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Supplemental information

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in very-low-birth-weight infants**

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Mother's Milk Microbiota is Associated with the Developing Gut Microbial Consortia in Very-Low-Birth-Weight Infants

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Online Supplemental Material

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Supplemental Table 1. Characteristics of infants and mothers included from the OptiMoM Fortifier Study related to STAR Methods

Characteristic	Value
Infant Characteristics (n=94)	
Birth weight	
Grams	850 (730, 1047)
<1000 g, No. (%)	62 (66.0)
1000-1249 g, No. (%)	32 (34.0)
Gestational age, weeks	27.4 (25.7, 29.1)
Small for gestational age, No. (%)	7 (7.4)
SNAP-II score	12 (5, 21)
APGAR score at 5 min	8 (7, 9)
Sex assigned at birth, No. (%)	
Female	50 (53.2)
Male	44 (46.8)
Delivery mode, No. (%)	
C-section	60 (63.8)
Vaginal	34 (36.2)
Multiple birth status, No. (%)	
Singleton	61 (64.9)
Twin	23 (24.5)
Triplet	10 (10.6)
Hospital recruitment site, No. (%)	
The Hospital for Sick Children	11 (11.7)
Sinai Health	83 (88.3)
Antibiotic Exposure	
Total exposure, No. (%)	87 (92.6)
Total number of days	6 (3, 15)
Infants with >3 days of exposure, No. (%)	61 (64.9)
Morbidity/mortality, No. (%)	
Late-onset sepsis	28 (29.8)
Necrotizing enterocolitis stage \geq II	2 (2.1)
Chronic lung disease ^a	26 (27.7)
Severe retinopathy of prematurity	5 (5.3)
Severe brain injury	12 (12.8)
Patent ductus arteriosus	49 (52.1)
Mortality	5 (5.3)
Feeding type, No. (%)	
Predominantly mother's milk	63 (67.0)
Mixed feeding with supplemental donor milk	31 (33.0)
Fortifier type, No. (%)	
Human milk-based fortifier	51 (54.3)
Bovine milk-based fortifier	43 (45.7)
Infant's fed at the breast prior to discharge, No. (%)	79 (84.0)
First postnatal day infants fed at breast, days	44 (29, 62)
Postmenstrual age of infants at first direct breastfeed	34 (33, 35)
Paired milk-stool samples per infant	5 (3, 6)
Maternal Characteristics (n=82)	
Maternal age, years	33 (31, 37)
Pre-pregnancy BMI ^b	24.2 (21.5, 28.1)
Maternal antibiotic use	

Total exposure, No. (%)	49 (59.8)
Total number of days	1 (0, 4)
Mother's with >1 day of antibiotic exposure, No. (%)	33 (40.2)

Values are median (Q1, Q3), unless otherwise stated.

^aOxygen support provided at 36 weeks post-conception, as defined by the Canadian Neonatal Network.

^bMissing BMI data for one mother (n=81).

Supplemental Table 2. Genera of all shared zOTUs between paired milk-stool samples related to Figure 3

Genus	Paired milk-stool samples n (%)	Mother-infant dyads n (%)
Unclassified Enterobacteriaceae	420 (99.5)	94 (100)
Staphylococcus	381 (90.3)	92 (97.9)
Enterococcus	201 (47.6)	74 (78.7)
Streptococcus	162 (38.4)	70 (74.5)
Veillonella	138 (32.7)	58 (61.7)
Clostridium sensu stricto	121 (28.7)	59 (62.8)
Acinetobacter	67 (15.9)	29 (30.9)
Corynebacterium	67 (15.9)	44 (46.8)
Pseudomonas	67 (15.9)	40 (42.6)
Haemophilus	52 (12.3)	32 (34)
Fingoldia	46 (10.9)	29 (30.9)
Bifidobacterium	40 (9.5)	26 (27.7)
Propionibacterium	30 (7.1)	19 (20.2)
Pantoea	30 (7.1)	24 (25.5)
Bacteroides	27 (6.4)	15 (16)
Stenotrophomonas	27 (6.4)	16 (17)
Peptoniphilus	20 (4.7)	12 (12.8)
Gemella	18 (4.3)	13 (13.8)
Prevotella	14 (3.3)	11 (11.7)
Actinomyces	12 (2.8)	9 (9.6)
Neisseria	12 (2.8)	7 (7.4)
Faecalibacterium	11 (2.6)	5 (5.3)
Lachnospiracea incertae sedis	8 (1.9)	7 (7.4)
Akkermansia	6 (1.4)	5 (5.3)
Lactococcus	6 (1.4)	6 (6.4)
Rothia	6 (1.4)	5 (5.3)
Lactobacillus	5 (1.2)	3 (3.2)
Parabacteroides	5 (1.2)	4 (4.3)
Bacillus	5 (1.2)	4 (4.3)
Blautia	5 (1.2)	4 (4.3)
Anaerococcus	4 (0.9)	4 (4.3)
Negativicoccus	4 (0.9)	3 (3.2)
Dialister	3 (0.7)	2 (2.1)
Peptostreptococcus	3 (0.7)	3 (3.2)
Actinobacillus	3 (0.7)	2 (2.1)
Alloscardovia	3 (0.7)	2 (2.1)
Clostridium XI	3 (0.7)	2 (2.1)
Aeromonas	2 (0.5)	2 (2.1)
Clostridium XVIII	2 (0.5)	2 (2.1)
Collinsella	2 (0.5)	2 (2.1)
Dermabacter	2 (0.5)	2 (2.1)
Granulicatella	2 (0.5)	1 (1.1)
Listeria	2 (0.5)	2 (2.1)
Morganella	2 (0.5)	1 (1.1)
Romboutsia	2 (0.5)	1 (1.1)
Alistipes	1 (0.2)	1 (1.1)
Anaeroglobus	1 (0.2)	1 (1.1)
Arthrobacter	1 (0.2)	1 (1.1)

