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What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically^{1,2}

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ABSTRACT

Background: Human milk is a complex fluid comprised of myriad substances, with one of the most abundant substances being a group of complex carbohydrates referred to as human milk oligosaccharides (HMOs). There has been some evidence that HMO profiles differ in populations, but few studies have rigorously explored this variability.

Objectives: We tested the hypothesis that HMO profiles differ in diverse populations of healthy women. Next, we examined relations between HMO and maternal anthropometric and reproductive indexes and indirectly examined whether differences were likely related to genetic or environmental variations.

Design: In this cross-sectional, observational study, milk was collected from a total of 410 healthy, breastfeeding women in 11 international cohorts and analyzed for HMOs by using high-performance liquid chromatography.

Results: There was an effect of the cohort ($P < 0.05$) on concentrations of almost all HMOs. For instance, the mean 3-fucosyllactose concentration was >4 times higher in milk collected in Sweden than in milk collected in rural Gambia (mean \pm SEM: 473 ± 55 compared with 103 ± 16 nmol/mL, respectively; $P < 0.05$), and disialyllacto-*N*-tetraose (DSLNT) concentrations ranged from 216 ± 14 nmol/mL (in Sweden) to 870 ± 68 nmol/mL (in rural Gambia) ($P < 0.05$). Maternal age, time postpartum, weight, and body mass index were all correlated with several HMOs, and multiple differences in HMOs [e.g., lacto-*N*-neotetraose and DSLNT] were shown between ethnically similar (and likely genetically similar) populations who were living in different locations, which suggests that the environment may play a role in regulating the synthesis of HMOs.

Conclusions: The results of this study support our hypothesis that normal HMO concentrations and profiles vary geographically, even in healthy women. Targeted genomic analyses are required to determine whether these differences are due at least in part to genetic variation. A careful examination of sociocultural, behavioral, and environmental factors is needed to determine their roles in this regard. This study

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Keywords: breastfeeding, carbohydrates, human milk, lactation, oligosaccharides

INTRODUCTION

Human milk oligosaccharides (HMOs)²³ are complex glycans that are highly abundant in human milk (1). Mature human milk

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²Supplemental Tables 1–15 and Supplemental Figures 1–3 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at <http://ajcn.nutrition.org>.

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²³Abbreviations used: DFLNT, difucosyllacto-*N*-tetraose; DSLNH, disialyllacto-*N*-hexaose; DSLNT, disialyllacto-*N*-tetraose; ET_R, rural Ethiopia; ET_U, urban Ethiopia; FDSLNT, fucodisialyllacto-*N*-hexaose; FLNH, fucosyllacto-*N*-hexaose; *FUT2*, galactoside 2- α -L-fucosyltransferase 2; *FUT3*, galactoside 3/4-L-fucosyltransferase; GB_R, rural Gambia; GB_U, urban Gambia; HMO, human milk oligosaccharide; LNFP, lacto-*N*-fucopentaose; LNT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LSTb, sialyl-lacto-*N*-tetraose b; LSTc, sialyl-lacto-*N*-tetraose c; NMF, nonnegative matrix factorization; US_C, United States–California (Hispanic); US_W, United States–Washington.

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contains from 5 to 20 g HMOs/L, which often exceed concentrations of protein (2–7); concentrations are even higher in colostrum (2, 8). In contrast, bovine milk contains lesser amounts of oligosaccharides, and their structures differ greatly from those in human milk (9–12). Decades of research have suggested that HMOs may be important for nourishing health-promoting bacteria in the breastfed infant's gastrointestinal tract (13–17), and emerging research suggests that HMOs act as antiadhesives, thereby reducing pathogen attachment and infectivity (18–22). HMOs also appear to act as antimicrobials that prevent pathogen proliferation (23) and as epithelial and immune cell modulators that affect host responses (24–26). HMOs may even be involved in brain development (27). As such, an understanding of HMO origins and functions, many of which are structure specific (28), as well as variations in intake by infants may lend key insights into the optimization of infant health and wellbeing during this critical phase of the life cycle.

Although there are substantial variations in HMO concentrations and profiles in women (29), very little is known about the basis of this variability aside from the activity of galactoside 2- α -L-fucosyltransferase 2 (*FUT2*) and galactoside 3/4-L-fucosyltransferase (*FUT3*) genes, which influence the presence or absence of α 1-2-fucosylated and α 1-3/4-fucosylated HMOs, respectively, as well as many other HMO structures (30, 31). Perhaps the most relevant study to date in which the interpopulation variation in HMO profiles was investigated was conducted by Erney et al. (32), who compared neutral oligosaccharides in milk that were collected from 435 women who were living in 10 countries. Although the authors recognized that, in some cases, small sample sizes within a country made comparisons and generalizations difficult, several findings were of significance. For instance, 2'-fucosyllactose was quantifiable in every milk sample that was collected in Mexico ($n = 156$) and Sweden ($n = 7$), but in only 46% of samples that were collected in the Philippines ($n = 22$). Sweden presented a particularly interesting picture with all samples containing 8 of 9 HMOs studied; none of the samples contained 3-fucosyllactose.

The primary objective of this study was to expand on the work of Erney et al. (32) to reexamine, with the use of more-advanced methods and rigorous sampling approaches, the hypothesis that HMO concentrations and profiles differ in diverse populations. As our secondary objective, we explored relations between selected maternal variables and HMO concentrations; in a subset of samples, we also indirectly examined whether differences in HMOs were more likely related to genetic or environmental factors.

METHODS

Experimental design, subjects, and ethics approvals

This investigation took place between May 2014 and April 2016 and was carried out as a cross-sectional, epidemiologic cohort study that involved multiple international sites. To be eligible for participation, women had to be breastfeeding or pumping ≥ 5 times/d (to ensure adequate milk production), have self-reported having healthy and nursing healthy infants, be ≥ 18 y of age, and be between 2 wk and 5 mo postpartum. Women did not need to be exclusively breastfeeding. Exclusion criteria included a current indication of a breast infection or breast pain that the woman did not consider normal for lactation, the maternal

use of antibiotics in the previous 30 d, or the nursing of a child with signs or symptoms of an acute illness in the previous 7 d or having taken antibiotics in the previous 30 d.

Our sample included 2 European (Spanish and Swedish), 1 South American (Peruvian), 2 North American, and 6 sub-Saharan African (rural and urban Ethiopian, rural and urban Gambian, Ghanaian, and Kenyan) populations and cohorts. Spanish subjects were recruited in Madrid, Zaragoza, Huesca, and Vizcaya with no additional requirements in terms of ethnicity. Swedish subjects were recruited in or near Helsingborg and had self-reported as Nordic (both parents and all grandparents were self-described as having only Swedish, Finnish, Danish, Icelandic, or Norwegian heritage). Peruvian subjects resided in a peri-urban area of Lima. North American subjects were recruited in Southeastern Washington and Northwestern Idaho [United States–Washington (US_W)] and Southern California [United States–California (Hispanic) (US_C)]; the former group was of unspecified ethnicity, and the latter group was self-identified as Hispanic. Both rural and urban Ethiopian subjects were self-identified as Sidama and were assumed to be genetically similar. Rural Ethiopian participants resided in the highlands of the Southern Nations, Nationalities, and Peoples' Region, whereas urban participants resided in Hawassa, which is also in the Southern Nations, Nationalities, and Peoples' Region. Rural and urban Gambian subjects had self-identified as Mandinka and were assumed to be genetically similar. Urban Gambian participants resided in the Bakau region, whereas the rural cohort stemmed from the West Kiang region. Ghanaian subjects were Krobo or Dangme and lived in southeastern Ghana. Kenyan subjects were recruited from the multiethnic city of Nakuru. Our goal was to obtain data and human milk samples from 40 women in each cohort, which was a number that was primarily chosen to fit within the available resources and time.

On enrollment, each woman completed several questionnaires including one questionnaire that ensured eligibility and another questionnaire that was related to general maternal and infant health and anthropometric measures. Ethics approvals were obtained for all procedures from each participating institution and with overarching approval from the Washington State University Institutional Review Board (13264). After being translated from English (when needed), informed, verbal, or written consent (depending on the locale and the subject's literacy level) was acquired from each participating woman.

Milk collection and preservation

With the use of gloved hands, research personnel or the mother (depending on cultural acceptability) cleaned the study breast (chosen by the subject) twice with the use of prepackaged castile soap towelettes (Professional Disposables International Inc.) and with a newly opened package each time. When deemed appropriate, this step was preceded by a general cleansing with water (and soap if needed) to remove noticeable soil. In the cohorts in Peru, Sweden, US_C, and US_W, ≤ 200 -mL (typically 40–60-mL) milk samples were collected into a single-use, sterile, polypropylene milk-collection container with a polybutylene terephthalate cap (Medela Inc.) with the use of an electric breast pump. In Spain, milk samples were collected via manual expression (with the use of a gloved hand) into single-use, sterile, polypropylene milk-collection containers with polybutylene

terephthalate caps (Medela Inc.). At the remaining sites, milk was manually expressed (with the use of a gloved hand) into sterile, polypropylene specimen containers with polyethylene caps (VWR International LLC.). When necessary to collect the desired volume or because the mother requested to switch breasts, milk was expressed from both breasts; when this occurred, the previously detailed methods were repeated with the other breast. To help control for known and unknown biases that might have been introduced through the use of different materials, all milk-collection supplies (e.g., gloves, wipes, and collection containers) were standardized and provided to study personnel at each site.

