported our observation that milk mineral concentrations underscored changes in the milk microbiome in contrast to maternal dietary intakes in mothers experiencing multiple micronutrient deficiencies during both early and established lactation.

Others have suggested that bioavailability of minerals from food sources may contribute to the negative and weak correlations between maternal mineral intake and milk mineral concentrations. (Fairweather-Tait & Hurrell, 1996). The *Mam*-Mayan community follows the "three sisters" diet, a diet based on corn, squash and beans, where most of their food sources are plant sources rather than animal sources and is based on starchy staples (mostly maize), legumes where only one third consumed flesh foods (mostly chicken or beef), and dark green leafy vegetables (Chomat et al., 2015). In our analysis, we did not consider the source of the maternal mineral intake (i.e., from animal or plant sources), which can affect the bioavailability of minerals. Therefore, the limited mineral bioavailability, due to plant foods as the main source of nutrients and the demonstrated inadequate intakes for multiple minerals, may be linked to to widespread low availability and inadequate intakes of minerals and trace elements.

The weak correlations of maternal sodium and potassium at early lactation, and maternal copper and selenium at established lactation with milk mineral concentrations observed in all mothers could be associated with homeostatic processes, such as active transport mechanisms in the mammary gland. These have been described for iron and copper (Domellöf et al., 2004; Samuel et al., 2020), suggesting

that regardless of the maternal mineral intake, the milk mineral concentrations remain independent from the maternal diet due to these homeostatic processes. Other factors could influence more milk mineral concentrations than maternal diet, such as lactation stage, maternal and infant age, parity, maternal physiological status (e.g. bone metabolism, renal function, hormonal regulation), maternal chronic diseases (e.g. diabetes, thyroid disorders), infections, geographic location (e.g. soil composition) and maternal genetic variants (Dror & Allen, 2018a; Mandiá et al., 2021).

**Associations of maternal diet with milk microbiome composition**

As for maternal diet and milk microbiome, some associations have been reported with macronutrients, fiber, poly- and mono-unsaturated fatty acids and vitamins (Ajeeb et al., 2024; Babakobi et al., 2020; Cortes-Macías et al., 2021; LeMay-Nedjelski et al., 2021; Padilha et al., 2019; Sindi et al., 2021; Williams et al., 2017). Yet, our results show no direct association between maternal mineral intake and milk microbiome; only dietary selenium was positively correlated with *E. americana,* and dietary zinc was positively correlated with *S. hominis*. Moreover, it is possible that statements about a strong link of maternal diet with milk microbiome might apply to other nutrients, but not minerals.

We found that the mineral intake below the reference values for iron, manganese, calcium, selenium, and potassium in our population highlighted a milk ecosystem with multiple co-existing deficiencies in minerals and trace elements. Previous studies reporting associations between maternal diet and milk microbiome had not described dietary adequacy (Cortes-Macías et al., 2021; LeMay-Nedjelski et al., 2021; Padilha et al., 2019); with the exception of one study , which focused on fatty acids and vitamins in well-nourished lactating women (Williams et al., 2017). Further research is needed regarding the differences in milk microbiota and micronutrient intakes in communities already experiencing multiple micronutrient deficiencies.

**Associations of milk mineral concentrations with milk microbiome**

Milk mineral concentrations and milk microbiome composition are dynamic and vary throughout lactation with the nutritional composition of human milk linked to the composition of the milk microbiota (Moossavi, Atakora, et al., 2019; Sanjulián et al., 2021). Only one study had analyzed the association between milk minerals and milk bacteria, but had not considered the difference between early and established lactation (Sanjulián et al., 2021). Sanjulián et al. observed that calcium was

negatively correlated with *Streptococcus, Prevotella,* Actinomycetota, and Bacteroidota; magnesium positively correlated with *Streptococcus* abundance; and selenium negatively correlated with *Staphylococcus.* In contrast, we observed a positive correlation of calcium with *Amaricoccus spp* and *Gardnerella spp* at established lactation, no significant correlations with magnesium, and selenium was positively correlated with *Staphylococcus caprae* at early lactation and with *Turicibacter bilis* at established lactation. In the gut microbiome, it has been emphasized that levels of mineral intake must be tightly regulated to prevent colonization of gut pathogens while maintaining a healthy immune response to invading bacteria (Murdoch & Skaar, 2022). This tight regulation may also be relevant in the mammary gland to prevent the colonization of microbial pathogens in the milk.

**Individual Minerals and Trace Elements Associated with the Milk Microbiome.**

In our study we uncovered 3 minerals associated with the milk microbiome in both early and established lactation: iron, manganese, and selenium. We further detail these associations below, as iron and manganese have been associated with nutritional immunity (Hood & Skaar, 2012; Lopez & Skaar, 2018; Murdoch & Skaar, 2022; Palmer & Skaar, 2016; Sheldon & Skaar, 2019). Nutritional immunity is a process by which the host reduces mineral availability to the bacteria, intended to inhibit bacterial growth (Murdoch & Skaar, 2022). This process has been reported in the gut for iron, manganese, zinc and copper (Hood & Skaar, 2012; Lopez & Skaar, 2018; Murdoch & Skaar, 2022; Palmer & Skaar, 2016; Sheldon & Skaar, 2019).

Iron: In the human body, iron is essential for oxygen transport, DNA metabolism, and mitochondrial function (Seyoum et al., 2021). For bacteria, iron is also a co-factor in iron-containing proteins in redox reactions, metabolic pathways, and electron transport chain mechanisms (Seyoum et al., 2021). In early lactation, iron was correlated with species from the Pseudomonadota phylum (in particular *Pseudomonas),* while in established lactation it was correlated with Bacillota and Actinomycetota. Previous studies have not associated milk iron with *Pseudomonas* but there is evidence of iron affinity in some *Pseudomonas* species, such as the opportunistic species *P*. *aeruginosa* that has a high capacity to acquire iron and use it as a virulence determinant (Sánchez-Jiménez et al., 2023; Schalk & Perraud, 2023). This iron affinity might be shared with other taxa in the Pseudomonadota phylum, which could explain its several associations with iron in this study. Other potential pathogens like *E. coli, Salmonella,* and *Shigella* are also siderophilic species, while beneficial bacteria such as *Bifidobacteria* or *Lactobacillus* have low iron requirements (Puga et al., 2022). Our analyses support

negative correlations between *Bifidobacteria* and *Lactobacilli* and milk iron concentrations, and the slightly higher mean concentration of milk iron could explain the low abundance of these bacterial taxa in our milk samples (Ballini et al., 2019; Bergillos-Meca et al., 2015). Importantly, Pseudomonadota are the first gut colonizers, helping the gut reach a full anaerobic status (Bäckhed et al., 2015; Gómez-Martín et al., 2022). Thus, iron in human milk may play a role in facilitating gut early colonization by Pseudomonadota, and may have implications for infant growth and nutritional status through alterations in the gut microbiome (Puga et al., 2022).

Manganese: In our study, manganese was correlated with species of the Bacillota (*Staphylococcus hominis* and *Staphylococcus spp.)* and Pseudomonadota (*Ewingella americana*) phyla in early lactation, and in established lactation it continued to be correlated with Bacillota (*Streptococcus, Planococcus chinensis* and *Turicibacter bilis)* but also with Bacteroidota (*Prevotella spp.* and *Prevotella jejuni)* and Actinomycetota (*Luteococcus peritonei* and *Pseudarthrobacter polychromogenes*). Manganese is an essential nutrient for intracellular activities in the human; it functions as a cofactor for a variety of enzymes involved in development, digestion, reproduction, antioxidant defense, energy production, immune response and regulation of neuronal activities (Chen P. et al., 2018). However, at elevated levels, manganese can have neurotoxic effects (Martinez-Morata et al., 2023). Evidence on its association with bacteria is scarce but, in the gut, manganese has been associated with Bacillota and Bacteroidota (Chi et al., 2017), which aligns with our findings. Manganese has also been associated with immune function, bacterial survival, and a capacity to promote virulence of pathogens (Lopez & Skaar, 2018; Pajarillo et al., 2021). In the gut, deficiencies in dietary manganese have been linked to increased risk of infection by *Staphylococcus aureus* (Murdoch & Skaar, 2022). In cows, it has been observed that manganese is needed by *S. uberis* (Smith A. J. et al., 2003) and *Lactobacillus rhamnosus* (Nugroho et al., 2022) to grow. Manganese seems to stop acidification within 1 day of nongrowing cells by affecting the lactococcal acidification rate (Nugroho et al., 2022). Similarly, also in cows, there is evidence that the supplementation of manganese decreased the relative abundance of *Treponema* within phylum *Spirochaetes* (Faulkner et al., 2017). Although none of these species were observed in our results, the evidence in cow's milk shows that manganese interacts with bacteria and it is necessary for the growth of certain species, which might occur in human milk. However further research is needed to understand their specific role in milk microbiome and analyze if the observations in the gut microbiome and in animals are similar in human milk.

Iron and manganese: We report that milk iron and manganese together made 64% and 75% of the correlations with bacterial community structure at early and established lactation, respectively, although these minerals were not reported in previous studies. Interestingly, in the gut, iron and manganese can influence each other's absorption as both metals use the same transporters (Hansen et al., 2009; Pajarillo et al., 2021; Shannon & Hill, 2019). For example, in the presence of *Mycobacterium tuberculosis* and *Salmonella enterica,* the host reduces mineral availability by redirecting storage of cellular iron, manganese, and magnesium from the phagolysosome to the cytoplasm. Although not investigated in milk, this nutritional immunity could be operational in milk specifically with iron and manganese, as they are the minerals with the most associations with bacteria at early and established lactation.

Selenium: Previous research in milk microbiome reported that selenium was negatively correlated with *Staphylococcus* (Sanjulián et al., 2021), which partially aligns with our findings, as selenium correlated positively with *S. caprae*. A recent study in rats deficient in selenium reported higher levels of Bacillota, although at genus level they did not identify *Staphylococcus* (Wu 2023). A similar association could be happening in our samples, as selenium was below the INCAP reference values at both stages of lactation and it was positively correlated with *S. caprae* at early lactation and with *Turicibacter bilis* at established lactation, both members of the Bacillota phylum. There is evidence in this same population that SCM is associated with higher milk selenium (Li et al., 2018). Selenium was one of the minerals that showed associations with milk microbiome in both stages of lactation so possibly SCM is a factor interfering in this association and further research that evaluates the association between milk selenium and milk bacteria in SCM and non-SCM mothers is needed. Selenium is an essential micronutrient for humans, as it is a component of selenoproteins. Selenoproteins are important for thyroid hormone metabolism, several immune system antioxidant defense processes (e.g., glutathione peroxidase), and for the intracellular control of oxidation and reduction reactions (Martinez-Morata et al., 2023). Importantly, an excessive exposure to selenium can have adverse effects on health (Martinez-Morata et al., 2023). It has been further reported that selenium has inhibitory effects on Staphylococci growth, as the supplementation of selenium inhibits the growth of *S. aureus* in bovine milk. As selenium is essential for the normal function of the immune system, it has also been suggested that mothers with higher levels of milk selenium could have an immune system more capable of reducing levels of *Staphylococcus* (Sanjulián et al., 2021). Similarly,

there is evidence that selenium supplementation increases the diversity and richness of gut microbiota (Arias-Borrego et al., 2023), including health-relevant taxa, which highlights its importance in milk microbiome. Of note, it has been reported that ¼ of all bacteria have selenoproteins, so they require selenium for their growth and metabolism (Kasaikina et al., 2011). This could explain why this was one of the minerals that mainly correlated with milk bacteria at both stages of lactation. In fact, a study evaluated *in vivo* the nutritional availability of selenometabolites and concluded that gut microbiota might contribute to the selenium nutritional availability (Takahashi et al., 2017), which again might also happen in the milk microbiome.

We recognize this study had certain limitations. A potential weakness of the dataset was the necessary inclusion of mothers with subclinical mastitis (SCM) to increase sample size, as the presence of SCM can affect the milk mineral concentrations (Li et al., 2018). Our mothers were not supplemented with any micronutrients, but they consumed fortified foods which could modify the milk mineral concentrations. We acknowledge the value of more advanced models for future research, that should monitor larger cohorts of lactating mothers over time and include more detailed dietary intake data, to explore the independent contribution of each variable to milk mineral concentration and milk microbial diversity using more advanced statistical modeling. The transferability of our results is limited due to the unique characteristics of the *Mam*-Mayan population, including their traditional diet, environmental exposures, and cultural practices, previously described in detail (Chomat et al., 2015; Lopez Leyva et al., 2020; Wren et al., 2015). However, our findings may still be valuable to other indigenous communities with similar dietary patterns, lifestyle factors, and socioeconomic conditions. Additionally, populations experiencing nutrient deficiencies or residing in low- and middle-income countries (LMICs) with comparable nutritional and environmental challenges may also benefit from these insights. Future research involving diverse populations could help determine the extent to which these associations hold across different settings.

**3.6 Conclusion**

In summary, our findings reveal a complex and dynamic interplay between milk minerals and the milk microbiome at early and established lactation. Results revealed a milk ecosystem where concentrations in milk minerals and trace elements were differentially associated with the milk microbiome. Only milk iron, manganese, and selenium were associated with milk bacteria in both stages of lactation including members of the phyla Pseudomonadota with Fe and Mn in early lactation,

Actinomycetota with Fe and Mn in established lactation, and Bacillota in both stages of lactation with Mn and Se. These bidirectional microbial correlations with milk minerals revealed that certain bacteria may thrive or be inhibited depending on the presence of specific minerals during early and established lactation.

Subsequent research will be required to better understand causation and the mechanisms by which milk minerals and milk microbiome interact and if these interactions are the same in nutrient-deficient and nutrient-adequate mothers. These future investigations should include milk volume in their analyses, distinguish between the food sources (animal or plant foods), and consider the timing between maternal meals and the milk sample collection. Additionally, future studies should explore if these modifications between milk minerals, trace elements and the milk microbiome might be linked to the high rates of growth faltering in breastfed infants in Guatemala and to impaired growth in other LMIC countries.

## 3.7 Figures

**Figure 3.1 – Maternal mineral intake at early and established lactation**

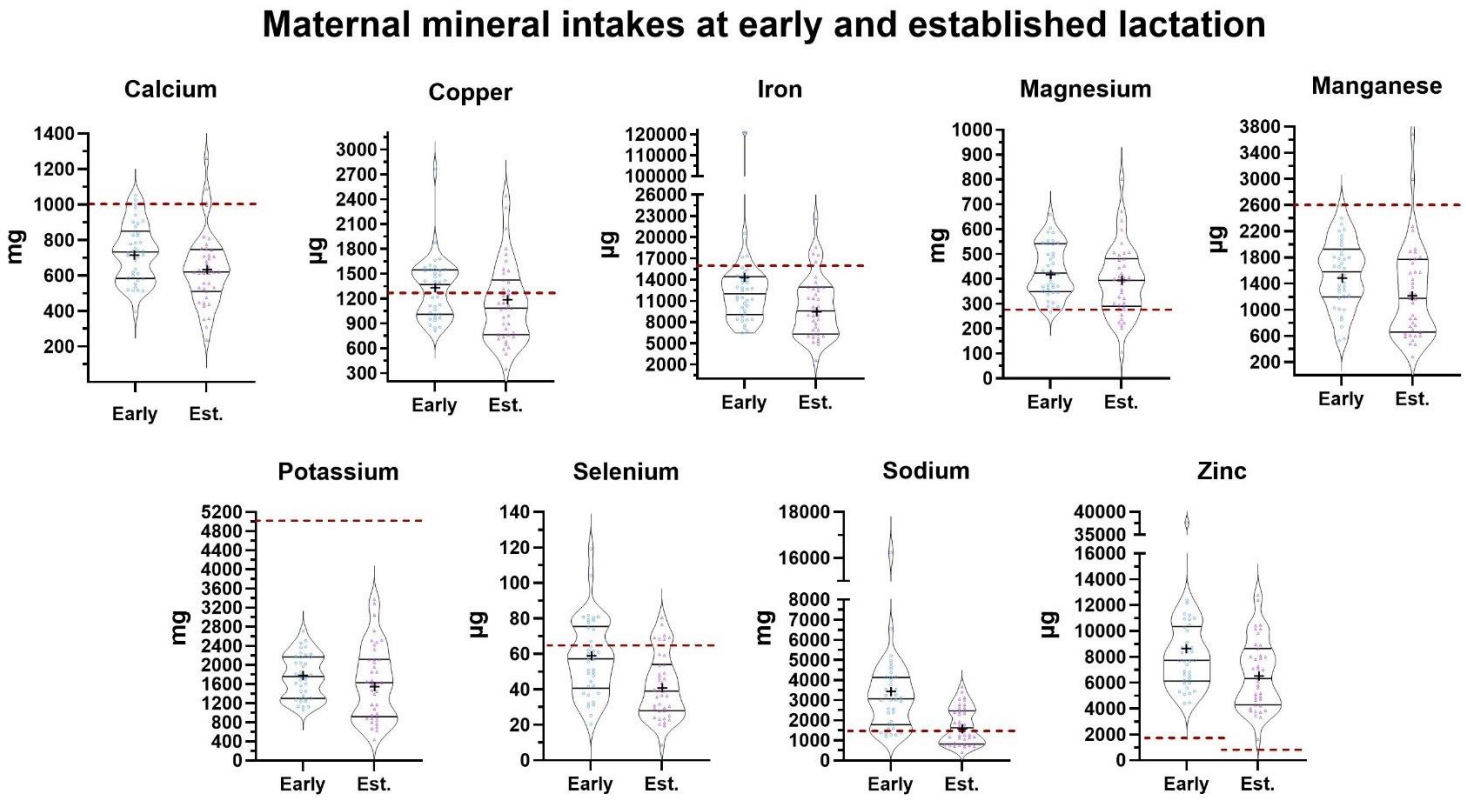



**Figure 3.1 – Maternal mineral intake at early and established lactation.** Each maternal mineral intake is shown at early (blue) and established (purple) lactation. In each violin plot, the middle line shows the median and all the points represent the spread of the maternal mineral intakes. The length between the top and bottom line represents the interquartile range (IQR, 75% and 25%). The width of the plot represents a more frequent value where it is wider and a less frequent value where it is narrower. The bottom line in the violin plot represents the first quartile and the top line represents the third quartile. The mean is shown as (+). The daily dietary recommendations by the INCAP for the Guatemalan population are shown as a reference in a dashed red line. The reference values for each mineral are the following: (Ca: 1000 mg, Cu: 1300 µg, Fe: 15600 µg, Mg: 275 mg, Mn: 2.6 µg, K: 5100 mg, Se: 65 µg, Na: 1500 mg, Zn: 1700 µg. (Menchú et al., 2012).

**Figure 3.2 – Milk mineral concentrations at early and established lactation.**

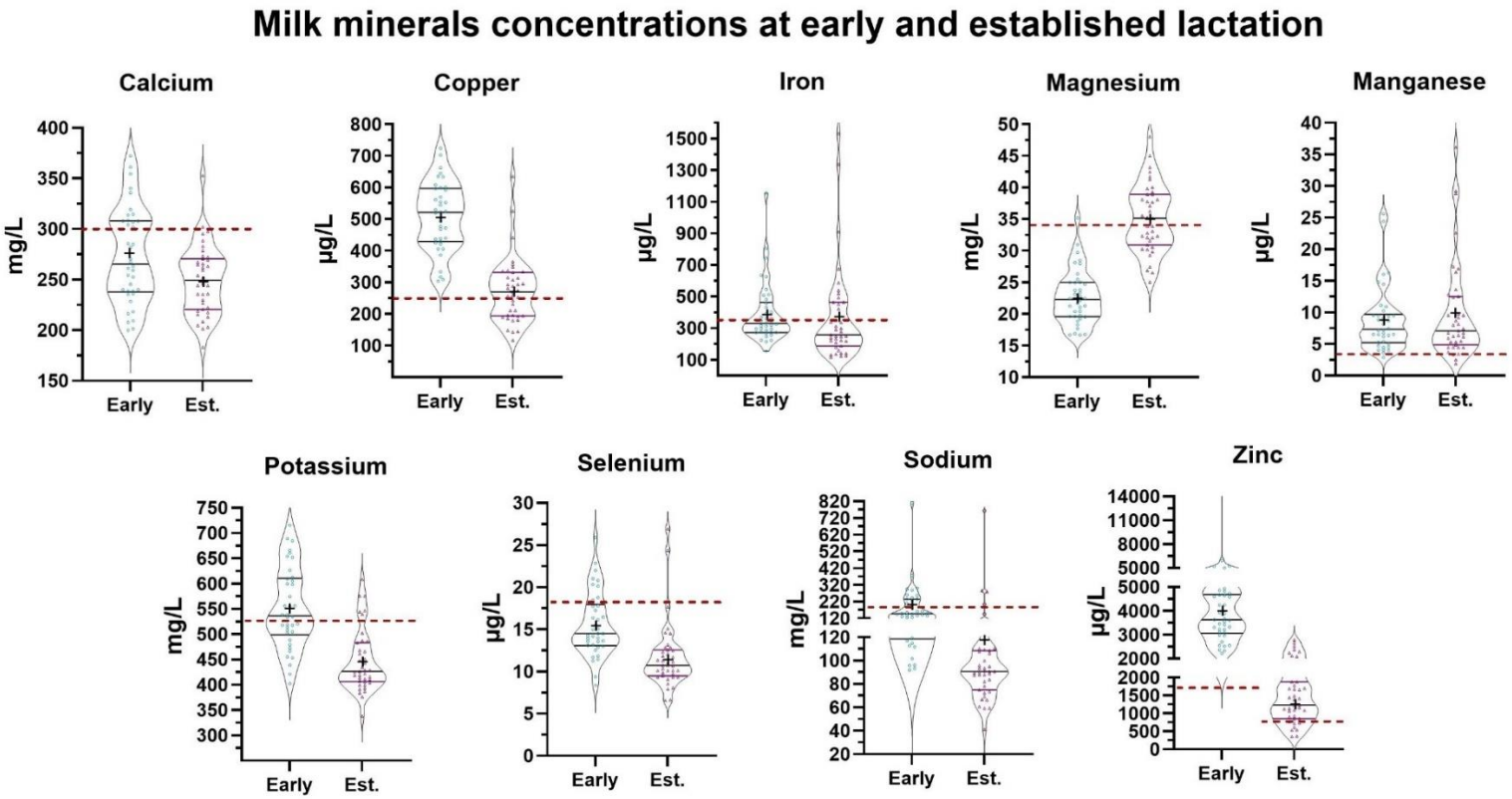



**Figure 3.2 – Milk mineral concentrations at early and established lactation.** Each milk mineral concentration is shown at early (blue) and established (purple) lactation. In each violin plot, the middle line shows the median and all the points represent the spread of the maternal mineral intakes. The length between the top and bottom line represents the interquartile range (IQR, 75% and 25%). The width of the plot represents a more frequent value where it is wider and a less frequent value where it is narrower. The bottom line in the violin plot represents the first quartile and the top line represents the third quartile. The mean is shown as (+). The average mineral content in human milk considered by the INCAP for the Guatemalan population is shown as a reference in a dashed red line. The reference values for each mineral are the following: Ca: 300 mg/L, Cu: 250 µg/L, Fe: 350 µg/L, Mg: 34 mg/L, Mn: 3 µg/L, K: 525 mg/L, Se: 18 µg/L, Na: 160 mg/L, Zn Early: 1750 µg/L, Zn Established: 700 µg/L. (Menchú et al., 2012).

**Figure 3.3 - Human milk microbiome composition**

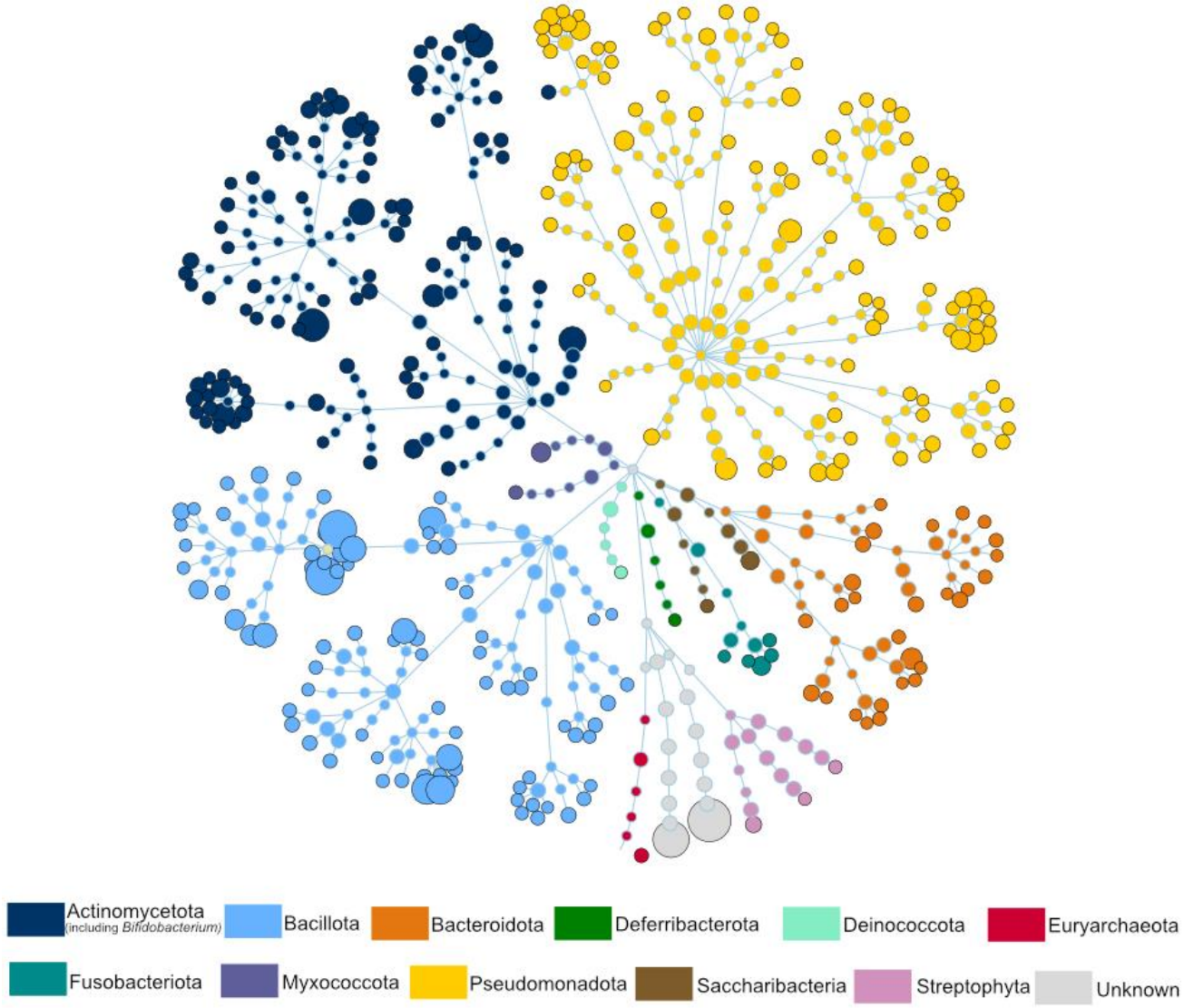



**Figure 3.3 - Human milk microbiome composition.** The total microbial community colored at phylum level of all the samples.

**Figure 3.4 – Alpha Diversity**

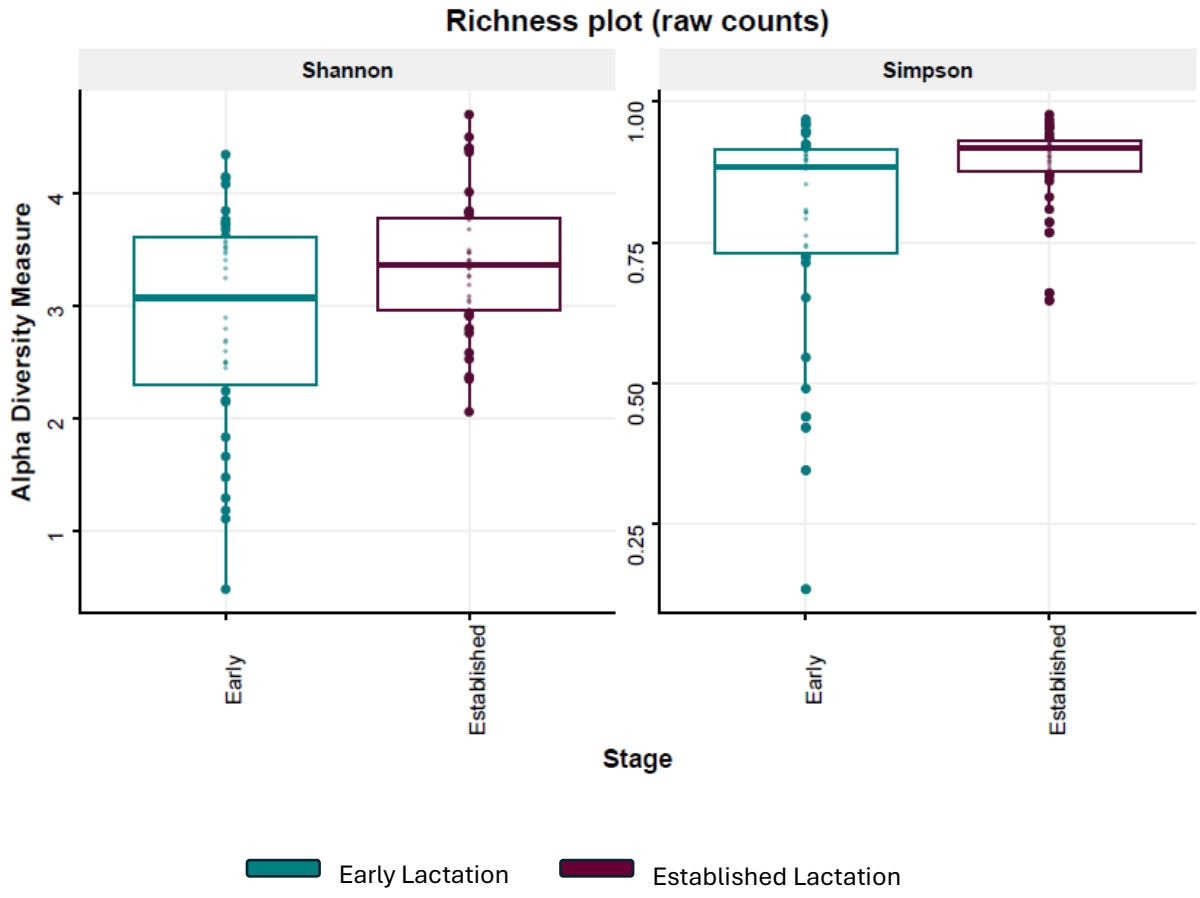



**Figure 3.4 –Alpha diversity -** Microbial diversity indices, Shannon (FDR = 0.01), and Simpson (FDR = 0.008) identified significant differences between early and established lactation.

**Figure 3.5 - Beta Diversity**

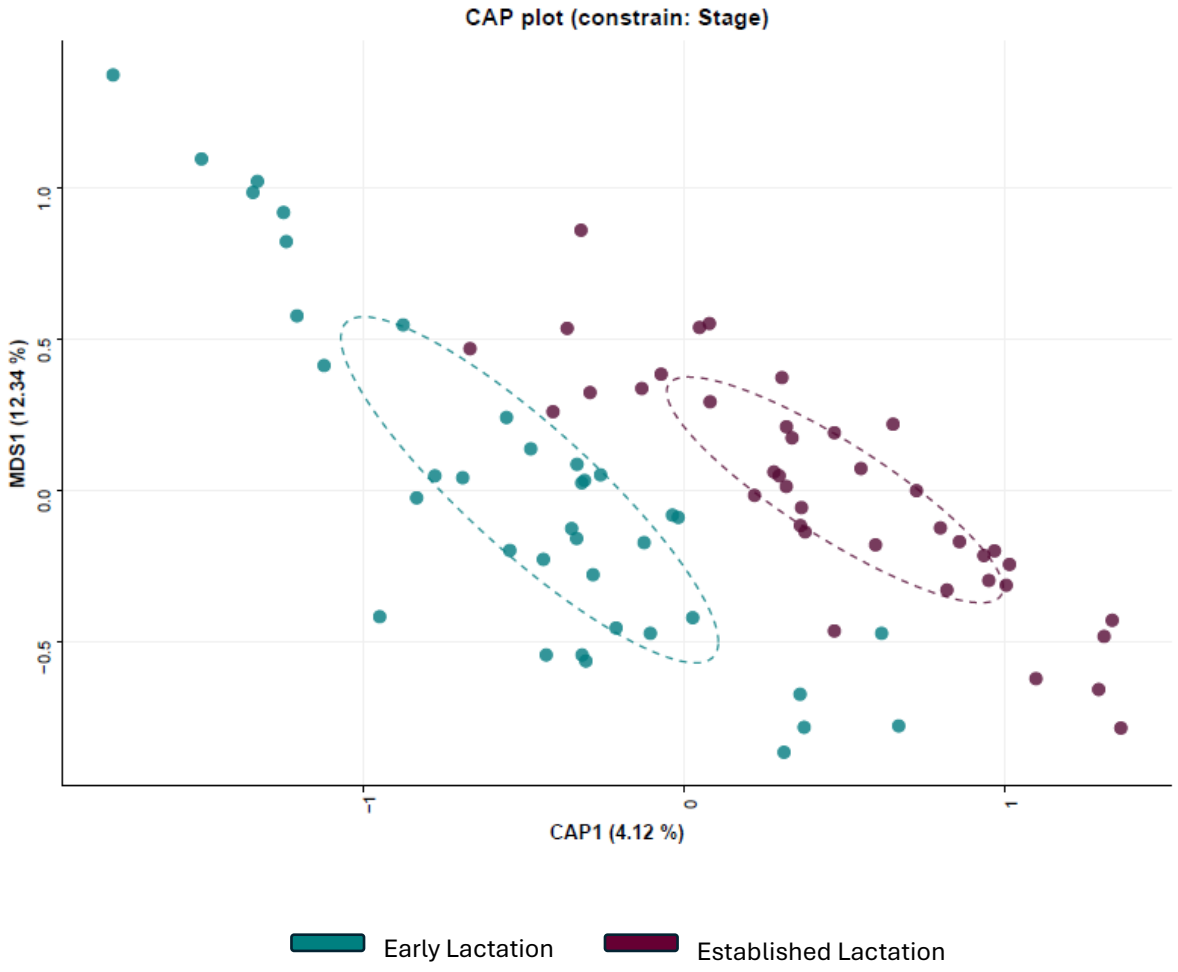



**Figure 3.5. Beta diversity -** Main factors were projected onto an unconstrained ordination diagram (NMDS) to reduce data dimensionality, allowing for the visualization of the relationships between samples in a lower-dimensional space (2D) while preserving the rank order of distances. The Canonical Analysis of Principal Coordinates (CAP) ordination segregated both lactation stages ($p < 0.001$).

**Figure 3.6 – Differentially abundant bacteria associated with the lactation stage**

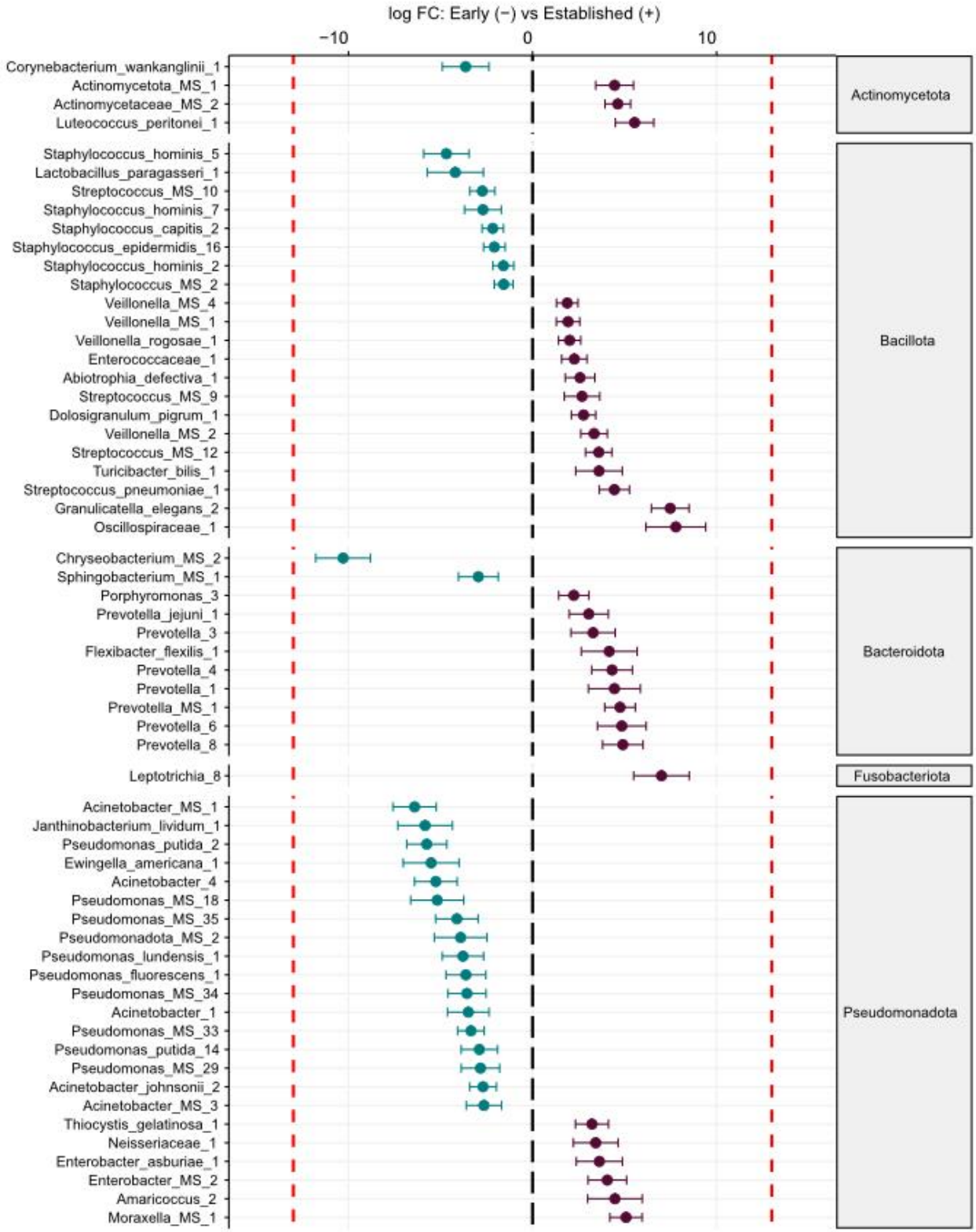


**Figure 3.6 – Differentially abundant bacteria associated with the lactation stage.** Significantly different ESVs between groups were estimated using DESeq (FDR < 0.1). Species are grouped by phylum and ordered by logFC in each group. The dashed red line indicates “infinite” log fold change, where an ESV had detectable counts in samples from only a single group. Differentially abundant ESVs between the early (*n* = 38) (blue) and established (*n* = 39) (purple) groups. 120 ESVs were differentially abundant, of which 50 were more abundant at early lactation and 70 at established lactation. ESVs labeled as "Unknown" were excluded from the final figure.