Chryseobacterium	1 (0.2)	1 (1.1)
Eisenbergiella	1 (0.2)	1 (1.1)
Flavonifractor	1 (0.2)	1 (1.1)
Fusicatenibacter	1 (0.2)	1 (1.1)
Fusobacterium	1 (0.2)	1 (1.1)
Howardella	1 (0.2)	1 (1.1)
Megasphaera	1 (0.2)	1 (1.1)
Parasutterella	1 (0.2)	1 (1.1)
Roseburia	1 (0.2)	1 (1.1)
Senegalimassilia	1 (0.2)	1 (1.1)
Sphingomonas	1 (0.2)	1 (1.1)
Sutterella	1 (0.2)	1 (1.1)
Average shared zOTU ratio per mother-infant dyad, mean % (95% CI)		34.4 (32.2, 36.5)

Shared zOTUs, between paired milk-stool samples, mapped back to 60 different genera. Genera shared in approximately 10% of paired milk stool samples were considered “commonly shared bacteria” and were included in all subsequent analyses. Abbreviations: CI, confidence interval.

Supplemental Table 3. Consistency of zOTUs shared in milk-stool pairs where a milk sample was paired with two stools from the same infant related to Figure 3

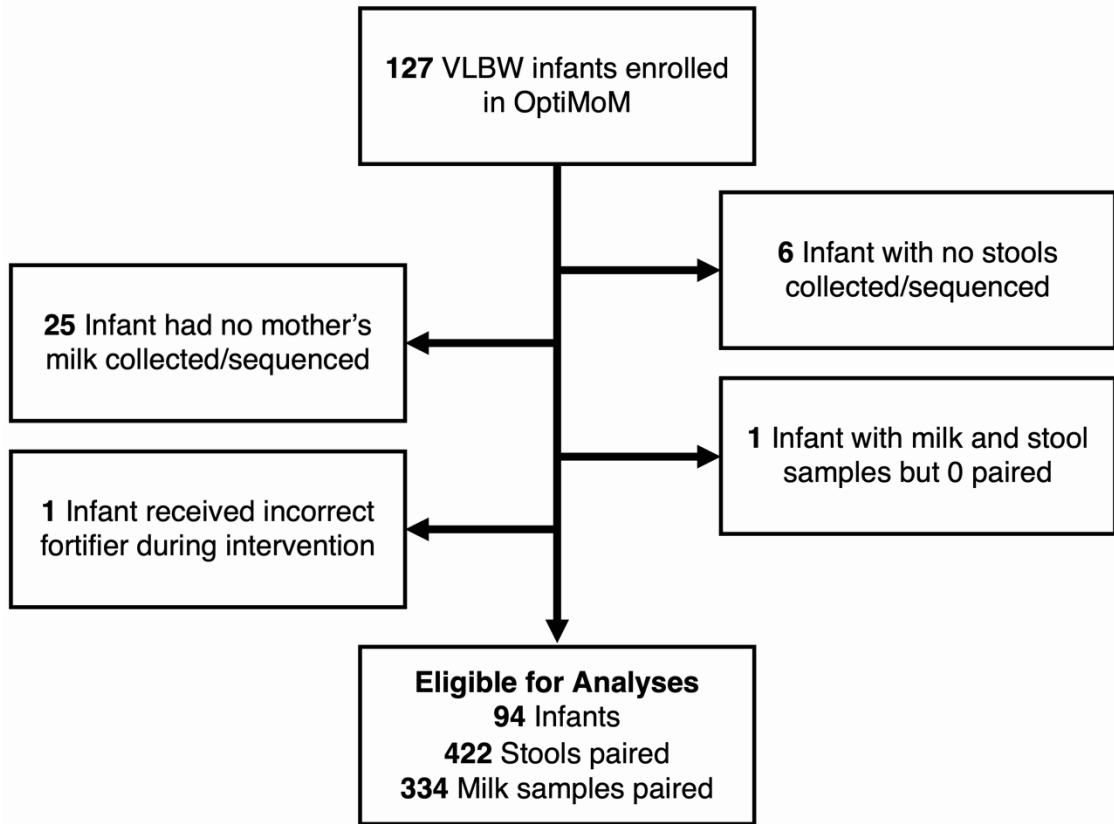
Milk Sample	Shared zOTUs in milk-stool 1	Shared zOTUs in milk-stool 2	Same shared zOTUs in both milk-stool 1 and milk-stool 2
1	7 (28.0)	6 (37.5)	5
2	13 (48.1)	13 (40.6)	10
3	19 (63.3)	12 (40.0)	12
4	8 (32.0)	6 (31.6)	4
5	8 (27.6)	6 (15.8)	6
6	4 (23.5)	6 (21.4)	4
7	2 (33.3)	2 (18.2)	2
8	4 (21.1)	5 (31.3)	3
9	5 (20.8)	5 (26.3)	4
10	4 (28.6)	4 (16.7)	3
11	5 (20.8)	6 (24.0)	4
12	7 (53.8)	6 (42.9)	5
13	3 (18.8)	2 (13.3)	2
14	6 (18.2)	8 (22.2)	6
15	7 (36.8)	5 (35.7)	4
16	7 (24.1)	5 (29.4)	5
17	2 (10.5)	4 (28.6)	2
18	5 (29.4)	5 (20.8)	4
19	4 (17.4)	6 (26.1)	4
20	13 (72.2)	14 (58.3)	8
21	11 (23.4)	7 (14.6)	6
22	13 (21.3)	12 (19.7)	7
23	5 (23.8)	4 (36.3)	2
24	7 (43.8)	5 (35.7)	4
25	6 (35.3)	6 (42.9)	5
26	5 (29.4)	5 (38.5)	5

