**ABBOTT NUTRITION SUBMISSION ON APPLICATION A1155 – 2'-FL AND LNnT IN INFANT FORMULA AND OTHER PRODUCTS**

January 17, 2019

## INTRODUCTION

This submission has been prepared by Abbott Nutrition. Abbott Nutrition believes that proper nutrition is the foundation for living the best live possible. Our aim is to make every stage of life a healthy one which is why we are dedicated to developing science-based nutrition products for people of all ages.

Abbott Nutrition is committed to ethically marketing our products and supports the voluntary restriction of marketing practices for infant formula products to support government policies which protect and promote breastfeeding. Abbott Nutrition believes that breastfeeding provides the best nutrition for infants and supports, educates and encourages mothers to breastfeed for as long as possible. When breastmilk is not given to an infant, infant formula is the only safe and recommended alternative.

We have reviewed Application A1155 – 2'-FL and LNnT in infant formula and other products and welcome the opportunity to provide comments to the Food Standards Australia New Zealand (FSANZ) in response to the Call for Submissions.

## COMMENTS

### Prohibition of use with Existing Oligosaccharide Permissions

FSANZ proposes to prohibit the addition of 2'-FL alone, or with LNnT, in combination with existing permissions for GOS and inulin-type fructans (ITF) for infant formula products and formulated supplementary foods for young children (FSFYC). FSANZ states this preliminary position is based on the evidence available to assess the tolerance of infants to the total combination of oligosaccharides.

Abbott Nutrition does NOT support the position to prohibit the addition of 2'-FL alone, or with LNnT, in combination with existing oligosaccharide permissions. In our view, this position is inconsistent with existing permissions of these novels foods. As summarized in section 1.3.2 of

the Call for Submissions, 2'-FL alone, or with LNnT, is permitted for use in infant formula products in numerous countries (*e.g.*, European Union, United States, Singapore, Israel) without conditions related to other oligosaccharides. Developing permissions for novel foods that are inconsistent with existing permissions presents challenges to the recognized importance of harmonized food standards as globalization of the food supply continues.

The concentration of HMOs present in human milk averages between 7 and 11 g/L, depending on geographical location (1). The variety of HMOs present in human milk also provides extensive historical evidence that these concentrations, as well as infinite combinations of individual HMOs, present no safety concerns. Additionally, studies evaluating the tolerance of infants fed milk-based formulations containing 2'-FL and GOS, as well as 2'-FL and FOS have shown the formulas were well tolerated (2, 3). Marriage et al., 2015 included three experimental milk-based formulas each contained a total of 2.4 g oligosaccharides/L as GOS alone or combined with 2'-FL. All formulas were well tolerated (2). Kajzer et al., 2016 evaluated the tolerance of infants fed a milk-based formula containing 2'-FL and FOS. The study concluded that the experimental formula was safe and well tolerated based on reported outcomes similar to those of infants fed human milk or formula without oligosaccharides (3).

If a combined limit for HMOs and existing GOS and IFT in infant formula products and FSFYC is preferred Abbott Nutrition proposes referencing the limit established for the combined use of GOS and IFT for infant formula products and FSFYC (e.g., 8 g/L).

**Specifications for 2'-FL and LNnT**

FSANZ proposes to set specification for 2'-FL and LNnT using those provided by the applicant. Abbott Nutrition supports the inclusion of specifications but notices that the proposed specification for 2'-FL (presented in sections 2.3 and 2.4 of FSANZ SD1 for this Call for Submissions) are no longer aligned with the specifications in the revised EU regulation. Abbott Nutrition requests that the 2'-FL specifications incorporated into Schedule 3 are aligned with Commission Implementing Regulation (EU) 2018/1023 of 23 July 2018 correcting Implementing Regulation (EU) 2017/2470 establishing the Union list of novel foods. And, that both sets of specifications be included. If one set of specifications is preferred Abbott Nutrition proposes the most inclusive set be adopted.

Additionally, Abbott Nutrition does NOT support the inclusion of methods of analysis to Schedule 3. Eliminating the methods of analysis would further align ingredient permissions with the revised EU regulation.

**CONCLUSION**

In the absence of public health and safety concerns and considering the evidence supporting health benefits, Abbott Nutrition:

- agrees with FSANZ's conclusion regarding public health and safety concerns associated with 2'-FL and LNnT
- supports FSANZ's proposal to permit a maximum of 2.4 g/L for 2'-FL alone, or in combination with LNnT, with no more than 0.6 g/L of LNnT
- does NOT support the prohibition of use with existing oligosaccharide permissions
- requests that the specifications within Schedule 3 are aligned with EU 2017/2470
- does NOT support the inclusion of methods of analysis within Schedule 3.

**References**


1. McGuire MK, Meehan CL, McGuire MA, et al. What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. *Am J Clin Nutr* 2017;105:1086–100.
2. Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA. Infants Fed a Lower Calorie Formula With 2'FL Show Growth and 2'FL Uptake Like Breast-Fed Infants. *Journal of pediatric gastroenterology and nutrition*. Dec 2015;61(6):649-658.
3. Kajzer J, Oliver J, Marriage B. Gastrointestinal tolerance of formula supplemented with oligosaccharides. *FASEB J*.2016;30(1Supp):671.

See corresponding editorial on page 1027.

# 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 ± 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*-neotetrose 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 was registered at clinicaltrials.gov as NCT02670278. *Am J Clin Nutr* 2017;105:1086–100.