**Figure 3.7 – Multifactorial Analysis between differentially abundant ESVs, milk minerals, and maternal mineral intake.**

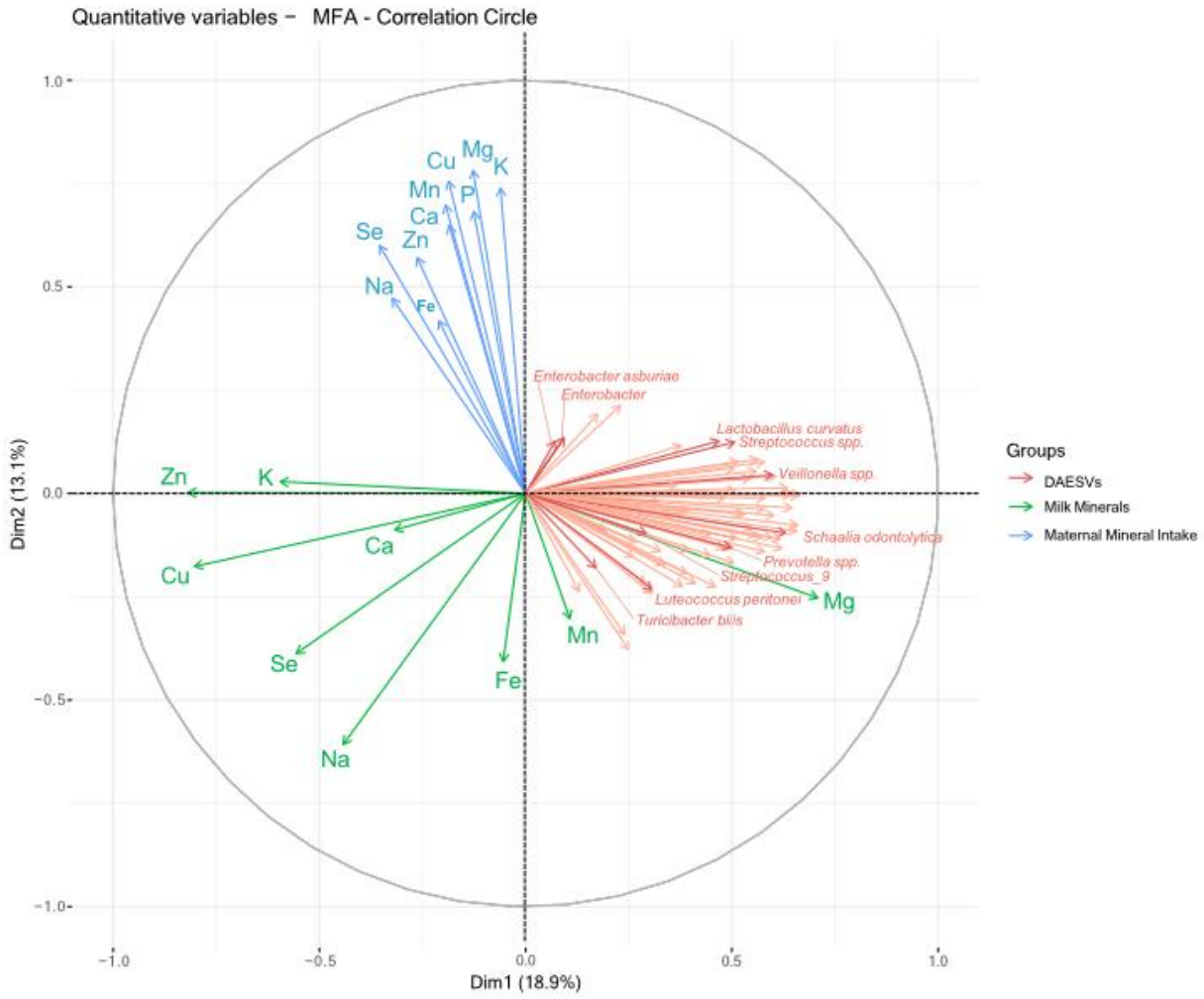



**Figure 3.7 — Multifactorial Analysis between differential abundant ESVs, milk minerals and maternal mineral intake.** The correlation circle represents the multifactorial analysis between the differential abundant ESVs (pink), milk mineral concentrations (green) and maternal mineral intake (blue). The interpretation of a correlation circle considers 3 elements: distance from the center, proximity to the edge of the circle and the angles between the variable vectors. Therefore, the closer a variable is to the center of the correlation circle, the weaker its correlation with the principal components being displayed. The closer a variable is to the edge of the correlation circle,

the better it is represented by the principal components being displayed. The angle between the vectors (from the origin to the variable points) of two variables on the correlation circle reflects their correlation with each other. An angle of 90° shows no correlation between variables, an angle > 90° shows a negative correlation and an angle <90° shows a negative correlation. Thus, no direct associations were observed between milk mineral concentrations (green) and maternal minerals intake (blue) (90° angle). However, most milk mineral concentrations (green) were negatively associated with milk bacteria (pink), as vectors are pointing in opposite directions (180° angle). Milk manganese and milk magnesium are in the same direction and close to the ESVs vectors (angle < 90°), meaning these milk minerals are positively correlated with milk bacteria. Milk bacteria is represented by the ESVs that were differentially abundant between early and established lactation. (DA: Differentially abundant; ESV: Exact Sequence variant).

**Figure 3.8 – Correlation matrix between differentially abundant ESVs, milk mineral concentrations and maternal mineral intake at early lactation**

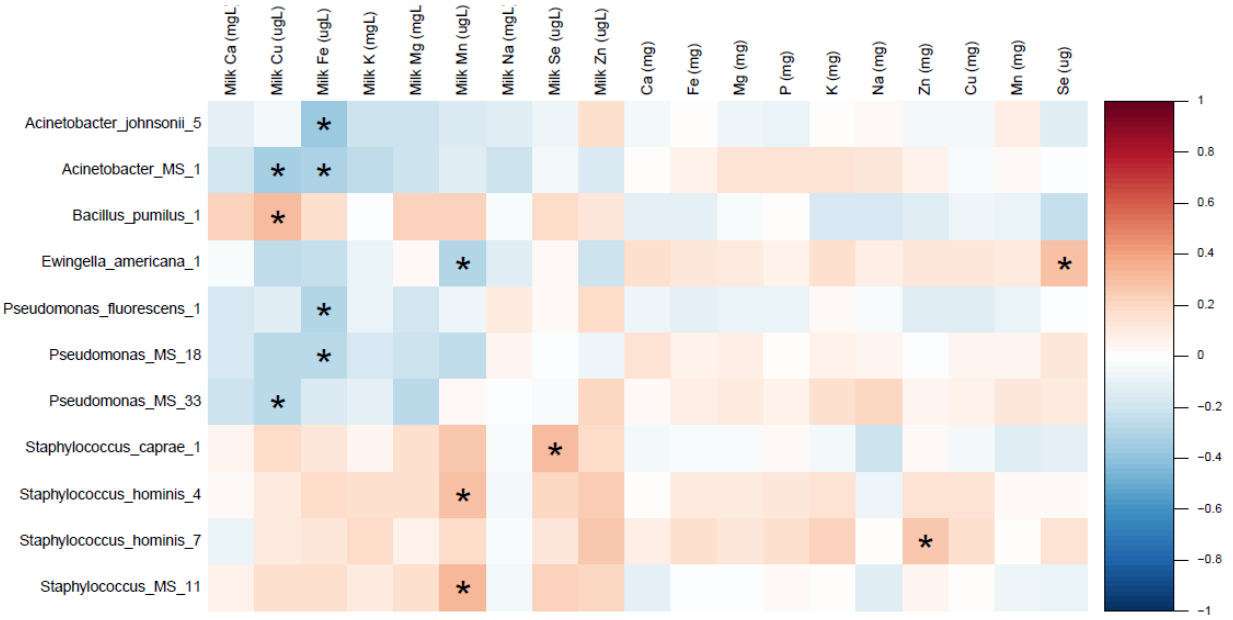



**Figure 8 – Correlation matrix between differentially abundant ESVs, milk mineral concentrations, and maternal mineral intake at early lactation**. Heatmap representing the bivariate Spearman correlation matrix between differentially abundant ESVs**,** milk mineral concentrations, and maternal mineral intake at early lactation. In the heatmap, the y-axis represents the differentially abundant ESV's. In the x-axis the minerals preceded by "milk" and with the units (mg/L or µg/L) represent the milk mineral concentrations and the minerals with the units in (mg) represent the maternal mineral intake. The red squares represent positive correlations, and the blue squares represent negative correlations. The intensity of the colors represents the degree of association between the ESVs and the milk mineral concentrations as measured by Spearman's correlations. The stars represent significant correlations (FDR < 0.1).

**Figure 3.9 - Correlation matrix between differentially abundant ESVs, milk mineral concentrations, and maternal mineral intake at established lactation**

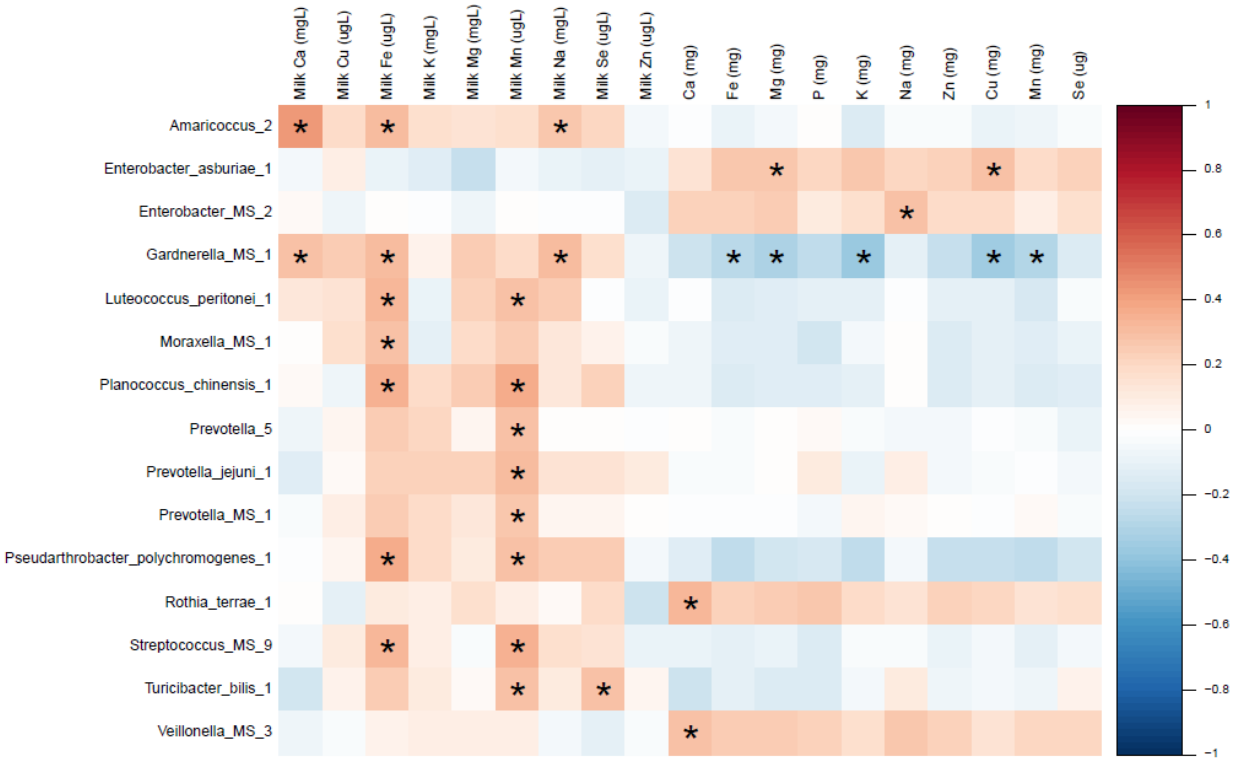



**Figure 9 - Correlation matrix between differentially abundant ESVs, milk mineral concentrations, and maternal mineral intake at established lactation**. Heatmap representing the bivariate Spearman correlation matrix between differentially abundant ESVs**,** milk mineral concentrations, and maternal mineral intake at established lactation. In the heatmap, the y-axis represents the differentially abundant ESV's. In the x-axis the minerals preceded by "milk" and with the units (mg/L or µg/L) represent the milk mineral concentrations and the minerals with the units in (mg) represent the maternal mineral intake. The red squares represent positive correlations, and the blue squares represent negative correlations. The intensity of the colors represents the degree of association between the ESVs and the milk mineral concentrations as measured by Spearman's correlations. The stars represent significant correlations (FDR < 0.1).

### 3.8 Supplementary Files

**Supplementary Table 3.1 - Population Characteristics, Maternal Nutrient Intake and Milk Mineral Concentrations**

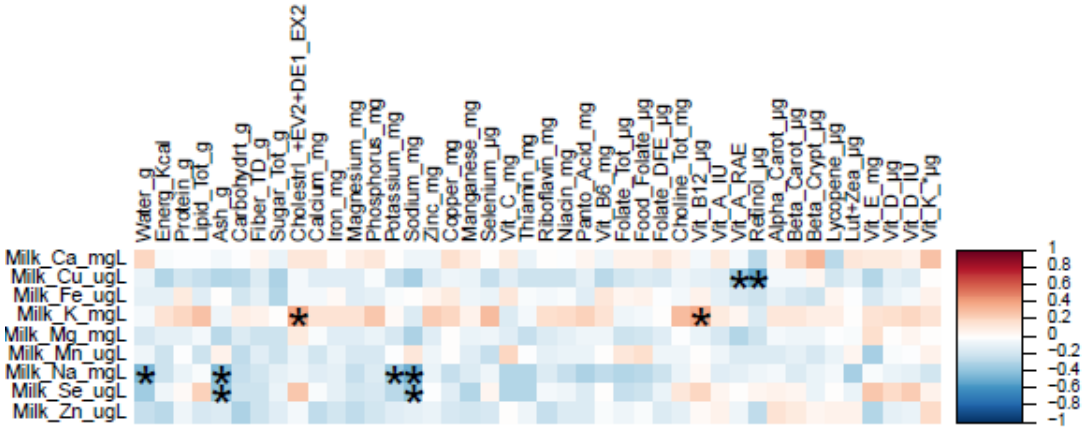
| | Total<br>N = 77 | Early Stage [1]<br>n = 38 | Established Stage [2]<br>n = 39 | p-value |
|---|---|---|---|---|
| **INFANT CHRACTERISTICS** | | | | |
| Age, days | 83 ± 66 | 18 ± 9 | 147 ± 17 | <0.001*** |
| **Sex, n (%)** | | | | 0.3 |
| Male, | 43 (55.84) | 21 (55.26) | 22 (56.41) | |
| Female | 34 (34.69) | 17 (44.74) | 17 (43.59) | |
| **MATERNAL CHARACTERISTICS** | | | | |
| **Age, y (%)** | | | | |
| <19 | 17 ± 1 (27.3) | 17 ± 2 (21.1) | 17 ± 1 (33.3) | 0.638 |
| ≥19 | 27 ± 6 (72.7) | 27 ± 6 (78.9) | 28 ± 6 (66.7) | 0.523 |
| Average (± stdev) | 24 ± 7 | 25 ± 7 | 24 ± 7 | 0.724 |
| Height, cm | 146.6 ± 5.5 | 148.2 ± 5.3 | 145.1 ± 5.3 | 0.014* |
| Weight, kg | 50.6 ± 8 | 51.2 ± 7.6 | 49.9 ± 8.4 | 0.521 |
| **BMI, kg/m$^2$ (% of mothers)** | | | | |
| Underweight (BMI:<18.5) | 17.0 ± 0.7 (3.9) | 17.4 (2.6) | 16.8 ± 0.9 (5.1) | 0.667 |
| Normal (BMI:18.5 – 24.9) | 22.4 ± 3.3 (70.1) | 22 ± 3.1 (71.1) | 22.8 ± 3.5 (69.2) | 0.096 |
| Overweight, (BMI:24.9 – 29.9) | 26.6 ± 3.3 (23.4) | 26.6 ± 3.1 (23.7) | 26.6 ± 3.5 (23.1) | 1.0 |
| Obesity, (BMI: <30) | 35.1 ± 2.7 (2.6) | 33.2 (2.6) | 37.0 (2.6) | - |
| Average (± stdev) | 23.5 ± 3.3 | 23.3 ± 3.1 | 23.8 ± 3.5 | 0.541 |
| **Parity** | | | | 0.108 |
| Primiparous, n (%) | 31 (40.8) | 15 (39.5) | 16 (42.1) | |
| Multiparous, n (%) | 45 (59.2) | 23 (60.5) | 22 (57.9) | |
| **MATERNAL NUTRIENT INTAKE [3]** | | | | |
| Water, g | 1435 ± 483 | 1604 ± 398[a] | 1270 ± 506 [b] | 0.002** |
| Energy, kcal | 1292.9 ± 373.2 | 1422.7 ± 319.9 [a] | 1166.4 ± 381.5 [b] | 0.002** |
| Protein, g | 39.9 ± 17.5 | 45.9 ± 18.2 [a] | 34 ± 14.8 [b] | 0.002** |
| Lipids, g | 19.7 ± 7.9 | 22.5 ± 7.1 [a] | 16.9 ± 7.7 [b] | 0.002** |
| Saturated fats, g | 3.6 ± 1.9 | 4.3 ± 2 [a] | 2.9 ± 1.6 [b] | 0.001*** |
| Monounsaturated fats, g | 5.8 ± 2.8 | 6.8 ± 2.6 [a] | 4.8 ± 2.6 [b] | <0.001*** |
| Polyunsaturated fats, g | 7.5 ± 2.8 | 8.1 ± 2.1 [a] | 6.9 ± 3.3 [b] | 0.047* |
| Cholesterol, mg | 72.1 ± 93.8 | 97.9 ± 107.2 [a] | 47 ± 71.4 [b] | 0.017* |
| Carbohydrates, g | 257.9 ± 71.7 | 279.5 ± 63.4 [a] | 236.9 ± 73.9 [b] | 0.008* |
| Sugars, g | 59.5 ± 23.4 | 67.8 ± 24.3 [a] | 51.4 ± 19.6 [b] | 0.002* |
| Fiber, g | 27.3 ± 9.3 | 28.5 ± 7 | 26.2 ± 11.0 | 0.283 |

| | | | | |
|---|---|---|---|---|
| Calcium, mg | 682.3 ± 193.5 | 728.6 ± 165.2 [a] | 637.1 ± 210 [b] | 0.037* |
| Copper, µg | 1238 ± 437.3 | 1325 ± 372.5 | 1153 ± 482.6 | 0.085 |
| Iron, µg | 12435.6 ± 13141.3 | 14748 ± 18002 | 10182 ± 4391 | 0.128 |
| Magnesium, mg | 412.4 ± 125.9 | 433.1 ± 104.7 | 392.3 ± 142 | 0.156 |
| Manganese, mg | 1.4 ± 0.6 | 1.5 ± 0.52 | 1.3 ± 0.7 | 0.068 |
| Potassium, mg | 1704.1 ± 640.2 | 1766.2 ± 457.9 | 1643.5 ± 779.7 | 0.401 |
| Selenium, µg | 49.7 ± 21.5 | 57.8 ± 21.8 [a] | 41.8 ± 18.3 [b] | <0.001*** |
| Sodium, mg | 2561.2 ± 2053.7 | 3422.1 ± 2510.8 [a] | 1722.4 ± 913 [b] | <0.001*** |
| Zinc, µg | 7714.2 ± 4299.2 | 8781 ± 5311 [b] | 66676 ± 2694 [a] | 0.031* |
| **MILK MINERAL CONCENTRATION [3]** | | | | |
| Calcium, mg/L | 261.1 ± 42.5 | 273.2 ± 47 [a] | 249.4 ± 34.3 [b] | 0.013* |
| Copper, µg/L | 389.8 ± 158.6 | 509 ± 108.7 [a] | 273.6 ± 103.5 [b] | <0.001* |
| Iron, µg/L | 381.2 ± 254.8 | 393.5 ± 193.2 [a] | 369.1 ± 305.4 [b] | 0.678 |
| Magnesium, mg/L | 29 ± 7.9 | 22.7 ± 4.3 [b] | 35.1 ± 5.4 [a] | <0.001*** |
| Manganese, µg/L | 9.4 ± 6.6 | 8.8 ± 5.1 [b] | 9.9 ± 7.8 [a] | 0.443 |
| Potassium, mg/L | 498.3 ± 88.2 | 551.2 ± 80 [a] | 446.8 ± 61.2 [b] | <0.001*** |
| Selenium, µg/L | 13.6 ± 4.4 | 15.6 ± 3.9 [a] | 11.7 ± 4 [b] | <0.001*** |
| Sodium, mg/L | 153 ± 125.4 | 188.5 ± 124.8 [a] | 118.4 ± 117.3 [b] | 0.013* |
| Zinc, µg/L | 2707.3 ± 1989.9 | 4035.5 ± 2004.1 [a] | 1395.7 ± 656.6 [b] | <0.001*** |

Note: [1] Early stage is defined from 5 to 46 days postpartum; [2] Late stage is defined from 109 – 184 days postpartum. [3] Values are arithmetic means ± SDs. Means labelled with an "a" are higher than means labelled with a "b" among the values in the same row.

**Supplementary Figure 3.1 – Heatmap of a univariate Spearman correlation matrix between milk mineral concentrations and the maternal mineral intake at early lactation**

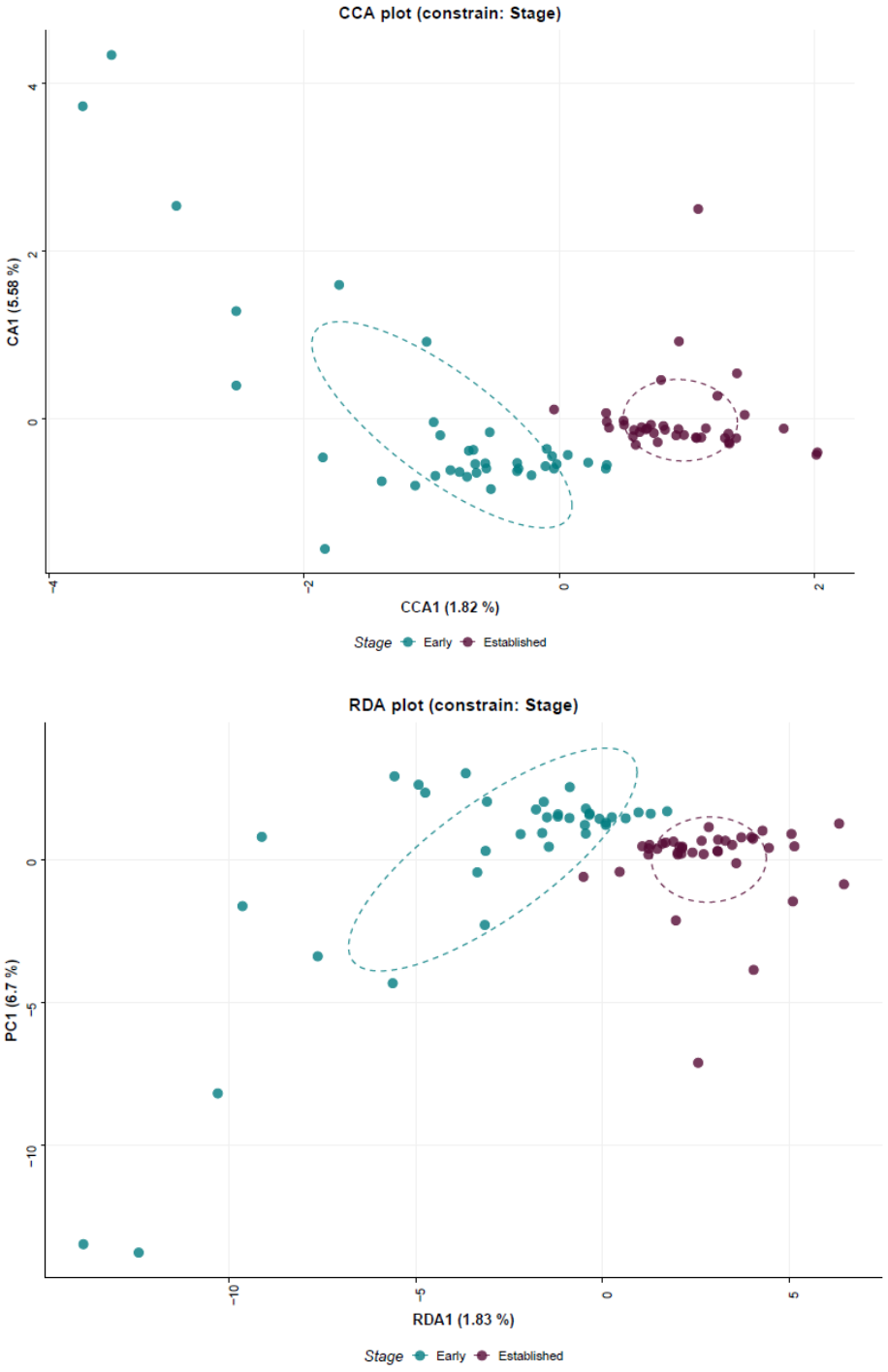
Red squares represent positive correlations, and blue squares represent negative correlations. The intensity of the colors represents the degree of association between the milk mineral concentrations and the maternal diet as measured by Spearman's correlations. The stars represent significant correlations (FDR < 0.1).

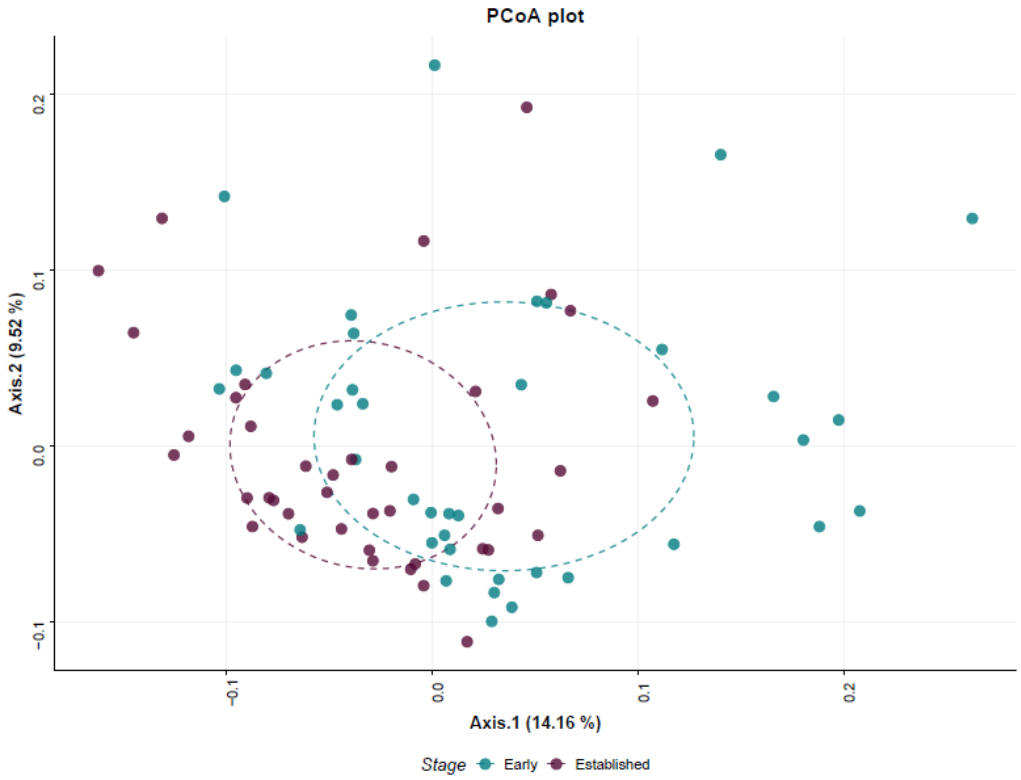



**Supplementary Figure 3.2 – Heatmap of a univariate Spearman correlation matrix between milk mineral concentrations and the maternal mineral intake at established lactation**

**Supplementary Figure 3.3 – Beta diversity**

Beta-diversity analyses segregated both lactation stages, along the Canonical Correspondence Analysis (CCA) ($p$ = <0.001), Redundancy Analysis (RDA) ($p$ < 0.001) and Principal Coordinate Analysis (PCoA) ($p$ = <0.001) ordinations






### 3.9 Data availability statement

The raw sequence data has been deposited at the European Genome-Phenome Archive (EGAD00001004160) and may be available upon request to KK, kristine.koski@mcgill.ca.

### 3.10 Ethics statement

The study was reviewed and approved by the Institutional Review Board (IRB) of McGill University and the Center for Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM). Participants provided their written informed consent to participate in this study. Women were recruited with the assistance of the community staff (CHWs and *comadronas*) from June 2012 to March 2013 in an effort to include all women until the predetermined samples sizes were achieved. Recruitment methods included home visits, loudspeaker announcements, and word-of-mouth invitations to attend meetings at the community health post where the study was explained, questions were answered, and women were invited to provide fully informed written consent (thumbprint if

unable to sign) if they wished to participate. Women who had delivered within the previous 40 days were observing a period of *cuarentena*; therefore, all procedures were carried out during a home visit. The institutional review boards of both McGill University and the Center for Studies of Sensory Impairment, Aging and Metabolism in Guatemala, approved the study, and permission was obtained from indigenous community leaders and the local authorities of the Ministry of Health. Participants were referred to the public health system whenever medically indicated. Laboratory results were communicated to participants directly, and treatment was provided free of charge for any diagnosed infection. At each sampling period, community staff administered a structured questionnaire in Spanish or *Mam*.

### 3.11 Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### 3.12 Author contributions

KK, EG, and LL: conceptualization, writing, reviewing, and editing of the manuscript. CM: writing, reviewing, and editing of the manuscript. LL: DNA extractions. EG: conducted bioinformatics and statistical analyses. KK: funding. All authors contributed to the article and approved the submitted version.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**3.15 References**

Abdi, H., & Williams, L. J. (2010). Principal component analysis. *WIREs Computational Statistics*, *2*(4), 433–459. https://doi.org/10.1002/wics.101

Ajeeb, T. T., Gonzalez, E., Solomons, N. W., Vossenaar, M., & Koski, K. G. (2024). Human milk microbiome: Associations with maternal diet and infant growth. *Frontiers in Nutrition*, *11*, 1341777. https://doi.org/10.3389/fnut.2024.1341777

Allen, L. H. (1994). Maternal micronutrient malnutrition: Effects on breast milk and infant nutrition, and priorities for intervention. *SCN News*, *11*, 21–24.

Allen, L. H. (2012). B vitamins in breast milk: Relative importance of maternal status and intake, and effects on infant status and function. *Advances in Nutrition (Bethesda, Md.)*, *3*(3), 362–369. https://doi.org/10.3945/an.111.001172

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Arias-Borrego, A., Callejón-Leblic, B., Collado, M. C., Abril, N., & García-Barrera, T. (2023). Omics insights into the responses to dietary selenium. *PROTEOMICS*, *23*(23–24), 2300052. https://doi.org/10.1002/pmic.202300052

Aumeistere, L., Ciproviča, I., Zavadska, D., Bavrins, K., & Borisova, A. (2018). Zinc Content in Breast Milk and Its Association with Maternal Diet. *Nutrients*, *10*(10), 1438. https://doi.org/10.3390/nu10101438

Babakobi, M. D., Reshef, L., Gihaz, S., Belgorodsky, B., Fishman, A., Bujanover, Y., & Gophna, U. (2020). Effect of Maternal Diet and Milk Lipid Composition on the Infant Gut and Maternal Milk Microbiomes. *Nutrients*, *12*(9), 2539. https://doi.org/10.3390/nu12092539

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., Zhong, H., Khan, M. T., Zhang, J., Li, J., Xiao, L., Al-Aama, J., Zhang, D., Lee, Y. S., Kotowska, D., Colding, C., … Wang, J. (2015). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host & Microbe*, *17*(5), 690–703. https://doi.org/10.1016/j.chom.2015.04.004

Ballini, A., Gnoni, A., De Vito, D., Dipalma, G., Cantore, S., Gargiulo Isacco, C., Saini, R., Santacroce, L., Topi, S., Scarano, A., Scacco, S., & Inchingolo, F. (2019). Effect of probiotics on the occurrence of nutrition absorption capacities in healthy children: A randomized double-blinded placebo-controlled pilot study. *European Review for Medical and Pharmacological Sciences*, *23*(19), 8645–8657. https://doi.org/10.26355/eurrev_201910_19182

Barone, M., D'Amico, F., Brigidi, P., & Turroni, S. (2022). Gut microbiome-micronutrient interaction: The key to controlling the bioavailability of minerals and vitamins? *BioFactors (Oxford, England)*, *48*(2), 307–314. https://doi.org/10.1002/biof.1835

Bates, C. J., & Tsuchiya, H. (1990). Zinc in breast milk during prolonged lactation: Comparison between the UK and the Gambia. *European Journal of Clinical Nutrition*, *44*(1), 61–69.

Bergillos-Meca, T., Cabrera-Vique, C., Artacho, R., Moreno-Montoro, M., Navarro-Alarcón, M., Olalla, M., Giménez, R., Seiquer, I., & Ruiz-López, M. D. (2015). Does Lactobacillus plantarum or ultrafiltration process improve Ca, Mg, Zn and P bioavailability from fermented goats' milk? *Food Chemistry*, *187*, 314–321. https://doi.org/10.1016/j.foodchem.2015.04.051

Bostock, M., Ogievetsky, V., & Heer, J. (2011). D3 Data-Driven Documents. *IEEE Transactions on Visualization and Computer Graphics*, *17*(12), 2301–2309. https://doi.org/10.1109/TVCG.2011.185

Bravi, F., Wiens, F., Decarli, A., Dal Pont, A., Agostoni, C., & Ferraroni, M. (2016). Impact of maternal nutrition on breast-milk composition: A systematic review. *The American Journal of Clinical Nutrition*, *104*(3), 646–662. https://doi.org/10.3945/ajcn.115.120881

Brown, D. (2006). Do Food Frequency Questionnaires Have Too Many Limitations? *Journal of the American Dietetic Association*, *106*(10), 1541–1542. https://doi.org/10.1016/j.jada.2006.07.020

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Chen, P., Bornhorst, J. B., & Aschner, M. (Invalid date). Manganese metabolism in humans. *Frontiers in Bioscience-Landmark*, *23*(9), Article 9. https://doi.org/10.2741/4665

Chi, L., Gao, B., Bian, X., Tu, P., Ru, H., & Lu, K. (2017). Manganese-induced sex-specific gut microbiome perturbations in C57BL/6 mice. *Toxicology and Applied Pharmacology*, *331*, 142–153. https://doi.org/10.1016/j.taap.2017.06.008

Chomat, A. M., Solomons, N. W., Koski, K. G., Wren, H. M., Vossenaar, M., & Scott, M. E. (2015). Quantitative Methodologies Reveal a Diversity of Nutrition, Infection/Illness, and Psychosocial Stressors During Pregnancy and Lactation in Rural *Mam* -Mayan Mother–Infant Dyads From the Western Highlands of Guatemala. *Food and Nutrition Bulletin*, *36*(4), 415–440. https://doi.org/10.1177/0379572115610944

Christian, P., Smith, E. R., Lee, S. E., Vargas, A. J., Bremer, A. A., & Raiten, D. J. (2021). The need to study human milk as a biological system. *The American Journal of Clinical Nutrition*, *nqab075*. https://doi.org/10.1093/ajcn/nqab075

Cortes-Macías, E., Selma-Royo, M., García-Mantrana, I., Calatayud, M., González, S., Martínez-Costa, C., & Collado, M. C. (2021). Maternal Diet Shapes the Breast Milk Microbiota

Composition and Diversity: Impact of Mode of Delivery and Antibiotic Exposure. *The Journal of Nutrition*, *151*(2), 330–340. https://doi.org/10.1093/jn/nxaa310

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., & Knight, R. (2009). Bacterial Community Variation in Human Body Habitats Across Space and Time. *Science*, *326*(5960), 1694–1697. https://doi.org/10.1126/science.1177486

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M., & Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. *Science (New York, N.Y.)*, *336*(6086), 1255–1262. https://doi.org/10.1126/science.1224203

Daniels, L., Gibson, R. S., Diana, A., Haszard, J. J., Rahmannia, S., Luftimas, D. E., Hampel, D., Shahab-Ferdows, S., Reid, M., Melo, L., Lamers, Y., Allen, L. H., & Houghton, L. A. (2019). Micronutrient intakes of lactating mothers and their association with breast milk concentrations and micronutrient adequacy of exclusively breastfed Indonesian infants. *The American Journal of Clinical Nutrition*, *110*(2), 391–400. https://doi.org/10.1093/ajcn/nqz047

Domellöf, M., Lönnerdal, B., Dewey, K. G., Cohen, R. J., & Hernell, O. (2004). Iron, zinc, and copper concentrations in breast milk are independent of maternal mineral status. *The American Journal of Clinical Nutrition*, *79*(1), 111–115. https://doi.org/10.1093/ajcn/79.1.111

Donohue, J. A., Solomons, N. W., Hampel, D., Shahab-Ferdows, S., Orozco, M. N., & Allen, L. H. (2020). Micronutrient supplementation of lactating Guatemalan women acutely increases infants' intake of riboflavin, thiamin, pyridoxal, and cobalamin, but not niacin, in a randomized crossover trial. *The American Journal of Clinical Nutrition*, *112*(3), 669–682. https://doi.org/10.1093/ajcn/nqaa147

Dror, D. K., & Allen, L. H. (2018). Overview of Nutrients in Human Milk. *Advances in Nutrition*, *9*(suppl_1), 278S-294S. https://doi.org/10.1093/advances/nmy022

Erick, M. (2018). Breast milk is conditionally perfect. *Medical Hypotheses*, *111*, 82–89. https://doi.org/10.1016/j.mehy.2017.12.020

Fairweather-Tait, S., & Hurrell, R. F. (1996). Bioavailability of minerals and trace elements. *Nutrition Research Reviews*, *9*(1), 295–324. https://doi.org/10.1079/NRR19960016

Fan, L., Xia, Y., Wang, Y., Han, D., Liu, Y., Li, J., Fu, J., Wang, L., Gan, Z., Liu, B., Fu, J., Zhu, C., Wu, Z., Zhao, J., Han, H., Wu, H., He, Y., Tang, Y., Zhang, Q., … Ren, W. (2023). Gut microbiota bridges dietary nutrients and host immunity. *Science China. Life Sciences*, *66*(11), 2466–2514. https://doi.org/10.1007/s11427-023-2346-1

Faulkner, M. J., Wenner, B. A., Solden, L. M., & Weiss, W. P. (2017). Source of supplemental dietary copper, zinc, and manganese affects fecal microbial relative abundance in lactating dairy cows. *Journal of Dairy Science*, *100*(2), 1037–1044. https://doi.org/10.3168/jds.2016-11680

Fehr, K., Moossavi, S., Sbihi, H., Boutin, R. C. T., Bode, L., Robertson, B., Yonemitsu, C., Field, C. J., Becker, A. B., Mandhane, P. J., Sears, M. R., Khafipour, E., Moraes, T. J., Subbarao, P., Finlay, B. B., Turvey, S. E., & Azad, M. B. (2020). Breastmilk Feeding Practices Are Associated with the Co-Occurrence of Bacteria in Mothers' Milk and the Infant Gut: The CHILD Cohort Study. *Cell Host & Microbe*, *28*(2), 285-297.e4. https://doi.org/10.1016/j.chom.2020.06.009

Gibson, R. S., Rahmannia, S., Diana, A., Leong, C., Haszard, J. J., Hampel, D., Reid, M., Erhardt, J., Suryanto, A. H., Sofiah, W. N., Fathonah, A., Shahab-Ferdows, S., Allen, L. H., & Houghton, L. A. (2020). Association of maternal diet, micronutrient status, and milk volume with milk micronutrient concentrations in Indonesian mothers at 2 and 5 months postpartum.

