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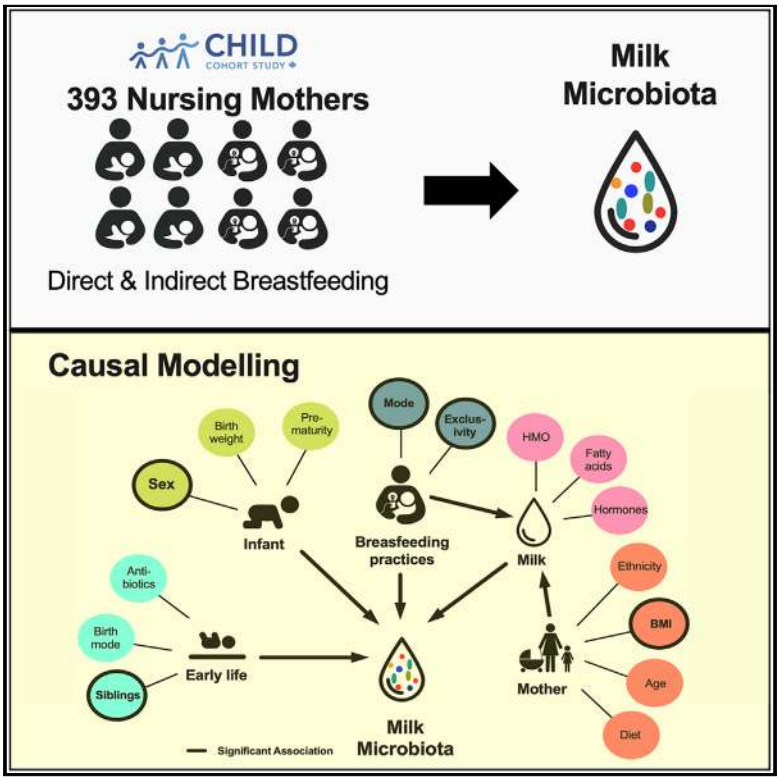
Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors

Graphical Abstract



Highlights

- Milk microbiota variability is affected by maternal factors and other milk components
- Some factors have phylum-specific effects
- Some variations in milk microbiota are sex-specific
- Feeding method (at the breast versus pumped) was strongly associated with milk microbiota

Authors

Shirin Moossavi, Shadi Sepehri, Bianca Robertson, ..., Malcolm R. Sears, Ehsan Khafipour, Meghan B. Azad

Correspondence

meghan.azad@umanitoba.ca

In Brief

Moossavi et al. examine human milk microbiota in the CHILD birth cohort and use causal modeling to describe sex-specific associations with maternal, infant, and early-life factors. A strong association with feeding method (i.e., pumped versus directly at the breast) suggests some milk bacteria originate from the infant oral cavity.

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Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors

Shirin Moossavi,[1,2,3,4] Shadi Sepehri,[2] Bianca Robertson,[5] Lars Bode,[5,6] Sue Goruk,[7] Catherine J. Field,[7] Lisa M. Lix,[8] Russell J. de Souza,[9,10,11] Allan B. Becker,[2,3] Piushkumar J. Mandhane,[12] Stuart E. Turvey,[13] Padmaja Subbarao,[14,15] Theo J. Moraes,[14] Diana L. Lefebvre,[16] Malcolm R. Sears,[16] Ehsan Khafipour,[2,17] and Meghan B. Azad[2,3,18,*]

[1]Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada
[2]Children's Hospital Research Institute of Manitoba, Pediatrics and Child Health, Winnipeg, MB, Canada
[3]Developmental Origins of Chronic Diseases in Children Network (DEVOTION), Winnipeg, MB, Canada
[4]Digestive Oncology Research Center, Digestive Disease Research Institute, Tehran University of Medical Sciences, Tehran, Iran
[5]Pediatrics, University of California, San Diego, La Jolla, CA, USA
[6]Larsson-Rosenquist Foundation Mother-Milk-Infant Center of Research Excellence, University of California, San Diego, La Jolla, CA, USA
[7]Agricultural Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada
[8]Community Health Sciences, University of Manitoba, Winnipeg, MB, Canada
[9]Clinical Epidemiology and Biostatistics, McMaster University, Hamilton, ON, Canada
[10]Nutritional Sciences, University of Toronto, Toronto, ON, Canada
[11]Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, ON, Canada
[12]Pediatrics, University of Alberta, Edmonton, AB, Canada
[13]Pediatrics, University of British Columbia, Vancouver, BC, Canada
[14]Division of Respiratory Medicine, Pediatrics, Hospital for Sick Children, University of Toronto, Toronto, ON, Canada
[15]Physiology, University of Toronto, Toronto, ON, Canada
[16]Medicine, McMaster University, Hamilton, ON, Canada
[17]Animal Science, University of Manitoba, Winnipeg, MB, Canada
[18]Lead Contact
*Correspondence: meghan.azad@umanitoba.ca
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SUMMARY

Breastmilk contains a complex community of bacteria that may help seed the infant gut microbiota. The composition and determinants of milk microbiota are poorly understood. Among 393 mother-infant dyads from the CHILD cohort, we found that milk microbiota at 3–4 months postpartum was dominated by inversely correlated Proteobacteria and Firmicutes, and exhibited discrete compositional patterns. Milk microbiota composition and diversity were associated with maternal factors (BMI, parity, and mode of delivery), breastfeeding practices, and other milk components in a sex-specific manner. Causal modeling identified mode of breastfeeding as a key determinant of milk microbiota composition. Specifically, providing pumped breastmilk was consistently associated with multiple microbiota parameters including enrichment of potential pathogens and depletion of bifidobacteria. Further, these data support the retrograde inoculation hypothesis, whereby the infant oral cavity impacts the milk microbiota. Collectively, these results identify features and determinants of human milk microbiota composition, with potential implications for infant health and development.

INTRODUCTION

Although previously considered sterile, breastmilk is now known to contain a complex community of bacteria that helps establish the infant gut microbiota (Parigi et al., 2015). If this process is disrupted, the infant may develop a dysbiotic microbiota, causing predisposition to chronic diseases such as allergy, asthma, and obesity (Gomez-Gallego et al., 2016). Recent studies on human milk microbiota suggest that it might be affected by local pathologies of the breast, mode of delivery, antibiotics, maternal health, and gestational age (Bode et al., 2014). However, these findings have not been reproduced in large-scale studies, and the determinants of milk microbiota are still mostly unknown.

Two main pathways have been proposed to explain the origin of milk microbiota: entero-mammary translocation of the maternal gut microbiota and retrograde inoculation by the infant's oral microbiota (McGuire and McGuire, 2017). The fact that colostrum collected even before the first infant feeding already contains a microbial community (Damaceno et al., 2017) supports the entero-mammary pathway, while the similarity of infant oral microbiota to breastmilk microbiota (Bisanz et al., 2015) supports the retrograde pathway. It is plausible that both pathways are contributing to the bacterial content of human milk.

Depending on the source of bacteria, different factors could contribute to shaping the milk microbiota. Factors influencing the mother's gut microbiota such as obesity (Collado et al.,

2008) or diet (Wu et al., 2011) could affect the bacteria originating from the maternal gut, while factors influencing the infant's microbiota (Laforest-Lapointe and Arrieta, 2017) such as mode of delivery, older siblings, complementary feeding, and mode of breastfeeding (directly at the breast versus pumped and bottled breastmilk) could potentially alter the bacteria derived from the infant's oral cavity. Other milk components such as human milk oligosaccharides (HMOs), milk fatty acids, hormones, immune cells, and antibodies could also modulate the milk "microenvironment" and create a niche constraint affecting composition of the microbial community. Additionally, factors that could impact the overall milk composition such as circadian rhythm (Nozad et al., 2012) and lactation stage could indirectly affect the milk microbial community.

Studies addressing these potential determinants of milk microbiota have been limited by small sample size, low sequencing depth, and lack of control for confounding factors. The objective of this study was to profile the milk microbiota in a large sample of healthy mother-infant dyads and examine the association of maternal, infant, early-life, and milk factors with milk microbiota composition.

RESULTS

We studied a representative subset of 393 breastfeeding dyads from the CHILD birth cohort (Table S1). The majority were Caucasian (74%), about half (54%) were primiparous, and a quarter (24%) delivered by Cesarean section. The mean ± SD duration of any breastfeeding was 13 ± 6 months and the mean lactation stage at sample collection was 17 ± 5 weeks.

We obtained a mean of 47,710 ± 18,643 high-quality sequencing reads per sample, compared with 46,770 ± 13,479 reads from the mock community and 627 ± 1,034 reads in sequencing negative controls. Profiles were significantly different by sample type, but not by sequencing runs or PCR reactions (Figure S1A). Sequencing contaminants (n = 173 from 9,884 total amplicon sequence variants [ASVs]) were identified and removed using the *decontam* package (Davis et al., 2018); this did not measurably affect sequencing depth (Figure S1B) or microbiota structure (Figure S1C). We observed strong consistency between the observed and expected composition of the mock community (Figure S1D).

Milk Microbiota Is Dominated by Inversely Correlated Proteobacteria and Firmicutes with High Inter-individual Variability

Following removal of rare taxa with <20 reads in total (Figure S1E), 18 unique phyla comprising 1,972 ASVs were detected, with the majority of taxa detected belonging to Proteobacteria (mean ± SD relative abundance: 67% ± 24%, range 3%–99%), Firmicutes (26% ± 22%, range 0.1%–91%), Actinobacteria (4% ± 4%, range 0%–61%), and Bacteroidetes (1% ± 3%, range 0%–44%) (Figure 1A). Proteobacteria and Firmicutes relative abundances were inversely correlated (Pearson $r = -0.97$, $p < 0.001$). At the genus level (Table S2), the most abundant taxa were *Streptococcus* (16% ± 17%), *Ralstonia* (5% ± 3%), and *Staphylococcus* (5% ± 11%). We defined core milk microbiota as ASVs present in at least 95% of individuals with minimum 1% mean relative abundance (Astudillo-García et al., 2017; Shade and Handelsman, 2012). Overall, 12 core ASVs were identified (Table S3); the five most abundant belonged to unclassified Burkholderiales (6% ± 3%), *Staphylococcus* (5% ± 12%), *Ralstonia* (5% ± 3%), unclassified Comamonadaceae (4% ± 3%), and *Acidovorax* (4% ± 2%). These and other core ASVs were present in 100% of samples compared to 0%–20% of sequencing negative controls (Table S3).

Milk Microbiota Profiles Exhibit Discrete Compositional Patterns

To identify potential inherent patterns in the milk microbiota, hierarchical clustering was performed on the core microbiota (Figure 1B). Four clusters (C1–C4) were identified (Tibshirani et al., 2001) (Figure S2) and were predominantly separated based on the relative abundances of *Moraxellaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae* (enriched in C1, n = 42); *Streptococcaceae*, *Staphylococcaceae*, and *Oxalobacteraceae* (C2, n = 98); *Oxalobacteriaceae* and *Comamonadaceae* (C3, n = 161); and *Streptococcaceae* and *Comamonadaceae* (C4, n = 92) (Figure 1C; Table S4). C1 and C2 had the lowest α diversity, while C3 was the most diverse (Figure 1D). Clusters were well separated on PCoA plots of the core (Figure 1E) and overall (>0.01% mean relative abundance; Figure S2C) milk microbiota. C1 and C2 had higher heterogeneity compared to C3 and C4 (Figure S2B), suggesting they may be more enriched with exogenously derived bacteria from maternal skin or breast pump microbiota. Indeed, the majority of mothers in C1 (86%) fed their infants pumped milk (indirect breastfeeding), compared to 66%, 53%, and 48% in C2, C3, and C4, respectively (Table S5; Figure 1F). C1 also had the highest rate of pump versus manual expression (94%, compared to 78%–83% in the other clusters) (Table S5).

