

# Oligosaccharides and Microbiota in Human Milk Are Interrelated at 3 Months Postpartum in a Cohort of Women with a High Prevalence of Gestational Impaired Glucose Tolerance

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## ABSTRACT

**Background:** Human milk is a rich source of human milk oligosaccharides (HMOs) and bacteria. It is unclear how these components interact within the breast microenvironment.

**Objectives:** The objectives were first, to investigate the association between maternal characteristics and HMOs, and second, to assess the association between HMOs and microbial community composition and predicted function in milk from women with high rates of gestational glucose intolerance.

**Methods:** This was an exploratory analysis of a previously completed prospective cohort study (NCT01405547) where milk samples ( $n = 107$ ) were collected at 3 mo postpartum. Milk microbiota composition was analyzed by V4-16S ribosomal RNA gene sequencing and HMOs by rapid high-throughput HPLC. Data were stratified and analyzed by maternal secretor status phenotype and associations between HMOs and microbiota were determined using linear regression models ( $\alpha$ -diversity), Adonis (B-diversity), Poisson regression models (differential abundance), and general linear models (predicted microbial function).

**Results:** Prepregnancy BMI, race, and frequency of direct breastfeeding, but not gestational glucose intolerance, were found to be significantly associated with a number of HMOs among secretors and non-secretors. Fucosyllacto-N-hexaose was negatively associated with microbial richness (Chao1) among secretors [B-estimate (SE):  $-9.3 \times 10^2$  ( $3.4 \times 10^2$ );  $P = 0.0082$ ] and difucosyllacto-N-hexaose was negatively associated with microbiota diversity (Shannon index) [ $-1.7$  (0.78);  $P = 0.029$ ] among secretors. Lacto-N-neotetraose (LNnT) was associated with both microbial B-diversity (weighted UniFrac  $R^2 = 0.040$ ,  $P = 0.036$ ) and KEGG ortholog B-diversity (Bray-Curtis  $R^2 = 0.039$ ,  $P = 0.043$ ) in secretors. Additionally, difucosyllactose in secretors and disialyllacto-N-hexaose and LNnT in non-secretors were associated with enrichment of predicted microbial genes encoding for metabolism- and infection-related pathways ( $P$ -false discovery rate  $< 0.1$ ).

**Conclusions:** HMOs are associated with the microbial composition and predicted microbial functions in human milk at 3 mo postpartum. Further research is needed to investigate the role these relations play in maternal and infant health. *J Nutr* 2021;151:3431–3441.

**Keywords:** human milk microbiota, human milk oligosaccharides, gestational diabetes, gestational glucose intolerance, microbiome, maternal BMI, secretor status, breastfeeding, lactation, human milk

## Introduction

Human milk is home to a myriad of microorganisms, including bacteria. The bacteria in human milk, termed the human milk microbiota, are believed to play a role in the colonization of the infant's gastrointestinal (GI) microbiota and may contribute to both short- and long-term health outcomes (1, 2). In addition to these microbes, human milk is also a rich source of biologically active components, including growth factors, antibodies, oligosaccharides, hormones, cytokines, chemokines, and antimicrobial compounds (3). Previous studies have focused on potential external modifiers of the milk microbiota, such as maternal diet, antibiotic exposure, and mode of delivery (4–8); however, very few reports have examined how components within human milk may act as potential internal modifiers by interacting with the milk microbiota and vice versa.

One of the bioactive components of interest are human milk oligosaccharides (HMOs). HMOs are unconjugated, structurally diverse carbohydrates, which are the third most abundant solid component in human milk after lactose and lipids (9). HMO composition is dictated in large part by the expression of a single gene, fucosyltransferase-2 (*FUT2*), and mothers with an active allele of this gene are known as “secretors,” whereas those lacking an active copy (homozygous for the inactive allele) are “non-secretors” (9). Emerging literature has reported that maternal secretor status is associated with a number of parameters of human health, from maternal risk of infection and chronic disease to her child's GI microbiota composition (10, 11). Maternal characteristics including secretor status, parity, lactation period, race, breastfeeding exclusivity, geographical region, maternal BMI, and mode of delivery have been shown to be associated with specific HMOs (12–14). Only 1 group has examined the association between gestational glucose metabolism in metabolically healthy women and specific HMOs in milk, but none have investigated the association between gestational diabetes mellitus (GDM) and individual HMOs, along with the interplay with the milk microbiota (15–17). GDM has been found to be associated with altered activities of cellular glycosyltransferases, but its impact on individual HMOs is unknown (16). Due to the risk of maternal glucose intolerance impacting the HMOs present

in milk, further investigation into this association is necessary (16).

When consumed by an infant, HMOs can act as prebiotics, or fermentable fibers, and act as a carbon source for specific bacteria in the infant GI tract (9). Within the infant GI tract, HMOs have been shown to be utilized by *Bifidobacterium* and *Bacteroides* species, while in vitro, they have also been shown to interact with other bacteria, such as *Staphylococcus* (18–21). While it has been hypothesized that bacteria in human milk may interact with HMOs within the mammary gland in a similar manner as to how they interact within the GI tract, this has not been systematically investigated. Only 4 studies to date have examined the association between HMOs and milk microbiota in mature human milk (22–25). These 4 studies have shown varying associations between specific HMOs and particular taxa within the milk microbiota; however, 3 of these 4 studies were limited by small sample sizes ( $n = 16$ ,  $n = 25$ ,  $n = 30$ ,  $n = 393$ ) and homogeneous cohorts in terms of race and metabolically healthy women. Additionally, none of these 4 studies examined the associations between HMOs and predicted microbial functions of the milk microbiota (22–25).

To address these gaps in the literature, the objective of this current study was to determine the association between HMOs and the human milk microbiota (microbial community structure and predicted functions) in a racially diverse prospective cohort of women with high rates of gestational glucose intolerance. Additionally, we aimed to determine how maternal demographic, metabolic, and obstetrical clinical parameters were associated with HMO composition. To the best of our knowledge, this is the first study to assess the relation between the milk microbiota, HMOs, and gestational impaired glucose tolerance (IGT).

