Title

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Journal

Microbial Ecology, 83(1)

ISSN

0095-3628

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Publication Date

2022

DOI

10.1007/s00248-021-01758-z

Peer reviewed

HHS Public Access

Author manuscript

Microb Ecol. Author manuscript; available in PMC 2023 January 01.

Published in final edited form as:

Microb Ecol. 2022 January ; 83(1): 246–251. doi:10.1007/s00248-021-01758-z.

Breastmilk, Stool, and Meconium: Bacterial Communities in South Africa

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Abstract

Human milk optimizes gut microbial richness and diversity, and is critical for proper immune development. Research has shown differing microbial composition based on geographic location, providing evidence that diverse biospecimen data is needed when studying human bacterial communities. Yet, limited research describes human milk and infant gut microbial communities in Africa. Our study uses breastmilk, stool, and meconium samples from a South African birth cohort to describe the microbial diversity, identify distinct taxonomic units, and determine correlations between bacterial abundance in breastmilk and stool samples. Mother-infant dyads (N=20) were identified from a longitudinal birth cohort in the Vhembe district of Limpopo Province, South Africa. Breastmilk, meconium, and stool samples were analyzed using 16S ribosomal RNA sequencing of the V4–V5 gene region using the MiSeq platform for identification

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Contribution Statement: Dr. Jordyn Wallenborn conceptualized and designed the study. Drs. Wallenborn,Pappas, and Gunier completed all analyses. Drs. Eskenazi, and Chevrier conceptualized and designed the study, designed data collection instruments, and coordinated and supervised data collection. The first draft of the manuscript was written by Dr. Wallenborn and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest/Competing Interests: The authors declare that they have no conflict of interest.

Ethics Approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Institutional Review Boards at the University of California, Berkeley; McGill University; the University of Pretoria; the Limpopo Department of Health and Social Development; and the Ethics Committee of Tshilidzini Hospital approved the study.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Consent for publication: The authors affirm that human research participants provided informed consent for publication.

and relative quantification of bacterial taxa. A non-metric multidimensional scaling using Bray-Curtis distances of sample Z-scores showed that meconium, stool, and breastmilk microbial communities are distinct with varying genus. Breastmilk was mostly comprised of *Streptococcus, Staphylococcus, Veillonella, and Corynebacterium*. Stool samples showed the highest levels of *Bifidobacterium, Faecalibacterium, Bacteroides*, and *Streptococcus*. Alpha diversity measures found that stool samples have the highest Shannon index score compared to breastmilk and meconium. The abundance of *Bifidobacterium* (r=0.57), *Blautia* (r=0.59) and *Haemophilus* (r=0.69) were correlated ($p<0.1$) between breastmilk and stool samples. Despite the importance of breastmilk in seeding the infant gut microbiome, we found evidence of distinct bacterial communities between breastmilk and stool samples from South African mother-infant dyads.

Keywords

Microbiome; Breast Milk; Human Milk; Meconium; Child Development; Gut Microbiome

Research adamantly supports that human milk saves lives [1], improves well-being [2, 3], and reduces societal costs [4] associated with illness and disability. Breastfeeding may also be a viable strategy for optimizing gut microbial richness and diversity [5]. In the immediate period following birth, the infant's immune system is undeveloped due to the near-sterile environment of the mother's womb [6–8]. The first stages of gut colonization and immune system maturation begin with exposure to vaginal and breastmilk microbial communities [9]. A lack of exposure to these microbial communities may disrupt infant gut microbial development, resulting in analtered immune function and increased risk of disease [10]. Deviations of bacterial communities from a healthy state (i.e. dysbiosis) of the infant gut microbiome is associated with necrotizing enterocolitis, inflammatory bowel diseases, malnutrition, metabolic conditions (e.g. obesity), and atopic diseases such as allergies and asthma [11].

Rehabilitating infant gut dysbiosis in early childhood is a potential strategy for promotinghealth and well-being; therefore, it is important to understand the microbial profile of breastmilk, and its link to infant gut microbiome. Yet, research on breastmilk microbiome is limited and little is known about the transfer of breastmilk microbes from mother to infant. Preliminary evidence suggests that breastmilk microbiota seeds the infant gut and strongly influences the lifelong gut microbiome trajectory [5, 12]. A twelve month prospective study of 107 infants reported that 25% of breastmilk bacteria were vertically transferred to the infant's gut microbiome [5]. Another small study (n=7) provided evidence that breastfeeding transfers gut-associated anaerobes [13, 14]. To date, seven studies have provided consistent results for the impact of exclusive breastfeeding on infant gut microbiota [5, 15–20], but with varying degrees of association and different research questions [21]. For example, Azad et al. (2015) reported that breastfeeding exclusivity and duration modified infant gut dysbiosis caused by intrapartum antibiotics [16]. Wood et al. (2018) reported that exclusive breastfeeding results in lower infant gut microbial diversity and distinct microbial composition; however, this study only assessed gut microbiota at six and fourteen weeks postpartum before the gut microbiome reached the transition phase [18, 22].