Milk Microbiota Diversity Is Associated with Mode of Breastfeeding, Method of Milk Expression, and Other Maternal Factors

Overall, milk samples had a mean ± SD richness (observed ASVs) of 147 ± 44 and diversity (inverse Simpson index) of 15.8 ± 8.7. We explored the association of α diversity with multiple maternal and infant factors (Tables 1 and S6). In multivariable linear regression adjusting for infant sex, mode of feeding, mode of delivery, and parity (number of older siblings), indirect breastfeeding was independently associated with lower milk bacterial richness (adjusted $\hat{\beta}$–18.9, 95% CI, −27.9, −9.9, $p < 0.001$) and diversity (adjusted $\hat{\beta}$−2.08, 95% CI, −3.91, −0.25, $p < 0.05$; Table 1). In a subset with data on milk expression method, pump versus manual expression was associated with significantly lower bacterial richness ($\hat{\beta} = -39.6$, 95% CI, −60.5, −18.7, $p < 0.001$). Within the range of lactation that we studied (17 ± 5 weeks), we did not detect significant associations of lactation stage with α diversity. In addition, we found no association between overall α diversity and mode of delivery, maternal ethnicity, history of atopy, smoking, BMI, secretor status, HMO diversity, or total HMO concentration (Table S6). There were some notable differences when examining within-phylum α diversity (Figure 2). For example, while maternal BMI was not associated with overall α diversity, it was inversely associated with Proteobacteria diversity and positively associated with

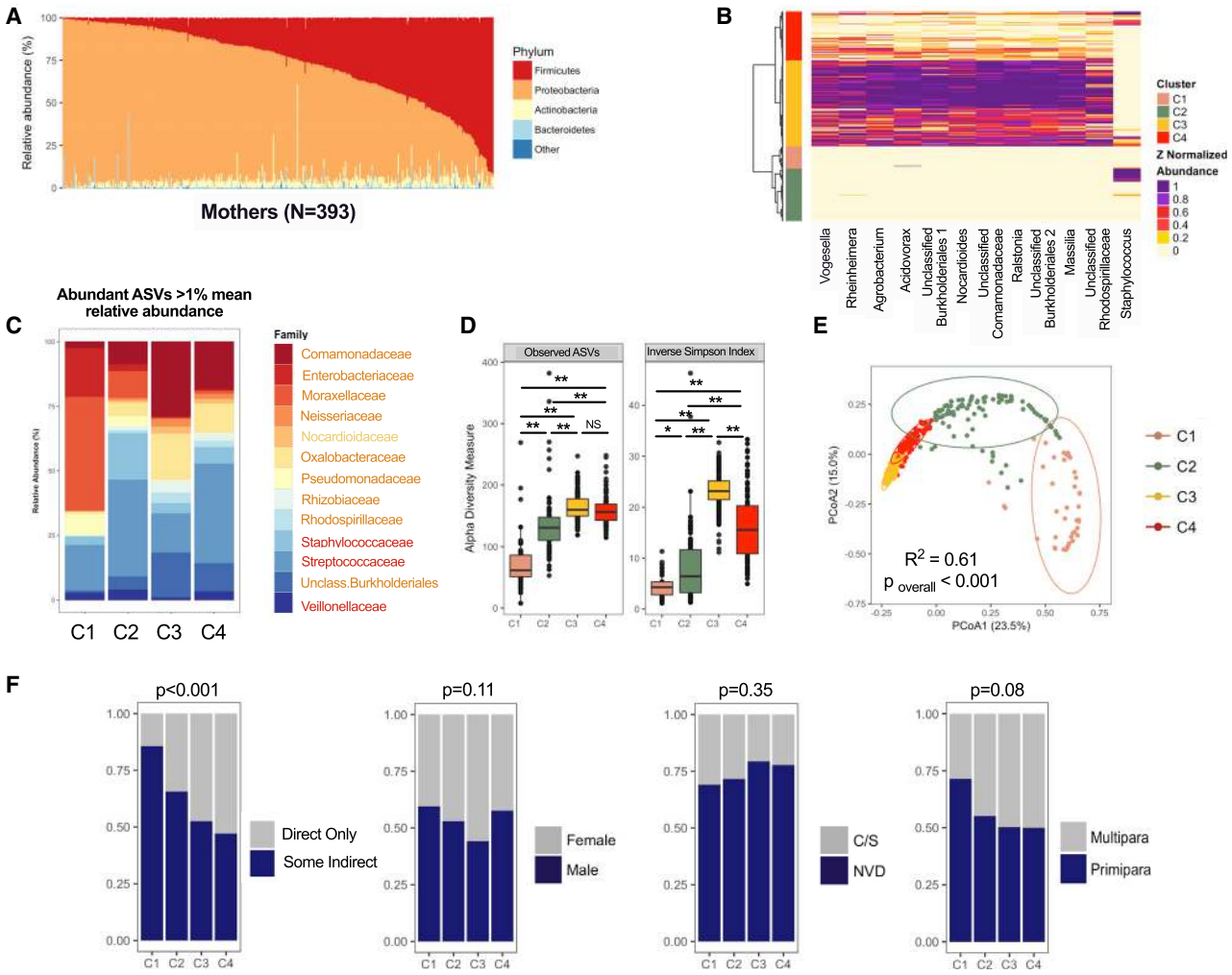

Figure 1. Milk Microbiota Profiles Are Highly Variable between Women, and Exhibit Discrete Compositional Patterns

(A) Milk microbiota profile at phylum level across samples.

(B) Hierarchical clustering of the core milk ASVs defined as present in at least 95% of samples with minimum 1% mean relative abundance; see also Figure S2.

(C) Relative abundance of dominant bacterial families in different clusters; see also Table S5.

(D) Comparison of α diversity between clusters *p < 0.05, **p < 0.001; NS, not significant.

(E) Comparison of β diversity between clusters using PERMANOVA.

(F) Prevalence of mode of breastfeeding, infant sex, mode of delivery (C/S, cesarean section; NVD, normal vaginal delivery), and parity in different clusters.

Firmicutes diversity. Maternal atopy and multiparity were associated with higher Actinobacteria richness.

Milk Microbiota Composition Is Associated with Breastfeeding Practices, Multiparity, and Other Maternal Factors in a Sex-Specific Manner

To identify factors associated with milk microbiota composition, we first examined the relative abundance of taxa at species and genus levels. Among the many factors examined, only mode of breastfeeding was associated with differential relative abundance for a few individual taxa (Figure 5D). *Gemellaceae*, *Vogesella*, and *Nocardioides* had higher relative abundances with direct breastfeeding whereas *Enterobacteriaceae* and *Pseudomonas* were relatively more abundant with indirect breastfeeding. No associations were observed between individual taxa and other factors examined, including maternal secretor status, ethnicity, atopy, parity, mode of delivery, or intrapartum antibiotics (data not shown).

Next, we assessed associations with core milk microbiota cluster membership (Figure 1F; Table S5). Clusters C3 and C4 had lower frequency of indirect breastfeeding compared to clusters C1 and C2 while exclusive breastfeeding and primaparity were more frequent in cluster C1 (Table S5). Maternal age, atopy, infant birth weight, gestational age, and intrapartum antibiotics were not associated with cluster membership (data not shown).

Next, we explored the association of each factor with the entire microbiota composition using redundancy analysis (RDA; Figure 3). Consistent with the cluster analyses, breastfeeding mode (p = 0.001) was significantly associated with the overall composition. Several additional factors including

Table 1. Factors Associated with Milk Microbiota α Diversity

		Richness: Observed OTUs			Diversity: Inverse Simpson			
			Univariate[a]	Model 1[b]		Univariate[a]	Model 1[b]	Model 2[c]
Factor	n (%)	Richness (Mean ± SD)	Crude β (95% CI)	Adjusted β (95% CI)	Diversity (Mean ± SD)	Crude β (95% CI)	Adjusted β (95% CI)	Adjusted β (95% CI)
Breastfeeding Mode								
All direct	162 (41.9)	157.8 ± 37.0	Ref.	Ref.	17.1 ± 8.2	Ref.	Ref.	Ref.
Some indirect	225 (58.1)	138.7 ± 46.9	−19.1 (−27.8, −10.4)[f]	−18.7 (−27.7, −9.64)[f]	14.9 ± 9.2	- 2.2 (−3.9, −0.36)[e]	−2.04 (−3.88, −0.20)[e]	−2.20 (−4.01, −0.40)[e]
Milk Expression[d]								
Manual	20 (18.8)	170.1 ± 40.0	Ref.	–	15.8 ± 7.9	Ref.	–	–
Pump	91 (81.2)	130.5 ± 43.2	−39.6 (−60.5, −18.7)[f]		13.8 ± 9.0	−2.08 (−6.40, 2.25)		
Mode of Delivery								
Vaginal	294 (76.0)	148.5 ± 43.9	Ref.	Ref.	16.3 ± 8.8	Ref.	Ref.	Ref.
C/S emergency	47 (12.1)	133.5 ± 47.3	−14.9 (−28.5, −1.5)[e]	−11.3 (−25.0, 2.5)	14.5 ± 9.2	−1.74 (−4.46, 0.99)	−1.02 (−3.81, 1.78)	−1.29 (−4.01, 1.44)
C/S elective	46 (11.9)	151.1 ± 39.0	2.6 (−11.0, 16.3)	2.5 (−11.2, 16.1)	14.2 ± 8.6	−2.09 (−4.84, 0.67)	−2.03 (−4.81, 0.76)	−1.89 (−4.65, 0.88)
Infant Sex								
Female	192 (48.9)	151.0 ± 43.3	Ref.	Ref.	16.7 ± 8.8	Ref.	Ref.	Ref.
Male	201 (51.1)	142.8 ± 43.9	−8.2 (−16.9, 0.4)	−7.3 (−16.0, 1.4)	14.9 ± 8.9	−1.81 (−3.56, −0.06)[e]	−1.65 (−3.43, 0.12)	−1.62 (−3.39, 0.15)
Older Siblings								
No	211 (53.7)	143.7 ± 47.5	Ref.	Ref.	15.2 ± 9.1	Ref.	Ref.	–
One	126 (32.1)	147.1 ± 39.7	3.4 (−6.3, 13.0)	−2.2 (−12.3, 7.8)	16.4 ± 8.6	1.22 (−0.74, 3.18)	0.68 (−1.37, 2.73)	
Two or more	56 (14.2)	157.7 ± 35.8	13.9 (1.1, 26.8)[e]	7.5 (−6.0, 20.9)	16.7 ± 8.5	1.47 (−1.15, 4.08)	1.11 (−1.64, 3.85)	

N = 393 dyads from the CHILD cohort. Data are presented as mean ± SD. BMI, body mass index; C/S, Caesarean section.

[a]All factors showing an association ($p < 0.05$) on univariate analysis are shown. For other factors examined without significant associations, see Table S6: maternal BMI, lactation stage (interquartile range 14–19 weeks), maternal age, maternal atopy, maternal secretor status (defined based on the presence or absence of 2′-FL or LNFP HMOs), maternal prenatal smoking, maternal ethnicity, milk fatty acids PC1, HMO diversity, and total HMO concentration.

[b]Linear regression adjusted for breastfeeding mode, infant sex, mode of delivery, older siblings, and sample processing time; N = 381.

[c]Linear regression adjusted for breastfeeding mode, infant sex, mode of delivery, and sample processing time; N = 381.

[d]n = 111. Method of milk expression was not systematically captured but was analyzed for samples where it was noted.

[e]$p < 0.05$.

[f]$p < 0.001$.

exclusive breastfeeding, lactation stage, parity, maternal BMI, ethnicity, and infant sex were significantly associated with the milk microbiota composition, albeit with very low redundancy values (each accounting for <1% of the variation in the milk microbiota). The fact that these factors were associated with overall microbiota composition, but not with cluster membership, suggests their influence on the non-core (inter-individual variable) component of the milk microbiota. There was a trend toward association of HMO composition with milk microbiota ($R^2 = 0.055$, $p = 0.074$) (Figure 3).

A multivariable RDA including mode of breastfeeding and lactation stage accounted for 2.2% of variation in the milk microbiota ($p = 0.001$) while additional inclusion of HMO composition increased the total variation explained to 7.3% ($p = 0.001$). Interestingly, the amount of variation explained was considerably higher when RDA models were stratified by infant sex (male, 13.3%, $p = 0.005$; female, 11.6%, $p = 0.17$). Sex specificity was also observed for univariate RDA associations for mode of breastfeeding and lactation stage (higher R^2 in males), as well as parity, mode of delivery, and maternal BMI (significant in females only) in stratified analyses. The combined contribution of all the factors accounted for 35.5% of variation in males ($p = 0.001$) and 34.1% in female infants ($p = 0.079$) (Figure 3).