## Methods

### Study participants and design

This was an exploratory analysis of bio-banked human milk samples and maternal demographic, metabolic, and obstetrical health data from a previously completed prospective cohort study (ClinicalTrials.gov: NCT01405547) (26, 27). The study protocol and findings of associations between these maternal clinical variables and the microbial composition of human milk have been previously published (7, 8, 26). Pregnant participants ( $n = 216$ ) were recruited from Sinai Health (formerly, Mount Sinai Hospital) in Toronto, Canada, and in late pregnancy completed a 3-h 100-g oral-glucose-tolerance test (OGTT) between March 2009 and July 2010. For this present analysis, women were included if sufficient volumes of their mature milk sample from 3 mo postpartum was available for both HMO and milk microbiota analyses. The study protocol was approved by the Sinai Health Human Research Ethics Board and consent was obtained from all participants (26). A total of 117 women with term-born infants provided a mature milk sample at 3 mo postpartum, with 111 samples available for the present study (Supplemental Figure 1).

### Maternal demographic, metabolic, and obstetrical data collection

A standardized self-report questionnaire was given during the first study visit (occurring at 30 wk of gestation; 95% CI: 25, 33 wk) to gather demographic, anthropometric, and obstetrical data. Prepregnancy BMI ( $\text{kg}/\text{m}^2$ ) was calculated and categorized as normal weight (18.5–24.9), overweight (25.0–29.9), and obese ( $\geq 30$ ). Maternal race was categorized as White, Asian, or other (pooled Black, South Asian, other); the latter was combined due to low frequency of these races in the cohort. Mode of delivery was categorized as vaginal, scheduled cesarean delivery, unscheduled cesarean delivery). Glucose tolerance status was

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Supplemental Figures 1–4 and Supplemental Tables 1–8 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents available on <https://academic.oup.com/jn>.

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Abbreviations used: DFLac, difucosyllactose; DFLNH, difucosyllacto-N-hexaose; DFLNT, difucosyllacto-N-tetraose; DSLNH, disialyllacto-N-hexaose; DSLNT, disialyllacto-N-tetraose; FDR, false discovery rate; FDSLNH, fucodisialyllacto-N-hexaose; FLNH, fucosyllacto-N-hexaose; *FUT2*, fucosyltransferase-2; *FUT3*, fucosyltransferase-3; GDM, gestational diabetes mellitus; GI, gastrointestinal; HMO, human milk oligosaccharide; IGT, impaired glucose tolerance; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, KEGG ortholog; LNFP, lacto-N-fucopentaose; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; LSTb/c, sialyl-lacto-N-tetraose-b/c; OGTT, oral-glucose-tolerance test; OTU, operational taxonomic unit; rRNA, ribosomal RNA; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

categorized as GDM, gestational IGT, or normoglycemia, and was diagnosed based on the results of the OGTT (26).

### Collection of human milk samples

Mothers were asked to pump a complete breast expression of milk using a double electric breast pump (Medela, Inc.) with a sterile pumping kit at their 3-mo postpartum visit. No pumping or breastfeeding was to take place 2 h prior to the study visit. The collected mature whole-milk samples were placed in aliquots and stored at  $-80^{\circ}\text{C}$ . Information regarding frequency of direct breastfeeding per day and human milk exclusivity (yes/no) was also collected at this visit.

### HMO analysis

HPLC was used to characterize HMOs in human milk as previously described (28). Briefly, oligosaccharides were extracted by high-throughput solid-phase extraction over C18 and Carbohydrate microcolumns and fluorescently labeled with 2-aminobenzamide. Labeled oligosaccharides were analyzed by HPLC on an amide-80 column (Tosoh Bioscience) with online fluorescence detection. Absolute concentrations were calculated based on standard response curves for each of the annotated HMOs. Total concentration of HMOs was calculated as the sum of the annotated oligosaccharides (milligrams per milliliter). HMO-bound fucose and HMO-bound sialic acid were calculated on a molar basis (nanomoles per milliliter). HMO Simpson's diversity index was calculated based on relative abundances of all annotated HMOs. Maternal secretor status was determined by the high abundance (secretor) or near absence (non-secretor) of the HMO 2'-fucosyllactose (2'FL) in the respective milk samples. The 19 HMOs examined in these analyses included the following: 2'FL, 3-fucosyllactose (3FL), 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL), difucosyllactose (DFLac), difucosyllactose-N-hexaose (DFLNH), difucosyllactose-N-tetraose (DFLNT), disialyllactose-N-hexaose (DSLNH), disialyllactose-N-tetraose (DSLNT), fucodisialyllactose-N-hexaose (FD-SLNH), fucosyllactose-N-hexaose (FLNH), lacto-N-fucopentaose-III/III (LNFP III/III), lacto-N-hexaose (LNH), lacto-N-neotetraose (LNnT), lacto-N-tetraose (LNT), sialyl-lacto-N-tetraose-b/c (LSTb/c).

### DNA extraction, amplification, and sequencing of human milk microbiota

DNA from the human milk samples that had never been previously thawed was extracted using the NucleoSpin Food DNA isolation kit (Macherey-Nagel), as described previously (7, 8, 29). Briefly, the 515F and 806R primers were used during PCR (28 cycles) to amplify the V4 hypervariable region (30, 31). To confirm amplification quality, a negative control (sterile water), positive control (*Pseudomonas aeruginosa*), and mock community were also run alongside the DNA samples. An Illumina MiSeq instrument (MiSeq-V2-300 cycle chemistry) was used to sequence the quantified library to produce 150-bp paired end reads.

### Bioinformatic and data analyses of the milk microbiota

Raw paired end sequences have been deposited in the NCBI Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/sra>) under the BioProject accession number PRJNA516669. The UPARSE pipeline in USEARCH processed the sequencing reads. The Ribosomal Database Project (RDP) 16S gold database (USEARCH) was used to detect and discard chimeric sequences (32). Sequences were grouped together into operational taxonomic units (OTUs) at 97% similarity using de novo clustering and the RDP 16S gold database was used to assign taxonomy to these OTUs. A FastTree QIIME python script was run to produce a phylogenetic tree (33).

A specialized package in R (version 3.6.1; R Foundation for Statistical Computing) intended for microbiome analysis, Phyloseq (1.25.2), was used to analyze microbiota composition (34). Singleton and doubleton OTUs were removed, as were OTUs that mapped to cyanobacteria/chloroplasts. Samples with low read counts were removed ( $n = 4$ ) and data were rarefied to 20,000 reads per sample. Phyloseq was subsequently used to calculate and visualize relative

abundances of predominant taxa (phylum and genus levels),  $\alpha$ -diversity (Chao1 and Shannon indices), and  $\beta$ -diversity (weighted UniFrac distance, Bray-Curtis dissimilarity) of the milk microbiota.