*The American Journal of Clinical Nutrition*, *112*(4), 1039–1050. https://doi.org/10.1093/ajcn/nqaa200

Gómez-Martín, M., Saturio, S., Arboleya, S., Herrero-Morín, D., Calzón, M., López, T., González, S., & Gueimonde, M. (2022). Association between diet and fecal microbiota along the first year of life. *Food Research International*, *162*, 111994. https://doi.org/10.1016/j.foodres.2022.111994

Gonzalez, E., Pitre, F. E., & Brereton, N. J. B. (2019). ANCHOR: A 16S rRNA gene amplicon pipeline for microbial analysis of multiple environmental samples. *Environmental Microbiology*, *21*(7), 2440–2468. https://doi.org/10.1111/1462-2920.14632

Hansen, S. L., Trakooljul, N., Liu, H.-C., Moeser, A. J., & Spears, J. W. (2009). Iron transporters are differentially regulated by dietary iron, and modifications are associated with changes in manganese metabolism in young pigs. *The Journal of Nutrition*, *139*(8), 1474–1479. https://doi.org/10.3945/jn.109.105866

Hood, M. I., & Skaar, E. P. (2012). Nutritional immunity: Transition metals at the pathogen-host interface. *Nature Reviews. Microbiology*, *10*(8). https://doi.org/10.1038/nrmicro2836

Kasaikina, M. V., Kravtsova, M. A., Lee, B. C., Seravalli, J., Peterson, D. A., Walter, J., Legge, R., Benson, A. K., Hatfield, D. L., & Gladyshev, V. N. (2011). Dietary selenium affects host selenoproteome expression by influencing the gut microbiota. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *25*(7), 2492–2499. https://doi.org/10.1096/fj.11-181990

Keikha, M., Bahreynian, M., Saleki, M., & Kelishadi, R. (2017). Macro- and Micronutrients of Human Milk Composition: Are They Related to Maternal Diet? A Comprehensive Systematic Review. *Breastfeeding Medicine: The Official Journal of the Academy of Breastfeeding Medicine*, *12*(9), 517–527. https://doi.org/10.1089/bfm.2017.0048

Lê, S., Josse, J., & Husson, F. (2008). FactoMineR: An R Package for Multivariate Analysis. *Journal of Statistical Software*, *25*, 1–18. https://doi.org/10.18637/jss.v025.i01

LeMay-Nedjelski, L., Asbury, M. R., Butcher, J., Ley, S. H., Hanley, A. J., Kiss, A., Unger, S., Copeland, J. K., Wang, P. W., Stintzi, A., & O'Connor, D. L. (2021). Maternal Diet and Infant Feeding Practices Are Associated with Variation in the Human Milk Microbiota at 3 Months Postpartum in a Cohort of Women with High Rates of Gestational Glucose Intolerance. *The Journal of Nutrition*, *151*(2), 320–329. https://doi.org/10.1093/jn/nxaa248

Li, C., Solomons, N. W., Scott, M. E., & Koski, K. G. (2016). Minerals and Trace Elements in Human Breast Milk Are Associated with Guatemalan Infant Anthropometric Outcomes within the First 6 Months. *The Journal of Nutrition*, *146*(10), 2067–2074. https://doi.org/10.3945/jn.116.232223

Li, C., Solomons, N. W., Scott, M. E., & Koski, K. G. (2018). Subclinical mastitis (SCM) and proinflammatory cytokines are associated with mineral and trace element concentrations in human breast milk. *Journal of Trace Elements in Medicine and Biology*, *46*, 55–61. https://doi.org/10.1016/j.jtemb.2017.11.010

Li, C., Solomons, N. W., Scott, M. E., & Koski, K. G. (2019). Anthropometry before Day 46 and Growth Velocity before 6 Months of Guatemalan Breastfed Infants Are Associated with Subclinical Mastitis and Milk Cytokines, Minerals, and Trace Elements. *The Journal of Nutrition*, *149*(9), 1651–1659. https://doi.org/10.1093/jn/nxz109

Lopez, C. A., & Skaar, E. P. (2018). The Impact of Dietary Transition Metals on Host-Bacterial Interactions. *Cell Host & Microbe*, *23*(6), 737–748. https://doi.org/10.1016/j.chom.2018.05.008

Lopez Leyva, L., Brereton, N. J. B., & Koski, K. G. (2020). Emerging frontiers in human milk microbiome research and suggested primers for 16S rRNA gene analysis. *Computational*

*and Structural Biotechnology Journal*, *19*, 121–133. https://doi.org/10.1016/j.csbj.2020.11.057

Lopez Leyva, L., Gonzalez, E., Solomons, N. W., & Koski, K. G. (2022). Human milk microbiome is shaped by breastfeeding practices. *Frontiers in Microbiology*, *13*. https://www.frontiersin.org/articles/10.3389/fmicb.2022.885588

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Lyons, K. E., Fouhy, F., O' Shea, C.-A., Ryan, C. A., Dempsey, E. M., Ross, R. P., & Stanton, C. (2021). Effect of storage, temperature, and extraction kit on the phylogenetic composition detected in the human milk microbiota. *MicrobiologyOpen*, *10*(1), e1127. https://doi.org/10.1002/mbo3.1127

Mandiá, N., Bermejo-Barrera, P., Herbello, P., López-Suárez, O., Fraga, J. M., Fernández-Pérez, C., & Couce, M. L. (2021). Human Milk Concentrations of Minerals, Essential and Toxic Trace Elements and Association with Selective Medical, Social, Demographic and Environmental Factors. *Nutrients*, *13*(6), 1885. https://doi.org/10.3390/nu13061885

Marín, M. L., Arroyo, R., Jiménez, E., Gómez, A., Fernández, L., & Rodríguez, J. M. (2009). Cold storage of human milk: Effect on its bacterial composition. *Journal of Pediatric Gastroenterology and Nutrition*, *49*(3), 343–348. https://doi.org/10.1097/MPG.0b013e31818cf53d

Martinez-Morata, I., Sobel, M., Tellez-Plaza, M., Navas-Acien, A., Howe, C. G., & Sanchez, T. R. (2023). A State-of-the-Science Review on Metal Biomarkers. *Current Environmental Health Reports*, *10*(3), 215–249. https://doi.org/10.1007/s40572-023-00402-x

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Menchú, M. T., Torún, B., & Elias, L. G. (2012). *Recomendaciones Dietéticas Diarias del INCAP*. INCAP. https://inec.cr/wwwisis/documentos/INCAP/Recomendaciones_Dieteticas_Diarias_del_INCAP.pdf

Moossavi, S., Atakora, F., Miliku, K., Sepehri, S., Robertson, B., Duan, Q. L., Becker, A. B., Mandhane, P. J., Turvey, S. E., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Subbarao, P., Field, C. J., Bode, L., Khafipour, E., & Azad, M. B. (2019). Integrated Analysis of Human Milk Microbiota With Oligosaccharides and Fatty Acids in the CHILD Cohort. *Frontiers in Nutrition*, *6*, 58. https://doi.org/10.3389/fnut.2019.00058

Moossavi, S., Sepehri, S., Robertson, B., Bode, L., Goruk, S., Field, C. J., Lix, L. M., de Souza, R. J., Becker, A. B., Mandhane, P. J., Turvey, S. E., Subbarao, P., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Khafipour, E., & Azad, M. B. (2019). Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors. *Cell Host & Microbe*, *25*(2), 324-335.e4. https://doi.org/10.1016/j.chom.2019.01.011

Murdoch, C. C., & Skaar, E. P. (2022). Nutritional immunity: The battle for nutrient metals at the host-pathogen interface. *Nature Reviews. Microbiology*, *20*(11), 657–670. https://doi.org/10.1038/s41579-022-00745-6

Nugroho, A. D. W., van Olst, B., Bachtiar, S. A., Boeren, S., Kleerebezem, M., & Bachmann, H. (2022). Manganese Modulates Metabolic Activity and Redox Homeostasis in Translationally Blocked Lactococcus cremoris, Impacting Metabolic Persistence, Cell Culturability, and Flavor Formation. *Microbiology Spectrum*, *10*(3), e0270821. https://doi.org/10.1128/spectrum.02708-21

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2020). *Vegan: Community Ecology Package* (Version 2.5-7) [Computer software]. https://CRAN.R-project.org/package=vegan

Ortega, R. M., Andrés, P., Martínez, R. M., López-Sobaler, A. M., & Quintas, M. E. (1997). Zinc levels in maternal milk: The influence of nutritional status with respect to zinc during the third trimester of pregnancy. *European Journal of Clinical Nutrition*, *51*(4), 253–258. https://doi.org/10.1038/sj.ejcn.1600393

Padilha, M., Danneskiold-Samsøe, N. B., Brejnrod, A., Hoffmann, C., Cabral, V. P., Iaucci, J. de M., Sales, C. H., Fisberg, R. M., Cortez, R. V., Brix, S., Taddei, C. R., Kristiansen, K., & Saad, S. M. I. (2019). The Human Milk Microbiota is Modulated by Maternal Diet. *Microorganisms*, *7*(11). https://doi.org/10.3390/microorganisms7110502

Pajarillo, E. A. B., Lee, E., & Kang, D.-K. (2021). Trace metals and animal health: Interplay of the gut microbiota with iron, manganese, zinc, and copper. *Animal Nutrition*, *7*(3), 750–761. https://doi.org/10.1016/j.aninu.2021.03.005

Palmer, L. D., & Skaar, E. P. (2016). Transition Metals and Virulence in Bacteria. *Annual Review of Genetics*, *50*, 67–91. https://doi.org/10.1146/annurev-genet-120215-035146

Perez, P. F., Dore, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., Segura-Roggero, I., Schiffrin, E. J., & Donnet-Hughes, A. (2007). Bacterial Imprinting of the Neonatal Immune System: Lessons From Maternal Cells? *PEDIATRICS*, *119*(3), e724–e732. https://doi.org/10.1542/peds.2006-1649

Petersohn, I., Hellinga, A. H., van Lee, L., Keukens, N., Bont, L., Hettinga, K. A., Feskens, E. J. M., & Brouwer-Brolsma, E. M. (2023). Maternal diet and human milk composition: An updated systematic review. *Frontiers in Nutrition*, *10*, 1320560. https://doi.org/10.3389/fnut.2023.1320560

*Population Growth in Low vs. High Income Countries | Wilson Center*. (2023). https://www.wilsoncenter.org/blog-post/population-growth-low-vs-high-income-countries

Puga, A. M., Samaniego-Vaesken, M. de L., Montero-Bravo, A., Ruperto, M., Partearroyo, T., & Varela-Moreiras, G. (2022). Iron Supplementation at the Crossroads of Nutrition and Gut Microbiota: The State of the Art. *Nutrients*, *14*(9), 1926. https://doi.org/10.3390/nu14091926

Resnicow, K., Odom, E., Wang, T., Dudley, W. N., Mitchell, D., Vaughan, R., Jackson, A., & Baranowski, T. (2000). Validation of Three Food Frequency Questionnaires and 24-Hour Recalls with Serum Carotenoid Levels in a Sample of African-American Adults. *American Journal of Epidemiology*, *152*(11), 1072–1080. https://doi.org/10.1093/aje/152.11.1072

Revelle, W. (Director). (2017). *psych: Procedures for Personality and Psychological Research* [Video recording]. https://CRAN.R-project.org/package=psych

Rizzatti, G., Lopetuso, L. R., Gibiino, G., Binda, C., & Gasbarrini, A. (2017). Proteobacteria: A Common Factor in Human Diseases. *BioMed Research International*, *2017*(1), 9351507. https://doi.org/10.1155/2017/9351507

Rodríguez, J. M. (2014). The Origin of Human Milk Bacteria: Is There a Bacterial Entero-Mammary Pathway during Late Pregnancy and Lactation? *Advances in Nutrition*, *5*(6), 779–784. https://doi.org/10.3945/an.114.007229

Samuel, T. M., Zhou, Q., Giuffrida, F., Munblit, D., Verhasselt, V., & Thakkar, S. K. (2020). Nutritional and Non-nutritional Composition of Human Milk Is Modulated by Maternal, Infant, and Methodological Factors. *Frontiers in Nutrition*, *7*, 172. https://doi.org/10.3389/fnut.2020.576133

Sánchez-Jiménez, A., Marcos-Torres, F. J., & Llamas, M. A. (2023). Mechanisms of iron homeostasis in Pseudomonas aeruginosa and emerging therapeutics directed to disrupt

this vital process. *Microbial Biotechnology*, *16*(7), 1475. https://doi.org/10.1111/1751-7915.14241

Sanjulián, L., Lamas, A., Barreiro, R., Cepeda, A., Fente, C. A., & Regal, P. (2021). Bacterial Diversity of Breast Milk in Healthy Spanish Women: Evolution from Birth to Five Years Postpartum. *Nutrients*, *13*(7), Article 7. https://doi.org/10.3390/nu13072414

Schalk, I. J., & Perraud, Q. (2023). Pseudomonas aeruginosa and its multiple strategies to access iron. *Environmental Microbiology*, *25*(4), 811–831. https://doi.org/10.1111/1462-2920.16328

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Horn, D. J. V., & Weber, C. F. (2009). Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541. https://doi.org/10.1128/AEM.01541-09

Seyoum, Y., Baye, K., & Humblot, C. (2021). Iron homeostasis in host and gut bacteria – a complex interrelationship. *Gut Microbes*, *13*(1), 1874855. https://doi.org/10.1080/19490976.2021.1874855

Shannon, M. C., & Hill, G. M. (2019). Trace Mineral Supplementation for the Intestinal Health of Young Monogastric Animals. *Frontiers in Veterinary Science*, *6*. https://doi.org/10.3389/fvets.2019.00073

Sheldon, J. R., & Skaar, E. P. (2019). Metals as phagocyte antimicrobial effectors. *Current Opinion in Immunology*, *60*, 1–9. https://doi.org/10.1016/j.coi.2019.04.002

Sian, L., Krebs, N. F., Westcott, J. E., Fengliang, L., Tong, L., Miller, L. V., Sonko, B., & Hambidge, M. (2002). Zinc homeostasis during lactation in a population with a low zinc intake. *The*

*American Journal of Clinical Nutrition*, *75*(1), 99–103. https://doi.org/10.1093/ajcn/75.1.99

Simonyté Sjödin, K., Domellöf, M., Lagerqvist, C., Hernell, O., Lönnerdal, B., Szymlek-Gay, E. A., Sjödin, A., West, C. E., & Lind, T. (2019). Administration of ferrous sulfate drops has significant effects on the gut microbiota of iron-sufficient infants: A randomised controlled study. *Gut*, *68*(11), 2095–2097. https://doi.org/10.1136/gutjnl-2018-316988

Sindi, A. S., Geddes, D. T., Wlodek, M. E., Muhlhausler, B. S., Payne, M. S., & Stinson, L. F. (2021). Can we modulate the breastfed infant gut microbiota through maternal diet? *FEMS Microbiology Reviews*, *45*(5), fuab011. https://doi.org/10.1093/femsre/fuab011

Smith, A. J., Ward, P. N., Field, T. R., Jones, C. L., Lincoln, R. A., & Leigh, J. A. (2003). MtuA, a Lipoprotein Receptor Antigen from Streptococcus uberis, Is Responsible for Acquisition of Manganese during Growth in Milk and Is Essential for Infection of the Lactating Bovine Mammary Gland. *Infection and Immunity*, *71*(9), 4842–4849. https://doi.org/10.1128/IAI.71.9.4842-4849.2003

Sprockett, D., Fukami, T., & Relman, D. (2018). Role of priority effects in the early-life assembly of the gut microbiota. *Nature Reviews. Gastroenterology & Hepatology*, *15*(4). https://doi.org/10.1038/nrgastro.2017.173

Takahashi, K., Suzuki, N., & Ogra, Y. (2017). Bioavailability Comparison of Nine Bioselenocompounds In Vitro and In Vivo. *International Journal of Molecular Sciences*, *18*(3), 506. https://doi.org/10.3390/ijms18030506

Thum, C., Cookson, A. L., Otter, D. E., McNabb, W. C., Hodgkinson, A. J., Dyer, J., & Roy, N. C. (2012). Can Nutritional Modulation of Maternal Intestinal Microbiota Influence the Development of the Infant Gastrointestinal Tract? *The Journal of Nutrition*, *142*(11), 1921–1928. https://doi.org/10.3945/jn.112.166231

Trautvetter, U., Camarinha-Silva, A., Jahreis, G., Lorkowski, S., & Glei, M. (2018). High phosphorus intake and gut-related parameters—Results of a randomized placebo-controlled human intervention study. *Nutrition Journal*, *17*(1), 23. https://doi.org/10.1186/s12937-018-0331-4

Wei, T., & Simko, V. (2021). *Package "corrplot."* R Core Team. https://github.com/taiyun/corrplot

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer International Publishing. https://doi.org/10.1007/978-3-319-24277-4

Williams, J. E., Carrothers, J. M., Lackey, K. A., Beatty, N. F., York, M. A., Brooker, S. L., Shafii, B., Price, W. J., Settles, M. L., McGuire, M. A., & McGuire, M. K. (2017). *Human Milk Microbial Community Structure Is Relatively Stable and Related to Variations in Macronutrient and Micronutrient Intakes in Healthy Lactating Women*. *147*(9), 1739–1748.

Wren, H. M., Solomons, N. W., Chomat, A. M., Scott, M. E., & Koski, K. G. (2015). Cultural Determinants of Optimal Breastfeeding Practices among Indigenous *Mam* -Mayan Women in the Western Highlands of Guatemala. *Journal of Human Lactation*, *31*(1), 172–184. https://doi.org/10.1177/0890334414560194

Wren-Atilola, H. M., Solomons, N. W., Scott, M. E., & Koski, K. G. (2018). Infant growth faltering linked to subclinical mastitis, maternal faecal-oral contamination, and breastfeeding. *Maternal & Child Nutrition*, *15*(3), e12756. https://doi.org/10.1111/mcn.12756

Yersin, S., & Vonaesch, P. (2024). Small intestinal microbiota: From taxonomic composition to metabolism. *Trends in Microbiology*, *32*(10), 970–983. https://doi.org/10.1016/j.tim.2024.02.013

## Connecting Statement 2

One of the main findings of Chapter 3 was that milk mineral composition, but not maternal mineral intake, was strongly associated with the human milk microbiome composition. The previous chapter also showed that the maternal dietary intake of several minerals and concentrations of milk minerals were below reference values.

We were thus interested on the impacts of these inadequate mineral concentrations on infant growth and analyzed the associations of milk minerals and milk microbiome diversity on infant growth. Considering that Guatemala has one of the highest rates in stunting worldwide, we explored through a longitudinal study if the interactions between milk minerals and milk microbiome diversity were playing a relevant role on infant growth.

# Chapter 4: Manuscript 3

## Milk Microbiome and Minerals: A Bidirectional Relationship Shaping Infant Growth

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**4.1 Abstract**

Currently, there is a knowledge gap in relation to the interaction of milk components and their association with infant growth. Our aim was to analyze the associations between minerals and the milk microbiome and how they affect infant growth in the *Mam*-Mayan indigenous population of Guatemala. In this longitudinal study, a total of 114 milk samples were collected from 57 mothers during early (5 – 46 days) and established (4 – 6 months) lactation. Concentrations of 12 milk minerals were analyzed. Infant growth was assessed according to the infant growth parameters [weight-for-age (WAZ) and head circumference-for-age (HCAZ) into ≥-1 SD and <-1 SD and length-for-age (LAZ), into ≥-2 SD and <-2 SD]. The milk microbiome diversity was obtained by 16S rRNA gene amplicon sequencing of the V5-V6 region. Infants with normal and impaired growth in weight and length were ingesting different milk microbiomes in early lactation but not at established lactation. In established lactation, only infants with normal and impaired head circumference were ingesting different milk microbiomes. Manganese and magnesium emerged as important minerals in relation to milk microbiome and growth as they had the most correlations with members of the milk microbiome in normal and impaired growth. Namely, *Cutibacterium acnes* was differentially abundant (DA) in the mild underweight and stunted infants and was negatively associated with manganese. On the other hand, in non-stunted and normal head circumference infants Phascolarctobacterium and *Streptococcus agalactiae* were positively associated with magnesium, and *Streptococcus pneumoniae* was positively associated with manganese. This suggests that milk microbial composition could alter milk minerals bioavailability and concentrations and vice-versa and ultimately influence infant growth.

**4.2 Introduction**

Impaired growth is emerging as a concern in breastfed infants (de Onis & Branca, 2016; Li et al., 2016, 2019). For decades, research on infant growth focused on children older than 6 months of age because of the assumption that milk was providing all the necessary nutrients for adequate infant growth (Marquis et al., 1997; Mata et al., 1976; Ruel & Menon, 2002; Simondon et al., 2002).

The influence of breastfeeding on infant growth has recently been recognised and diverse milk components have been identified as critical for growth, including macronutrients, oligosaccharides, short-chain fatty acids, and endocrine factors (Brockway et al., 2023, 2024; Geddes et al., 2021; Perrella et al., 2021). Milk minerals for their part have typically been overlooked (Eriksen et al., 2018), yet evidence of associations between milk minerals and infant growth is accumulating (Azad et al., 2024; Reyes et al., 2023). For instance, a recent systematic review reported a positive correlation between infant growth and milk concentrations of iodine, manganese, calcium, and zinc (Reyes et al., 2023). Two studies conducted by our research group reported that low mineral concentrations of calcium, magnesium, potassium, rubidium, and strontium in human milk were associated with impaired infant weight-for-age *z* score (WAZ), length-for-age *z* score (LAZ), and head circumference-for-age *z* score (HCAZ) (Li et al., 2016); while milk magnesium in early lactation (5- 46 days) was associated with linear growth velocity in breastfed infants younger than 6 mo (Li et al., 2019). Milk concentrations of zinc for its part has been described as fundamental for infant growth, development, and cell differentiation (Doneray et al., 2017), as well as in the metabolism of macronutrients and the synthesis of hormones involved in bone growth and metabolism (Perrella et al., 2021). Therefore, while there is evidence that minerals could play an important role in infant growth, more research is needed to characterize their association with other milk components, including the microorganisms found in human milk - the milk microbiome (Robertson et al., 2019).

Indeed, evidence from the gut microbiome indicates several associations between gut microbial composition and infant growth. For example, healthy growth in the first 6 months is associated with greater *Bifidobacterium longum* and *Streptococcus thermophilus* in the infant gut (Robertson et al., 2019), taxa typically associated with breastfeeding, immunomodulatory characteristics and polysaccharide and oligosaccharide metabolism (O'Callaghan & van Sinderen, 2016; Zafar & Saier, 2021). Data are more limited in milk, but a recent cross-sectional study showed that a higher prevalence of *Streptococcus* and *Actinobacteria* species in the milk microbiome positively correlated with infant head circumference, particularly during established lactation (Ajeeb et al.,

2022a). Another cross-sectional study led by the same group reported that the milk microbiome of mildly stuntend infants (LAZ < 1.5 SD) had more *Streptococcus mitis* and *Stenotrophomonas maltophilia* in early lactation, and *Brevundimonas_MS_1, Paracoccus_MS_1,* and *Streptococcus mitis* in established lactation. In contrast, non-stunted infants (LAZ ≥ 1.5 SD) had more *Paracoccus aminovorans* and *Pseudomonas cedrina* in early lactation, and *Lactococcus lactis* in established lactation (Ajeeb et al., 2022b).

The interactions between members of the human milk microbiome with other nutrients such as milk minerals remain poorly explored, despite the fact that some are essential for bacterial growth and survival. In a recent study, we reported a complex and dynamic interplay between milk minerals and the milk microbiome, which seems to operate independently of maternal diet (Lopez Leyva et al., 2025). Milk iron, manganese and selenium emerged as key minerals for milk microbiome composition (Lopez Leyva et al., 2025). Iron, manganese, and selenium were correlated with members of the phyla Pseudomonadota (Fe and Mn) in early lactation, with Actinomycetota (Fe and Mn) in established lactation, and with Bacillota in both stages of lactation (Mn and Se). Another study reported that calcium was negatively correlated with *Streptococcus, Prevotella, Actinobacteria*, and *Bacteroidetes*; magnesium showed a positive relationship with *Streptococcus* abundance in human milk; and selenium negatively correlated with *Staphylococcus* (Sanjulián et al., 2021). Similarly, as with infant growth and the microbiome, the association between minerals and the microbiome has been better documented in the gut than in human milk. For instance, a review on the human gut microbiome reported that bacterial strains of *Streptococcus, Lactobacillus* and *Bifidibacteria* increased the bioaccessibility and bioavailability of foods high in iron, zinc, calcium, selenium, magnesium (Bielik & Kolisek, 2021). Thus, more studies in human milk microbiome are needed to understand the existing associations between minerals and the milk microbiome and how they affect infant growth.

Guatemala has one of the highest exclusive breastfeeding rates in the World, approaching 58.5% (UNICEF, 2023) and is also home to a unique population of *Mam*-Mayan mothers which complies with the World Health Organization (WHO) recommendations to exclusively breastfeed for 6 months. Yet, in some rural Mayan regions, the prevalence of child stunting is estimated to be

> 58% (UNICEF/WHO/World Bank Group, 2023). Guatemala has simultaneously high rates of exclusive breastfeeding and impaired growth. We thus hypothesize that impaired infant growth will be associated with higher loads of opportunistic bacteria linked to the mineral deficiencies in human milk. By sampling longitudinally, a cohort of 57 *Mam*-Mayan mothers and the growth of their infants at 5 –46 d postpartum (early lactation) and 4–6 mo postpartum (established lactation), we will 1) associate milk mineral concentrations and the human milk microbiome diversity at early and established lactation and 2) associate milk minerals and milk microbiome diversity with adequate or impaired infant growth.

### 4.3 Materials and methods

**Study site and participants.** This longitudinal study was part of a collaboration between McGill University and the Center for Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM) in the Republic of Guatemala. Field studies were conducted from June 2012 through January 2013 in eight rural *Mam*-speaking communities of the San Juan Ostuncalco region in Guatemala (Chomat et al. 2015). The inclusion criteria were indigenous lactating women with infants at 1) early stage of lactation (5 - 46 days), and 2) established stage of lactation (109 – 184 days) and who had a vaginal delivery. The exclusion criteria were: 1) mothers with colostrum (milk < 4 days postpartum), 2) mothers treated with antibiotics during postpartum period and 3) mothers with subclinical mastitis. Maternal age, BMI, parity and infant sex were controlled for in the analyses. Ethical approval was obtained from Institutional Review Boards of both institutions and permission was obtained from community leaders and the local authorities of the Ministry of Health. All mothers provided written informed consent for participation in the study.

**Study Design.** In this longitudinal study, collected milk samples were categorized into 2 lactation stages: early (5 –46 d postpartum, n = 57) and established (4–6 mo postpartum, n = 57). A subsequent division was done according to infant growth parameters. Infants were classified according to the infant growth parameters [length-for-age (LAZ), into ≥-2 SD and <-2 SD, weight-for-age (WAZ) and head circumference-for-age (HCAZ) into ≥-1 SD and <-1 SD]. These ranges were chosen to be consistent with our previous studies that measured infant growth (Li et al., 2016,

2019; Wren-Atilola et al., 2018). The milk mineral concentrations and milk microbiome composition were analyzed.

**Human milk sample collection and milk composition.** Prior to collection, the nipple and areola of the breast were cleaned with 70% ethyl alcohol. Human milk samples were collected during a 3-hour time window in the morning from the breast not recently used for breastfeeding via full manual expression by a trained midwife, who used hand sanitizer before and after collection. This is important since other studies have shown differences in milk microbiome diversity with the use of breast pumps (Fehr et al., 2020; Marín et al., 2009; Moossavi et al., 2019). Milk was collected into 60 ml plastic vials and immediately stored on ice. Samples were partitioned into 15 ml tubes at the field laboratory (-30°C) prior to transfer on dry ice to McGill University where they were stored at -80°C, which is known to preserve the milk microbiome integrity (Lyons et al., 2021), until DNA extraction for microbiome analysis was performed.

**Milk mineral concentrations**. Human milk concentrations of 12 minerals (Na, Ca, Cu, Fe, Mg, Mn, P, K, Rb Se, Sr, and Zn) were measured as described previously (Li et al., 2016, 2018). Milk samples were thawed and thoroughly homogenized. Overnight digestion of the samples was completed in plastic DigiTUBEs (SCP Science) through use of trace metal–grade concentrated nitric acid followed by a 3-h reaction with trace metal–grade hydrogen peroxide (30%, Ultrex II, JT Baker), followed by heating at 90°C for 3 h, as previously described (Li et al., 2016). Minerals were quantified by inductively coupled mass spectrometry (ICP-MS) using a Varian ICP-820MS (Analytik Jena, Germany) equipped with a Collision Reaction Interface. Calibration standards and quality controls have been previously described (Li et al., 2016). The limits of detection (LOD) for each of the 12 minerals measured on 8 replicates of the lowest calibration standard, were calcium, 1.505 mg/L; copper, 0.396 mg/L; iron, 1.34 mg/L; magnesium, 0.232 mg/L; manganese, 0.005 mg/L; potassium, 4.887 mg/L; rubidium, 0.032 mg/L; selenium, 0.007 mg/L; sodium, 1.816 mg/L; strontium, 0.026 mg/L; and zinc 0.116 mg/L. Milk mineral concentrations were compared to the average mineral content in human milk considered by the INCAP (Institute of Nutrition of Central America and Panama) for the Guatemalan population (Menchú et al., 2012).

**Anthropometric measurements.** Infant length, weight, and head circumference were measured as described previously (Wren et al., 2015). Infant WAZ, LAZ, and HCAZ were calculated according to WHO Growth Reference Standards (*WHO Child Growth Standards*, 2006) through use of WHO Anthro software version 3.2. The international definition of "moderate malnutrition" (two SD from the WHO standards median) defined moderate stunting as LAZ <-2SD. For WAZ and HCAZ, it was considered impaired growth the international definition of "mild malnutrition" <-1SD and adequate ≥-1SD. This was in order to align with previous studies of the same research group (Ajeeb et al., 2022a) and because there were too few children who were under -2SD.

**DNA extraction.** DNA extraction was done using 1ml of milk with the Quick-DNA Fungal/Bacterial Miniprep kit from Zymo Research according to the manufacturer's protocol. DNA was sent to the McGill Genome Centre for quality control, library preparation, and sequencing. Upon reception of the samples, a Picogreen test was done to measure the DNA concentration. As the concentration of the samples was very low, a speed vac was used to concentrate the DNA, and then it was resuspended in 12uL DNAse/RNAse free water. The V5-V6 region was amplified in two steps using the P609F and P699R primers. PCR amplification was performed in triplicate with controls using the NEB Phusion Taq DNA Polymerase with the following cycle conditions: initial denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 58°C for 30 s, 72°C for 30 s, and concluded by a final extension at 72°C for 5 min. The barcoding was done using the Kapa Hifi 2X ready mix with the following conditions: initial denaturation at 95°C for 3 min, followed by 12 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and concluded by a final extension at 72°C for 5 min. Following amplicon amplifications, the samples that amplified were pooled and normalized. The normalization was followed by two 1X AMPure clean-up to remove primer-dimers before quantification using qPCR and the LabChip. The resulting amplicons were sequenced using an Illumina MiSeq platform at the McGill Genome Centre and assembled from 300 paired-end reads; reagent controls were below the detection limit.

**Microbial Community Characterization.** The sequencing data were processed using the ANCHOR pipeline for microbial analysis (Gonzalez et al., 2019). Reads were aligned and

dereplicated using Mothur (Schloss et al., 2009). Exact sequence variants (ESVs) were selected based on a count threshold of 36 across all samples. Taxonomic annotations were performed using BLASTn against multiple databases, including NCBI 16S RefSeq and NCBI nr/nt, applying criteria of >99% identity and coverage (Altschul et al., 1990; Callahan et al., 2016). In cases of multiple hits with 100% identity, NCBI 16S rRNA RefSeq was prioritized due to the high standard of curation. All annotations, particularly species calls, should be considered putative even when sharing 100% sequence identity to a single species due to potential errors in databases (Gonzalez et al., 2019). Given the low biomass nature of these samples, contamination was controlled for at all stages: experimentally with the use of PCR controls and reagent controls (Gonzalez et al., 2019).

**Microbial Community Assessment** To visualize the microbial community composition at the phylum level, the relative abundances of exact sequence variants (ESVs) were computed from the normalized data. A custom JavaScript script utilizing the D3.js library (Bostock et al., 2011) was used to create the flower diagram representing cumulative microbial abundance. Alpha and beta diversity analyses were conducted to compare microbial diversity between early and established lactation stages. Alpha diversity was calculated using the Shannon and Simpson indices, while beta diversity was evaluated using Bray-Curtis dissimilarity. The vegan (Oksanen et al., 2020), phyloseq (McMurdie & Holmes, 2013), and ggplot2 (Wickham, 2016) packages in R were used for these calculations and visualizations. Canonical Analysis of Principal Coordinates (CAP) was employed to assess beta diversity and to visualize the separation between the two lactation stages. The significance of group separation was tested using ANOVA-like permutation tests, with p-values corrected using the Benjamini-Hochberg procedure.

**Differential Abundance Analysis.** Differential abundance of microbial taxa between normal and impaired growth at early and established lactation was assessed using DESeq2 (Love et al., 2014). Raw read counts were imported into DESeq2, and regularized log transformation (rlog) was applied as the normalization method to stabilize variance across samples. Differentially abundant exact sequence variants (ESVs) were identified with a significance threshold set at a

false discovery rate (FDR) of 0.05. For visualization of the results, the phyloseq (McMurdie & Holmes, 2013) and ggplot2 (Wickham, 2016) R packages were used. Differentially abundant taxa were represented by grouping species by phylum and ordering them according to their log fold change. A dashed red line was used to denote “infinite” log fold changes, indicating that some ESVs were detected in samples from only one group (either normal or impaired growth) and absent in the other.

**Correlation Analysis.** Spearman's rank correlation was used to examine the relationships between milk mineral concentrations and differentially abundant bacterial species (ESVs) in normal and impaired growth at early and established lactation. The Kendall rank correlation coefficient was calculated using the psych (Revelle, 2017) and corrplot (Wei & Simko, 2021) R packages. Bacterial abundance data consisted of ESVs identified as differentially abundant. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method, and results with an adjusted p-value (FDR) < 0.05 were retained. ESVs labeled as "Unknown" were excluded from the final heatmap. The correlation matrix was visualized using the corrplot package, where the intensity of color represented the strength of the correlation (blue = negative correlation, red: positive correlation), and statistical significance was indicated with circles without a cross.

## 4.4 Results

### Population characteristics and milk composition at early and established stage of lactation

This longitudinal study included 57 lactating mothers who provided milk samples at early (5 – 46 days) and established (4 – 6 mo) lactation, for a total of 114 milk samples. Milk samples were classified by lactation stage and according to the 3 growth parameters (WAZ, LAZ and HCAZ) z-scores categories, resulting in the following 6 groups: 1) normal weight (WAZ ≥ –1 SD), 2) mildly underweight (WAZ < –1 SD), 3) non-stunted (LAZ ≥ –2 SD), 4) stunted (LAZ < –2 SD), 5) normal (HCAZ ≥ –1 SD) and 6) mild-impaired head circumference (HCAZ < –1 SD). **Table 4.1** shows the population characteristics at early (A) and established (B) lactation, respectively, and compares differences between infants with normal and impaired growth, as defined with WAZ, LAZ, and HCAZ parameters. At early lactation, the maternal age differed between infants with normal and

impaired WAZ, and maternal height differed between the groups of stunted and non-stunted infants. At established lactation, maternal weight differed between the groups of infants with normal and impaired WAZ and LAZ, and maternal height differed between stunted and non-stunted infants**.**

Milk mineral concentrations that infants with normal and impaired growth received at early and established lactation are shown in **Figures 4.1** for WAZ, **Figure 4.2** for LAZ and **Figure 4.3** for HCAZ and **Appendix 1 and 2.** The distribution of milk mineral concentrations was similar in all the growth parameters. In early and established lactation, average milk mineral concentrations above the reference values were chromium and zinc, while those below the reference values were calcium, sodium and selenium.

**Bacterial characteristics of the human milk at early and established lactation**

A total of 765 ESVs were assembled and captured 2,883,818 sequence reads across all 114 human milk samples**.** These could be annotated as 545 species, 171 genera, and 96 family, as well as 220 which could not be recognized as 99% similar (in both identity and coverage) to any known taxa and were labeled as “unknown”. However, these ESVs were retained in the statistical analyses to ensure a comprehensive assessment of microbiome composition. Notably, a subset of these ‘unknown’ taxa that were found to be significant in our differential abundance analyses were subsequently identified (100% identity) using BLASTn against a broader reference database. To ensure robust and reliable microbiome comparisons, we used regularized log (RLOG) transformation for normalization. This approach was chosen for its ability to stabilize variance across different abundance levels. To validate the normalization process, we generated abundance vs. variance plots, which confirmed the absence of stochastic effects and ensured appropriate variance stabilization. As contamination is a known challenge in microbiome studies, we included negative (blank) controls during sample processing to monitor potential contaminants. Of the 545 ESVs annotated as putative species, the average BLASTn return identity was 99.9% including 458 perfect hits (100% identity). The most abundant ESVs across all samples

were *Streptococcus salivarius_1, Streptococcus spp.,* and *Agrococcus carbonis_1,* making up 12.4%, 7%, and 5.5% of the microbial community, respectively **(Figure 4.4).**

Of the six alpha-diversity indices used to characterize and compare human milk microbiome communities for WAZ, LAZ and HCAZ, only differences were observed during early lactation. Simpson (FDR = 0.08) index revealed significant differences in microbial community richness between infants with WAZ < -1 SD and WAZ ≥ -1 SD. Similarly, between infants with LAZ < -2 SD and LAZ ≥ -2 SD, the Simpson index (FDR = 0.09) showed significant differences at early lactation. Finally, the Shannon (FDR = 0.08) and Simpson (FDR = 0.04) indices showed significant differences between infants with HCAZ < -1 SD and HCAZ ≥ -1 SD **(Appendix 3)**.

Beta-diversity analyses segregated the normal weight (WAZ < –1 SD) and the mildly underweight (WAZ ≥ –1 SD) infant groups, the stunted (LAZ < –2 SD) and non-stunted (LAZ ≥ –2 SD) infant groups and normal (HCAZ < –1 SD) and infants with mild-impaired head circumference (HCAZ ≥ –1 SD) at early lactation. At established lactation, only the groups of infants with normal (HCAZ < –1 SD) and mild-impaired head circumference (HCAZ ≥ –1 SD) were segregated along the Canonical Analysis of Principal Coordinates (CAP) ($p$ < 0.001) and Redundancy Analysis (RDA) ($p$ < 0.001) **(Figure 4.5** and **Appendix 4).**

**Differentially abundant bacteria between infants with normal and impaired growth according to weight, length, and head circumference Z-scores at early and established lactation**

Infants with normal and impaired growth in weight and length ingested different milk microbiomes in early lactation, but not at established lactation. In established lactation, only infants with normal and impaired head circumference were exposed to different milk microbiomes. Therefore, results for bacterial differential abundance analysis at established lactation are only shown for head circumference.

***Infant weight-for-age Z-score (WAZ)***

In early lactation, 16 ESVs were differentially abundant (DA) (FDR < 0.05) between normal weight (WAZ ≥ –1 SD) and mildly underweight (WAZ < –1 SD) infants. The normal weight group (WAZ ≥ –1 SD) had more DA taxa with 15 ESVs. The only species DA in the mildly underweight (WAZ < –1 SD) group was *Cutibacterium acnes_1* (FC = 2.44) **(Supplementary figure 4.1.A)**.

***Infant length-for-age Z-score (LAZ)***

In early lactation, 24 ESVs were DA between non-stunted (LAZ ≥ –2 SD) and stunted (LAZ < –2 SD) infants. The non-stunted infants (WAZ ≥ –1 SD) had more DA taxa with 19 ESVs vs 5 ESVs in the stunted group **(Supplementary figure 4.1.B)**.

***Infant head circumference -for-age Z-score (HCAZ)***

In early lactation, 32 ESVs were DA between infants with normal (HCAZ ≥ –1 SD) and mild-impaired head circumference (HCAZ < –1 SD). The normal group (HCAZ ≥ –1 SD) had more DA taxa with 25 DA ESVs, while the group of infants with mild-impaired head circumference (HCAZ < –1 SD) had 7 DA ESVs **(Supplementary figure 4.1.C)**.

In established lactation, HCAZ was the only growth parameter that had DA ESVs. The 2 DA ESVs observed were both in the group of infants with mild-impaired head circumference (HCAZ < –1 SD) group **(Supplementary figure 4.1.D)**.

**Correlation of milk minerals with DA bacteria of infants with normal and impaired growth.**

***Infant weight-for-age Z-score***

In early lactation in the group of infants with normal weight (WAZ ≥ –1 SD), 4 of the 5 significant negative correlations of DA ESVs with milk minerals, were with magnesium: *Pseudomonas spp.* (FDR = 0.02), *Pseudomonas fluorescens* (FDR = 0.04), *Janthinobacterium lividum_1* (FDR = 0.02), and *Pseudomonas fluorescens* (FDR = 0.02). *Janthinobacterium lividum_1* was also negatively correlated with calcium (FDR = 0.01). In the mildly underweight group (WAZ < –1 SD), *Cutibacterium acnes,* which was the only ESV DA, was negatively correlated with manganese (FDR = 0.04). There were not any positive correlations at early lactation. In established lactation there were not any DA ESVs **(Figure 4.6)**

***Infant length-for-age Z-score***

In early lactation in the group of non-stunted infants (LAZ ≥ –2 SD) *Streptococcus pneumoniae_1* had a positive correlation with manganese (FDR = 0.04) and *Sphingobacterium faecium* had a negative correlation with magnesium (FDR = 0.03). In early lactation in the group of stunted infants (LAZ < –2 SD), *Cutibacterium acnes_1* had a negative correlation with manganese (FDR = 0.04), as was the case in the group of infants mildly underweight (WAZ < –1 SD). In established lactation there were no DA ESVs **(Figure 4.7).**

***Infant head circumference -for-age Z-score***

In early lactation in the group of infants with normal head circumference (HCAZ ≥ –1 SD), *Streptococcus pneuomoniae* had a positive correlation with manganese (FDR = 0.04), as was the case in the non-stunted group (LAZ ≥ –2 SD). In addition, *Streptococcus spp.* was positively correlated with magnesium (FDR = 0.04) and *Lactobacillus johnsonii* was negatively correlated with selenium (FDR = 0.03). Also, in early lactation but in the group of infants with mild-impaired head circumference (HCAZ < –1 SD), Pseudomonas spp. was the only ESV negatively correlated with magnesium (FDR = 0.02) **(Figure 4.8A)**.