**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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[2] Supplemental 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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[23] Abbreviations used: DFLNT, difucosyllacto-*N*-tetrose; DSLNH, disialyllacto-*N*-hexaose; DSLNT, disialyllacto-*N*-tetraose; $ET_R$, rural Ethiopia; $ET_U$, urban Ethiopia; FDSLNH, 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; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetrose; 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-$\alpha$-L-fucosyltransferase 2 (*FUT2*) and galactoside 3/4-L-fucosyltransferase (*FUT3*) genes, which influence the presence or absence of $\alpha$1-2-fucosylated and $\alpha$1-3/4-fucosylated HMOs, respectively, as well as many other HMO structures (30, 31). Perhaps the most relevant study to date in which the interpopulation variation in HMO profiles was investigated was conducted by Erney et al. (32), who compared neutral oligosaccharides in milk that were collected from 435 women who were living in 10 countries. Although the authors recognized that, in some cases, small sample sizes within a country made comparisons and generalizations difficult, several findings were of significance. For instance, 2′-fucosyllactose was quantifiable in every milk sample that was collected in Mexico ($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*-tetrose (DFLNT), disialyllacto-*N*-hexaose (DSLNH), disialyllacto-*N*-tetraose (DSLNT), fucodisialyllacto-*N*-hexaose (FDSLNH), fucosyllacto-*N*-hexaose (FLNH), lacto-*N*-fucopentaose (LNFP) I, LNFP II, LNFP III, lacto-*N*-hexaose, lacto-*N*-neotetraose (LNnT), lacto-*N*-tetrose (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). $\alpha$-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/m$^2$)].

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 population[1]

| | Ethiopia | | Gambia | | | | | | United States | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | 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 ($n = 19$) |
| 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$^{a–c}$ | 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$^{a–d}$ | 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 | 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$^{a,b}$ | 60.1 ± 1.5$^{b,c}$ | 65.5 ± 2.0$^{a,b}$ | 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$^{c,d}$ | 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$^{a–d}$ | 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 world[1]

| | Ethiopia | | Gambia | | | | | | | United States | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| HMO | 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$^{a,b,c}$ | 184 ± 42$^{b,c}$ | 103 ± 16$^{c}$ | 162 ± 21$^{b,c}$ | 192 ± 33$^{b,c}$ | 195 ± 28$^{b,c}$ | 209 ± 32$^{a,b,c}$ | 206 ± 25$^{a,b,c}$ | 473 ± 55$^{a}$ | 122 ± 14$^{b,c}$ | 388 ± 47$^{a,b}$ |
| LNnT, nmol/mL | 838 ± 60$^{a,b,c}$ | 927 ± 67$^{a,b,c}$ | 1423 ± 117$^{a}$ | 781 ± 61$^{b,c}$ | 866 ± 70$^{a,b,c}$ | 1073 ± 103$^{a,b}$ | 588 ± 56$^{c}$ | 548 ± 45$^{c}$ | 854 ± 74$^{a,b,c}$ | 776 ± 42$^{b,c}$ | 793 ± 82$^{a,b,c}$ |
| 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$^{a,b}$ | 462 ± 35$^{a,b,c}$ | 585 ± 61$^{a,b}$ | 890 ± 88$^{a}$ | 435 ± 34$^{b,c}$ | 636 ± 63$^{a,b}$ | 504 ± 40$^{a,b,c}$ | 200 ± 24$^{d}$ | 402 ± 34$^{b,c,d}$ | 294 ± 49$^{c,d}$ |
| LNT, nmol/mL | 1304 ± 131$^{b,c}$ | 1408 ± 113$^{a,b}$ | 2265 ± 222$^{a}$ | 1576 ± 143$^{a}$ | 1882 ± 209$^{a}$ | 1632 ± 158$^{a}$ | 953 ± 139$^{b}$ | 1570 ± 119$^{a}$ | 2132 ± 210$^{a}$ | 1135 ± 91$^{a,b}$ | 1438 ± 198$^{a,b}$ |
| 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$^{a,b}$ | 1925 ± 201$^{a,b}$ | 1551 ± 190$^{a,b}$ | 1133 ± 89$^{a,b}$ | 1667 ± 167$^{b}$ | 1115 ± 105$^{a,b}$ | 2001 ± 227$^{a,b}$ | 1893 ± 205$^{a,b}$ | 2125 ± 216$^{a,b}$ | 1240 ± 154$^{a}$ |
| 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$^{a,b}$ |
| 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$^{a}$ | 82 ± 7$^{a}$ | 79 ± 14$^{a,b}$ |
| LSTc, nmol/mL | 101 ± 19$^{c}$ | 169 ± 15$^{a,b}$ | 159 ± 13$^{a,b}$ | 146 ± 20$^{a,b,c}$ | 246 ± 25$^{a}$ | 158 ± 22$^{a,b,c}$ | 182 ± 17$^{a,b}$ | 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$^{a,b}$ | 870 ± 68$^{a}$ | 477 ± 45$^{b,c}$ | 561 ± 57$^{a,b}$ | 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$^{a,b}$ | 35 ± 5$^{a,b}$ | 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$^{a,b,c}$ | 81 ± 11$^{b,c}$ | 64 ± 10$^{c}$ | 195 ± 21$^{a,b}$ | 115 ± 17$^{a,b,c}$ | 285 ± 32$^{a}$ | 98 ± 12$^{b,c}$ | 93 ± 11$^{a,b,c}$ |
| FDSLNH, nmol/mL | 158 ± 30$^{a,b}$ | 240 ± 26$^{a,b}$ | 197 ± 24$^{a,b}$ | 199 ± 33$^{a,b}$ | 182 ± 25$^{a,b}$ | 204 ± 25$^{a,b}$ | 245 ± 33$^{a,b}$ | 314 ± 38$^{a}$ | 83 ± 15$^{b}$ | 370 ± 48$^{a}$ | 70 ± 9$^{b}$ |
| DSLNH, nmol/mL | 50 ± 7$^{b}$ | 136 ± 12$^{a}$ | 109 ± 12$^{a,b}$ | 129 ± 20$^{a,b}$ | 126 ± 18$^{a,b}$ | 101 ± 11$^{a,b}$ | 108 ± 16$^{a,b}$ | 103 ± 11$^{a,b}$ | 55 ± 11$^{a,b}$ | 93 ± 12$^{a,b}$ | 33 ± 6$^{b}$ |
| Total, nmol/mL | 9748 ± 626$^{c}$ | 12,187 ± 519$^{a,b,c}$ | 13,732 ± 497$^{a,b}$ | 13,435 ± 613$^{a,b}$ | 11,307 ± 631$^{b,c}$ | 12,480 ± 628$^{a,b,c}$ | 14,474 ± 539$^{a,b}$ | 13,349 ± 645$^{a,b}$ | 15,998 ± 768$^{a}$ | 13,035 ± 502$^{a,b}$ | 15,606 ± 727$^{a,b}$ |