### Statistical analyses

#### Associations between maternal characteristics, secretor status, and HMO composition.

Chi-square tests (R version 3.6.1) were used to examine associations between categorical maternal clinical data and secretor status (secretor vs. non-secretor), whereas Wilcoxon rank-sum tests were run to determine associations between continuous maternal clinical data and secretor status. The categorical maternal data of interest included prepregnancy BMI (healthy, overweight, obese), glucose tolerance status (normoglycemic, IGT, GDM), mode of delivery (vaginal, scheduled cesarean delivery, unscheduled cesarean delivery), race (White, Asian, other), and human milk exclusivity [exclusive vs. nonexclusive (mixed formula feeding)]. The continuous maternal data included frequency of direct breastfeeding per day ( $n/\text{day}$ ) and BMI ( $\text{kg}/\text{m}^2$ ). Wilcoxon rank-sum tests were also run to determine the relation between secretor status and total HMO concentration (milligrams per milliliter), HMO-bound sialic acid concentration (nanomoles per milliliter), and HMO-bound fucose concentration (nanomoles per milliliter). The significance level for these tests was set at  $P < 0.05$ .

After assessing relations between secretor status and HMO composition patterns, data were stratified by secretor status (secretor,  $n = 78$ ; non-secretor,  $n = 29$ ) and univariable linear regression models (SAS version 9.4; SAS Institute) were run to determine associations between maternal clinical data and each of the 19 HMOs within each secretor status stratum. Clinical data included the following: categorical prepregnancy BMI (healthy, overweight, obese), prepregnancy BMI as a continuous variable ( $\text{kg}/\text{m}^2$ ), glucose tolerance status (normoglycemic, IGT, GDM), mode of delivery (vaginal, scheduled cesarean delivery, unscheduled cesarean delivery), race (White, Asian, other), human milk exclusivity (vs. nonexclusive), and frequency of direct breastfeeding ( $n/\text{day}$ ). To avoid skewing the data in the analyses, *FUT2*-dependent HMOs (2'FL, LNFP I, DFLac, DFLNH), known to be almost exclusively found in secretor milk, were not examined within the non-secretor strata for the present, and all subsequent, analyses.

#### HMO composition and milk microbiota.

Using the total cohort for this present study ( $n = 107$ ), associations between secretor status and microbial  $\alpha$ -diversities (Chao1 and Shannon indices) were determined by multivariable linear regression models (PROC MIXED) in SAS version 9.4, while adjusting for microbial DNA extraction batch and PCR sequencing batch. The data were then stratified by secretor status and models were built to assess the association between the top 19 HMOs (milligrams per milliliter), HMO diversity (Simpson's index), total HMO concentration (milligrams per milliliter), HMO-bound sialic acid concentration (nanomoles per milliliter), and HMO-bound fucose concentration (nanomoles per milliliter) and  $\alpha$ -diversity (Chao1 and Shannon indices). All models were adjusted by microbial DNA extraction and PCR batch effects. Due to sample size constraints from stratifying on the basis of secretor status, we were not able to adjust for any additional maternal covariates of interest. The significance level for these analyses was set at  $P < 0.05$ .

$\beta$ -Diversities (weighted UniFrac distance, Bray-Curtis dissimilarity) of the human milk microbiota were calculated and visualized using principal coordinate analysis. All HMO concentrations were made categorical (Bisect 1 or 2) and run grouped as Bisect 1 (concentrations below the median) and Bisect 2 (concentrations above the median) for  $\beta$ -diversity analyses. Associations within the overall cohort ( $n = 107$ ) between secretor status and microbial  $\beta$ -diversities (weighted UniFrac distance, Bray-Curtis dissimilarity) were determined by Adonis (vegan package version 2.5-3 in R) (30) while adjusting for microbial DNA extraction batch and PCR sequencing batch. Data were then stratified by secretor status and associations between HMOs and  $\beta$ -diversity measures were ascertained using Adonis (vegan package version 2.5-3 in R), as previously described (7, 8, 30). All HMO models were first

**TABLE 1** Summary of maternal demographic, metabolic, and obstetric characteristics stratified by secretor status phenotype<sup>1</sup>

Baseline characteristics	Cohort	Secretors	Non-secretors
Race			
White	60 (56)	47 (60)	13 (45)
Asian	26 (24)	16 (21)	10 (34)
Other	21 (20)	15 (19)	6 (21)
Prepregnancy BMI, kg/m <sup>2</sup>			
Mean ± SD	24.4 ± 4.6	24.1 ± 4.5	25.4 ± 5.0
Healthy (18.5–24.9)	67 (63)	51 (65)	16 (55)
Overweight (25–29.9)	29 (27)	21 (27)	8 (28)
Obese (>30)	11 (10)	6 (8)	5 (17)
Gestational glucose tolerance status			
Normoglycemia	65 (61)	50 (64)	15 (52)
Impaired glucose tolerance	18 (17)	13 (17)	5 (17)
Gestational diabetes	24 (22)	15 (19)	9 (31)
Mode of delivery <sup>2</sup>			
Unscheduled cesarean delivery	27 (25)	13 (17)	14 (48)
Scheduled cesarean delivery	21 (20)	18 (23)	3 (10)
Vaginal	59 (55)	47 (60)	12 (41)
Human milk exclusivity			
Exclusive	54 (50.5)	39 (50)	15 (52)
Nonexclusive	53 (49.5)	39 (50)	14 (48)
Frequency of direct breastfeeding, <sup>3</sup> n/d	7.0 (5.5, 8.5)	7.0 (5.5, 8.0)	7.0 (4.9, 8.1)

<sup>1</sup>Values are *n* (%) unless otherwise specified. Maternal race was categorized as white, Asian, or other (pooled Black, South Asian, other); the latter was combined due to low frequency of these races. Cohort *n* = 107; secretors, *n* = 78; non-secretors, *n* = 29.

<sup>2</sup>A statistically significant association was observed between mode of delivery and secretor status phenotype following a chi-square test (*P* = 0.0032) of categorical data (vaginal vs. scheduled cesarean delivery vs. unscheduled cesarean delivery) (*P* < 0.05).

<sup>3</sup>Frequency of direct breastfeeding is expressed as median (IQR).

run unadjusted in the Adonis model and then run adjusted for microbial DNA extraction and PCR batch effects. The significance level was set at *P* < 0.05.

