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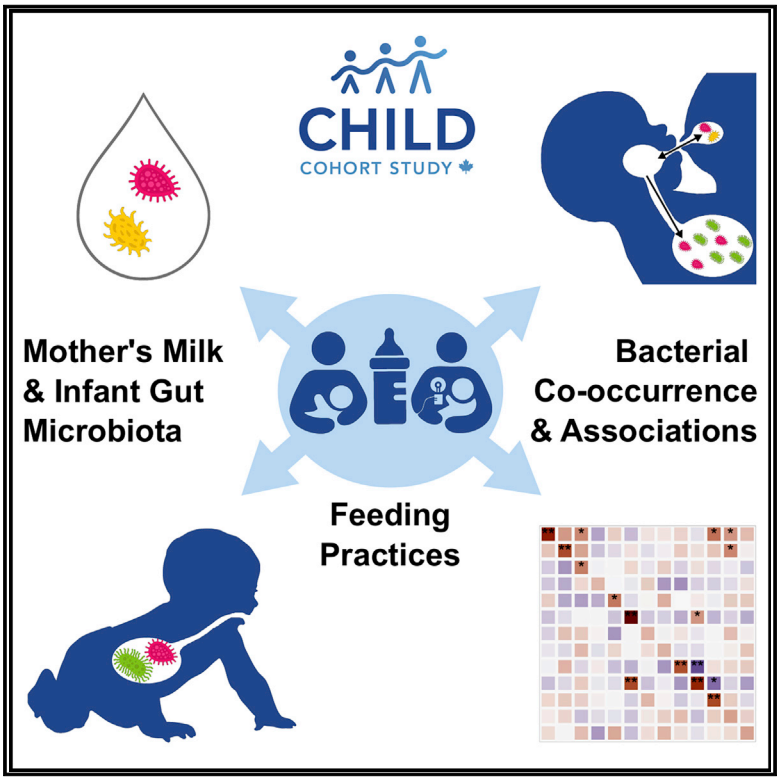
# Breastmilk Feeding Practices Are Associated with the Co-Occurrence of Bacteria in Mothers' Milk and the Infant Gut: the CHILD Cohort Study

## Graphical Abstract




## Highlights

- Breastfeeding exclusivity and duration strongly influence infant gut microbiota
- A few bacterial species co-occur in mothers' milk and their infants' stool
- This co-occurrence is reduced when breastmilk is pumped and fed from a bottle
- Bacteria in breastmilk contribute to overall infant gut microbiota composition

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## In Brief

Fehr et al. analyze infant gut microbiota in the CHILD cohort and identify associations with breastmilk feeding practices (exclusivity, duration, and pumping) and breastmilk microbiota. Within mother-infant dyads, co-occurrence of bacteria in breastmilk and stool suggests bacteria in breastmilk may transfer to the infant and influence the developing gut microbiota.

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# Breastmilk Feeding Practices Are Associated with the Co-Occurrence of Bacteria in Mothers' Milk and the Infant Gut: the CHILD Cohort Study

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## SUMMARY

Gut microbiota play a critical role in infant health. It is now accepted that breastmilk contains live bacteria from endogenous and exogenous sources, but it remains unclear whether these bacteria transfer to the infant gut and whether this process is influenced by breastmilk feeding practices. Here, we show that certain bacteria, including *Streptococcus* spp. and *Veillonella dispar*, co-occur in mothers' milk and their infants' stool, and co-occurrence is reduced when infants receive pumped breastmilk. The relative abundances of commonly shared species are positively correlated between breastmilk and stool. Overall, gut microbiota composition is strongly associated with breastfeeding exclusivity and duration but not breastmilk feeding mode (nursing versus pumping). Moreover, breastmilk bacteria contributed to overall gut microbiota variation to a similar extent as other modifiers of the infant microbiome, such as birth mode. These results provide evidence that breastmilk may transfer bacteria to the infant gut and influence microbiota development.

## INTRODUCTION

Gut microbiota development in early life impacts long-term health (Ihekweazu and Versalovic, 2018; Milani et al., 2017) and breastfeeding is among the most influential factors affecting this process (Azad et al., 2013; Bäckhed et al., 2015; Stewart et al., 2018). It is well established that breastfeeding provides prebiotic human milk oligosaccharides (HMOs) to support the developing infant gut microbiota (Sela et al., 2008; Vatanen et al., 2019). In addition, human milk contains a diverse bacterial community (Heikkilä and Saris, 2003; Jost et al., 2014; Martín et al., 2003; Togo et al., 2019), although the origins and functional significance of bacteria in human milk remain unclear (Moossavi and Azad, 2019). Human milk has been explored as a potential source or vehicle for bacteria that colonize the infant gut (Biagi et al., 2018; Murphy et al., 2017; Pannaraj et al., 2017); however, the role of milk bacteria in seeding the infant gut and the potential modifying effect of breastmilk feeding practices on this process are poorly understood.

According to the priority effect hypothesis, infant gut microbiota composition depends on the type and order in which

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bacteria colonize the intestinal tract (Fukami, 2015; Martínez et al., 2018). Newborns are typically inoculated with maternal vaginal and fecal bacteria during delivery (Bäckhed et al., 2015; Dominguez-Bello et al., 2010; Shao et al., 2019), and further transmission occurs postnatally from other maternal body sites during frequent oral and skin-to-skin contact (Ferretti et al., 2018; Williams et al., 2019), as well as other household members and the home environment (Martin et al., 2016). The role of human milk bacteria in this process has not been extensively studied. A few reports have identified shared taxa between maternal milk and infant stool (Asnicar et al., 2017; Biagi et al., 2017; Duranti et al., 2017; Jost et al., 2014; Milani et al., 2015; Pannaraj et al., 2017), suggesting that milk could provide pioneering species to the infant gut. However, these studies mainly focused on the sharing of individual taxa without exploring the global impact of milk bacteria on the overall gut microbiota, and most did not account for other known modifiers of the infant microbiota.

While the effect of breastfeeding on the infant gut microbiota is well established (Azad, 2019; Bäckhed et al., 2015; Pannaraj et al., 2017; Stewart et al., 2018), the impact of breastmilk feeding mode (i.e., nursing at the breast or feeding pumped breastmilk from a bottle) is unknown. Pumping is common and allows many mothers to overcome challenges related to breastfeeding and continue providing breastmilk to their infant; however, pumping can affect the bacterial composition of breastmilk (Moossavi et al., 2019; Weiss, 2005), and the consequences of these practices for the infant gut microbiota have not been studied. Conceivably, breastmilk feeding mode could also influence the transfer of other, non-milk-derived bacteria (e.g., from maternal skin, infant mouth, breast pumps, or bottles) to the infant gut (McGuire and McGuire, 2017; Moossavi and Azad, 2019; Moossavi et al., 2019; Williams et al., 2019). To our knowledge, despite the large and growing proportion of infants receiving pumped breastmilk (O'Sullivan et al., 2019; Rasmussen and Geraghty, 2011), no prior studies have assessed the potential impact of feeding mode on the gut microbiota of infants fed with breastmilk.

It remains unclear whether bacteria found in human milk are correlated with infant gut microbiota composition. The impact of breastmilk feeding practices on these correlations is also uncertain, and the relative importance of milk bacteria compared with other known modifiers of the infant microbiome is not known. We were able to address these questions with data from the longitudinal CHILD Cohort Study.

## RESULTS

Among the subset of 1,249 mother-infant dyads from the CHILD Cohort Study included in our analysis, the mean duration of any breastmilk feeding was 9.4 ± 3.3 months (3.3 ± 2.3 months exclusive breastmilk feeding), and 61% of mothers reported feeding some pumped milk around the time of sample collection at 3–4 months. Over half (56%) were first-time mothers and 25% delivered by cesarean section. These and other sociodemographic characteristics were similar across the different subsets used for the milk, gut, and paired milk-gut analyses (Table S1; Figure S1). Breastfeeding practices differed by study site, and exclusive breastfeeding was more common among multiparous mothers who delivered vaginally (Table S2).

### Infant Gut Microbiota Composition, Diversity, and Maturity Are Associated with Breastfeeding Exclusivity and Duration, But Not Occasional Feeding of Pumped Milk

The association between breastmilk feeding practices and gut microbiota composition was first assessed using redundancy analysis (RDA) (Figures 1A–1C). At 3 months, in pairwise univariate analyses, infants no longer fed with breastmilk differed in microbiota composition compared with those exclusively ($R^2$ = 1.16%, p = 0.001) or partially ($R^2$ = 0.84%, p = 0.001) fed with breastmilk (Figure 1A). A subtler difference was observed between infants fed with breastmilk partially versus exclusively ($R^2$ = 0.44%, p = 0.001). In a multivariable adjusted model (Table S3), breastfeeding exclusivity explained more variation ($R^2$ = 1.08%, p = 0.001) than other cofactors, namely, birth mode ($R^2$ = 0.63%, p = 0.001), having older siblings ($R^2$ = 0.37%, p = 0.001), and intrapartum antibiotics ($R^2$ = 0.24%, p = 0.005). Among breastmilk-fed infants, breastmilk feeding mode was not associated with gut microbiota composition in the univariate ($R^2$ = 0.20%, p = 0.217) (Figure 1B) or adjusted ($R^2$ = 0.20%, p = 0.174, Table S3) models, nor with overall gut microbiota diversity (p = 0.093, Figure 1E) or microbiota maturity (p = 0.55, Figure 1E). The effect of breastmilk feeding mode remained insignificant in a sensitivity analysis limited to exclusively breastfed infants (not shown). At 1 year, breastfeeding duration explained 1.01% of observed variation in infant gut microbiota composition in both univariate and adjusted models (p = 0.001) (Figure 1C; Table S3). This was comparable to variation explained by having older siblings ($R^2$ = 0.95%, p = 0.001, Table S3) in the adjusted model.

We also observed some differences in gut microbiota diversity and maturity according to breastmilk feeding practices (Figures 1D and 1F). At 3 months, infants who were no longer fed with breastmilk had higher microbiota diversity (mean ± SD: 2.11 ± 0.56 Shannon index) and maturity (265 ± 55 days) compared with those who were partially (1.78 ± 0.72 Shannon index, p < 0.001; 238 ± 62 days, p = 0.002) or exclusively fed with breastmilk (1.69 ± 0.67 Shannon index, p < 0.001; 217 ± 69 days, p < 0.001) (Figure 1D). Diversity increased between 3 months and 1 year of age among breastmilk-fed infants, whereas no further increase was observed among the infants who had ceased breastfeeding before 3 months (Figure 1G); similar results were observed for microbiota maturity (Figure 1G). Together, these results suggest a dose-dependent impact of breastmilk feeding on gut microbiota diversity, and an early or accelerated maturation and diversification in non-breastfed infants. Overall, results were similar in a sensitivity analysis including the small number of never-breastfed infants as a separate group (Figure S2).

### Specific Infant Gut Bacteria Are Associated with Breastfeeding Exclusivity and Duration

After assessing the overall infant gut microbiota composition and diversity, we tested the association of breastmilk feeding practices with the prevalence (Figure S3) and relative abundance (Figure 2; Table S4) of different bacteria (represented by amplicon sequencing variants, ASVs) in the infant gut. Clear differences in the overall relative abundance profile of ASVs were observed according to breastfeeding exclusivity at 3 months (Figure 2A). Using multivariable linear models, many differences

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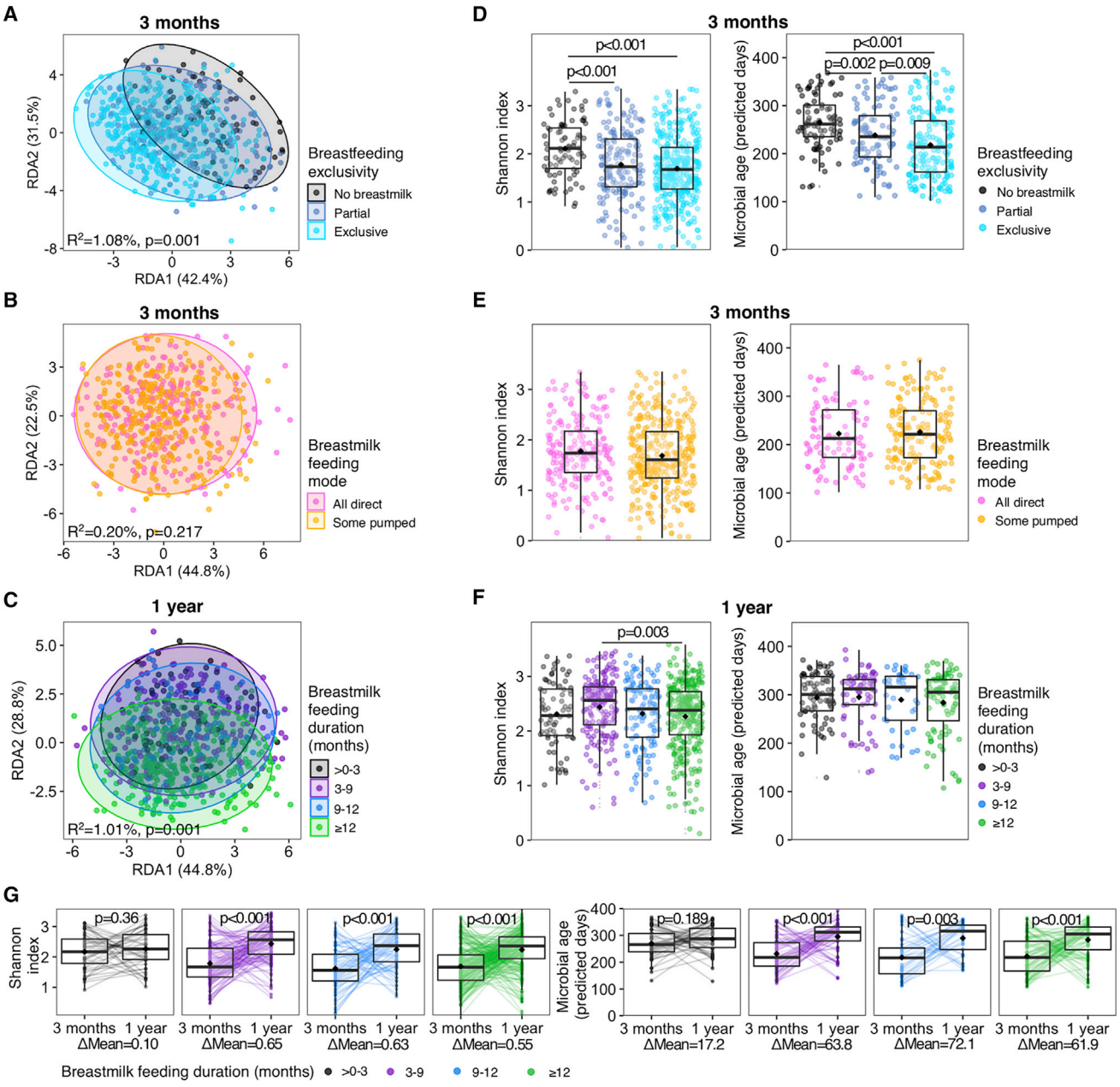


**Figure 1. Infant Gut Microbiota Composition, Diversity, and Maturity Are Associated with Breastmilk Feeding Practices**

(A–C) Redundancy analyses showing univariate associations of breastfeeding exclusivity (A) and breastmilk feeding mode (B) with 3-month infant gut microbiota composition (n = 653) and breastfeeding duration (C) with 1-year infant gut microbiota composition (n = 698).