In all sites except rural Ethiopia (ET_R) and Peru, milk was immediately placed in ice or in a cold box (4°C) where it remained until it was partitioned, within 1 h, into aliquots. Milk was frozen (−20°C), shipped on dry ice (if necessary; −78.5°C), and again frozen (−20°C) until it was analyzed. In Peru, milk was immediately partitioned into aliquots and frozen (−20°C), shipped on dry ice, and again frozen (−20°C) until it was analyzed. Because the ET_R site did not have consistent access to electricity, milk that was collected in this cohort was preserved with a milk-preservation solution (one-to-one ratio) that was contained in a Milk DNA Preservation and Isolation Kit (Norgen Biotek Corp.); this preserved milk was stored at an ambient temperature for ≤1 wk after which it was transferred to a freezer (−20°C), shipped on dry ice, and again frozen (−20°C) until it was analyzed. Unpublished data from our research group confirmed that the use of this preservation method did not influence the HMO analysis (L Bode, MK McGuire, June 2016).

Oligosaccharide analysis

HPLC was used to characterize HMO in breast milk as previously described (33). Briefly, human milk (20 μL) was spiked with raffinose (a non-HMO carbohydrate) as an internal standard to allow for absolute quantification. Oligosaccharides were extracted with the use of high-throughput solid-phase extraction over C18 and carbograph microcolumns (Thermo Scientific HyperSep) and fluorescently labeled with 2-aminobenzamide. Labeled oligosaccharides were analyzed with the use of HPLC on an amide-80 column with an ammonium formate–acetonitrile buffer system at a concentration of 50-mmol/L. Separation was performed at 25°C and was monitored with the use of a fluorescence detector at a 360-nm excitation and 425-nm emission. The peak annotation was based on standard retention times and a mass spectrometric analysis with the use of a duo ion-trap mass spectrometer (Thermo LCQ) that was equipped with a nano-electrospray ionization source. Absolute concentrations were calculated on the basis of standard response curves for each of the annotated HMOs. The following 19 HMOs were identified and quantified: 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, difucosyllactose, difucosyllacto-*N*-hexaose, difucosyllacto-*N*-tetraose (DFLNT), disialyllacto-*N*-hexaose (DSLNH), disialyllacto-*N*-tetraose (DSLNT), fucodisialyllacto-*N*-hexaose (FDLSNH), fucosyllacto-*N*-hexaose (FLNH), lacto-*N*-fucopentaose (LNFP I, LNFP II, LNFP III, lacto-*N*-hexaose, lacto-*N*-neotetraose (LNnT), lacto-*N*-tetraose (LNT), sialyl-lacto-*N*-tetraose b (LSTb), and sialyl-lacto-*N*-tetraose c (LSTc). HMOs were also grouped according to common structural elements. Secretor milk was defined as having a 2'-fucosyllactose concentration that was greater than a

natural, very low break in the data. The total concentration of HMOs was calculated as the sum of the annotated oligosaccharides. The proportion of each HMO that made up the total HMO concentration was also calculated. HMO concentrations were analyzed with the use of both a molar-based unit of measure (nanomoles per milliliter) and a weight-based unit of measure (micrograms per milliliter). However, in the interest of space and coherence, only the molar data are presented and discussed in this article. Data that were analyzed on a weight basis (micrograms per milliliter) are shown in **Supplemental Tables 1–9**.

Statistical analysis

All exploratory and descriptive statistical analyses were performed with the use of R software (version 3.3.2; R Foundation for Statistical Computing) (34). To correct for nonnormal (right-skewness) distributions, HMO quantities were log transformed before analyses. The effect of the cohort on total, individual, and grouped HMO concentrations was tested via 1-factor ANOVA procedures with the use of the AOV option in the stats package in R software. Multiple comparisons were carried out with the use of Bonferroni adjustment [LSD.test in the agricolae package (35)] to assess differences in populations. Differences in proportions of each cohort that were characterized as being secretors were tested with the use of a chi-square post hoc procedure in the NCStats package (36) with Benjamini and Hochberg false-discovery-rate corrections (37). α -Diversity metrics including richness, the Shannon diversity index, the inverse Simpson index, Shannon evenness, Simpson evenness, and Pielou evenness were computed (38). The AOV procedure was also used to examine the effect of the cohort on richness, evenness, and diversity indexes and to examine the effect of the cohort on selected metadata [maternal age, parity, time postpartum, and BMI (in kg/m²)].

To visualize and characterize associations between individual HMO or HMO profiles and selected metadata, heat maps of Spearman-rank correlation coefficients were constructed with the use of the corrplot package (39). To help control for the many correlations in which we were interested while also wanting to fully explore the many relations that might have been of interest in this exploratory component of our data analysis, associations were deemed significant with the assumption of $\alpha = 0.01$.

Multivariate analyses to explore patterns and similarities in complex HMO profiles were followed and included nonmetric multidimensional scaling analyses with the use of a Bray-Curtis dissimilarity matrix [metaMDS procedure in the vegan package (38) and ggplot2 package (40) and a principle components analysis princomp procedure in the stats base package of R software]. Within these analyses, potential groupings of HMO profiles by cohort, continent and ethnicity, BMI, time postpartum, parity, and maternal age were examined. In this evaluation, continuous variables were categorized as follows: BMI (<18.5, 18.5–24.9, and ≥25); time postpartum (quartiles: 20–46, 47–63, 64–78, and 79–161 d); parity (1, 2, and ≥3 children); and maternal age (quartiles: 18–22, 23–27, 28–32, and 33–46 y). Nonnegative matrix factorization (NMF) was also used to discern potential patterns in the HMO profile data (41). In this set of analyses, data were processed with the use of the Brunet method (42), and 6 basis components were retained on the basis of the rank estimate that was determined from the same package.

Heat maps of the NMF feature scores were created with the heatmap.2 procedure in the gplots package (43) to look for patterns within the data structure (distinct from the correlation maps and shown in **Supplemental Figures 1 and 2**).

RESULTS

Description of participating women

A total of 413 women were enrolled; 41, 40, 40, 40, 42, 42, 43, 41, 24, 41, and 19 women were from ET_R, urban Ethiopia (ET_U), rural Gambia (GB_R), urban Gambia (GB_U), Ghana, Kenya, Peru, Spain, Sweden, US_W, and US_C, respectively. Milk samples from all of these women, except for 2 women in Ghana and 1 woman in ET_R, were successfully obtained and analyzed. Consequently, data from a total of 410 women were included in our analysis. Basic anthropometric and demographic information of these participants is shown in **Table 1**. Several of these classifications differed in the cohorts. For example, women in ET_U were younger than all other groups except for their counterparts in ET_R, GB_R, Kenya, and Peru. Parity in women in ET_R and GB_R was higher than that of women in ET_U, Spain, Sweden, and US_W. Body weight also varied greatly in the cohorts whereby women in Peru, Sweden, US_C, US_W, Spain, Ghana, and GB_U were relatively heavier and had higher BMI, and women in ET and GB_R were lighter and had lower BMI. Note that groups in ET_R than ET_U as well as cohorts in GB_R than GB_U were, for the most part, similar in terms of these variables although parity was higher in women in ET_R than in ET_U; there were no differences in these factors between the 2 US cohorts.

Effects of cohort on individual HMO concentrations and HMO groupings

Mean values for individual and total HMO concentrations for each cohort are provided in **Table 2** (all women) and visually depicted in **Figure 1** (all women, secretors, and nonsecretors). Relative abundances of each HMO in all women, secretors, and nonsecretors in each cohort are shown in **Figure 2**. There was an effect of the cohort on the total HMO concentration and the concentrations of all the HMO types except for LNFP I. For instance, DSLNT concentrations ranged from a low of 216 ± 14 nmol/mL in Sweden to a high of 870 ± 68 nmol/mL in GB_R (*P* < 0.05). LNFP III was significantly higher in milk that was produced by Swedish women than by all other cohorts (*P* < 0.05) except for women in the US_C; and LSTb was lower (*P* < 0.05) in milk that was produced by women in Peru and the US_C than by all other cohorts. In addition, although they did not reach significance with the use of Bonferroni correction for multiple comparisons, 2'-fucosyllactose concentrations were 4–5 times higher in samples that were collected in the US_C (7043 ± 858 nmol/L) and Peru (6528 ± 435 nmol/L) than in those that were collected in Ghana (1428 ± 207 nmol/mL).