[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, difucosyllacto-*N*-hexaose; DFLNT, difucosyllacto-*N*-tetrose; 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*-tetrose; LSTb, sialyl-lacto-*N*-tetraose b; LSTc, sialyl-lacto-*N*-tetraose c; 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

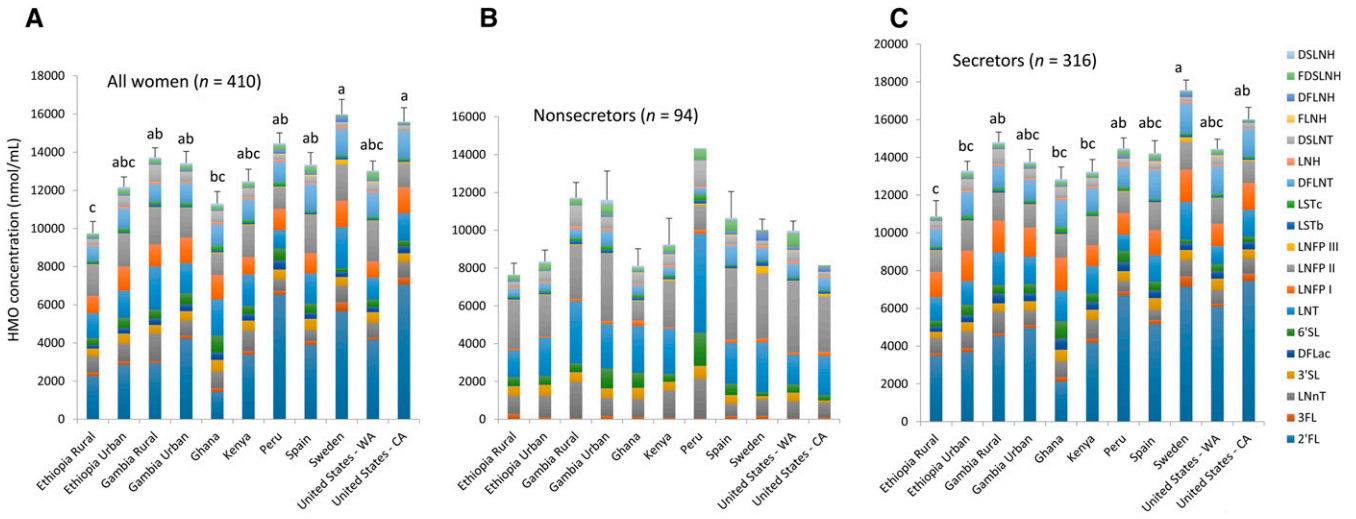


**FIGURE 1** Mean ± SEM absolute total and HMO isoform concentrations of all women combined (A), nonsecretors (B), and secretors (C). (A and B) Bars without a common lowercase letter represent total HMO values that differed with the use of Bonferroni-correction procedures for multiple comparisons. All 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*-tetrose; 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*-tetrose; 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

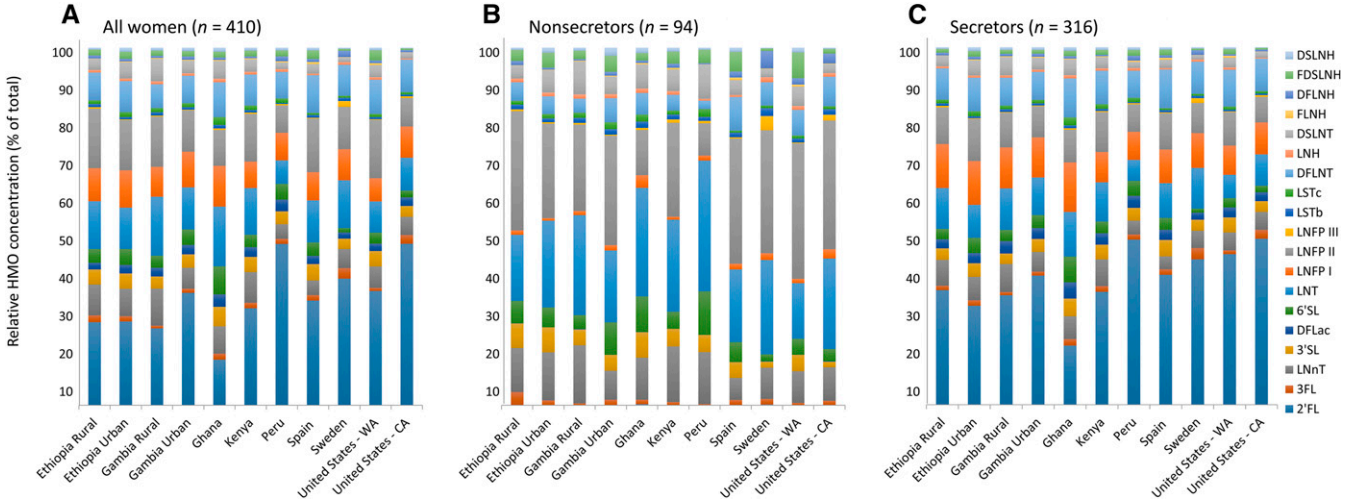


**FIGURE 2** Mean ± SEM relative abundance of HMO concentrations of all women combined (A), nonsecretors (B), and secretors (C) in each cohort. Note that there was only one nonsecretor subject each in Peru and United States - CA. CA, California; DFLac, difucosyllactose; DFLNH, difucosyllacto-*N*-hexaose; DFLNT, difucosyllacto-*N*-tetrose; 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*-tetrose; 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 world[1]