To date, studies on breastmilk and stool microbiome are predominately conducted in developed nations in North America — which does not address the significant variability in the microbiome found across racial groups and geographic locations [23]. Country-specific microbial data are vital for interpretation of large studies, such as the Human Microbiome Project, that attempt to characterize healthy microbial states and understand the link with human health. Our study uses breastmilk, stool, and meconium samples from a South African birth cohort to describe the microbial diversity, identify distinct taxonomic units, and determine correlations between bacterial abundance in breastmilk and stool samples from mother-infant dyads in a geographic region and population that is vastly understudied.

The Venda Health Examination of Mothers, Babies and their Environment (VHEMBE) is a longitudinal birth cohort in the Vhembe district of Limpopo Province, South Africa. Between August 2012 and December 2013, pregnant women presenting to give birth at Tshilizidini Hospital were screened and recruited. To be eligible, women had to meet the following criteria: ≥18 years old, spoke TshiVenda at home, lived within 20 kilometers of the hospital and did not plan to move away, did not have a malaria diagnosis during pregnancy, and gave birth to a live singleton infant. VHEMBE staff identified 920 eligible participants; of whom 752 completed the baseline survey and 706 provided breastmilk samples at 1 week postpartum [24]. A detailed description of study participants, including variables that may influence the microbiome, can be found elsewhere [24]. This study was performed in line with the principles of the Declaration of Helsinki. The Institutional Review Boards at the University of California, Berkeley; McGill University; the University of Pretoria; the Limpopo Department of Health and Social Development; and the Ethics Committee of Tshilidzini Hospital approved the study.

We analyzed breastmilk samples collected when the child was 1y (n=10), meconium samples collected at birth (n=10) and stool samples collected at 1y (n=20), using 16S ribosomal RNA (rRNA) sequencing at the Vincent J. Coates Genomics Sequencing Laboratory, University of California, Berkeley. Meconium and stool samples were collected from diapers into sterile containers. Breastmilk samples were collected using hand expression into a sterile container. All mothers used soap and water to wash their hands and breast. All samples were temporarily stored on ice packs or in a refrigerator until transferred to a −20° C freezer for short-term storage and a −80° C freezer for long-term storage. Our study population consists of HIV negative mother-infant dyads, ranging from 18–40 years old. Half of the mother-infant dyads in our study (n=10) had all three samples analyzed — maternal breastmilk, and infant stool and meconium. The other half of mother-infant dyads (n=10) had only stool samples analyzed. Prior to DNA isolation, the samples were subjected to mechanical bead-beating pretreatment using ZymoBiomics D4300 DNA miniprep kits (Zymo Research, Irvine, CA). DNA was then extracted using QIAamp Ultraclean Production Pathogen Mini Kits (Qiagen, Valencia, CA). We performed 16S ribosomal RNA amplicon sequencing of the V4–V5 gene region using the MiSeq platform (Illumina, San Diego, CA) for identification and relative quantification of bacterial taxa [25]. An outline of the 16S Illumina Amplicon protocol we used can be found here [26].

All raw sequence analysis, taxonomic identification, and visualization were processed using dbcAmplicons and R (packages ggplot2, vegan, phyloseq). A taxonomic threshold of 97% was used to bin reads into operational taxonomic units (OTUs) [27]. OTU abundance is described at the taxonomic units of genus levels. Alpha rarefaction was determined using the phylogenetic distance [28] and Shannon index [29]. A non-metric multidimensional scaling (NMDS) using Bray-Curtis distances of sample Z-scores was used to minimize variance. We calculated Spearman correlation coefficients for bacterial total and relative abundance between breastmilk and stool samples collected from the mother-child dyads. Bacterial total load was generated using dbcAmplicons for each sample. Analyses was performed in R and corrected for multiple comparisons using the Benjamini-Hochberg procedure to control for the false discovery rate ($q<0.05$) [30].