(D–F) Associations of breastfeeding exclusivity (D) and breastmilk feeding mode (E) at 3 months (n = 653) and breastfeeding duration (F) at 1 year (n = 698) with gut microbiota diversity (Shannon index) and maturity (predicted age in days, n = 349 3-month and n = 264 1-year stool; this n excludes infants used to train the microbiota maturity prediction algorithm). Tested using Wilcoxon signed-rank test, no p value shown indicates no significance.

(G) Change in gut microbiota diversity and maturity from 3 months to 1 year stratified by breastfeeding duration for infants sampled at both time points. Tested using Wilcoxon signed-rank test for matched pairs on infants breastfeed >0–3 months (n = 98 for diversity; n = 98 for maturity), 3–9 months (n = 224; 130), 9–12 months (n = 150; 76), and >12 months (n = 432; 174). ΔMean, change in mean diversity from 3 months to 1 year. See also Figure S2; Table S3.

were observed for infants exclusively fed breastmilk versus those who were no longer fed breastmilk at 3 months, including 4 enriched with exclusive breastmilk feeding and 8 enriched with cessation of breastmilk feeding (Figure 2B). The 4 taxa enriched with exlusive breastmilk feeding were *Haemophilus parainfluenzae*, *Streptococcus*(3), and two ASVs classified as *Veillonella dispar*. Taxa enriched among infants who were no longer fed breastmilk included ASVs classified as *Blautia*, *Streptococcus*(2) and two unclassified *Lachnospiraceae*(2,3) (Figure 2B). Six of these 12 ASVs also differed in relative abundance between

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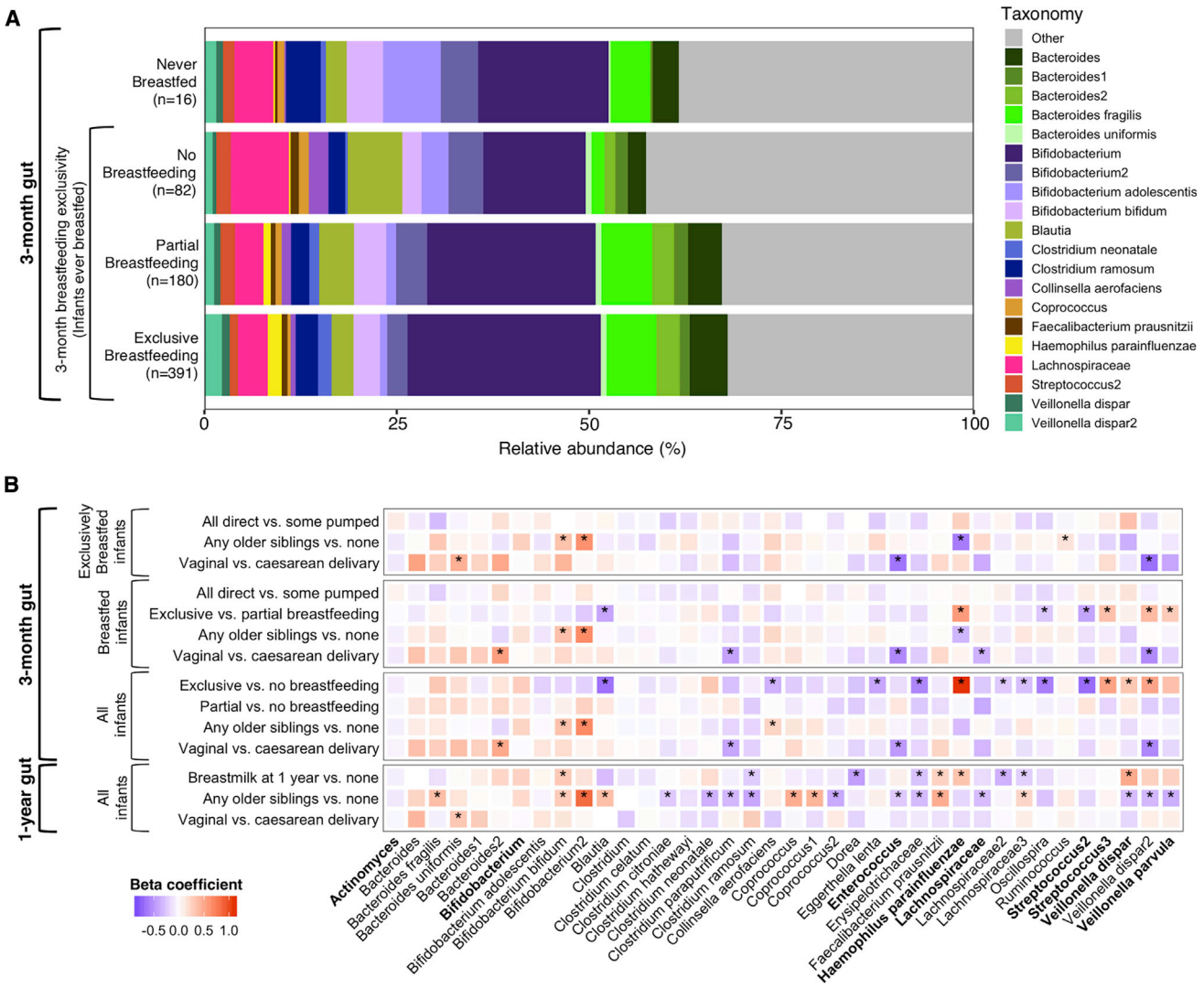


**Figure 2. Specific Bacteria in the Infant Gut Are Associated with Breastmilk Feeding Practices**

(A) Average gut microbiota composition of infants exclusively, partially, and not receiving breastmilk at 3 months. Showing the top 20 most abundant ASVs. (B) Association of breastmilk feeding practices with centered log-ratio transformed ASV relative abundances in the infant gut, using adjusted linear regression permutation tests. Covariates known to influence infant gut microbiota (birth mode and older siblings) are shown for comparison; models were additionally adjusted for intrapartum antibiotics. Separate models were generated for: exclusively breastmilk-fed infants (n = 391); all breastmilk-fed infants (n = 571); and all infants (n = 653) at 3 months; and all infants at 1 year (n = 698). Showing ASVs present in at least 20% of samples (see Table S4 for full results). *$p_{(FDR)} < 0.05$. Bold taxa indicate ASVs commonly shared with mothers' milk.

infants exclusively and partially breastfed. Breastmilk feeding mode was not associated with ASV relative abundances, even within exclusively breastmilk-fed infants.

At 1 year, 4 prevalent ASVs were enriched in the gut with breastmilk feeding, including *H. parainfluenzae* and *V. dispar*, while 5 were enriched with cessation of breastmilk feeding, including *Dorea*, and two unclassified *Lachnospiraceae*(2,3) (Figure 2B). Notably, *Bifidobacterium bifidum* was enriched with breastmilk feeding, while other prevalent ASVs assigned to *Bifidobacterium* were not.

Along with breastmilk feeding practices, we assessed additional factors known to influence infant gut microbiota for comparison (Figure 2B). Consistent with previous studies (Bäckhed et al., 2015; Dominguez-Bello et al., 2010; Shao et al., 2019), cesarean delivery was associated with depletion of Bacteroides and other taxonomic differences at 3 months. Interestingly, presence of older siblings had a relatively strong association with ASV relative abundances at 1 year, affecting 19 of the 38 prevalent ASVs, including enrichment of *B. bifidum* and *Bifidobacterium*(2).

## Breastmilk and the Infant Gut Have Distinct Microbiota Compositions, but Some Bacteria Are Shared and Correlated between a Mother's Milk and Her Own Infant's Gut

The most prevalent genera of the infant gut were generally distinct from the most prevalent genera of breastmilk (Figure 3A; Table S5). Only *Streptococcus* and *Veillonella* were present in

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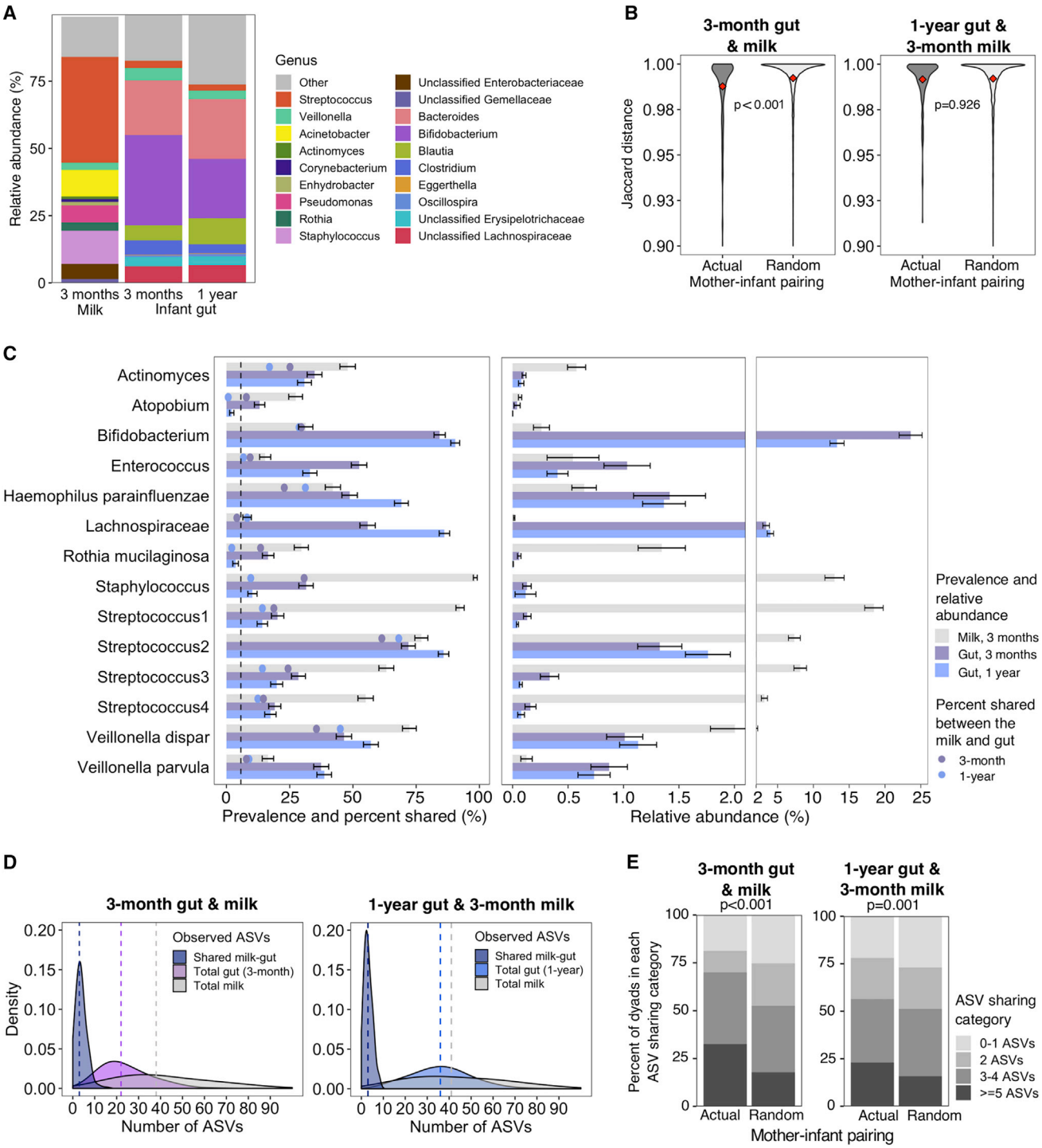


**Figure 3. Infant Gut and Milk Microbiota Are Distinct But Some Bacteria Are Shared between a Mother's Milk and Their Own Infant's Gut**

(A) Average breastmilk microbiota composition (n = 799) and infant gut microbiota composition at 3 months (n = 669) and 1 year (n = 718), showing prevalent genera of each sample type (present in >60% of samples).

(B) Dissimilarity between milk and infant gut microbiota of actual mother-infant dyads compared with random dyads, tested by a linear regression permutation test. Jaccard distances range from 0 (completely similar) to 1 (completely dissimilar); red dots indicate means.

(C) For commonly shared bacteria (ASVs shared in at least 15 dyads), prevalence in milk and stool, the percent of dyads sharing the ASV (left panel), and average relative abundance in milk and stool (right panel) are shown. For the percent shared, any points that fall below the dotted line (<15 dyads) are not considered commonly shared at that time-point. Error bars for prevalence and relative abundance represent standard error of the proportion and mean, respectively. See also Table S5.