Values are the number of shared zOTUs (shared zOTU ratio expressed as a percentage). Shared zOTU ratio was calculated as the number of shared zOTUs between a paired milk-stool sample divided by the total number of zOTUs in the respective stool sample. Mother's milk samples (n=26) were paired with two stool samples from the sample infant provided they fell within the +/- 7-day range; this was done to maximize the number of milk-stool pairs included in our analyses.

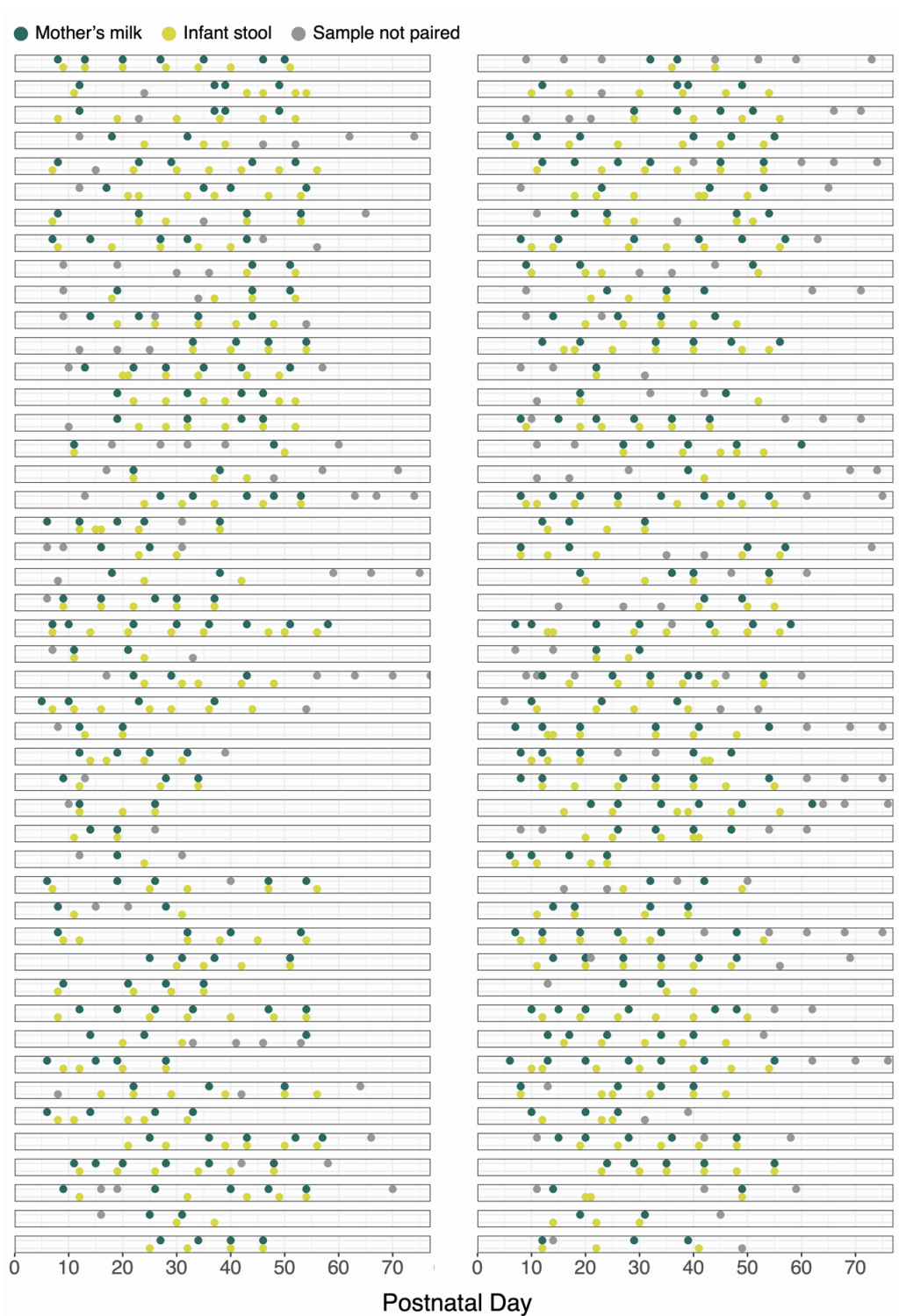
Supplemental Table 4. Summary of P values from linear mixed-effects models examining relationships between milk bacterial intakes of commonly-shared genera and their concentrations in infant stools related to Figure 5