| | Ethiopia | | Gambia | | | | | | | United States | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | 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) |
| 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 ± 73b$^{b,c}$ | 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$^{a,b,c}$ |
| $\alpha$-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 |
| $\alpha$-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$^{a,b}$ |
| $\alpha$-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 ± 53$^{b,c}$ | 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-correction 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, difucosyllactose; DFLNH, difucosyllacto-*N*-hexaose; DFLNT, difucosyllacto-*N*-tetrose; 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*-tetrose; LSTb, sialyl-lacto-*N*-tetraose b; LSTc, sialyl-lacto-*N*-tetraose c; 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

[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 LNnT + 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 $a$2,6-linked oligosaccharides ($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 $\alpha$1-2-fucosylated HMO were correlated ($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

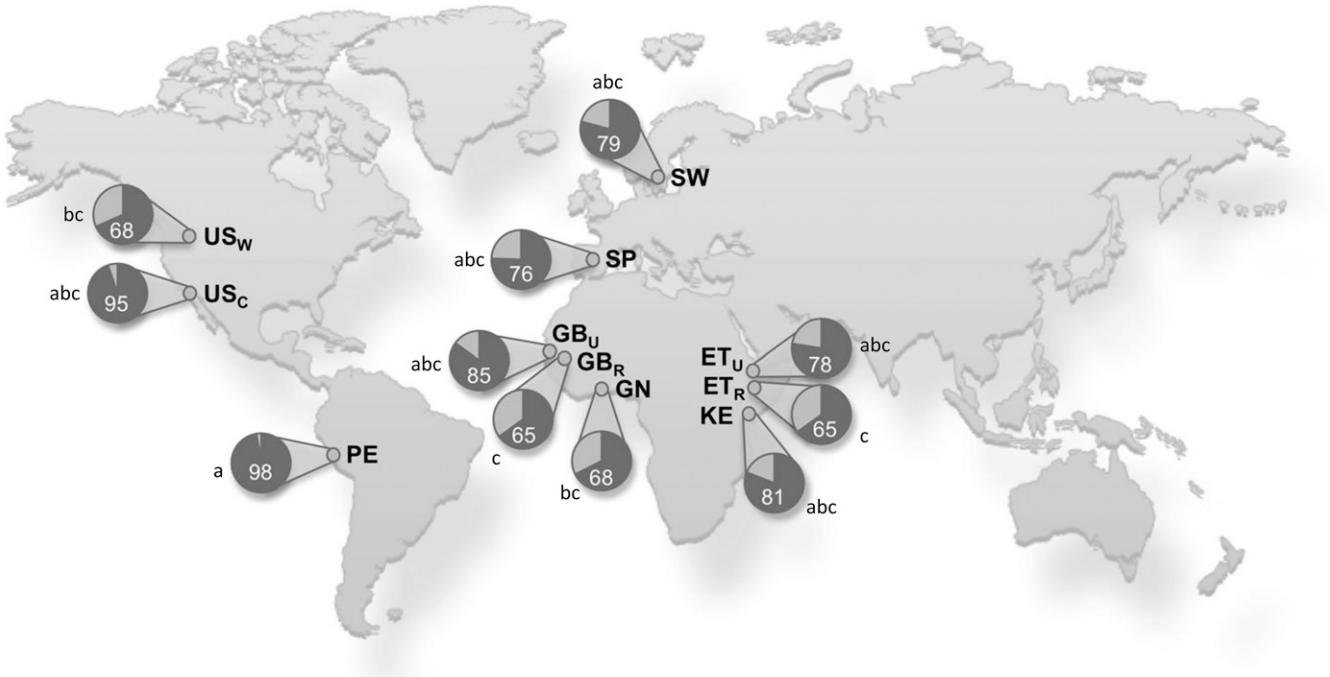


**FIGURE 3** Percentages of women in each cohort categorized as secretors. Cohorts that do not share a common lowercase letter differ ($P < 0.05$) in terms of their percentages of women who were secretors with the use of a chi-square test with Benjamini and Hochberg false-discovery-rate corrections. $ET_R$, 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.