Several differences were also shown between rural and urban sites in Ethiopia and between rural and urban sites in The Gambia despite the fact that, within each country, the populations studied were expected to have been genetically related. For instance, in The Gambia, the LNnT concentration of milk that was produced

TABLE 1
Characteristics of women (n = 410) participating in the study and effects of population¹

Variable	Ethiopia				Gambia				United States			
	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Spain (n = 41)	Sweden (n = 24)	Washington (n = 41)	California (n = 19)
Age, ² y	24.6 ± 0.8 ^{c,d}	21.7 ± 0.5 ^d	26.9 ± 1.2 ^{b,d}	27.0 ± 0.8 ^{b,c}	28.9 ± 0.9 ^{a,c}	28.9 ± 0.9 ^{a,c}	25.4 ± 0.8 ^{b,d}	26.7 ± 1.0 ^{b,d}	34.3 ± 0.6 ^a	30.9 ± 1.1 ^{a,b}	29.0 ± 0.8 ^{a,c}	29.0 ± 1.1 ^{a,c}
Parity, n	3.6 ± 0.3 ^a	1.7 ± 0.2 ^{c,d}	4.2 ± 0.5 ^a	3.3 ± 0.3 ^{a,b}	2.3 ± 0.2 ^{a,c}	2.3 ± 0.2 ^{a,c}	2.5 ± 0.2 ^{a,c}	2.0 ± 0.1 ^{a,d}	1.3 ± 0.1 ^d	1.6 ± 0.2 ^{b,d}	1.8 ± 0.2 ^{b,d}	1.8 ± 0.2 ^{a,d}
Time postpartum, ³ d	71 ± 5	59 ± 2	65 ± 3	62 ± 3	58 ± 3	58 ± 3	73 ± 4	60 ± 3	70 ± 4	49 ± 4	68 ± 3	62 ± 5
Weight, ⁴ kg	51.0 ± 1.3 ^c	55.6 ± 1.2 ^{b,c}	56.3 ± 1.3 ^{b,c}	64.2 ± 1.7 ^{a,b}	63.3 ± 1.7 ^{a,b}	63.3 ± 1.7 ^{a,b}	60.1 ± 1.5 ^{b,c}	65.5 ± 2.0 ^{b,b}	64.3 ± 1.5 ^{a,b}	73.6 ± 2.6 ^a	75.0 ± 2.4 ^a	76.3 ± 3.0 ^a
Height, ⁵ cm	155 ± 1 ^{d,e}	159 ± 1 ^{c,d}	162 ± 1 ^{a,c}	167 ± 1 ^a	159 ± 1 ^{b,d}	159 ± 1 ^{c,d}	159 ± 1 ^{b,d}	153 ± 1 ^e	165 ± 1 ^{a,b}	169 ± 1 ^a	167 ± 1 ^a	162 ± 1 ^{a,d}
BMI, ⁵ kg/m ²	21.3 ± 0.4 ^d	22.1 ± 0.5 ^{c,d}	21.4 ± 0.5 ^{c,d}	23.0 ± 0.6 ^{b,d}	25.0 ± 0.6 ^{a,d}	25.0 ± 0.6 ^{a,d}	23.6 ± 0.6 ^{b,d}	28.1 ± 0.8 ^a	23.5 ± 0.6 ^{b,d}	25.8 ± 1.0 ^{a,c}	26.8 ± 0.8 ^{a,b}	29.1 ± 1.1 ^a

¹ All values are means ± SEMs. Values in a row that do not share a common superscript letter differed with the use of Bonferroni-correction procedures for multiple comparisons, *P* < 0.05. All statistical inferences were based on log-transformed data.

² Because of missing data (although we are confident that all women were of the appropriate age range), n = 38 and 39 for rural and urban Gambia cohorts, respectively.

³ Because of missing data, n = 39 for the rural Gambia cohort.

⁴ Because of missing data, n = 39, 41, and 37 in rural Gambia, Kenya, and United States–Washington cohorts, respectively.

⁵ Because of missing data, n = 40 in the Kenya cohort.

TABLE 2
Variation in total and individual HMOs in 410 healthy women living in selected locations around the world¹

HMO	Ethiopia				Gambia				United States				
	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Ghana (n = 40)	Kenya (n = 42)	Peru (n = 43)	Spain (n = 41)	Sweden (n = 24)	Washington (n = 41)	California (n = 19)
2'FL, nmol/mL	2264 ± 370	2853 ± 369	2950 ± 455	4220 ± 530	1438 ± 207	3380 ± 422	6528 ± 435	3906 ± 464	5661 ± 728	4159 ± 531	7043 ± 858		
3FL, nmol/mL	189 ± 22 ^{ab,c}	184 ± 42 ^{b,c}	103 ± 16 ^c	162 ± 21 ^{b,c}	192 ± 33 ^{b,c}	195 ± 28 ^{b,c}	209 ± 32 ^{ab,c}	206 ± 25 ^{ab,c}	473 ± 55 ^a	122 ± 14 ^{b,c}	388 ± 47 ^{ab}		
LNNt, nmol/mL	838 ± 60 ^{ab,c}	927 ± 67 ^{ab,c}	1423 ± 117 ^a	781 ± 61 ^{b,c}	866 ± 70 ^{ab,c}	1073 ± 103 ^{ab}	588 ± 56 ^c	548 ± 45 ^c	854 ± 74 ^{a,b,c}	776 ± 42 ^{b,c}	793 ± 82 ^{ab,c}		
3'SL, nmol/mL	413 ± 33	526 ± 43	465 ± 36	505 ± 45	618 ± 56	528 ± 44	528 ± 51	607 ± 42	467 ± 65	562 ± 40	473 ± 55		
DFLac, nmol/mL	179 ± 27	290 ± 33	338 ± 59	355 ± 42	393 ± 65	338 ± 38	470 ± 49	307 ± 40	275 ± 40	270 ± 38	374 ± 57		
6'SL, nmol/mL	374 ± 63 ^{c,d}	545 ± 45 ^{ab}	462 ± 35 ^{ab,c}	585 ± 61 ^{ab}	890 ± 88 ^a	435 ± 34 ^{b,c}	636 ± 63 ^{ab}	504 ± 40 ^{ab,c}	200 ± 24 ^d	402 ± 34 ^{b,c,d}	294 ± 49 ^{c,d}		
LNT, nmol/mL	1304 ± 131 ^{b,c}	1408 ± 113 ^{ab}	2265 ± 222 ^a	1576 ± 143 ^a	1882 ± 209 ^a	1632 ± 158 ^a	953 ± 139 ^b	1570 ± 119 ^a	2132 ± 210 ^a	1135 ± 91 ^{ab}	1438 ± 198 ^{ab}		
LNFP I, nmol/mL	904 ± 167	1276 ± 205	1153 ± 208	1343 ± 193	1292 ± 224	921 ± 158	1116 ± 112	1056 ± 167	1395 ± 220	850 ± 144	1368 ± 143		
LNFP II, nmol/mL	1618 ± 165 ^b	1713 ± 151 ^{ab}	1925 ± 201 ^{ab}	1551 ± 190 ^{ab}	1133 ± 89 ^{ab}	1667 ± 167 ^b	1115 ± 105 ^{ab}	2001 ± 227 ^{ab}	1893 ± 205 ^{ab}	2125 ± 216 ^{ab}	1240 ± 154 ^d		
LNFP III, nmol/mL	44 ± 8 ^{b,c}	24 ± 3 ^c	40 ± 7 ^{b,c}	30 ± 4 ^{b,c}	47 ± 7 ^{b,c}	46 ± 10 ^{b,c}	53 ± 8 ^{b,c}	32 ± 4 ^{b,c}	269 ± 22 ^a	25 ± 5 ^c	76 ± 10 ^{ab}		
LSTb, nmol/mL	86 ± 7 ^a	79 ± 7 ^a	132 ± 10 ^a	96 ± 12 ^a	115 ± 10 ^a	86 ± 1 ^a	41 ± 5 ^b	105 ± 15 ^a	140 ± 15 ^e	82 ± 7 ^a	79 ± 14 ^{ab}		
LSTc, nmol/mL	101 ± 19 ^c	169 ± 15 ^{ab}	159 ± 13 ^{ab}	146 ± 20 ^{ab,c}	246 ± 25 ^a	158 ± 22 ^{ab,c}	182 ± 17 ^{ab}	72 ± 7 ^c	92 ± 16 ^{b,c}	112 ± 12 ^{b,c}	103 ± 12 ^{b,c}		
DFLNT, nmol/mL	758 ± 127	1057 ± 147	913 ± 135	1032 ± 139	1105 ± 188	1082 ± 134	1076 ± 95	1406 ± 120	1388 ± 147	1246 ± 105	1418 ± 197		
LNH, nmol/mL	68 ± 8	86 ± 9	112 ± 17	92 ± 15	109 ± 13	92 ± 10	107 ± 10	58 ± 4	113 ± 12	93 ± 7	39 ± 5		
DSLNT, nmol/mL	310 ± 30 ^{c,d}	553 ± 42 ^{ab}	870 ± 68 ^a	477 ± 45 ^{b,c}	561 ± 57 ^{ab}	444 ± 47 ^{b,c,d}	274 ± 33 ^d	357 ± 31 ^{b,c,d}	216 ± 14 ^d	443 ± 28 ^{b,c,d}	275 ± 14 ^{b,c,d}		
FLNH, nmol/mL	5 ± 1 ^c	27 ± 5 ^b	30 ± 5 ^{ab}	35 ± 5 ^{ab}	32 ± 7 ^b	33 ± 6 ^b	50 ± 7 ^{b,c}	83 ± 9 ^a	10 ± 3 ^{b,c}	73 ± 7 ^a	7 ± 1 ^{b,c}		
DFLNH, nmol/mL	84 ± 15 ^c	94 ± 16 ^c	87 ± 13 ^c	123 ± 18 ^{ab,c}	81 ± 11 ^{b,c}	64 ± 10 ^c	195 ± 21 ^{ab}	115 ± 17 ^{ab,c}	285 ± 32 ^a	98 ± 12 ^{b,c}	93 ± 11 ^{ab,c}		
FDSLNT, nmol/mL	158 ± 30 ^{ab}	240 ± 26 ^{ab}	197 ± 24 ^{ab}	199 ± 33 ^{ab}	182 ± 25 ^{ab}	204 ± 25 ^{ab}	245 ± 33 ^{ab}	314 ± 38 ^a	83 ± 15 ^b	370 ± 48 ^a	70 ± 9 ^b		
DSLNT, nmol/mL	50 ± 7 ^b	136 ± 12 ^a	109 ± 12 ^{ab}	129 ± 20 ^{ab}	126 ± 18 ^{ab}	101 ± 11 ^{ab}	108 ± 16 ^{ab}	103 ± 11 ^{ab}	55 ± 11 ^{ab}	93 ± 12 ^{ab}	33 ± 6 ^b		
Total, nmol/mL	9748 ± 626 ^c	12,187 ± 519 ^{ab,c}	13,732 ± 497 ^{ab}	13,435 ± 613 ^{ab}	11,307 ± 631 ^{b,c}	12,480 ± 628 ^{ab,c}	14,474 ± 539 ^{ab}	13,349 ± 645 ^{ab}	15,998 ± 768 ^a	13,035 ± 502 ^{ab}	15,606 ± 727 ^{ab}		