Figure 1 shows results from the NMDS for meconium collected at birth, and stool and breastmilk samples from 1 year postpartum. Based on this dimensional reduction plot, meconium, stool, and breastmilk microbial communities are distinct with varying genus. Results from the permutational multivariate analysis of variance (PERMANOVA) analysis provides evidence of a significant difference in clustering between breastmilk and stool samples (*p*-value 0.001) (not shown). A Sheppard plot of the residuals (i.e. dissimilarities to the original data) showed minimal scatter, suggesting that original dissimilarities are well preserved in the reduced number of dimensions (not shown).

Figure 2 displays the abundance plot of breastmilk, stool, and meconium samples. Breastmilk was mostly comprised of *Streptococcus, Staphylococcus, Veillonella, and Corynebacterium*. Stool samples showed the highest levels of *Bifidobacterium, Faecalibacterium, Bacteroides*, and *Streptococcus*. Despite general perceptions that meconium is sterile, all 10 meconium samples were home to bacterial communities; albeit small counts. Meconium samples were mostly comprised of *Staphylcoccus, Streptococus, Prevotella, and Bifidobacterium*. Further, four out of ten samples had relatively high levels of *Escherichia/Shigella*.

Figure 3 displays three alpha diversity measures for breastmilk, stool, and meconium samples. On average, stool samples have the highest Shannon index score, indicating stool has the highest amount of richness and microbial community consistency, with most samples having an index score above 2. Breastmilk samples also have rich and consistent microbial communities, with an index score falling slightly below 2. As expected, meconium samples have low diversity, with Shannon index scores hovering around 0. The abundance of *Bifidobacterium* (r=0.57), *Blautia* (r=0.59) and *Haemophilus* (r=0.69) were correlated ($p<0.1$) between breastmilk and stool samples collected from mother-child dyads (data not shown).

Our study describes the distinct microbial communities in breastmilk, stool, and meconium samples in South African mother-infant dyads. The main limitation is lack of generalizability to other populations due to the South African cohort; however, research has demonstrated variability in the microbiome across geographic locations demonstrating the need for microbiome research across multiple countries and ethnic groups [23]. Previous studies in South Africa have independently studied breastmilk, stool, and meconium

microbial profiles [31, 32]. Breastmilk and stool samples from A multi-ethnic United States study that analyzed breastmilk and stool samples using 16S and similar bioinformatics were dominated with *Proteobacteria*, and *Enterobacteriaceae* and *Bifidobacteriaceae*, respectively [5]. However, our study reported mostly different bacterial species dominance in both breastmilk and stool samples. Due to the differences in geographic location and ethnic backgrounds, this comparison may lend to the argument of microbial variations between individuals from different geographic locations, and highlights the need of continued research. An additional limitation of our study is that extractions of meconium and breastmilk samples provided low DNA yield and samples could have been contaminated by contact with diapers or skin during collection.

The first years of life are critical for optimal gut microbial colonies [9]. A number of factors may impact the human gut microbiome including antibiotic use [16], mode of delivery [16], and geographic location [23]. Limited evidence also suggests that breastfeeding can modify gut dysbiosis [16] and breastmilk microbiome may impact immunologic programming [33–36]. To our knowledge, our pilot study is among the first to document breastmilk and infant gut microbial communities in South Africa. We also provide an important description of microbial communities that can be directly compared to previous studies and suggests geographic variations in stool and breastmilk microbial communities. Future research on the impact of breastmilk microbial colonies on infant gut dysbiosis is needed, and investigations should examine the importance of geographic location, diet, and other lifestyle differences in microbial composition.

Acknowledgments:

This study was funded by the National Institute of Environmental Health Sciences (grant number 1R01ES020360-01, PI Eskenazi). J. Chevrier is supported by a Canada Research Chair in Global Environmental Health and Epidemiology.