Relative abundances of the top 5 phyla and top 8 genera of milk microbiota were explored as these were the predominant taxa consistent in both secretor and non-secretor milk samples. Using the overall cohort (*n* = 107), Poisson regression models (PROC GENMOD) were built in SAS (version 9.4) to evaluate the association between secretor status as a covariate and the relative abundance of predominant microbial taxa as outcomes, while adjusting for microbial DNA extraction and PCR batch effects. Data were then stratified by secretor status and the associations between HMOs and predominant microbial taxa were assessed. All models were adjusted for microbial DNA extraction and PCR batch effects. To address overdispersion, the SEs of these models were adjusted using Pearson scaling. To account for multiple comparisons, the Benjamini-Yekutieli cut-point approach was used (35); based on 5 phyla (5 tests), significance was set at *P* ≤ 0.022 for the phylum models, and for 8 genera (8 tests), significance was set at *P* ≤ 0.018 for the genus models.

### HMO composition and predicted microbial functions

Microbial functions were inferred using Piphillin (<https://piphillin.secondgenome.com/>) (36). The Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.genome.jp/kegg/>) was used as a reference database to create a gene feature table from the 16S ribosomal RNA (rRNA) sequence data. A random subset of samples was used to accommodate the Piphillin analysis (*n* < 100 samples required), resulting in 93 samples available for statistical analyses. KEGG pathways not expressed in bacteria were subsequently removed. All KEGG analyses were run stratified by secretor status (secretor, *n* = 67; non-secretor, *n* = 26). Statistically significant associations between HMOs and KEGG pathways were assessed in 2 ways: first, examining the association between HMOs and B-diversity of predicted KEGG orthologs (Bray-Curtis dissimilarity) using Adonis models in R adjusted for microbial DNA extraction and PCR sequencing batch effects (*P* < 0.05); second, individual HMOs associated with differentially expressed predicted

functional KEGG pathways were assessed using MaAsLin2 in R [false discovery rate (FDR) adjusted, *P* < 0.1], while adjusting for microbial DNA extraction and PCR sequencing batch effects.

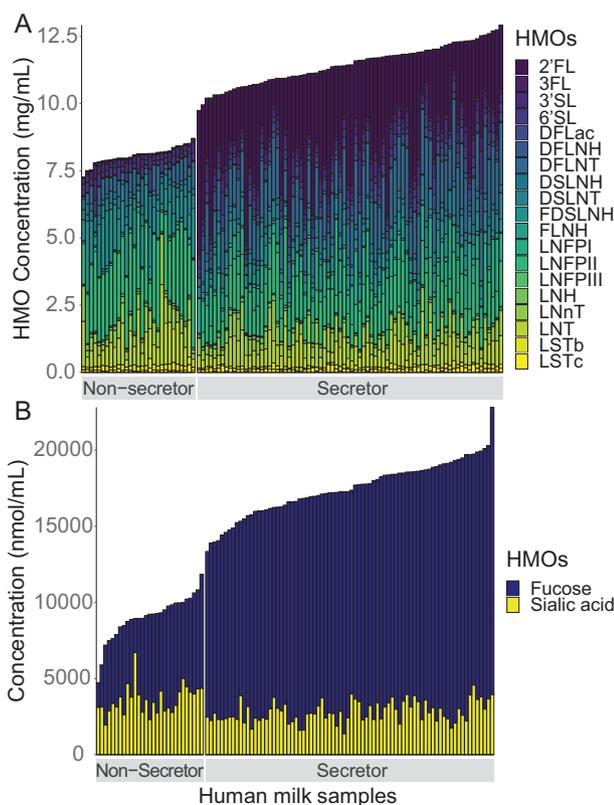
## Results

### Participant description

Human milk samples were expressed and collected at 3 ± 1 mo postpartum (mean ± SD). Maternal clinical data, including prepregnancy BMI, gestational glucose tolerance, mode of delivery, race, frequency of direct breastfeeding, and human milk exclusivity (vs. nonexclusive) are described in Table 1 according to secretor status. Mode of delivery was significantly associated with secretor status following chi-square analyses (*P* = 0.0032; Table 1); no other statistically significant differences were identified between maternal clinical data and secretor status.

### Secretor status is a predictor of HMO composition at 3 mo postpartum

Among secretor mothers, the top 3 most abundant HMOs by concentration (milligrams per milliliter) included 2'FL, DFLNT, and LNFPII (Figure 1; Supplemental Table 1). Among non-secretor mothers, the most abundant HMOs by concentration (milligrams per milliliter) included LNFPII, FDSLNH, and LNT. Secretor mothers expressed significantly higher concentrations (milligrams per milliliter) of total HMOs in their milk compared with non-secretor mothers [median (IQR): 11.4 (10.9, 12.0) vs. 8.13 (7.91, 8.22) mg/mL; *P* < 0.0001], mainly due to the higher quantities of 2'FL. Secretor mothers also expressed over double the concentration (nanomoles per milliliter) of HMO-bound fucose in their milk compared with non-secretors [ $1.5 \times 10^4$



**FIGURE 1** (A) HMO concentrations (mg/mL) and (B) HMO-bound sialic acid and fucose concentrations (nmol/mL) in mature human milk by secretor status phenotype (secretor,  $n = 78$ ; non-secretor,  $n = 29$ ). HMOs measured were 2'FL, 3FL, 3'SL, 6'SL, DFLac, DFLNH, DFLNT, DSLNH, DSLNT, FDSLNH, FLNH, LNFP I/II/III, LNH, LNnT, LNT, and LSTb/c. DFLac, difucosyllactose; DFLNH, difucosyllacto-N-hexaose; DFLNT, difucosyllacto-N-tetraose; DSLNH, disialyllacto-N-hexaose; DSLNT, disialyllacto-N-tetraose; FDSLNH, fucodisialyllacto-N-hexaose; FLNH, fucosyllacto-N-hexaose; HMO, human milk oligosaccharide; LNFP I/II/III, lacto-N-fucopentaose-I/II/III; LNH, lacto-N-hexaose; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; LSTb/c, sialyl-lacto-N-tetraose-b/c; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

( $1.4 \times 10^4$ ,  $1.6 \times 10^4$ ) vs.  $5.7 \times 10^3$  ( $4.8 \times 10^3$ ,  $6.5 \times 10^3$ );  $P < 0.0001$ ] (Figure 1; Supplemental Table 1).