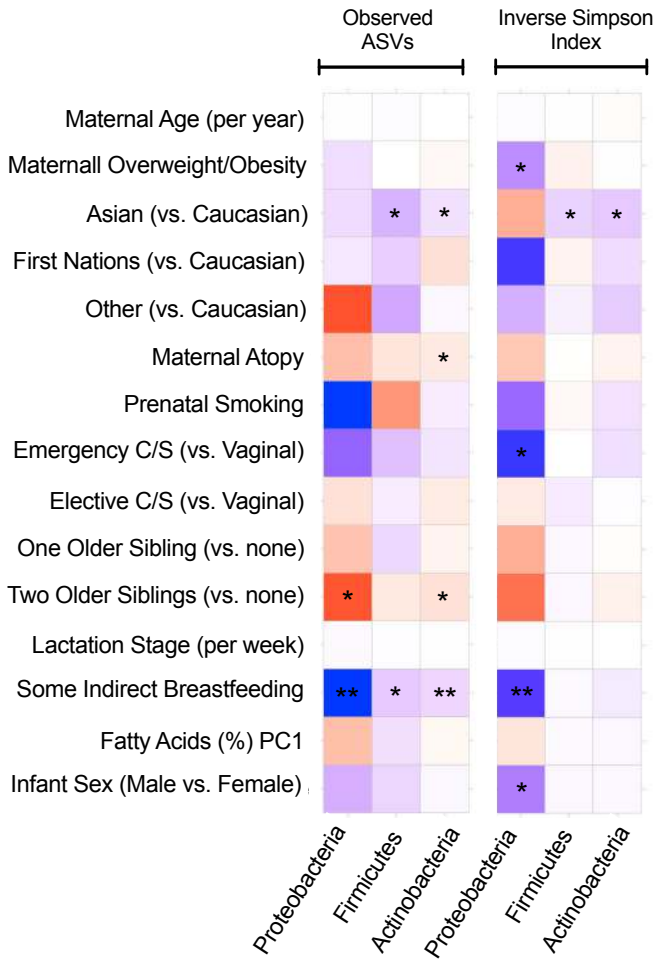

Figure 2. Factors Associated with Milk Microbiota Within-Phylum α Diversity

β coefficients of univariate associations with linear regression are visualized for observed ASVs and inverse Simpson index for major milk microbiota phyla. *p < 0.05, **p < 0.001.C/S, Ceasarean section.

Structural Equation Modeling Identifies Mode of Breastfeeding as a Key Determinant of Milk Microbiota, and Defines Other Causal Pathways Influencing Milk Composition

None of the results above account for the theoretical framework of causal pathways by which different factors could affect milk microbiota (Figure 4A) We performed confirmatory factor analysis (CFA) (Kline, 2016), a variant of structural equation modeling (SEM), to evaluate and compare different plausible frameworks (Figures 4B and S3). We modeled the milk environment as a latent (unobserved) construct with HMOs, fatty acids, insulin, and leptin as indicators. Our variable selection in the CFA was guided by the current literature on proposed mechanisms of mammary gland colonization. Variables potentially important for the retrograde pathway are mode of delivery, infant sex, and older siblings (factors shaping the infant microbiota), as well as mode of breastfeeding (because indirect feeding eliminates contact with the infant oral cavity). Variables potentially important for the enteromammary route include maternal BMI and dietary pattern, which could be correlated with the maternal gut microbiota.

In our final model, breastfeeding mode was directly associated with milk microbiota with β coefficient of 0.19 ($p < 0.001$) (Figure 4). There was a trend in the direct association of infant sex with milk microbiota ($\beta = 0.09$, $p = 0.072$), whereas no association was detected for birth mode or maternal BMI ($\beta < 0.10$, $p > 0.05$). Maternal diet influenced maternal BMI, and BMI was significantly associated with the milk environment latent construct. Overall, we did not detect a significant association between the milk environment and the milk microbiota ($\beta = -0.049$, $p = 0.45$) (Figures 4, S3A, and S3B).

We assessed other plausible causal pathways in equivalent models (Figure S3; Table S7). For example, we removed the indirect effect of BMI on milk microbiota mediated by the milk environment (Figure S3C); however, the model was not a good fit, suggesting that BMI both directly and indirectly affects the milk microbiota. Furthermore, we observed that the effect of mode of breastfeeding on the milk microbiota was not mediated via the milk environment (Figure S3D). We also incorporated some known associations such as the direct effect of diet on milk fatty acids (Figure S3E) and potential direct effect of HMOs on milk microbiota (Figure S3F). Controlling for these additional associations, mode of breastfeeding was the only consistent factor directly associated with the milk microbiota composition. Overall, the CFA suggests that (1) mode of breastfeeding directly influences the milk microbiota, and (2) maternal diet influences BMI, which affects non-bacterial milk components that do not directly influence milk microbiota.

Indirect Breastfeeding Is Associated with Enrichment of Potential Pathogens and Depletion of Bifidobacteria in Milk Microbiota

Given the consistent association of breastfeeding mode with different milk microbiota measures using different analytical approaches (above), we conducted further analyses to explore this relationship (Figure 5). Indirect breastfeeding was associated with lower α (within sample) diversity and higher β (between sample) diversity (Figures 5A–5C). Using a standard ANOVA test, several taxa were differentially abundant based on mode of breastfeeding. *Enterobacteriaceae* and *Enterococcaceae* were more abundant with indirect breastfeeding while *Gemellaceae* and *Vogesella* were enriched with direct breastfeeding (FDR $p < 0.05$). Notably, although *Enterobacteriaceae* was present in 70% of samples regardless of the mode of breastfeeding, it had a 5-fold lower mean relative abundance in direct versus indirect breastfeeding (1.1% ± 3.5% versus 5.0% ± 13.5%, $p = 0.002$). Linear discriminant analysis (Segata et al., 2011) (Figure 5D)

Analysis Variables	All Dyads (n=393) R^2	p	Male (n=201) R^2	p	Female (n=192) R^2	p
Univariable						
Maternal BMI	0.43	*	0.54		1.02	*
Maternal secretor status	0.38	~	0.62		0.61	
Mode of delivery	0.62		0.73		1.71	*
Number of older siblings	0.84	*	1.08		1.53	*
Child antibiotics at sample collection	0.25		0.80	~	0.67	
Mother antibiotics at sample collection	0.21		0.70		0.51	
Lactation stage	0.59	**	0.84	~	0.60	
Mode of breastfeeding (BF)	1.34	***	2.17	***	0.97	*
Exclusive breastfeeding	0.93	***	1.38	**	0.87	*
Total HMO concentration	0.31		0.50		0.60	
HMO compositional profile	5.50	~	10.40		10.50	
Milk insulin	0.32		0.69		0.50	
Milk leptin	0.28		0.42		0.63	
Milk fatty acid profile	7.80		15.50		15.50	
Multivariable						
Model 1 Mode of BF, older siblings, total HMO	2.27	***	3.63	***	2.89	*
Model 2 Mode of BF, lactation stage	2.15	***	3.58	***	1.55	*
Model 3 Mode of BF, lactation stage, HMO profile	7.29	***	13.25	**	11.57	
Model 4 All factors in table	18.19	**	35.47	***	34.11	~

Figure 3. Redundancy Analysis of Associations of Maternal and Infant Factors with Overall Milk Microbiota Composition among 393 Dyads in the CHILD Cohort

Redundancy values (R^2) indicate the percent (%) variation explained by each individual factor (in univariate analyses) or each multivariable model. Shading reflects magnitude of R^2. Maternal age, history of atopy, infant birth weight, gestational age, and intrapartum antibiotics were not significantly associated with milk microbiota composition in RDA and are not shown. BMI, body mass index; HMO, human milk oligosaccharide. ~p < 0.10, *p < 0.05, **p < 0.01, ***p < 0.001.

identified several discriminant taxa that were not differentially abundant in conventional analysis. For example, members of the Actinobacteria phylum and *Veillonellaceae* (a member of oral microbiota) were enriched with direct breastfeeding while *Stenotrophomonas* and *Pseudomonadaceae* (potential opportunistic pathogens) were enriched with indirect breastfeeding (Figure 5D), further suggesting that direct breastfeeding facilitates acquisition of oral microbiota while indirect breastfeeding leads to enrichment by environmental (pump-associated) bacteria.

We next examined association of mode of breastfeeding with *Bifidobacterium* spp. as they constitute the majority of shared taxa between mother's milk and infant stool (Biagi et al., 2017). We identified *Bifidobacterium bifidum* and *B. animalis*, as well as two unclassified *Bifidobacterium* spp., in the milk microbiota Figure 5. The most prevalent *Bifidobacterium* spp. in breastmilk was present in 48% of direct versus 30% of indirect breastfeeding ($p < 0.001$) (Figure 5E).

To assess whether associations with breastfeeding mode were related to the method of milk sample collection, we performed a sensitivity analysis among samples with available information on method of milk collection (n = 111; Figure S4). Overall, pumping versus manual expression was associated with lower milk microbiota richness (Table 1). Stratified analyses showed that richness was significantly lower with indirect breastfeeding regardless of the method of sample collection (Figure S4A), suggesting that feeding mode and collection mode independently influence milk microbiota composition.

DISCUSSION

Using multiple analytic approaches to study the human milk microbiota in a general population cohort, we provide evidence that its composition and diversity are influenced by maternal factors, early life events, breastfeeding practices, and other milk components. To our knowledge, this is among the largest studies of human milk microbiota performed to date, and it is the only study to apply a multivariable causal modeling approach. We identified four main clusters within the milk microbiota and found that mode of breastfeeding was significantly associated with milk microbiota composition. Some additional factors (e.g., maternal BMI and parity) were associated with microbiota composition in a sex-specific manner, while others (e.g., maternal atopy and smoking) were associated with microbiota diversity in a phylum-specific manner. We also uniquely integrated other milk components (e.g., HMOs and fatty acids) in our analyses. Together, these results considerably expand upon existing knowledge about milk microbiota, providing evidence for the importance of breastfeeding practices, maternal factors, and potential sex differences in the pathways determining milk microbiota composition.

Milk Microbiota Clusters Reflect Different Sources of Exogenous Milk Bacteria

Dominant milk bacteria in our cohort were in accordance with previously published results (McGuire and McGuire, 2017). Also in agreement with previous reports (Bode et al., 2014), milk microbiota demonstrated a high degree of inter-individual variability with only a minority of taxa shared across the study population. Though we acknowledge that discrete clusters might not exist within the milk microbiota (Knights et al., 2014), we used hierarchical clustering to identify inherent patterns in the milk microbial community, finding four main clusters. While C1 was dominated by *Enterobacteriaceae*, *Moraxellaceae*, and *Pseudomonadaceae*, C2 had the highest relative abundances of *Streptococcaceae* and *Staphylococcaceae* as well as low abundance of potential reagent contaminants. Given the decreasing proportions of indirect breastfeeding as well as pump-expressed milk from C1 to C2, it is plausible that the clusters, at least partially, reflect a gradient of infant oral versus environmental (e.g., breast pump apparatus biofilm) sources of exogenous milk bacteria (Jiménez et al., 2017). In a previous

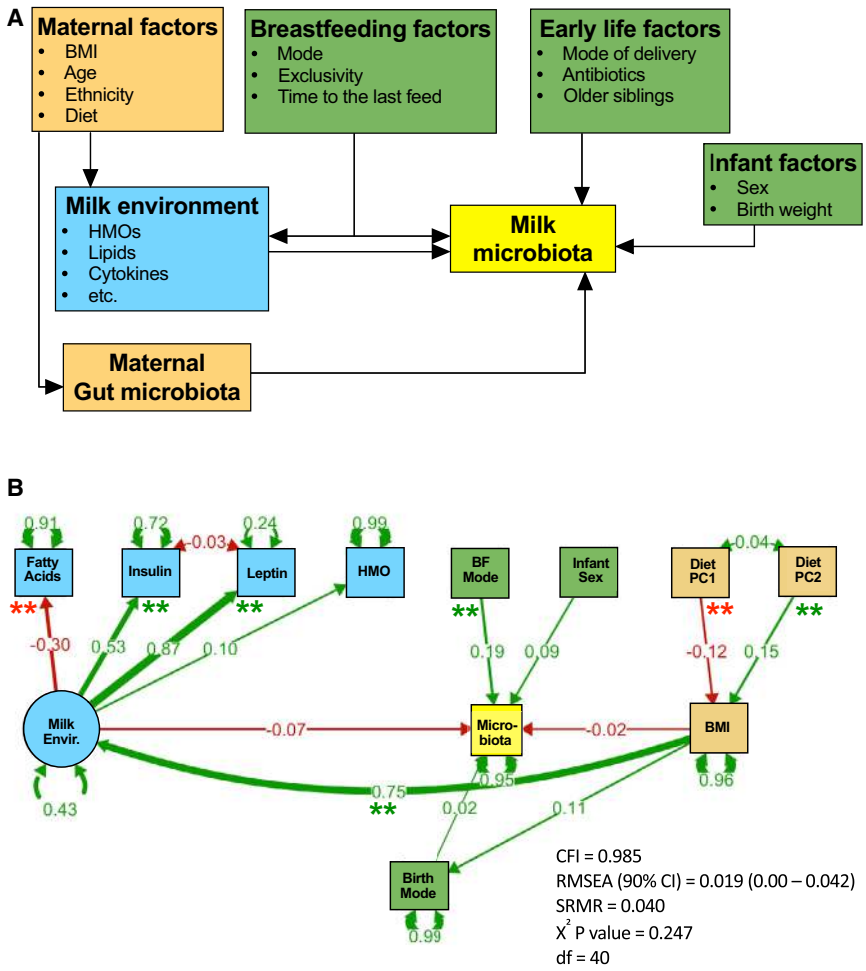

Figure 4. Theoretical Framework and Causal Modeling of Maternal and Infant Factors Influencing the Milk Microbiota

(A) Theoretical framework of how different factors are associated with milk microbiota composition. Maternal factors are more likely to indirectly influence the milk microbiota via modulating other milk components or the maternal gut microbiota, while early-life and infant factors could more directly shape the milk microbiota by influencing the infant oral microbiota.