	Intake	Intake*time	Intake*feed type	Intake*fortifier	Intake*fed at breast
Total Bacteria	<0.001	0.04	0.002	0.048	0.55
Unclass. Enterobacteriaceae	<0.001	0.02	0.005	0.29	0.63
Staphylococcus	0.44	0.25	0.63	0.09	0.88
Veillonella	<0.001	0.12	0.13	0.41	<0.001
Streptococcus	<0.001	0.21	0.98	0.10	0.19
Enterococcus	0.01	0.26	0.15	0.047	0.43
Clostridium sensu stricto	0.005	0.92	0.06	0.73	0.02
Acinetobacter	0.02	0.61	0.53	0.61	0.41
Corynebacterium	0.35	0.71	0.39	0.73	0.83
Pseudomonas	<0.001	0.02	0.63	0.25	0.78
Haemophilus	0.31	0.63	0.49	0.24	0.89
Finegoldia	0.03	0.99	0.86	0.80	0.69
Bifidobacterium	0.37	0.72	0.64	0.43	0.61

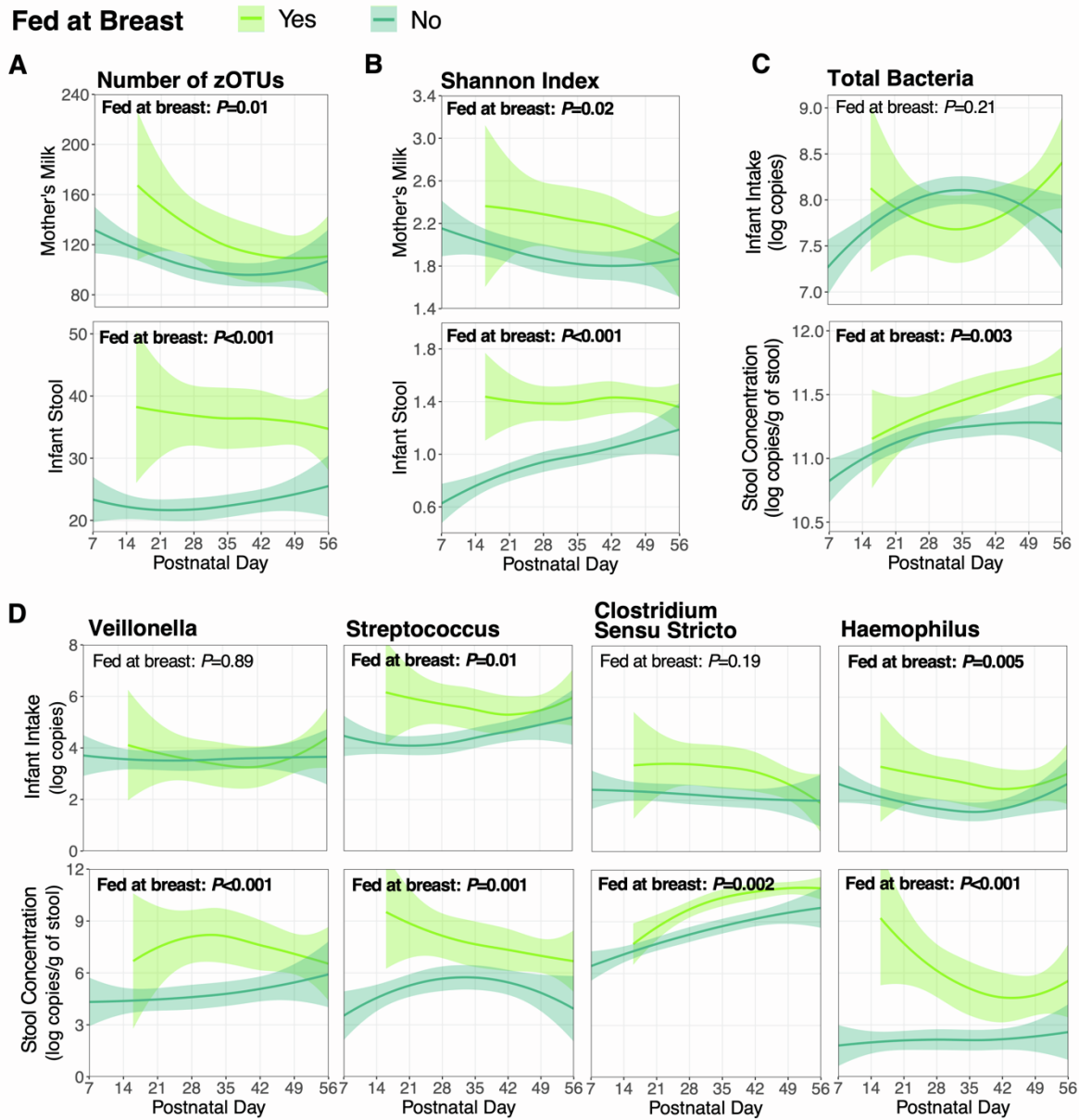
The modulatory potential of postnatal period and feeding practices on the relationships between milk bacterial intakes of commonly-shared genera and their concentrations in infant stools was examined and *P*-values of interaction terms are displayed. *P* values from the main effects were FDR-adjusted to account for multiple comparisons.