*(legend continued on next page)*

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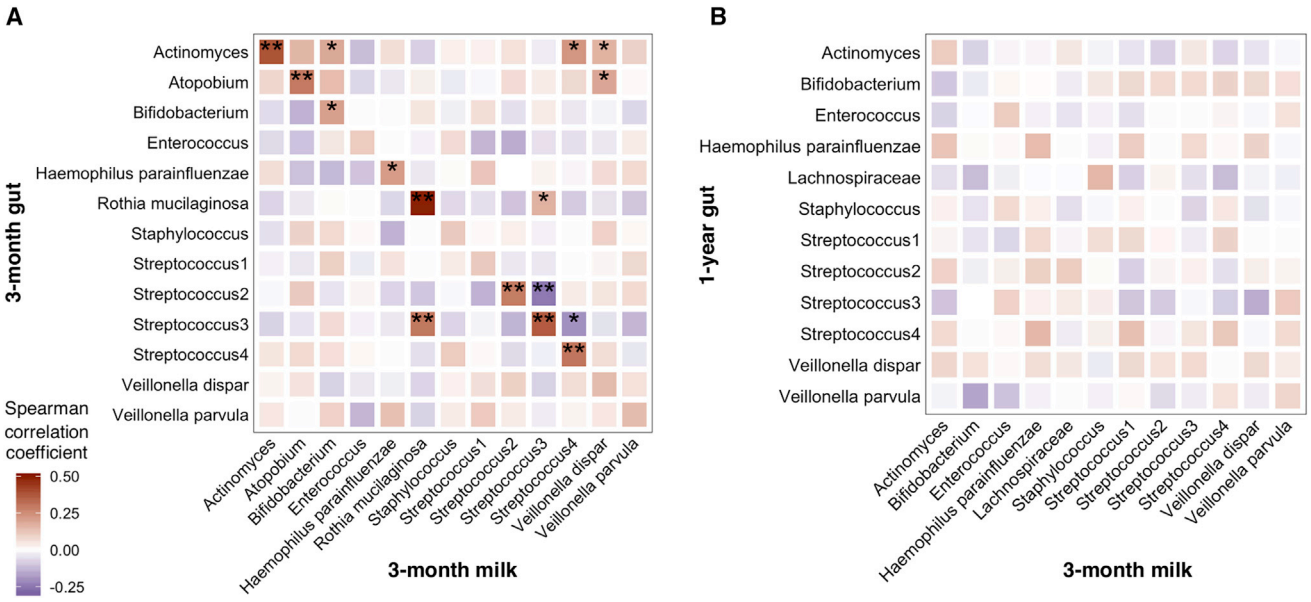


**Figure 4. Commonly Shared Bacteria Are Correlated between a Mother's Milk and Infant's Gut**

Spearman rank correlation between relative abundances of commonly shared bacteria (ASVs shared in at least 15 dyads) in the infant gut and milk, for the 3-month (A) and 1-year (B) dataset. *$p_{(FDR)} < 0.1$, **$p_{(FDR)} < 0.001$.

over 60% of both sample types. Overall, *Streptococcus* and *Staphylococcus* dominated milk, while *Bifidobacterium* and *Bacteroides* dominated the infant gut (Figure 3A). As expected, the microbiota composition of milk and the infant gut were almost completely dissimilar using the Jaccard distance (Figure 3B)—but interestingly, at 3 months, the infant gut and milk microbiota of actual mother-infant dyads were slightly more similar than the microbiota of random mother-infant pairings ($p < 0.001$). In contrast, there was no detectable difference in dissimilarity between actual and random pairings of 3-month mother's milk and 1-year infant gut.

Next, we assessed the sharing of individual ASVs among dyads. Bacteria shared most commonly at 3 months included *Streptococcus*(2) (shared in 61.4% of dyads), *V. dispar* (35.6%), *Staphylococcus* (30.7%), and *Bifidobacterium* (27.1%) (Figure 3C; Table S5). Overall, a median of 3 ASVs were shared within actual dyads at 3 months (range: 0–16) and 1 year (range: 0–9). This contrasts with the large number and wide range of total ASVs in milk (median 38, range 5–115) and the infant gut (median 22, range 5–93 at 3 months; median 36, range 9–87 at 1 year) (Figure 3D). Notably, actual dyads were more likely to share 5 or more ASVs than random milk-gut pairings at 3 months ($p < 0.001$) and 1 year ($p = 0.001$) (Figure 3E), supporting the hypothesis that some bacteria are transferred between a mother's milk and her own infant's gut.

We further assessed correlations between relative abundances of commonly shared bacteria in breastmilk and the infant gut within dyads (Figure 4). Generally, any given ASV in milk was correlated with that same ASV in the infant gut at 3 months. These correlations were always positive, and 8/13 were noteworthy ($p_{(FDR)} < 0.1$). Milk-gut correlations were strongest for *Rothia mucilaginosa*, three *Streptococcus* ASVs, and one *Actinomyces* ASV ($r \geq 0.30$, $p_{(FDR)} < 0.001$, Figure 4A). In contrast, only a small proportion of correlations were significant among all other possible milk-gut ASV pairs (6 positive and 2 negative correlations among 156 possible pairs). Notably, there were negative correlations between different unclassified species of Streptococci that suggest possible competitive relationships ($r < -0.20$, $p_{(FDR)} < 0.1$). Additionally, a strong correlation was observed between *R. mucilaginosa* in milk and *Streptococcus*(3) in the infant gut ($r = 0.30$, $p_{(FDR)} < 0.001$). No significant correlations were observed between relative abundances of commonly shared bacteria in 3-month milk and the 1-year infant gut (Figure 4B).

## The Extent of Sharing Bacteria between Breastmilk and the Infant Gut Is Associated with Breastmilk Feeding Practices

Next, we assessed whether breastmilk feeding practices were associated with the extent of milk-gut bacterial sharing within dyads (Figure 5A). At 3 months, breastfeeding exclusivity was not associated with the number of shared ASVs ($p = 0.19$); however, the mode of breastmilk feeding was important: The proportion of infants sharing 5 or more ASVs with their own mother's milk was higher among those who were directly breastfed compared with those receiving some pumped milk (44.3%

(D) Density plots showing distributions for the number of ASVs shared between milk and the infant gut of actual mother-infant dyads, and the total number of ASVs per milk and stool sample. Medians are shown as dotted lines. The y axis shows density, with higher density indicating more dyads with a given value.
(E) The percent of dyads in each ASV sharing category among actual and random dyads. Tested by the chi-square test, p values compare ≥5 to <5 shared ASVs.

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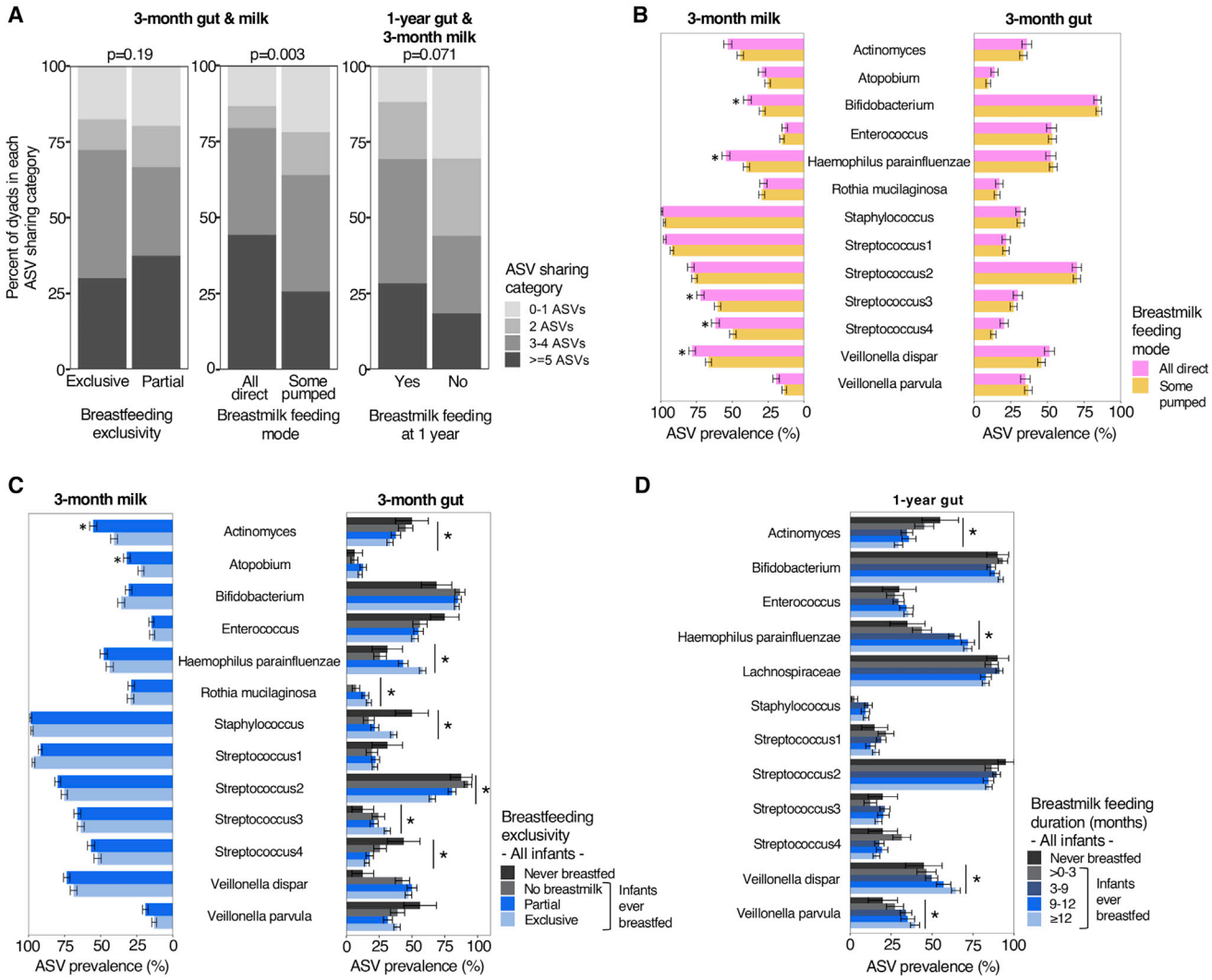


**Figure 5. Breastmilk Feeding Practices Are Associated with Shared Bacteria in Milk and the Infant Gut**

(A) The percent of dyads in each ASV sharing category (0–1, 2, 3–4, and ≥5 ASVs shared) associated with breastmilk feeding practices. Tested by the chi-square test, p values compare ≥5 to <5 shared ASVs. See also Figure S4.

(B–D) Associations of breastmilk feeding mode (B) and exclusivity (C) with the prevalence of commonly shared bacteria (ASVs shared in at least 15 dyads) in milk (n = 802) and the 3-month infant gut (n = 669). (D) Associations of breastfeeding duration at 1 year with the prevalence of commonly shared bacteria in the infant gut at 1 year (n = 718). Error bars show the estimated standard error for proportions. *$p_{(FDR)} < 0.05$, tested using the chi-square test, including the chi-square test for linear trends to assess differences across breastfeeding exclusivity and duration groups (line indicates linear trend). Note that never-breastfed infants were included specifically for this analysis (C and D). Despite the low sample size of this group (n = 16 for 3-month and n = 20 for 1-year infant stool), it was of particular interest to observe whether commonly shared ASVs were present in never-breastfed infants. See Figure S5 for relative abundances.

versus 25.6%; p = 0.003). At 1 year, the proportion of dyads sharing 5 or more ASVs tended to be higher among those who were still breastfeeding compared with those who had stopped (28.4% versus 18.4%; p = 0.071) (Figure 5A). Similar associations were observed when the number of shared ASVs was assessed as a continuous variable (Figure S4).

We then focused on the most commonly shared ASVs (Figure 3C; Table S5), and assessed the potential for breastmilk feeding mode and exclusivity to influence their prevalence (Figures 5B and 5C) and relative abundance (Figures S5A and S5B) in the infant gut and milk at 3 months. In milk, the prevalence and relative abundance of several commonly shared bacteria was lower in mothers who sometimes pumped compared with those who only directly breastfed ($p_{(FDR)} < 0.05$), including *V. dispar*, *H. parainfluenzae*, and a *Streptococcus*(3) (Figures 5B and S5B). A *Bifidobacterium* sp. was also less prevalent in milk from mothers who pumped, although no difference in relative abundance was detected. No associations were observed between breastmilk feeding mode and commonly shared bacteria in the infant gut (Figures 5B and S5B). Conversely, for breastfeeding exclusivity, we observed few associations with shared ASVs in milk but many associations with ASVs in the infant gut: There was an increase in prevalence of *H. parainfluenzae*, *R. mucilaginosa*, *Staphylococcus*, and *Streptococcus*(3) with

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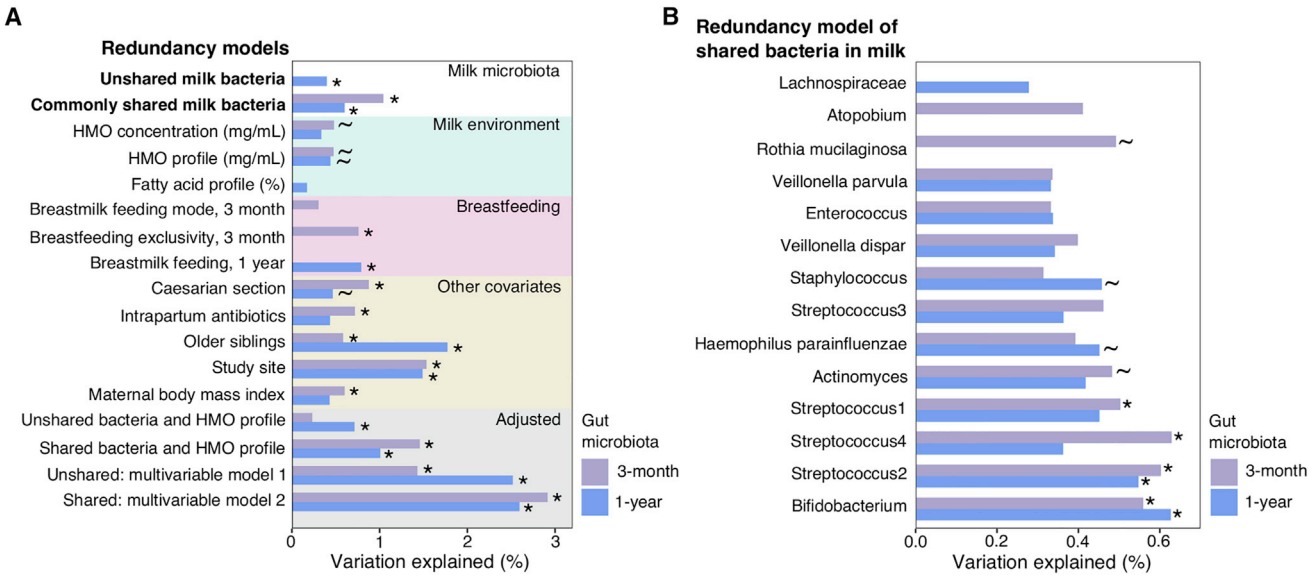


**Figure 6. Bacteria in Milk Are Associated with Infant Gut Microbiota Composition with a Strength Comparable to Other Factors in Early-Life**

Redundancy analyses showing the percent of variation in gut microbiota explained ($R^2$ in percent) by (A) milk bacteria, other milk components, breastmilk feeding practices, and other relevant covariates, and (B) commonly shared bacteria in milk. The adjusted $R^2$ is used for all multivariable models. All participants in the milk-gut paired datasets were analyzed (Figure S1). Centered log-ratio transformed abundances were used for unshared milk bacteria (10 ASVs with an average relative abundance >1%) and commonly shared milk bacteria (13 ASVs shared in at least 15 dyads at 3 months, and 12 at 1 year). Variation explained only considers covariates in the model for the specified multivariable models 1 and 2. Multivariable model 1: unshared milk bacteria and covariates (breastfeeding exclusivity [or breastfeeding at 1 year for the 1-year dataset], HMO profile [matrix of concentrations for 19 HMOs], birth mode, intrapartum antibiotics, older siblings, and study site). Multivariable model 2: shared milk bacteria and covariates, as above. Adjusted $R^2$ were used for multivariable models. *$p < 0.05$, ~$p < 0.1$. See also Table S6.

increased breastfeeding exclusivity, along with lower prevalence of two *Streptococcus* ASVs, and an *Actinomyces* ($p_{(FDR)} < 0.05$) (Figure 5C). Similar findings were observed for relative abundances (Figure S5A). Including never-breastfed infants in univariate prevalence analysis revealed a few clear trends. For instance, *R. mucilaginosa* was present in 0% of never-breastfed infants compared with 7.3%, 14.4%, and 17.1% of infants that no longer, partially, and exclusively breastmilk fed, respectively (Figure 5C). Overall, it appears that breastmilk feeding mode primarily influenced the commonly shared bacteria in milk, whereas breastfeeding exclusivity primarily influenced those in the infant gut.