(B) Mode of breastfeeding is significantly associated with the milk microbiota in structural equation modeling. Standardized β coefficients are reported. BMI, maternal body mass index; CFI, comparative fix index; CI, confidence interval; RSMEA, root-mean-square error of approximation; SRMR, standardized root-mean residuals; *p < 0.05, **p < 0.01. Green, positive; red, negative.

See also Figure S3 and Table S7.

report, three clusters were identified in the milk microbiota of Chinese women with dominance of *Streptococcaceae*, *Staphylococcaceae*, and *Pseudomonadaceae* defining the three clusters (Li et al., 2017). Differences in the cluster-dominating species compared to our study could be the consequence of different clustering methods, inclusion criteria (e.g., restriction to "core" taxa in our study), prevalence of key exposure variables (e.g., indirect breastfeeding), milk collection methods (e.g., aseptic protocol with electric pump in the Chinese study), or geographic variations in the milk microbiota. Further research is warranted to explore the existence and biological relevance of compositional patterns in milk microbiota.

Support for the Exogenous Sources of Milk Colonization

Our study contributes evidence to the ongoing debate regarding the origins of milk microbiota. Two main pathways have been proposed to explain the origin of milk microbiota: entero-mammary translocation of the maternal gut microbiota and retrograde inoculation by the infant's oral microbiota. While it has been hypothesized that the mammary gland may be colonized by a complex microbial community, it has also been suggested that milk microbiota is merely the result of contamination at multiple steps of collection, processing, and storage (Rainard, 2017). Identification of microbial communities within the breast tissue of non-lactating women suggests that the mammary gland is indeed colonized (Urbaniak et al., 2014), and recent data confirm that bacteria are present in human milk collected under aseptic conditions (Sakwinska et al., 2016). However, the same study found a higher abundance and different composition of microbiota in milk collected using a non-aseptic protocol, indicating a substantial contribution from skin microbiota or other externally acquired bacteria. The "retrograde inoculation" hypothesis is also supported by the observation that bacterial load gradually decreases over the course of each feed (West et al., 1979).

Our study provides intriguing evidence to further support the retrograde hypothesis, showing that indirect breastfeeding (defined as at least one serving of pumped milk in the preceding 2 weeks) was significantly associated with milk microbiota diversity and composition. This striking association was consistently replicated using different analytical methods (multivariable regression, redundancy analysis, discriminant analysis, and structural equation modeling), providing confidence that these observed associations could be causal. Our results suggest that both the act of pumping and the lack of contact with the infant oral cavity may independently influence the milk microbiota, although we cannot definitively distinguish between these related factors, nor identify the sources of the exogenously derived bacteria in the expressed breast milk. Nevertheless, based on lower richness associated with indirect versus direct breastfeeding in both manually expressed and pumped milk samples, we speculate that exposure to the infant oral cavity has a persistent impact on shaping the milk microbiota community. Further research is needed to characterize this "retrograde inoculation" process, and to explore the possible impact of pumping on the microbiota of expressed milk.

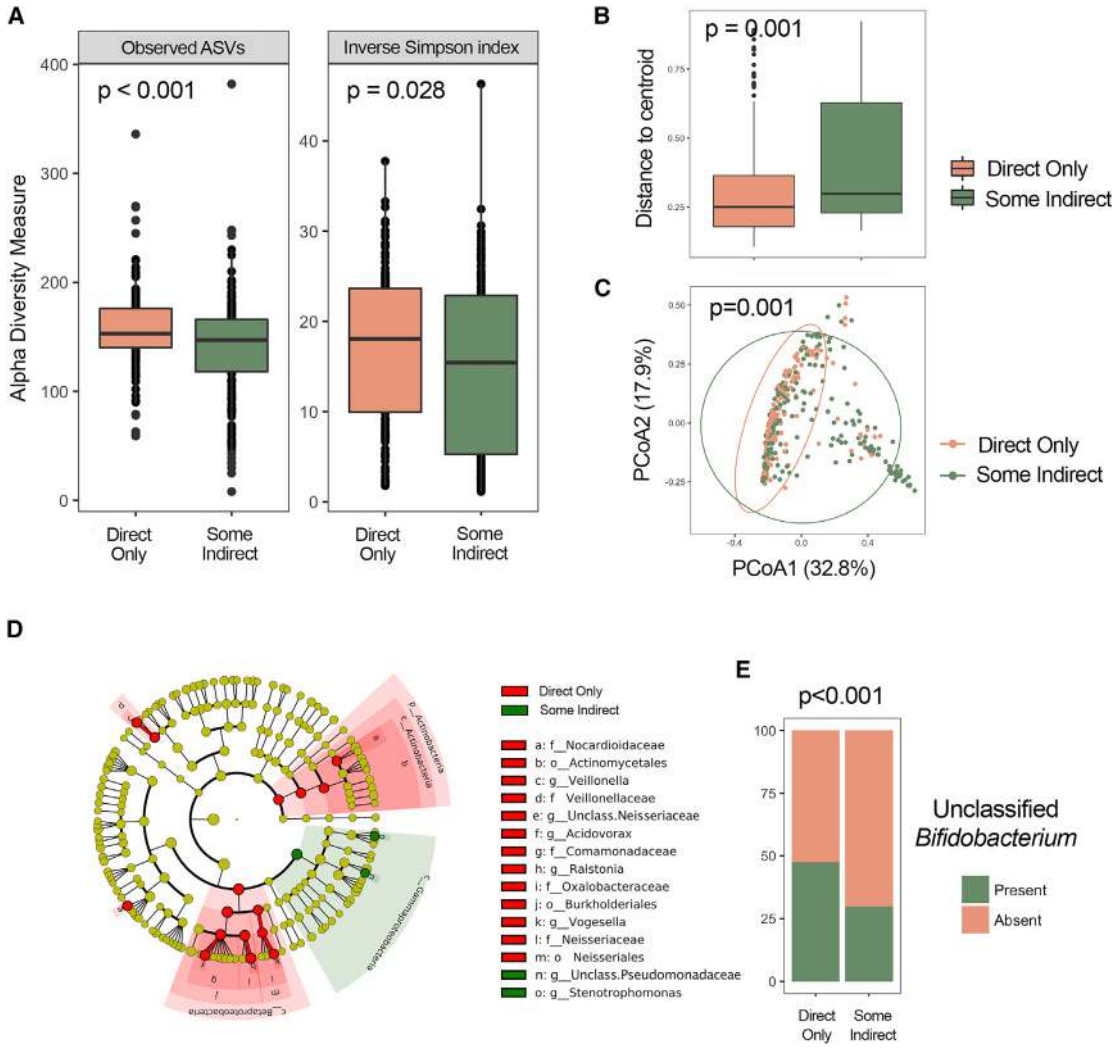

Figure 5. Milk Microbiota Is Associated with Mode of Breastfeeding

(A) α diversity.

(B) Homogeneity of dispersion.

(C) β diversity.

(D) Discriminant analysis of taxa enrichment.

(E) Prevalence of *Bifidobacterium* species.

See also Figure S4.

Mechanistic Explanation for a Clinical Observation

Our current findings on indirect breastfeeding also suggest a possible explanation for our recent observation that infants fed pumped milk are at increased risk for pediatric asthma compared to those fed exclusively at the breast (Klopp et al., 2017). In the current study of milk microbiota, we found that one *Bifidobacterium* spp. existed in milk at relatively high prevalence and was significantly depleted with indirect breastfeeding. This could have important implications for infant immune development and asthma risk because *Bifidobacterium* spp. constitute the majority of shared taxa between mother's milk and infant stool (Biagi et al., 2017), which is typically dominated by *Bifidobacterium* spp. (Asnicar et al., 2017; Biagi et al., 2017). Gut microbiota is crucial in the development and education of the infant immune system (Li et al., 2014), and disruption of gut microbiota in the first few months of life is associated with atopy and asthma later in childhood (Azad et al., 2015; Bridgman et al., 2016). We acknowledge that bifidobacteria may be underrepresented in our study due to known biases against detecting this species using 16S rRNA gene sequencing (Walker et al., 2015); however, the lower prevalence of *Bifidobacterium* spp. associated with indirect breastfeeding among the uniformly processed samples in our study suggests that modifiable factors could indeed impact the proportions of this bacterium in milk. We also found that *Enterobacteriaceae* and potential pathogens were enriched with indirect breastfeeding, consistent with culture-dependent studies where pump expression increased the abundance of *Enterobacteriaceae* and other gram-negative bacteria in milk (Jiménez et al., 2017). Increased exposure to potential pathogens in breastmilk could pose a risk of respiratory infection in the infant, representing

another possible mechanism for increasing asthma risk (Beigelman and Bacharier, 2016). Further research is warranted to explore the impact of pump expression and indirect breastfeeding on milk microbiota and its subsequent effect on infant gut microbiota, immune development, and related health outcomes.

Sex-Specific Variations in Milk Microbiota

We observed intriguing differences in milk microbiota α diversity and overall community structure according to infant sex. There was a trend in direct association of infant sex with milk microbiota in our SEM analysis, and we also found sex-dependent associations of breastfeeding mode, exclusivity, and lactation stage (higher R^2 in males), as well as parity, mode of delivery, and maternal BMI (significant in females only) with milk microbiota in RDA. Lack of sex-stratified analysis could potentially explain why previous studies have reported inconclusive results on the association of the above-mentioned factors with milk microbiota.

The mechanism for the observed sex differences in milk microbiota remains to be determined. Sex differences in gut microbiota have been reported, and are usually attributed to hormonal differences between male and female hosts (Markle et al., 2013). While the host is always female in the case of milk microbiota, it is suspected (as described above) that milk microbiota is partially derived from the infant oral cavity, which may differ in male versus female infants (Takeshita et al., 2016). The sex differences we have observed in milk microbiota could therefore be interpreted as additional evidence supporting the retrograde inoculation hypothesis. Other mechanisms for these sex differences are also possible, as sex-dependent variations have been observed in other milk components including calcium, cortisol, and fat (Fujita et al., 2012; Hinde, 2007; Hinde et al., 2013; Sullivan et al., 2011).

Milk Microbiota and Other Milk Components

Our study uniquely investigated the relationship of milk microbiota and other milk components such as fatty acids, maternal hormones, and HMOs, which may shape the overall milk environment and create the niche for the milk microbiota (Williams et al., 2017). Using RDA, we observed a trend in the association of milk fatty acid and HMO profiles with milk microbiota composition. However, using SEM we did not detect a directional or correlational association of the overall milk environment with the milk microbiota composition, perhaps because our study lacked information on other key components of the milk environment (e.g., immune cells, cytokines, and micro- and macro-nutrients). Further research is warranted to explore the interaction between milk microbiota and other milk components, and to study their combined impact on infant development.