### Maternal prepregnancy BMI, race, and frequency of direct breastfeeding are associated with the HMO profiles of human milk

Relations between maternal clinical data and HMO composition were examined separately for secretors and non-secretors. Within the secretor strata, prepregnancy BMI ( $\text{kg}/\text{m}^2$ ) was found to be positively associated with DFLac ( $P = 0.011$ ), while higher concentrations of DSLNT were observed in the milk of Asian mothers compared with other races ( $P = 0.045$ ) (Table 2). Negative dose-dependent relations were also observed for secretor mothers with frequency of direct breastfeeding ( $n/\text{day}$ ) for LNFP I ( $P = 0.0034$ ), LSTb ( $P = 0.043$ ), DSLNT ( $P = 0.0088$ ), and total HMO ( $P = 0.021$ ) concentrations.

Among non-secretors, race was a strong predictor of individual HMO concentrations (Table 2). Lower concentrations of LNT were observed among both White ( $P = 0.021$ ) and Asian ( $P = 0.032$ ) mothers; in addition, reduced concentrations of 6'SL, LNnT, and DSLNT were observed in White and Asian mothers compared with other races. Conversely, a

higher concentration of LNFP II was observed among White (vs. other) mothers ( $P = 0.0009$ ) and Asian (vs. other) mothers ( $P = 0.013$ ), along with a greater concentration of HMO-bound fucose compared with other races (Table 2). Additionally, dose-dependent relations were again observed with frequency of direct breastfeeding among the non-secretor strata; lower concentrations of 3FL ( $P = 0.049$ ) and increased concentrations of FDSLNH ( $P = 0.015$ ) were observed with each additional time an infant was fed directly at the breast.

### Individual HMOs are associated with microbial diversity in human milk

No statistically significant associations were found between secretor status as a variable and microbial  $\alpha$ -diversity as an outcome measure when examining the overall cohort ( $n = 107$ ) [Chao1 index, B-estimate (SE):  $-12.7$  (35.8),  $P = 0.72$ ; Shannon index:  $0.07$  (0.12),  $P = 0.55$ ]. However, within the secretor strata, 3'SL and FLNH were negatively associated with milk microbiota richness (Chao1 index;  $P = 0.049$  and  $P = 0.0082$ , respectively) and DFLNH was negatively associated with milk microbiota diversity (Shannon index;  $P = 0.029$ ) (Supplemental Figure 2; Supplemental Table 2). Among non-secretor samples, LNT was also negatively associated with milk microbiota richness ( $P = 0.025$ ).

No statistically significant associations were found between secretor status as a variable and microbial B-diversity as an outcome measure when examining the overall cohort ( $n = 107$ ; weighted UniFrac distance:  $R^2 = 0.018$ ,  $P = 0.11$ ; Bray-Curtis dissimilarity:  $R^2 = 0.013$ ,  $P = 0.19$ ). Among secretors, LNnT was associated with milk microbiota B-diversity (adjusted Weighted UniFrac distance:  $R^2 = 0.040$ ,  $P = 0.036$ ) (Supplemental Figure 3, Supplemental Table 3); however, among the non-secretor strata, no individual HMOs were associated with milk microbiota B-diversity (Supplemental Table 4).

### Individual HMOs are associated with microbial taxa in human milk

Milk microbiota composition stratified by secretor strata is outlined in Supplemental Table 5 and visualized in Supplemental Figure 4. Milk microbiota composition was found to be relatively consistent with minor statistically significant differences between the 2 secretor strata. When looking at the overall cohort, secretor status (secretor vs. non-secretor) was associated with a reduced incidence of Actinobacteria ( $P = 0.016$ ) (Supplemental Table 6); however, no associations were observed between secretor status and predominant genera within the overall cohort.

Within the secretor strata, DFLNH concentration was associated with a significantly reduced incidence of Proteobacteria ( $P = 0.013$ ) and an increased incidence of Firmicutes ( $P = 0.017$ ), while LNFP II ( $P = 0.001$ ), FDSLNH ( $P = 0.0064$ ), and HMO-bound sialic acid concentration (nanomoles per milliliter) ( $P = 0.019$ ) were all associated with a significantly reduced incidence of Bacteroidetes (Figure 2, Supplemental Table 6). Total HMO concentration was associated with a significantly increased incidence of *Staphylococcus* ( $P = 0.0054$ ) and *Corynebacterium* ( $P = 0.012$ ). LNFP I was associated with a significantly reduced incidence of *Rothia* ( $P = 0.0057$ ) (Figure 2, Supplemental Table 6).

Among the non-secretor strata, total HMO concentration was associated with a significantly reduced incidence of Actinobacteria ( $P = 0.0029$ ), Fusobacteria ( $P = 0.002$ ), and

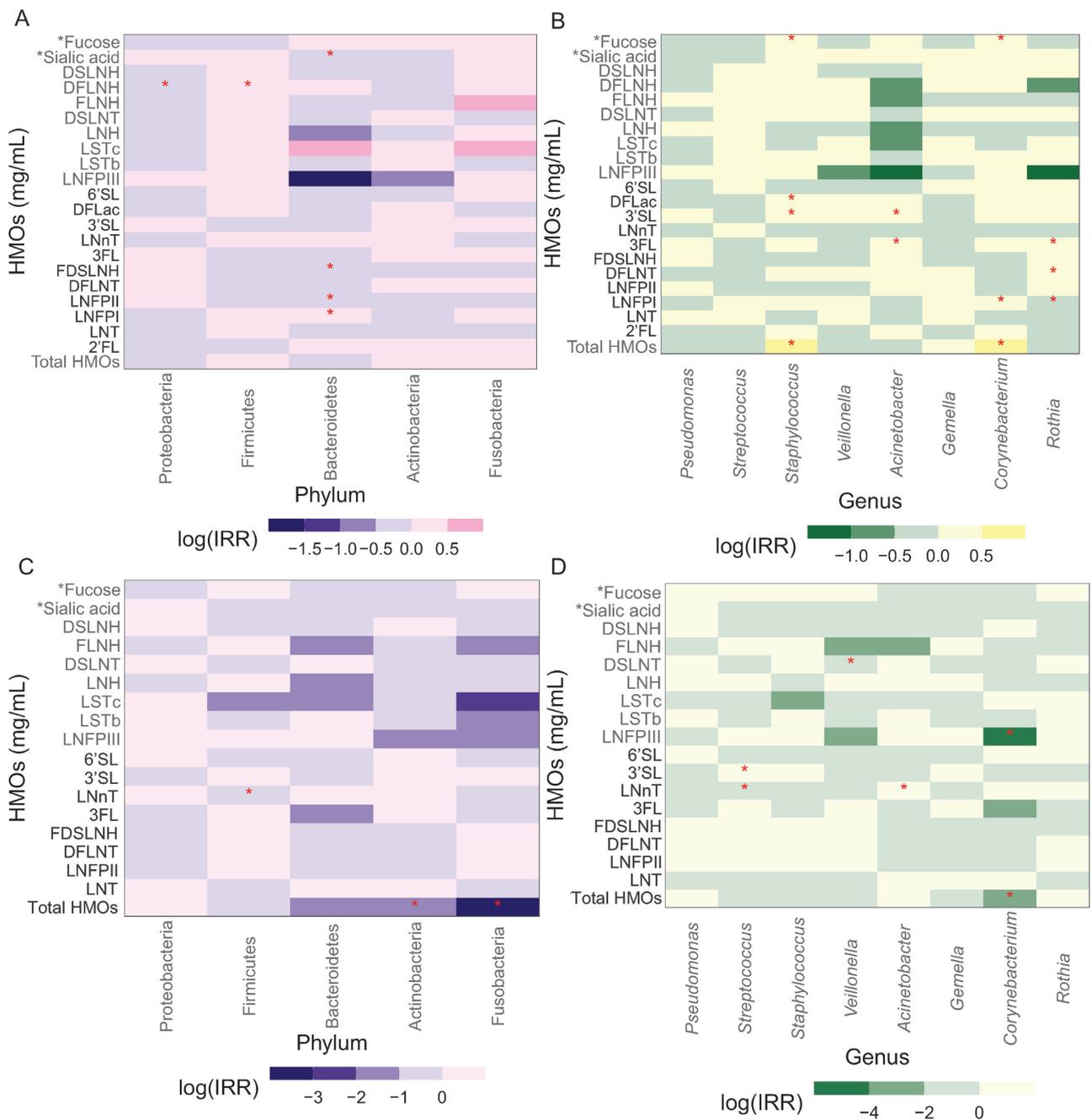
**TABLE 2** Significant associations between maternal characteristics and individual HMOs stratified by secretor status<sup>1</sup>