Supplemental Figure 1. Inclusion of infants, mothers, and samples in the present analysis from the OptiMoM Fortifier Study related to STAR Methods

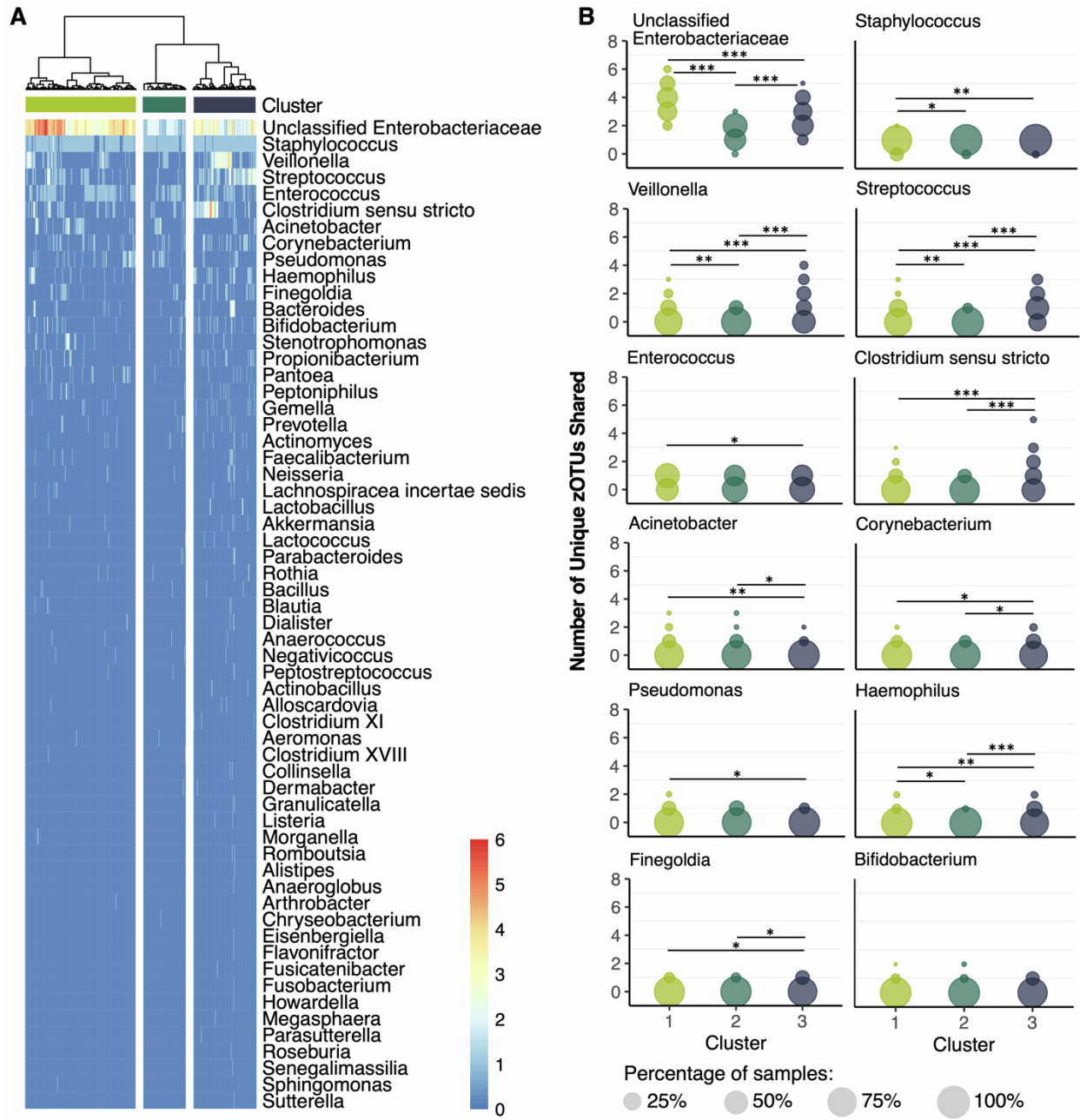


Supplemental Figure 2. Pairing of milk and stool samples for each mother-infant dyad included in our analysis related to STAR Methods. Each box represents an individual mother-infant dyad ($n = 94$). Green and yellow circles represent mother's milk and infant stool samples, respectively. Grey circles represent both mother's milk or infant stool samples not paired and therefore excluded from our analyses. See STAR methods for the pre-defined criteria set to pair mother's milk with infant stool samples.