Modifiable Maternal Factors and Milk Microbiota

There is great interest in identifying modifiable factors influencing the milk microbiota, with studies to date reporting inconsistent associations for mode of delivery, maternal diet, and maternal BMI (Cabrera-Rubio et al., 2012; Li et al., 2017; McGuire and McGuire, 2017; Sakwinska et al., 2016; Urbaniak et al., 2016). Maternal diet and BMI are interrelated, and both can modify gut microbiota composition (Collado et al., 2008; Wu et al., 2011) as well as the macro- and micro-nutrient profile of human milk (Mazurier et al., 2017; McGuire et al., 2017), altering the niche for milk microbiota. Maternal BMI is also positively associated with Caesarean section delivery (Pettersen-Dahl et al., 2018). In our SEM analysis, maternal diet, maternal BMI, and delivery mode did not directly affect milk microbiota. However, controlling for the maternal dietary pattern as a factor either affecting the maternal BMI or directly modulating milk fatty acid composition, we observed that BMI could indirectly (via the effect on the overall milk environment) influence the milk microbiota. Using RDA, we found that mode of delivery and maternal BMI were associated with milk microbiota in female infants only, although the effect sizes were small (<2% of variation explained). We also observed a trend toward lower bacterial richness following emergency Caesarean section after controlling for relevant confounding factors. Overall, these associations were relatively subtle and sex-specific, which may explain why previous smaller studies have not found a consistent overall effect of delivery mode on milk microbiota composition.

Strengths and Limitations

The main strength of this study is our unique multi-variable and multi-method approach to assessing the effect of diverse maternal, infant, early-life, and milk factors on the milk microbiota, using the rich data and large sample size afforded by the CHILD cohort. Notably, however, the many factors we evaluated collectively explained less than a third of the total variation observed in milk microbiota composition, indicating that other unmeasured factors are contributing to the large inter-individual variation in milk microbiota profiles. The main limitation of our study is that milk samples were pooled from multiple feeds and were not collected aseptically. While this collection protocol precludes analysis of potential diurnal variations and limits our ability to strictly study the milk microbiota (without skin or other "contaminants"), it provides an accurate representation of the microbiota that infants ingest, which is arguably more biologically relevant to infant health outcomes. Although refrigeration could potentially impact the microbial profile of the milk samples (Sosa and Barness 1987), we did not find any association between sample processing time and milk microbiota composition. We collected a single sample from each mother, so we could not examine longitudinal changes in milk microbiota composition over time. Although we identified potential reagent contaminants at the sequencing step, sequencing results were not available for DNA extraction negative controls, and thus we were not able to identify and remove potential contaminants that might have been introduced during the extraction. Finally, as with all culture-independent microbiota studies, we could not quantify bacterial load or confirm the viability of bacteria identified in our samples (McGuire and McGuire, 2015). As 16S rRNA gene sequencing has limited capacity to resolve the taxa to species and strain levels, further metagenomic and/or culturomic studies are required to confirm and validate the results of this study.

Future Directions

Our results indicate that fixed and modifiable factors can influence the milk microbiota in a sex-specific manner. Whether milk provides bacteria to colonize the infant gut or merely provides selective nutrients to foster a permissive environment is still an open question. Further investigation is needed to determine if milk effectively transfers maternal microbiota or simply enriches and protects the infant oral microbiota for a safe passage to the infant's distal gut. Studies are also needed to identify sources of exogenously derived bacteria; characterize other

elements of the milk microbiota, including fungi and viruses; and determine the impact of pumping on the microbiota of expressed milk. Finally, the impact of milk microbiota on infant gut microbiota development and health remains to be explored and could have important implications for microbiota-based strategies for early-life prevention of chronic conditions.

Conclusions

In this large and comprehensive study, we have used multivariable approaches to explore many features and potential determinants of human milk microbiota composition. Our results suggest that multiple maternal, infant, and environmental factors interactively influence milk microbiota composition. Most strikingly, indirect breastfeeding and pump expression were consistently associated with milk microbiota composition, highlighting the importance of breastfeeding practices. Interesting sex differences were also identified, as well as potential associations between microbiota and other milk components. Further research is warranted to replicate these findings in other populations and explore their implications for infant health and development.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and seven tables and can be found with this article online at https://doi.org/10.1016/j.chom.2019.01.011.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.B.A.; Methodology, S.M., E.K., S.S., L.B., and L.M.L.; Investigation, S.M., B.R., and S.G.; Writing – Original Draft, S.M. and M.B.A.; Writing – Review & Editing, S.S., B.R., L.B., S.G., C.J.F., L.M.L., R.J.S., A.B.B., P.J.M., S.E.T., P.S., T.J.M., D.L.L., M.R.S., and E.K.; Funding Acquisition, M.B.A.; Resources, R.J.S., A.B.B., P.J.M., S.E.T., P.S., D.L.L., and M.R.S.; Supervision, M.B.A. and E.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Breastmilk (1ml)	Moraes et al., 2015	CHILD study http://childstudy.ca
Commercial Kits		
Quick-DNA Fungal/Bacterial extraction kit	Zymo Research	Cat# D6005
MiSeq Reagent Kit v3 (600-cycle)	Illumina	Cat# MS-102-3003
Deposited Data		
Raw data	This paper	16S rRNA sequence data (NCBI) BioProject: PRJNA481046, Sequence Read Archive, SRA: SRP153543
Primers		
16S rRNA-Forward Primer 515F: GTGCCAGCMGCCGCGGTAA	Caporaso et al., 2012	N/A
16S rRNA-Reverse Primer 806R: GGACTACHVGGGTWTCTAAT	Caporaso et al., 2012	N/A
Software and Algorithms		
QIIME2 v.2018.6	Caporaso et al., 2010	https://qiime2.org
Greengenes v. 13.8	DeSantis et al., 2006	http://greengenes.secondgenome.com
Phyloseq v. 1.19.1	McMurdie and Holmes, 2013	https://joey711.github.io/phyloseq/index.html
Decontam v. 1.1.0	(Davis et al., 2018)	https://benjjneb.github.io/decontam/vignettes/decontam_intro.html
MICE v. 2.30	van Buuren and Groothuis-Oudshoorn, 2011	https://github.com/stefvanbuuren/mice
CoDaSeq v. 0.99.1	Gloor and Reid, 2016	https://github.com/ggloor/CoDaSeq
Vegan v. 2.4-4	Oksanen et al., 2017	https://cran.r-project.org/web/packages/vegan/vegan.pdf
LEFSe	Segata et al., 2011	http://huttenhower.sph.harvard.edu/galaxy/
Lavaan v. 0.5-23.1097	Rosseel, 2012	N/A
R v. 3.3.3 and 3.5.1	R Core Team	https://www.r-project.org

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Meghan Azad (meghan.azad@umanitoba.ca).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Inclusion and Exclusion of Study Participants

We studied a representative subset of 428 mothers in the Canadian Healthy Infant Longitudinal Development (CHILD) cohort, a population-based birth cohort designed to study the developmental origins of pediatric asthma and allergy (Subbarao et al., 2015). Women with singleton pregnancies were enrolled between 2008 and 2012 (n = 3407) and remained eligible if they delivered a healthy infant > 35 weeks gestation (n = 3264). For the current study, we selected a representative subset of 428 mother-infant dyads with available milk samples, ensuring equal representation across the 4 study sites, excluding dyads missing key maternal (e.g., diet, BMI) or infant (1y clinical) data, and then randomly selecting among the rest (Table S1). This study was approved by the Human Research Ethics Boards at McMaster University and the Universities of Manitoba, Alberta, Toronto and British Columbia.

Maternal, Infant and Early-Life Factors

Infant feeding was reported by standardized questionnaire at 3, 6, 12, 18 and 24 months. At the time of sample collection (3-4 months), breastfeeding status was classified as exclusive (human milk only) or partial (human milk supplemented with infant formula or solid food). The mode of breastmilk feeding was reported for breastfed infants at three months of age and classified as "direct only" (no feeding of pumped milk), or "some indirect" (at least one serving of pumped milk in the past two weeks) (Klopp et al., 2017). Maternal age, infant sex, birth weight, gestational age, method of birth, parity, and intrapartum antibiotic use were documented from hospital records. Mode of delivery was categorised as normal vaginal delivery (NVD), emergency, or elective Caesarean section. Maternal ethnicity, tobacco smoking, history of asthma diagnosis, and maternal and child intravenous and/or oral antibiotic before sample collection use were reported by standardized questionnaire. Maternal dietary patterns were determined by principal components analysis (PCA) from a modified food frequency questionnaire (de Souza et al., 2016).

METHOD DETAILS

Sample Collection and Processing

Each mother provided one sample of milk at 3-4 months postpartum [mean (SD) 17 (5) weeks postpartum] in a sterile milk container provided by CHILD study. To control for differences in the milk composition of fore- and hindmilk (Hytten, 1954) as well as the diurnal variation (Nozad et al., 2012); a mix of foremilk and hindmilk from multiple feeds during a 24-hour period was collected. Hand expression was recommended, but pumping was also acceptable. The sample was not collected aseptically. Samples were refrigerated at home for up to 24 hours before being collected and processed by study staff (Moraes et al., 2015). Samples were stored at −80°C until analysis. The time between sample pick-up from home and processing by the CHILD laboratory personnel was recorded. We did not observe any associations of sample processing time with milk microbiota richness, diversity, taxa relative abundance, overall composition, and cluster membership (not shown).

Milk Microbiota Analysis

Genomic DNA was extracted from 1 mL breastmilk using Quick-DNA Fungal/Bacterial extraction kit following the manufacturer's instructions (Zymo Research, USA). The samples were centrifuged (13,000 g at 4°C for 20 min), the fat rim was carefully removed using a sterile swab, and the supernatant stored for future analysis. Total DNA was extracted from the pellet. Samples were sequenced following amplification of V4 hypervariable region of the 16S rRNA gene with modified F515/R806 primers (Caporaso et al., 2012) on a MiSeq platform (Illumina, San Diego, CA, USA) as previously described (Derakhshani et al., 2016). Sterile DNA-free water was used as negative controls in sequencing library preparation. A mock community consisting of DNA extracted from of 10 species with known theoretical relative abundances (Zymo Research, USA) were also run as positive control. Overlapping paired-end reads were processed with dada2 pipeline (Callahan et al., 2016) using the open-source software QIIME 2 v.2018.6 (https://qiime2.org) (Caporaso et al., 2010). Unique amplicon sequence variants (ASVs) were assigned a taxonomy and aligned to the 2013 release of the Greengenes reference database at 99% sequence similarity (DeSantis et al., 2006). Demultiplexed sequencing data was deposited into the Sequence Read Archive (SRA) of NCBI and can be accessed via accession number SRA: SRP153543.

Analysis of Other Milk Components

Human milk oligosaccharides (HMOs), milk fatty acids (MFA), and milk metabolic hormones (insulin and leptin) were measured in the same milk samples. HMO analysis was performed at the University of California, San Diego, as previously described (Fields and Demerath, 2012). Briefly, raffinose was added to each sample as an internal standard for absolute quantification. HMOs were isolated by high-throughput solid-phase extraction, fluorescently labeled, and analyzed by high-performance liquid chromatography with fluorescence detection. 19 HMOs were detected and quantified on the basis of retention time comparison with commercial standard oligosaccharides and mass spectrometry analysis. These 19 HMOs typically account for > 90% of total HMO content; their concentrations were summed to estimate total HMO concentration. The relative abundance of each HMO was calculated. Maternal secretor status was defined by the presence of 2′-fucosyllactose (2′-FL) or lacto-N-fucopentaose (LNFP) (Alderete et al., 2015). Milk metabolic hormones (leptin and insulin) were measured using the Mesoscale Discovery System at University of Alberta as previously described (Chan et al., 2018). Briefly, measurements were done in duplicate using kits precoated with the antibody to each hormone following the manufacturer's instructions (MesoScale Discovery, Gaithersburg, MD, USA). Results were measured on the MesoScale Discovery Sector Imager 2400 plate reader and the Discovery Workbench 3.0 software was used to generate standard curves and calculate analyte concentrations in each sample. MFAs were analyzed by gas liquid chromatography at University of Alberta as previously described (Cruz-Hernandez et al., 2013). Briefly, total milk lipids were extracted using a modified Folch protocol. The total lipid extracted was resuspended in 500 mL fresh hexane and injected into a gas liquid chromatograph. Fatty acid methyl esters were prepared using HCl/Methanol (3N) as a catalyst followed by gas liquid chromatography. An internal triglyceride standard was added to identify the fatty acids and determine their relative concentration. Fatty acids were identified according to commercial standards 502 and 643 (Nu-Chek Prep, Elysian, MN, USA) and expressed as a relative percent of total identified fatty acids.