In established lactation, only one ESV, *Streptococcus_MS_10,* which could represent one or more of the species: *Streptococcus_miti, Streptococcus_oralis* or *Streptococcus_pneumoniae*) in the group of infants with mild-impaired head circumference (HCAZ < –1 SD) was positively correlated with manganese (FDR = 0.08) **(Figure 4.8B).**

**4.5 Discussion**

The current study aimed to analyze the associations between milk minerals and milk microbiome and their impact on infant growth in the *Mam*-Mayan indigenous population of Guatemala. Our longitudinal design allowed us to explore the dynamic associations between milk microbiome composition and infant growth at early and established lactation. Our novel findings were 1) Infants with normal and impaired growth in weight and length ingested different milk microbiomes in early lactation but not at established lactation, 2) In established lactation, only infants with normal and impaired head circumference ingested different milk microbiomes, 3)

Some bacteria could be associated with normal growth, such as *Luteococcus peritonei* (for all growth parameters measured, that is WAZ, LAZ and HCAZ), *Bifidobacterium longum, Lancefieldella parvula,* Phascolarctobacterium*, Rothia mucilaginosa, Streptococcus agalactiae* and *Streptococcus pneumoniae* (only for the LAZ and HCAZ parameters), *Janthinobacterium lividum* (only WAZ parameter) and *Lactobacillus johnsonii* (only HCAZ parameter)*,* 4) Similarly, *C. acnes* could be associated with impaired growth as it was identified in infants with impaired WAZ and LAZ parameters and 5) Milk manganese and magnesium emerge as important minerals due to the multiple associations with milk bacteria in infants with both impaired and normal growth.

**Milk microbiome in early lactation is critical in infant growth**

Infants with normal and impaired growth in weight and length ingested different milk microbiomes in early lactation but not at established lactation. In established lactation, only infants with normal and impaired head circumference ingested different milk microbiome, which emphasizes the importance of early lactation in the length and weight of infants. Previous research describing associations of the gut microbiome with infant growth characterized 2 vulnerable periods of infant growth as the first 6 months of life and from 6 months to 2 years (Robertson et al., 2019). However, rather than the broad classification of 0 – 6 months of life, our results showed that the first days of life (5 – 46 days) are more critical in contributing to impaired infant length and weight. In fact, age has been reported as the most influential factor defining gut microbiome composition and function in early life (Robertson et al., 2023), and possibly the same applies to milk microbiome composition. Furthermore, a systematic review on linear growth failure in low- and middle-income countries concluded that the highest incidence of stunting onset occurred from birth to 3 months and accounted for 23% of all children who experienced stunting by age 24 months (Benjamin-Chung et al., 2020). This aligns with our results and underscores the importance of finding ways to detect, treat and possibly prevent impaired growth before the commonly suggested timing of 6 months of age; the current interventions recommended by the WHO to treat impaired growth between birth and 6 months of age are limited (Benjamin-Chung et al., 2020). These interventions in the neonatal period only include delayed cord clamping, neonatal vitamin K administration, and kangaroo mother care (WHO,

2018). After the neonatal period, the unique recommendation is exclusive breastfeeding (WHO, 2018), which our study and others indicate that it does not reduce infant stunting in a population experiencing chronic undernutrition, like our study population. (Benjamin-Chung et al., 2020; Bhutta et al., 2008; Eriksen et al., 2017; Giugliani et al., 2015). Further nutrition recommendations that improve milk composition in early lactation are needed to prevent infant impaired growth.

**Milk microbiome and infant growth**

The associations between infant growth and the milk microbiome remain underexplored, with only cross-sectional studies performed to date (Ajeeb et al., 2022a, 2022b, 2024; Cheema et al., 2022). One of the cross-sectional studies reported that *S. mitis* was negatively associated with head circumference, LAZ and HCAZ scores, while *S. epidermidis* was negatively associated with infant length, and *S. parasanguis* was positively associated with infant weight and WAZ score (Cheema et al., 2022). Cheema et. al. found a mix of positive and negative associations of *Streptococcus* and *Staphylococcus* species with infant growth. In comparison, most of our associations of *Streptococcus* and *Staphylococcus* were in infants with impaired growth LAZ and WAZ and HCAZ parameters. This might be associated to different species, as we identified *S. pneumoniae* and *S. agalactiae* and Cheema et. al. identified *S. mitis* and *S. epidermis.* A cross-sectional study in milk microbiome and infant growth conducted in the same study population reported similar results (Ajeeb et al., 2022a, 2022b). In early lactation, diverse species of *Pseudomonas* and *Streptococcus* were differentially abundant in infants with normal LAZ, WAZ and HCAZ scores (Ajeeb et al., 2022a, 2022b). In comparison to our study, *Pseudomonas* also predominated in the normal growth groups (particularly in LAZ), while *Streptococcus* were differentially abundant in both groups depending on the species. These similar results were expected as milk samples come from the same population, and they were confirmed through this longitudinal study.

***Bacteria associated with normal growth***

We observed more differentially abundant ESVs in the milk of infants with normal growth in WAZ, LAZ and HCAZ parameters in comparison to the milk of infants with impaired growth, indicating

more microbial diversity in normal growth. This aligns with previous evidence in the gut microbiome, where microbial diversity increases as infants age, which is typically associated with good health outcomes, including adequate growth (Shamash & Maurice, 2022).

Infants with normal weight ingested milk dominated by ESVs from the Pseudomonadota phyla including *P. fluorescens, P. putida and P. trittici*. In a cross-sectional study, *Pseudomonas* were also associated with normal LAZ, WAZ and HCAZ parameters, including the species *P. flurosescens, P. koreensis* and *P. cedrina* (Ajeeb et al., 2022a, 2022b). However, in our study, *Pseudomonas* were also present in milk ingested by infants with impaired growth, so we cannot exclusively associate them with normal growth. *Luteococcus peritonei* also stands out as it was present in the milk that infants with normal WAZ, LAZ and HCAZ parameters, although it has not been identified in previous studies that have associated bacteria with infant growth. *Luteococcus peritonei* was originally isolated from the human peritoneum so it could be considered part of the human microbiome (Collins et al., 2000), but further research is needed to confirm its role in infant growth. Other important species observed in the milk ingested by infants with normal growth were *Janthinobacterium lividum* (WAZ) and *Lactobacillus johnsonii* (HCAZ). *Janthinobacterium lividum* is recognized for its antibacterial, antiviral, and antifungal properties (Baricz et al., 2018) and *Lactobacillus johnsonii* is a typical intestinal probiotic known for its beneficial abilities as anti-inflammatory, immunomodulatory, intestinal microflora balance, and intestinal barrier protection (Zhang et al., 2023).

Interestingly, several ESVs were present in the milk ingested by infants with both normal LAZ and normal HCAZ scores including *Bifidobacterium longum, Lancefieldella parvula,* Phascolarctobacterium*, Rothia mucilaginosa, Streptococcus agalactiae,* and *Streptococcus pneumoniae.* This could indicate the presence of a "core" milk microbiome supporting healthy infant growth in this specific population. In addition, the genera *Limosilactobacillus* and *Corynebacterium* were present in the milk of infants with normal LAZ and normal HCAZ, but the species observed in each growth parameter differed. In normal LAZ, the species differentially abundant were *Limosilactobacillus_fermentum* and *Corynebacterium fermentum* while *Limosilactobacillus gastricus* and *Corynebacterium gastricus* were differentially abundant in

HCAZ. Importantly, *B. longum* is known for its probiotic properties and it is a prevalent member of the gut microbiota of breastfed infants (Ding et al., 2024). It was recently described as positively implicated in early growth in infants (Vatanen et al., 2022) and as regulator of bone formation (Ding et al., 2024), which could positively impact infant growth. Our results confirm this positive role of *B. longum* in infant growth as it was associated with infants with normal LAZ and HCAZ. *Lancefieldella parvula* is a lesser-known bacterium, but it has been isolated from human sources, specifically from the oral cavity, suggesting some association with the human microbiome (Copeland et al., 2009). However, it has not been previously associated with infant growth. *Rothia mucilaginosa* is typically part of the healthy oral microbiome but can cause opportunistic infections, particularly in immunocompromised individuals (Ekkelenkamp et al., 2010). *Phascolarctobacterium* is prevalent in the gastrointestinal tract and can produce short-chain fatty acids with beneficial immunomodulatory characteristics (Wu et al., 2017). *Streptococcus agalactiae* can be found in the gastrointestinal and genito-urinary tracts and can commonly cause infections in newborns, pregnant women, and immunocompromised individuals (Hanna & Noor, 2023). Similarly, *Streptococcus pneumoniae* is also an identified pathogen, responsible for pneumonia, meningitis, and other serious infections. Surprisingly, both latter species were associated with normal infant growth despite being known for their pathogenicity. None of these species have been associated previously with infant growth, but their presence in milk ingested by infants with normal growth according to LAZ and HCAZ could indicate a positive association with infant growth, or maybe that their pathogenicity changes when they interact with other milk components, such as milk minerals.

The mechanisms by which milk bacteria can affect infant growth remain unknown, but in the gut microbiome there is evidence that growth-promoting taxa, such as *R. gnavus* and *C. symbosium,* can improve the impaired growth phenotype by driving amino acids away from oxidation in favor of protein synthesis and lean mass formation (Blanton et al., 2016). It's possible that the species associated with normal growth identified in this study act through similar mechanisms to promote growth, but further research is needed.

***Bacteria associated with impaired growth***

On the other hand, *Cutibacterium acnes* was present in the milk ingested by infants with impaired WAZ and LAZ parameters and *Pseudomonas* and *Streptococcus* were associated with infants with impaired HCAZ at both stages of lactation. In a previous cross-sectional study in the same population, *C. acnes* was one of the most abundant species reported in the milk microbiome, although it was not associated with any infant growth parameters (Cheema et al., 2022). *Cutibacterium acnes* and *Streptococcus* are commonly found on human skin, in the mouth, and gut (Platsidaki & Dessinioti, 2018). *Pseudomonas* species are often environmental bacteria found in soil, water, and plant surfaces, but can also be opportunistic pathogens. For example, *Cutibacterium* acnes is linked to acne, *Streptococcus* species can cause skin and respiratory infections, and *Pseudomonas* species are known for opportunistic infections. The fact that *C. acnes* is not DA in infants with normal HCAZ, but *Pseudomonas* are, could be explained by the role of *C. acnes* in homeostasis, as it has been described in the skin's microbiome. In the skin, *C. acnes* plays a homeostatic role interacting with other cutaneous microorganisms such as *Staphylococcus epidermidis*, *Streptococcus pyogenes*, and *Pseudomonas* species. On one hand, *S. epidermis* may limit the over colonization of *C. acnes* and on the other hand, *C. acnes* may limit the proliferation of *S. aureus* and *S. pyogenes.* These changes in the skin microbiome composition may lead to inflammation (Platsidaki & Dessinioti, 2018). Similar associations could be happening in milk and *C. acnes* might be differentially abundant in impaired growth because it is actually preventing the proliferation of other pathogens  In fact, it has been shown in cutaneous samples that when *C. acnes* was present, *Pseudomonas* species typically were not, and vice versa (Hall et al., 2018), as we observed in our samples. Further investigation is needed to better understand the interactions between bacteria in the milk along with other milk components.

**Associations of milk minerals and milk microbiome on infant growth**

Manganese and magnesium emerged as important minerals in relation to milk microbiome composition and infant growth and their associations with bacteria are the same in the different growth parameters (WAZ, LAZ and HCAZ). For instance, *S. penumoniae* was positively associated with manganese and *S. agalactiae* and Phascolarbacterium were positively associated with

magnesium, in infants with normal LAZ and HCAZ at early lactation. *Pseudomonas* was negatively associated with magnesium in infants with normal WAZ and HCAZ also at early lactation. On the other hand and also at early lactation, *C. acnes* was negatively associated with manganese, but in infants with impaired weight and length. Previous studies in milk microbiome have not reported associations of magnesium and manganese with these bacteria, although other associations have been observed between magnesium and manganese in the gut microbiota with mostly positive effects.

In relation to magnesium, evidence in the gut microbiome shows that adequate levels of magnesium might increase microbial diversity (Devi et al., 2022; Kour et al., 2021). Its supplementation can help in remodeling the gut microbiota in patients with obesity and type 2 diabetes (Piuri et al., 2021) and improved bone mineral density in postmenopausic osteopenic women (Lambert et al., 2017). However, an unnecessary supplementation can decrease gut microbial diversity (García-Legorreta et al., 2020). Magnesium deficiency in mice reduced *Bifidobacteria* levels which promotes an inflammatory response (Pachikian et al., 2010) and interestingly, low dietary magnesium was associated with microbiota with a higher capacity to harvest energy from the diet (García-Legorreta et al., 2020). Importantly, *Lactobacillus spp*. increased the bioavailability of magnesium in the gut (Aljewicz et al., 2014; Bergillos-Meca et al., 2015). It seems that different levels of magnesium can have different effects on the gut microbiome. In our study, milk magnesium was below the reference values in early lactation, and it was mostly negatively associated with bacteria in normal growth. It seems that the interaction of magnesium with different taxa such as *S. agalactiae* and *Phascolarbacterium* is promoting infant growth. Perhaps as described by García – Legorreta et. al. (2020), the low levels of milk magnesium promote the interaction of bacteria with high capacity to harvest energy and therefore promote infant growth.

As pertaining to manganese, we recently reported its association with the milk microbiome—specifically, with Pseudomonadota in early lactation, Actinomycetota in established lactation, and Bacillota across both stages (Lopez Leyva et al., 2025). There is evidence that manganese is essential for bacterial growth (Faulkner et al., 2017; Nugroho et al., 2022; Smith et al., 2003), and

it has also been linked to immune function, bacterial survival, and a capacity to promote the virulence of certain pathogens (Lopez & Skaar, 2018; Pajarillo et al., 2021). Since manganese has the capacity to make a pathogen more aggressive, and in our study, it was negatively correlated with *C. acnes* in infants with impaired weight and height, one possibility is that the interaction between manganese and *C. acnes* may be influencing infant growth in a detrimental way. On the other hand, manganese was positively associated with *S. pneumoniae* in infants with normal head circumference during early lactation, suggesting that in this context, manganese might instead be supporting immune development and thereby benefiting growth. These interpretations remain speculative and require further investigation

There is also evidence in the gut that microorganisms can regulate the levels of micronutrients by modulating their metabolism and absorption (Barone et al., 2022; Fan et al., 2023), which could also be the case in human milk whereby controlling their concentration, infant growth would be affected. Further mechanistic research exploring the bioavailability of minerals in milk are needed.

Surprisingly, minerals known to play important roles in infant growth and to interact extensively with gut bacteria—such as calcium, iron, and zinc (Barone et al., 2022; Bielik & Kolisek, 2021) were not associated with DA bacteria for any of the growth parameters evaluated. One possible explanation is that the effect of these minerals on the milk microbiome may depend on whether their concentrations in milk are adequate or deficient, potentially influencing their interactions with bacteria and, in turn, their bioavailability. In our population, several milk minerals were found to be below established reference values, which could help explain why some of these key minerals showed no associations with the milk microbiome, despite their known importance for infant growth.

We acknowledge the value of more advanced statistical models for future research, that should monitor larger cohorts of lactating mothers and include more time-points to evaluate changes over time. Only having data of two time-points made it impossible to identify trends. Further research is needed to explain the underlying mechanisms of the associations between milk minerals, milk microbiome and infant growth.

## Conclusion

In summary, the associations between bacteria and minerals are complex, mutually influential, and are correlated with infant growth. This suggests that milk microbial composition could alter milk mineral bioavailability and concentrations and vice-versa and ultimately influence infant growth. Therefore, these associations could be an infant growth modulator along with other factors such as maternal health, social and physical environments.

**4.6 Tables**

**Table 4.1 (A) - Population Characteristics for the 3 growth parameters (LAZ, WAZ, HCAZ) at early lactation**

| | Early stage (5 - 46 days)[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **WAZ < -1SD (17)** | **WAZ ≥ -1SD (40)** | *P*-value[3] | **LAZ < -2SD (19)** | **LAZ ≥ -2SD (38)** | *P*-value[3] | **HCAZ < -1SD (20)** | **HCAZ ≥ -1SD (37)** | *P*-value[3] |
| **Maternal Characteristics[2]** | | | | | | | | | |
| Age, y | 21.1 ± 5.4 | 25.3 ± 7.4 | *0.04* | 22 ± 5.2 | 25.1 ± 7.7 | 0.08 | 22.1 ± 5.7 | 25.1 ± 7.6 | 0.12 |
| Height, cm | 145.6 ± 5.2 | 147.3 ± 5.2 | 0.29 | 144.8 ± 5 | 147.8 ± 5 | *0.04* | 145.9 ± 4.3 | 147.2 ± 5.6 | 0.38 |
| Weight, kg | 48.4 ± 6.1 | 51 ± 7.4 | 0.21 | 49 ± 5.4 | 50.8 ± 7.7 | 0.38 | 48.1 ± 5.6 | 51.3 ± 7.6 | 0.11 |
| BMI, kg/m$^2$ | 22.8 ± 2.3 | 23.5 ± 3.1 | 0.39 | 23.4 ± 1.9 | 23.3 ± 3.3 | 0.86 | 22.6 ± 2 | 23.7 ± 3.3 | 0.2 |
| Parity | | | 0.71 | | | 0.51 | | | 0.39 |
| Primiparous, n (%) | 7 (41.2) | 14 (35.9 | | 6 (31.6) | 15 (40.5) | | 6 (30) | 15 (41.7) | |
| Multiparous, n (%) | 10 (58.8) | 25 (64.1) | | 13 (68.4) | 22 (59.5) | | 14 (70) | 21 (58.3) | |
| **Infant Characteristics [2]** | | | | | | | | | |
| Age, days | 19 ± 8 | 19 ± 8 | 0.77 | 19 ± 9 | 19 ± 8 | 0.91 | 18 ± 9 | 19 ± 8 | 0.69 |
| Sex | | | 0.93 | | | 0.57 | | | 0.75 |
| Male, n (%) | 10 (58.8) | 23 (57.5) | | 10 (52.6) | 23 (60.5) | | 11 (55) | 22 (59.5) | |
| Female, n (%) | 7 (41.2) | 17 (42.5) | | 9 (47.4) | 15 (39.5) | | 9 (45) | 15 (40.5) | |
| Length, cm | 48 ± 1.8 | 50.3 ± 2 | ***<0.001*** | 47.6 ± 1.6 | 50.6 1.7± | ***< 0.001*** | 48.2 ± 1.9 | 50.4 ± 2 | ***<0.001*** |
| Weight, kg | 3.1 ±0.4 | 3.8 ± 0.5 | ***<0.001*** | 3.2 ± 0.5 | 3.8 ± 0.5 | ***< 0.001*** | 3.2 ± 0.5 | 3.8 ± 0.5 | ***<0.001*** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Head circumference, cm | 34.5 ± 1.6 | 35.9 ± 1.5 | **0.002** | 34.8 ± 1.8 | 35.8 ± 1.5 | *0.03* | 34 ± 1.2 | 36.3 ± 1.2 | ***<0.001*** |
| Weight-for-age Z-score (WAZ) | -1.7 ± 0.6 | -0.1 ± 0.7 | ***<0.001*** | -1.4 ± 0.9 | -0.2 ± 0.8 | ***< 0.001*** | -1.4 ± 0.8 | -0.1 ± 0.8 | ***< 0.001*** |
| Length-for-age Z-score (LAZ) | -2.5 ± 0.7 | -1.2 ± 0.8 | ***<0.001*** | -2.6 ± 0.5 | -1.1 ± 0.6 | ***< 0.001*** | -2.3 ± 0.8 | -1.2 ± 0.8 | ***<0.001*** |
| Head circumference-for-age Z-score (HCAZ) | -1.3 ± 1 | -0.1 ± 1.1 | ***<0.001*** | -1.0 ± 1.1 | -0.2 ± 1.1 | *0.01* | -1.6 ± 0.5 | 0.2 ± 0.9 | ***<0.001*** |

Note: [1] Early stage is defined from 5 to 46 days postpartum; [2] Values are arithmetic means ± SD.[3] The level of significance of the *p*-values follows this format: *p < 0.05* are italicized denoting significance, **p < 0.01** are bold denoting stronger significance, ***p < 0.001*** are bold and italicized denoting very strong significance.

**Table 4.1 (B) - Population Characteristics for the 3 growth parameters (LAZ, WAZ, HCAZ) at established lactation**

| | Established stage (109 – 184 days)[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **WAZ < -1SD (33)** | **WAZ ≥ -1SD (24)** | *P*-value[3] | **LAZ < -2SD (23)** | **LAZ ≥ -2SD (34)** | *P*-value[3] | **HCAZ < -1SD (25)** | **HCAZ ≥ -1SD (31)** | *P*-value[3] |
| **Maternal Characteristics[2]** | | | | | | | | | |
| Age, y | 23.1 ± 6.3 | 26.3 ± 7.8 | 0.1 | 23 ± 6.8 | 25.5 ± 7.1 | 0.19 | 24.4 ± 6.8 | 24.4 ± 7.5 | 0.99 |
| Height, cm | 145.6 ± 5.1 | 148 ± 5.1 | 0.08 | 144.4 ± 4.7 | 148 ± 5.1 | **0.009** | 146.7 ± 5.4 | 146.3 ± 5 | 0.77 |
| Weight, kg | 47.7 ± 7.6 | 52.7 ± 7.5 | *0.02* | 46.7 ± 6.7 | 51.9 ± 8.0 | *0.01* | 48.5 ± 6.5 | 50.9 ± 8.9 | 0.26 |
| BMI, kg/m$^2$ | 22.5 ± 3.6 | 24.1 ± 3.1 | 0.09 | 22.3 ± 2.6 | 23.8 ± 3.8 | 0.13 | 22.5 ± 2.6 | 23.8 ± 3.9 | 0.15 |
| Parity | | | 0.64 | | | 0.16 | | | 0.45 |
| Primiparous, n (%) | 13 (39.4) | 8 (33.3) | | 11 (47.8) | 6 (25) | | 8 (32) | 13 (41.9) | |
| Multiparous, n (%) | 20 (60.6) | 16 (66.7) | | 12 (52.2) | 18 (75) | | 17 (68) | 18 (58.1) | |
| **Infant Characteristics [2]** | | | | | | | | | |
| Age, days | 119 ± 17 | 151 ± 22 | 0.95 | 151 ± 22 | 150 ± 17 | 0.36 | 149 ± 20 | 150 ± 18 | 0.81 |
| Sex | | | 0.3 | | | 0.76 | | | 0.22 |
| Male, n (%) | 21 (63.6) | 12 (50) | | 15 (65.2) | 18 (52.9) | | 17 (68) | 16 (51.6) | |
| Female, n (%) | 12 (36.4) | 12 (50) | | 8 (34.8) | 16 (47.1) | | 8 (32) | 15 (48.4) | |
| Length, cm | 59.9 ± 2.4 | 62.4 ± 2.3 | ***< 0.001*** | 58.9 ± 2.3 | 62.3 ± 2 | ***<0.001*** | 60.5 ± 2.8 | 61.2 ± 2.5 | 0.3 |
| Weight, kg | 5.9 ± 0.6 | 7.1 ± 0.6 | ***<0.001*** | 5.9 ± 0.8 | 6.7 ± 0.7 | ***<0.001*** | 6.2 ± 0.9 | 6.5 ± 0.7 | 0.11 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Head circumference, cm | 40.6 ± 1.8 | 41.7 ± 1.5 | *0.02* | 40.5 ± 1.8 | 41.4 ± 1.6 | 0.06 | 39.7 ± 1.4 | 42.1 ± 1.1 | ***<0.001*** |
| Weight-for-age Z-score (WAZ) | -1.8 ± 0.7 | -0.1 ± 0.5 | ***<0.001*** | -1.8 ± 0.9 | -0.6 ± 0.8 | ***<0.001*** | -1.4 ± 1 | -0.8 ± 1 | *0.03* |
| Length-for-age Z-score (LAZ) | -2.4 ± 0.9 | -1.1 ± 0.9 | ***<0.001*** | -2.9 ± 0.6 | -1.2 ± 0.7 | ***<0.001*** | -2.1 ± 1.1 | -1.7 ± 1 | 0.1 |
| Head circumference-for-age Z-score (HCAZ) | -1.2 ± 1.3 | -0.2 ± 1.1 | **0.003** | -1.3 ± 1.2 | -0.4 ± 1.3 | *0.01* | -2 ± 0.9 | 0.2 ± 0.7 | ***<0.001*** |

Note: [1] Late stage is defined from 109 – 184 days postpartum. [2] Values are arithmetic means ± SD.[3] The level of significance of the *p*-values follows this format: *p < 0.05* are italicized denoting significance, **p < 0.01** are bold denoting stronger significance, ***p < 0.001*** are bold and italicized denoting very strong significance

## 4.7 Figures

**Figure 4.1 – Mineral concentrations by lactation stage and normal and impaired growth in weight for age (WAZ).**

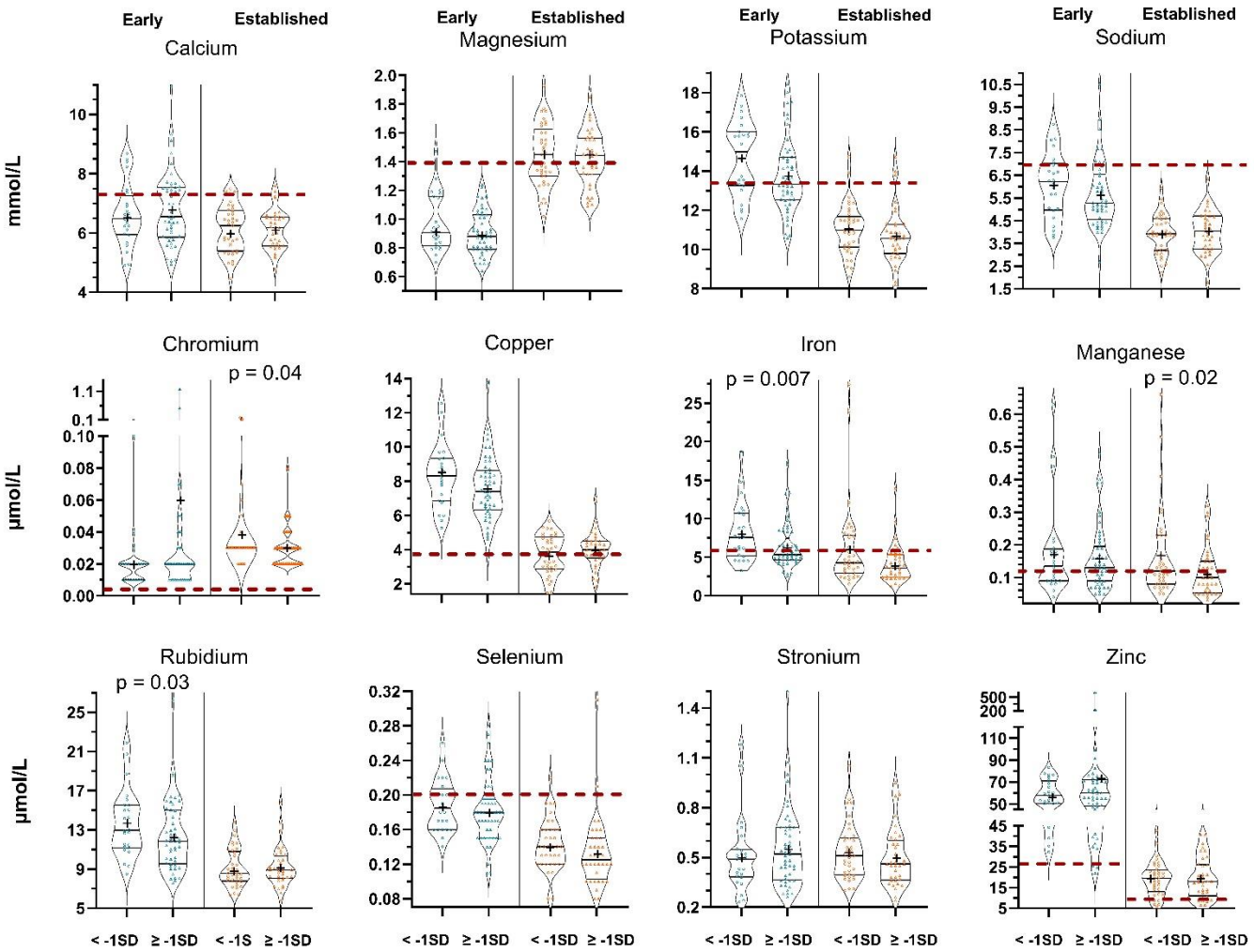


**Figure 4.1 – Mineral concentrations by lactation stage and normal and impaired growth in weight for age (WAZ).** Each milk mineral concentration is shown by early lactation (blue) and established lactation (purple) and mildly underweight (impaired growth) (< -1 SD) and normal weight (normal growth) (≥ -1 SD). Significant differences (p – value <0.05) in milk mineral concentrations between milk from infants with normal and impaired growth are shown indicating the corresponding p-value. In each violin plot, the middle line shows the median and the extreme lines represent the 25th and 75th quartile. The mean is shown as (+). The average mineral content in human milk considered by the INCAP for the Guatemalan population is shown as a reference in a dashed red line [Ca: 300 mg/L (7.4 mmol/L), Cu: 250 µg/L (3.9 µmol/L) , Fe: 350 µg/L (6.3 µmol/L), Mg: 34 mg/L (1.4 mmol/L), Mn: 3 µg/L (0.1µmol/L), K: 525 mg/L (13.4 mmol/L), Se: 18 µg/L (0.2 µmol/L), Na: 160 mg/L (7.0 mmol/L), Zn Early: 1750 µg/L (26.8 µmol/L), Zn Established: 700 µg/L (10.7 µmol/L)] (Menchú et al., 2012).No references values were identified for rubidium and strontium. Data show in mmol/L or µmol/L for practical scaling.

**Figure 4.2– Mineral concentrations by lactation stage and normal and impaired growth in length for age (LAZ).**

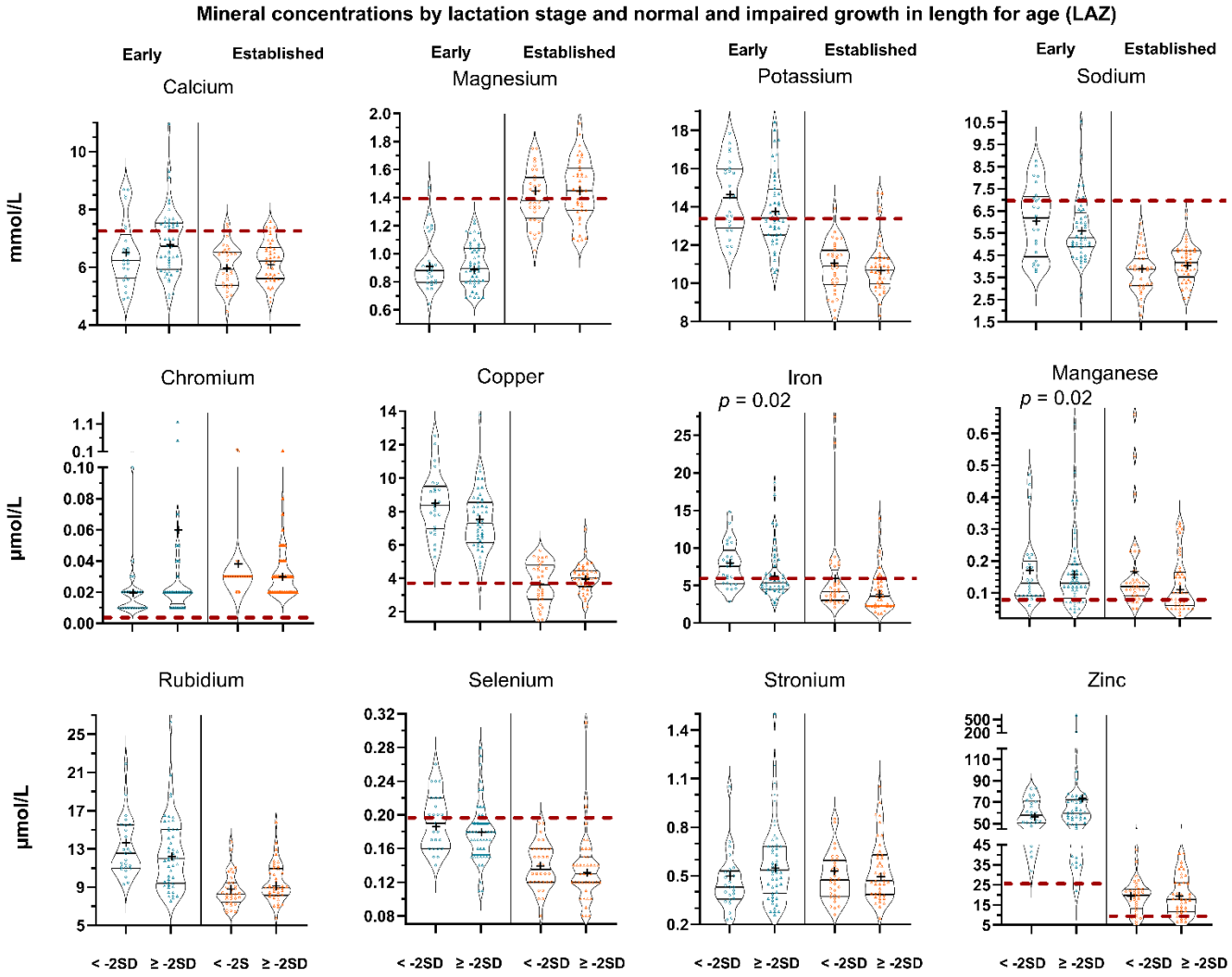


**Figure 4.2– Mineral concentrations by lactation stage and normal and impaired growth in length for age (LAZ).** Each milk mineral concentration is shown by early lactation (blue) and established lactation (purple) and stunted (impaired growth) (< -2 SD) and non-stunted (normal growth) (≥ -2 SD). Significant differences (p – value <0.05) in milk mineral concentrations between milk from infants with normal and impaired growth are shown indicating the corresponding p-value. In each violin plot, the middle line shows the median and the extreme lines represent the 25th and 75th quartile. The mean is shown as (+). The average mineral content in human milk considered by the INCAP for the Guatemalan population is shown as a reference in a dashed red line [Ca: 300 mg/L (7.4 mmol/L), Cu: 250 µg/L (3.9 µmol/L) , Fe: 350 µg/L (6.3 µmol/L), Mg: 34 mg/L (1.4 mmol/L), Mn: 3 µg/L (0.1µmol/L), K: 525 mg/L (13.4 mmol/L), Se: 18 µg/L (0.2 µmol/L), Na: 160 mg/L (7.0 mmol/L), Zn Early: 1750 µg/L (26.8 µmol/L), Zn Established: 700 µg/L (10.7 µmol/L)] (Menchú et al., 2012).No references values were identified for rubidium and strontium. Data show in mmol/L or µmol/L for practical scaling.

**Figure 4.3 –Mineral concentrations by lactation stage and normal and impaired growth in head circumference for age (HCAZ).**

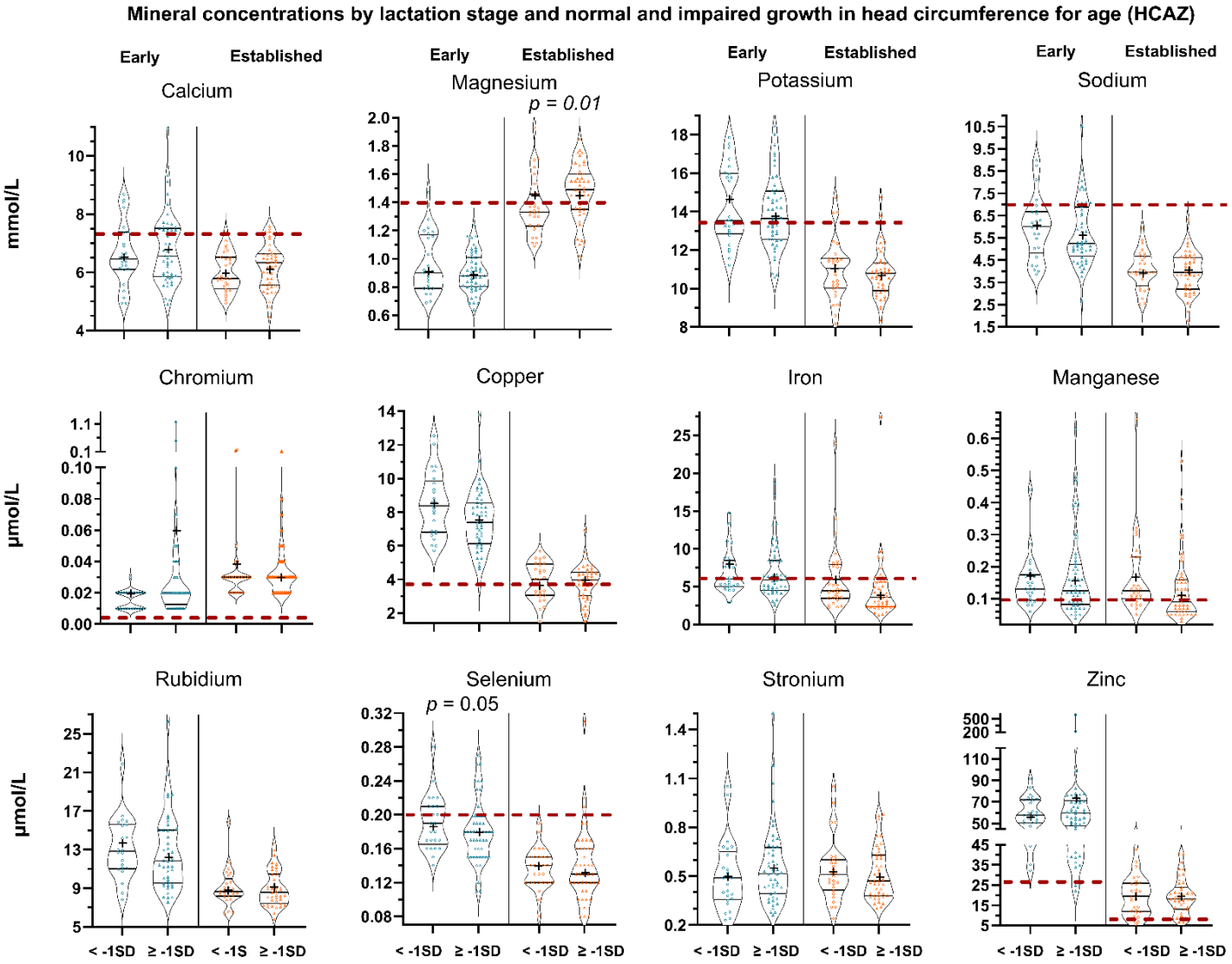


**Figure 4.3 –Mineral concentrations by lactation stage and normal and impaired growth in head circumference for age (HCAZ).** Each milk mineral concentration is shown by early lactation (blue) and established lactation (purple) and mildly impaired head circumference (< -1 SD) and normal head circumference (≥-1 SD). Significant differences (p – value <0.05) in milk mineral concentrations between milk from infants with normal and impaired growth are shown indicating the corresponding p-value. In each violin plot, the middle line shows the median and the extreme lines represent the 25th and 75th quartile. The mean is shown as (+). The average mineral content in human milk considered by the INCAP for the Guatemalan population is shown as a reference in a dashed red line [Ca: 300 mg/L (7.4 mmol/L), Cu: 250 µg/L (3.9 µmol/L) , Fe: 350 µg/L (6.3 µmol/L), Mg: 34 mg/L (1.4 mmol/L), Mn: 3 µg/L (0.1µmol/L), K: 525 mg/L (13.4 mmol/L), Se: 18 µg/L (0.2 µmol/L), Na: 160 mg/L (7.0 mmol/L), Zn Early: 1750 µg/L (26.8 µmol/L), Zn Established: 700 µg/L (10.7 µmol/L)] (Menchú et al., 2012).No references values were identified for rubidium and strontium. Data show in mmol/L or µmol/L for practical scaling.