¹ All values are means ± SEMs. Values in a row that do not share a common superscript letter differed with the use of Bonferroni-correction procedures for multiple comparisons, $P < 0.05$. All statistical inferences were based on log-transformed data. DFLac, difucosyllactose; DFLNH, difucosyllactose-N-tetraose; DFLNT, difucosyllactose-N-hexaose; DFLNH, difucosyllactose-N-hexaose; DSLNH, disialyllactose-N-tetraose; FDSLNT, fucosialyllactose-N-tetraose; FLNH, fucosyllactose-N-hexaose; FLNT, fucosyllactose-N-tetraose; HMO, human milk oligosaccharide; LNFP, lacto-N-fucopentaose; LNH, lacto-N-hexaose; LNNt, lacto-N-neotetraose; LNT, lacto-N-tetraose; LSTb, sialyllactose-N-tetraose b; LSTc, sialyllactose-N-tetraose c; 2'FL, 2'-fucosyllactose; 3'FL, 3'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

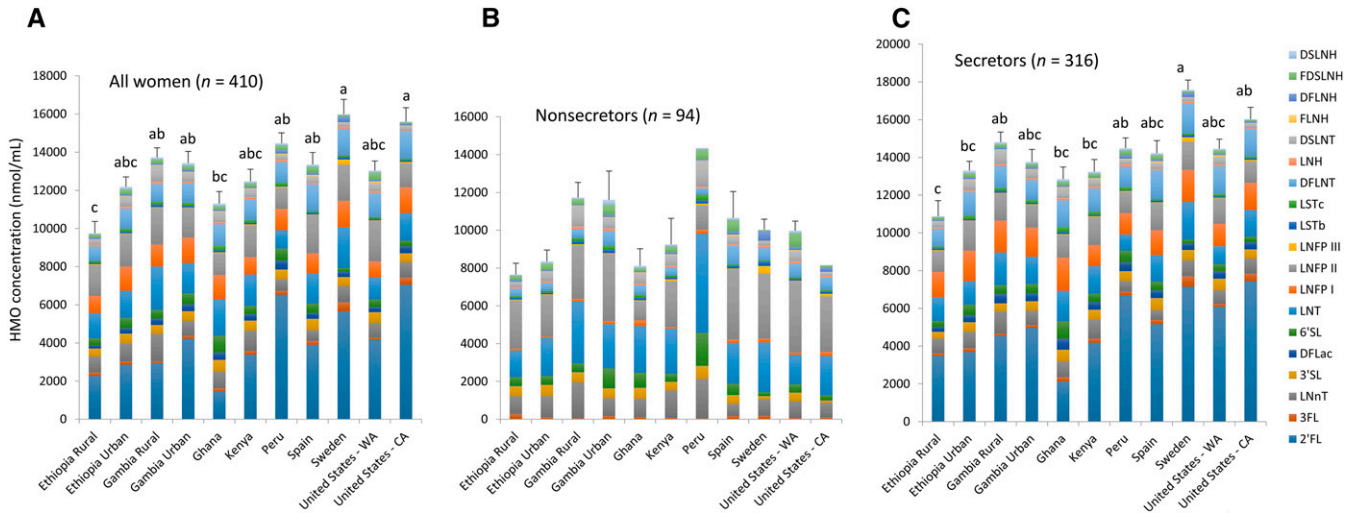


FIGURE 1 Mean \pm SEM absolute total and HMO isoform concentrations of all women combined (A), nonsecretors (B), and secretors (C). (A and B) Bars without a common lowercase letter represent total HMO values that differed with the use of Bonferroni-correction procedures for multiple comparisons. All statistical inferences were carried out on log-transformed data. Note that there was only one nonsecretor subject each in Peru and United States - CA. CA, California; 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, lacto-*N*-fucopentaose; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LSTb, sialyl-lacto-*N*-tetraose b; LSTc, sialyl-lacto-*N*-tetraose c; WA, Washington; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

by the rural cohort was higher than that produced by the urban cohort (1423 ± 117 compared with 781 ± 61 nmol/mL, respectively; $P < 0.05$). The same difference was shown for DSLNT (870 ± 68 compared with 477 ± 45 nmol/mL, respectively; $P < 0.05$). Conversely, although they were similar between rural and urban Gambian cohorts, concentrations of 6'-sialyllactose, LSTc, and FLNH were higher in milk that was produced by mothers in ET_U than by mothers in ET_R.

There were also several differences between the 2 US populations despite the fact that they were very similar in terms of anthropometric and reproductive variables. For instance, FDSLNH was higher in the US_W group than in the US_C group

(370 ± 48 compared with 70 ± 9 nmol/mL, respectively; $P < 0.05$). Because both ethnicity and location, both of which are likely related to environmental variables such as the diet, were different between these groups; however, further work will be required to tease apart potentially causative factors.

Other groupings of HMOs that are based on factors such as HMO-bound sialic acid, chain type, and linkage type also revealed differences in cohorts (Table 3). For instance, milk that was produced by women in Sweden and the US_C was the most fucosylated and the least sialylated; milk from mother in ET_R was less sialylated than that of women in ET_U; and milk produced by women in Peru was highly enriched with small HMOs

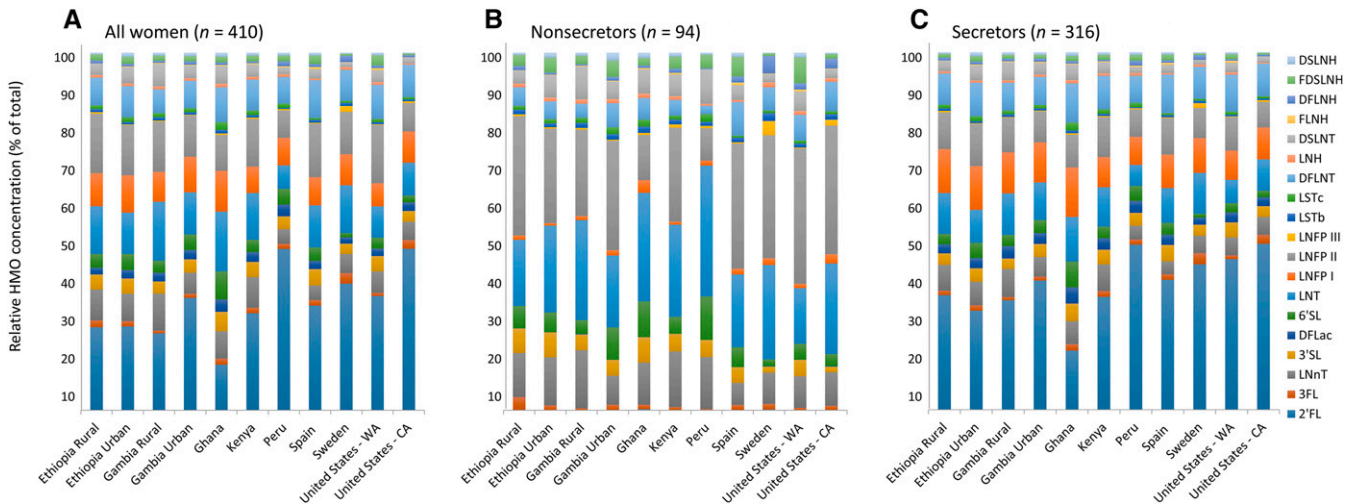


FIGURE 2 Mean \pm SEM relative abundance of HMO concentrations of all women combined (A), nonsecretors (B), and secretors (C) in each cohort. Note that there was only one nonsecretor subject each in Peru and United States - CA. CA, California; DFLac, difucosyllactose; DFLNH, difucosyllacto-*N*-hexaose; 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, lacto-*N*-fucopentaose; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LSTb, sialyl-lacto-*N*-tetraose b; LSTc, sialyl-lacto-*N*-tetraose c; WA, Washington; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

TABLE 3
Variation in HMO groupings in 410 healthy women living in selected locations around the world¹