QUANTIFICATION AND STATISTICAL ANALYSIS

Microbial Data Pre-processing and Reagent Contaminant Removal

Data analysis was conducted in R (R Core Team, 2017). Initial preprocessing of the ASV table was conducted using the Phyloseq package (McMurdie and Holmes, 2013). Potential reagent contaminants (Salter et al., 2014) were identified using decontam package based on either the frequency of the ASV in the negative control or the negative correlation with DNA concentration (Davis et al., 2018). Decontam package could remove 70%–90% of contaminants specifically when the source of contamination was not well-defined (Karstens et al., 2018). Overall, 9,711 unique ASVs were detected and 173 were identified as contaminants and excluded. Mock community composition was assessed and agreement with theoretical composition verified (Figure S1D). Samples with less than 25,000 sequencing reads were excluded (n = 35) and the remaining samples (n = 393) were rarefied to the minimum 25,000 sequencing reads per sample. ASVs only present in the mock community or negative controls (n = 894) and ASVs belonging to phylum Cyanobacteria, family of mitochondria, and class of chloroplast (n = 240) were removed. ASVs with less than 20 reads across the entire dataset (n = 6,173) were also removed, resulting in 1,972 remaining ASVs. The contribution of the excluded rare ASVs to the total reads per sample was deemed negligible (Figure S1E). The numbers of sequencing reads of taxa were then relativized to the total sum of 25,000. This dataset was used for analysis unless otherwise specified.

Handling Missing Data

Missing data in HMOs (n = 4) and MFA (n = 5) matrices was imputed using multivariate imputation by chained equations (MICE) package (van Buuren and Groothuis-Oudshoorn, 2011). Dimension reduction of ASVs, HMOs, and MFAs was achieved by principal component analysis (PCA). Samples with missing data for other covariates were excluded from multivariable analyses.

Exclusion of Data

Sequencing reads were excluded if they were deemed to be potential reagent contaminants. Samples were excluded if they had less than 25,000 sequencing reads. Additionally, samples were excluded from multivariable analyses if they were missing essential covariate data.

Linear Association of α Diversity with Maternal, Infant, and Milk Factors

α diversity was assessed by the observed ASVs (richness) and inverse Simpson Index (diversity). Association of α diversity with maternal, infant, early life, and milk factors was assessed by linear regression adjusting for factors with p value of < 0.05 in univariate analysis.

Linear Relationships between Taxa Abundance and Maternal, Infant, and Milk Factors

To control for the compositional nature of the data, ASV counts were center log-ratio transformed following zero-replacement (Gloor and Reid, 2016; Palarea-Albaladejo and Martin-Fernandez, 2015). After this transformation, taxa relative abundances were compared at species, genus, and phylum levels by one-way analysis of variance (ANOVA). P values were corrected with Benjamini-Hochberg's false discovery rate (FDR) method (Benjamini and Hochberg, 1995).

Redundancy Analysis

The association of maternal, infant, early life, and milk factors with milk microbiota composition was assessed by redundancy analysis (RDA) with 1000 permutations using the vegan package (Oksanen et al., 2017).

Hierarchical Clustering of the Core Microbiota

Hierarchical clustering was performed on Bray-Curtis dissimilarity matrix of core ASVs (n = 12, defined as being present in at least 95% of samples with minimum of 1% mean relative abundance), with ward sum-of-square algorithm. The optimal number of clusters was determined using Gap statistics, which compares the observed change in within-cluster dispersion versus the expected change under an appropriate reference null distribution (Tibshirani et al., 2001). Dissimilarity (β diversity) of clusters was assessed by permutational ANOVA (PERMANOVA) using the vegan package (Oksanen et al., 2017). Association of factors with cluster membership was assessed using ANOVA (Tukey post hoc) for continuous and $\chi 2$ (post hoc) for categorical variables.

Structural Equation Modeling

Structural equation modeling (SEM) was performed using confirmatory factor analysis (CFA) to assess the direct versus indirect association of determinant factors of the milk microbiota. SEM was conducted using the lavaan package (Rosseel, 2012) and path diagrams were visualized using semPlot package (Epskamp and Stuber, 2017). Variable selection was informed by the results of statistical tests explained above. The milk environment was modeled as a latent construct with principal component (PC) 1 axes of HMOs, MFAs, and hormones as indicators. The milk microbiota PC1 was used as the milk microbiota variable. Multivariate normality was assessed by Henze-Zirkler's tests (Korkmaz et al., 2014). Given the non-normality of the data and inclusion of dichotomous categorical variables, the model was estimated using maximum likelihood (ML) parameter estimation with bootstrapping (n = 1000) (Kline, 2016). The latent variable was scaled to have variance of one. Model fit was assessed by $\chi 2$ test, the comparative fix index (CFI), root mean square error of approximation (RSMEA) and its 90% confidence interval (CI), and the standardized root

mean residuals (SRMR). Non-significant $\chi 2$ test, CFI $\geq$ 0.9, RMSEA < 0.05, and SRMR < 0.08 were considered as indications of good model fit (Kline, 2016). Equivalents of the final model were explored and defined by changing the directionality of associations or implying co-variation instead of causation.

Linear Discriminant Analysis

Taxa enrichment based on mode of breastfeeding was assessed by linear discriminant analysis (LDA) effect size (LEfSe) with default parameters and logarithmic LDA score threshold of three (Segata et al., 2011).

Presence/Absence Analysis by Mode of Breastfeeding

Bifidobacterium and *Enterobacteriaceae* prevalence according to the mode of breastfeeding was assessed by $\chi 2$ test.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the 16S rRNA sequence data reported in this paper are BioProject: PRJNA481046 and SRA: SRP153543.

Cell Host & Microbe, Volume *25*

Supplemental Information

Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors

Shirin Moossavi, Shadi Sepehri, Bianca Robertson, Lars Bode, Sue Goruk, Catherine J. Field, Lisa M. Lix, Russell J. de Souza, Allan B. Becker, Piushkumar J. Mandhane, Stuart E. Turvey, Padmaja Subbarao, Theo J. Moraes, Diana L. Lefebvre, Malcolm R. Sears, Ehsan Khafipour, and Meghan B. Azad

Table S1. Characteristics of mother-infant dyads from the CHILD cohort included in this analysis (n=393) in comparison with all eligible dyads (n=2536) related to STAR Methods.

Table S2. Most abundant bacterial genera (>1% mean relative abundance) in the human milk microbiota among 393 mothers in the CHILD cohort related to Figure 1.

Table S3. Core milk microbiota[a] among 393 mothers in the CHILD cohort in comparison to the negative controls related to Figure 1.

Table S4. Comparison of milk microbiota composition at family level, by cluster related to Figure 1.

Table S5. Univariate associations of maternal and infant factors with milk microbiota composition and cluster membership among 393 dyads in the CHILD cohort related to Figure 1.

Table S6. Factors not associated with milk microbiota α diversity among 393 mothers in the CHILD cohort related to Table 1.

Table S7. Comparison the equivalent models of the final structural equation model related to Figure 4.

Figure S1. Data quality control related to STAR Methods**.** .

Figure S2. Hierarchical clustering of core milk microbiota among 393 mothers in the CHILD cohort related to Figure 1**.**

Figure S3. Equivalent structural equation models assessing other potentially plausible models of associations of milk microbiota determinants among 393 mothers in the CHILD cohort related to Figure 4.

Figure S4. Expressed milk microbiota is influenced by collection method related to Figure 5.

Table S1. Characteristics of mother-infant dyads from the CHILD cohort included in this analysis (n=393) in comparison with all eligible dyads (n=2536) related to STAR Methods.

Factor	Characteristics	Mean ± SD or n (%)[a]	
		Subset for this study N=393	All eligible dyads N=2536[b]
Maternal	Age (years)	33.0 ± 4.2	32.7 ± 4.2
	Pre-pregnancy BMI (Kg/m^2)	24.3 ± 5.2	24.5 ± 6.3
	History of atopy	251 (63.9)	1624 (65.4)
	Secretor status	279 (71.7)	901 (74.7)[c]
	Ethnicity		
	Caucasian	287 (73.0)	1870 (74.3)
	Asian	73 (18.6)	403 (16.0)
	First Nations	15 (3.8)	87 (3.5)
	Other	18 (4.6)	158 (6.3)
Infant	Birth weight (g)	3469 ± 469	3450 ± 479
	Female sex	192 (48.9)	1198 (47.2)
	Gestational age (weeks)	39.2 ± 1.3	39.2 ± 1.2
Early life	Mode of delivery		
	Elective C/S	46 (11.9)	264 (10.6)
	Emergency C/S	47 (12.1)	339 (13.6)
	Vaginal	294 (76.0)	1893 (75.8)
	Maternal intrapartum antibiotics	139 (35.8)	996 (39.9)
	Maternal postpartum antibiotics before 3-4 months	41 (10.6)	265 (10.8)
	Child antibiotics before 3-4 months	11 (2.8)	75 (3.1)
	Older siblings		
	None	211 (53.7)	1345 (53.1)
	One	126 (32.1)	860 (33.9)
	Two or more	56 (14.2)	330 (13.0)
Breastfeeding	Lactation stage at sample collection (weeks)	17.3 ± 5.3	16.5 ± 5.0
	Exclusive BF (breast milk only) at sample collection	190 (48.3)	1393 (55.9)
	Direct BF (at the breast) only	162 (41.9)	1018 (40.1)
	Duration of BF (months)	13.2 ± 5.8	12.5 ± 5.6
	Duration of exclusive BF (months)	3.5 ± 2.3	3.7 ± 2.3
Milk	HMO concentration (mg/mL)	10.2 ± 2.1	10.3 ± 2.1[c]
	HMO Simpson's diversity	4.9 ± 1.4	4.9 ± 1.3[c]
	Insulin (pg/mL)	760 ± 664	Unknown
	Leptin (pg/mL)	556 ± 601	Unknown

[a]Percentages are calculated after excluding dyads with missing data. BF, breastfeeding; BMI, body mass index; C/S, Caesarean section; HMO, human milk oligosaccharide; NVD, normal vaginal delivery

[b] Those who breastfed ≥12 weeks and provided a milk sample

[c] available for N=1206

Table S2. Most abundant bacterial genera (>1% mean relative abundance) in the human milk microbiota among 393 mothers in the CHILD cohort related to Figure 1.

Lineage	Genus	Prevalence (%)[a]	Relative abundance (%)		
			Mean±SD	CV	Range
Firmicutes - *Streptococcaceae*	*Streptococcus*	98.7	16.2 ± 16.7	1.0	87.4
Firmicutes - *Staphylococcaceae*	*Staphylococcus*	99.8	4.9 ± 11.5	2.4	87.5
Proteobacteria - *Oxalobacteraceae*	*Ralstonia*	99.2	4.8 ± 2.8	0.6	9.4
Proteobacteria - *Comamonadaceae*	*Acidovorax*	99.5	3.9 ± 2.3	0.6	13.3
Proteobacteria - *Moraxellaceae*	*Acinetobacter*	48.6	3.7 ± 12.0	3.2	87.3
Proteobacteria - *Comamonadaceae*	*Aquabacterium*	99.5	3.2 ± 1.9	0.6	7.6
Proteobacteria - *Oxalobacteraceae*	*Massilia*	99.8	2.4 ± 1.4	0.6	6.5
Proteobacteria - *Rhizobiaceae*	*Agrobacterium*	99.2	1.9 ± 1.1	0.6	4.5
Proteobacteria - Uncl. Alteromonadales	*Rheinheimera*	99.5	1.9 ± 1.2	0.6	4.7
Firmicutes - *Veillonellaceae*	*Veillonella*	78.1	1.5 ± 2.7	1.8	21.0
Proteobacteria - *Neisseriaceae*	*Vogesella*	99.2	1.2 ± 0.7	0.6	3.0
Actinobacteria - *Nocardioidaceae*	*Nocardioides*	99.2	1.1 ± 0.6	0.6	2.6
Proteobacteria - *Pseudomonadaceae*	*Pseudomonas*	33.3	1.0 ± 7.2	6.9	72.8

[a] Percentage of samples where the taxa was present.

CV, coefficient of variation; Uncl, unclassified.

Table S3. Core milk microbiota[a] among 393 mothers in the CHILD cohort in comparison to the negative controls related to Figure 1.