**Figure 4.4 – Human milk microbiome composition.**

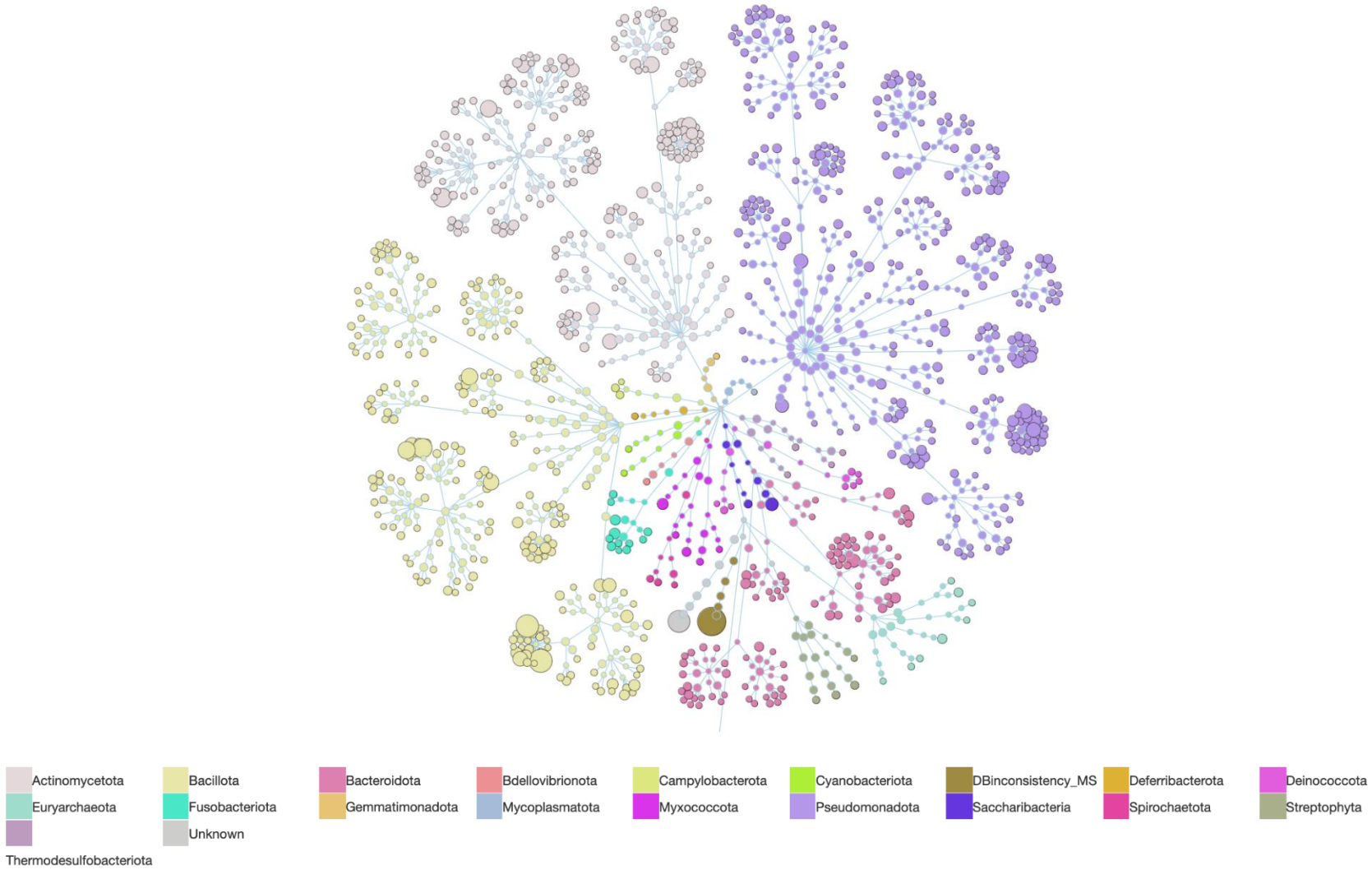
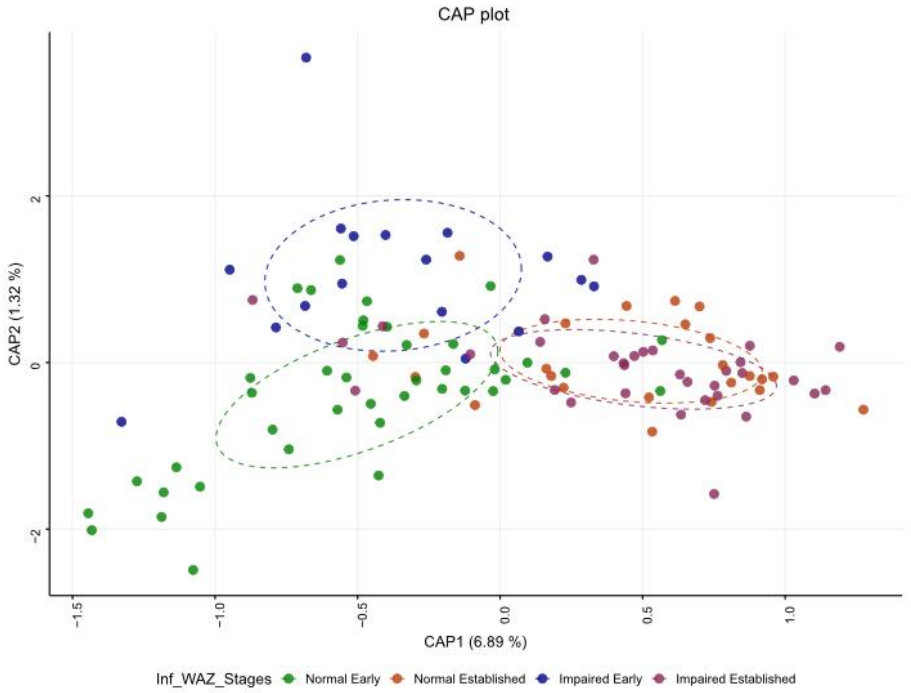


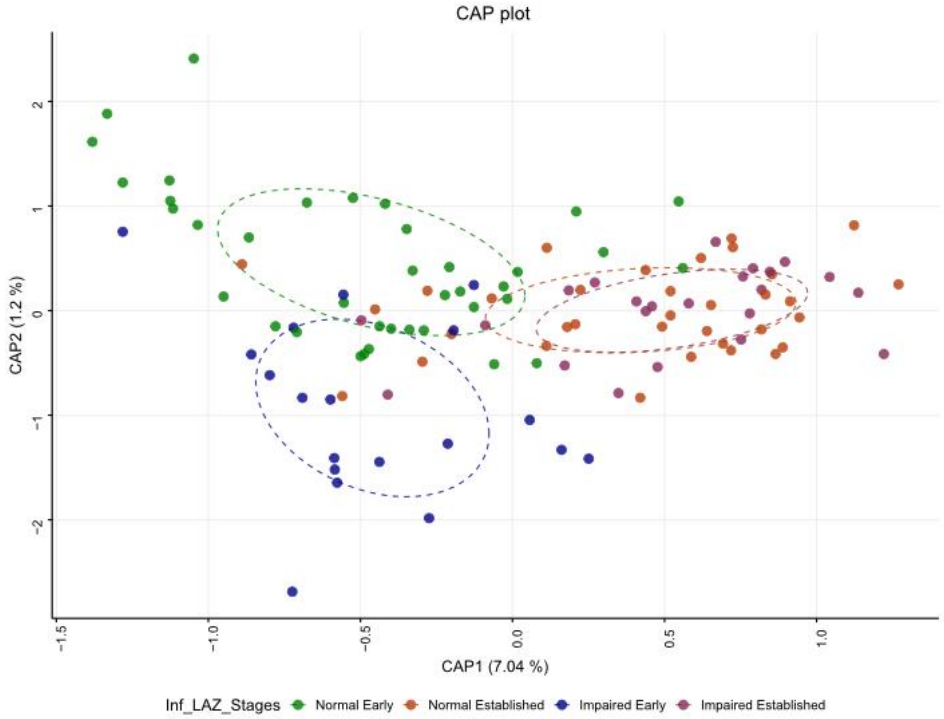

**Figure 4.4 –Human milk microbiome composition**. The total microbial community colored at phylum level of all samples.

**Figure 4.5 – Microbial diversity.**

**A) Beta diversity for WAZ**




**B) Beta diversity for LAZ**



**C) Beta diversity for HCAZ**

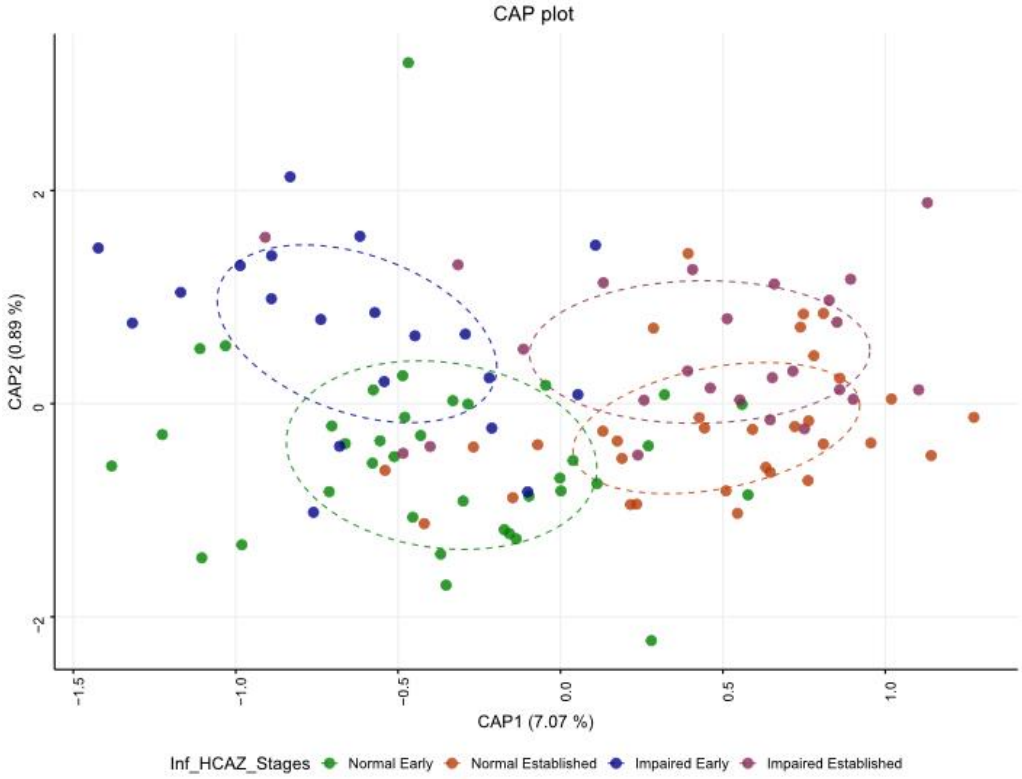



**Figure 4.5 – Microbial diversity.** The Canonical Analysis of Principal Coordinates (CAP) ordination segregated bacteria from normal and impaired growth infants at early and established lactation (Green: normal growth at early lactation, orange: normal growth at established lactation, blue: impaired growth at early lactation and purple: impaired growth at established lactation) ($p$ < 0.001). **A)** Beta diversity for WAZ**. B)** Beta diversity for LAZ, **C)** Beta diversity for HCAZ. (The other ordination plots corresponding to RDA are included as an appendix).

**Figure 4.6 – Correlation of DA bacteria in normal and impaired growth with milk minerals for WAZ at early lactation.**

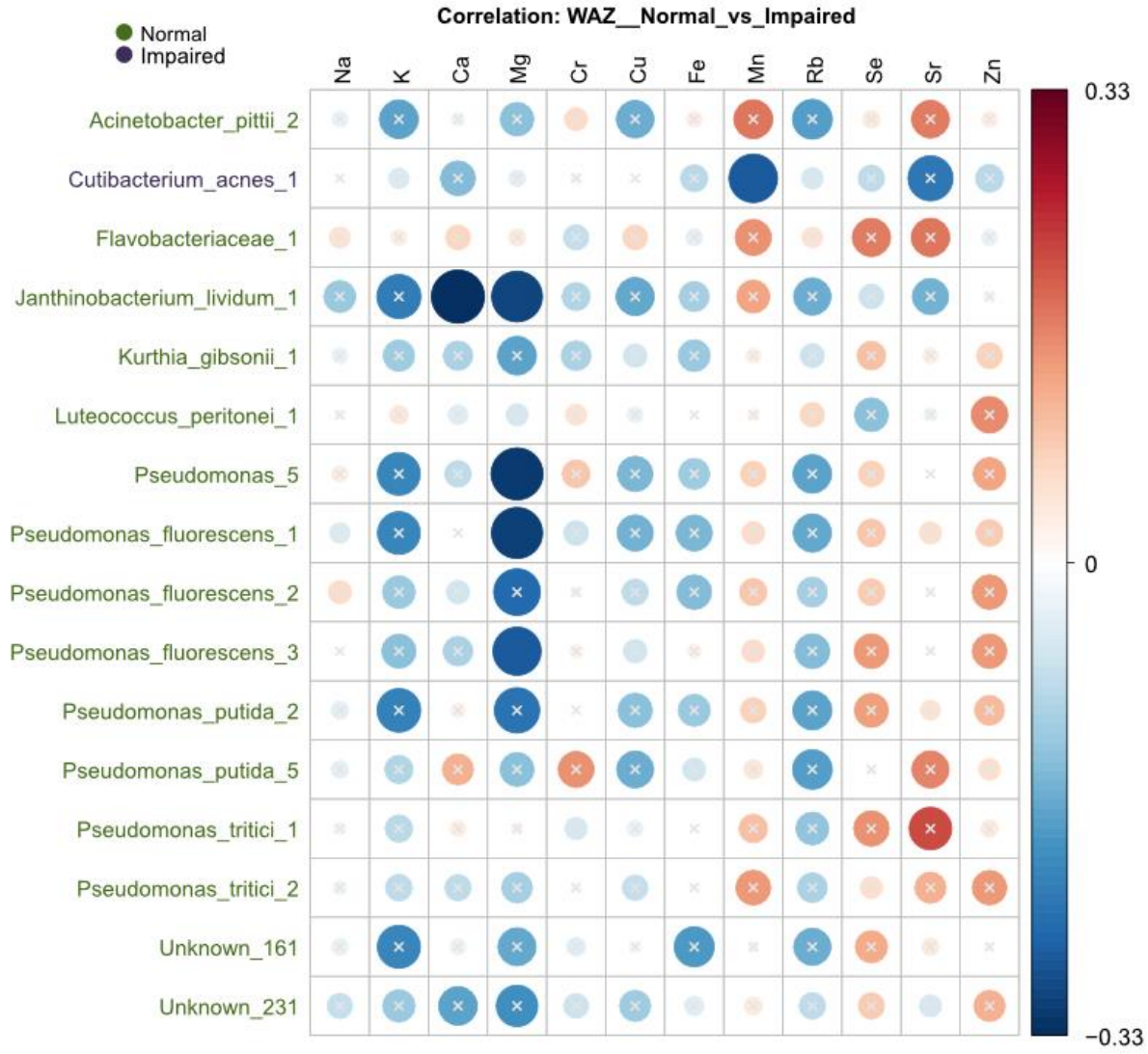



**Figure 4.6 – Heatmap correlation for WAZ at early lactation.** Heatmap of a bivariate Spearman correlation matrix between milk mineral concentrations and differentially abundant ESVs according to normal weight/normal growth (green) and mildly underweight/impaired growth (blue) infants at early lactation. Red circles represent positive correlations, and blue squares represent negative correlations. The intensity of the colors represents the degree of association between the ESVs and the milk mineral concentrations as measured by Spearman's correlations. The crosses represent non-significant correlations and circles with no crosses represent significant correlations (FDR < 0.05).

**Figure 4.7 – Correlation of DA bacteria in normal and impaired growth with milk minerals for LAZ at early lactation.**

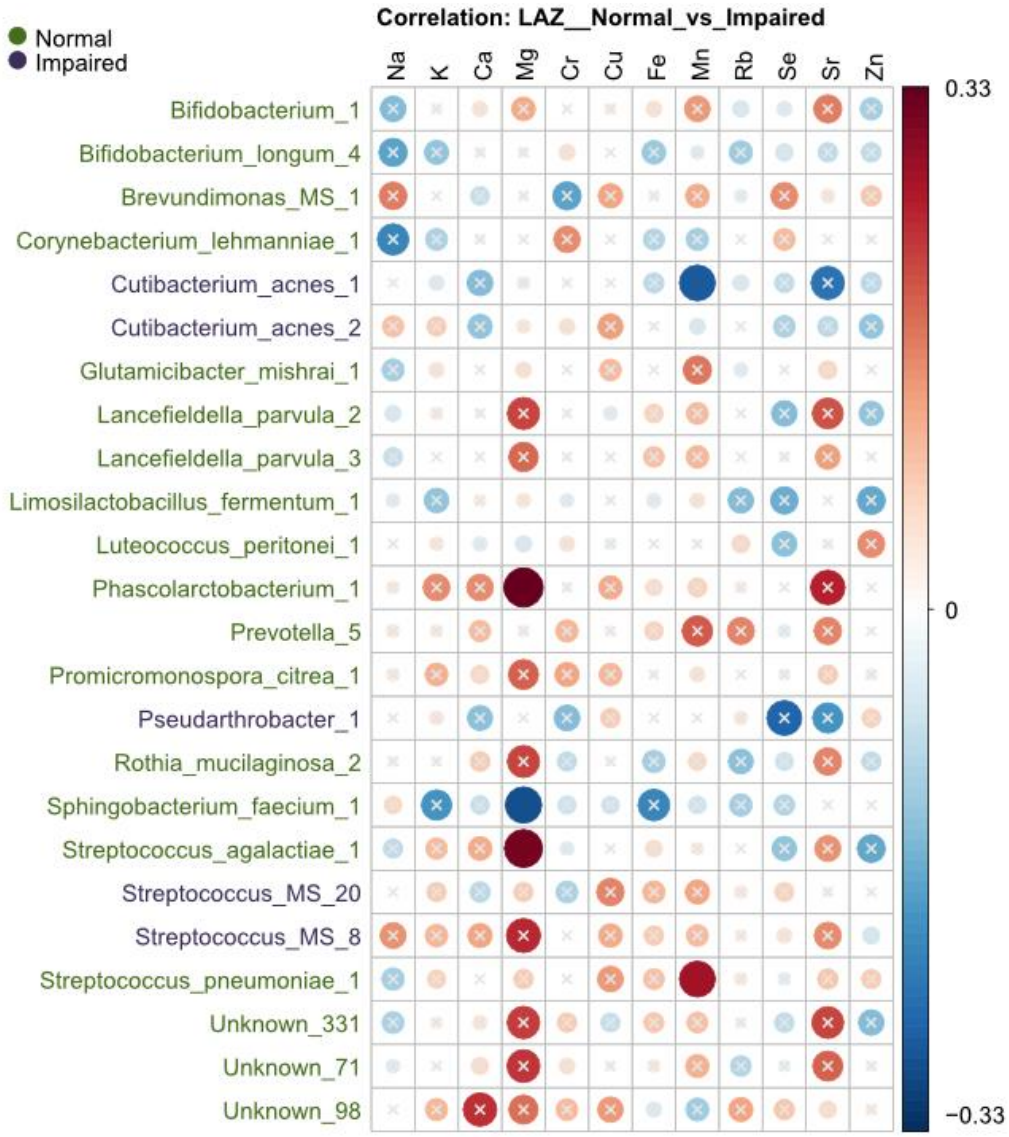
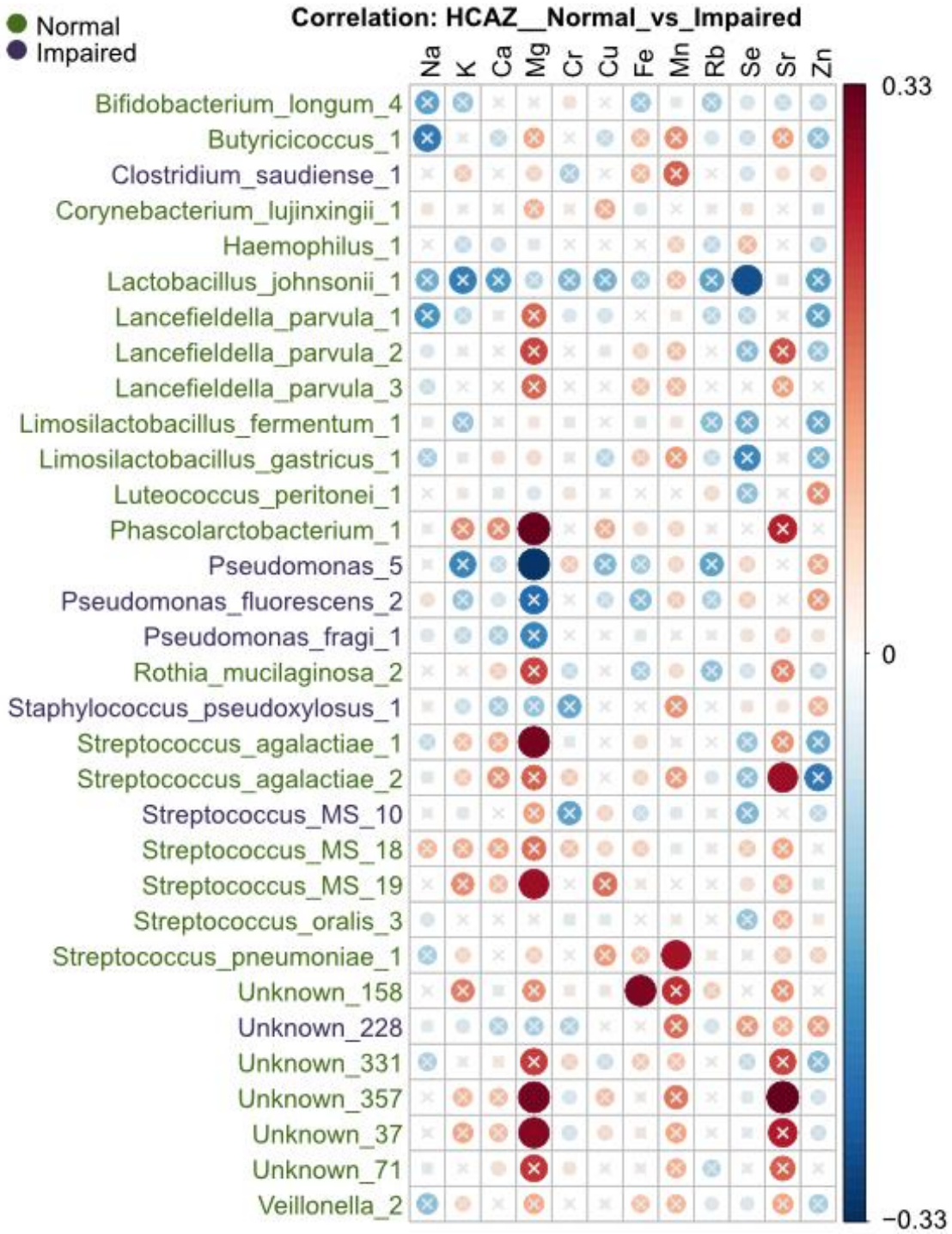



**Figure 4.7 – Heatmap correlation for LAZ at early lactation.** Heatmap of a bivariate Spearman correlation matrix between milk mineral concentrations and differentially abundant ESVs according to non-stunted/normal growth (green) and stunted/impaired growth (blue) infants at early lactation. Red circles represent positive correlations, and blue squares represent negative correlations. The intensity of the colors represents the degree of association between the ESVs and the milk mineral concentrations as measured by Spearman's correlations. The crosses represent non-significant correlations and circles with no crosses represent significant correlations (FDR < 0.05).

**Figure 4.8 – Correlation of DA bacteria in normal and impaired growth with milk minerals for HCAZ at early and established lactation.**

**A) Early lactation**



**B) Established lactation**

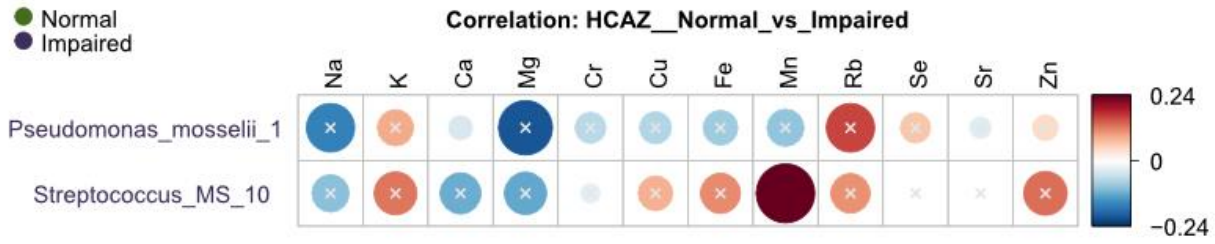
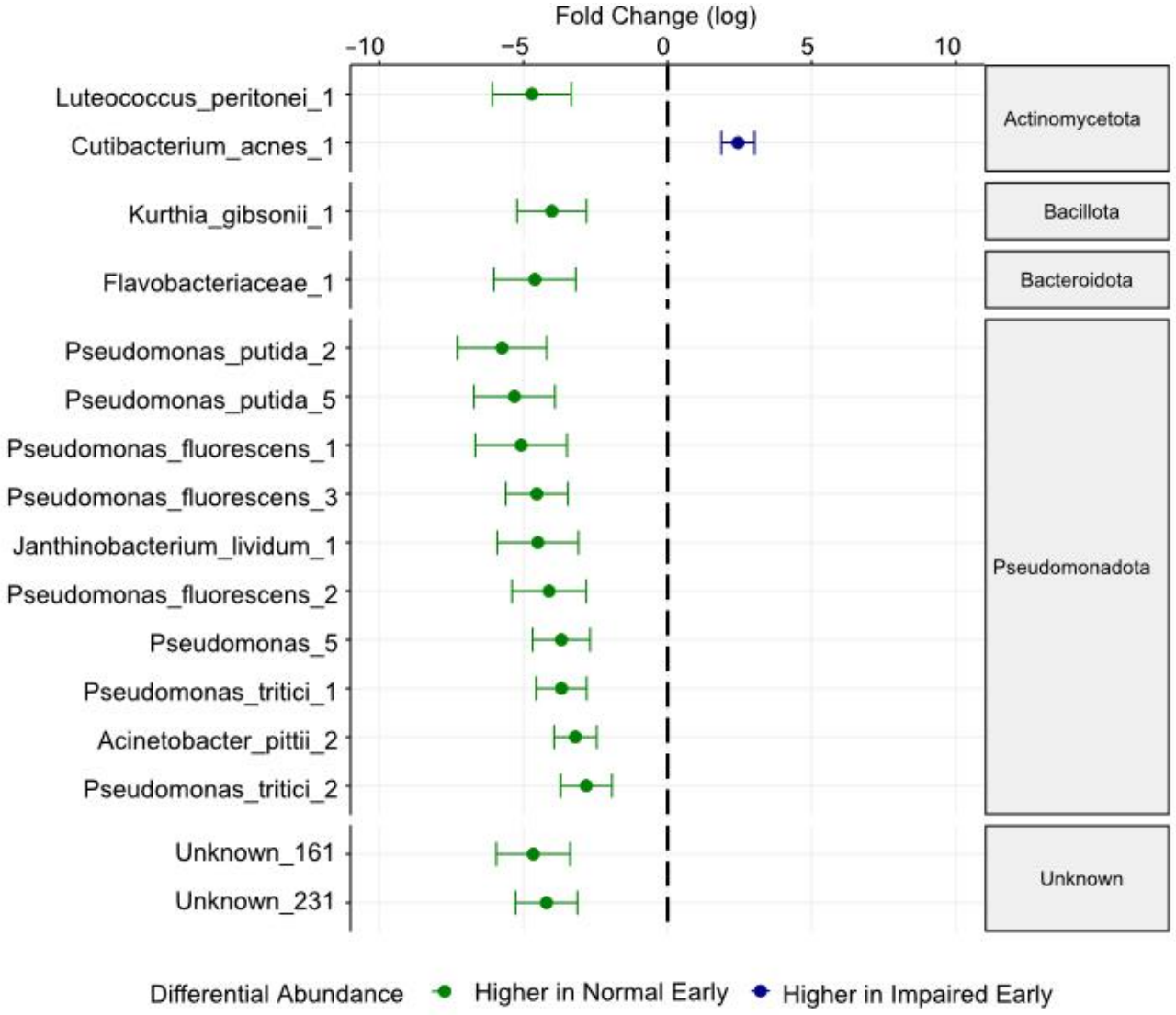



**Figure 4.8 – Heatmap correlation for HCAZ at early and established lactation.** Heatmap of a bivariate Spearman correlation matrix between milk mineral concentrations and differentially abundant ESVs according to normal head circumference (green) and mildly impaired head circumference (blue) infants at (A) early lactation and (B) established lactation. Red circles represent positive correlations, and blue circles represent negative correlations. The intensity of the colors represents the degree of association between the ESVs and the milk mineral concentrations as measured by Spearman's correlations. The crosses represent non-significant correlations and circles with no crosses represent significant correlations (FDR < 0.05).

### 4.8 Supplementary Figures

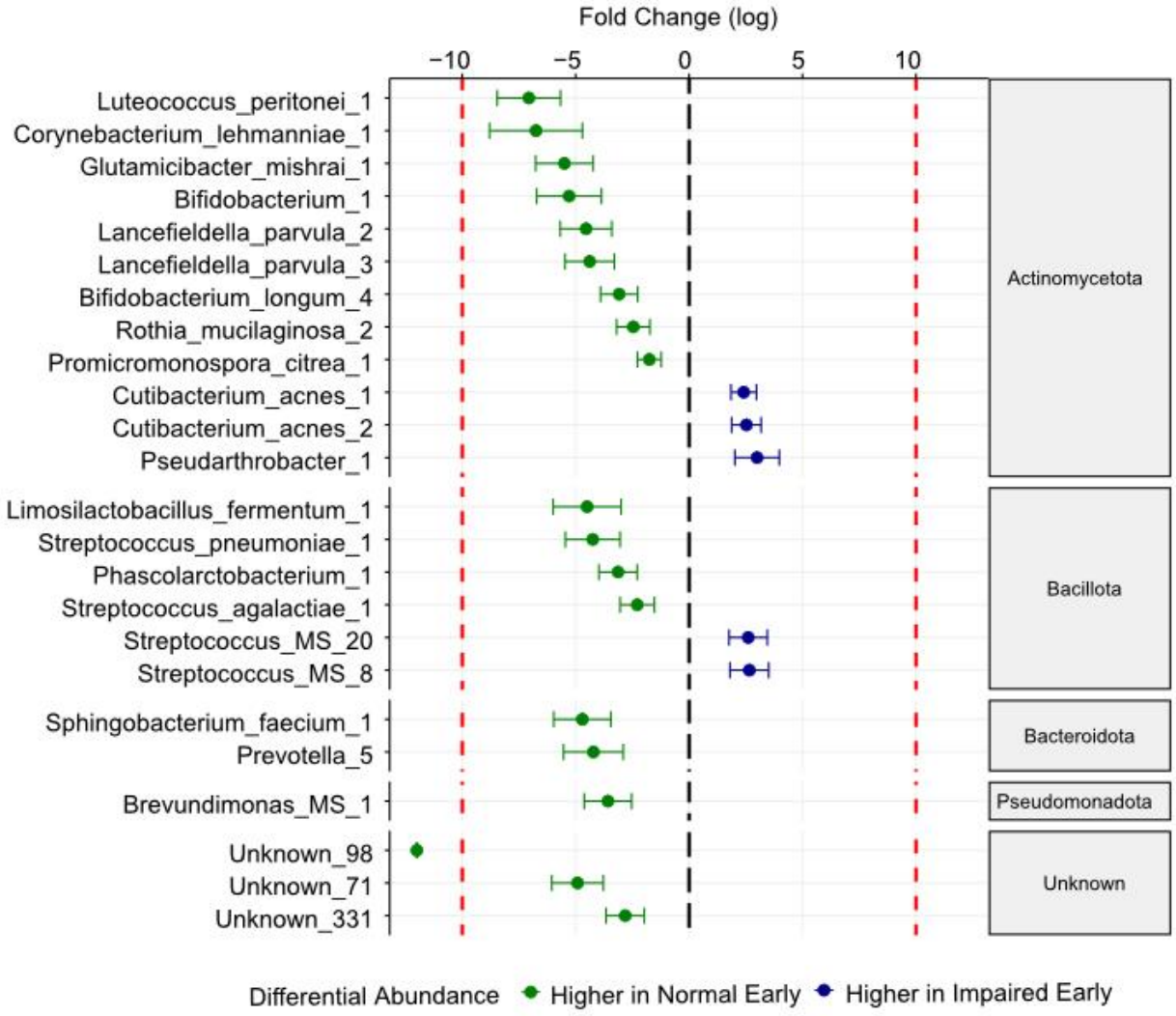
**Supplementary Figure 4.1.A – Differential abundance analysis in WAZ at early lactation.**

Significantly different ESVs between normal weight/normal growth and mildly underweight/impaired growth were estimated using DESeq (FDR < 0.05). Species are grouped by phylum and ordered by logFC in each group. There were not differential abundant bacteria in at established lactation in WAZ.



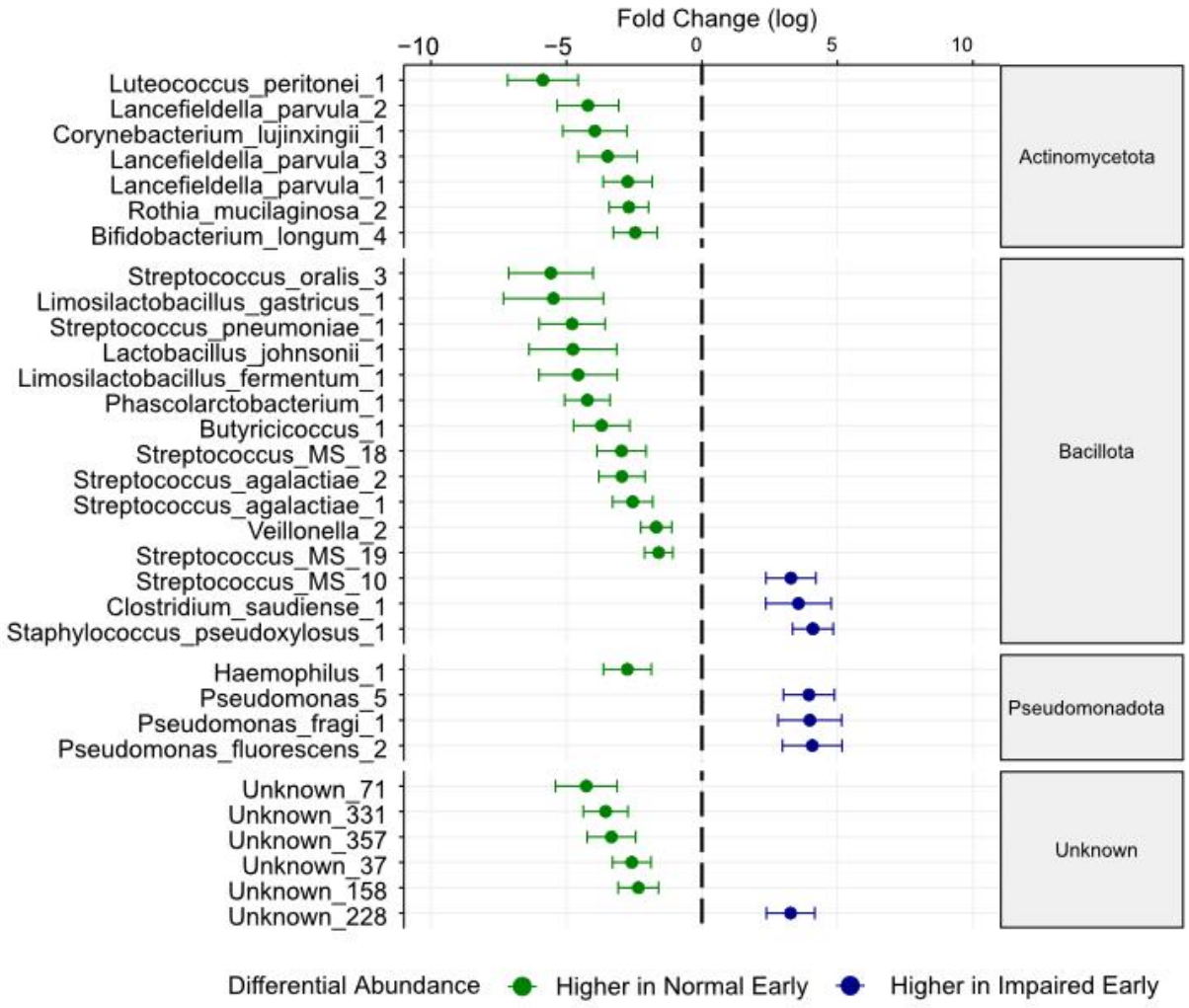
**Supplementary Figure 4.1.B – Differential abundance analysis in LAZ at early lactation.**

Significantly different ESVs between groups were estimated using DESeq (FDR < 0.05). Species are grouped by phylum and ordered by logFC in each group. The dashed red line indicates "infinite" log fold change, where an ESV had detectable counts in samples from only a single group. There were not differential abundant bacteria in at established lactation in LAZ.



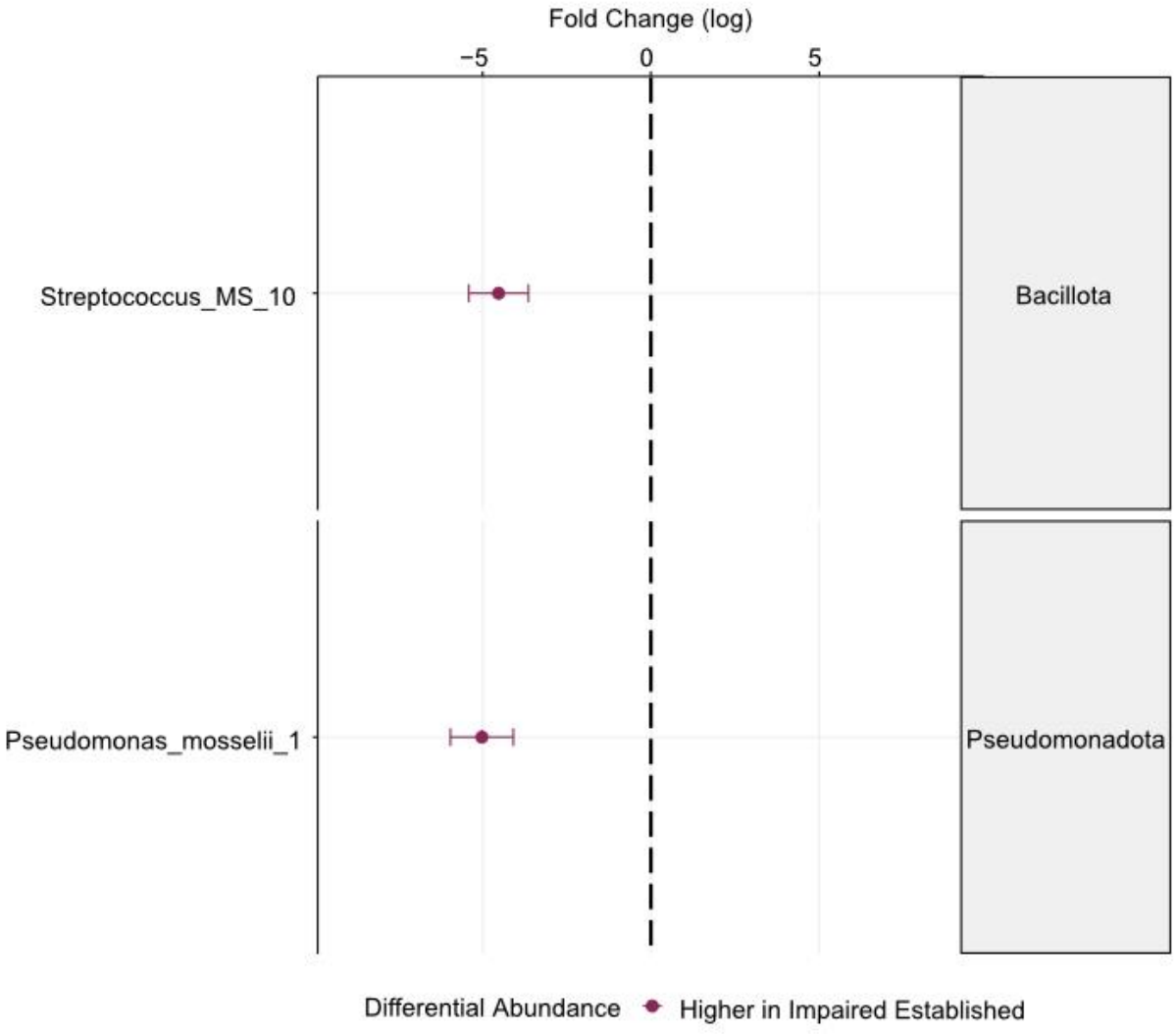
**Supplementary figure 4.1.C – Differential abundance analysis in HCAZ at early lactation.**

Significantly different ESVs between groups were estimated using DESeq (FDR < 0.05). Species are grouped by phylum and ordered by logFC in each group. The dashed red line indicates "infinite" log fold change, where an ESV had detectable counts in samples from only a single group.