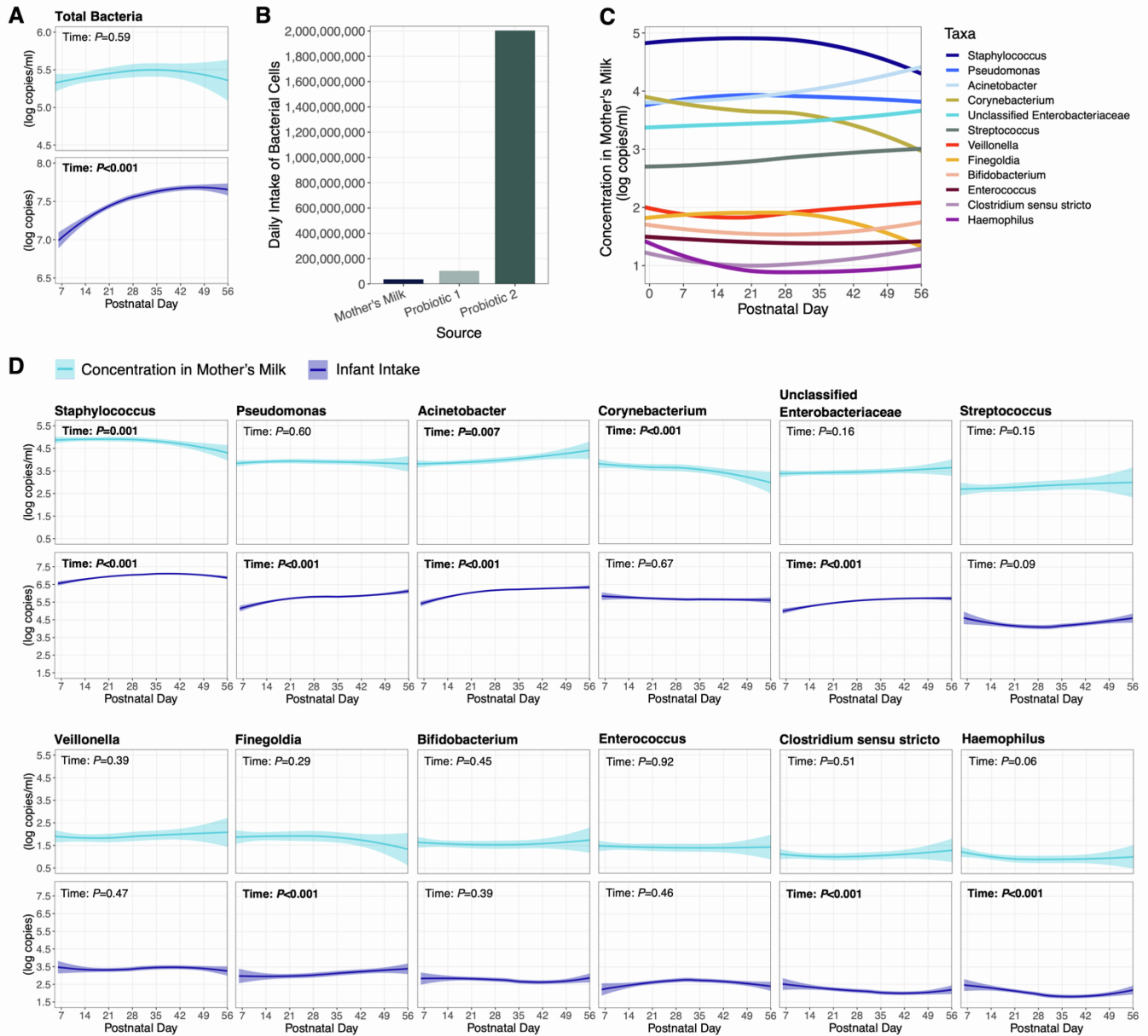
Supplemental Figure 3. Microbiota in mother's milk and infant stool across hospitalization stratified by feeding at the breast related to Figures 1 and 4. (A) Number of zOTUs and (B) Shannon index over time, stratified by feeding at the breast in mother's milk (top row, $n=334$ mother's milk samples) and infant stool samples (bottom row, $n=422$ stools). Solid lines represent the mean and shaded areas represent the 95% confidence interval. Models were adjusted for postnatal week, DNA extraction batch, infant sex assigned at birth, birth weight stratum, and feeding variables of interest. (C) 3-day total bacterial intake from mother's milk (top row) and total bacterial concentration in stools (bottom row) over time, stratified by feeding at the breast. P values are from linear mixed-effects models adjusted as described for panel (A) and (B). (D) 3-day milk intake and stool concentrations of commonly shared genera over time stratified by feeding at the breast; only taxa that exhibited significant differences in the concentration of infant stools before and after direct breastfeeding was initiated are shown. P

values are from linear mixed-effects models adjusted as described in panel (A). Abbreviations: zOTU, zero-radius operational taxonomic unit.