Variable	Ethiopia				Gambia				United States					
	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Kenya (n = 42)	Peru (n = 43)	Spain (n = 41)	Sweden (n = 24)	Washington (n = 41)	California (n = 19)
HMO-bound sialic acid, ² nmol/mL	2010 ± 163 ^{b,c}	3179 ± 158 ^a	3570 ± 181 ^a	2941 ± 212 ^a	3605 ± 235 ^a	2704 ± 157 ^{a,b}	2640 ± 180 ^{a,b}	2835 ± 150 ^a	1606 ± 96 ^c	2968 ± 153 ^a	1705 ± 106 ^{b,c}			
HMO-bound fucose, ³ nmol/mL	7226 ± 608 ^b	9198 ± 621 ^{a,b}	9074 ± 665 ^{a,b}	10,558 ± 614 ^{a,b}	7474 ± 679 ^b	9415 ± 659 ^{a,b}	12,797 ± 1090 ^a	11,254 ± 640 ^{a,b}	13,679 ± 866 ^a	10,953 ± 603 ^{a,b}	13,963 ± 872 ^a			
Small HMOs, ⁴ nmol/mL	3240 ± 357 ^b	4108 ± 383 ^{a,b}	3980 ± 466 ^b	5471 ± 520 ^{a,b}	3138 ± 266 ^b	4538 ± 440 ^{a,b}	7900 ± 441 ^a	5223 ± 481 ^{a,b}	6800 ± 813 ^{a,b}	5245 ± 552 ^{a,b}	8199 ± 947 ^{a,b}			
Type 1, ⁵ nmol/mL	4222 ± 281 ^{b,c}	5029 ± 242 ^{a,b}	6344 ± 345 ^a	5043 ± 317 ^{a,b,c}	4984 ± 346 ^{a,b,c}	4751 ± 297 ^{a,b,c}	3499 ± 249 ^c	5089 ± 337 ^{a,b,c}	5776 ± 380 ^{a,b}	4634 ± 268 ^{a,b,c}	4400 ± 297 ^{a,b,c}			
Type 2, ⁶ nmol/mL	983 ± 730 ^{b,c}	1120 ± 64 ^{a,b}	1622 ± 124 ^a	957 ± 69 ^{b,c}	1159 ± 81 ^{a,b}	1277 ± 109 ^{a,b}	824 ± 65 ^{b,c}	652 ± 48 ^c	1215 ± 93 ^{a,b}	913 ± 48 ^{b,c}	972 ± 90 ^{a,b,c}			
α-1,2, ⁷ nmol/mL	3169 ± 520	4129 ± 515	4103 ± 609	5562 ± 690	2731 ± 386	4302 ± 546	7644 ± 505	4962 ± 605	7056 ± 855	5009 ± 634	8412 ± 904			
α-1,3, ⁸ nmol/mL	232 ± 26 ^{b,c}	208 ± 41 ^c	143 ± 16 ^c	192 ± 20 ^c	239 ± 33 ^c	241.2 ± 26.5 ^{b,c}	262 ± 32 ^{b,c}	238 ± 24 ^{b,c}	742 ± 47 ^a	147 ± 13 ^c	464 ± 44 ^{a,b}			
α-2,6, ⁹ nmol/mL	561 ± 80 ^c	793 ± 58 ^{a,b}	753 ± 46 ^{a,b,c}	828 ± 84 ^{a,b}	1251 ± 110 ^a	680 ± 49 ^{b,c}	859 ± 75 ^{a,b}	681 ± 55 ^{b,c}	431 ± 32 ^c	595 ± 44 ^{b,c}	476 ± 59 ^{b,c}			

¹ All values are means ± SEMs. Values in a row that do not share a common superscript letter differed with the use of Bonferroni-corrected procedures for multiple comparisons, $P < 0.05$. All statistical inferences were based on log-transformed data. *Overall effect of cohort, $P < 0.01$ (1-factor ANOVA). DFLac, difucosylactose; DFLNH, difucosylactose-*N*-hexaose; DFLNT, difucosylactose-*N*-tetraose; DSLNH, disialylactose-*N*-hexaose; DSLNT, disialylactose-*N*-tetraose; FDSLNT, fucodisialylactose-*N*-hexaose; FLNH, fucosylactose-*N*-hexaose; FLNT, lacto-*N*-tetraose; LNT, lacto-*N*-tetraose; LSTb, sialyl-lactose-*N*-tetraose b; LSTc, sialyl-lactose-*N*-tetraose c; 2'FL, 2'-fucosylactose; 3FL, 3-fucosylactose; 3'SL, 3'-sialylactose; 6'SL, 6'-sialylactose.

² Calculated as the sum of all sialic acid moieties bound to each HMO.

³ Calculated as the sum of all fucose moieties bound to each HMO.

⁴ Calculated as 2'FL + 3FL + 3'SL + 6'SL.

⁵ Calculated as LNT + LNFP I + LNFP II + LSTb + DSLNT.

⁶ Calculated as LNT + LNFP III + LSTc.

⁷ Calculated as LNFP I + 2'FL.

⁸ Calculated as LNFP III + 3FL.

⁹ Calculated as LSTb + LSTc + 6'SL.

(defined as the sum of 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, and trisaccharides).

Effects of cohort on secretor status and of secretor status on individual HMO concentrations

The proportion of women who were categorized as being secretors (defined as having a 2'-fucosyllactose concentration that was greater than a natural, very low break in the data) was also substantially different in populations (**Figure 3**) and ranged from 65% in populations in GB_R and ET_R to 98% in the cohort in Peru ($P < 0.01$). The percentage of secretors in the cohort in Peru was also higher than that in the cohorts in Ghana and the US_W (98% compared with 68%, respectively; $P < 0.01$) but was similar to that in the cohort in the US_C (self-identified as Hispanic) (98% compared with 95%; $P = 1.00$). As anticipated and as illustrated in Figures 1 and 2, absolute and relative HMO concentrations in secretors and nonsecretors were substantially different (HMO concentrations by secretor status are shown in **Supplemental Tables 10–13**).

Relations in selected maternal anthropometric, demographic, or reproductive variables and individual HMO concentrations

Variations in maternal age, time postpartum, BMI, and weight were associated with several of the HMO types and groups (**Figure 4**). For instance, age was negatively correlated with concentrations of LNnT, LSTc, and DSLNH ($r = -0.14$, -0.17 , and -0.15 , respectively) and was positively correlated with the concentration of FLNH ($r = 0.15$). Maternal weight and BMI were positively correlated with 2'-fucosyllactose ($r = 0.20$ for both), FLNH ($r = 0.19$ and 0.15 , respectively), HMO-bound fucose

($r = 0.21$ for both), and small HMOs ($r = 0.21$ and 0.23 , respectively); and maternal weight was positively correlated with LNFP III ($r = 0.20$) and DFLNT ($r = 0.14$). Conversely, maternal weight and BMI were inversely correlated with LNnT and DSLNT ($r = -0.16$ and -0.21 , respectively; and $r = -0.20$ and -0.24 , respectively). The time postpartum was inversely correlated with several HMOs including 6'-sialyllactose, LNFP III, LSTc, lacto-*N*-hexaose, DSLNT, and α 2,6-linked oligosaccharides ($r = -0.31$, -0.23 , -0.40 , -0.26 , -0.13 , and -0.36 , respectively).

Relations between HMO concentrations

Several correlations also existed in the concentrations of different HMOs and groups thereof. For instance, concentrations of 2'-fucosyllactose, difucosyllactose, and LNFP I were all correlated ($r = 0.23$ – 0.54); this correlation was expected because their synthesis in the mammary gland is dependent on FUT2 activity. Similarly, concentrations of HMO-bound fucose and α 1-2-fucosylated HMO were correlated ($r = 0.82$) as were 2'-fucosyllactose and combined small HMOs ($r = 0.98$). There was also a positive association between LNT and LNnT concentrations ($r = 0.75$). LNFP I and LNFP II were negatively correlated ($r = -0.46$); and 2'-fucosyllactose was negatively correlated with LNFP II ($r = -0.52$). Other associations of interest were that both LNT and LNnT were positively correlated with DSLNT ($r = 0.60$ and 0.62 , respectively), and LSTb was positively correlated with DSLNT ($r = 0.55$).

Effect of cohort on HMO diversity

Diversity metrics also differed in cohorts, and mean values are provided in **Table 4**. In general, HMO diversity and evenness were lowest in milk that was produced by women in Peru and the US_C and

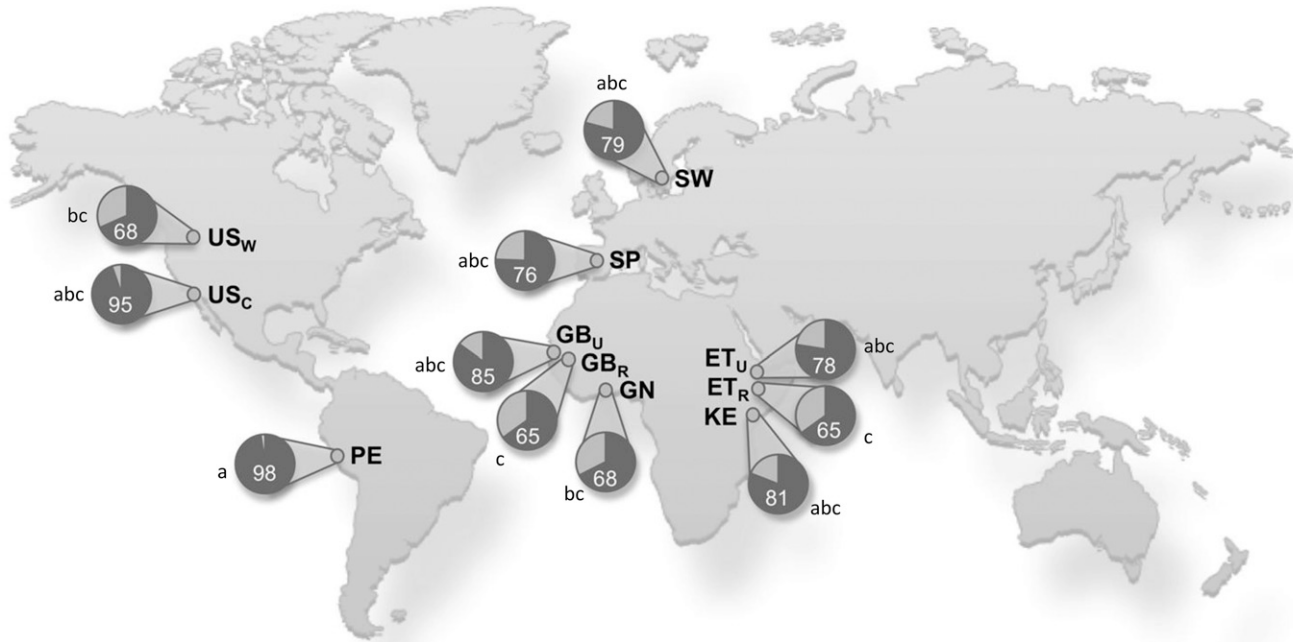


FIGURE 3 Percentages of women in each cohort categorized as secretors. Cohorts that do not share a common lowercase letter differ ($P < 0.05$) in terms of their percentages of women who were secretors with the use of a chi-square test with Benjamini and Hochberg false-discovery-rate corrections. ET_R, rural Ethiopia; ET_U, urban Ethiopia; GB_R, rural Gambia; GB_U, urban Gambia; GN, Ghana; KE, Kenya; PE, Peru; SP, Spain; SW, Sweden; US_C, United States–California (Hispanic); US_W, United States–Washington.