HMOs and maternal characteristics	B-Estimate (SE)	P
Secretors		
DFLac, mg/mL		
Prepregnancy BMI (kg/m <sup>2</sup> )	0.0099 (0.0038)	0.011
DSLNT, mg/mL		
Race		0.031 <sup>2</sup>
White vs. other	− 0.0076 (0.060)	0.90 <sup>3</sup>
Asian vs. other	0.15 (0.073)	0.045 <sup>3</sup>
LNFPI, mg/mL		
Frequency of direct breastfeeding (n/d)	− 0.072 (0.024)	0.0034
LSTb, mg/mL		
Frequency of direct breastfeeding (n/d)	− 0.0051 (0.0025)	0.043
DSLNT, mg/mL		
Frequency of direct breastfeeding (n/d)	− 0.025 (0.0091)	0.0088
Total HMOs, mg/mL		
Frequency of direct breastfeeding (n/d)	− 0.075 (0.032)	0.021
Non-secretors		
FLNH, mg/mL		
Prepregnancy BMI (kg/m <sup>2</sup> )	− 0.0030 (0.0012)	0.024
LNT, mg/mL		
Race		0.029 <sup>2</sup>
White vs. other	− 0.67 (0.27)	0.021 <sup>3</sup>
Asian vs. other	− 0.65 (0.29)	0.032 <sup>3</sup>
LNFPII, mg/mL		
Race		0.0022 <sup>2</sup>
White vs. other	1.04 (0.28)	0.0009 <sup>3</sup>
Asian vs. other	0.77 (0.29)	0.013 <sup>3</sup>
DFLNT, mg/mL		
Race		0.0047 <sup>2</sup>
White vs. other	0.43 (0.13)	0.0025 <sup>3</sup>
Asian vs. other	0.36 (0.13)	0.013 <sup>3</sup>
LNT, mg/mL		
Race		0.0024 <sup>2</sup>
White vs. other	− 0.46 (0.15)	0.0044 <sup>3</sup>
Asian vs. other	− 0.52 (0.15)	0.0024 <sup>3</sup>
6'SL, mg/mL		
Race		0.041 <sup>2</sup>
White vs. other	− 0.16 (0.072)	0.035 <sup>3</sup>
Asian vs. other	− 0.17 (0.075)	0.036 <sup>3</sup>
DSLNT, mg/mL		
Race		0.012 <sup>2</sup>
White vs. other	− 0.35 (0.12)	0.0079 <sup>3</sup>
Asian vs. other	− 0.32 (0.13)	0.020 <sup>3</sup>
HMO-bound fucose, nmol/mL		
Race		0.0002 <sup>2</sup>
White vs. other	$2.4 \times 10^3 (5.4 \times 10^2)$	0.0001 <sup>3</sup>
Asian vs. other	$2.1 \times 10^3 (5.7 \times 10^2)$	0.0008 <sup>3</sup>
FDSLNH, mg/mL		
Frequency of direct breastfeeding (n/d)	0.10 (0.040)	0.015
3FL, mg/mL		
Frequency of direct breastfeeding (n/d)	− 0.014 (0.0067)	0.049
3'SL, mg/mL		
Frequency of direct breastfeeding (n/d)	− 0.020 (0.0091)	0.042

<sup>1</sup> Secretors  $n = 78$ ; non-secretors,  $n = 29$ . P values less than 0.05 were significant. DFLac, difucosyllactose; DFLNH, difucosyllacto-N-hexaose; DFLNT, difucosyllacto-N-tetraose; DSLNT, disialyllacto-N-tetraose; FDSLNH, fucodisialyllacto-N-hexaose; FLNH, fucosyllacto-N-hexaose; HMO, human milk oligosaccharide; LNFPI, lacto-N-fucopentaose-I/II; LNH, lacto-N-hexaose; LNT, lacto-N-neotetraose; LNT, lacto-N-tetraose; LSTb, sialyl-lacto-N-tetraose-b; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

<sup>2</sup> Values are overall group-effect P value. Associations between maternal clinical data and HMOs stratified by secretor status were assessed via univariable linear regression models (PROC MIXED; SAS Institute). Maternal race was categorized as White, Asian, or other (pooled Black, South Asian, other); the latter was combined due to low frequency of these races.

<sup>3</sup> Values are the pairwise comparison P-value.