Lineage	Genus	Samples (n=393)			Negative controls (n=15)
		Mean±SD	Maximum	Prevalence	Prevalence
Proteobacteria - Burkholderiales	Unclassified	5.86 ± 3.43	12.56	100%	13%
Firmicutes - *Staphylococcaceae*	*Staphylococcus*	4.86 ± 11.5	87.5	100%	20%
Proteobacteria - *Oxalobacteraceae*	*Ralstonia*	4.79 ± 2.76	9.41	100%	7%
Proteobacteria - *Comamonadaceae*	Unclassified	4.42 ± 2.58	9.75	100%	7%
Proteobacteria - *Comamonadaceae*	*Acidovorax*	3.95 ± 2.34	13.33	100%	20%
Proteobacteria - *Oxalobacteraceae*	*Massilia*	2.37 ± 1.40	6.47	100%	13%
Proteobacteria – Uncl. Alteromonadales	*Rheinheimera*	1.89 ± 1.15	4.74	100%	0
Proteobacteria - *Rhizobiaceae*	*Agrobacterium*	1.85 ± 1.08	4.51	100%	7%
Proteobacteria - *Rhodospirillaceae*	Unclassified	1.61 ± 1.07	5.24	100%	7%
Proteobacteria - *Neisseriaceae*	*Vogesella*	1.23 ± 0.74	3.04	100%	0
Actinobacteria - *Nocardioidaceae*	*Nocardioides*	1.09 ± 0.65	2.61	100%	13%
Proteobacteria - Burkholderiales	Unclassified	1.07 ± 0.64	2.63	100%	0

[a] Defined as amplicon sequence variants (ASVs) present in at least 95% of samples with mean relative abundance of more than 1% after removing potential reagent contaminants. Uncl, unclassified.

Table S4. Comparison of milk microbiota composition at family level, by cluster related to Figure 1.

Family	C1 (n=42)	C2 (n=98)	C3 (n=161)	C4 (n=92)	q value
Unclassified Alteromonadales	0.08 ± 0.08	0.85 ± 0.4	2.95 ± 0.6	1.98 ± 0.5	<0.001
Veillonellaceae	1.18 ± 3.38	2.5 ± 3.8	0.67 ± 1.1	2.2 ± 2.4 c	<0.001
Neisseriaceae	0.06 ± 0.05	0.55 ± 0.3	1.9 ± 0.4	1.29 ± 0.3	<0.001
Pseudomonadaceae	**3.31 ± 11.3**	2.40 ± 12.1	0.14 ± 0.9	0.07 ± 0.2	0.007
Oxalobacteraceae	0.38 ± 0.29	**3.27 ± 1.5**	**11.2 ± 1.5**	7.37 ± 1.1	<0.001
Moraxellaceae	**17.8 ± 22.2**	6.31 ± 15.6	0.18 ± 1.2	0.75 ± 2.9	<0.001
Enterobacteriaceae	**7.6 ± 13.9**	1.59 ± 4.9	0.31 ± 1.8	0.41 ± 1.8	<0.001
Comamonadaceae	0.9 ± 2.1	5.20 ± 2.4	**17.9 ± 2.3**	**11.9 ± 1.8**	<0.001
Rhizobiaceae	0.1 ± 0.1	0.85 ± 0.38	2.9 ± 0.5	1.91 ± 0.4	<0.001
Nocardioidaceae	0.06 ± 0.06	0.49 ± 0.23	1.7 ± 0.3	1.13 ± 0.2	<0.001
Streptococcaceae	**7.1 ± 17.8**	**22.8 ± 22.8**	**9.45 ± 7.2**	**25.1 ± 12.6**	<0.001
Staphylococcaceae	1.3 ± 2.1	**11.0 ± 20.8**	2.48 ± 3.3	4.12 ± 5.6	<0.001
Other	60.0 ± 26.8	41.5 ± 22.8	45.6 ± 4.8	40.2 ± 9.9	-

Families with >1% overall mean relative abundance are shown. Relative abundances of dominant families within each cluster are in bold. Data is presented as mean ± SD. Relative abundances were compared by one-way analysis of variance (ANOVA).

Table S5. Univariate associations of maternal and infant factors with milk microbiota composition and cluster membership among 393 dyads in the CHILD cohort related to Figure 1. p values <0.05 are in bold. Data are presented as mean±SD.

Factor	Microbiota Cluster Membership [a]				
	C1 (n = 42)	**C2 (n = 98)**	**C3 (n = 161)**	**C4 (n = 92)**	**Overall p-value**
BMI (Kg/m^2)	25.8 ± 7.4	24.6 ± 4.8	24.2 ± 4.7	23.8 ± 4.5	0.17
Secretor status, n (%)					
Secretor	30 (71.4)	76 (79.2)	104 (65.4)	69 (75.0)	0.10
Non-secretor	12 (28.6)	20 (20.8)	55 (34.6)	23 (25.0)	
Ethnicity, n (%)					
Asian	6 (14.2)	21 (21.4)	36 (22.4)	10 (10.9)	
Caucasian	33 (78.6)	68 (69.4)	112 (69.6)	74 (80.4)	0.61
First Nation	1 (2.4)	5 (5.1)	6 (3.7)	3 (3.3)	
Other	2 (4.8)	4 (4.1)	7 (4.3)	5 (5.4)	
Lactation stage at sample collection (weeks)	17.6 ± 5.2	17.7 ± 6.16	16.8 ± 4.9	17.5 ± 5.3	0.51
Mode of breastfeeding, n (%)					
Indirect	36 (85.7)	63 (65.6)	83 (52.5)	43 (47.3)	**<0.001**
Direct	6 (14.3) [cde]	33 (34.4) [cfg]	75 (47.5) [dg]	48 (52.7) [ef]	
Milk expression [b]					
Manual	1 (6.3)	6 (19.4)	9 (29.6)	4 (16.7)	0.54
Pump	15 (93.7)	25 (80.6)	31 (70.4)	20 (83.3)	
Exclusive breastfeeding at sample collection, n (%)					
No	29 (69.1)	54 (55.1)	74 (46.0)	46 (50.0)	0.052
Yes	13 (30.9) [g]	44 (44.9)	87 (54.0) [g]	46 (50.0)	
Total HMO (mg/mL)	9.9 ± 1.9	10.6 ± 2.1	10.1 ± 2.1	10.2 ± 2.2	0.18
Insulin (pg/mL)	741 ± 591	788 ± 552	748 ± 687	760 ± 765	0.97
Leptin (pg/mL)	612 ± 615	564 ± 581	554 ± 604	528 ± 617	0.91
Number of older siblings, n (%)					
None	30 (71.4)	54 (55.1)	81 (50.3)	46 (50.0)	0.08
One	9 (21.4)	31 (31.6)	55 (34.2)	31 (33.7)	
Two or more	3 (7.2) [gh]	13 (13.3)	25 (15.5) [g]	15 (16.3) [h]	
Infant sex					
Female	17 (40.5)	46 (46.9)	90 (55.9)	39 (42.4)	0.11
Male	25 (59.5)	52 (53.1)	71 (44.1)	53 (57.6)	
Mode of delivery					
Vaginal	29 (69.1)	68 (80.0)	127 (79.3)	70 (77.8)	0.22
Caesarean section - emergency	9 (21.4)	11 (12.9)	19 (11.9)	8 (8.9)	
Caesarean section - elective	4 (9.5)	6 (7.1)	14 (8.8)	12 (13.3)	

[a] Associations were tested by ANOVA (Tukey post hoc) for continuous and $\chi 2$ (post hoc) for categorical variables.

[b] n= 112. Method of milk expression was not systematically captured, but was analyzed for samples where it was noted. The frequency is calculated in the subset with available data.

[c, d, e, f] $p<0.05$ for overall and pairwise comparison of clusters. [gh] $p<0.1$. Same superscript denotes pairwise significant difference.

Not Shown: Maternal age, history of atopy, infant birth weight, gestational age, intrapartum antibiotics, and antibiotics before the time of sample collection by mother or child were not significantly associated with the clusters. BMI, body mass index; HMO, human milk oligosaccharide

Table S6. Factors not associated with milk microbiota α diversity among 393 mothers in the CHILD cohort related to Table 1.

Factor	n (%)	Observed ASVs	Inverse Simpson
Maternal age (years)			
20-30	100 (25.3)	149 ± 43	16.1 ± 9.0
30-40	270 (68.4)	147 ± 44	15.8 ± 8.7
> 40	25 (6.3)	140 ± 42	14.5 ± 9.7
Maternal weight class			
Normal	260 (65.8)	147 ± 43	15.9 ± 8.8
Overweight/obese	135 (34.2)	146 ± 45	15.6 ± 8.9
Maternal ethnicity			
Caucasian	276 (73.6)	148 ± 45	15.9 ± 8.9
Asian	70 (18.7)	139 ± 38	15.3 ± 8.5
First Nation	15 (4.0)	148 ± 56	16.4 ± 10.5
Other	14 (3.7)	153 ± 33	16.0 ± 7.4
Maternal atopy			
No	134 (35.7)	142 ± 44	15.2 ± 9.1
Yes	241 (64.3)	150 ± 43	16.1 ± 8.7
Maternal secretor status			
Secretor	269 (71.7)	147 ± 43	15.4 ± 8.8
Non-secretor	106 (28.3)	145 ± 40	16.6 ± 8.7
Maternal ever smoking			
No	307 (77.7)	147 ± 42	15.9 ± 8.8
Yes	88 (22.3)	145 ± 48	15.4 ± 9.2
Maternal prenatal smoking			
No	377 (95.4)	147 ± 43	15.9 ± 8.9
Yes	18 (4.6)	140 ± 61	12.6 ± 8.3
Lactation stage (weeks)			
10-15	175 (44.5)	149 ± 45	16.1 ± 8.9
15-21	133 (33.8)	144 ± 39	15.4 ± 8.8
21-27	56 (14.2)	142 ± 47	14.9 ± 8.9
27-32	21 (5.4)	152 ± 50	17.5 ± 9.6
>32	8 (2.1)	144 ± 26	14.2 ± 5.5

Univariate linear regression, all $p > 0.05$

Sample processing time, HMO diversity, or total HMO concentration were also not significantly associated with α diversity (not shown).

Table S7. Comparison the equivalent models of the final structural equation model related to Figure 4. See also Figure S5.

Model	Difference from final model	Df	χ2 p value	CFI	RMSEA (90% CI)	SRMR	AIC
Final model	Causative effect of milk on microbiota	40	0.247	0.985	0.019 (0.00 – 0.042)	0.040	11401
A	Causative effect of microbiota on milk	40	0.251	0.985	0.019 (0.00 – 0.042)	0.040	11401
B	Milk environment and microbiota correlation	40	0.247	0.985	0.019 (0.00 – 0.042)	0.040	11401
C	Effect of BMI on milk removed	41	**< 0.001**	**0.404**	**0.119 (0.106 – 0.133)**	**0.108**	11614
D	Effect of mode of breastfeeding on the milk environment	40	**0.028**	0.949	0.035 (0.012 – 0.054)	0.046	11414
E	Effect of maternal diet on MFA	38	0.210	0.982	0.022 (0.00 – 0.044)	0.040	11404
F	Direct effect of HMO on microbiota	39	0.241	0.984	0.020 (0.00 – 0.043)	0.040	11402

AIC, Akaike information criterion; BMI, body mass index; CFI, comparative fix index; CI, confidence interval; HMO, human milk oligosaccharide; MFA, milk fatty acid; RSMEA, root mean square error of approximation; SRMR, standardized root mean residuals.