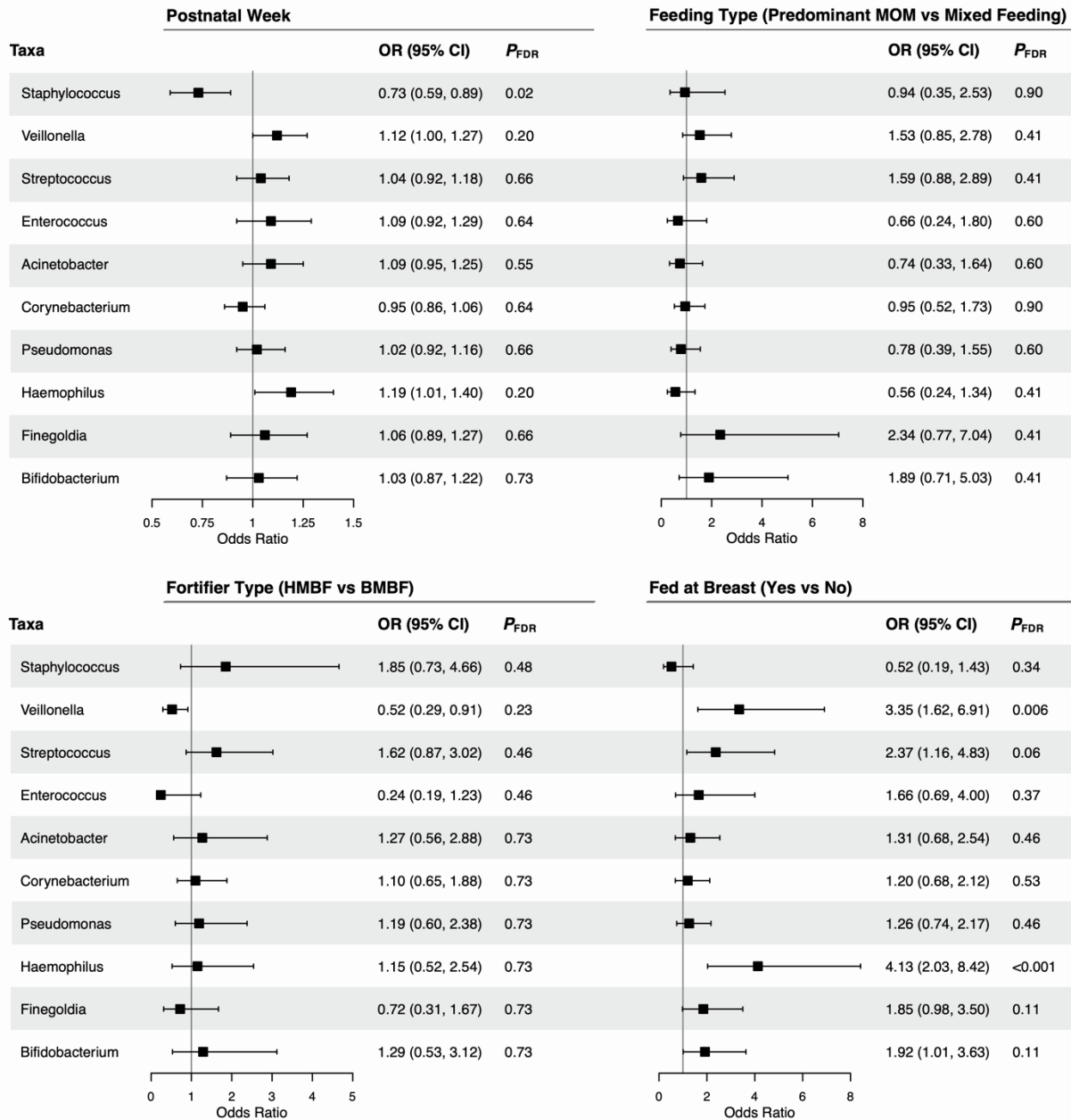
Supplemental Figure 4. Hierarchical clustering analysis of shared taxa in paired milk-stool samples related to Figure 4. (A) Heatmap showing the number of unique zOTUs shared,

mapped back to the genus level, for all paired milk-stool samples (n=422). Milk-stool sample pairs were clustered into 3 clusters by assessing the within-cluster sum of squares. **(B)** Count dot plots showing differences between the 3 clusters for the number of unique zOTUs shared, mapping back to the commonly shared genera. Commonly shared genera were shared in approximately 10% of all paired milk-stool samples. The y-axis represents the number of unique zOTUs shared between paired milk-stool samples that map back to each respective genera. Circle sizes are proportional to the percentage of samples, with the legend providing a reference range from 25% to 100%. Repeated measures Poisson regression models were used to determine differences between clusters. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(C)** Table showing the odds of being in a certain cluster based on postnatal week, feeding type, fortifier group and feeding at the breast. A multinomial logistic regression model with repeated measures was used and adjusted for postnatal week, feeding type, fortifier group, and feeding at the breast. Due to the size of the smallest cluster, a maximum of 5 covariates could be included in this model. Abbreviations: zOTU, zero-radius operational taxonomic unit; MOM, mother's milk; HMBF, human milk-based fortifier; BMBF, bovine milk-based fortifier.