**FIGURE 2** Differential abundance of the top 5 most abundant phyla and top 8 most abundant genera in human milk microbiota according to HMO composition by secretor strata. (A) Phylum secretor strata, (B) genus secretor strata, (C) phylum non-secretor strata, (D) genus non-secretor strata. Secretor,  $n = 78$ ; non-secretor,  $n = 29$ . \*Sialic acid and \*Fucose denote that these HMO-bound molecules were run in the Poisson regression model as nmol/mL; all other HMOs were run as mg/mL, as indicated in the y-axis label. Heatmaps represent the results from multivariable Poisson regression models (PROC GENMOD; SAS Institute), adjusted for microbial DNA extraction and PCR sequencing batches with the top 5 phyla and top 8 genera as the outcome variables; the colored boxes refer to the incidence rate ratio on a log scale, while asterisks refer to statistically significant results.  $*P \leq 0.022$  for phylum,  $P \leq 0.018$  for genus. To account for multiple comparisons, the Benjamini-Yekutieli cut-point approach was used. DFLac, difucosyllactose; DFLNH, difucosyllacto-N-hexaose; DFLNT, difucosyllacto-N-tetraose; DSLNH, disialyllacto-N-hexaose; DSLNT, disialyllacto-N-tetraose; FDSLNH, fucodisialyllacto-N-hexaose; FLNH, fucosyllacto-N-hexaose; HMO, human milk oligosaccharide; LNFP I/II/III, lacto-N-fucopentaose-I/II/III; LNH, lacto-N-hexaose; LNNt, lacto-N-neotetraose; LNT, lacto-N-tetraose; LSTb/c, sialyl-lacto-N-tetraose-b/c; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

*Corynebacterium* ( $P < 0.0001$ ) (Figure 2, Supplemental Table 6). 3'SL was associated with a significantly increased incidence of *Streptococcus* ( $P = 0.006$ ), while LNNt was associated with a reduced incidence of *Streptococcus* ( $P = 0.010$ ). LNNt

was associated with a significantly increased incidence of *Acinetobacter* ( $P = 0.017$ ), while DSLNT and LNFP III were associated with reduced incidences of *Veillonella* ( $P = 0.010$ ) and *Corynebacterium* ( $P = 0.014$ ), respectively.

### Individual HMOs are associated with the predicted functional capabilities of microbiota in human milk

A number of changes were observed in the abundance of predicted KEGG pathways in relation to individual HMOs. Among the secretor strata, DFLac was significantly associated with an enrichment of predicted pathways encoding for metabolism and infection, including lipoic acid metabolism, bacterial invasion of epithelial cells, and *Staphylococcus aureus* infection ( $P\text{-FDR} < 0.1$ ) (Table 3). When examining the non-secretor strata, LNnT and DSLNH were associated with an enrichment in microbial pathways involved in the degradation of aromatic hydrocarbons and biosynthesis of antibiotics ( $P\text{-FDR} < 0.1$ ).

In addition to KEGG pathways, associations between HMOs and KEGG ortholog (KO) B-diversity (Bray-Curtis dissimilarity) were also explored. Similar to our findings with microbial B-diversity, LNnT was significantly associated with KO B-diversity among secretors ( $R^2 = 0.039$ ,  $P = 0.043$ ) (Supplemental Table 7). Within the non-secretor strata, DFLNT was also found to be significantly associated with KO B-diversity in adjusted models ( $R^2 = 0.14$ ,  $P = 0.007$ ) (Supplemental Table 8).

## Discussion

Maternal glucose intolerance during pregnancy increases a mother's risk of developing type 2 diabetes post-pregnancy as well as increases the risk of morbidities in her neonate, such as hypoglycemia (37–40). Little is known about the role of perturbed glucose metabolism on human milk components; however, our results are the first to show that 1) not GDM but prepregnancy BMI, race, frequency of direct breastfeeding ( $n/\text{day}$ ), and secretor status are associated with specific HMOs in mature human milk and 2) that HMOs are associated with microbial diversity, abundance of predominant taxa, and predicted microbial functions, regardless of glucose tolerance status.

Of the maternal characteristics we examined, race (White, Asian, other) appeared to be the most consistent determinant of HMO composition in our cohort, and this association has also been reported by others (13). Although there does appear to be a relation between race and *FUT2* status, associations appear to differ geographically, perhaps reflecting different single nucleotide polymorphisms in the *FUT2* gene (41–43). We also found maternal prepregnancy BMI to be positively associated with DFLac, an HMO that has previously been shown to be positively correlated with increased weight gain, weight velocity, and length gain in infants at 5 mo postpartum (44). Despite associations with prepregnancy BMI, it is interesting that no associations were observed between glucose tolerance status and HMO composition, given the potential for altered glucose tolerance to modify glycosylation patterns of human milk glycoproteins (16, 45). Interestingly, the 1 study to date ( $n = 24$ , milk collected 2 wk postpartum, United States) to examine associations between GDM and total HMO abundances also observed no differences in total HMO concentrations in milk from mothers with or without GDM; however, the authors did not examine individual HMOs (16). Recently, third-trimester glucose homeostasis in normoglycemic women ( $n = 136$ , United States) was found to be associated with secretor status, with non-secretors displaying significantly greater fasting insulin and HOMA-IR values than secretors; however, these values were still within a healthy range for pregnant women in their third trimester (17). The non-secretor group was also shown to

have a significantly higher BMI than secretors. It is unknown why these different associations were observed compared with our study findings, but it is important to emphasize that the women in the study by Saben et al. (17) were metabolically healthy and did not meet the diagnostic criteria for either gestational IGT or GDM. Finally, Azad et al. (13) also found associations between frequency of direct breastfeeding and HMO composition, hypothesizing that the regularity of milk removal and the volume of milk production both have the potential to alter the quantities and types of HMOs in human milk (13, 46, 47). This hypothesis continues to hold true for our study, as we also found a number of associations with this variable, regardless of secretor strata, indicating that the frequency of direct breastfeeding is a probable modulator of HMO composition.

Minor variations in microbiota composition were observed based on secretor status, a finding that is in line with previous investigations (48, 49); however, we did identify differences in milk microbiota diversity according to specific HMOs. FLNH was associated with milk microbiota richness as previously reported by Moossavi et al. (22), although our directionality was opposite. LNnT was also found to be associated with human milk microbiota B-diversity, although the magnitude was quite small; however, this finding may suggest that the microbial communities as a whole may differ depending on the HMO concentrations that a mother expresses in her milk. Differences in the B-diversity of the overall composition of predicted KEGG orthologs were also observed with LNnT. LNnT has been previously studied for its role in infant nutrition, especially as a supplemental addition to infant formula, as well as its inverse association with weight and height in children at 5 y of age (50, 51). For this reason, this observed association between LNnT and human milk microbiota B-diversity at both the taxonomic and predicted functional levels requires further investigation due to its potential implications in infant and child health.