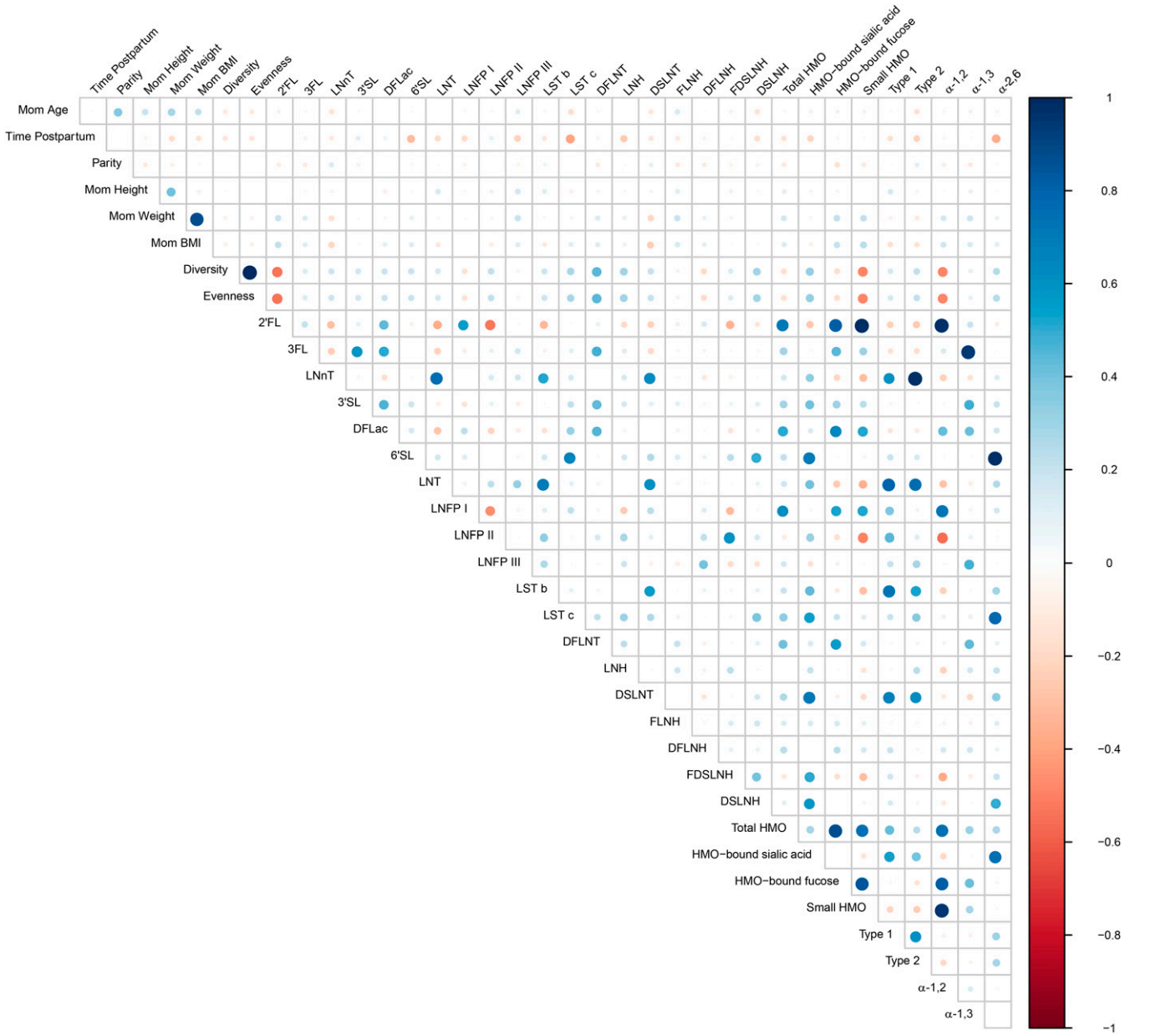


**FIGURE 4** Spearman rank correlations between selected maternal anthropometric, demographic, and reproductive variables and HMO types and groupings. Sizes of dots and colors indicate directionality (blue denotes positive; red denotes negative) and the strength of the association. Total HMO-bound sialic acid; total HMO-bound fucose; small HMO; type 1; type 2; $\alpha$-1,2; $\alpha$-1,3; and $\alpha$-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 + LNFPI + LNFPII + LSTb + DSLNT; LNnT + LNFPIII + LSTc; LNFP I + 2′FL; LNFP III + 3FL; and LSTb + LSTc + 6′SL, respectively. DFLac, difucosyllactose; DFLNH, difucosyllacto-*N*-hexaose; DFLNT, difucosyllacto-*N*-tetrose; 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*-tetrose; 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 world[1]

| | Ethiopia | | Gambia | | | | | | | United States | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | 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) |
| Shannon entropy | 2.06 ± 0.03$^{a,b,c}$ | 2.15 ± 0.03$^{a,b}$ | 2.07 ± 0.03$^{a,b,c}$ | 2.06 ± 0.04$^{a,b,c}$ | 2.27 ± 0.03$^{a}$ | 2.10 ± 0.04$^{a,b,c}$ | 1.94 ± 0.04$^{b,c}$ | 2.08 ± 0.03$^{a,b,c}$ | 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$^{a,b}$ | 8.14 ± 0.26$^{a,b}$ | 8.14 ± 0.31$^{a,b}$ | 9.84 ± 0.29$^{a}$ | 8.38 ± 0.27$^{a,b}$ | 7.25 ± 0.29$^{b}$ | 8.14 ± 0.20$^{a,b}$ | 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$^{a,b}$ | 5.74 ± 0.23$^{a,b,c}$ | 5.56 ± 0.29$^{b,c}$ | 7.31 ± 0.29$^{a}$ | 5.78 ± 0.26$^{a,b,c}$ | 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$^{a,b}$ | 0.43 ± 0.01$^{a,b}$ | 0.43 ± 0.02$^{a,b}$ | 0.52 ± 0.02$^{a}$ | 0.44 ± 0.01$^{a,b}$ | 0.38 ± 0.02$^{b}$ | 0.43 ± 0.01$^{a,b}$ | 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$^{a,b}$ | 0.30 ± 0.01$^{a,b,c}$ | 0.30 ± 0.02$^{b,c}$ | 0.38 ± 0.02$^{a}$ | 0.30 ± 0.01$^{a,b,c}$ | 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$^{a,b,c}$ | 0.73 ± 0.01$^{a,b}$ | 0.70 ± 0.01$^{a,b,c}$ | 0.70 ± 0.01$^{a,b,c}$ | 0.77 ± 0.10$^{a}$ | 0.71 ± 0.01$^{a,b,c}$ | 0.66 ± 0.01$^{b,c}$ | 0.71 ± 0.01$^{a,b,c}$ | 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 HMO[1]

| | Ethiopia | | Gambia | | | | | | | United States | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HMO | 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) | Overall |
| 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*-tetrose; 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*-tetrose; 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 HMOs[1]

| | BMI, kg/m$^2$ | | |
|---|---|---|---|
| HMO | 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*-tetrose; 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*-tetrose; 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.