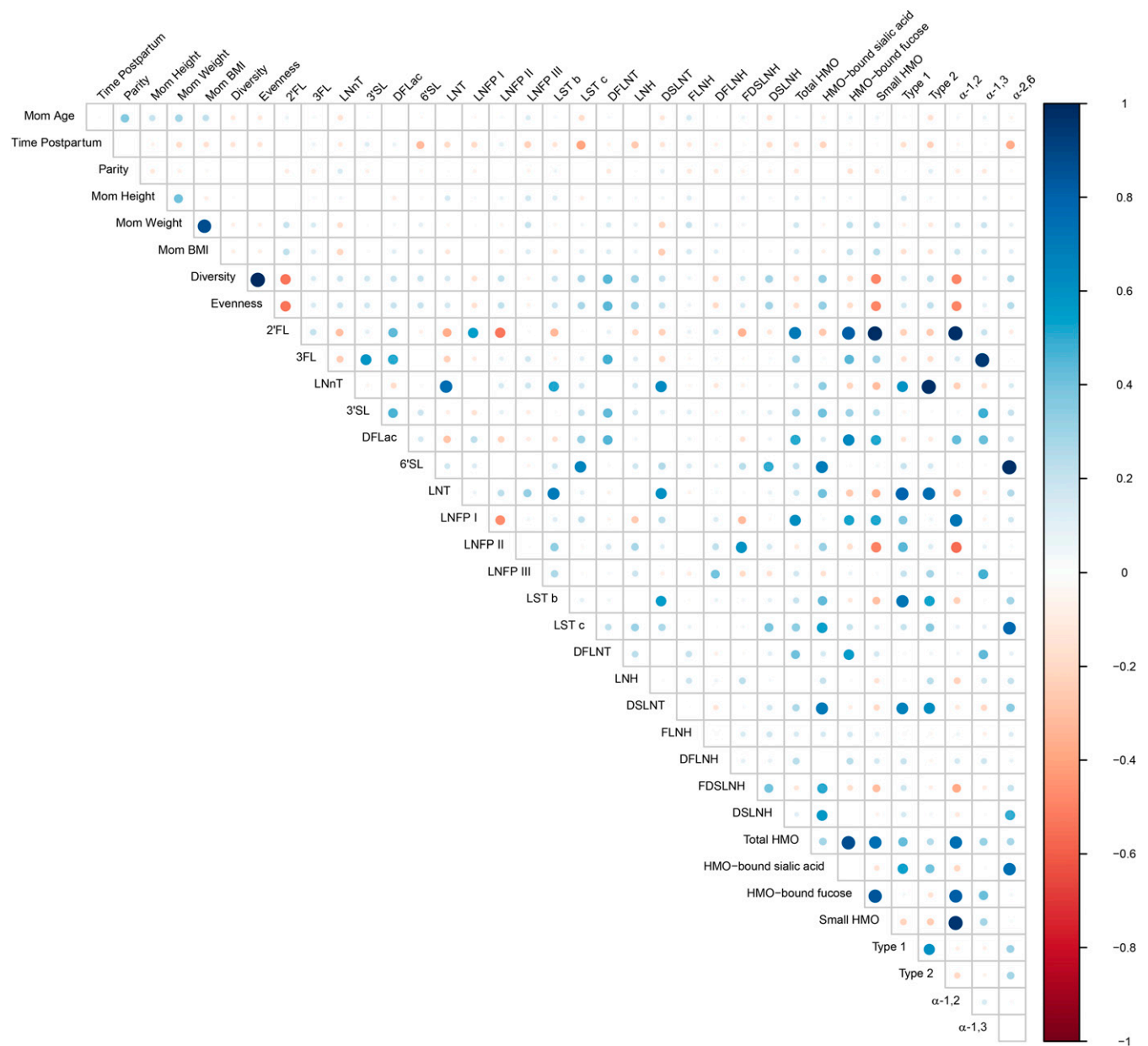


FIGURE 4 Spearman rank correlations between selected maternal anthropometric, demographic, and reproductive variables and HMO types and groupings. Sizes of dots and colors indicate directionality (blue denotes positive; red denotes negative) and the strength of the association. Total HMO-bound sialic acid; total HMO-bound fucose; small HMO; type 1; type 2; α -1,2; α -1,3; and α -2,6 were calculated as: the sum of all sialic acid moieties bound to each HMO; all fucose moieties bound to each HMO; 2'FL + 3FL + 3'SL + 6'SL; LNT + LNFP I + LNFP II + LSTb + DSLNT; LNnT + LNFP III + LSTc; LNFP I + 2'FL; LNFP III + 3FL; and LSTb + LSTc + 6'SL, respectively. 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, lacto-*N*-fucopentaose; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LST, sialyl-lacto-*N*-tetraose; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

were highest in milk that was produced by women in Ghana. There were no differences in HMO diversity between rural and urban cohorts in either ET or GB cohorts or between US cohorts (see **Supplemental Tables 14** and **15** for diversity metrics by secretor status).

Effects of cohort and other factors on complex milk oligosaccharide profiles

A visual and numerical evaluation of nonmetric dimensional scaling and principle components analyses and plots (color coded

by cohort, continent and ethnicity, BMI, time postpartum, parity, and maternal age) provided no discernible evidence that these factors accounted for an appreciable variability in the overall HMO profiles (MK McGuire, SL Brooker, WJ Price, B Shafii, unpublished results, June 2016). To account for the multivariate nature of the data, the NMF method was considered (43). The basic purpose of the NMF analysis was to decompose the data matrix into metacomponents and to determine their potential probabilities of contribution to the underlying variability structure. For example, as shown in **Table 5**, overall scores were used

TABLE 4
Variation in HMO diversity and evenness indexes in 410 healthy women living in selected locations around the world¹

Variable	Ethiopia						Gambia					United States			
	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)	Urban (n = 40)	Ghana (n = 40)	Kenya (n = 42)	Peru (n = 43)	Spain (n = 41)	Sweden (n = 24)	Washington (n = 41)	California (n = 19)	
Shannon entropy	2.06 ± 0.03 ^{ab,c}	2.15 ± 0.03 ^{ab}	2.07 ± 0.03 ^{ab,c}	2.06 ± 0.04 ^{ab,c}	2.27 ± 0.03 ^a	2.10 ± 0.04 ^{ab,c}	1.94 ± 0.04 ^{b,c}	2.08 ± 0.03 ^{ab,c}	1.99 ± 0.03 ^{b,c}	2.02 ± 0.03 ^{b,c}	1.86 ± 0.07 ^c				
Shannon diversity	7.98 ± 0.24 ^b	8.79 ± 0.26 ^{ab}	8.14 ± 0.26 ^{ab}	8.14 ± 0.31 ^{ab}	9.84 ± 0.29 ^a	8.38 ± 0.27 ^{ab}	7.25 ± 0.29 ^b	8.14 ± 0.20 ^{ab}	7.40 ± 0.24 ^b	7.70 ± 0.24 ^b	6.69 ± 0.41 ^b				
Inverse Simpson	5.51 ± 0.25 ^{b,c}	6.22 ± 0.27 ^{ab}	5.74 ± 0.23 ^{ab,c}	5.56 ± 0.29 ^{b,c}	7.31 ± 0.29 ^a	5.78 ± 0.26 ^{ab,c}	4.33 ± 0.25 ^c	5.38 ± 0.20 ^{b,c}	4.73 ± 0.25 ^{b,c}	4.92 ± 0.23 ^{b,c}	4.30 ± 0.39 ^c				
Shannon evenness	0.42 ± 0.01 ^b	0.46 ± 0.01 ^{ab}	0.43 ± 0.01 ^{ab}	0.43 ± 0.02 ^{ab}	0.52 ± 0.02 ^a	0.44 ± 0.01 ^{ab}	0.38 ± 0.02 ^b	0.43 ± 0.01 ^{ab}	0.39 ± 0.01 ^b	0.41 ± 0.01 ^b	0.35 ± 0.02 ^b				
Simpson evenness	0.29 ± 0.01 ^{b,c}	0.33 ± 0.01 ^{ab}	0.30 ± 0.01 ^{ab,c}	0.30 ± 0.02 ^{b,c}	0.38 ± 0.02 ^a	0.30 ± 0.01 ^{ab,c}	0.23 ± 0.01 ^c	0.28 ± 0.01 ^{b,c}	0.25 ± 0.01 ^{b,c}	0.26 ± 0.01 ^{b,c}	0.23 ± 0.02 ^c				
Pielou evenness	0.70 ± 0.01 ^{ab,c}	0.73 ± 0.01 ^{ab}	0.70 ± 0.01 ^{ab,c}	0.70 ± 0.01 ^{ab,c}	0.77 ± 0.10 ^a	0.71 ± 0.01 ^{ab,c}	0.66 ± 0.01 ^{b,c}	0.71 ± 0.01 ^{ab,c}	0.67 ± 0.01 ^{b,c}	0.69 ± 0.01 ^{b,c}	0.63 ± 0.02 ^c				