Overall, the associations between HMOs and differentially abundant taxa in the milk microbiota were modest. Previous research has found total HMO content of human milk to be positively associated with *Staphylococcus* relative abundance (21). This supports our findings showing a positive association between overall HMO concentration and *Staphylococcus* genus relative abundance among secretor mothers. Intriguingly, a previous in vitro analysis reported that HMOs can stimulate the growth of *Staphylococcus* in a variety of media (21). Future directions validating this mechanism in vitro are warranted to determine whether HMOs are able to stimulate the growth of *Staphylococcus*.

No other study to our knowledge has investigated beyond taxa associations with HMOs and evaluated how predicted microbial functions differ depending on HMO composition. DFLac was found within the secretor strata to be associated with a number of predicted pathways encoding for bacterial invasion of epithelial cells and *S. aureus* infection. Fascinatingly, previous work examining secretor status and the risk of infection has shown a differing susceptibility for risk of a number of infections (i.e., mumps, measles, and norovirus), with either an increased or decreased risk based on secretor genotype and the illness in question (10, 52, 53). *S. aureus* is one of the causative agents of mastitis, and interestingly, previous work has shown an increased abundance of infection-related genes in the milk microbiota of women with a confirmed diagnosis of mastitis (54, 55). Although we were unable to assess if secretor status was associated with the risk of mastitis in our cohort,

**TABLE 3** Significant relations between individual HMOs and predicted microbial KEGG pathways in human milk stratified by secretor status<sup>1</sup>

HMO and KEGG pathway	Coefficient (SD) <sup>2</sup>	P <sup>3</sup>
Secretors		
DFLac		
Bacterial invasion of epithelial cells	$1.97 \times 10^{-5}$ ( $5.06 \times 10^{-6}$ )	0.056
<i>Staphylococcus aureus</i> infection	0.00041 (0.00012)	0.095
Lipoic acid metabolism	$4.66 \times 10^{-5}$ ( $1.18 \times 10^{-5}$ )	0.056
Pyruvate metabolism	0.00018 ( $4.71 \times 10^{-5}$ )	0.059
Secondary bile acid biosynthesis	$1.31 \times 10^{-5}$ ( $3.55 \times 10^{-6}$ )	0.065
Tuberculosis	$-3.31 \times 10^{-5}$ ( $8.32 \times 10^{-6}$ )	0.056
Biosynthesis of secondary metabolites	0.00056 (0.00016)	0.077
Biosynthesis of vancomycin group antibiotics	$-1.34 \times 10^{-5}$ ( $3.83 \times 10^{-6}$ )	0.077
Carbon fixation pathways in prokaryotes	0.00014 ( $3.89 \times 10^{-5}$ )	0.077
<i>Vibrio cholerae</i> infection	$4.52 \times 10^{-6}$ ( $1.31 \times 10^{-6}$ )	0.077
Carbon metabolism	0.00025 ( $7.73 \times 10^{-5}$ )	0.095
D-Arginine and D-ornithine metabolism	$1.43 \times 10^{-5}$ ( $4.29 \times 10^{-6}$ )	0.095
Non-secretors		
LNnT		
Toluene degradation	0.00017 ( $3.51 \times 10^{-5}$ )	0.015
Chlorocyclohexane and chlorobenzene degradation	0.00012 ( $3.06 \times 10^{-5}$ )	0.094
DSLNH		
Biosynthesis of enediyne antibiotics	$9.38 \times 10^{-7}$ ( $1.93 \times 10^{-7}$ )	0.019

<sup>1</sup>Associations between individual HMOs and secretor status stratified differentially expressed predicted functional KEGG pathways were assessed using MaAsLin2. All models were adjusted for microbial DNA extraction batch and PCR sequencing batch. Secretors,  $n = 67$ ; non-secretors,  $n = 26$ . DFLac, difucosyllactose; DSLNH, disialyllacto-N-hexaose, HMO, human milk oligosaccharide; KEGG, Kyoto Encyclopedia of Genes and Genomes; LNnT, lacto-N-neotetraose.

<sup>2</sup>Values are shown as coefficient (SD) from MaAsLin2 models that were adjusted for microbial DNA extraction and PCR batch effects.

<sup>3</sup>P values are false discovery rate (FDR) adjusted. A cutoff of  $P_{FDR} < 0.1$  was deemed statistically significant.

assessing this relation in future studies may be warranted given that the HMO glycosylation pattern may directly impact the development of mastitis.

The present study had many strengths, including a metabolically heterogeneous population, along with secretor phenotype stratification to help us better understand HMO composition. Our cohort was also enriched with mothers diagnosed with altered gestational glucose tolerance, allowing us to investigate the role (or lack thereof) this metabolic variant plays in HMO composition. We recognize there are also limitations to the present study. First, we were unable to assess for the *FUT3* genotype in our cohort, which would have provided further resolution into the genetic components of HMO composition by classifying mothers based on Lewis blood group, which is not as straightforward to determine based on HMO composition alone. Future research integrating the genetic heterozygosity and classifications of all 4 potential milk subgroups of *FUT2/FUT3* would provide a deeper understanding of how maternal demographic, metabolic, and obstetrical factors are associated with HMO composition, when accounting for known genetic factors. Second, although a large proportion of our cohort had gestational IGT (~40%), there is the potential that this sample size of women, especially when stratified by secretor status, was inadequate to detect some differences for some comparisons. Third, our use of 16S rRNA gene sequencing data to infer microbial functions using Piphillin may not reflect the true microbiota function and requires confirmation with shotgun metagenomic sequencing. In addition, our milk samples were stored at  $-80^{\circ}\text{C}$  for approximately 6 y before 16S rRNA gene sequencing was conducted; although the literature suggests this does not result in a substantial change to the microbiota or HMO compositions, this is a potential limitation of our study (56, 57). Last, our study was cross-sectional, meaning we

were not able to investigate longitudinal changes in HMOs over time, or how their relation with maternal clinical variables or microbiota may change over the postpartum period.

Our study is the first to analyze the interplay between HMOs and the human milk microbiota composition in a cohort of women with varying degrees of gestational glucose intolerance, a metabolic profile that deserves greater attention given its potential association with altered glycosylation patterns of HMOs. Our microbial predicted function results may point to the need to investigate risk of infections, such as mastitis, during lactation, and to determine whether secretor status may confer protection from risk of additional postpartum morbidities in both the mother and infant.

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