We further assessed whether breastfeeding duration influenced commonly shared bacteria in the infant gut at 1 year, including never-breastfed infants in univariate analysis (Figure 5D). Similar to trends for breastfeeding exclusivity, *H. parainfluenzae* and *Actinomyces* also showed increased prevalence with increasing breastfeeding duration, along with *V. dispar* and *Veillonella parvula*. *H. parainfluenzae* and *V. dispar* were also relatively more abundant in breastmilk-fed infants compared with those no longer breastfed (Figures 2B and S5C).

## Commonly Shared Milk Bacteria Are Associated with Overall Infant Gut Microbiota Composition

Next, we hypothesized that at least some commonly shared bacteria represent milk bacteria with the ability to transfer to the infant gut, and that such bacteria could affect the overall infant gut microbiota composition. To test this, we used RDA to assess the relative abundances of commonly shared (n = 13 ASVs) and unshared (n = 10 ASVs) milk bacteria, as possible sources of variation in the gut microbiota composition of breastmilk-fed infants (Figure 6A). The contribution of other milk and early-life factors was also assessed for comparison. We found that commonly shared milk bacteria were associated with the 3-month infant gut microbiota ($R^2_{adj}$ = 1.04% variation explained, p = 0.003), whereas unshared milk bacteria were not (p = 0.873). Both shared and unshared milk bacteria explained a similar lower level of variation at 1 year ($R^2_{adj}$ = 0.55%, p = 0.024 and $R^2_{adj}$ = 0.40%, p = 0.033, respectively). Notably, shared milk bacteria explained more variation in the 3-month infant gut microbiota than several well-known determinants of this microbial community, including breastfeeding exclusivity (exclusive versus partial, 0.76%), birth mode (0.87%), intrapartum antibiotics (0.72%), and older siblings (0.58%). In a sensitivity analysis, the variation explained by commonly shared milk bacteria was similar using alternative thresholds to define "commonly shared bacteria" (Table S6).

Lastly, we investigated the contribution of each commonly shared bacterium in milk to the observed variation in infant gut microbiota (Figure 6B). *Streptococcus*(4), *Streptococcus*(2), and *Bifidobacterium* were associated with 3-month gut microbiota composition ($R^2$ = 0.63%, 0.60% and 0.56%, respectively, $p < 0.05$). Only *Bifidobacterium* and *Streptococcus*(2) were significantly associated with gut microbiota at 1 year ($R^2$ = 0.63% and 0.55%, respectively, $p < 0.05$).

## DISCUSSION

Our findings provide evidence that bacteria are shared and potentially transferred from mothers (or other exogenous

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sources) to infants through breastmilk and further suggest that early weaning or feeding pumped breastmilk may disrupt this process. Breastfeeding is a well-known determinant of the infant gut microbiota (Azad et al., 2013; Bäckhed et al., 2015; Ho et al., 2018; Stewart et al., 2018), and the microbiota of breastmilk and the infant gut have been compared in a few previous studies (Biagi et al., 2018, 2017; Lackey et al., 2019; Pannaraj et al., 2017). Our study adds to this previous work by evaluating the association of multiple breastmilk feeding practices (mode, exclusivity, and duration), milk bacteria, and milk components with infant gut microbiota composition at multiple time points. Here, we confirm that breastfeeding exclusivity and duration are major drivers of infant gut microbiota composition, and provide evidence that (1) infant gut and milk microbiota within mother-infant dyads are related, despite very distinct gut and milk microbial communities; (2) a few commonly shared bacteria associated with breastmilk feeding practices may be transferred within breastmilk to the infant gut or from the infant to breastmilk; and (3) commonly shared bacteria in milk may influence overall infant gut microbiota composition independent of (and to a similar degree as) other early-life factors, including HMOs and birth mode.

### Breastmilk Feeding Practices Shape the Infant Gut Microbiota and Influence Maternal-Infant Sharing of Bacteria

Our study confirms that breastmilk is a major driver of infant gut microbiota development (Azad et al., 2013; Bäckhed et al., 2015; Pannaraj et al., 2017) and provides evidence that breastmilk bacteria contribute to this effect. These results advance the accumulating body of evidence that breastmilk may seed the infant gut with bacteria originating from the mother or other exogenous sources (Biagi et al., 2017; Jost et al., 2014; Lackey et al., 2019; Pannaraj et al., 2017; Ramani et al., 2018). For overall infant gut microbiota composition and some individual gut taxa, we observed dose-dependent associations according to breastfeeding exclusivity and duration. We also observed higher co-occurrence of ASVs in dyads who were still breastmilk fed at 12 months, suggesting that sustained breastfeeding supports continuous bacterial transfer via mothers' milk.

We did not detect clear gut taxonomic differences according to breastmilk feeding mode; however, pumping was associated with depletion of some shared bacteria in milk and appeared to reduce the amount of mother-infant bacterial sharing. This suggests that some shared bacteria may transfer to milk during direct breastfeeding, supporting the hypothesis that the infant mouth could be colonized by environmental bacteria that disperse to both the milk (via retrograde transfer during direct breastfeeding) and the infant gut (McGuire and McGuire, 2017; Moossavi et al., 2019). Indeed, a few shared bacteria identified in our study, including *V. dispar* and *H. parainfluenzae*, are common oral bacteria (Könönen, 2000; Williams et al., 2019) and were enriched in breastmilk of mothers that only breastfed directly. Regardless of where milk bacteria originate, our data support the hypotheses that (1) breastmilk may act as an incubator that enriches certain bacteria and/or a protective vehicle to transport bacteria to the lower segments of the intestinal tract, and (2) that habitual pumping alters the composition of bacteria in breastmilk. It is also possible that the viability of breastmilk bacteria may be compromised by the process of pumping, storing (e.g., freezing, thawing), and bottle feeding, but this was not assessed in our study. Future research would benefit from longitudinal sampling of milk, oral, and gut microbiota to clearly define the dynamic relationship between these microbial communities and determine how they are impacted by milk expression, storage, and feeding practices.

### Bacteria in Breastmilk Could Seed the Infant Gut and Influence Health

The importance of milk microbiota for gut microbiota development is a matter of ongoing debate, but the presence of microbes in milk from phylogenetically diverse animal species (Derakhshani et al., 2018; Muletz-Wolz et al., 2019; Pannaraj et al., 2017) indicates its evolutionary conservation and functional significance. It is hypothesized that breastmilk provides bacteria to the infant gut either through vertical transmission from the mother or horizontal transfer from exogenous sources of milk bacteria, such as the home environment or the infant mouth (Moossavi and Azad, 2019). Consistent with previous research (Biagi et al., 2017; Jost et al., 2014; Lackey et al., 2019; Pannaraj et al., 2017; Ramani et al., 2018), we also found that breastmilk may specifically provide species of *Veillonella*, *Rothia*, *Streptococcus*, *Bifidobacterium*, *Haemophilus*, and *Staphylococcus* to the infant gut. It is noteworthy that no bacteria were commonly shared in all dyads, potentially reflecting inter-individual variability of milk microbiota profiles. Nevertheless, the increased sharing with prolonged and direct breastfeeding and strong positive correlations between the milk and gut relative abundances of shared bacteria provide evidence that some degree of bacterial seeding is plausible.

The transfer of bacteria in breastmilk might contribute to the beneficial health effects of breastfeeding observed in the CHILD cohort and other studies, including lower rates of childhood asthma (Dogaru et al., 2014; Klopp et al., 2017) and obesity (Azad et al., 2018b; Horta et al., 2015; Wang et al., 2017)—conditions that have also been linked to early-life perturbations of gut microbiota (Arrieta et al., 2015; Bervoets et al., 2013; Forbes et al., 2018). Notably in the CHILD cohort, we observed slightly weaker protection against asthma and being overweight among breastfed infants receiving some pumped milk (Azad et al., 2018b; Klopp et al., 2017). Our current findings suggest this might be due to reduced sharing or transfer of bacteria via mothers' milk. Shared bacteria identified in our study that are of particular interest and may influence immune homeostasis include *Rothia*, *Veillonella*, and *Bifidobacterium*. *Rothia* and *Veillonella* in 3-month stool were previously shown to be protective against atopic wheeze in the CHILD cohort (Arrieta et al., 2015). Interestingly, *Veillonella* has the ability to convert lactate to propionate and butyrate (Zoetendal et al., 2012), and these short chain fatty acids (SCFA) are associated with positive health outcomes and immunoregulatory effects (Sivaprakasam et al., 2016). Similarly, some *Bifidobacterium* spp. are specialized in the breakdown of HMOs and produce metabolites utilized by other microbiota (Fanning et al., 2012; Ruiz et al., 2017), such as members of *Lachnospiraceae*, which subsequently produce SCFAs (Patterson et al., 2017; Uematsu et al., 2008). Further

research is warranted to explore therapeutic applications of bacteria that co-occur in milk and stool, which may intrinsically withstand transit through the gastrointestinal tract.

It is important to consider alternative explanations for the identification of bacteria shared between breastmilk and the infant gut: That is, alternatively or in addition to reflecting the transmission of bacteria (of exogenous or endogenous origin) within milk to the infant gut, it is possible that both are separately seeded by the same exogenous sources, such as the infant mouth or home environment. It is also possible that breastmilk and its consortium of bacteria (regardless of their origins) influence oral and airway microbial communities and impact infant health independently of the gut microbiota. Further mechanistic research is required to confirm if and how milk transfers bacteria to the infant gut and to determine the health impact of maternal and infant microbiota.

### Breastmilk Bacteria Globally Influence the Infant Gut Microbiota Community Independently of HMOs

If viable bacteria in milk can reach the infant gut, they could conceivably influence the gut microbiota composition through colonization and/or interactions with resident gut microbes. Early gut microbiota communities, for instance, alter the gut environment, and thereby promote the growth of other bacteria and result in successional community shifts (Lozupone et al., 2012). Our results support this hypothesis and suggest that bacteria in milk account for a similar degree of variability as other known determinants of the infant microbiota (e.g., birth mode and intrapartum antibiotics)—although it is difficult to directly compare these effect estimates because milk microbiota were assessed on the same day as stool collection, whereas birth mode and intrapartum antibiotics represent exposures occurring several months prior. Importantly, the associations we observed were independent of the HMO profile, suggesting a dual mechanistic role of breastmilk in modulating the infant gut microbiota by providing both prebiotic and probiotic factors. Further studies are needed to confirm that breastmilk is a source of bacteria while controlling for other important milk components, which could modulate the physiological and immunological niche in the infant gut. In addition, studies integrating data from other maternal and infant microbiota communities could provide further insight into the routes, mechanisms, and impacts of mother-infant bacterial transfer.

### Strengths and Limitations

The main strengths of this study include access to rich metadata in the CHILD cohort and our ability to take a multi-analytic approach to associate breastfeeding practices and milk microbiota with infant gut microbiota, while controlling for relevant cofactors. The main limitations are the observational study design and the fact that milk microbiota were not analyzed at 1 year, limiting our ability to directly assess the sharing of milk bacteria at 1 year as was done at 3 months. However, we believe the sharing of bacteria in early infancy is especially important, because gut microbial communities are particularly dynamic and sensitive to the introduction of new species during this critical period of development. Milk and stool samples were analyzed in separate laboratories, although we used the same sequencing primers and bioinformatics pipelines. Also, we did not collect quantitative information on feeding practices (e.g., proportion or frequency of breastmilk versus formula, or direct versus pumped breastmilk), so it is possible that differences associated with predominant formula feeding or predominant and/or exclusive pumping were missed. In addition, we could not evaluate other potential sources of gut and milk microbiota, such as the maternal skin or infant oral cavity, because these were not sampled in the CHILD cohort. As with all culture-independent microbiota studies, we could not confirm the viability or absolute abundances of the bacteria identified in our samples. Lastly, 16S rRNA gene sequencing has limited capacity to resolve taxa to species and strain levels, and further research (e.g., metagenomic and/or culture-based studies) is required to validate the results of this study by confirming strain-level sharing of milk and gut microbiota. An additional advantage to shotgun metagenomic analysis would be the ability to assess the functional capacity of shared bacteria.

### Conclusions and Future Directions

Our results provide evidence that specific bacteria may be transferred from a mother's milk to her infant's gut, and that bacteria in milk influence the overall infant gut microbiota composition to a similar extent as other known modifiers of the gut microbiota. This process appears to be influenced by breastmilk feeding practices and could have long-lasting health implications by influencing gut microbiota development. Further research is warranted to replicate these findings in other populations, determine their clinical significance, and study the mechanisms and potential therapeutic applications of bacterial transmission through breastmilk.

## 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 can be found online at https://doi.org/10.1016/j.chom.2020.06.009.

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## AUTHOR CONTRIBUTIONS

Conceptualization, M.B.A., B.B.F., and S.E.T.; Methodology, K.F., S.M., R.C.T.B., H.S., L.B., E.K., and C.J.F.; Investigation & Formal Analysis, K.F., S.M., H.S., and R.C.T.B.; Visualization, K.F. and S.M.; Writing – Original Draft, K.F., S.M., and M.B.A.; Writing – Review & Editing, H.S., R.C.T.B., L.B., B.R., C.Y., C.J.F., A.B.B., P.J.M., M.R.S., E.K., T.J.M., P.S., B.B.F., and S.E.T. (all authors).; Funding Acquisition, M.B.A., B.B.F., and S.E.T.; Resources, A.B.B, P.J.M., S.E.T., P.S., B.B.F., and M.R.S.; Supervision, M.B.A., S.E.T., and B.B.F.