**Supplementary figure 4.1.D – Differential abundance analysis in HCAZ at established lactation.**

Significantly different ESVs between groups were estimated using DESeq (FDR < 0.05). Species are grouped by phylum and ordered by logFC in each group. The dashed red line indicates “infinite” log fold change, where an ESV had detectable counts in samples from only a single group. There were not differential abundant bacteria in at established lactation in HCAZ.



**4.9 References**

Ajeeb, T. T., Gonzalez, E., Solomons, N. W., & Koski, K. G. (2022a). Human milk microbial species are associated with infant head-circumference during early and late lactation in Guatemalan mother-infant dyads. *Frontiers in Microbiology*, *13*, 908845. https://doi.org/10.3389/fmicb.2022.908845

Ajeeb, T. T., Gonzalez, E., Solomons, N. W., & Koski, K. G. (2022b). Human milk microbial species are associated with mild growth deficits during early infancy among Guatemalan mother–infant dyads. *Frontiers in Microbiomes*, *1*. https://www.frontiersin.org/articles/10.3389/frmbi.2022.1008467

Ajeeb, T. T., Gonzalez, E., Solomons, N. W., Vossenaar, M., & Koski, K. G. (2024). Human milk microbiome: Associations with maternal diet and infant growth. *Frontiers in Nutrition*, *11*, 1341777. https://doi.org/10.3389/fnut.2024.1341777

Aljewicz, M., Siemianowska, E., Cichosz, G., & Tońska, E. (2014). The effect of probiotics (Lactobacillus rhamnosus HN001, Lactobacillus paracasei LPC-37, and Lactobacillus acidophilus NCFM) on the availability of minerals from Dutch-type cheese. *Journal of Dairy Science*, *97*(8), 4824–4831. https://doi.org/10.3168/jds.2014-8240

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Azad, M. B., Brockway, M. M., & Reyes, S. M. (2024). Human milk composition and infant anthropometrics: Overview of a systematic review with clinical and research implications. *International Breastfeeding Journal*, *19*(1), 45. https://doi.org/10.1186/s13006-024-00652-x

Baricz, A., Teban, A., Chiriac, C. M., Szekeres, E., Farkas, A., Nica, M., Dascălu, A., Oprișan, C., Lavin, P., & Coman, C. (2018). Investigating the potential use of an Antarctic variant of

Janthinobacterium lividum for tackling antimicrobial resistance in a One Health approach. *Scientific Reports*, *8*(1), 15272. https://doi.org/10.1038/s41598-018-33691-6

Barone, M., D'Amico, F., Brigidi, P., & Turroni, S. (2022). Gut microbiome-micronutrient interaction: The key to controlling the bioavailability of minerals and vitamins? *BioFactors (Oxford, England)*, *48*(2), 307–314. https://doi.org/10.1002/biof.1835

Benjamin-Chung, J., Mertens, A., Colford, J. M., Hubbard, A. E., Laan, M. J. van der, Coyle, J., Sofrygin, O., Cai, W., Nguyen, A., Pokpongkiat, N. N., Djajadi, S., Seth, A., Jilek, W., Chung, E. O., Dayal, S., Hejazi, N., Malenica, I., Li, H., Hafen, R., … Consortium, members of the ki C. G. (2020). *Early childhood linear growth failure in low- and middle-income countries* (p. 2020.06.09.20127001). medRxiv. https://doi.org/10.1101/2020.06.09.20127001

Bergillos-Meca, T., Cabrera-Vique, C., Artacho, R., Moreno-Montoro, M., Navarro-Alarcón, M., Olalla, M., Giménez, R., Seiquer, I., & Ruiz-López, M. D. (2015). Does Lactobacillus plantarum or ultrafiltration process improve Ca, Mg, Zn and P bioavailability from fermented goats' milk? *Food Chemistry*, *187*, 314–321. https://doi.org/10.1016/j.foodchem.2015.04.051

Bhutta, Z., Ahmed, T., Black, R., Cousens, S., Dewey, K., E, G., Ba, H., B, K., Ss, M., Hp, S., & M, S. (2008). What works? Interventions for maternal and child undernutrition and survival. *Lancet (London, England)*, *371*(9610). https://doi.org/10.1016/S0140-6736(07)61693-6

Bielik, V., & Kolisek, M. (2021). Bioaccessibility and Bioavailability of Minerals in Relation to a Healthy Gut Microbiome. *International Journal of Molecular Sciences*, *22*(13), 6803. https://doi.org/10.3390/ijms22136803

Blanton, L. V., Charbonneau, M. R., Salih, T., Barratt, M. J., Venkatesh, S., Ilkaveya, O., Subramanian, S., Manary, M. J., Trehan, I., Jorgensen, J. M., Fan, Y.-M., Henrissat, B., Leyn, S. A., Rodionov, D. A., Osterman, A. L., Maleta, K. M., Newgard, C. B., Ashorn, P., Dewey, K. G., & Gordon, J. I. (2016). Gut bacteria that prevent growth impairments

transmitted by microbiota from malnourished children. *Science (New York, N.Y.)*, *351*(6275), aad3311. https://doi.org/10.1126/science.aad3311

Bostock, M., Ogievetsky, V., & Heer, J. (2011). D3 Data-Driven Documents. *IEEE Transactions on Visualization and Computer Graphics*, *17*(12), 2301–2309. https://doi.org/10.1109/TVCG.2011.185

Brockway, M. (Merilee), Daniel, A. I., Reyes, S. M., Gauglitz, J. M., Granger, M., McDermid, J. M., Chan, D., Refvik, R., Sidhu, K. K., Musse, S., Patel, P. P., Monnin, C., Lotoski, L., Geddes, D. T., Jehan, F., Kolsteren, P., Bode, L., Eriksen, K. G., Allen, L. H., … Azad, M. B. (2024). Human Milk Bioactive Components and Child Growth and Body Composition in the First 2 Years: A Systematic Review. *Advances in Nutrition*, *15*(1), 100127. https://doi.org/10.1016/j.advnut.2023.09.015

Brockway, M. (Merilee), Daniel, A. I., Reyes, S. M., Granger, M., McDermid, J. M., Chan, D., Refvik, R., Sidhu, K. K., Musse, S., Patel, P. P., Monnin, C., Lotoski, L., Geddes, D., Jehan, F., Kolsteren, P., Allen, L. H., Hampel, D., Eriksen, K. G., Rodriguez, N., & Azad, M. B. (2023). Human Milk Macronutrients and Child Growth and Body Composition in the First Two Years: A Systematic Review. *Advances in Nutrition*, *15*(1), 100149. https://doi.org/10.1016/j.advnut.2023.100149

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cheema, A. S., Gridneva, Z., Furst, A. J., Roman, A. S., Trevenen, M. L., Turlach, B. A., Lai, C. T., Stinson, L. F., Bode, L., Payne, M. S., & Geddes, D. T. (2022). Human Milk Oligosaccharides and Bacterial Profile Modulate Infant Body Composition during Exclusive Breastfeeding. *International Journal of Molecular Sciences*, *23*(5), Article 5. https://doi.org/10.3390/ijms23052865

Collins, M. D., Lawson, P. A., Nikolaitchouk, N., & Falsen, E. (2000). Luteococcus peritonei sp. Nov., isolated from the human peritoneum. *International Journal of Systematic and Evolutionary Microbiology*, *50 Pt 1*, 179–181. https://doi.org/10.1099/00207713-50-1-179

Copeland, A., Sikorski, J., Lapidus, A., Nolan, M., Del Rio, T. G., Lucas, S., Chen, F., Tice, H., Pitluck, S., Cheng, J.-F., Pukall, R., Chertkov, O., Brettin, T., Han, C., Detter, J. C., Kuske, C., Bruce, D., Goodwin, L., Ivanova, N., … Detter, J. C. (2009). Complete genome sequence of Atopobium parvulum type strain (IPP 1246T). *Standards in Genomic Sciences*, *1*(2), 166–173. https://doi.org/10.4056/sigs.29547

de Onis, M., & Branca, F. (2016). Childhood stunting: A global perspective. *Maternal & Child Nutrition*, *12 Suppl 1*, 12–26. https://doi.org/10.1111/mcn.12231

Devi, R., Kaur, T., Kour, D., Yadav, A., Yadav, A. N., Suman, A., Ahluwalia, A. S., & Saxena, A. K. (2022). Minerals solubilizing and mobilizing microbiomes: A sustainable approach for managing minerals' deficiency in agricultural soil. *Journal of Applied Microbiology*, *133*(3), 1245–1272. https://doi.org/10.1111/jam.15627

Ding, M., Li, B., Chen, H., Liang, D., Ross, R. P., Stanton, C., Zhao, J., Chen, W., & Yang, B. (2024). Human breastmilk-derived Bifidobacterium longum subsp. Infantis CCFM1269 regulates bone formation by the GH/IGF axis through PI3K/AKT pathway. *Gut Microbes*, *16*(1), 2290344. https://doi.org/10.1080/19490976.2023.2290344

Doneray, H., Olcaysu, E., Yildirim, A., & Ozden, A. (2017). The effect of the zinc concentration in breast milk on neonatal weight gain. *Journal of Trace Elements in Medicine and Biology*, *41*, 32–35. https://doi.org/10.1016/j.jtemb.2017.02.006

Ekkelenkamp, M. B., Rooijakkers, S. H. M., & Bonten, M. J. M. (2010). Chapter 165—Staphylococci and micrococci. In J. Cohen, S. M. Opal, & W. G. Powderly (Eds.), *Infectious*

*Diseases (Third Edition)* (pp. 1632–1644). Mosby. https://doi.org/10.1016/B978-0-323-04579-7.00165-9

Eriksen, K. G., Christensen, S. H., Lind, M. V., & Michaelsen, K. F. (2018). Human milk composition and infant growth. *Current Opinion in Clinical Nutrition and Metabolic Care*, *21*(3), 200–206. https://doi.org/10.1097/MCO.0000000000000466

Eriksen, K. G., Johnson, W., Sonko, B., Prentice, A. M., Darboe, M. K., & Moore, S. E. (2017). Following the World Health Organization's Recommendation of Exclusive Breastfeeding to 6 Months of Age Does Not Impact the Growth of Rural Gambian Infants. *The Journal of Nutrition*, *147*(2), 248–255. https://doi.org/10.3945/jn.116.241737

Fan, L., Xia, Y., Wang, Y., Han, D., Liu, Y., Li, J., Fu, J., Wang, L., Gan, Z., Liu, B., Fu, J., Zhu, C., Wu, Z., Zhao, J., Han, H., Wu, H., He, Y., Tang, Y., Zhang, Q., … Ren, W. (2023). Gut microbiota bridges dietary nutrients and host immunity. *Science China. Life Sciences*, *66*(11), 2466–2514. https://doi.org/10.1007/s11427-023-2346-1

Fehr, K., Moossavi, S., Sbihi, H., Boutin, R. C. T., Bode, L., Robertson, B., Yonemitsu, C., Field, C. J., Becker, A. B., Mandhane, P. J., Sears, M. R., Khafipour, E., Moraes, T. J., Subbarao, P., Finlay, B. B., Turvey, S. E., & Azad, M. B. (2020). Breastmilk Feeding Practices Are Associated with the Co-Occurrence of Bacteria in Mothers' Milk and the Infant Gut: The CHILD Cohort Study. *Cell Host & Microbe*, *28*(2), 285-297.e4. https://doi.org/10.1016/j.chom.2020.06.009

García-Legorreta, A., Soriano-Pérez, L. A., Flores-Buendía, A. M., Medina-Campos, O. N., Noriega, L. G., Granados-Portillo, O., Nambo-Venegas, R., Tovar, A. R., Mendoza-Vargas, A., Barrera-Oviedo, D., Pedraza-Chaverri, J., & Palacios-González, B. (2020). Effect of Dietary Magnesium Content on Intestinal Microbiota of Rats. *Nutrients*, *12*(9), 2889. https://doi.org/10.3390/nu12092889

Geddes, D. T., Gridneva, Z., Perrella, S. L., Mitoulas, L. R., Kent, J. C., Stinson, L. F., Lai, C. T., Sakalidis, V., Twigger, A.-J., & Hartmann, P. E. (2021). 25 Years of Research in Human Lactation: From Discovery to Translation. *Nutrients*, *13*(9), 3071. https://doi.org/10.3390/nu13093071

Giugliani, E. R. J., Horta, B. L., Loret de Mola, C., Lisboa, B. O., & Victora, C. G. (2015). Effect of breastfeeding promotion interventions on child growth: A systematic review and meta-analysis. *Acta Paediatrica (Oslo, Norway: 1992)*, *104*(467), 20–29. https://doi.org/10.1111/apa.13160

Gonzalez, E., Pitre, F. E., & Brereton, N. J. B. (2019). ANCHOR: A 16S rRNA gene amplicon pipeline for microbial analysis of multiple environmental samples. *Environmental Microbiology*, *21*(7), 2440–2468. https://doi.org/10.1111/1462-2920.14632

Hall, J., Cong, Z., Imamura-Kawasawa, Y., Kidd, B., Dudley, J., Thiboutot, D., & Nelson, A. (2018). Isolation and Identification of the Follicular Microbiome: Implications for Acne Research. *The Journal of Investigative Dermatology*, *138*(9). https://doi.org/10.1016/j.jid.2018.02.038

Hanna, M., & Noor, A. (2023). Streptococcus Group B. In *StatPearls [Internet]*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK553143/

Kour, D., Rana, K. L., Kaur, T., Yadav, N., Yadav, A. N., Kumar, M., Kumar, V., Dhaliwal, H. S., & Saxena, A. K. (2021). Biodiversity, current developments and potential biotechnological applications of phosphorus-solubilizing and -mobilizing microbes: A review. *Pedosphere*, *31*(1), 43–75. https://doi.org/10.1016/S1002-0160(20)60057-1

Lambert, M. N. T., Thybo, C. B., Lykkeboe, S., Rasmussen, L. M., Frette, X., Christensen, L. P., & Jeppesen, P. B. (2017). Combined bioavailable isoflavones and probiotics improve bone status and estrogen metabolism in postmenopausal osteopenic women: A randomized

controlled trial. *The American Journal of Clinical Nutrition*, *106*(3), 909–920. https://doi.org/10.3945/ajcn.117.153353

Li, C., Solomons, N. W., Scott, M. E., & Koski, K. G. (2016). Minerals and Trace Elements in Human Breast Milk Are Associated with Guatemalan Infant Anthropometric Outcomes within the First 6 Months. *The Journal of Nutrition*, *146*(10), 2067–2074. https://doi.org/10.3945/jn.116.232223

Li, C., Solomons, N. W., Scott, M. E., & Koski, K. G. (2018). Subclinical mastitis (SCM) and proinflammatory cytokines are associated with mineral and trace element concentrations in human breast milk. *Journal of Trace Elements in Medicine and Biology*, *46*, 55–61. https://doi.org/10.1016/j.jtemb.2017.11.010

Li, C., Solomons, N. W., Scott, M. E., & Koski, K. G. (2019). Anthropometry before Day 46 and Growth Velocity before 6 Months of Guatemalan Breastfed Infants Are Associated with Subclinical Mastitis and Milk Cytokines, Minerals, and Trace Elements. *The Journal of Nutrition*, *149*(9), 1651–1659. https://doi.org/10.1093/jn/nxz109

Lopez Leyva, L., Gonzalez, E., Maurice, C. F., & Koski, K. G. (2025). Milk mineral composition is strongly associated with the human milk microbiome. *Frontiers in Nutrition*.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Lyons, K. E., Fouhy, F., O' Shea, C.-A., Ryan, C. A., Dempsey, E. M., Ross, R. P., & Stanton, C. (2021). Effect of storage, temperature, and extraction kit on the phylogenetic composition detected in the human milk microbiota. *MicrobiologyOpen*, *10*(1), e1127. https://doi.org/10.1002/mbo3.1127

Marín, M. L., Arroyo, R., Jiménez, E., Gómez, A., Fernández, L., & Rodríguez, J. M. (2009). Cold storage of human milk: Effect on its bacterial composition. *Journal of Pediatric*

*Gastroenterology and Nutrition*, *49*(3), 343–348. https://doi.org/10.1097/MPG.0b013e31818cf53d

Marquis, G. S., Habicht, J. P., Lanata, C. F., Black, R. E., & Rasmussen, K. M. (1997). Association of breastfeeding and stunting in Peruvian toddlers: An example of reverse causality. *International Journal of Epidemiology*, *26*(2), 349–356. https://doi.org/10.1093/ije/26.2.349

Mata, L. J., Kronmal, R. A., García, B., Butler, W., Urrutia, J. J., & Murillo, S. (1976). Breast-feeding, weaning and the diarrhoeal syndrome in a Guatemalan Indian village. *Ciba Foundation Symposium*, *42*, 311–338. https://doi.org/10.1002/9780470720240.ch17

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Menchú, M. T., Torún, B., & Elias, L. G. (2012). *Recomendaciones Dietéticas Diarias del INCAP*. INCAP. https://inec.cr/wwwisis/documentos/INCAP/Recomendaciones_Dieteticas_Diarias_del_INCAP.pdf

Moossavi, S., Sepehri, S., Robertson, B., Bode, L., Goruk, S., Field, C. J., Lix, L. M., de Souza, R. J., Becker, A. B., Mandhane, P. J., Turvey, S. E., Subbarao, P., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Khafipour, E., & Azad, M. B. (2019). Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors. *Cell Host & Microbe*, *25*(2), 324-335.e4. https://doi.org/10.1016/j.chom.2019.01.011

O'Callaghan, A., & van Sinderen, D. (2016). Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Frontiers in Microbiology*, *7*, 925. https://doi.org/10.3389/fmicb.2016.00925

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2020). *Vegan: Community Ecology Package* (Version 2.5-7) [Computer software]. https://CRAN.R-project.org/package=vegan

Pachikian, B. D., Neyrinck, A. M., Deldicque, L., De Backer, F. C., Catry, E., Dewulf, E. M., Sohet, F. M., Bindels, L. B., Everard, A., Francaux, M., Guiot, Y., Cani, P. D., & Delzenne, N. M. (2010). Changes in Intestinal Bifidobacteria Levels Are Associated with the Inflammatory Response in Magnesium-Deficient Mice. *The Journal of Nutrition*, *140*(3), 509–514. https://doi.org/10.3945/jn.109.117374

Perrella, S., Gridneva, Z., Lai, C. T., Stinson, L., George, A., Bilston-John, S., & Geddes, D. (2021). Human milk composition promotes optimal infant growth, development and health. *Seminars in Perinatology*, *45*(2), 151380. https://doi.org/10.1016/j.semperi.2020.151380

Piuri, G., Zocchi, M., Della Porta, M., Ficara, V., Manoni, M., Zuccotti, G. V., Pinotti, L., Maier, J. A., & Cazzola, R. (2021). Magnesium in Obesity, Metabolic Syndrome, and Type 2 Diabetes. *Nutrients*, *13*(2), 320. https://doi.org/10.3390/nu13020320

Platsidaki, E., & Dessinioti, C. (2018). Recent advances in understanding Propionibacterium acnes ( Cutibacterium acnes) in acne. *F1000Research*, *7*, F1000 Faculty Rev-1953. https://doi.org/10.12688/f1000research.15659.1

Revelle, W. (Director). (2017). *psych: Procedures for Personality and Psychological Research* [Video recording]. https://CRAN.R-project.org/package=psych

Reyes, S. M., Brockway, M. (Merilee), McDermid, J. M., Chan, D., Granger, M., Refvik, R., Sidhu, K. K., Musse, S., Monnin, C., Lotoski, L., Geddes, D. T., Jehan, F., Kolsteren, P., Allen, L. H., Hampel, D., Eriksen, K. G., Rodriguez, N., & Azad, M. B. (2023). Human Milk Micronutrients and Child Growth and Body Composition in the First 2 years: A Systematic

Review. *Advances in Nutrition*, *15*(1), 100082. https://doi.org/10.1016/j.advnut.2023.06.005

Robertson, R. C., Edens, T. J., Carr, L., Mutasa, K., Gough, E. K., Evans, C., Geum, H. M., Baharmand, I., Gill, S. K., Ntozini, R., Smith, L. E., Chasekwa, B., Majo, F. D., Tavengwa, N. V., Mutasa, B., Francis, F., Tome, J., Stoltzfus, R. J., Humphrey, J. H., … Manges, A. R. (2023). The gut microbiome and early-life growth in a population with high prevalence of stunting. *Nature Communications*, *14*(1), 654. https://doi.org/10.1038/s41467-023-36135-6

Robertson, R. C., Manges, A. R., Finlay, B. B., & Prendergast, A. J. (2019). The Human Microbiome and Child Growth—First 1000 Days and Beyond. *Trends in Microbiology*, *27*(2), 131–147. https://doi.org/10.1016/j.tim.2018.09.008

Ruel, M. T., & Menon, P. (2002). Child feeding practices are associated with child nutritional status in Latin America: Innovative uses of the demographic and health surveys. *The Journal of Nutrition*, *132*(6), 1180–1187. https://doi.org/10.1093/jn/132.6.1180

Sanjulián, L., Lamas, A., Barreiro, R., Cepeda, A., Fente, C. A., & Regal, P. (2021). Bacterial Diversity of Breast Milk in Healthy Spanish Women: Evolution from Birth to Five Years Postpartum. *Nutrients*, *13*(7), Article 7. https://doi.org/10.3390/nu13072414

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Horn, D. J. V., & Weber, C. F. (2009). Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541. https://doi.org/10.1128/AEM.01541-09

Shamash, M., & Maurice, C. F. (2022). Phages in the infant gut: A framework for virome development during early life. *The ISME Journal*, *16*(2), 323–330. https://doi.org/10.1038/s41396-021-01090-x

Simondon, K. B., Diallo, A., & Simondon, F. (2002). Breastfeeding and Growth in Rural Senegalese Toddlers. In B. Koletzko, K. F. Michaelsen, & O. Hernell (Eds.), *Short and Long Term Effects of Breast Feeding on Child Health* (pp. 173–181). Springer US. https://doi.org/10.1007/0-306-46830-1_15

UNICEF. (2023). *Breastfeeding*. UNICEF DATA. https://data.unicef.org/topic/nutrition/breastfeeding/

UNICEF/WHO/World Bank Group. (2023, May 18). *UNICEF-WHO-The World Bank: Joint Child Malnutrition Estimates (JME) — Levels and Trends – 2023 edition*. UNICEF DATA. https://data.unicef.org/resources/jme-report-2023/

Vatanen, T., Ang, Q. Y., Siegwald, L., Sarker, S. A., Le Roy, C. I., Duboux, S., Delannoy-Bruno, O., Ngom-Bru, C., Boulangé, C. L., Stražar, M., Avila-Pacheco, J., Deik, A., Pierce, K., Bullock, K., Dennis, C., Sultana, S., Sayed, S., Rahman, M., Ahmed, T., … Xavier, R. J. (2022). A distinct clade of Bifidobacterium longum in the gut of Bangladeshi children thrives during weaning. *Cell*, *185*(23), 4280-4297.e12. https://doi.org/10.1016/j.cell.2022.10.011

Wei, T., & Simko, V. (2021). *Package "corrplot."* R Core Team. https://github.com/taiyun/corrplot

WHO. (2018). *Reducing stunting in children: Equity considerations for achieving the global targets 2025*. https://www.who.int/publications/i/item/9789241513647

*WHO child growth standards: Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: methods and development*. (2006). https://www.who.int/publications-detail-redirect/924154693X

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer International Publishing. https://doi.org/10.1007/978-3-319-24277-4

Wren, H. M., Solomons, N. W., Chomat, A. M., Scott, M. E., & Koski, K. G. (2015). Cultural Determinants of Optimal Breastfeeding Practices among Indigenous *Mam* -Mayan Women in the Western Highlands of Guatemala. *Journal of Human Lactation*, *31*(1), 172–184. https://doi.org/10.1177/0890334414560194

Wren-Atilola, H. M., Solomons, N. W., Scott, M. E., & Koski, K. G. (2018). Infant growth faltering linked to subclinical mastitis, maternal faecal-oral contamination, and breastfeeding. *Maternal & Child Nutrition*, *15*(3), e12756. https://doi.org/10.1111/mcn.12756

Wu, F., Guo, X., Zhang, J., Zhang, M., Ou, Z., & Peng, Y. (2017). Phascolarctobacterium faecium abundant colonization in human gastrointestinal tract. *Experimental and Therapeutic Medicine*, *14*(4), 3122–3126. https://doi.org/10.3892/etm.2017.4878

Zafar, H., & Saier, M. H. (2021). Gut Bacteroides species in health and disease. *Gut Microbes*, *13*(1), 1848158. https://doi.org/10.1080/19490976.2020.1848158

Zhang, Z., Zhao, L., Wu, J., Pan, Y., Zhao, G., Li, Z., & Zhang, L. (2023). The Effects of Lactobacillus johnsonii on Diseases and Its Potential Applications. *Microorganisms*, *11*(10), 2580. https://doi.org/10.3390/microorganisms11102580

# Chapter 5: General Discussion and Conclusions

## 5.1 Main outcomes

Despite the well-known benefits of breastfeeding, impaired growth remains prevalent among Guatemalan infants under six months (UNICEF/WHO/World Bank Group, 2023). While human milk is often considered the 'ideal food' for infants, evidence suggests that it may lack sufficient vitamins and minerals in some populations (Daniels et al., 2019; Donohue et al., 2020; Li et al., 2016). However, milk minerals in particular have received limited attention. The association between maternal mineral intake and human milk mineral content remains inconclusive, and little is known about the interactions between various milk components—specifically, the relationship between milk minerals and the milk microbiome. Furthermore, there is a lack of longitudinal studies examining how the milk microbiome might influence infant growth, leaving important knowledge gaps in understanding the full impact of human milk composition on early development, especially in developing countries and indigenous communities. Additionally, to date there had been no direct comparison of existing bacterial primers for milk microbiome analyzes to ensure successful detection of the expected genera commonly reported in milk such as *Cutibacterium* and *Bifidobacterium*. In milk microbiome research, the common targeted regions were V1 – V4 with the primers 27F – 338R and U515F – 806R, but these primers are not capable of detecting both *Cutibacterium* and *Bifidobacterium;* they only identify one or the other. To fill these knowledge gaps, we reviewed the history and sources of milk microbiome, as well as the factors that affect milk microbiome composition and the methodological developments for studying it (Chapter 2). We next conducted an observational cross-sectional study to associate maternal diet mineral intake, milk mineral concentrations, and milk microbiome diversity at early and established lactation periods (Chapter 3). Finally, through a longitudinal study, we aimed to determine if human milk minerals are associated with human milk microbiome diversity at early and established lactation and associate them with infant growth using the Growth Reference Standards WAZ, LAZ, and HCAZ parameters (Chapter 4).

We identified a set of primers capable of capturing the expected genera in milk microbiome and we validated their effectiveness in the milk microbiome field of research. Our novel observations uncovered strong associations of milk mineral concentrations with milk microbial diversity but a weak association with maternal diet. We reported the highly dynamic nature of mineral and microbial composition of human milk, where iron, manganese and selenium stand out as minerals associated with milk microbiome diversity at early and established lactation. When looking into the association of minerals with infant growth, manganese continued to have an important role, and magnesium also emerged as a mineral with several correlations with milk bacteria and with an important association with infant growth. When determining the association between milk microbiome and infant growth, we observed that infants with normal and impaired growth in weight and length were exposed to different milk microbiomes in early lactation, but not at established lactation. In established lactation, only infants with normal and impaired head circumference were exposed to different milk microbiomes, suggesting that at established lactation milk bacteria has an association with head circumference but not weight nor length. When looking into the specific taxa differentially abundant in impaired growth, *Cutibacterium acnes* stood out as it was present in infants with low LAZ and WAZ values. In normal growth, the bacteria differentially abundant were *Luteococcus peritonei* (correlating with WAZ, LAZ and HCAZ values), *Bifidobacterium longum, Lancefieldella parvula,* Phascolarctobacterium*, Rothia mucilaginosa, Streptococcus agalactiae* and *Streptococcus pneumoniae* (correlating with LAZ and HCAZ values), *Janthinobacterium lividum* (correlating with WAZ) and *Lactobacillus johnsonii* (correlating with HCAZ). Only some of these bacterial taxa further correlated with milk minerals. For instance, *C. acnes* was negatively correlated with milk manganese, while *S. pneumoniae* was positively correlated with milk manganese, *S. aglactiae* and *Phascolarbacterium* were positively correlated with magnesium, *Janthinobacterium lividum* was negatively correlated with calcium, and *Lactobacillus johnsonii* was negatively correlated with selenium. In particular, magnesium and manganese had several correlations with milk bacteria and infant growth. The patterns observed allowed us to identify bacteria associated with normal and impaired growth.

### 5.2 Maternal mineral intake and milk minerals

The mother's role on infants' health is crucial as highlighted in the concepts of "the triad" and the "lactocrine programming" (Bartol et al., 2013; Bode et al., 2020; de Weerth et al., 2023; Verduci et al., 2021). These concepts emphasize the close relationship the mother and infant have and how they are linked by human milk. Therefore, any variation in any of the components of this triad will have an impact on another. Since one of our main goals was to explore the importance of milk minerals, we were interested in understanding their association with maternal mineral intake, as the current evidence linking the two is inconclusive (Bravi et al., 2016; Bzikowska-Jura et al., 2023; Dror & Allen, 2018; Maru et al., 2013; Petersohn et al., 2023). Some minerals such as calcium, iron, and zinc are considered to be affected by maternal diet in some studies (Butts et al., 2018; Gibson et al., 2020), whereas other studies report no association (Daniels et al., 2019; Dror & Allen, 2018).

Since there is evidence that maternal micronutrient deficiencies may continue during lactation and alter milk composition (Dror & Allen, 2018), it was important to evaluate maternal mineral intake to determine if it met the recommended intake by the INCAP nutrition guidelines (Menchú et al., 2012). For several minerals, we observed that over 75% of the mothers had intakes below the reference values including for calcium, iron, manganese, and potassium at both stages of lactation, and selenium at established lactation. We did not collect information about the nutritional status through biochemical tests and clinical examinations but used diet as a proxy of the nutritional status. Other studies in the same population have reported nutrient deficiencies (Chomat et al., 2015; Kanapuram, 2023). It is important to consider the insufficient mineral intake when interpreting the associations between maternal mineral intake and milk mineral concentrations, as it is a factor that could explain our reported negative correlations.

There is evidence of similar negative correlations between milk minerals and maternal diet in low socioeconomic populations (Geissler et al., 1978; Gibson et al., 2020; Krebs, 1998). It has been hypothesized that the negative correlation is a compensatory mechanism that occurs in mothers living in low socioeconomic status, where an inadequate nutrient intake leads the body to increase the secretion of the deficient mineral and transport it to the mammary gland, increasing

the nutrient milk concentration of the deficient nutrient (Geissler et al., 1978; Gibson et al., 2020; Krebs, 1998). Other factors linked to the negative associations between maternal mineral intake and milk minerals could be prolonged breastfeeding and low complementary food intake, which have been associated with higher concentrations of milk zinc despite low maternal zinc plasma concentrations (Bates & Tsuchiya, 1990). This seems to be another compensatory mechanism of the maternal body to balance the low mineral content in milk due to prolonged breastfeeding, because as lactation progresses, the nutritional content of milk declines. If an inadequate complementary food intake is added to this, the need to compensate for the milk mineral concentrations increases.

Besides observing negative correlations between milk minerals and maternal mineral intake, we also report that the associations were weak. Weak correlations have been reported previously (Butts et al., 2018; Gibson et al., 2020; Samuel et al., 2020), and the main explanation could be associated to the well-regulated homeostatic processes that some minerals like zinc, iron, and copper have, such as active transport mechanisms in the mammary gland (Domellöf et al., 2004). Other factors include maternal physiological changes during lactation, such as changes in calcium and bone metabolism (Olausson et al., 2012). Another factor to consider is milk volume, which has been suggested to be more associated to variations in iron and zinc milk concentrations than iron and zinc maternal status (Domellöf et al., 2004). We did not analyze the association of milk volume and milk mineral concentrations, but the average milk concentration of iron and zinc were both above the reference values. However, this factor might be affecting other minerals and should be considered in future research. Additional factors affecting milk mineral concentrations could be the interactions among milk minerals or even other milk components such as bacteria.

Our results report an interconnection between minerals, where the intake of a mineral will affect the milk concentration of other minerals as well. These types of interactions between nutrients have been reported before: milk calcium concentration can be affected by maternal iron deficiency or anemia (Dror & Allen, 2018), or by water and thiamin (Butts et al., 2018), milk copper concentrations can be affected by selenium intake (Dror & Allen, 2018), milk magnesium can be altered by energy (calories), carbohydrates, iodine, iron, and potassium intake (Butts et al., 2018)

and milk zinc can also be variable according to energy intake (Vuori et al., 1980), magnesium and potassium (Butts et al., 2018). We also observed a negative association between milk copper and maternal intake of selenium, as reported by Dror & Allen (2018), and milk zinc was not associated with magnesium nor potassium, but it was negatively associated with calcium.

Since maternal mineral intake is not associated with milk mineral concentrations, how can milk mineral content be improved? Could other milk components modify the milk mineral concentrations? We hypothesized that milk minerals are associated with milk microbiome, as these associations have been described in the gut, where it has been reported that bacteria increase the bioavailability and bioaccessibility of minerals (Bielik & Kolisek, 2021).

**5.3 Suggested primer pairs for milk microbiome research**

The first publication from this research titled "Emerging frontiers in human milk microbiome and suggested primers for 16S rRNA gene analysis" was presented in Chapter 2 as the literature review of this dissertation. This publication provided a brief history of milk microbiome research, described the possible sources of milk microbiome, and highlighted a variety of factors that influence the human milk microbiome, including stage of lactation, maternal BMI and diet, and the use of antibiotics and probiotics. We also described existing inconsistencies in the most prevalent bacterial taxa found in milk and suggested that this was associated with primer bias for microbial amplicon sequencing.

We compared the most common primers currently used in milk microbiome research and the percentage of detection of the expected genera present in human milk microbiome, most notably *Bifidobacteria*, a bacteria important for its ability to convert aromatic amino acids to their lactic acid derivatives and their colonization of the gut in early life, which has critical impact on the immune system (Gensollen et al., 2016; Laursen et al., 2021). We concluded that the 784F/1061R primer pair (V5-V6 targeting) or the 27F YM + 3 high degeneracy variant (within 27F/533R, V1-V3 targeting) were more suitable for human milk research due to high coverage of all genera currently considered as ''core" in human milk (Groer et al., 2020; Hunt et al., 2011; Lackey et al., 2019; McGuire & McGuire, 2015).

The results of this analysis supported our choice to use a pair of primers targeting the V5-V6 regions. As all the characteristics of the primer pair 784F/1061R were present in the primer pair P609F/P699R, another primer pair optimized and in use by the sequencing facility of the McGill Genome Center, we proceeded with the primer pair P609F/P699R.

**5.4 Milk microbiome observed in V1 – V3 vs V5 – V6 regions**

Previous studies in our research team analyzed the milk microbiome of the same population in cross-sectional studies but with different primers (Ajeeb et al., 2022a, 2022b; Gonzalez et al., 2021; Lopez Leyva et al., 2022). These studies used the V1 – V3 regions and the primer pair 27F/533R. For the studies presented in this research, we used the V5 – V6 regions and the primer P609F/P699R.

More ESVs (2122 vs 503) and genera (145 vs137) were identified with V5-V6. This can be in part related to the selected primers and regions. However, this can also be due to an updated version of the pipeline ANCHOR and differences in the data set, despite coming from the same study population.

At phylum level, both sets of primers amplified *Actinomycetota, Bacillota, Bacteroidota, Deinococcota, Fusobacteriota, Pseudomonadota, Saccaribacteria* and *Streptophyta.* However, the primer pair V5-V6 (P609F/P699R) amplified 10 more phyla including *Bdellovibrionota, Campylobacterota, Cyanobacteriota, Deferribacterota, Euryarchaeota, Gemmatimonadota, Mycoplasmatota, Myxococcota, Spirochaetota* and *Thermodesulfobacteriota.* At genera level, both ESVs tables have 80 ESVs in common, whereas V5-V6 (P609F/P699R) has 322 unique ESVs and V1 – V3 (27F/533R) only has 55. As expected, the primer pair 27F/533R of regions V1 – V3 was not able to capture *Bifidobacteria,* as explained previously (Lopez Leyva et al., 2020), since the percentage of sequences amplified within *Bifidobacteria* is only of 13.5% in the V1 – V3 (27F/533R) versus 91.3% in the V5-V6 (P609F/P699R). Other relevant genera that were differentially abundant in our analyses were amplified by V5-V6 (P609F/P699R) but not by V1 – V3 (27F/533R) including *Ewingella, Gardnerella, Limosilacotbacillus, Luteococcus, Phascolarctobacterium* and *Planococcus*. At the ESV level, both studies identified *S. salivarius* as the most abundant ESV.

Further analyses comparing both primer pairs should be performed with the same data set and the same pipeline version (e.g. ANCHOR) in order to better understand their differences. The suggested analyses should include alpha diversity metrics, beta diversity and community structure, precise taxonomic resolution and statistical comparisons such as differential abundance analysis or correlation analyses to assess consistency.

**5.5 Milk microbiome in same mothers between early and established lactation**

It has been widely described that one of the main factors affecting milk microbiome composition is the stage of lactation (Cabrera-Rubio et al., 2012; Gonzalez et al., 2021; Khodayar-Pardo et al., 2014). Specifically, one of the first studies from our research group highlighted the difference in milk microbiome composition in early and established lactation (Gonzalez et al., 2021). In consequence, all our subsequent analyses have been designed considering the lactation stage. The third manuscript in this dissertation in Chapter 4 is the first study in our research group to conduct a longitudinal study in these milk samples from the Guatemalan population, as all the previous studies had been cross-sectional.

In this longitudinal study, the milk microbiome composition of the same mothers at early and established lactation shared some similarities and differences. In both stages of lactation, the three main phyla were Bacillota, Actinomycetota, and Pseudomonadota. Pseudomonadota was slightly more abundant in early lactation (4% vs 2% in established) while Bacillota was more abundant in established lactation (48% vs 37% in early). Both lactation stages had similar abundance of Actinomycetota (17% in early vs 18% in established) and Saccharibacteria (0.14% in early vs 0.22% in established). Bacteroidota was slightly higher in established lactation (0.7% vs 0.16 in early lactation), Myxococcota was higher in early lacatation and the least abundant phylum in established lactation and Fusobacteriota was higher in established lactation and the least abundant phylum in early lactation.

Considering the 10 most abundant genera, *Streptococcus* was the most abundant genus in both stages of lactation. In early lactation *Staphylococcus, Cutibacterium, Actinomycetes MS, Corynebacterium, Gemella, Pseudomonadota* and *Kocuria* had a higher relative abundance in

comparison to established lactation. The genera *Agrococcus, Veillonella, Promicromonospora* and *Granulicatella* had a higher relative abundance in established lactation than in early lactation.

At species level, *Streptoccous salivarius* was the most abundant in both stages of lactation. Followed by *Staphylococcus hominis* in early lactation and by *Agrococcus carbonis* in established lactation. *Staphylococcus epidermis* had a slightly higher relative abundance in early lactation than in established lactation (1.5% vs 1.21%) while *Veillonella* had a higher relative abundance in established lactation than in early lactation (3.08% vs 0.67%). These results confirmed the differences in milk microbial composition from the same mothers at early and established lactation.