**TABLE 7**
NMF scores describing parity for individual HMOs[1]

| | Parity, children, $n$ | | |
|---|---|---|---|
| HMO | 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*-tetrose; 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*-tetrose; 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 $\alpha$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 $\beta$1-3-linkage to derive type 1 chains (e.g., LNT) or attached in $\beta$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 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 HMOs[1]

| | Time postpartum, d | | | |
|---|---|---|---|---|
| HMO | 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*-tetrose; 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*-tetrose; 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.

**TABLE 9**
NMF scores describing maternal age quartiles for individual HMOs[1]

| | Maternal age, y | | | |
|---|---|---|---|---|
| HMO | 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*-tetrose; 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*-tetrose; 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.

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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# Infants Fed a Lower Calorie Formula With 2′FL Show Growth and 2′FL Uptake Like Breast-Fed Infants

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**ABSTRACT**

**Objectives:** The aim of the present study was to examine the growth and tolerance of infants fed infant formulas with a caloric density closer to human milk (HM) supplemented with human milk oligosaccharides (HMOs) and to study uptake of the HMOs.

**Methods:** A prospective, randomized, controlled, growth and tolerance study was conducted in healthy, singleton infants (birth weight ≥2490 g), who were enrolled by day of life (DOL) 5. Formula-fed infants were randomized to 1 of 3 formulas with a caloric density of 64.3 kcal/dL. Each formula contained galactooligosaccharides, and the 2 experimental formulas contained varying levels (0.2 and 1.0 g/L) of the HMO 2′-fucosyllactose (2′FL). The 3 formula groups were compared with an HM-fed reference group. Infants were exclusively fed either formula (n = 189) or HM (n = 65) from enrollment to 119 DOL. 2′FL was measured in the blood and urine collected from a subset of infants at DOL 42 and 119, and in HM collected from breast-feeding mothers at DOL 42.

**Results:** There were no significant differences among any groups for weight, length, or head circumference growth during the 4-month study period. All of the formulas were well tolerated and comparable for average stool consistency, number of stools per day, and percent of feedings associated with spitting up or vomit. 2′FL was present in the plasma and urine of infants fed 2′FL, and there were no significant differences in 2′FL uptake relative to the concentration fed.

**Conclusions:** This is the first report of infants fed 2′FL-fortified formulas with a caloric density similar to HM. Growth and 2′FL uptake were similar to those of HM-fed infants.

**Key Words:** galactooligosaccharides, growth, human milk oligosaccharides, infant formula, tolerance

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**What Is Known**

- Human milk oligosaccharides may provide protection and enhance the development of the gastrointestinal and immune systems of breast-fed infants.
- Human milk oligosaccharides represent the third largest solid component in human milk after lactose and lipids, levels ranging from 5 to 12 g/L in mature milk to >20 g/L in preterm colostrum.
- The caloric density of human milk is variable, and recent evidence suggests that its calorie content has been overestimated.

**What Is New**

- No significant differences in weight, length, and head circumference between infants fed human milk or 64.3 kcal/dL formulas from birth to 4 months of age have been noted.
- Formulas supplemented with 2′-fucosyllactose are well tolerated, and 2′-fucosyllactose absorption profiles are similar to those of breast-fed infants.

Human milk (HM) confers short- and long-term benefits to breast-fed infants, including reduced risk of otitis media, gastrointestinal (GI) and respiratory tract diseases, obesity, diabetes, atopic dermatitis, childhood leukemia, and sudden infant death syndrome (1). The precise features, however, of HM, which provide these advantages have not been clearly elucidated. Ongoing research is increasingly revealing the important role of human milk oligosaccharides (HMOs) in conferring protection and enhancing the development of breast-fed infants (2,3). HMOs represent the third largest solid component in HM after lactose and lipids, with levels ranging from ∼5 to 12 g/L in mature milk to >20 g/L in preterm colostrum (2,4,5). HMOs provide protection in a multitude of ways including enhancing the development of the immune system, binding pathogens and toxins to prevent their uptake, and enhancing the epithelial barrier function of the gut (3,6,7). These important biomolecules also play a role in shaping the intestinal microbiome. HMOs act as prebiotics, selectively promoting colonization by *Bifidobacterium bifidum*, which is prevalent in the intestines of HM-fed infants (2,5). Fucosylated HMOs also regulate neuronal dependent gut motility and may enhance cognition via the gut-brain axis (8).

Although the types and levels of HMOs vary considerably among women and between the stages of lactation, the major portion of HMOs include ∼20 structures, including 2′-fucosyllactose (2′FL) (2,9). The fucosylation of HMOs is determined by an individual's histo-blood group antigen status, specifically the Secretor and Lewis groups. Approximately 80% of the European and American populations are considered secretors, and women with this status secrete HMO structures containing (α1,2)-linked fucose, such as 2′FL (2,10,11). 2′FL is the most abundant HMO in this group, with levels ranging from 0.06 to 4.65 g/L (12,13).

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HMOs are largely indigestible by the intestinal enzymes of infants (14). In breast-fed infants, low levels of intact HMOs are found in the stools, having passed through the gut, and in their urine and plasma, having been absorbed into the bloodstream and excreted via the kidneys (9,10,15,16). Goehring et al (10) recently reported that HMOs, including 2′FL, are found in urine and plasma of breast-fed but not formula-fed infants, and that the levels of HMOs in plasma and urine correlate with those in mothers' breast milk; 2′FL was not present in the circulation of infants fed breast milk devoid of 2′FL.