## DECLARATION OF INTERESTS

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Biological Samples | | |
| Breastmilk (1 mL) | 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 16S rRNA sequence data, Milk Batch 1 | This paper | 16S rRNA sequence data (NCBI) BioProject accession:PRJNA481046 |
| Raw 16S rRNA sequence data, Milk Batch 2 | This paper | 16S rRNA sequence data (NCBI) BioProject accession:PRJNA597997 |
| Raw 16S rRNA sequence data, Stool | CHILD database | Access upon request to Stuart E. Turvey |
| Primers | | |
| 16S rRNA-Forward Primer 515F: GTGCCAGCMGCCGCGGTAA | Caporaso et al., 2012 | N/A |
| 16S rRNA-Reverse Primer 806R:GGACTACHVGGG TWTCTAAT | 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. 1.26.1 | McMurdie and Holmes, 2013 | https://joey711.github.io/phyloseq/index.html |
| Decontam v. 1.2.1 | Davis et al., 2018 | https://benjjneb.github.io/decontam/vignettes/decontam_intro.html |
| CoDaSeq v. 0.99.3 | Gloor and Reid, 2016 | https://github.com/ggloor/CoDaSeq |
| Vegan v. 2.5.4 | Oksanen et al., 2019 | https://cran.r-project.org/web/packages/vegan/vegan.pdf |
| lmPerm v. 2.1.0 | Wheeler and Torchiano, 2016 | https://cran.r-project.org/web/packages/lmPerm/lmPerm.pdf |
| zCompositions v. 1.3.2 | Palarea-Albaladejo and Martín-Fernández, 2015 | https://cran.r-project.org/web/packages/zCompositions/zCompositions.pdf |
| R v. 3.5.2 | R Core Team | https://www.r-project.org |

### RESOURCE AVAILABILITY

#### Lead Contact

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

#### Materials Availability

For breastmilk data, further requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Meghan Azad (meghan.azad@umanitoba.ca). For infant stool data, further requests for resources and reagents should be directed to and will be fulfilled by Stuart E. Turvey (sturvey@bcchr.ca) and Brett Finlay (bfinlay@msl.ubc.ca).

#### Data and Code Availability

The accession numbers for the 16S rRNA gene sequence data reported in this paper are BioProject accession (NCBI): PRJNA481046 and BioProject (NCBI): PRJNA597997 (breastmilk). Gut microbiota data are deposited in the CHILD database and can be accessed upon request to Stuart E. Turvey.

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## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Study Population

Women with singleton pregnancies were enrolled in the CHILD cohort study between 2008 and 2012 (n=3621) and remained eligible if they delivered a healthy infant >35 weeks gestation (n=3455) (Subbarao et al., 2015). Breastmilk and infant stool were collected at a home visit scheduled for age 3 months and an additional infant stool sample was collected at a clinic visit scheduled for age 1 year. Mothers gave written informed consent in accordance with the Declaration of Helsinki. The protocols were approved by the Human Research Ethics Boards at McMaster University, the Hospital for Sick Children, and the Universities of Manitoba, Alberta, and British Columbia.

Milk samples selected for microbiome analysis from a representative subset of 428 mothers were previously analyzed (Moossavi et al., 2019). An additional subset of 766 mothers enriched for maternal and infant health conditions (atopy, asthma, obesity) were selected in this study. After pre-processing of sequences, 16S rRNA data was available for a total of 877 breastmilk samples (Figure S1). Gut microbiota 16S rRNA data were available for an asthma-enriched subset of 702 and 742 infants at 3 months and 1 year, respectively (Patrick et al., 2020). Infants who never received any breast milk ('never-breastfed') were excluded from datasets and analyses (Figure S1) unless specified otherwise (for a few univariate analyses), because we had low power to examine this small group separately (n=16 with 3-month stool and n=20 with 1-year stool), and we felt it was biologically inappropriate to group them with infants who were breastfed, even for a short period of time. Figure S1 illustrates the sample selection process and sample size for downstream statistical analyses. Analyses evaluating breastmilk feeding practices and gut microbiota required only infant stool samples, allowing us to include all infant stool samples with available information on breastfeeding practices plus the following essential covariates: birth mode, intrapartum antibiotics, and older siblings (n=653 for 3-month stool and n=698 for 1-year stool). For the paired milk-gut analyses comparing mothers' milk microbiota to their own infants' gut microbiota, dyads with both milk and stool microbiota data were included [n=266 dyads with infant stool and breastmilk microbiota at 3 months postpartum (3-month dataset) and n=282 dyads with 3-month breastmilk and 1-year infant stool (1-year dataset)].

### Infant Feeding and Early-Life Factors

Infant feeding was reported by standardized questionnaire at 3, 6, and 12 months. At the time of milk sample collection (3-4 months), breastmilk feeding status was classified as exclusive (breastmilk only), partial (breastmilk supplemented with infant formula) or none (no longer breastfed). The mode of breastmilk feeding was reported for breastfed infants at sample collection and classified as ''all direct breastmilk" (nursing at the breast only, with no feeding of pumped milk), or "some pumped breastmilk" (at least one serving of pumped milk in the past two weeks) (Klopp et al., 2017). Breastmilk feeding at 1 year was classified as ''Yes'' (continuation of any breastmilk feeding) or ''No'' (ceased breastfeeding prior to 1 year). Breastmilk feeding duration was categorized as >0-3, 3-9, 9-12 or $\geq$12 months. ''Never breastfed'' was included as an additional category in a few univariate analyses. Maternal age, infant sex, birth weight, gestational age, birth mode (Cesarean section or normal vaginal delivery), parity (older siblings, yes vs. no), and intrapartum antibiotic use were documented from hospital records.

## METHOD DETAILS

### Sample Collection and Microbiota Analysis

Analysis of breastmilk microbiota (Moossavi et al., 2019) and sequencing data generation for infant stool microbiota (Patrick et al., 2020) has been previously described. Briefly, mothers provided one sample of milk collected during a 24-hour period (fore milk and hind milk from multiple feedings) at 3-4 months postpartum [mean (SD) 17 (5) weeks]. It is possible that some maternal skin microbiota were sampled along with breastmilk microbiota; however, this provides an accurate representation of the microbiota ingested by the infant. A soiled diaper was provided on the same day for infant stool collection. Samples were refrigerated at home for up to 24 hours before being collected and processed by study staff (Moraes et al., 2015). An additional infant stool sample was provided at the 1-year clinical assessment.

Milk microbiota was analyzed at the University of Manitoba by 16S rRNA gene sequencing of the V4 hypervariable region with modified F515/R806 primers (Caporaso et al., 2012) on a MiSeq platform (Illumina, San Diego, CA, USA) and processed as previously described (Derakhshani et al., 2016; Moossavi et al., 2019). Sterile DNA-free water was used for negative controls in sequencing library preparation. Mock communities consisting of DNA extracted from 8 species with known theoretical relative abundances (Zymo Research, USA) were also run as positive controls. Infant stool was analysed at the University of British Columbia by 16S rRNA gene sequencing of the V4 hypervariable region with F515/R806 primers as previously described (Patrick et al., 2020). Demultiplexed sequencing data used in this study are deposited into the Sequence Read Archive (SRA) of NCBI and can be accessed via accession numbers PRJNA481046 and PRJNA597997 (breastmilk). Gut microbiota data have been deposited in the CHILD database and can be accessed upon request to Stuart E. Turvey

### Other Milk Components

Human milk oligosaccharides (HMOs) were measured at the University of California, San Diego, using high-performance liquid chromatography (Azad et al., 2018a) and fatty acids were measured by gas liquid chromatography at the University of Alberta (Cruz-Hernandez et al., 2013), as previously described.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Microbiota Data Pre-processing

Breastmilk and gut data were processed similarly. 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). Data analysis was conducted in R (R Core Team, 2018) and preprocessing of the ASV table was conducted using the Phyloseq package (McMurdie and Holmes, 2013). Potential reagent contaminants of milk microbiota were identified and removed as previously described (Moossavi et al., 2020). ASVs belonging to the genus *Halomonas* were highly present in negative controls for the gut microbiota [median read-count (IQR), 214.0 (122.0-271.0)] and thus removed.

ASVs belonging to the phylum Cyanobacteria, family of mitochondria, and class of chloroplast, and ASVs only present in extraction/PCR controls (2,350 ASVs for milk and 1,833 for the gut) were removed. Samples were rarefied to 8,000 sequencing reads per sample, leaving 877 breastmilk samples [73% of total 1,194 samples (Figure S1)] with 9,985 unique ASVs, and 1,444 stool samples [83% of total 1,736 samples (Figure S1)] with 4,420 unique ASVs. To eliminate sequencing artifacts, ASVs with less than 60 reads across each dataset (0.001% of total reads per sample on average) were removed (Gloor and Reid, 2016), resulting in 1,122 and 1,102 remaining ASVs in breastmilk and stool, respectively. This also resulted in the removal of an average ± SD of 86 ± 201 reads per breastmilk sample and 23 ± 33 reads per stool sample. Overall, the contribution of reads discarded through rarefaction (Figure S6A), and through exclusion of rare ASVs (Figure S6B) was deemed negligible. The number of sequencing reads per sample was then relativized to a total sum of 8,000 for downstream analyses. For microbiota composition analyses (redundancy analysis) and differential abundance analyses, ASVs with an average relative abundance below 0.01% were additionally removed and abundances were centered log-ratio (CLR) transformed using the *CoDaSeq* package (Gloor and Reid, 2016) after zeros were imputed using a Bayesian-multiplicative replacement method (Palarea-Albaladejo and Martín-Fernández, 2015).

### Statistical Analysis

For selection of covariates used throughout analyses, initial univariate redundancy analyses were performed on covariates considered potentially important to infant gut microbiota composition based on previous literature (Azad et al., 2016; Bäckhed et al., 2015; Martin et al., 2016). These included birth mode, intrapartum antibiotics, maternal body mass index, infant sex, gestational weight gain and home environment variables, namely pet ownership, older siblings, and study center/geographic location. Based on these preliminary analyses, birth mode, intrapartum antibiotics and older siblings, with statistical significance and sufficiently large $R^2$ ($p<0.05$ & $R^2>0.7\%$), were included in final multivariable models to assess microbiota composition and abundances of individual ASVs in the infant gut. This approach was taken to avoid overfitting models, considering the relatively low sample size of the milk-gut paired datasets.

Redundancy analysis (RDA) was used to investigate associations between breastmilk feeding practices, other early-life factors and infant gut microbiota composition using the vegan package (Oksanen et al., 2019). Specifically, the effect of each breastmilk feeding practice on gut microbiota composition was assessed in a univariate and adjusted model (covariates: birth mode, intrapartum antibiotics, older siblings and study site). Associations between infant gut microbiota diversity (Shannon index) and breastmilk feeding practices were tested using the Wilcoxon signed-rank test. For infants with both a 3-month and 1-year stool sample (n=464), the change in diversity from 3 months to 1 year was also assessed. Microbiota maturity ("microbiota age") of the infant gut was predicted using a random forest model on log-transformed relative abundances of ASVs for a subset of infants (n=613). This model was trained on a random subset of breastfed infants (n=793), for which log-transformed relative abundances were regressed against chronologic age at the time of sampling using default parameters of the R randomForest algorithm, similar to methods described previously (Subramanian et al., 2014). A correlation between microbiota age predictions and actual chronologic age was verified (Pearson's r=0.42, $p<0.001$). Associations between infant gut microbiota maturity predictions and breastmilk feeding practices were tested using the Wilcoxon signed-rank test. Univariate RDA and diversity analyses were additionally replicated including "Never breastfed" infants as a separate category.

Associations of breastmilk feeding practices and ASV relative abundances were tested separately for breastmilk and the infant gut. Associations of CLR-transformed abundances were tested by linear regression permutation tests (Wheeler and Torchiano, 2016) for ASVs present in at least 10% of samples. All models were adjusted for birth mode, intrapartum antibiotics and older siblings. In addition to this set of covariates, the analysis for breastmilk controlled for batches that were analyzed separately. Models predicting abundance in the infant gut were used to assess the effect of: 1) Breastfeeding exclusivity for all infants at 3 months (n=669), 2) Breastmilk feeding mode and exclusivity for breastfed infants at 3 months (n=571), 3) Breastmilk feeding mode for exclusively breastfed infants at 3 months (n=98), 4) Breastfeeding continuation for all infants at 1 year (n=718). Breastmilk feeding practices were also associated with ASV prevalence (presence/absence) in the infant gut by adjusted logistic regression using the same input variables as the linear regression models described above. This was done for ASVs present in at least 10% of samples. For all models, the Benjamini-Hochberg procedure was used to correct p-values. Both prevalence and relative abundances were evaluated to answer different questions since abundances provide information about population growth whereas prevalence can be more of an indication for bacterial transfer (Karpinets et al., 2018; Mainali et al., 2017).

For dyads with available breastmilk and gut data (3-month and 1-year datasets, Figure S1), dissimilarity between milk-gut pairs was calculated using Jaccard distances (Oksanen et al., 2019). Dissimilarity between actual dyads was compared to dissimilarity

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between random pairings of breastmilk and stool samples using a linear regression permutation test as described previously (Pärnänen et al., 2018). For random milk-gut pairings, all possible pairwise permutations of breastmilk and stool samples except those from actual dyads were used (3-month dataset, n=71,022; 1-year dataset, n=79,242). Actual and random milk-gut pairings (dyads) were classified by the number of ASVs shared between their breastmilk and infant stool sample to estimate the level of bacterial sharing. Based on the distribution of ASV sharing, the following categories were generated: 0-1, 2, 3-4 and $\geq$5 shared ASVs (Figure S7). The proportion of dyads in each ASV sharing category was compared between actual and random dyads and associated with breastmilk feeding practices using the chi-square test. For comparison of actual and random dyads, p-values were obtained using Monti-Carlo simulation and 4000 replicates. The number of shared bacteria was also tested as a continuous variable. The number of total ASVs shared between the milk and stool sample of a dyad was contrasted with the total number of ASVs in milk and stool samples in probability density plots using ggplot2 (Wickham, 2016) for estimation of the relative likelihood for each value of a sample, estimated using Kernel density estimation.

Correlations between the relative abundance of commonly shared bacteria in breastmilk and the infant gut were tested using Spearman rank correlation and visualized using a heatmap. Benjamini-Hochberg procedure was used to correct p-values, and tests with $p_{(FDR)}<0.1$ were considered significant. Commonly shared bacteria were defined as ASVs present in both breastmilk and the infant gut of at least 15 dyads (n=13 ASVs at 3 months; n=12 ASVs at 1 year).