Indications of poor model fit are in **bold**: χ2 p-value <0.05, CFI <0.9, RMSEA > 0.05, and SRMR>0.08

Figure S1. Data quality control related to STAR Methods. A) Milk microbiota β diversity by sample type, sequencing run, and PCR plates, prior to removal of potential contaminant ASVs using the decontam package. β diversity was assessed on Bray-Curtis dissimilarity and tested by PERMANOVA. There was a significant difference based on sample type but not sequencing runs or PCR plates. Comparison of B) Sequencing depth and C) Composition of the top 500 most abundant ASVs before and after contaminant removal. D) Relative abundance of most abundant bacterial genera (>1% mean relative abundance) in the mock community in comparison with the theoretical composition, each bar represents a replicate. E) Sequencing output processing to remove low abundance ASVs. The 393 individual samples are on the X axis and relative abundances of the sums of sequence reads per sample are plotted along the Y axis. The initial ASV table following contaminant removal contained 8145 ASVs. Following exclusion of ASVs with less than 20 reads across the entire dataset, 1,972 ASVs remained. Exclusion of ASVs with less than 0.01% and 1% relative abundance resulted in 301 and 22 remaining ASVs, respectively. Analyses have been performed on the 1,972 ASVs with >20 reads in total, unless stated otherwise. N samples = 428, N mock =18, N negative control =15,

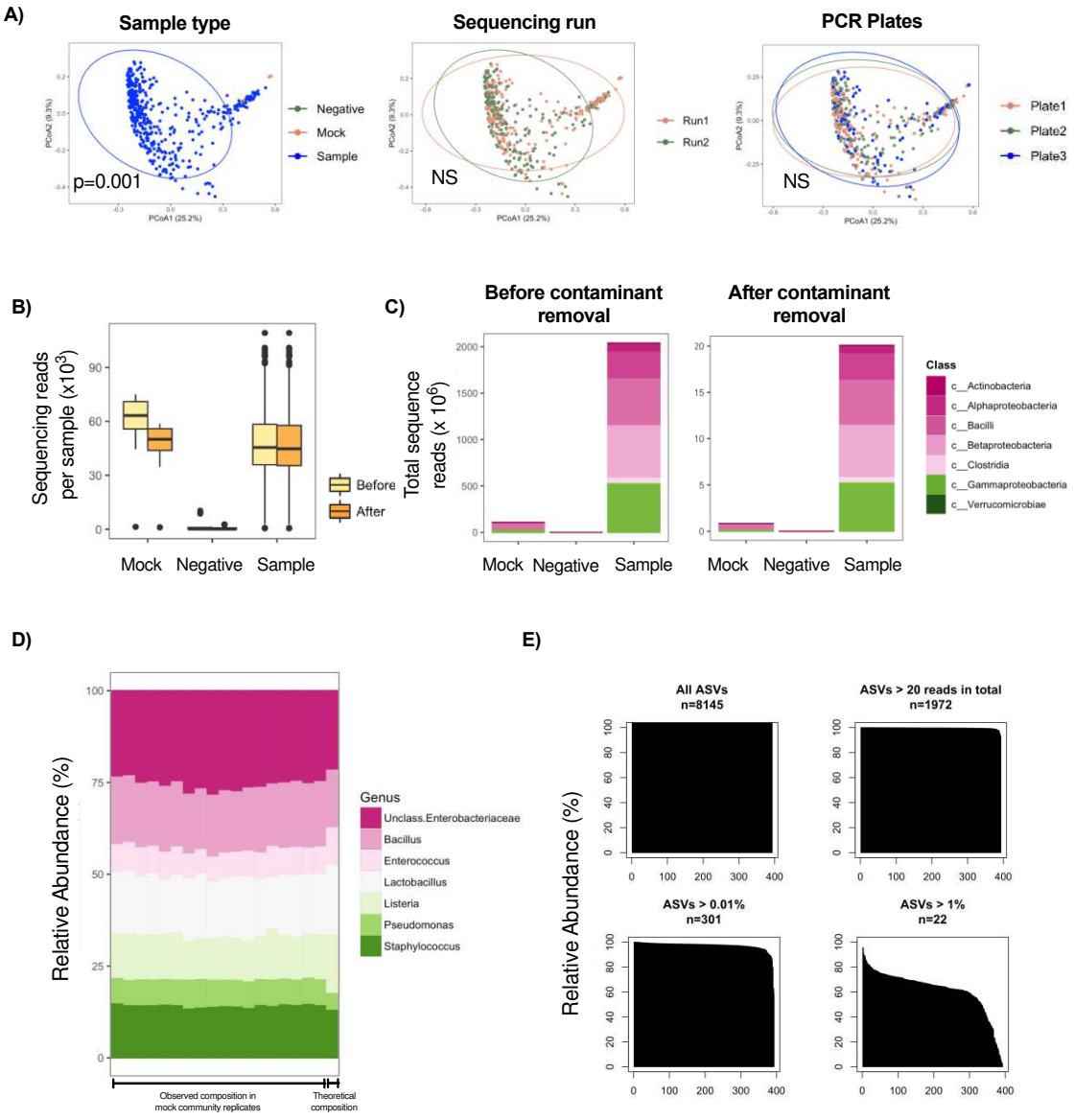

Figure S2. Hierarchical clustering of core milk microbiota among 393 mothers in the CHILD cohort related to Figure 1. The optimum numbers of clusters in our data were determined by Gap statistics. According to the gap statistics, there are four main clusters. A) Gap statistics plot, B) heterogeneity of clusters in terms of the milk microbiota compositional dispersion, C) PCoA plot on Bray-Curtis dissimilarity among ASVs with more than 0.01% mean relative abundance (n=301). β diversity between clusters was assessed by PERMANOVA; p-values are shown in panels B and C.

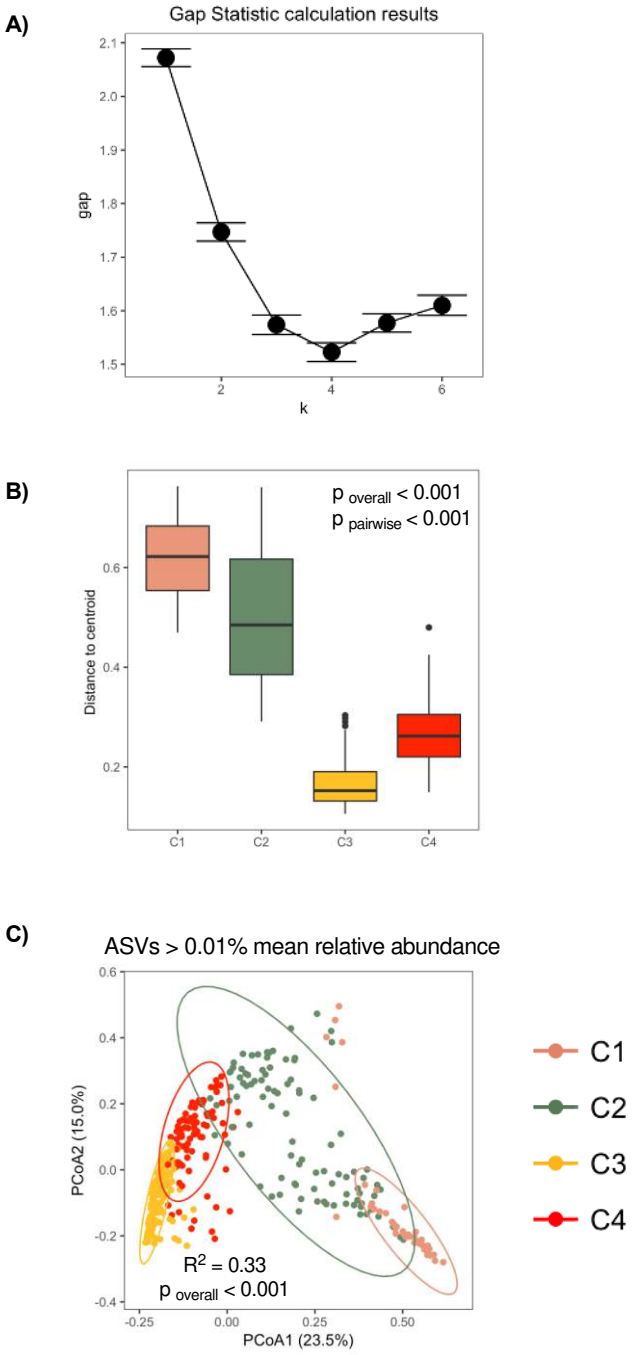

Figure S3. Equivalent structural equation models assessing other potentially plausible models of associations of milk microbiota determinants among 393 mothers in the CHILD cohort related to Figure 4. Compared to the final model depicted in Figure 3B, these models assessed: A) causative effect of milk microbiota on milk environment, B) correlation of milk microbiota and milk microenvironment, C) only direct effect of BMI on milk microbiota retained, D) effect of mode of breastfeeding on the milk environment, E) maternal diet effect on milk fatty acids, F) direct effect of HMOs on milk microbiota. Standardized β coefficients are reported. Abbreviations: mlk, milk environment; MFA, milk fatty acid PC1; INS, insulin; LEP, leptin; HMO, human milk oligosaccharide PC1; microbiota, milk microbiota PC1; BMI, maternal body mass; DEL, mode of delivery; SEX, infant sex; MOD: mode of breastfeeding; DRC1 and DRC2, maternal diet PC1 and PC2. * p<0.05, ** p<0.01. Green (positive), red (negative). See also Table S7.

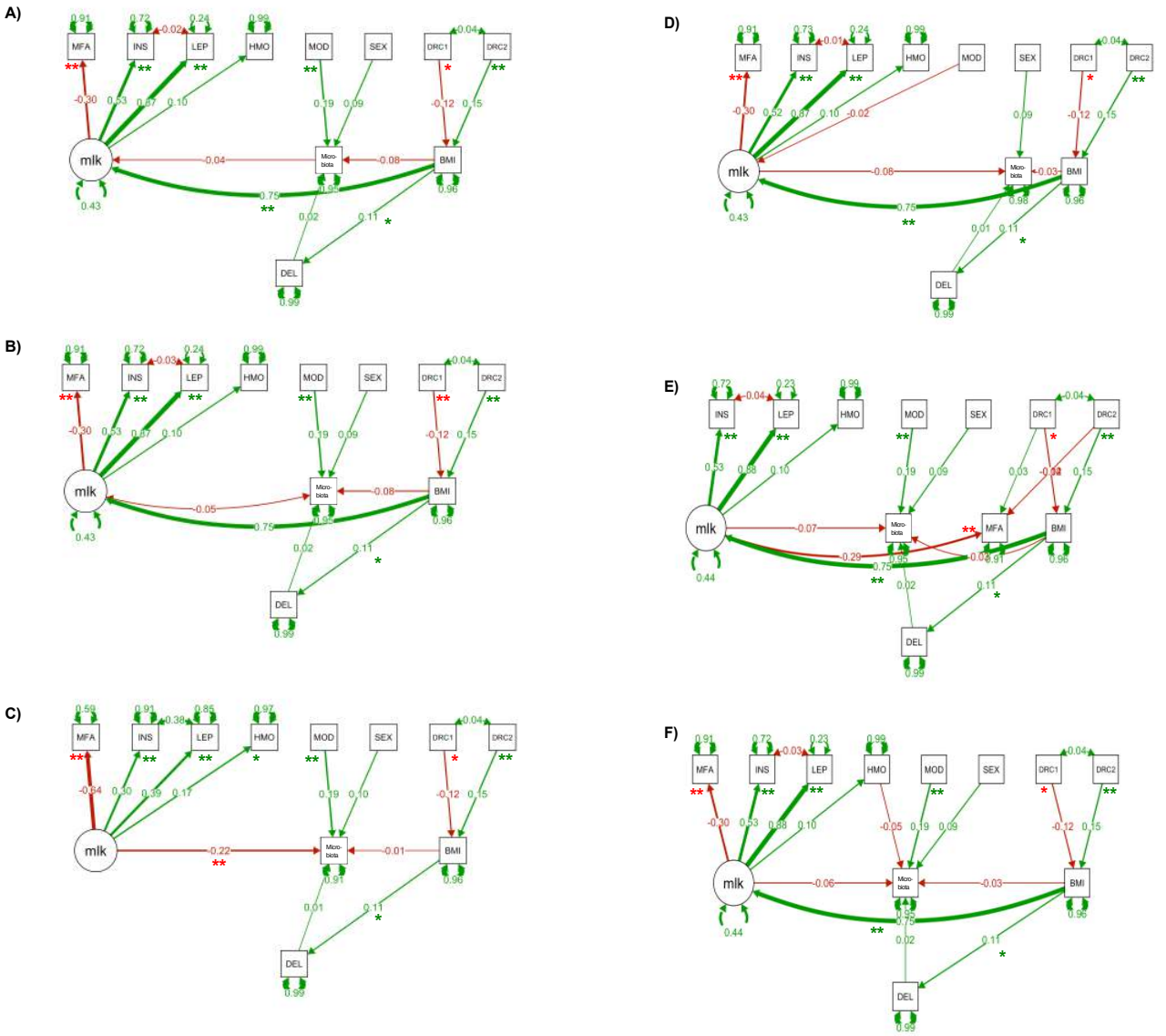

Figure S4. Expressed milk microbiota is influenced by collection method related to Figure 5. A) α diversity, B) β diversity.