An additional difference between HM and formula is caloric density and protein content. Currently, most commercially available infant formulas in the United States provide ~67.6 kcal/dL (20 kcal/fl oz), which was based on initial estimates of caloric density of mature HM. One of the earliest comprehensive reviews of HM composition from the National Research Council reported that HM calorie concentration ranged from 67.0 to 71.0 kcal/dL (17). The caloric density of HM is highly variable, and more evidence suggests that its calorie content has been overestimated (18). A systematic review of 22 studies that included 1088 HM samples revealed that the reported calorie content of mature HM ranges from $50.4 \pm 2.0$ to $78.2 \pm 3.5$ kcal/dL, with a mean of 65.2 kcal/dL (19). In addition, the review reported lower protein content (1.4 g/dL) in HM than that typically found in standard infant formulas (19). Reilly et al (20), in a review of 25 studies, reported the mean calorie content of HM to be 63.9 kcal/dL. These findings are consistent with HM values from the European Commission (65.1 kcal/dL) (21) and the Institute of Medicine (65.0 kcal/dL) (22). The American Academy of Pediatrics, on the advice of the Life Sciences Research Organization (LSRO), recommends that infant formula provide 63.0 to 71.0 kcal/dL (23). Regulations in Europe are based on the opinion of the Scientific Committee on Food (21), which specifies a minimum energy content of 60 kcal/dL and a maximum energy content of 70 kcal/dL for infant formula. At the request of the European Commission, however, the European Food Safety Authority recently delivered a scientific opinion stating that it is desirable that infant formulas be designed in a way that their calorie content tends toward the lower limit of the HM range (24).

We conducted a growth and tolerance trial that evaluated 3 infant formulas with a caloric density of ~64.3 kcal/dL (19 kcal/fl oz). This reduction in caloric density was achieved by reducing all of the macronutrients by ~5%; two of the formulas were also fortified with 2′FL. Plasma and urine samples were collected to quantitate systemic 2′FL in formula-fed and HM-fed infants. Results from the biological samples that were collected to evaluate immune and prebiotic effects associated with feeding 2′FL-fortified formulas will be reported separately.

# METHODS

## Study Design

This prospective growth and tolerance study was conducted at 28 sites throughout the United States from April 2013 through January 2014. Healthy, full-term infants were enrolled by 5 days of age. A subset of parents provided consent for optional biological sampling that included the collection of urine, stool, and blood from infants, and HM samples from breast-feeding mothers. Data are presented for the HMO levels in HM, urine, and plasma samples.

Infants whose parents intended to feed their infants formula exclusively were randomized to be fed a control formula (CF) or 1 of 2 experimental formulas (EFs) that were similar to the CF, except they contained levels of galactooligosaccharides (GOS) different from those in the CF, and they contained 2′FL at 0.2 or 1.0 g/L. 2′FL is a white powdered oligosaccharide (Inalco SpA, Milan, Italy) produced through a proprietary chemical synthesis. The total amount of oligosaccharides was 2.4 g/L in all of the formula groups. A nonrandomized HM-fed group was also enrolled.

Parents were asked to feed the assigned study formula or HM as their infant's sole source of nutrition until 119 days of age. The primary outcome variable was weight gain per day from day of life (DOL) 14 to 119, whereas secondary variables included measures of tolerance and other anthropometric measures. Supportive variables included additional infant and maternal demographics, formula intake, parents' responses to questions related to their satisfaction with the formula and their infant's behavior, the concentrations of 2′FL in HM, and infant plasma and urine and their relative absorptions.

Before enrollment, a parent or legally authorized representative of each enrolled infant signed a consent form approved by a central institutional review board for the protection of human subjects.

## Subjects

Inclusion criteria were singleton birth, gestational age 37 to 42 weeks and birth weight ≥2490 g. Subjects were eligible if they were between 0 and 5 days of age at enrollment, had exclusively been fed either formula or HM since birth, were judged to be in good health, as determined from the infant's medical history and parental report, and were from smoke-free homes. Mothers of infants in the HM-fed group were instructed not to smoke during the study period, and other household members for all of the subjects were not to smoke in the home. Exclusion criteria were an adverse maternal, fetal, or infant medical history considered by investigators to have potential effects on tolerance, growth, and/or development. This included, but was not limited to, suspected maternal substance abuse. Gestational diabetes was acceptable if the infant's birth weight was equal to or less than the 2010 World Health Organization (WHO) Growth Charts 95th percentile. Infants of mothers who intended to use a combination of breast- and formula-feeding, and infants who had been treated with antibiotics other than those administered in eye drops at birth were excluded. Infants receiving medications (including over-the-counter medications such as Mylicon for gas [McNeil Consumer Pharmaceuticals, Washington, PA]), home remedies (such as juice for constipation), herbal preparations, probiotics, or rehydration fluids that might affect GI tolerance were not to be enrolled unless both the parent and the physician agreed to discontinue the use of these agents before enrollment. The use of these products was discouraged for the duration of the study, as was the provision of solid foods.

## Diets and Concomitant Treatments

Infants were fed 1 of the 4 diets. The 3 formulas were targeted to contain 64.3 kcal/dL (19 kcal/fl oz) (Table 1), and their composition was similar to that of a milk-based commercially available formula. The CF contained 2.4 g/L GOS. The 2 EFs were similar to the CF but contained either 0.2 g/L 2′FL and 2.2 g/L GOS (EF1) or 1.0 g/L 2′FL and 1.4 g/L GOS (EF2). The total amount of nondigestible oligosaccharides was similar for all of the 3 formulas (ie, 2.4 g/L). Infants in the HM-fed group were fed their mothers' own milk by breast and/or bottle. The 3 formulas were similar in appearance, consistency, and odor. The formulas were provided in ready-to-feed 32 fl oz bottles, each of which had a unique 7- character product code to ensure that parents and investigators were not aware of the formula identification. Parents were instructed to feed the assigned formulas ad libitum and to supplement infants with water ad libitum. All of the formulas met the levels of nutrients for the population as recommended by the

TABLE 1. Energy, macronutrient, GOS, and 2′FL concentrations in the control and EFs

| Ingredient | CF | EF 1 | EF 2 |
|---|---|---|---|
| Energy, kcal/dL | 64.3 | 64.3 | 64.3 |
| Protein | 13.3 | 13.3 | 13.3 |
| Fat | 34.7 | 34.7 | 34.7 |
| Total carbohydrate | 69.0 | 69.0 | 69.0 |
| GOS | 2.4 | 2.2 | 1.4 |
| 2′FL | — | 0.2 | 1.0 |

All values are expressed as g/L unless otherwise indicated. 2′FL = 2′-fucosyllactose; CF = control formula; EF = experimental formula; GOS = galactooligosaccharides.