For commonly shared ASVs, univariate associations between breastmilk feeding practices and prevalence in breastmilk and the infant gut were determined using the chi-squared test. The chi-squared test for trend was used to assess breastfeeding exclusivity (never breastfed, and none, partial or exclusive breastfeeding at 3-months) and breastfeeding duration (never breastfed, and >0-3, 3-9, 9-12, or $\geq$12 months) in an ordinal manner. Note that despite the relatively few infants never breastfed, it was of particular interest to identify whether commonly shared ASVs were present in the gut of infants that were never breastfed and observe whether there may be a dose-dependent trend in prevalence.

Among dyads with paired samples, RDA was used to explore associations between commonly shared milk bacteria (defined above), unshared milk bacteria (10 ASVs >1% average abundance not classified as commonly shared), other milk components (HMO and fatty acid profile), breastmilk feeding practices and other relevant covariates (birth mode, intrapartum antibiotics, older siblings and study site) with infant gut microbiota composition. These variables were assessed individually and in models adjusted for the most relevant covariates (HMO profile, breastmilk feeding practices, birth mode, intrapartum antibiotics, older siblings and study site). For all multivariable models the $R^2$ adjusted for the addition of multiple variables was reported ($R^2_{adj}$). Our goal was to estimate the contribution of potentially transferable (shared) bacteria in breastmilk to variation in infant gut microbiota relative to other relevant covariates. We selected redundancy analysis (Oksanen et al., 2019) because it allows for the assessment of associations between complex data (i.e. gut microbiota) as the dependent variable and various distinct covariates as independent variables.

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## Supplemental Information

# Breastmilk Feeding Practices Are Associated with the Co-Occurrence of Bacteria in Mothers' Milk and the Infant Gut: the CHILD Cohort Study

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**Supplementary Tables and Figures**

**Table S1.** Characteristics of dyads from the CHILD cohort included in each dataset used for analyses, related to STAR Methods. See also Figure S1.

**Table S2** Participant characteristics stratified by breastfeeding exclusivity at 3 months and breastfeeding continuation at 1 year, related to STAR Methods. See also Table S1.

**Table S3.** Multivariate redundancy analyses showing associations between breastmilk feeding practices, other cofactors, and infant gut microbiota composition, related to Figure 1A, B & C.

**Table S4.** Linear models associating centered log-ratio transformed abundances of amplicon sequence variants in the gut with breastmilk feeding practices and other cofactors, related to Figure 2. *See Excel file.*

**Table S5.** Prevalence and relative abundance of commonly shared bacteria in breastmilk and infant gut microbiota, related to Figure 3C.

**Table S6.** Sensitivity analysis: variation in infant gut microbiota composition at 3 months explained by commonly shared bacteria in breastmilk using alternative thresholds to define commonly shared bacteria. See also Figure 6.

**Figure S1.** Study flow chart, related to STAR Methods.

**Figure S2.** Sensitivity analysis isolating infants who were never breastfed: microbiota composition and diversity associated with breastmilk feeding exclusivity and duration. Replication of main Figure 1A, D, C & F.

**Figure S3.** Prevalence of bacteria in the infant gut associated with breastfeeding practices in multivariable models, related to Figure 2.

**Figure S4.** Breastmilk feeding practices associated with the number of amplicon sequence variants (ASVs) shared between milk and the infant gut, related to Figure 5A.

**Figure S5.** Associations between breastmilk feeding practices and relative abundances of commonly shared bacteria, related to Figure 2 and Figure 5.

**Figure S6.** Rarefaction and rare ASV removal, related to STAR Methods.

**Figure S7.** Histograms showing the number of shared amplicon sequence variants (ASVs) per dyad, related to Figure 3E and Figure 5A.

**Table S1. Characteristics of dyads from the CHILD cohort included in each dataset used for analyses, related to STAR Methods. See also Figure S1.**

| | Factor | Mean ± SD or n (%)[a] | | | | |
|---|---|---|---|---|---|---|
| | | Paired milk-gut datasets | | 3-month datasets | | 1-year dataset |
| | | 3-month gut & milk n=266 | 1-year gut & 3-month milk n=282[a] | 3-month gut n=653[a] | 3-month milk n=802 | 1-year gut n=698 |
| Maternal | Age (years) | 33.0 ± 4.1 | 33.4 (4.2) | 32.60 ± 4.6 | 32.92 ± 4.3 | 32.74 ± 4.6 |
| | Ethnicity | | | | | |
| | Caucasian | 204 (76.4) | 214 (75.9) | 486 (74.9) | 593 (73.9) | 519 (74.7) |
| | Asian | 37 (13.9) | 43 (15.2) | 103 (15.9) | 128 (16.0) | 114 (16.4) |
| | First Nations | 11 (4.1) | 6 (2.1) | 24 (3.7) | 26 (3.2) | 25 (3.6) |
| | Other | 15 (5.6) | 19 (6.7) | 36 (5.5) | 55 (6.9) | 37 (5.3) |
| Infant | Birth weight (g) | 3,483 ± 474 | 3,476 ± 473 | 3487 ± 478 | 3456 ± 485 | 3469 ± 484 |
| | Female sex | 103 (38.6) | 107 (37.9) | 279 (42.7) | 359 (44.8) | 289 (41.4) |
| | Gestational age (weeks) | 39.1 ± 1.3 | 39.1 ± 1.3 | 39.2 ± 1.4 | 39.1 ± 1.3 | 39.2 ± 1.4 |
| Early life | Caesarean section | 64 (24.3) | 72 (25.7) | 157 (24.0) | 213 (26.6) | 168 (24.1) |
| | Maternal intrapartum antibiotics | 103 (39.2) | 115 (40.9) | 247 (37.8) | 328 (40.9) | 262 (37.5) |
| | Maternal antibiotics before 3 months | 23 (8.7) | 33 (11.9) | 70 (10.8) | 86 (10.8) | 80 (11.7) |
| | Child antibiotics before 3 months | 6 (2.3) | 6 (2.2) | 20 (3.1) | 16 (2.0) | 21 ( 3.1) |
| | Multiparity (older siblings) | 133 (42.3) | 123 (43.6) | 284 (43.5) | 347 (43.3) | 314 (45.0) |
| Infant feeding | Only Direct breastmilk feeding (BF)[b] | 97 (37.0) | 104(37.9) | 217 (38.0) | 314 (39.2) | 238 (39.9) |
| | Duration of any BF (months)[c] | 9.7 ± 2.8 | 9.7 ± 2.8 | 8.9 ± 3.9 | 9.9 ± 2.7 | 9.0 ± 3.8 |
| | Duration of exclusive BF (months) | 3.3 ± 2.3 | 3.2 ± 2.3 | 3.2 ± 2.3 | 3.4 ± 2.3 | 3.2 ± 2.3 |
| | BF exclusivity at 3 months | | | | | |
| | Exclusive | 170 (63.7) | 173 (61.3) | 391 (59.9) | 522 (65.1) | 417 (59.7) |
| | Partial | 96 (36.0) | 108 (38.3) | 180 (27.6) | 280 (34.9) | 208 (29.8) |
| | BF continuation at 1 year | - | 134 (48.7) | - | - | 318 (45.6) |
| | Solids in diet at 3 months | 3 (1.1) | 3 (1.1) | 20 (3.1) | 12 (1.5) | 19 (2.7) |
| Other | HMO concentration (mg/mL) | 10.2 ± 2.1 | 10.3 ± 2.0 | 10.0 ± 2.1) | 10.3 ± 2.1) | 10.1 ± 2.1 |
| | Study city | | | | | |
| | Edmonton | 50 (18.8) | 46 (16.3) | 112 (17.2) | 166 (20.7) | 119 (17.0) |
| | Toronto | 79 (29.7) | 80 (28.4) | 167 (25.6) | 225 (28.1) | 159 (22.8) |
| | Vancouver | 77 (28.9) | 83 (29.4) | 179 (27.4) | 198 (24.7) | 179 (25.6) |
| | Winnipeg | 60 (22.6) | 73 (25.9) | 195 (29.9) | 213 (26.6) | 241 (34.5) |
| | Milk collection season | | | | | |
| | Winter | 64 (24.0) | 76 (27.0) | 141 (21.6) | 193 (24.1) | 172 (24.7) |
| | Spring | 68 (25.5) | 63 (22.3) | 182 (27.9) | 212 (26.4) | 179 (25.7) |
| | Summer | 64 (24.0) | 73 (25.9) | 164 (25.2) | 196 (24.4) | 180 (25.8) |
| | Fall | 71 (26.6) | 70 (24.8) | 165 (25.3) | 201 (25.1) | 166 (23.8) |

[a]Percent excludes samples with missing data. [b]Percent out of breastfeeding infants. [c]12 month BF duration = continued BF at 1-year sampling (12 month maximum duration).

**Table S2. Participant characteristics stratified by breastfeeding exclusivity at 3 months and breastfeeding continuation at 1 year, related to STAR Methods. See also Table S1.**

| Factor | Mean ± SD or n (%)[a] | | | | |
|---|---|---|---|---|---|
| | 3-month gut dataset, Breastfeeding exclusivity at 3 months | | | 1-year gut dataset, Breastfeeding continuation at 1 year | |
| | Exclusive n=391 | Partial n=180 | No breastmilk n=82 | Yes n=318 | No n=380 |
| Age (years) | 33.0 ± 4.2 | 32.8 ± 4.8 | 30.3 ± 5.5 | 33.6 ± 4.2 | 32.1 ± 4.7 |
| Ethnicity | | | | | |
| Caucasian | 301 (77.2) | 124 (69.7) | 61 (75.3) | 243 (76.7) | 276 (73.0) |
| Asian | 59 (15.1) | 33 (18.5) | 11 (13.6) | 50 (15.8) | 64 (16.9) |
| First Nations | 14 (3.6) | 7 (3.9) | 3 (3.7) | 10 (3.2) | 15 (4.0) |
| Other | 16 (4.1) | 14 (7.9) | 6 (7.4) | 14 (4.4) | 23 (6.1) |
| Female sex | 178 (45.5) | 60 (33.3) | 41 (50.0) | 135 (42.5) | 154 (40.5) |
| Gestational age (weeks) | 39.2 ± 1.3 | 39.1 ± 1.4 | 39.0 ± 1.3 | 39.4 ± 1.4 | 39.0 ± 1.4 |
| Caesarean section | 84 (21.5) | 52 (28.9) | 21 (25.6) | 67 (21.1) | 101 (26.6) |
| Intrapartum antibiotics | 134 (34.3) | 76 (42.2) | 37 (45.1) | 126 (39.6) | 136 (35.8) |
| Maternal antibiotics before 3 months | 44 (11.3) | 14 (7.8) | 12 (15.4) | 40 (12.7) | 40 (10.9) |
| Child antibiotics before 3 months | 12 (3.1) | 5 (2.8) | 3 (3.7) | 11 (3.5) | 10 (2.7) |
| Multiparity (older siblings) | 178 (45.5) | 78 (43.3) | 28 (34.1) | 140 (44.0) | 174 (45.8) |
| Only Direct breastmilk (BF)[b] | 156 (39.9) | 61 (33.9) | 0 (0.0) | 137 (43.8) | 101 (35.6) |
| Study city | | | | | |
| Edmonton | 62 (15.9) | 35 (19.4) | 15 (18.3) | 43 (13.5) | 76 (20.0) |
| Toronto | 107 (27.4) | 42 (23.3) | 18 (22.0) | 64 (20.1) | 95 (25.0) |
| Vancouver | 110 (28.1) | 56 (31.1) | 13 (15.9) | 110 (34.6) | 69 (18.2) |
| Winnipeg | 112 (28.6) | 47 (26.1) | 36 (43.9) | 101 (31.8) | 140 (36.8) |

[a]Percent excludes samples with missing data. [b]Percent out of breastfeeding infants

**Table S3. Multivariate redundancy analyses showing associations between breastfeeding practices, other cofactors, and infant gut microbiota composition, related to Figure 1A, B & C.**

| | $R^2$ (%) | p |
|---|---|---|
| **Model 1: all infants at 3 months (n=653)** | | |
| Overall (Adjusted $R^2$) | 1.72 | 0.001 |
| Breastfeeding exclusivity (Exclusive, partial, or no breastmilk) | 1.08 | 0.001 |
| Birth mode | 0.63 | 0.001 |
| Intrapartum antibiotics | 0.24 | 0.005 |
| Older siblings | 0.37 | 0.001 |
| Study city | 0.61 | 0.003 |
| **Model 2: breastfed infants at 3 months (n=571)** | | |
| Overall (Adjusted $R^2$) | 1.20 | 0.001 |
| Breastfeeding mode (Exclusive or partial breastmilk) | 0.20 | 0.174 |
| Breastfeeding exclusivity | 0.44 | 0.001 |
| Birth mode | 0.68 | 0.001 |
| Intrapartum antibiotics | 0.27 | 0.007 |
| Older siblings | 0.34 | 0.001 |
| Study city | 0.65 | 0.024 |
| **Model 3: all infants at 1 year (n=698)** | | |
| Overall (Adjusted $R^2$) | 1.80 | 0.001 |
| Breastfeeding duration* | 1.01 | 0.001 |
| Birth mode | 0.26 | 0.001 |
| Intrapartum antibiotics | 0.22 | 0.013 |
| Older siblings | 0.95 | 0.001 |
| Study city | 0.61 | 0.002 |

*Breastfeeding duration was included as a categorical variable (<3, 3-9, 9-12 or ≥12 months).