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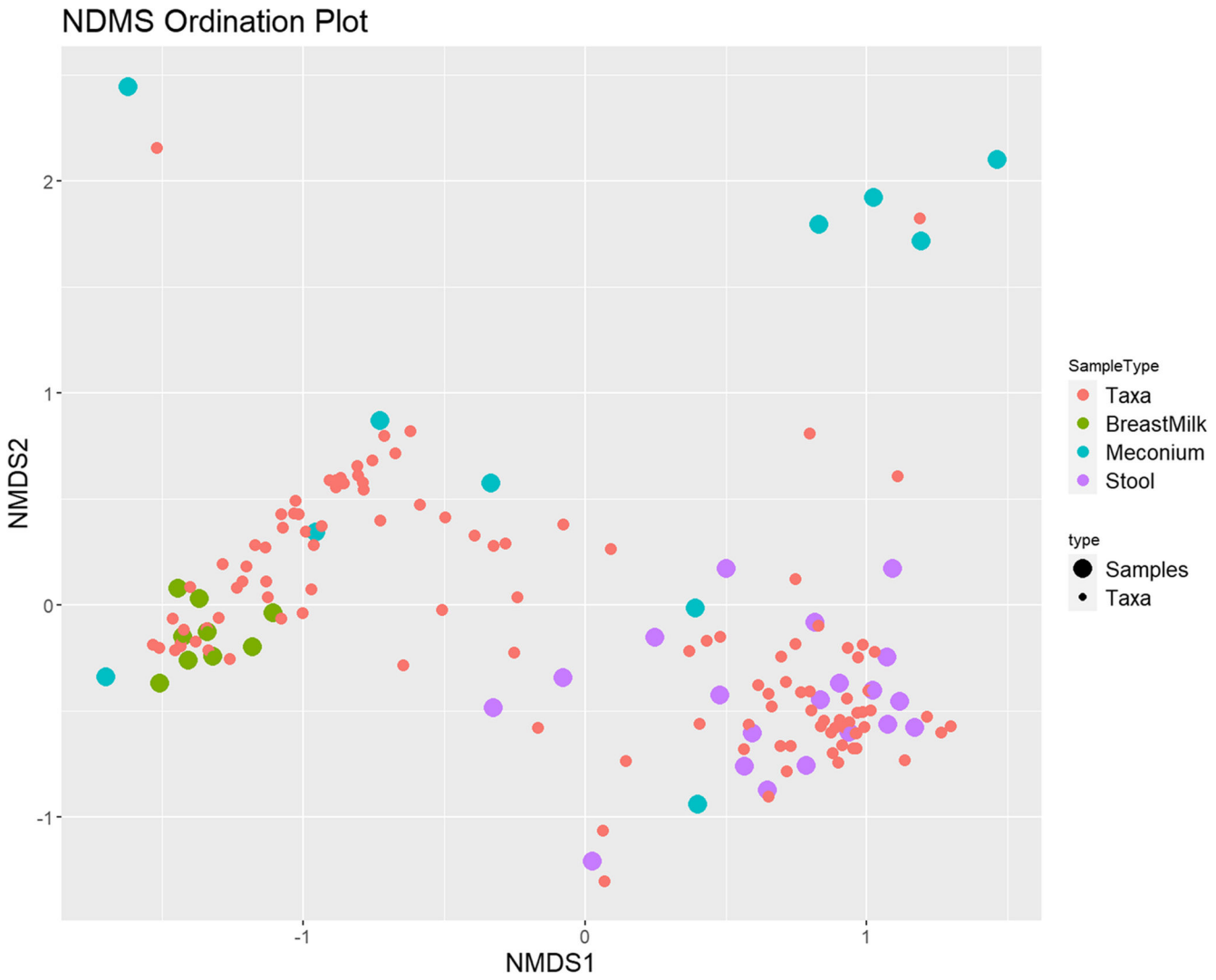

Figure 1.
Non-metric multidimensional scaling using Bray-Curtis distances of sample Z-scores for meconium at birth, and breastmilk and stool samples at one year postpartum