Finally, we were interested in comparing the milk microbiome composition by stage of lactation between the cross-sectional studies and this longitudinal study. Both studies identified similar bacteria. However, it is important to remember that they were amplified with different primer pairs, which influences the bacteria observed. In both studies the most abundant species was *Streptococcus salivarius.* The cross-sectional study identified *Streptococcus and Staphylococcus* as the main genera in early lactation and *Proteobacteria* in established lactation. This longitudinal study also identified *Streptococcus* and *Staphylococcus* as some of the main genera in early lactation but in contrast to the cross-sectional study, *Proteobacteria (Pseudomonadota)* were more abundant in early lactation than in established lactation. However, the differences observed might not only be associated with the study design but also to the primer pair selection and updated pipeline version. The results of the longitudinal study are more reliable on account of analyzing the milk of the same mothers at early and established lactation, the advantages of primer pair V5-V6 (P609F/P699R), and the use of updated software and pipeline versions for the milk microbiome analyses.

**5.6 Milk minerals and milk microbiome**

Lately, the importance of studying human milk as an ecosystem rather than independently has been recognized (Christian et al., 2021; Smilowitz et al., 2023). Milk composition highly varies in relation to different factors, and the interaction with components such as milk minerals and milk microbiome.

We analyzed the association between milk minerals and milk microbiome in a cross-sectional (Chapter 3) and longitudinal study (Chapter 4). Since in our cross-sectional study we observed a strong correlation between milk minerals and milk microbiome, we included this association in our longitudinal study and focused on infant growth. In the cross-sectional study, iron, manganese and selenium were the only milk minerals that had associations with the milk microbiome in both stages of lactation. In fact, iron and manganese had the greatest number of correlations with milk bacteria. However, in the longitudinal study, from those minerals only manganese continued to be associated with the milk bacteria and infant growth and magnesium emerged as a mineral with several associations with milk microbiome and infant growth

It is challenging to distinguish whether the associations between and among milk components are causal in nature or coincidental factors, especially because nutrient deficiencies tend to co-exist and might affect these associations (Smilowitz et al., 2023). In our studies, our population had milk mineral intakes and milk mineral concentrations below the reference values, so it is difficult to confirm these associations in other populations or in women with adequate nutritional status. However, others have explained that the interaction between milk nutrients and milk microbiome is plausible considering that microorganisms depend on their hosts to obtain essential nutrients, so variation in milk nutrients will impact which microorganisms survive in the mammary gland and constitute the milk microbiome (Smilowitz et al., 2023). Associations between milk nutrients and milk microbiome have been done mostly for proteins, lactose, fatty acids, and lipids. To our knowledge, this study is the second one to provide evidence in relation to the association between milk minerals and the milk microbiome. The mechanisms through which this happens remain unexplored in human milk although some evidence has been reported in the gut microbiome.

Microorganisms can regulate the levels of micronutrients and functional structure in the gut by intervening in the biosynthetic processes and by modulating their metabolism and absorption (Barone et al., 2022; Fan et al., 2023), which could also be the case in human milk. To illustrate this, calcium absorption is increased by short-chain fatty acids, bacterial metabolic byproducts that lower the gastrointestinal pH, facilitating the solubility of calcium and consequently

enhancing its transepithelial transport. Similarly, bacteria can synthesize enzymes that release minerals from dietary sources (Bielik & Kolisek, 2021). For instance, phytases can release inorganic forms of calcium, magnesium, iron, and phosphorous, after the hydrolysis of phytic acid present in many plant-based foods (Bohn et al., 2007). However, none of these mechanisms have been described in human milk, even though SCFA and *Lactococcus lactis*, a phytic acid-producing species, are found in human milk. Although phytases are rarely isolated from human milk, they might be obtained from the maternal diet through phytic-acid rich foods and promote the bioavailability of milk minerals (Zhou et al., 2021).

### 5.7 Milk minerals, milk microbiome and infant growth

In Chapter 4, we examined the associations between milk minerals, the milk microbiome, and infant growth outcomes. Our findings revealed compelling patterns, allowing us to identify specific bacterial taxa linked to both normal and impaired growth, as well as their relationships with key minerals—particularly magnesium and manganese. Notably, early lactation emerged as a critical period, during which both the composition of the milk microbiome and infant growth trajectories, especially length and weight, appeared most sensitive.

Among the taxa identified, *Cutibacterium acnes* was differentially abundant in infants with impaired WAZ and LAZ scores **(Figure 5.1)**. Since it is a common skin bacterium, it could be argued that this is contamination. However, it has been identified previously in human milk (Jost et al., 2013) and in fact, in one study it was one of most abundant species (Cheema et al., 2022). In our study, contamination was minimized through careful disinfection of the breast and hands of the midwife during sample collection. The presence of *C. acnes* might be associated to the retrograde flow, one of the recognized routes of bacterial colonization of the milk, where skin bacteria colonize the infant oral cavity and become part of the milk microbiome (Fernández et al., 2013; Ramsay et al., 2004; Thum et al., 2012). Its potential role in infant growth may be linked to its interactions with other microbes such as *Pseudomonas* and *S. aureus*. Similar to its role in skin microbiota, *C. acnes* may compete with pathogenic bacteria, thereby mitigating negative effects on growth (Platsidaki & Dessinioti, 2018). Interestingly, it showed a consistent negative

correlation with manganese in relation to both LAZ and WAZ parameters **(Figure 5.2)**, suggesting that manganese availability could influence its proliferation or pathogenicity, thereby indirectly

Several bacterial taxa were also associated with normal LAZ and HCAZ scores **(Figure 5.3).** Many of these bacteria are commonly found in the human microbiome. For example, *Bifidobacterium longum*, previously linked to healthy growth (Vatanen et al., 2022), appeared in the milk ingested by infants with both normal LAZ and HCAZ profiles. While some bacteria shared between these two growth parameters were associated with milk minerals—*Phascolarctobacterium* and *Streptococcus agalactiae* showed positive associations with magnesium, and *S. pneumoniae* with manganese—others, including *B. longum*, *Luteococcus peritonei*, *Lancefieldella parvula*, and *Rothia mucilaginosa*, did not display any mineral correlations.

Overall, magnesium and manganese were the only minerals consistently associated with microbial composition across all three growth parameters: WAZ, LAZ, and HCAZ. These two minerals also showed the highest number of bacterial correlations during early lactation **(Figure 5.4)**, underscoring their potential relevance to both microbiome dynamics and infant growth. Additional minerals—such as calcium (associated with WAZ), selenium, and strontium (associated with HCAZ)—also emerged in our analyses, though their correlations were more limited. For instance, calcium in infants with normal WAZ was linked to *Janthinobacterium lividum*, known for its broad antimicrobial properties (Baricz et al., 2018). Selenium in infants with normal HCAZ correlated with *Lactobacillus johnsonii*, a well-documented probiotic with immunomodulatory and gut barrier-enhancing functions (Zhang et al., 2023). Strontium and magnesium were associated with *S. agalactiae* in both LAZ and HCAZ parameters.

It is noteworthy that despite expectations based on previous literature, only magnesium and manganese demonstrated strong and consistent associations. For example, a recent systematic review identified positive correlations between infant growth and milk levels of iodine, manganese, calcium, and zinc (Reyes et al., 2023). Moreover, the roles of iron and zinc in supporting healthy growth have been extensively documented (Bhutta et al., 2008; Black et al., 2013; Petry et al., 2016; Savarino et al., 2021). While our results reinforce the importance of manganese, they also highlight magnesium as a potentially underrecognized factor, particularly

when viewed through the lens of its interaction with the milk microbiome. The absence of iron and zinc associations in our findings may reflect complex microbial dynamics that modulate mineral bioavailability or host utilization.

The interactions between milk minerals and milk microbiome composition may influence infant growth by enhancing mineral absorption and utilization, supporting bone development, and promoting energy storage, all of which contribute to healthy growth (Gopalakrishna & Hand, 2020). However, alterations in the milk microbiome composition or the presence of opportunistic bacteria could lead to adverse outcomes, such as infections, increased risk of diarrhea and inflammation, and competition for host minerals (Ramani et al., 2018). These factors can negatively impact an infant's nutritional status and hinder growth. Further research is needed to elucidate how these interactions between milk components affect infant growth.

**5.8 Strengths and Limitations**

To the best of our knowledge, the current research is the first one to analyze collectively associations between maternal mineral intake, milk minerals, milk microbiome, and infant growth. Our population also provided a unique opportunity to analyze the milk microbiome, as many of the factors known to affect its composition are homogenous in the *Mam*-Mayan mothers, such as exclusive or predominant breastfeeding for >6 months, the absence of therapeutic use of antibiotics, normal BMI, and manual milk extraction rather than the use of pump, which can modify the milk microbiome (Fehr et al., 2020; Marín et al., 2009; Moossavi et al., 2019). Maternal diet was assessed on the same day of the milk collection which provided us with reliable information, as diet and milk microbiome composition can vary throughout pregnancy and lactation. Additionally, our mothers were not supplemented with any micronutrients, which could modify the milk mineral concentrations. However, they consume fortified foods, which could have an effect on milk mineral concentrations, and we did not take it into account. The primers selected to analyze the milk microbiome allowed us to capture a wide range of bacteria, including *Bifidobacteria,* which are typically not captured when using other primers and regions such as V3-V4 (Lopez Leyva et al., 2020). The novel use of this set of primers in milk microbiome research mitigates the limitation of using 16S rRNA sequencing instead of

whole genome sequencing (WGS). However, WGS approaches remain a technical challenge for studies involving high sample numbers and human milk. The use of ESVs allowed us to have a higher resolution and a more precise identification of microorganisms down to species level and sometimes even strain level. In the longitudinal study, we collected data from the same mothers and infants at both stages of lactation, which allowed us to identify differences in early and established lactation in the milk composition and infant growth. Lastly, our results can be generalizable to other indigenous populations with similar characteristics or populations with deficient mineral intakes, as they could have similar associations between maternal mineral intake, milk mineral concentrations, and milk microbiome composition.

However, we recognize this research also had limitations including the cross-sectional design in the first study, which limited our capacity to establish causal inference and investigate the temporal relation between maternal diet, milk minerals, and milk microbiome. We also did not subdivide our milk mineral concentrations between below and above the reference values for the analysis, which could have informed us if the level of milk mineral concentrations is associated to the milk microbiome composition. In the longitudinal study, we were not able to evaluate change over time which would have allowed us to evaluate if changes in the milk microbiome composition and milk minerals at early lactation affected the infants' growth category (normal or impaired) in established lactation. Only having data from two time-points limited our possibilities to conduct a proper longitudinal analysis, as it was impossible to identify trends. The lack of mineral maternal status (measured in blood samples) is another important limitation, as diet can only be a proxy and is not sufficient to determine a nutrient deficiency or level of absorption. We did not consider the type of breastfeeding (exclusive, predominant, or mixed) nor the milk volume which can modify the milk microbiome and milk mineral intake by the infant. We did not have information about the milk macronutrients concentrations nor human milk oligosaccharides (HMOs), which must be interacting with the milk minerals and milk microbiome. Finally, we did not measure iodine, a mineral recognized as being affected by maternal mineral intake, nor did we exclude mothers with subclinical mastitis in the cross-sectional study, which can also have an impact on the milk microbiome and milk mineral composition. We also did not exclude premature

infants, which have been reported in this study population in another study, as we did not collect the gestational age here.

### 5.9 Future directions of research

Future research in milk microbiome and milk minerals should study the milk microbiome composition in relation to adequate or inadequate levels of milk mineral concentrations as well as adequate or inadequate maternal mineral intake or nutritional status; different levels of milk minerals or maternal mineral intakes might affect in diverse ways the milk microbiome and vice-versa. We encourage the scientific community to conduct more longitudinal studies to better understand the effect of milk microbiome composition on infant growth and evaluate changes over time while accounting for milk volume and type of breastfeeding.

We consider that it is crucial to understand the cellular mechanisms by which minerals integrate into human milk, as we now recognize that maternal mineral intake is not one of the main drivers. Similarly, experimental studies must be conducted to understand the mechanisms by which milk minerals and the milk microbiome interact. A good starting point could be the minerals and bacteria identified as most relevant in this body of work. Considering our results that identified bacteria associated with impaired growth, we recommend disentangling cause vs correlation in future studies.

The same way we observed associations between minerals and microbiome, other milk components such as milk macronutrients and HMOs should be integrated.

Considering that maternal mineral intake does not affect milk mineral concentrations, but there rather is a strong correlation between milk microbiome and milk minerals, maybe milk minerals can be modulated by milk bacteria. Similarly, considering that the maternal gut bacteria could be colonizers of milk microbiome, modulating the maternal gut microbiome could be another pathway for improving milk mineral concentrations and their bioavailability, with the goal of promoting adequate infant growth. Future studies should aim to create concrete recommendations that can benefit the mother and the infant. Therefore, in the longer term, we suggest clinical trials or interventions that analyze the effect of probiotics intake by the mother

and/or infant. These interventions may need to consider maternal health pre-pregnancy, including nutrient-deficient and nutrient-adequate women to comprehend the bidirectional associations between milk minerals and milk microbiome. This type of study will be able to inform nutritional guidelines and public health interventions targeting maternal and infant health.

**5.10 Conclusion**

In summary, our findings reveal a complex and dynamic interplay between milk minerals and the milk microbiome, which seems to operate independently of maternal diet. Iron, manganese, and selenium correlate with milk microbiome composition at both stages of lactation. However, when we investigated the association of milk minerals and milk microbiome and their role in infant growth, magnesium and manganese emerged as crucial minerals. Specific bacteria associated with magnesium and manganese were correlated with normal and impaired growth. For instance, *Cutibacterium acnes* was differentially abundant in impaired growth in WAZ and LAZ while in normal growth the following species were differentially abundant: *Pseudomonas fluorescens* (in WAZ), *Streptococcus pneumoniae, Streptococcus agalactiae* and *Phascolarbacterium* (in LAZ and HCAZ). Finally, differences in milk microbiome on infant growth occur at early lactation. This suggests that associations between milk microbiome and milk minerals are dynamic and bidirectional, and they could be an infant growth modulator since early lactation. This PhD work advances knowledge in maternal and infant nutrition and provides direction for future studies in milk minerals, milk microbiome, and infant growth.

## 5.11 Figures

**Figure 5.1 – Bacteria associated with impaired growth in the growth parameters WAZ and LAZ.**

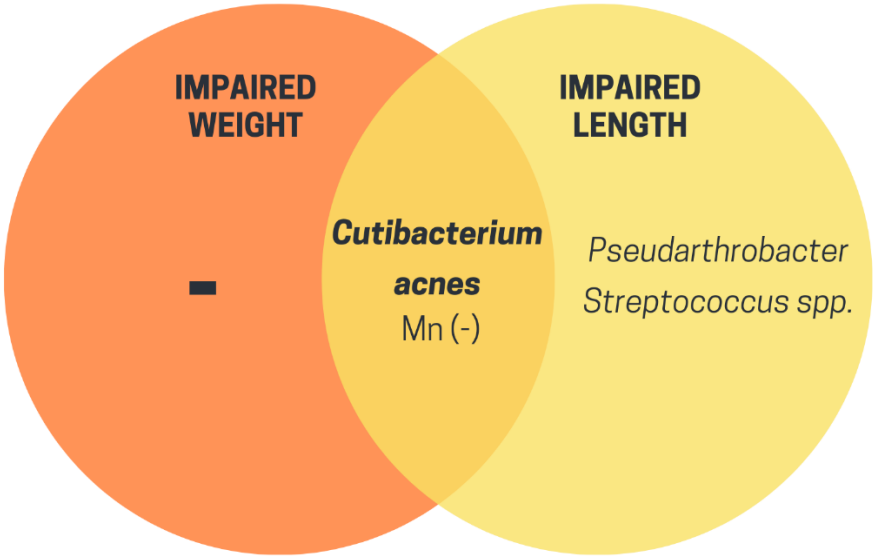



**Figure 5.1 – Bacteria associated with impaired growth in the growth parameters WAZ and LAZ.** Venn diagram showing the differentially abundant bacteria in infants with impaired growth in the growth parameters WAZ (left) and LAZ (right) and the bacteria shared between both growth parameters (centre).

**Figure 5.2 – Differentially abundant bacteria in normal and impaired growth in WAZ, LAZ, HCAZ, and their correlations with milk minerals.**

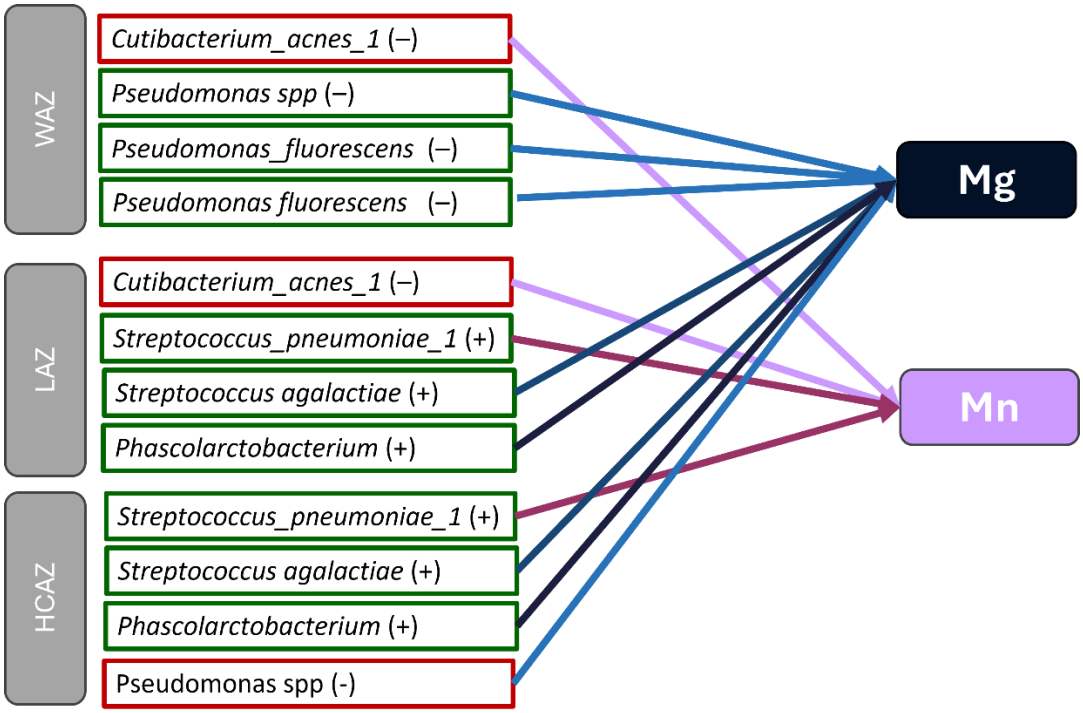



**Figure 5.2 – Differentially abundant bacteria in normal and impaired growth in WAZ, LAZ, HCAZ, and their correlations with milk minerals.** Bacteria differentially abundant in infants with normal (green boxes) and impaired (red boxes) growth for each growth parameter (WAZ, LAZ and HCAZ). The type of correlation [positive (+) or negative (-)] with milk minerals is shown next to each bacterium. Purple lines show a correlation with milk manganese and blue lines show a correlation with milk magnesium.

**Figure 5.3 – Bacteria associated with normal growth in the growth parameters LAZ and HCAZ.**

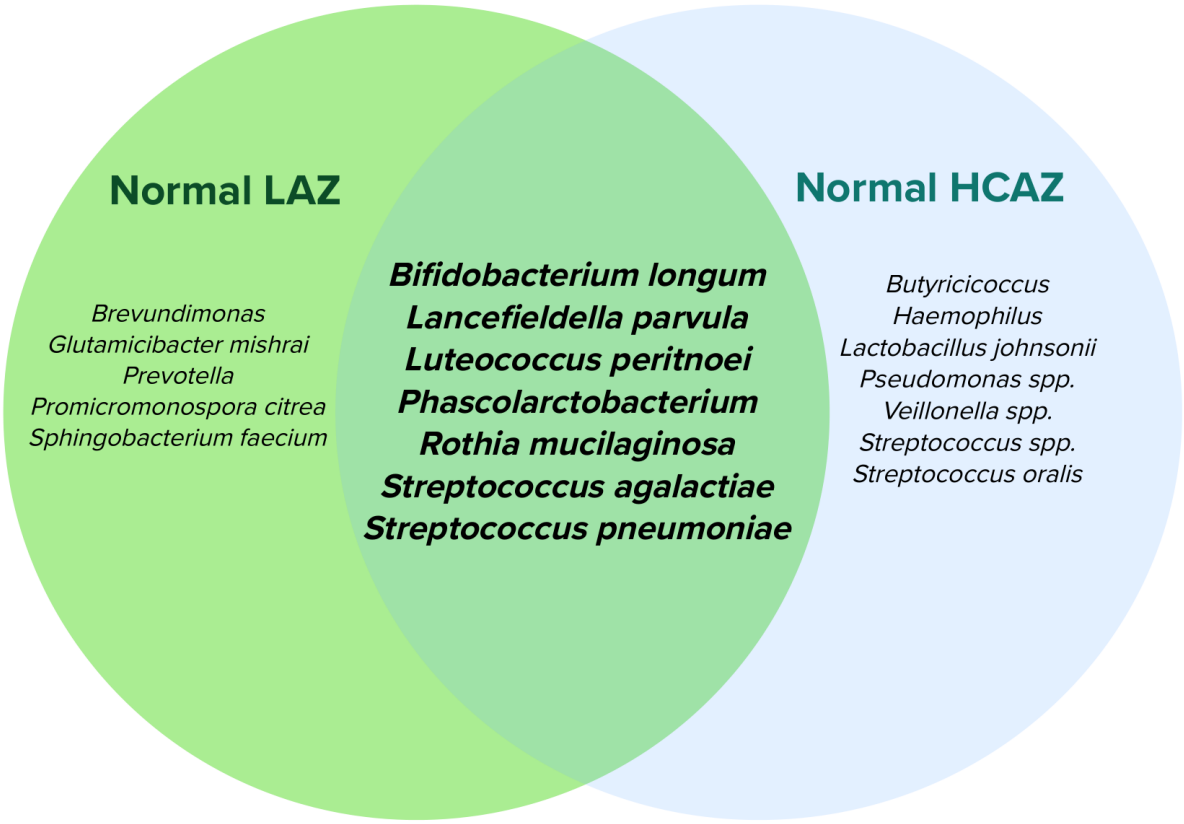



**Figure 5.3 – Bacteria associated with normal growth in the growth parameters LAZ and HCAZ.** Venn diagram showing the differentially abundant bacteria in normal growth in the growth parameters LAZ (left) and HCAZ (right) and the bacteria shared between both growth parameters (centre).

**Figure 5.4 – Bacteria and milk minerals shared in the associations with the growth parameters WAZ, LAZ, and HCAZ at early lactation.**

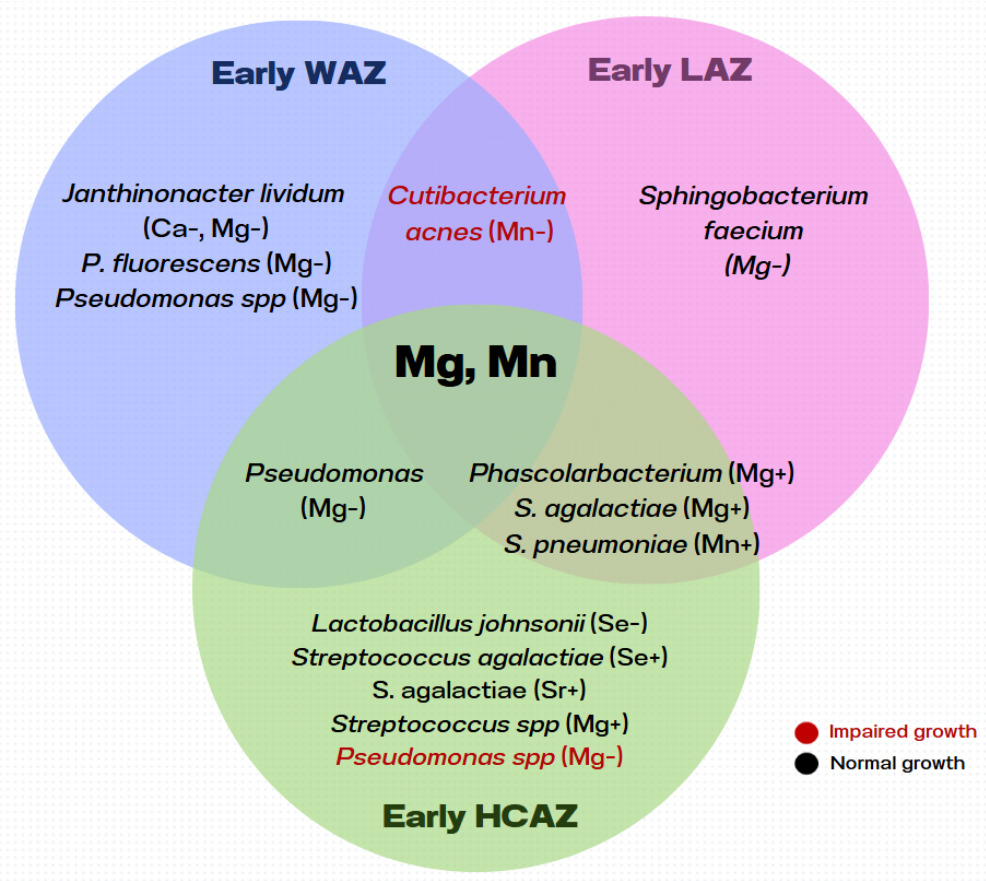



**Figure 5.4 – Bacteria and milk minerals associations in the growth parameters WAZ, LAZ, and HCAZ at early lactation.** Milk magnesium and manganese were associated with several taxa in WAZ, LAZ and HCAZ at early lactation. Taxa in red color were differentially abundant (DA) in infants with impaired growth and taxa in black color were DA in infants with normal growth. In the purple circle are shown the DA bacteria associated with a mineral in the growth parameter WAZ at early lactation. In the pink circle are shown the DA bacteria associated with a mineral in the growth parameter LAZ at early lactation. In the green circle are shown the DA bacteria associated with a mineral in the growth parameter HCAZ at early lactation. The Venn diagram also shows the shared bacteria between the different growth parameters that were also associated with milk magnesium and milk manganese. *C acnes* was DA in the growth parameters WAZ and LAZ and in both cases it was negatively associated with manganese in infants with impaired growth (red).

*Pseudomonas* was DA in the growth parameters WAZ and HCAZ and in both cases it was negatively correlated with magnesium. The bacteria *S. pneumoniae, S. agalactiae* and *Phascolarbacterium* were DA in the growth parameters HCAZ and LAZ and in both cases *S. pneumonia* was negatively correlated with manganese and *S. agalactiae* and *Phascolarbacterium* were positively correlated with magnesium.

## References


Ajeeb, T. T., Gonzalez, E., Solomons, N. W., & Koski, K. G. (2022a). Human milk microbial species are associated with infant head-circumference during early and late lactation in Guatemalan mother-infant dyads. *Frontiers in Microbiology*, *13*, 908845. https://doi.org/10.3389/fmicb.2022.908845

Ajeeb, T. T., Gonzalez, E., Solomons, N. W., & Koski, K. G. (2022b). Human milk microbial species are associated with mild growth deficits during early infancy among Guatemalan mother–infant dyads. *Frontiers in Microbiomes*, *1*. https://www.frontiersin.org/articles/10.3389/frmbi.2022.1008467

Ajeeb, T. T., Gonzalez, E., Solomons, N. W., Vossenaar, M., & Koski, K. G. (2024). Human milk microbiome: Associations with maternal diet and infant growth. *Frontiers in Nutrition*, *11*, 1341777. https://doi.org/10.3389/fnut.2024.1341777

Allen, L. H. (1994). Maternal micronutrient malnutrition: Effects on breast milk and infant nutrition, and priorities for intervention. *SCN News*, *11*, 21–24.

Allen, L. H. (2012). B vitamins in breast milk: Relative importance of maternal status and intake, and effects on infant status and function. *Advances in Nutrition (Bethesda, Md.)*, *3*(3), 362–369. https://doi.org/10.3945/an.111.001172

Baricz, A., Teban, A., Chiriac, C. M., Szekeres, E., Farkas, A., Nica, M., Dascălu, A., Oprișan, C., Lavin, P., & Coman, C. (2018). Investigating the potential use of an Antarctic variant of Janthinobacterium lividum for tackling antimicrobial resistance in a One Health approach. *Scientific Reports*, *8*(1), 15272. https://doi.org/10.1038/s41598-018-33691-6

Barone, M., D'Amico, F., Brigidi, P., & Turroni, S. (2022). Gut microbiome-micronutrient interaction: The key to controlling the bioavailability of minerals and vitamins? *BioFactors (Oxford, England)*, *48*(2), 307–314. https://doi.org/10.1002/biof.1835

Bartol, F. F., Wiley, A. A., Miller, D. J., Silva, A. J., Roberts, K. E., Davolt, M. L. P., Chen, J. C., Frankshun, A.-L., Camp, M. E., Rahman, K. M., Vallet, J. L., & Bagnell, C. A. (2013). Lactation Biology Symposium: Lactocrine signaling and developmental programming. *Journal of Animal Science*, *91*(2), 696–705. https://doi.org/10.2527/jas.2012-5764

Bates, C. J., & Tsuchiya, H. (1990). Zinc in breast milk during prolonged lactation: Comparison between the UK and the Gambia. *European Journal of Clinical Nutrition*, *44*(1), 61–69.

Bhutta, Z., Ahmed, T., Black, R., Cousens, S., Dewey, K., E, G., Ba, H., B, K., Ss, M., Hp, S., & M, S. (2008). What works? Interventions for maternal and child undernutrition and survival. *Lancet (London, England)*, *371*(9610). https://doi.org/10.1016/S0140-6736(07)61693-6

Bielik, V., & Kolisek, M. (2021). Bioaccessibility and Bioavailability of Minerals in Relation to a Healthy Gut Microbiome. *International Journal of Molecular Sciences*, *22*(13), 6803. https://doi.org/10.3390/ijms22136803

Black, R. E., Victora, C. G., Walker, S. P., Bhutta, Z. A., Christian, P., de Onis, M., Ezzati, M., Grantham-McGregor, S., Katz, J., Martorell, R., Uauy, R., & Maternal and Child Nutrition Study Group. (2013). Maternal and child undernutrition and overweight in low-income and middle-income countries. *Lancet (London, England)*, *382*(9890), 427–451. https://doi.org/10.1016/S0140-6736(13)60937-X

Blanton, L. V., Charbonneau, M. R., Salih, T., Barratt, M. J., Venkatesh, S., Ilkaveya, O., Subramanian, S., Manary, M. J., Trehan, I., Jorgensen, J. M., Fan, Y.-M., Henrissat, B., Leyn, S. A., Rodionov, D. A., Osterman, A. L., Maleta, K. M., Newgard, C. B., Ashorn, P., Dewey, K. G., & Gordon, J. I. (2016). Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. *Science (New York, N.Y.)*, *351*(6275), aad3311. https://doi.org/10.1126/science.aad3311

Bode, L., Raman, A. S., Murch, S. H., Rollins, N. C., & Gordon, J. I. (2020). Understanding the mother-breastmilk-infant "triad." *Science*, *367*(6482), 1070–1072. https://doi.org/10.1126/science.aaw6147

Bohn, L., Josefsen, L., Meyer, A. S., & Rasmussen, S. K. (2007). Quantitative analysis of phytate globoids isolated from wheat bran and characterization of their sequential dephosphorylation by wheat phytase. *Journal of Agricultural and Food Chemistry*, *55*(18), 7547–7552. https://doi.org/10.1021/jf071191t

Bravi, F., Wiens, F., Decarli, A., Dal Pont, A., Agostoni, C., & Ferraroni, M. (2016). Impact of maternal nutrition on breast-milk composition: A systematic review. *The American Journal of Clinical Nutrition*, *104*(3), 646–662. https://doi.org/10.3945/ajcn.115.120881

Butts, C. A., Hedderley, D. I., Herath, T. D., Paturi, G., Glyn-Jones, S., Wiens, F., Stahl, B., & Gopal, P. (2018). Human Milk Composition and Dietary Intakes of Breastfeeding Women of Different Ethnicity from the Manawatu-Wanganui Region of New Zealand. *Nutrients*, *10*(9), Article 9. https://doi.org/10.3390/nu10091231

Bzikowska-Jura, A., Czerwonogrodzka-Senczyna, A., Olędzka, G., Szostak-Węgierek, D., Weker, H., & Wesołowska, A. (2018). Maternal Nutrition and Body Composition During Breastfeeding: Association with Human Milk Composition. *Nutrients*, *10*(10), 1379. https://doi.org/10.3390/nu10101379

Bzikowska-Jura, A., Wesołowska, A., Sobieraj, P., Michalska-Kacymirow, M., Bulska, E., & Starcevic, I. (2023). Maternal diet during breastfeeding in correlation to calcium and phosphorus concentrations in human milk. *Journal of Human Nutrition and Dietetics: The Official Journal of the British Dietetic Association*, *36*(3), 798–809. https://doi.org/10.1111/jhn.13100

Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., & Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and

mode of delivery. *The American Journal of Clinical Nutrition*, *96*(3), 544–551. https://doi.org/10.3945/ajcn.112.037382

Cabrera-Rubio, R., Mira-Pascual, L., Mira, A., & Collado, M. C. (2016). Impact of mode of delivery on the milk microbiota composition of healthy women. *Journal of Developmental Origins of Health and Disease*, *7*(1), 54–60. https://doi.org/10.1017/S2040174415001397

Campos, A. P., Vilar-Compte, M., & Hawkins, S. S. (2020). Association Between Breastfeeding and Child Stunting in Mexico. *Annals of Global Health*, *86*(1), 145. https://doi.org/10.5334/aogh.2836

CDC. (2023). *Breastfeeding: Recommendations and Benefits*. Centers for Disease Control and Prevention. https://www.cdc.gov/nutrition/infantandtoddlernutrition/breastfeeding/recommendations-benefits.html

Cheema, A. S., Gridneva, Z., Furst, A. J., Roman, A. S., Trevenen, M. L., Turlach, B. A., Lai, C. T., Stinson, L. F., Bode, L., Payne, M. S., & Geddes, D. T. (2022). Human Milk Oligosaccharides and Bacterial Profile Modulate Infant Body Composition during Exclusive Breastfeeding. *International Journal of Molecular Sciences*, *23*(5), Article 5. https://doi.org/10.3390/ijms23052865

Chomat, A. M., Solomons, N. W., Koski, K. G., Wren, H. M., Vossenaar, M., & Scott, M. E. (2015). Quantitative Methodologies Reveal a Diversity of Nutrition, Infection/Illness, and Psychosocial Stressors During Pregnancy and Lactation in Rural *Mam* -Mayan Mother–Infant Dyads From the Western Highlands of Guatemala. *Food and Nutrition Bulletin*, *36*(4), 415–440. https://doi.org/10.1177/0379572115610944

Christian, P., Smith, E. R., Lee, S. E., Vargas, A. J., Bremer, A. A., & Raiten, D. J. (2021). The need to study human milk as a biological system. *The American Journal of Clinical Nutrition*, *nqab075*. https://doi.org/10.1093/ajcn/nqab075

Daniels, L., Gibson, R. S., Diana, A., Haszard, J. J., Rahmannia, S., Luftimas, D. E., Hampel, D., Shahab-Ferdows, S., Reid, M., Melo, L., Lamers, Y., Allen, L. H., & Houghton, L. A. (2019). Micronutrient intakes of lactating mothers and their association with breast milk concentrations and micronutrient adequacy of exclusively breastfed Indonesian infants. *The American Journal of Clinical Nutrition*, *110*(2), 391–400. https://doi.org/10.1093/ajcn/nqz047

de Weerth, C., Aatsinki, A.-K., Azad, M. B., Bartol, F. F., Bode, L., Collado, M. C., Dettmer, A. M., Field, C. J., Guilfoyle, M., Hinde, K., Korosi, A., Lustermans, H., Mohd Shukri, N. H., Moore, S. E., Pundir, S., Rodriguez, J. M., Slupsky, C. M., Turner, S., van Goudoever, J. B., … Beijers, R. (2023). Human milk: From complex tailored nutrition to bioactive impact on child cognition and behavior. *Critical Reviews in Food Science and Nutrition*, *63*(26), 7945–7982. https://doi.org/10.1080/10408398.2022.2053058

Domellöf, M., Lönnerdal, B., Dewey, K. G., Cohen, R. J., & Hernell, O. (2004). Iron, zinc, and copper concentrations in breast milk are independent of maternal mineral status. *The American Journal of Clinical Nutrition*, *79*(1), 111–115. https://doi.org/10.1093/ajcn/79.1.111

Donohue, J. A., Solomons, N. W., Hampel, D., Shahab-Ferdows, S., Orozco, M. N., & Allen, L. H. (2020). Micronutrient supplementation of lactating Guatemalan women acutely increases infants' intake of riboflavin, thiamin, pyridoxal, and cobalamin, but not niacin, in a randomized crossover trial. *The American Journal of Clinical Nutrition*, *112*(3), 669–682. https://doi.org/10.1093/ajcn/nqaa147

Dror, D. K., & Allen, L. H. (2018a). Overview of Nutrients in Human Milk. *Advances in Nutrition*, *9*(suppl_1), 278S-294S. https://doi.org/10.1093/advances/nmy022

Dror, D. K., & Allen, L. H. (2018b). Retinol-to-Fat Ratio and Retinol Concentration in Human Milk Show Similar Time Trends and Associations with Maternal Factors at the Population

Level: A Systematic Review and Meta-Analysis. *Advances in Nutrition (Bethesda, Md.)*, *9*(suppl_1), 332S-346S. https://doi.org/10.1093/advances/nmy021

Erick, M. (2018). Breast milk is conditionally perfect. *Medical Hypotheses*, *111*, 82–89. https://doi.org/10.1016/j.mehy.2017.12.020

Eriksen, K. G., Christensen, S. H., Lind, M. V., & Michaelsen, K. F. (2018). Human milk composition and infant growth. *Current Opinion in Clinical Nutrition and Metabolic Care*, *21*(3), 200–206. https://doi.org/10.1097/MCO.0000000000000466

Fan, L., Xia, Y., Wang, Y., Han, D., Liu, Y., Li, J., Fu, J., Wang, L., Gan, Z., Liu, B., Fu, J., Zhu, C., Wu, Z., Zhao, J., Han, H., Wu, H., He, Y., Tang, Y., Zhang, Q., … Ren, W. (2023). Gut microbiota bridges dietary nutrients and host immunity. *Science China. Life Sciences*, *66*(11), 2466–2514. https://doi.org/10.1007/s11427-023-2346-1

Fehr, K., Moossavi, S., Sbihi, H., Boutin, R. C. T., Bode, L., Robertson, B., Yonemitsu, C., Field, C. J., Becker, A. B., Mandhane, P. J., Sears, M. R., Khafipour, E., Moraes, T. J., Subbarao, P., Finlay, B. B., Turvey, S. E., & Azad, M. B. (2020). Breastmilk Feeding Practices Are Associated with the Co-Occurrence of Bacteria in Mothers' Milk and the Infant Gut: The CHILD Cohort Study. *Cell Host & Microbe*, *28*(2), 285-297.e4. https://doi.org/10.1016/j.chom.2020.06.009

Fernández, L., Langa, S., Martín, V., Maldonado, A., Jiménez, E., Martín, R., & Rodríguez, J. M. (2013). The human milk microbiota: Origin and potential roles in health and disease. *Pharmacological Research*, *69*(1), 1–10. https://doi.org/10.1016/j.phrs.2012.09.001

Geissler, C., Calloway, D. H., & Margen, S. (1978). Lactation and pregnancy in Iran. II. Diet and nutritional status. *The American Journal of Clinical Nutrition*, *31*(2), 341–354. https://doi.org/10.1093/ajcn/31.2.341

Gensollen, T., Iyer, S. S., Kasper, D. L., & Blumberg, R. S. (2016). How colonization by microbiota in early life shapes the immune system. *Science*, *352*(6285), 539–544. https://doi.org/10.1126/science.aad9378

Gibson, R. S., Rahmannia, S., Diana, A., Leong, C., Haszard, J. J., Hampel, D., Reid, M., Erhardt, J., Suryanto, A. H., Sofiah, W. N., Fathonah, A., Shahab-Ferdows, S., Allen, L. H., & Houghton, L. A. (2020). Association of maternal diet, micronutrient status, and milk volume with milk micronutrient concentrations in Indonesian mothers at 2 and 5 months postpartum. *The American Journal of Clinical Nutrition*, *112*(4), 1039–1050. https://doi.org/10.1093/ajcn/nqaa200

Gomez-Gallego, C., Garcia-Mantrana, I., Salminen, S., & Collado, M. C. (2016). The human milk microbiome and factors influencing its composition and activity. *Seminars in Fetal and Neonatal Medicine*, *21*(6), 400–405. https://doi.org/10.1016/j.siny.2016.05.003

Gonzalez, E., Brereton, N. J. B., Li, C., Lopez Leyva, L., Solomons, N. W., Agellon, L. B., Scott, M. E., & Koski, K. G. (2021). Distinct Changes Occur in the Human Breast Milk Microbiome Between Early and Established Lactation in Breastfeeding Guatemalan Mothers. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.557180

Gopalakrishna, K. P., & Hand, T. W. (2020). Influence of Maternal Milk on the Neonatal Intestinal Microbiome. *Nutrients*, *12*(3), 823. https://doi.org/10.3390/nu12030823

Grenov, B., Briend, A., Sangild, P. T., Thymann, T., Rytter, M. H., Hother, A.-L., Mølgaard, C., & Michaelsen, K. F. (2016). Undernourished Children and Milk Lactose. *Food and Nutrition Bulletin*, *37*(1), 85–99. https://doi.org/10.1177/0379572116629024

Groer, M. W., Morgan, K. H., Louis-Jacques, A., & Miller, E. M. (2020). A Scoping Review of Research on the Human Milk Microbiome. *Journal of Human Lactation: Official Journal of International Lactation Consultant Association*, *36*(4), 628–643. https://doi.org/10.1177/0890334420942768

Hermansson, H., Kumar, H., Collado, M. C., Salminen, S., Isolauri, E., & Rautava, S. (2019). Breast Milk Microbiota Is Shaped by Mode of Delivery and Intrapartum Antibiotic Exposure. *Frontiers in Nutrition*, *6*. https://doi.org/10.3389/fnut.2019.00004

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M. E., Beck, D. L., Abdo, Z., Fox, L. K., Williams, J. E., McGuire, M. K., & McGuire, M. A. (2011). Characterization of the Diversity and Temporal Stability of Bacterial Communities in Human Milk. *PLoS ONE*, *6*(6), e21313. https://doi.org/10.1371/journal.pone.0021313

Jost, T., Lacroix, C., Braegger, C., & Chassard, C. (2013). Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *British Journal of Nutrition*, *110*(7), 1253–1262. https://doi.org/10.1017/S0007114513000597

Kanapuram, M. (2023). *Associations of maternal diet with infant growth outcomes in eight indigenous communities of guatemala: Effect modification by maternal age (dissertation).* McGill University Libraries.