¹ All values are means ± SEMs. Values in a row that do not share a common superscript letter differed with the use of Bonferroni-correction procedures for multiple comparisons, $P < 0.05$. All statistical inferences were based on log-transformed data. HMO, human milk oligosaccharide.

to break down the probability of each HMO that contributed to the observed pattern in the data with higher numbers having a higher probability of contribution (i.e., 2'-fucosyllactose contributed the most to the overall observed variability). Further separation of the analysis, by dividing the data into subgroups (such as population or BMI range), allowed for analysis of how these same components contributed to specified subsets of the data. Several detectable patterns were apparent when the NMF was used to analyze subgroups of the data. The extraction of HMO components with the highest feature scores led to 6 compounds (2'-fucosyllactose, LNFP I, LNFP II, 6'-sialyllactose, DFLNT, and FDSLNH); NMF scores for these HMOs (Supplemental Figure 1, Table 5) suggested that they contributed differently to the overall structure of HMO profiles across populations. For instance, 2'-fucosyllactose appeared to be highly influential to the variability of HMO profiles in the groups in Ghana, GB_U, GB_R, ET_U, and ET_R but was less important in most other cohorts. DFLNT appeared to have a similar pattern, but it was also important in Kenya. Another example was 6'-sialyllactose, which contributed substantially to HMO profiles in the ET_R, US_W, US_C, and Peru cohorts but in the other cohorts. Contributions to HMO-profile variability also seemed to differ by maternal BMI, parity, and time postpartum (Tables 6–8) (see Supplemental Figures 2 and 3 for related basis maps). For example, 2'-fucosyllactose was relatively more important in healthy-weight and overweight women than in underweight women, whereas difucosyllacto-*N*-hexaose was more important to the data structure in underweight women. Difucosyllactose appeared to decrease in relative contribution as BMI increased; conversely, 6'-sialyllactose, FDSLNH, and DSLNH appeared to increase in contribution as BMI increased. LNFP III was more important to the overall data structure for primiparous women than for multiparous women, and DSLNH has the greatest contribution in milk that was collected between 20 and 46 d postpartum. FLNH and DFLHN both contributed to lower amounts of the data structure as women got older (Table 9) (see Supplemental Figure 2 for related basis maps).

DISCUSSION

Results from this study support our a priori hypothesis that concentrations of individual oligosaccharides and groupings thereof vary geographically in milk that is produced by healthy women. Indeed, absolute concentrations of all HMOs except for LNFP I varied in the studied cohorts. Because we took great care to collect and analyze the samples in a similar manner, we conclude that these differences are not a result of methodologic variation. In some cases (e.g., LNnT in GB_R compared with GB_U), differences occurred despite similar genetic backgrounds, thereby suggesting that environmental factors may be important. In other cases (e.g., 2'-fucosyllactose in the US_C compared with US_W), differences occurred across populations despite similar anthropometric and reproductive backgrounds, thereby suggesting that genetics, epigenetics, or other undocumented factors (e.g., micronutrient intake) also likely play important roles.

An understanding of the genesis and implications of HMO variation is important because increasing literature has suggested that individual HMOs might have particular structure-specific effects on infant health and risk of disease. For example, Mexican infants who received milk with low concentrations of

TABLE 5
Overall and population-specific NMF scores for each HMO¹

HMO	Ethiopia		Gambia		Ghana (n = 40)	Kenya (n = 42)	Peru (n = 43)	Spain (n = 41)	Sweden (n = 24)	United States		Overall
	Rural (n = 40)	Urban (n = 40)	Rural (n = 40)	Urban (n = 40)						Washington (n = 41)	California (n = 19)	
2'FL	0.43	0.30	0.39	0.41	0.43	0.45	0.98	0.63	0.68	0.60	0.62	1.00
3FL	0.26	0.13	0.19	0.42	0.42	0.44	0.46	0.58	0.36	0.55	0.60	0.50
LNnT	0.18	0.15	0.26	0.22	0.34	0.17	0.32	0.30	0.25	0.42	0.14	0.45
3'SL	0.19	0.08	0.27	0.14	0.09	0.14	0.17	0.33	0.20	0.07	0.26	0.19
DFLac	0.49	0.30	0.32	0.67	0.29	0.31	0.54	0.69	0.56	0.23	0.47	0.51
6'SL	0.45	0.49	0.36	0.18	0.46	0.28	0.40	0.33	0.13	0.16	0.74	0.70
LNT	0.34	0.29	0.48	0.22	0.65	0.22	0.44	0.23	0.33	0.29	0.40	0.55
LNFP I	0.44	0.30	0.42	0.43	0.60	0.61	0.66	0.52	0.53	0.59	0.52	0.79
LNFP II	0.34	0.36	0.51	0.39	0.32	0.49	0.57	0.46	0.65	0.31	0.47	0.63
LNFP III	0.22	0.18	0.16	0.20	0.34	0.61	0.15	0.13	0.39	0.37	0.33	0.42
LSTb	0.28	0.19	0.39	0.22	0.35	0.24	0.22	0.27	0.18	0.25	0.14	0.33
LSTc	0.34	0.23	0.23	0.17	0.21	0.17	0.21	0.32	0.06	0.14	0.55	0.46
DFLNT	0.44	0.28	0.38	0.72	0.46	0.72	0.74	0.85	0.56	0.59	0.71	0.72
LNH	0.25	0.35	0.39	0.19	0.19	0.30	0.41	0.43	0.51	0.28	0.20	0.22
DSLNT	0.15	0.16	0.19	0.20	0.32	0.23	0.23	0.28	0.14	0.22	0.25	0.23
FLNH	0.15	0.13	0.19	0.16	0.50	0.06	0.47	0.42	0.25	0.29	0.29	0.31
DFLNH	0.43	0.32	0.34	0.61	0.23	0.43	0.34	0.41	0.21	0.44	0.41	0.37
FDSLNH	0.47	0.45	0.48	0.52	0.36	0.60	0.55	0.64	0.56	0.39	0.42	0.63
DSLNH	0.54	0.24	0.50	0.28	0.41	0.46	0.35	0.61	0.09	0.18	0.14	0.52

¹ NMF scores represent the probability of contribution to (and importance of) a specified HMO variable to the basis component. 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, lacto-*N*-fucopentaose; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LSTb, sialyl-lacto-*N*-tetraose b; LSTc, sialyl-lacto-*N*-tetraose c; NMF, nonnegative matrix factorization; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

2'-fucosyllactose (nonsecretor milk) had higher diarrhea incidence (44) than of those who consumed appreciably higher amounts of 2'-fucosyllactose. Higher concentrations of FUT2-dependent HMOs such as 2'-fucosyllactose were also correlated with lower risk of allergy at 2 and 5 y of age in infants with high hereditary allergy risk (45), and HMOs including 2'-fucosyllactose attenuated food allergy symptoms in a mouse model (46). Data from our current study revealed significant variations in secretor status and 2'-fucosyllactose concentrations across global locations. Most strikingly, and similar to the reported high percentage of secretors in Mexico (32), we also showed very high percentages of secretors in women in PE and the US_C who self-identified as Hispanic. We hypothesize that this difference has been driven by evolutionary pressures that have conferred 2'-fucosyllactose-related health benefits in these populations, at least in their historical locations and within long-term behavioral and environmental constructs.

Other HMO isoforms are also likely related to health and disease risk in particular situations. For instance, a lower total HMO concentration and a higher proportion of 3'-sialyllactose were correlated with higher HIV transmission in Zambian infants (33), and HIV infection in lactating women was correlated with differences in HMOs both in Zambia and South Africa (33, 47). Moreover, HMO compositions have been associated with infant mortality and morbidity in HIV-exposed uninfected infants in Zambia (48). As such, increased consumption of these HMOs might be particularly important in this high-risk condition. Alderete et al. (49) have also shown that concentrations of individual HMOs in mother's milk were associated with infant weight as well as lean and fat body masses in a US cohort. Similarly,

Charbonneau et al. (50) reported that milk that was produced by Malawian mothers who were nursing severely stunted infants had lower HMO concentrations than in milk that was produced by mothers who were breastfeeding healthy-weight infants. Together, these studies suggest that the variation in HMO composition may affect the recipient infant's metabolic phenotype, which is likely mediated through the gastrointestinal microbiome.