**Table S4. Linear models associating centered log-ratio transformed abundances of amplicon sequence variants in the gut with breastmilk feeding practices and other cofactors. See also Figure 2.** *See Excel file.*

**Table S5. Prevalence and relative abundance of commonly shared bacteria in breastmilk and infant gut microbiota, related to Figure 3C.**

| **Commonly shared bacteria**[a] | **% Prevalence** Mean (SD) relative abundance | | | Percent of dyads sharing ASV | |
|---|---|---|---|---|---|
| | 3-month milk | 3-month gut | 1-year gut | 3-month milk & 3-month gut (n=266) | 3-month milk & 1 year gut (n=282) |
| *Streptococcus2* | **77.9** 7.8 (13.7) | **71.9** 1.3 (3.3) | **85.8** 1.8 (3.4) | 61.4 | 68.1 |
| *Veillonella dispar* | **71.5** 2.0 (3.6) | **46.4** 1.0 (2.7) | **57.1** 1.1 (2.8) | 35.6 | 45.0 |
| *Staphylococcus* | **98.1** 13.1 (22.0) | **31.5** 0.1 (0.6) | **10.3** 0.1 (1.6) | 30.7 | 9.6 |
| *Bifidobacterium* | **32.2** 0.2 (1.2) | **84.3** 23.6 (26.3) | **90.4** 13.3 (16.6) | 29.6 | 28.7 |
| *Actinomyces* | **47.2** 0.6 (1.3) | **34.8** 0.1 (0.3) | **30.8** 0.4 (0.1) | 25.1 | 17.0 |
| *Streptococcus3* | **62.9** 8.2 (14.5) | **28.5** 0.3 (1.4) | **19.9** 0.1 (0.2) | 24.3 | 14.2 |
| *Haemophilus parainfluenzae* | **40.5** 0.6 (1.8) | **48.7** 1.4 (5.3) | **69.2** 1.4 (3.3) | 22.8 | 31.2 |
| *Streptococcus1* | **92.1** 18.6 (21.2) | **20.2** 0.1 (0.6) | **14.2** 0.1 (0.2) | 18.7 | 14.2 |
| *Streptococcus4* | **54.3** 2.9 (7.11) | **19.1** 0.2 (0.8) | **17.4** 0.1 (0.5) | 14.6 | 12.4 |
| *Rothia mucilaginosa* | **29.6** 1.3 (3.48) | **16.5** 0.06 (0.26) | **3.6** 0.005 (0.040) | 13.5 | 2.1 |
| *Enterococcus* | **15.4** 0.6 (3.82) | **52.4** 1.0 (3.4) | **33.0** 0.4 (1.6) | 9.4 | 6.7 |
| *Veillonella parvula* | **15.4** 0.1 (0.85) | **37.5** 0.9 (2.7) | **38.7** 0.7 (2.4) | 7.9 | 8.9 |
| *Atopobium* | **27.3** 0.07 (0.24) | **13.1** 0.04 (0.45) | **2.1** 0.001 (0.009) | 7.9 | 0.7 |
| *Unclassified Lachnospiraceae* | **6.4** 0.01 (0.70) | **55.8** 3.4 (7.7) | **86.2** 4.0 (7.5) | 4.1 | 8.2 |

[a]Shared in at least 15 dyads in either dataset. Sorted from most to least sharing at 3 months.

**Table S6. Sensitivity analysis: variation in infant gut microbiota composition at 3 months explained by commonly shared bacteria in breastmilk using alternative thresholds to define commonly shared bacteria. See also Figure 6.**

| | **Relaxed definition: 18 ASVs shared by ≥8 dyads** | **Selected definition: 13 ASVs shared by ≥15 dyads** | **Strict definition: 8 ASVs shared by ≥45 dyads** |
|---|---|---|---|
| **Adjusted $R^2$ (used)** | 0.88% | 1.04% | 0.80% |
| **$R^2$** | 7.64% | 5.92% | 3.81% |
| **p-value** | 0.006 | 0.001 | 0.001 |

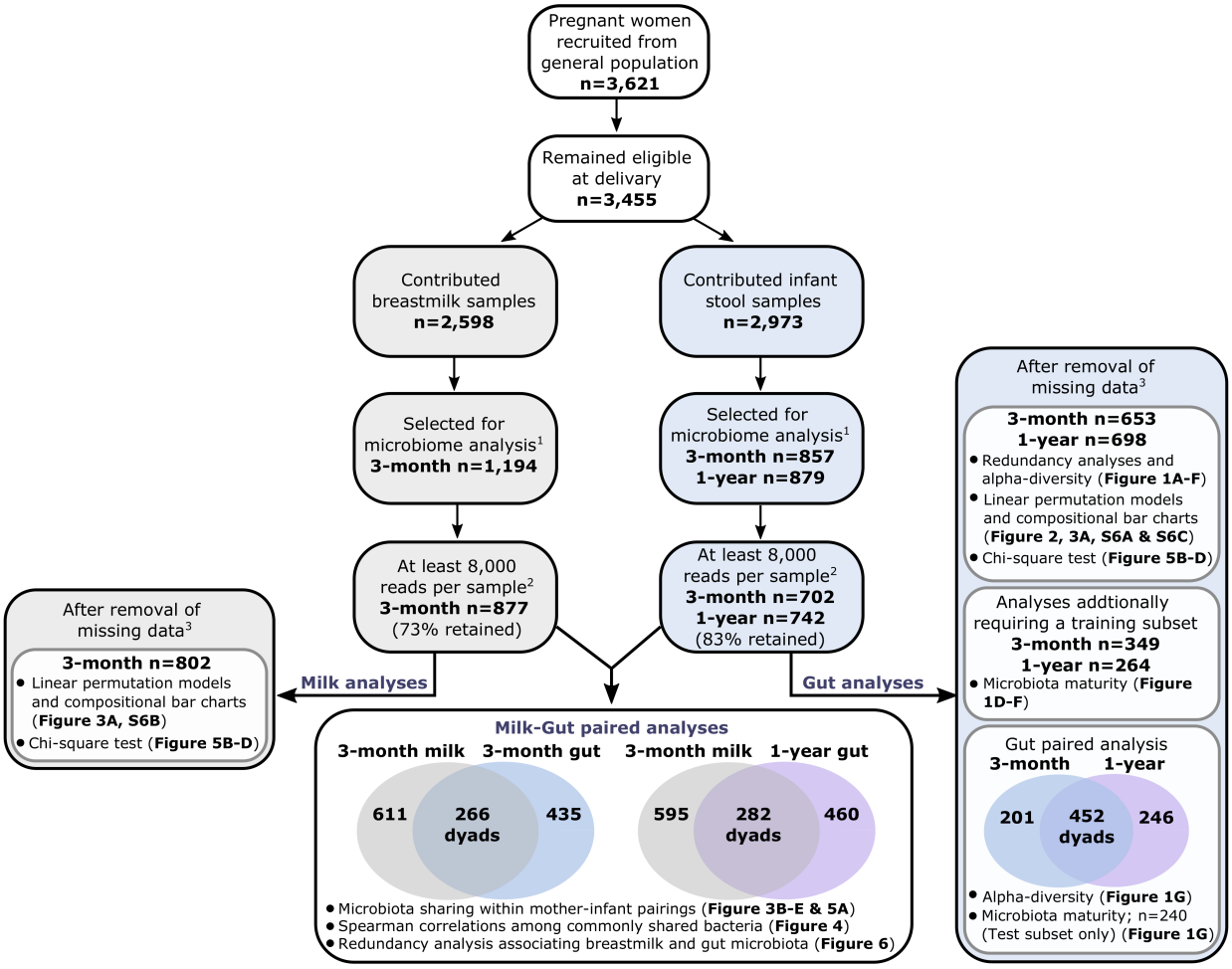


**Figure S1. Study flow chart, related to STAR Methods.** Mothers were recruited into the CHILD Cohort Study during pregnancy and remained eligible if they delivered a healthy infant >35 weeks gestation. All samples selected for microbiome analysis were submitted for 16S rRNA gene sequencing. [1]An initial representative subset of breastmilk samples (n=428) and a second subset enriched for childhood atopy, asthma and obesity (n=766) were selected for microbiome analysis. A subset of stool samples enriched for infant atopy and/or wheeze at age 1 year was selected based on the availability of infant blood samples. [2]After removal of reads that did not pass DADA2 filters (Callahan et al., 2016) and contaminant reads, samples with fewer than 8,000 reads (low read/DNA yield) were removed (27% of breastmilk and 17% of stool samples). [3]Samples were removed if they were missing information on main variables used for milk and gut specific analyses (breastfeeding, birth mode, intrapartum antibiotics, and older siblings). Note that **Figure 2A, 5C, 5D & S2** also included an additional group of infants that were never breastfed and had 3-month infant stool (n=16), and 1-year infant stool (n=20) available (not shown in the flow diagram).

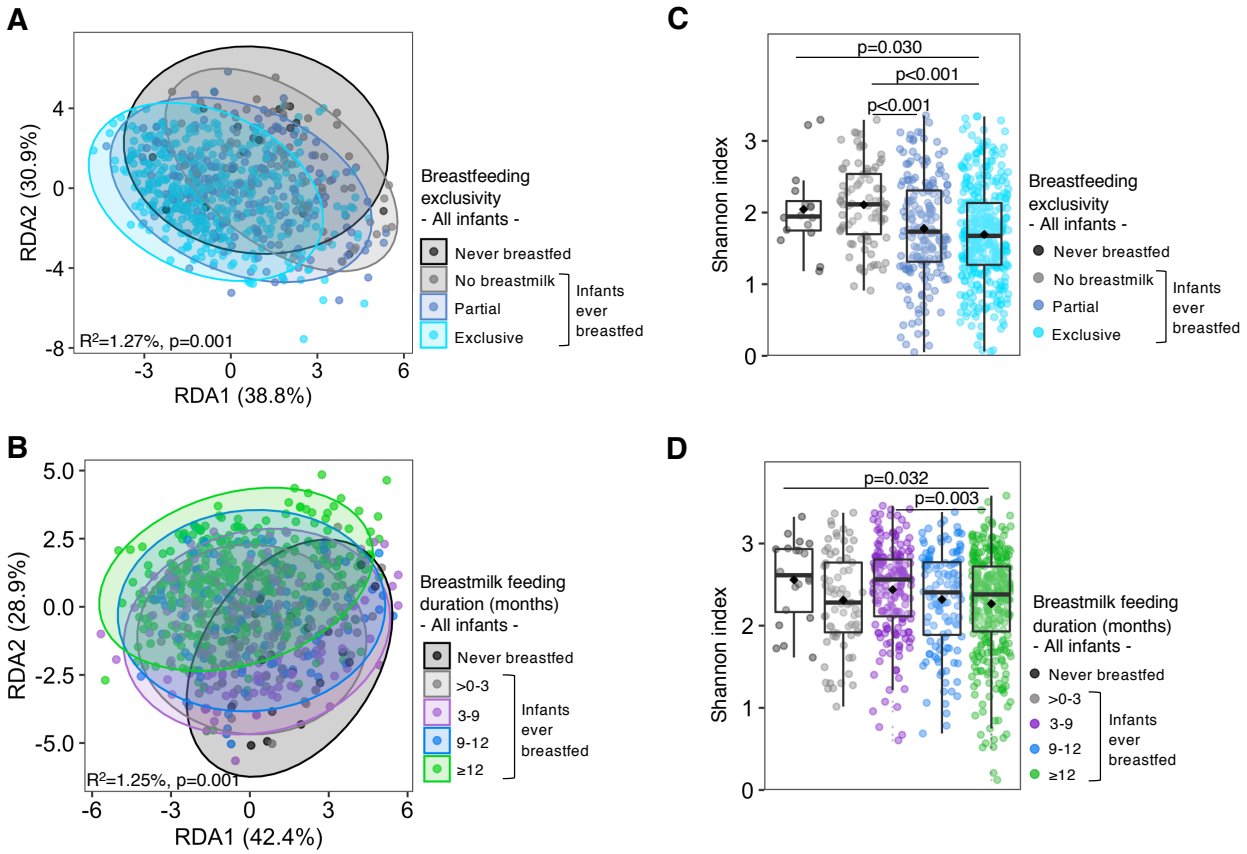


**Figure S2. Sensitivity analysis isolating infants who were never breastfed: microbiota composition and diversity associated with breastmilk feeding exclusivity and duration. Replication of main Figure 1A, C, D & F. A–B)** Redundancy analyses showing univariate associations of breastfeeding exclusivity at 3-months (n=669) **(A)** and breastfeeding duration at 1-year (n=718) **(B)** with infant gut microbiota composition. **C-D)** Associations of breastfeeding exclusivity at 3-months (n=669) **(C)** and breastfeeding duration at 1-year (n=718) **(D)** with gut microbiota diversity (Shannon index).

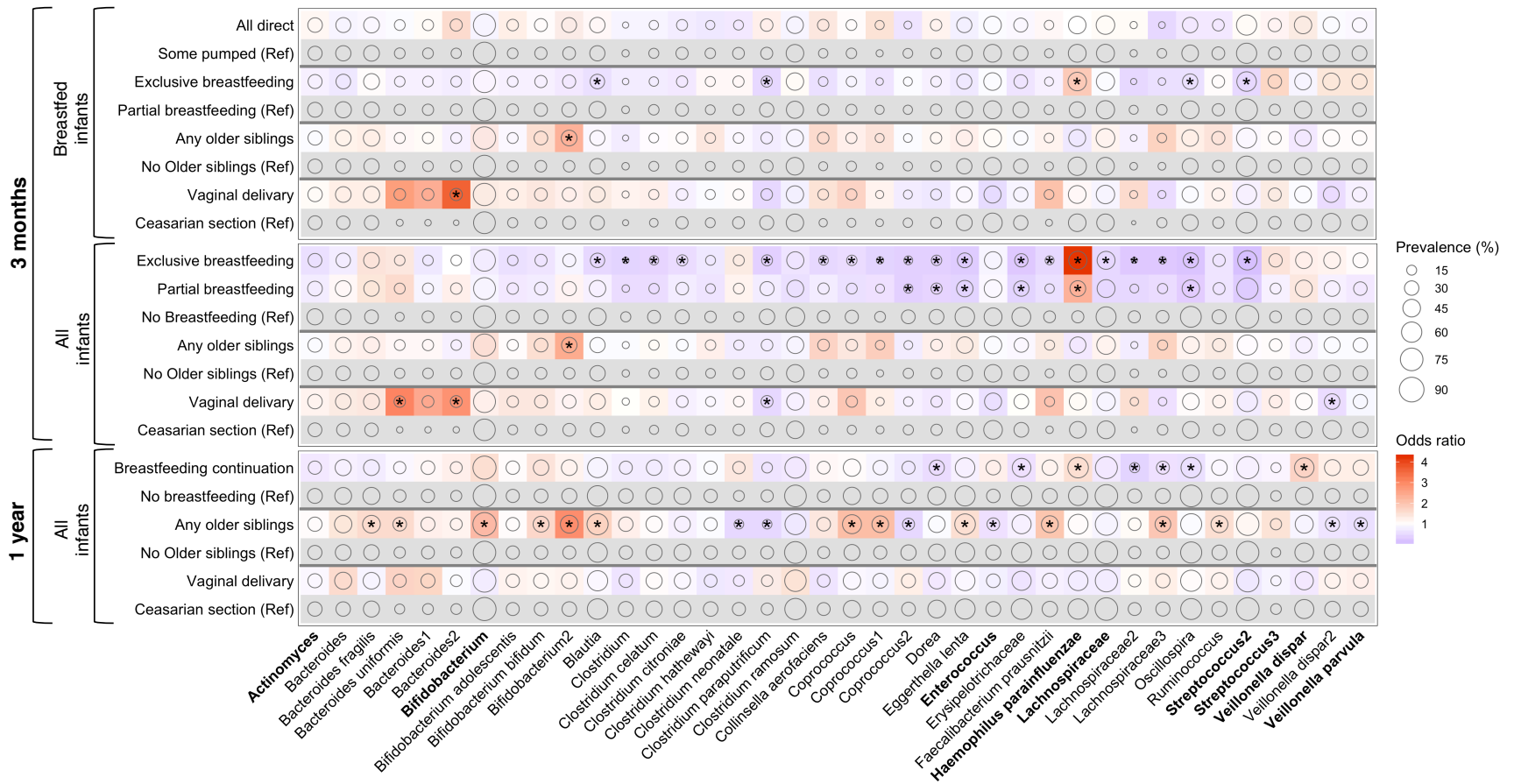


**Figure S3. Prevalence of bacteria in the infant gut associated with breastfeeding practices in multivariable models, related to Figure 2.** Association of breastfeeding practices with the prevalence of ASVs in the infant gut using multivariable logistic regression. Potentially important covariates known to influence the infant gut microbiota (birth mode and older siblings) are shown for comparison, and models were additionally adjusted for intrapartum antibiotics. Separate models were generated for: breastfed infants (n=571) and all infants (n=653) at 3 months; and all infants at 1 year (n=698). ASVs present in at least 20% of samples were analyzed. Bold taxa indicate bacteria commonly shared with mothers' breastmilk. The prevalence in each group is shown by varying dot size, including reference groups (Ref). Colours represent the direction (red for >1, blue for <1) and magnitude of odds ratios for the presence of each ASV compared to the reference group (grey). *$p_{(FDR)}$<0.05. Also see **Figure 2A** for results of CLR-transformed relative abundance.