Khodayar-Pardo, P., Mira-Pascual, L., Collado, M. C., & Martínez-Costa, C. (2014). Impact of lactation stage, gestational age and mode of delivery on breast milk microbiota. *Journal of Perinatology*, *34*(8), 599–605. https://doi.org/10.1038/jp.2014.47

Korpela, K., Blakstad, E. W., Moltu, S. J., Strømmen, K., Nakstad, B., Rønnestad, A. E., Brække, K., Iversen, P. O., Drevon, C. A., & de Vos, W. (2018). Intestinal microbiota development and gestational age in preterm neonates. *Scientific Reports*, *8*(1). Scopus. https://doi.org/10.1038/s41598-018-20827-x

Krebs, N. F. (1998). Zinc supplementation during lactation. *The American Journal of Clinical Nutrition*, *68*(2 Suppl), 509S-512S. https://doi.org/10.1093/ajcn/68.2.509S

Lackey, K. A., Williams, J. E., Meehan, C. L., Zachek, J. A., Benda, E. D., Price, W. J., Foster, J. A., Sellen, D. W., Kamau-Mbuthia, E. W., Kamundia, E. W., Mbugua, S., Moore, S. E., Prentice, A. M., K., D. G., Kvist, L. J., Otoo, G. E., García-Carral, C., Jiménez, E., Ruiz, L., …

McGuire, M. K. (2019). What's Normal? Microbiomes in Human Milk and Infant Feces Are Related to Each Other but Vary Geographically: The INSPIRE Study. *Frontiers in Nutrition*, *6*, 45. https://doi.org/10.3389/fnut.2019.00045

Laursen, M. F., Sakanaka, M., von Burg, N., Mörbe, U., Andersen, D., Moll, J. M., Pekmez, C. T., Rivollier, A., Michaelsen, K. F., Mølgaard, C., Lind, M. V., Dragsted, L. O., Katayama, T., Frandsen, H. L., Vinggaard, A. M., Bahl, M. I., Brix, S., Agace, W., Licht, T. R., & Roager, H. M. (2021). Bifidobacterium species associated with breastfeeding produce aromatic lactic acids in the infant gut. *Nature Microbiology*, *6*(11), 1367–1382. https://doi.org/10.1038/s41564-021-00970-4

Le Doare, K., Holder, B., Bassett, A., & Pannaraj, P. S. (2018). Mother's Milk: A Purposeful Contribution to the Development of the Infant Microbiota and Immunity. *Frontiers in Immunology*, *9*, 361. https://doi.org/10.3389/fimmu.2018.00361

Lewandowska-Pietruszka, Z., Figlerowicz, M., & Mazur-Melewska, K. (2022). The History of the Intestinal Microbiota and the Gut-Brain Axis. *Pathogens*, *11*(12), 1540. https://doi.org/10.3390/pathogens11121540

Li, C., Solomons, N. W., Scott, M. E., & Koski, K. G. (2016). Minerals and Trace Elements in Human Breast Milk Are Associated with Guatemalan Infant Anthropometric Outcomes within the First 6 Months. *The Journal of Nutrition*, *146*(10), 2067–2074. https://doi.org/10.3945/jn.116.232223

Li, C., Solomons, N. W., Scott, M. E., & Koski, K. G. (2019). Anthropometry before Day 46 and Growth Velocity before 6 Months of Guatemalan Breastfed Infants Are Associated with Subclinical Mastitis and Milk Cytokines, Minerals, and Trace Elements. *The Journal of Nutrition*, *149*(9), 1651–1659. https://doi.org/10.1093/jn/nxz109

Lif Holgerson, P., Esberg, A., West, C. E., & Johansson, I. (2023). The breast milk and childhood gastrointestinal microbiotas and disease outcomes: A longitudinal study. *Pediatric Research*, *93*(3), 570–578. https://doi.org/10.1038/s41390-022-02328-w

Liu, F., He, S., Yan, J., Yan, S., Chen, J., Lu, Z., Zhang, B., & Lane, J. (2022). Longitudinal changes of human milk oligosaccharides, breastmilk microbiome and infant gut microbiome are associated with maternal characteristics. *International Journal of Food Science & Technology*, *57*(5), 2793–2807. https://doi.org/10.1111/ijfs.15324

Liu, K., Ding, T., Fang, L., Cui, L., Li, J., Meng, X., Zhu, G., Qian, C., Wang, H., & Li, J. (2020). Organic Selenium Ameliorates Staphylococcus aureus-Induced Mastitis in Rats by Inhibiting the Activation of NF-κB and MAPK Signaling Pathways. *Frontiers in Veterinary Science*, *7*. https://www.frontiersin.org/article/10.3389/fvets.2020.00443

Lockyer, F., McCann, S., & Moore, S. E. (2021). Breast Milk Micronutrients and Infant Neurodevelopmental Outcomes: A Systematic Review. *Nutrients*, *13*(11), 3848. https://doi.org/10.3390/nu13113848

Lopez Leyva, L., Brereton, N. J. B., & Koski, K. G. (2020). Emerging frontiers in human milk microbiome research and suggested primers for 16S rRNA gene analysis. *Computational and Structural Biotechnology Journal*, *19*, 121–133. https://doi.org/10.1016/j.csbj.2020.11.057

Lopez Leyva, L., Gonzalez, E., Solomons, N. W., & Koski, K. G. (2022). Human milk microbiome is shaped by breastfeeding practices. *Frontiers in Microbiology*, *13*. https://www.frontiersin.org/articles/10.3389/fmicb.2022.885588

Ma, J., Palmer, D. J., Geddes, D., Lai, C. T., & Stinson, L. (2022). Human Milk Microbiome and Microbiome-Related Products: Potential Modulators of Infant Growth. *Nutrients*, *14*(23), 5148. https://doi.org/10.3390/nu14235148

Malbe, M., Attila, M., & Atroshi, F. (2006). Possible involvement of selenium in Staphylococcus aureus inhibition in cow's whey. *Journal of Animal Physiology and Animal Nutrition*, *90*(3–4), 159–164. https://doi.org/10.1111/j.1439-0396.2005.00582.x

Marín, M. L., Arroyo, R., Jiménez, E., Gómez, A., Fernández, L., & Rodríguez, J. M. (2009). Cold storage of human milk: Effect on its bacterial composition. *Journal of Pediatric Gastroenterology and Nutrition*, *49*(3), 343–348. https://doi.org/10.1097/MPG.0b013e31818cf53d

Martín, R., Langa, S., Reviriego, C., Jiménez, E., Marín, M. L., Olivares, M., Boza, J., Jiménez, J., Fernández, L., Xaus, J., & Rodríguez, J. M. (2004). The commensal microflora of human milk: New perspectives for food bacteriotherapy and probiotics. *Trends in Food Science & Technology*, *15*(3–4), 121–127. https://doi.org/10.1016/j.tifs.2003.09.010

Maru, M., Birhanu, T., & Tessema, D. (2013). Calcium, magnesium, iron, zinc and copper, compositions of human milk from populations with cereal and "enset" based diets. *Ethiopian Journal of Health Sciences*, *23*(2). https://pubmed.ncbi.nlm.nih.gov/23950625/

McGuire, M. K., & McGuire, M. A. (2015). Human Milk: Mother Nature's Prototypical Probiotic Food? *Advances in Nutrition*, *6*(1), 112–123. https://doi.org/10.3945/an.114.007435

Menchú, M. T., Torún, B., & Elias, L. G. (2012). *Recomendaciones Dietéticas Diarias del INCAP*. INCAP. https://inec.cr/wwwisis/documentos/INCAP/Recomendaciones_Dieteticas_Diarias_del_INCAP.pdf

Moossavi, S., Sepehri, S., Robertson, B., Bode, L., Goruk, S., Field, C. J., Lix, L. M., de Souza, R. J., Becker, A. B., Mandhane, P. J., Turvey, S. E., Subbarao, P., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Khafipour, E., & Azad, M. B. (2019). Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors. *Cell Host & Microbe*, *25*(2), 324-335.e4. https://doi.org/10.1016/j.chom.2019.01.011

Moukarzel, S., & Bode, L. (2017). Human Milk Oligosaccharides and the Preterm Infant: A Journey in Sickness and in Health. *Clinics in Perinatology*, *44*(1), 193–207. https://doi.org/10.1016/j.clp.2016.11.014

Notarbartolo, V., Giuffrè, M., Montante, C., Corsello, G., & Carta, M. (2022). Composition of Human Breast Milk Microbiota and Its Role in Children's Health. *Pediatric Gastroenterology, Hepatology & Nutrition*, *25*(3), 194–210. https://doi.org/10.5223/pghn.2022.25.3.194

Olausson, H., Goldberg, G. R., Laskey, M. A., Schoenmakers, I., Jarjou, L. M. A., & Prentice, A. (2012). Calcium economy in human pregnancy and lactation. *Nutrition Research Reviews*, *25*(1), 40–67. https://doi.org/10.1017/S0954422411000187

Pannaraj, P. S., Li, F., Cerini, C., Bender, J. M., Yang, S., Rollie, A., Adisetiyo, H., Zabih, S., Lincez, P. J., Bittinger, K., Bailey, A., Bushman, F. D., Sleasman, J. W., & Aldrovandi, G. M. (2017). Association Between Breast Milk Bacterial Communities and Establishment and Development of the Infant Gut Microbiome. *JAMA Pediatrics*, *171*(7), 647. https://doi.org/10.1001/jamapediatrics.2017.0378

Perez, P. F., Dore, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., Segura-Roggero, I., Schiffrin, E. J., & Donnet-Hughes, A. (2007). Bacterial Imprinting of the Neonatal Immune System: Lessons From Maternal Cells? *PEDIATRICS*, *119*(3), e724–e732. https://doi.org/10.1542/peds.2006-1649

Petersohn, I., Hellinga, A. H., van Lee, L., Keukens, N., Bont, L., Hettinga, K. A., Feskens, E. J. M., & Brouwer-Brolsma, E. M. (2023). Maternal diet and human milk composition: An updated systematic review. *Frontiers in Nutrition*, *10*, 1320560. https://doi.org/10.3389/fnut.2023.1320560

Petry, N., Olofin, I., Boy, E., Donahue Angel, M., & Rohner, F. (2016). The Effect of Low Dose Iron and Zinc Intake on Child Micronutrient Status and Development during the First

1000 Days of Life: A Systematic Review and Meta-Analysis. *Nutrients*, *8*(12), E773. https://doi.org/10.3390/nu8120773

Platsidaki, E., & Dessinioti, C. (2018). Recent advances in understanding Propionibacterium acnes ( Cutibacterium acnes) in acne. *F1000Research*, *7*, F1000 Faculty Rev-1953. https://doi.org/10.12688/f1000research.15659.1

Ramani, S., Stewart, C. J., Laucirica, D. R., Ajami, N. J., Robertson, B., Autran, C. A., Shinge, D., Rani, S., Anandan, S., Hu, L., Ferreon, J. C., Kuruvilla, K. A., Petrosino, J. F., Venkataram Prasad, B. V., Bode, L., Kang, G., & Estes, M. K. (2018). Human milk oligosaccharides, milk microbiome and infant gut microbiome modulate neonatal rotavirus infection. *Nature Communications*, *9*(1), 5010. https://doi.org/10.1038/s41467-018-07476-4

Ramsay, D. T., Kent, J. C., Owens, R. A., & Hartmann, P. E. (2004). Ultrasound Imaging of Milk Ejection in the Breast of Lactating Women. *PEDIATRICS*, *113*(2), 361–367. https://doi.org/10.1542/peds.113.2.361

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.-P., & Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology*, *2*(4), 361–367. https://doi.org/10.1038/86373

Reyes, S. M., Brockway, M. (Merilee), McDermid, J. M., Chan, D., Granger, M., Refvik, R., Sidhu, K. K., Musse, S., Monnin, C., Lotoski, L., Geddes, D. T., Jehan, F., Kolsteren, P., Allen, L. H., Hampel, D., Eriksen, K. G., Rodriguez, N., & Azad, M. B. (2023). Human Milk Micronutrients and Child Growth and Body Composition in the First 2 years: A Systematic Review. *Advances in Nutrition*, *15*(1), 100082. https://doi.org/10.1016/j.advnut.2023.06.005

Robertson, R. C., Manges, A. R., Finlay, B. B., & Prendergast, A. J. (2019). The Human Microbiome and Child Growth—First 1000 Days and Beyond. *Trends in Microbiology*, *27*(2), 131–147. https://doi.org/10.1016/j.tim.2018.09.008

Ronan, V., Yeasin, R., & Claud, E. C. (2021). Childhood Development and the Microbiome: The Intestinal Microbiota in Maintenance of Health and Development of Disease During Childhood Development. *Gastroenterology*, *160*(2), 495–506. https://doi.org/10.1053/j.gastro.2020.08.065

Ruegg, P. L. (2022). The bovine milk microbiome – an evolving science. *Domestic Animal Endocrinology*, *79*, 106708. https://doi.org/10.1016/j.domaniend.2021.106708

Samuel, T. M., Zhou, Q., Giuffrida, F., Munblit, D., Verhasselt, V., & Thakkar, S. K. (2020). Nutritional and Non-nutritional Composition of Human Milk Is Modulated by Maternal, Infant, and Methodological Factors. *Frontiers in Nutrition*, *7*, 172. https://doi.org/10.3389/fnut.2020.576133

Sanjulián, L., Lamas, A., Barreiro, R., Cepeda, A., Fente, C. A., & Regal, P. (2021). Bacterial Diversity of Breast Milk in Healthy Spanish Women: Evolution from Birth to Five Years Postpartum. *Nutrients*, *13*(7), Article 7. https://doi.org/10.3390/nu13072414

Sarangi, A. N., Goel, A., & Aggarwal, R. (2019). Methods for Studying Gut Microbiota: A Primer for Physicians. *Journal of Clinical and Experimental Hepatology*, *9*(1), 62–73. https://doi.org/10.1016/j.jceh.2018.04.016

Savarino, G., Corsello, A., & Corsello, G. (2021). Macronutrient balance and micronutrient amounts through growth and development. *Italian Journal of Pediatrics*, *47*(1), 109. https://doi.org/10.1186/s13052-021-01061-0

Selma-Royo, M., Calvo-Lerma, J., Bäuerl, C., Esteban-Torres, M., Cabrera-Rubio, R., & Collado, M. C. (2022). Human milk microbiota: What did we learn in the last 20 years? *Microbiome Research Reports*, *1*(3), 19. https://doi.org/10.20517/mrr.2022.05

Słyk-Gulewska, P., Kondracka, A., & Kwaśniewska, A. (2023). MicroRNA as a new bioactive component in breast milk. *Non-Coding RNA Research*, *8*(4), 520–526. https://doi.org/10.1016/j.ncrna.2023.06.003

Smilowitz, J. T., Allen, L. H., Dallas, D. C., McManaman, J., Raiten, D. J., Rozga, M., Sela, D. A., Seppo, A., Williams, J. E., Young, B. E., & McGuire, M. K. (2023). Ecologies, synergies, and biological systems shaping human milk composition—a report from "Breastmilk Ecology: Genesis of Infant Nutrition (BEGIN)" Working Group 2. *The American Journal of Clinical Nutrition*, *117*(Suppl 1), S28–S42. https://doi.org/10.1016/j.ajcnut.2022.11.027

Smith, M. I., Yatsunenko, T., Manary, M. J., Trehan, I., Mkakosya, R., Cheng, J., Kau, A. L., Rich, S. S., Concannon, P., Mychaleckyj, J. C., Liu, J., Houpt, E., Li, J. V., Holmes, E., Nicholson, J., Knights, D., Ursell, L. K., Knight, R., & Gordon, J. I. (2013). Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. *Science (New York, N.Y.)*, *339*(6119), 548–554. https://doi.org/10.1126/science.1229000

Soliman, A., De Sanctis, V., Alaaraj, N., Ahmed, S., Alyafei, F., Hamed, N., & Soliman, N. (2021). Early and Long-term Consequences of Nutritional Stunting: From Childhood to Adulthood. *Acta Bio Medica : Atenei Parmensis*, *92*(1), e2021168. https://doi.org/10.23750/abm.v92i1.11346

Thum, C., Cookson, A. L., Otter, D. E., McNabb, W. C., Hodgkinson, A. J., Dyer, J., & Roy, N. C. (2012). Can Nutritional Modulation of Maternal Intestinal Microbiota Influence the Development of the Infant Gastrointestinal Tract? *The Journal of Nutrition*, *142*(11), 1921–1928. https://doi.org/10.3945/jn.112.166231

Tran, P. A., & Webster, T. J. (2011). Selenium nanoparticles inhibit Staphylococcus aureus growth. *International Journal of Nanomedicine*, *6*, 1553–1558. https://doi.org/10.2147/IJN.S21729

UNICEF. (2021). *Malnutrition in Children*. UNICEF DATA. https://data.unicef.org/topic/nutrition/malnutrition/

UNICEF/WHO/World Bank Group. (2023, May 18). *UNICEF-WHO-The World Bank: Joint Child Malnutrition Estimates (JME) — Levels and Trends – 2023 edition*. UNICEF DATA. https://data.unicef.org/resources/jme-report-2023/

USAID. (2018). *Guatemala: Nutrition Profile*. https://2017-2020.usaid.gov/sites/default/files/documents/1864/Guatemala-Nutrition-Profile-Mar2018-508.pdf

Vatanen, T., Ang, Q. Y., Siegwald, L., Sarker, S. A., Le Roy, C. I., Duboux, S., Delannoy-Bruno, O., Ngom-Bru, C., Boulangé, C. L., Stražar, M., Avila-Pacheco, J., Deik, A., Pierce, K., Bullock, K., Dennis, C., Sultana, S., Sayed, S., Rahman, M., Ahmed, T., … Xavier, R. J. (2022). A distinct clade of Bifidobacterium longum in the gut of Bangladeshi children thrives during weaning. *Cell*, *185*(23), 4280-4297.e12. https://doi.org/10.1016/j.cell.2022.10.011

Verduci, E., Giannì, M. L., Vizzari, G., Vizzuso, S., Cerasani, J., Mosca, F., & Zuccotti, G. V. (2021). The Triad Mother-Breast Milk-Infant as Predictor of Future Health: A Narrative Review. *Nutrients*, *13*(2), 486. https://doi.org/10.3390/nu13020486

Vuori, E., Mäkinen, S. M., Kara, R., & Kuitunen, P. (1980). The effects of the dietary intakes of copper, iron, manganese, and zinc on the trace element content of human milk. *The American Journal of Clinical Nutrition*, *33*(2), 227–231. https://doi.org/10.1093/ajcn/33.2.227

WHO. (2020, August). *Infant and young child feeding*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/infant-and-young-child-feeding

*WHO | Breastfeeding*. (2018). WHO. http://www.who.int/nutrition/topics/exclusive_breastfeeding/en/

Wiciński, M., Sawicka, E., Gębalski, J., Kubiak, K., & Malinowski, B. (2020). Human Milk Oligosaccharides: Health Benefits, Potential Applications in Infant Formulas, and Pharmacology. *Nutrients*, *12*(1), 266. https://doi.org/10.3390/nu12010266

Wren, H. M., Solomons, N. W., Chomat, A. M., Scott, M. E., & Koski, K. G. (2015). Cultural Determinants of Optimal Breastfeeding Practices among Indigenous *Mam* -Mayan Women in the Western Highlands of Guatemala. *Journal of Human Lactation*, *31*(1), 172–184. https://doi.org/10.1177/0890334414560194

Wren-Atilola, H. M., Solomons, N. W., Scott, M. E., & Koski, K. G. (2018). Infant growth faltering linked to subclinical mastitis, maternal faecal-oral contamination, and breastfeeding. *Maternal & Child Nutrition*, *15*(3), e12756. https://doi.org/10.1111/mcn.12756

Wren-Atilola, H. M., Solomons, N. W., Scott, M. E., & Koski, K. G. (2021). Infant Anthropometry and Growth Velocity Before 6 Months are Associated with Breastfeeding Practices and the Presence of Subclinical Mastitis and Maternal Intestinal Protozoa in Indigenous Communities in Guatemala. *Current Developments in Nutrition*, *5*(9), nzab086. https://doi.org/10.1093/cdn/nzab086

Yi, D. Y., & Kim, S. Y. (2021). Human Breast Milk Composition and Function in Human Health: From Nutritional Components to Microbiome and MicroRNAs. *Nutrients*, *13*(9), 3094. https://doi.org/10.3390/nu13093094

Zhang, Z., Zhao, L., Wu, J., Pan, Y., Zhao, G., Li, Z., & Zhang, L. (2023). The Effects of Lactobacillus johnsonii on Diseases and Its Potential Applications. *Microorganisms*, *11*(10), 2580. https://doi.org/10.3390/microorganisms11102580

Zhou, D., Zhao, Y., Li, J., Ravichandran, V., Wang, L., Huang, Q., Chen, C., Ni, H., & Yin, J. (2021). Effects of Phytic Acid-Degrading Bacteria on Mineral Element Content in Mice. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.753195

# Appendix

**Appendix 1 - Milk Mineral Concentrations for the 3 growth parameters (LAZ, WAZ, HCAZ) at early lactation**

| | Early stage (5 - 46 days)[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **WAZ < -1SD (17)** | **WAZ ≥ -1SD (40)** | *P*-value[3] | **LAZ < -2SD (19)** | **LAZ ≥ -2SD (38)** | *P*-value[3] | **HCAZ < -1SD (20)** | **HCAZ ≥ -1SD (37)** | *P*-value[3] |
| **Milk Mineral Concentrations [2]** | | | | | | | | | |
| Calcium, mg/L | 269.9 ± 43 | 277 ± 54.1 | 0.63 | 257.4 ± 42.7 | 283.6 ± 52.7 | 0.66 | 261.9 ± 46 | 281.9 ± 52.4 | 0.16 |
| Copper, µg/L | 523.6 ± 100.7 | 489.3 ± 124.2 | 0.32 | 527.8 ± 104 | 485.4 ± 123.1 | 0.2 | 520.9 ± 108.7 | 488 ± 122.4 | 0.32 |
| Chromium, mg/L | 1120.4 ± 1091 | 3437.3 ± 10274 | 0.36 | 1127.7 ± 1068.2 | 3555.6 ± 10532.5 | 0.32 | 821.4 ± 318.3 | 3786.8 ± 10650.6 | 0.22 |
| Iron, µg/L | 494.9 ± 220.7 | 350 ± 156.1 | **0.007** | 473.5 ± 161.2 | 353 ± 189.5 | **0.02** | 411 ± 172.4 | 383.5 ± 197.6 | 0.6 |
| Magnesium, mg/L | 23.5 ± 4 | 21.7 ± 3.7 | 0.1 | 22.4 ± 4.1 | 22.2 ± 3.7 | 0.84 | 22.9 ± 4.3 | 21.9 ± 3.5 | 0.37 |
| Manganese, µg/L | 10.2 ± 8 | 9.3 ± 5.9 | 0.66 | 10.4 ± 6.6 | 9.1 ± 6.5 | 0.47 | 9.5 ± 5.9 | 9.6 ± 6.9 | 0.96 |
| Potassium, mg/L | 574.6 ± 73 | 538.9 ± 71.9 | 0.09 | 567.3 ± 74.7 | 540.7 ± 72.2 | 0.2 | 559.4 ± 81.9 | 544.2 ± 69.1 | 0.46 |
| Rubidium, µg/L | 1217.7 ± 286.8 | 1036.8 ± 272.9 | *0.03* | 1152.4 ± 295.1 | 1059.9 ± 281.7 | 0.25 | 1139.9 ± 232.4 | 1064.2 ± 312.3 | 0.35 |
| Selenium, µg/L | 14.8 ± 2.2 | 14.5 ± 3.2 | 0.72 | 15.3 ± 2.8 | 14.2 ± 3 | 0.21 | 15.6 ± 2.8 | 14 ± 2.9 | *0.05* |
| Sodium, mg/L | 138.3 ± 33.6 | 129.6 ± 30.7 | 0.34 | 138.9 ± 38.5 | 128.8 ± 27.4 | 0.32 | 134.4 36.3 | 131 ± 29.1 | 0.67 |
| Stronium, µg/L | 46.6 ± 22.9 | 48.3 ± 21.1 | 0.79 | 41.7 ± 16.9 | 50.8 ± 23 | 0.13 | 44.9 ± 19.9 | 49.4 ± 22.4 | 0.46 |
| Zinc, µg/L | 3779 ± 816.1 | 4891.2 ± 5889.5 | 0.44 | 3907.7 ± 1032.3 | 4885.4 ± 6033.5 | 0.49 | 3976.2 ± 953.9 | 4874.8 ± 6124.7 | 0.52 |

Note: [1] Early stage is defined from 5 to 46 days postpartum; [2] Values are arithmetic means ± SD.[3] The level of significance of the *p*-values follows this format: *p < 0.05* are italicized denoting significance, **p < 0.01** are bold denoting stronger significance, ***p < 0.001*** are bold and italicized denoting very strong significance.

**Appendix 2 – Table - Milk Mineral Concentrations for the 3 growth parameters (LAZ, WAZ, HCAZ) at established lactation**

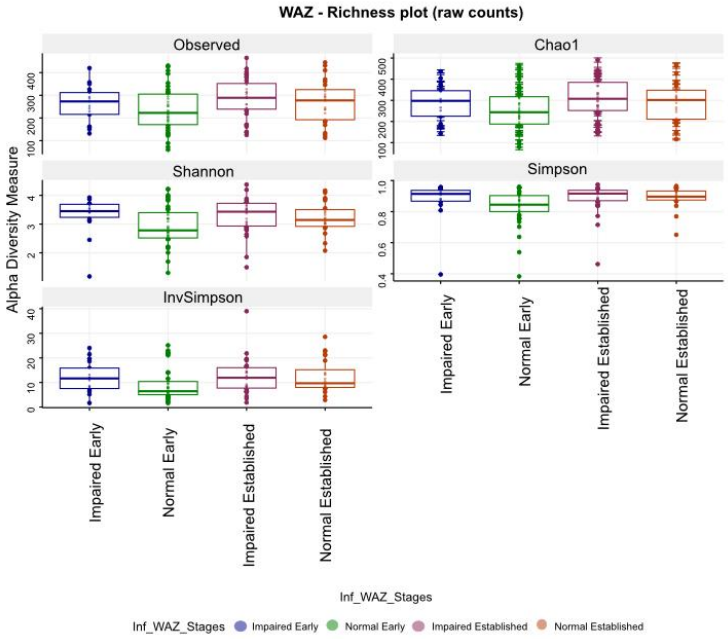
| | Established stage (109 – 184 days)[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **WAZ < -1SD (33)** | **WAZ ≥ -1SD (24)** | *P*-value[3] | **LAZ < -2SD (23)** | **LAZ ≥ -2SD (34)** | *P*-value[3] | **HCAZ < -1SD (25)** | **HCAZ ≥ -1SD (31)** | *P*-value[3] |
| **Milk Mineral Concentrations [2]** | | | | | | | | | |
| Calcium, mg/L | 251.2 ± 31.3 | 240.8 ± 23.9 | 0.18 | 240.3 ± 26.6 | 251.3 ± 29.5 | 0.16 | 241.7 ± 26.4 | 251.5 ± 30.3 | 0.21 |
| Copper, µg/L | 239.4 ± 72.5 | 261.6 ± 60.9 | 0.29 | 231.5 ± 79.3 | 260.4 ± 57.9 | 0.14 | 249.4 ± 69.2 | 248.2 ± 69.6 | 0.95 |
| Chromium, mg/L | 2196.4 ± 1864.2 | 1460.2 ± 553.7 | *0.04* | 1982.6 ± 1921.3 | 1821.4 ± 1157.7 | 0.69 | 1938.4 ± 1936.5 | 1876.9 ± 1075.7 | 0.88 |
| Iron, µg/L | 349.7 ± 314.8 | 226.8 ± 164.4 | 0.09 | 378.7 ± 351.2 | 243.4 ± 177.3 | 0.06 | 340.3 ± 270.4 | 267.1 ± 268.6 | 0.32 |
| Magnesium, mg/L | 35.3 ± 5.2 | 33.9 ± 4.7 | 0.3 | 34.2 ± 4.7 | 35.1 ± 5.3 | 0.52 | 32.8 ± 5 | 36.2 ± 4.6 | *0.01* |
| Manganese, µg/L | 9.8 ± 7.6 | 6.1 ± 4.3 | *0.02* | 10.7 ± 8.3 | 6.6 ± 4.7 | *0.02* | 9.6 ± 7.2 | 7.3 ± 6.2 | 0.21 |
| Potassium, mg/L | 429.6 ± 46.7 | 415.8 ± 52 | 0.3 | 423.5 ± 44.1 | 424 ± 52.8 | 0.97 | 425.2 ± 51.2 | 423.9 ± 48.5 | 0.92 |
| Rubidium, µg/L | 788.6 ± 168.8 | 778.8 ± 130.2 | 0.81 | 769.7 ± 164.9 | 794.5 ± 145.3 | 0.55 | 793 ± 138.9 | 780.6 ± 166.3 | 0.77 |
| Selenium, µg/L | 11.2 ± 2.2 | 10.4 ± 3.8 | 0.33 | 11 ± 1.9 | 10.7 ± 3.5 | 0.79 | 10.5 ± 1.9 | 11.1 ± 3.7 | 0.41 |
| Sodium, mg/L | 92 ± 18.9 | 91.6 ± 17.8 | 0.94 | 89.1 ± 19.8 | 93.7 ± 17.3 | 0.36 | 93.7 ± 19.9 | 90.9 ± 17.2 | 0.56 |
| Stronium, µg/L | 47.6 ± 15.3 | 40.5 ± 13.2 | 0.08 | 44.8 ± 12.8 | 44.5 ± 16.1 | 0.94 | 44.9 ± 16.4 | 44.8 ± 13.5 | 0.99 |
| Zinc, µg/L | 1270.4 ± 538.4 | 1233.7 ± 621.4 | 0.81 | 1229.2 ± 482.9 | 1272.4 ± 628.1 | 0.78 | 1210.2 ± 561.7 | 1297.9 ± 590.3 | 0.57 |

Note: [1] Established stage is defined from 109 – 184 days postpartum. [2] Values are arithmetic means ± SD.[3] The level of significance of the *p*-values follows this format: *p < 0.05* are italicized denoting significance, **p < 0.01** are bold denoting stronger significance, ***p < 0.001*** are bold and italicized denoting very strong significance.

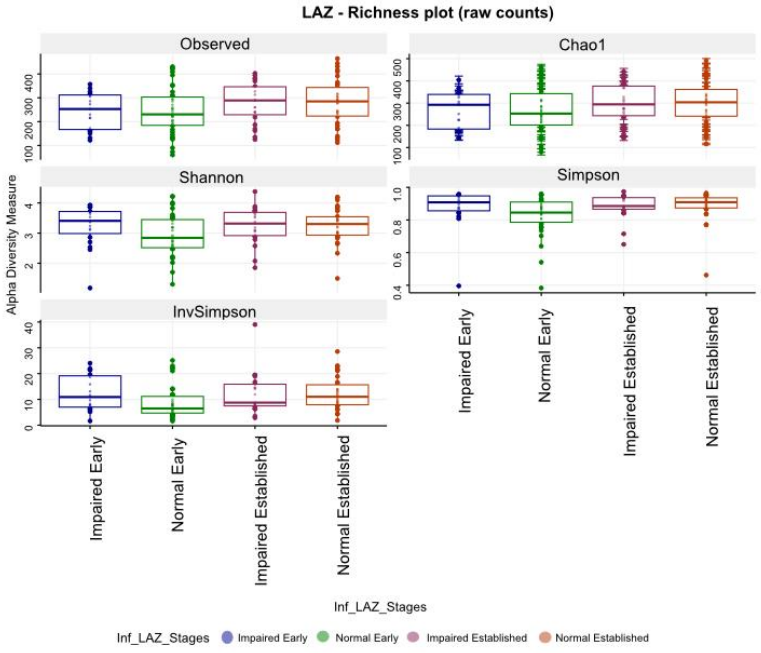
**Appendix 3 - Alpha Diversity**

Indices Observed, Shannon, chao 1 and Simpson are shown for A) WAZ, B) LAZ and C) HCAZ. Each index is shown for the groups of impaired and normal growth at early and established lactation.

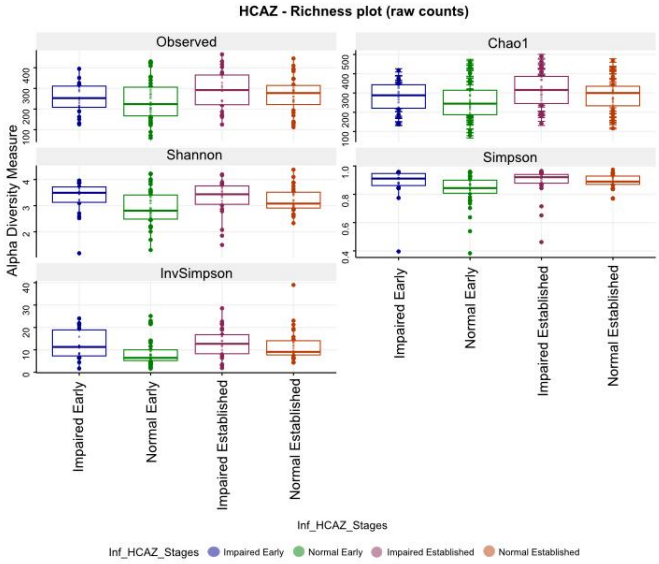
## A) Alpha diversity of LAZ




## B) Alpha diversity of WAZ



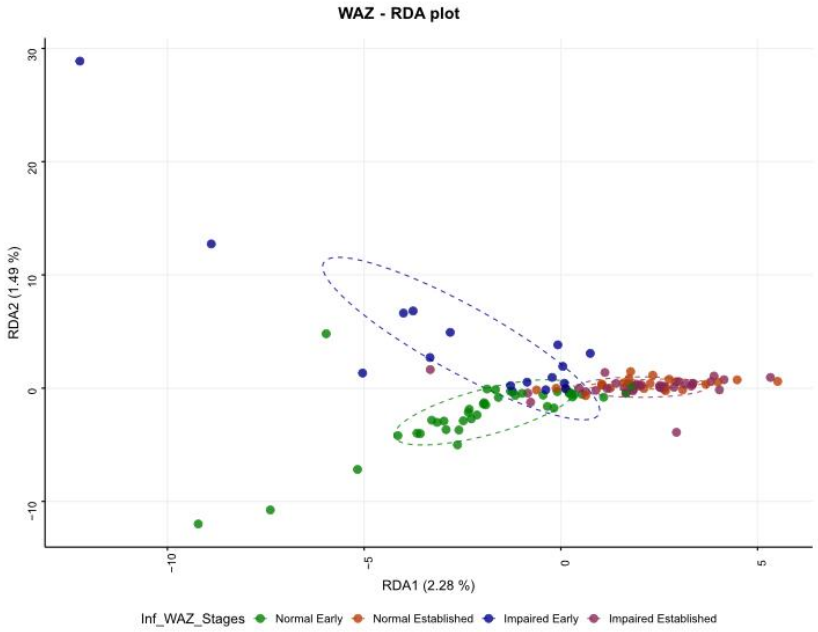
### C) Alpha diversity of HCAZ



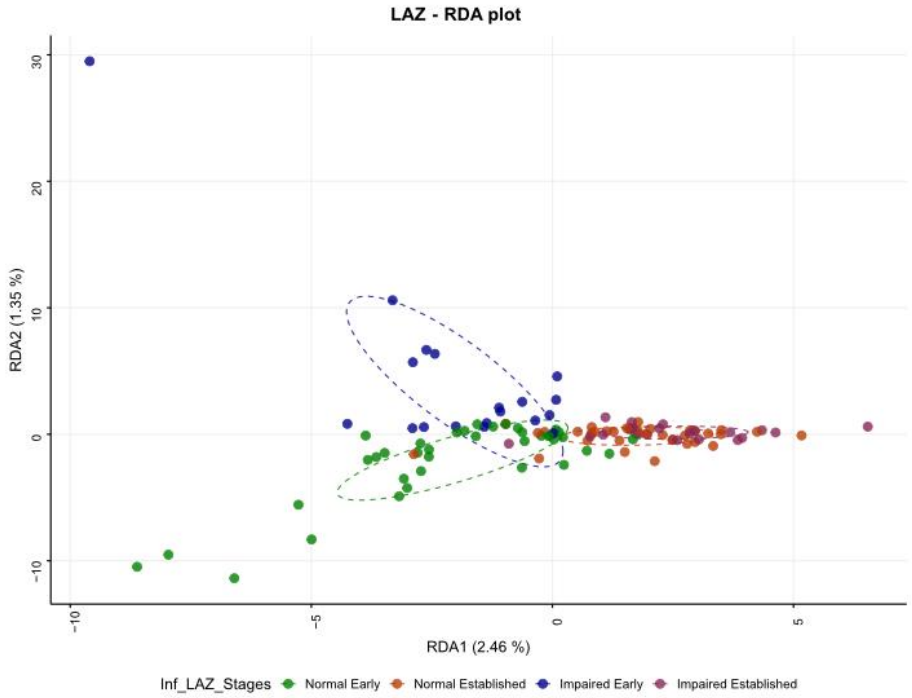

## Appendix 4 – Beta diversity

The RDA ordination plots are shown for A) WAZ, B) LAZ and C) HCAZ. Each plot segregated milk bacteria by impaired and normal growth at early and established lactation.

### A) RDA ordination plot for WAZ



**B) RDA ordination plot for LAZ**




**C) RDA ordination plot for HCAZ**