Research from the Bode laboratory (51) has also indicated that consumption of higher amounts of DSLNT, which is a sialylated HMO, may have been protective against the development of necrotizing enterocolitis-like symptoms in a rodent model. In the current study, we showed that maternal weight and BMI were inversely correlated with DSLNT concentrations, which suggest that maternal factors may partially contribute to HMO composition. The NMF analysis also suggested that there were somewhat different patterns in maternal BMI categories. Clearly, whether maternal adiposity is causally related to milk DSLNT (or any other HMO) concentration or HMO profiles or, instead, is a proxy for other maternal and environmental variables could not be ascertained from the current study. In addition, we recognize that body weight and BMI are not good indicators of adiposity during the postpartum period (52) and that other more sophisticated methods (e.g., dual-energy X-ray absorptiometry) will be needed to investigate this relation more thoroughly.

Note that, except for mothers in the US_C, the mean concentration of 3-fucosyllactose in milk that was produced by the Swedish mothers in the current study was >2 times that of milk that was produced by women in all other cohorts. This result is in contrast with the previous work of Erney et al. (32) who detected no 3-fucosyllactose in the milk of Swedish mothers. In

TABLE 6
NMF scores describing BMI for individual HMOs¹

HMO	BMI, kg/m ²		
	Underweight (<18.5 ; $n = 22$)	Healthy weight (18.5 – 24.9 ; $n = 242$)	Overweight (≥ 25 ; $n = 139$)
2'FL	0.48	1.00	0.64
3FL	0.43	0.42	0.32
LNnT	0.16	0.54	0.35
3'SL	0.19	0.16	0.22
DFLac	0.54	0.45	0.32
6'SL	0.34	0.62	0.69
LNT	0.35	0.44	0.55
LNFP I	0.56	0.75	0.60
LNFP II	0.50	0.59	0.52
LNFP III	0.42	0.42	0.50
LSTb	0.07	0.30	0.30
LSTc	0.27	0.48	0.43
DFLNT	0.69	0.70	0.73
LNH	0.18	0.25	0.18
DSLNT	0.26	0.24	0.13
FLNH	0.51	0.26	0.54
DFLNH	0.58	0.45	0.25
FDSLNH	0.41	0.63	0.62
DSLNH	0.28	0.48	0.61

¹ NMF scores represent the probability of contribution to (and importance of) a specified HMO variable to the basis component. 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, lacto-*N*-fucopentaose; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LSTb, sialyl-lacto-*N*-tetraose b; LSTc, sialyl-lacto-*N*-tetraose c; NMF, nonnegative matrix factorization; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

addition, although 3-fucosyllactose varied in the populations we studied, it was not the most variable, as suggested by Erney et al. (32). Instead, FLNH and LNFP III were 2- and 3-times more variable than 3-fucosyllactose. It is possible that methodologic differences between studies might explain this discrepancy.

Our data also revealed correlations between individual HMOs, which suggest that there are common synthetic pathways. Some of these correlations were anticipated; for instance, there were positive correlations between 2'-fucosyllactose, difucosyllactose, and LNFP I, which are HMOs that are all α 1-2-fucosylated and highly depend on FUT2 activity. Other associations, such as the positive correlation between LNT and LNnT, were surprising. We had anticipated that the terminal galactose is either attached in β 1-3-linkage to derive type 1 chains (e.g., LNT) or attached in β 1-4-linkage to derive type 2 chains (e.g., LNnT); however, this would have yielded a negative correlation between LNT and LNnT, which suggests that other factors determine and limit chain elongation. Future studies that include genomic and transcriptomic data sets will help delineate HMO biosynthetic pathways and unravel how the synthesis of different HMOs is controlled. Forthcoming studies should be designed to determine correlations between HMOs and other milk components including the diverse communities of bacteria that are known to be in human milk (53–55).

One of our secondary objectives was to compare and contrast HMO contents and profiles between ethnically similar (and likely

TABLE 7
NMF scores describing parity for individual HMOs¹

HMO	Parity, children, n		
	1 ($n = 159$)	2 ($n = 111$)	≥ 3 ($n = 250$)
2'FL	0.99	0.97	0.65
3FL	0.47	0.35	0.43
LNnT	0.29	0.43	0.38
3'SL	0.18	0.20	0.12
DFLac	0.51	0.31	0.42
6'SL	0.65	0.64	0.54
LNT	0.46	0.51	0.45
LNFP I	0.78	0.76	0.70
LNFP II	0.68	0.45	0.56
LNFP III	0.55	0.38	0.21
LSTb	0.29	0.29	0.26
LSTc	0.42	0.54	0.41
DFLNT	0.75	0.66	0.69
LNH	0.22	0.27	0.17
DSLNT	0.16	0.25	0.27
FLNH	0.37	0.27	0.28
DFLNH	0.30	0.30	0.35
FDSLNH	0.63	0.55	0.55
DSLNH	0.51	0.41	0.55

¹ NMF scores represent the probability of contribution to (and importance of) a specified HMO variable to the basis component. 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, lacto-*N*-fucopentaose; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LSTb, sialyl-lacto-*N*-tetraose b; LSTc, sialyl-lacto-*N*-tetraose c; NMF, nonnegative matrix factorization; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

genetically similar) populations who were living in different locations. In this regard, note that there were several differences between milk that was produced by women in GB_U and GB_R (both Mandinka) and between milk that was produced by women in ET_U and ET_R (both Sidama). This finding suggests that there may be some effect of a relatively recent migration on the composition of HMOs rather than all of the variation being related strictly to genetic factors or simple-to-measure anthropometric and demographic variables. However, note that, unlike in all other cohorts for which milk was preserved by cold storage, milk collected from women in ET_R was first chemically preserved. Although our unpublished data (MK McGuire, KA Lackey, June 2016) suggest that the preservation method does not influence our ability to accurately characterize microbial communities in human milk, additional studies should be conducted to verify this finding.

In conclusion, the current study presents foundational data on what can be considered normal with regard to the HMO composition of milk that is produced by relatively healthy women in different locations around the world. Future studies are needed to determine how the variation in HMO composition is related to maternal and infant health and to generate hypotheses on HMO structure-function relations that can be tested in preclinical and clinical studies. Our data also provide a solid, and relatively unique, foundation on which to assess the deviation from a normal milk composition when women are not healthy (e.g., with diabetes, mastitis, or HIV). Future studies concerning this topic

TABLE 8
NMF scores describing time-postpartum quartiles for individual HMOs¹

HMO	Time postpartum, d			
	20–46	47–63	64–78	79–161
2'FL	0.80	0.84	0.65	0.74
3FL	0.45	0.33	0.39	0.39
LNnT	0.38	0.44	0.26	0.55
3'SL	0.27	0.20	0.25	0.21
DFLac	0.48	0.27	0.39	0.39
6'SL	0.68	0.48	0.59	0.35
LNT	0.40	0.41	0.53	0.41
LNFP I	0.62	0.73	0.73	0.63
LNFP II	0.52	0.77	0.50	0.58
LNFP III	0.34	0.49	0.28	0.21
LSTb	0.29	0.31	0.31	0.29
LSTc	0.39	0.32	0.24	0.37
DFLNT	0.75	0.75	0.70	0.65
LNH	0.22	0.25	0.20	0.26
DSLNT	0.31	0.37	0.24	0.24
FLNH	0.60	0.31	0.17	0.35
DFLNH	0.40	0.45	0.24	0.30
FDSLNH	0.62	0.66	0.58	0.66
DSLNH	0.64	0.40	0.42	0.22

¹ NMF scores represent the probability of contribution to (and importance of) a specified HMO variable to the basis component. 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, lacto-*N*-fucopentaose; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LSTb, sialyllacto-*N*-tetraose b; LSTc, sialyllacto-*N*-tetraose c; NMF, nonnegative matrix factorization; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

should strive to include women from regions (e.g., Asia) that were not included in the current study and women who are not healthy or are nursing unhealthy infants. The identification of the variation of the normal HMO composition in healthy women is just the beginning of a broader attempt to understand how sociocultural, evolutionary, environmental, and genomic aspects affect human milk composition and, subsequently, infant health. We posit that there is likely no one-size-fits-all construct when it comes to human milk composition and, thus, infant nutrition. Instead, we hypothesize that human milk composition has likely evolved differentially in such a way as to optimally nourish infants who are born in various social, environmental, genetic, and behavioral contexts. Future studies should be designed in such a way to examine this possibility and to also test it experimentally.

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TABLE 9
NMF scores describing maternal age quartiles for individual HMOs¹

HMO	Maternal age, y			
	15–22	23–27	28–32	33–46
2'FL	0.97	0.71	0.83	0.69
3FL	0.43	0.43	0.46	0.43
LNnT	0.55	0.23	0.35	0.36
3'SL	0.19	0.26	0.18	0.15
DFLac	0.46	0.36	0.40	0.56
6'SL	0.66	0.58	0.63	0.71
LNT	0.54	0.30	0.53	0.44
LNFP I	0.75	0.62	0.82	0.67
LNFP II	0.60	0.48	0.63	0.49
LNFP III	0.24	0.54	0.36	0.43
LSTb	0.39	0.24	0.35	0.28
LSTc	0.47	0.39	0.44	0.46
DFLNT	0.71	0.78	0.68	0.65
LNH	0.27	0.27	0.22	0.21
DSLNT	0.36	0.15	0.32	0.29
FLNH	0.28	0.33	0.51	0.43
DFLNH	0.29	0.34	0.51	0.49
FDSLNH	0.48	0.63	0.64	0.64
DSLNH	0.48	0.44	0.62	0.59

¹ NMF scores represent the probability of contribution to (and importance of) a specified HMO variable to the basis component. 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, lacto-*N*-fucopentaose; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LSTb, sialyllacto-*N*-tetraose b; LSTc, sialyllacto-*N*-tetraose c; NMF, nonnegative matrix factorization; 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose.

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