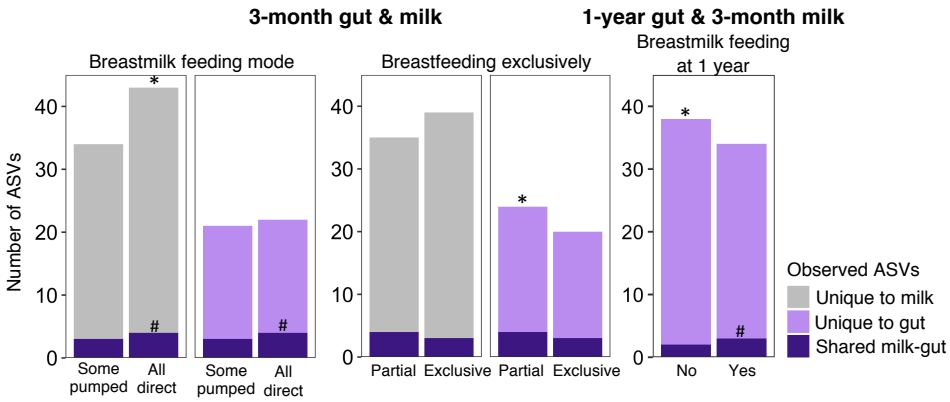


**Figure S4. Breastmilk feeding practices associated with the number of Amplicon sequence variants (ASVs) shared between milk and the infant gut, related to Figure 5A.** Total observed ASVs in milk and the infant gut (total height of bars) associated with breastmilk feeding mode and exclusivity at 3 months and breastmilk feeding continuation at 1 year. Tested using Wilcoxon signed-rank test. Note that the number of total milk and infant gut ASVs that are shared (dark purple) is compared as well and takes up the same number of ASVs in both milk and the infant gut. *$p<0.05$, comparisons for total observed ASVs. #$p<0.05$, comparisons for number of shared ASVs.

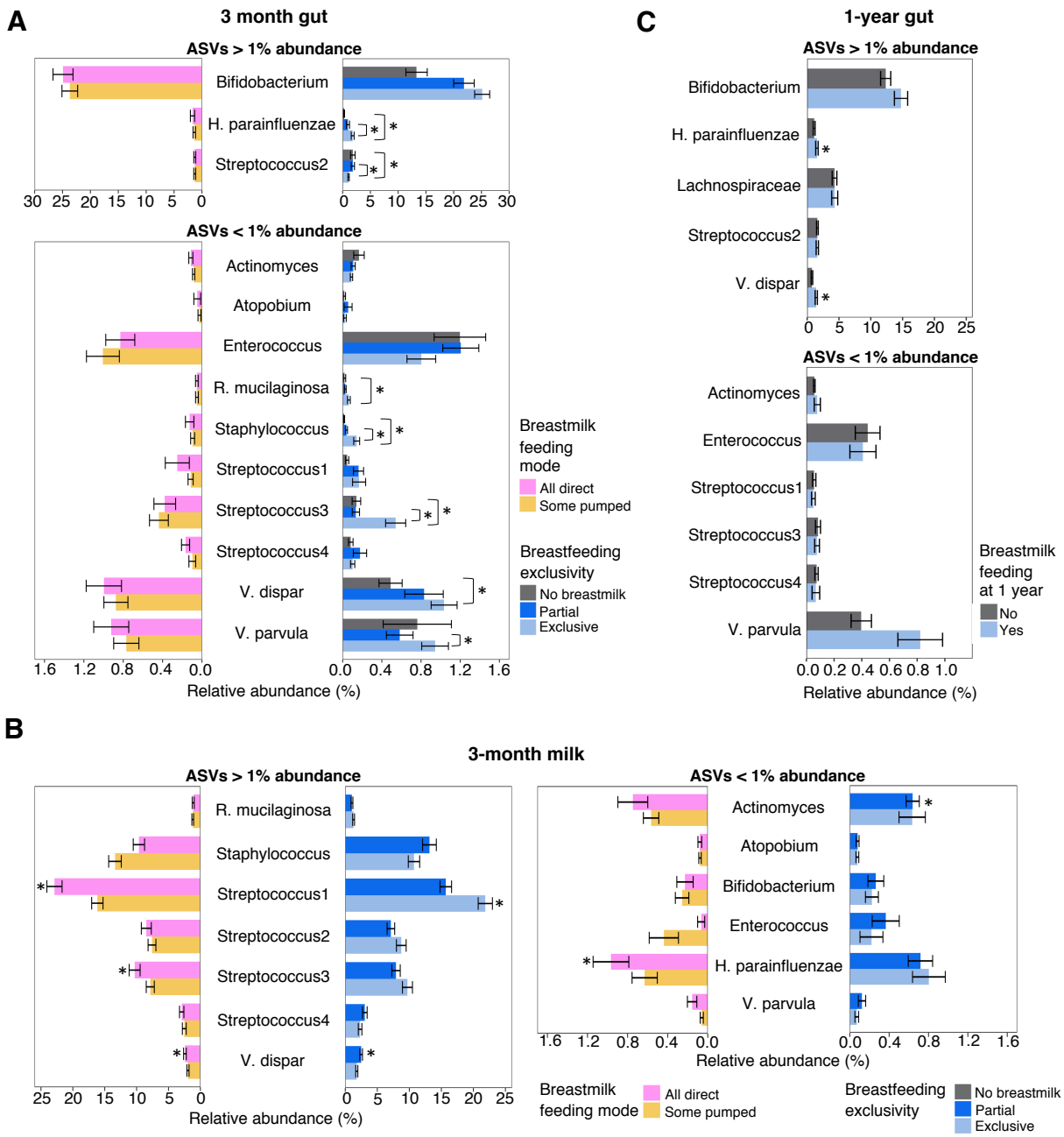


**Figure S5. Associations between breastmilk feeding practices and relative abundances of commonly shared bacteria, related to Figure 2 and Figure 5.** Breastmilk feeding mode and exclusivity at 3 months associated with relative abundances of commonly shared bacteria in the infant gut **(A)** and breastmilk **(B)**. Breastmilk feeding continuation associated with abundances of commonly shared bacteria in the infant gut at 1 year **(C)**. Showing differences in relative abundance and error bars showing the standard error of the mean. Associations tested using linear regression permutation tests on centered log-ratio abundances. Models were adjusted for birth mode, older sibling and intrapartum antibiotics (see also **Figure 2**), and the model for breastmilk **(B)** was additionally adjusted for a batch effect. *$p_{(FDR)}$<0.05. Full results of the tests can be found in **Table S4**. Abbreviations: *H. parainfluenzae, Haemophilus parainfluenzae; R. mucilaginosa, Rothia mucilaginosa; V. dispar, Veillonella dispar; V. parvula, Veillonella parvula.*

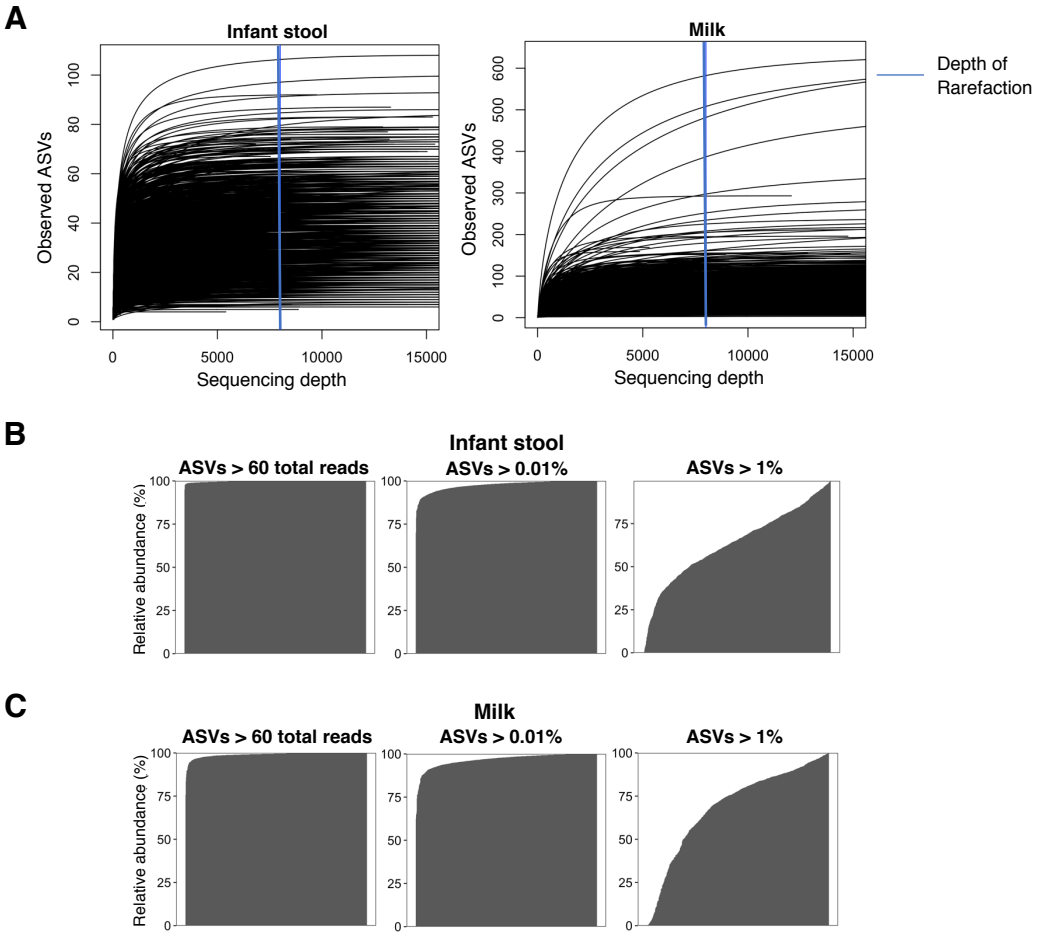


**Figure S6. Rarefaction and rare ASV removal, related to STAR Methods. A)** Rarefaction curves showing the number of observed ASVs at each sequence subsampling threshold for infant stool and milk. The depth of rarefaction is indicated with a blue line (8,000 reads/sample). The majority of samples reach or nearly reach saturation of observed ASVs per sample at a depth of 8,0000 reads per sample. **B-C)** Proportion of reads remaining per sample after removal of rare ASVs. Only rare taxa that made up a small fraction of reads in the total dataset were removed for microbiota diversity (ASVs > 60 total reads) and composition (>0.01%) analyses. Samples are across the x-axis, n=1,444 for infant stool **(B)** and n=1,194 for milk **(C).**

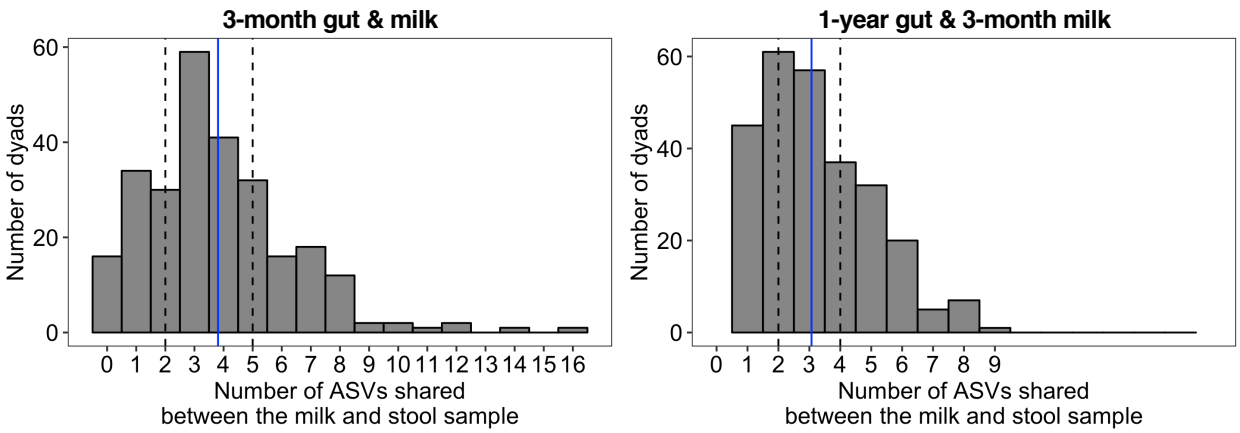


**Figure S7. Histograms showing the number of shared Amplicon sequence variants (ASVs) per dyad, related to Figure 3E and Figure 5A.** Dotted lines represent the interquartile range (2–5), and the blue solid line indicates the mean. The number of shared ASVs was categorized as: 0–1, 2, 3–4, ≥5, and for chi-square tests, <5 and ≥5 shard ASVs was compared to assess which dyads had the greatest bacterial sharing.