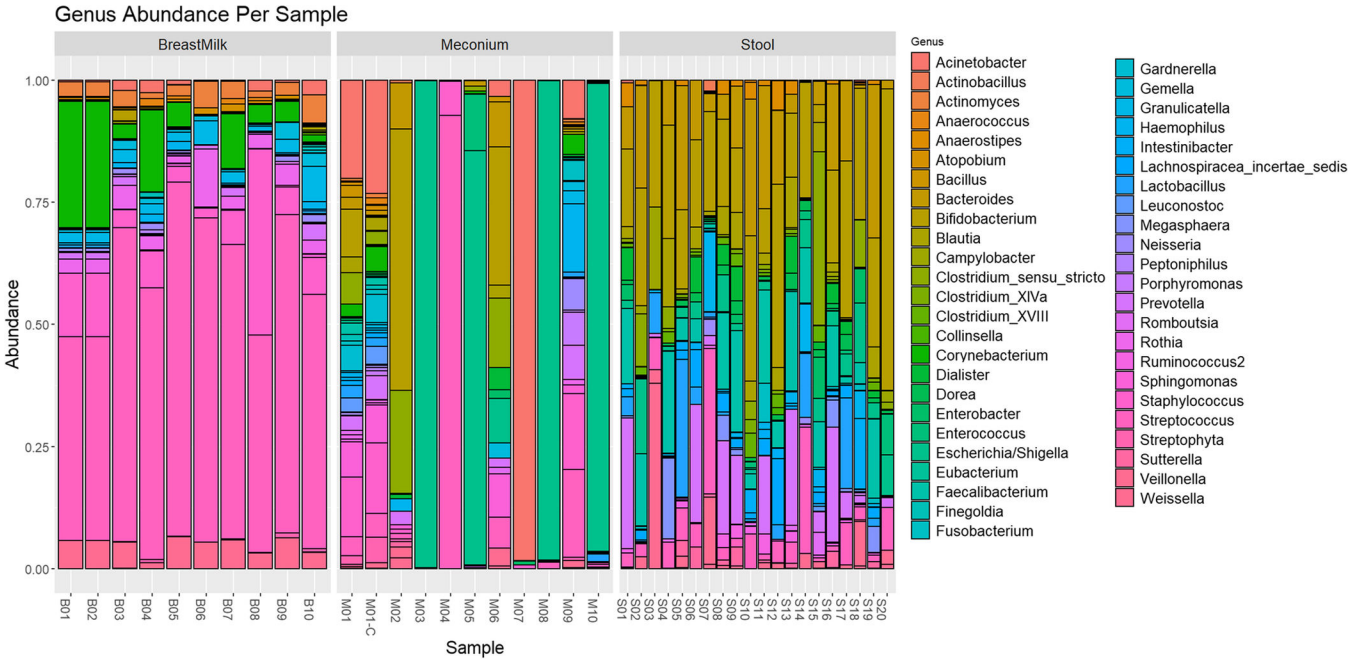

Figure 2.
Bacterial abundance plot for meconium at birth, and breastmilk and stool samples at one year postpartum.

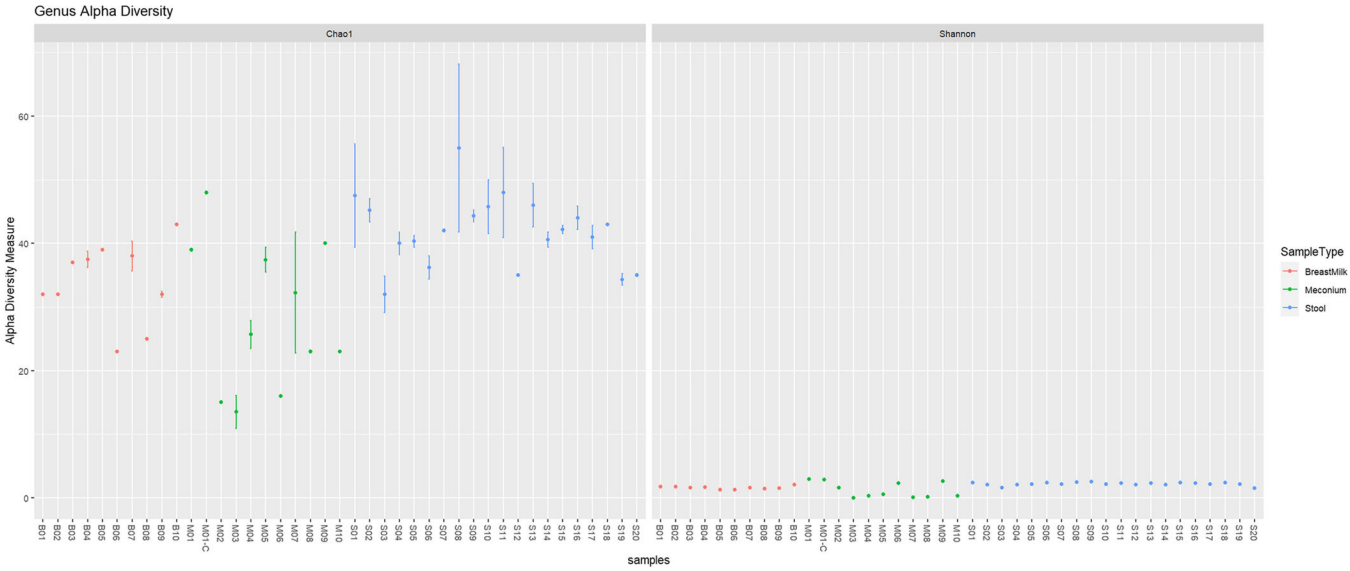

Figure 3.
Alpha diversity indices for meconium at birth, and breastmilk and stool samples at one year postpartum.