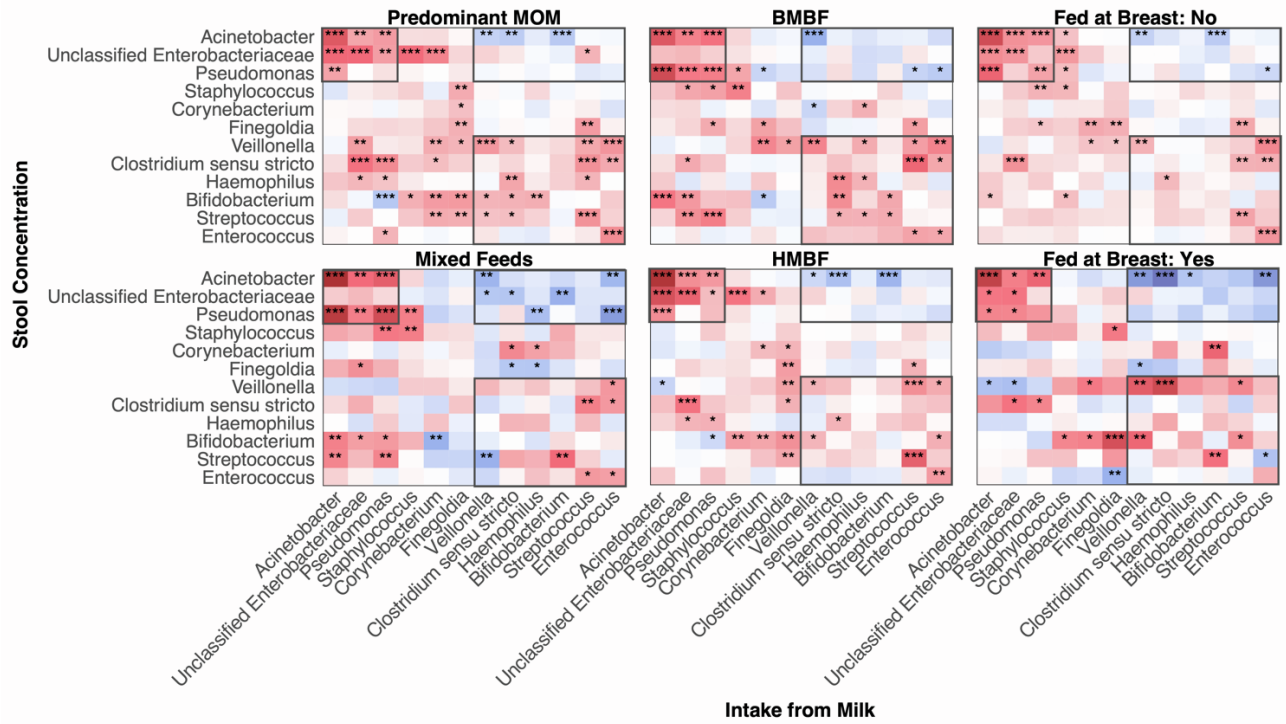


Supplemental Figure 5. Bacterial concentrations in mother's milk and VLBW infant milk bacterial intakes across initial hospitalization related to Figure 5 and Figure 6. (A) Total bacterial concentration of mother's milk (log copies/ml) and total milk bacterial intakes (n=3,508 days of milk bacterial intakes calculated for all infants) of infants (log copies) across initial hospitalization. Refer to STAR methods for a description of how daily bacterial intakes were calculated. Solid lines represent the mean and shaded areas represent the 95% confidence interval. A linear mixed-effects model was run to examine whether the total bacterial concentration of mother's milk changed over time; this model was adjusted for postpartum week of expression, DNA extraction batch, and gestational age. A linear mixed-effects model was also run to assess whether the total bacterial intake of infants changed over time. This model was adjusted for postnatal week, DNA extraction batch, infant sex assigned at birth, birth weight stratum, and feeding variables of interest (feeding type, fortifier group, and feeding at the breast).

(B) Comparison between the estimated total bacterial cells provided from mother's milk to daily doses provided by two probiotics currently used in North American neonatal intensive care units (Probiotic 1, BioGaia Protectis BABY - Probiotic Drops; Probiotic 2, Renew Life Flora Baby Probiotic Powder). Mean total bacterial cells in mother's milk were calculated by dividing the daily total bacterial intake (copies) of each infant by 5 copies (bacteria in mother's milk had an average of 5×10^6 copies), followed by taking the average. Information for Probiotic 1 and Probiotic 2 were retrieved from product monographs. **(C)** Concentration of the commonly shared bacterial genera in mother's milk (log copies/ml) across hospitalization. This figure depicts only the means as solid lines with 95% confidence intervals removed for visual clarity. **(D)** Concentrations of commonly shared bacterial genera in mother's milk (log copies/ml) and their daily intakes in infant (log copies) across hospitalization. P values for milk concentrations and infant milk bacterial intakes are from linear-mixed effects models adjusted as described for panel (A).



Supplemental Figure 6. Likelihood of a given taxa being observed in both milk-stool samples rather than milk alone depending on postnatal period and feeding practices related to Figure 5. Unadjusted repeated measures logistic regressions were used to examine how postnatal period and feeding practices influence the likelihood of observing a given genera in both milk-stool samples rather than milk alone. P-values from these analyses were FDR corrected for multiple comparisons. Models were not run for unclassified Enterobacteriaceae and *Clostridium sensu stricto* because almost all sample pairs had these taxa in both milk and stool samples. Abbreviations: OR, Odds ratio; CI, confidence interval; MOM, mother's milk; HMBF, human milk-based fortifier; BMBF, bovine milk-based fortifier.



Supplemental Figure 7. Correlations between milk bacterial intakes and concentrations in infant stools stratified by in-hospital feeding practices related to Figure 6. Heatmaps displaying spearman rank correlations between 3-day milk bacterial intakes (log copies) and concentrations in infant stools (log copies/g stool) stratified by in-hospital feeding practices (feeding type, fortifier type, feeding at the breast). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Black borders are used to highlight key findings across heatmaps. Abbreviations: MOM, mother’s milk; HMBF, human milk-based fortifier; BMBF, bovine milk-based fortifier.