American Academy of Pediatrics Committee on Nutrition (25) and as regulated by the Infant Formula Act of 1980 (26) and subsequent amendments (27).

At the time of enrollment, parents confirmed their intent to feed the study formula or HM as the sole source of nutrition for the duration of the study, unless instructed otherwise by their health care professional.

Before enrollment, infants in the formula-fed groups were not to have received any HM (mother's or donor milk), and infants in the HM-fed group were not to have received any formula or donor milk. Vitamin and mineral supplements (excluding vitamin or mineral supplements containing vitamin D for infants in the HM group or as recommended by a health care professional) were not to be given during the study period as the study formulas were nutritionally complete.

### Evaluable Data

The following criteria were used to define evaluable data: from enrollment throughout the study period, formula-fed infants were not to receive alternate feedings other than assigned study product for more than a total of 5 days, or consume rehydration or receive intravenous fluids for more than a total of 3 days. Foods, juices, vitamin, and/or mineral supplements (excluding vitamin or mineral supplements containing vitamin D for infants in the HM-fed group or as recommended by a health care professional) or other sources of nutrition were not to be used for >5 consecutive days or a total of 10 days. Medications (including over-the-counter medications such as Mylicon), home remedies, herbal preparations, or probiotics that may affect GI tolerance were not to be used for more than a total of 2 days.

For the optional biological sampling conducted in a subset of study infants, from enrollment throughout the study period, formula-fed infants were not to receive >8 fl oz of an alternate feeding (HM or formula other than their assigned study formula, or >2 feedings via breast) per week. The HM-fed infants were not to receive >8 fl oz of infant formula or donor milk per week. For 48 hours before the collection of urine samples, formula-fed infants were not to consume any feedings other than the assigned study formula, and HM-fed infants were not to consume any formula or donor milk.

## Randomization

Sealed envelopes containing the group assignment for formula-fed infants were prepared from computer-generated randomization schedules prepared by the sponsor. Randomization was stratified by site and sex, with each center having its own randomization schedule. Enrollment was competitive, and no goals were set for the individual sites.

## Study Visits

At the enrollment visit, prestudy feeding regimens, present infant medication/supplement use, birth anthropometric measurements, and gestational age were recorded, and present length, weight, and head circumference were measured. Demographic data were collected, including race, number, and ages of siblings in the home, and mode of delivery. Data regarding maternal medication/supplement use, prepregnancy height and weight, and maternal weight gain during pregnancy were recorded for the HM group. Eligible subjects were randomized to one of the formula groups or enrolled into the HM group. The parents were instructed to exclusively feed HM or begin feeding the assigned study formula as the first feeding following enrollment.

After enrollment, infants were seen at 5 additional clinic visits at DOL 14, 28, 42, 84, and 119. The DOL 14, 28, 42, and 84 visits had a window of ±3 days, and the DOL 119 visit had a window of ±5 days. At each visit, growth was measured, and detailed interval diet and clinical histories were taken that included any adverse events, changes in mother's intake of medications/supplements (HM group), smoking status in the home, and whether the infant had received any medications, home remedies, or nonstudy feedings.

### Anthropometric Measures

Research staff was trained to weigh and measure infants. A video explaining procedures for obtaining accurate anthropometric measures was provided to each site, and completion of staff training on measuring anthropometrics was documented. All of the measurements were made twice, with a third being made if the difference between the 2 measurements exceeded defined limits. Infant weights were measured to the nearest 10 g using a digital, electronic scale. The scale was calibrated annually by a qualified technician, accuracy testing was performed before infants were weighed, and a log of scale weight checks was maintained. Individual infant's growth was plotted on WHO growth charts. Infant length and head circumference were measured to the nearest 0.1 cm.

### Tolerance Measures

At enrollment and the DOL 42, 84, and 119 visits, the parents were given intake and stool records, and thorough instructions regarding their proper completion. The parents recorded detailed, 24-hour information about the volume of formula consumed at each feeding or the number of HM feedings, incidence of spitting up and vomiting associated with feedings, and each infant's stool characteristics (frequency, consistency, and color). Records were maintained by parents starting with the first feeding after enrollment, continuing until DOL 28, and for 3 consecutive days before the DOL 42, 84, and 119 visits. The study staff reviewed the completed forms with the parents at each visit to ensure they were completed correctly and thoroughly. Parents also completed infant feeding and stool patterns questionnaires and formula satisfaction questionnaires (formula-fed group only) at the DOL 28 and 119 visits. Parents completed an infant behavior questionnaire at the DOL 119 visit.

### Breast Milk Samples

At the DOL 28 visit, breast-feeding mothers who consented to the optional biological sampling were given kits for the collection of breast milk at home within 24 hours of the DOL 42 visit. Alternatively, samples were collected at the DOL 42 visit. Breast

milk samples were not collected at DOL 119. For each collection, a 20-mL mid-milk sample was collected from 1 breast starting ~5 minute after the infant had begun sucking or the breast had begun to be pumped. The goal was to collect the sample at the regular feeding time of the infant, with a 2-hour gap since the previous feeding. The time of HM collection, the time that the breast being used for collection was last used for feeding, and the time the mother ate her last meal were recorded. Samples collected at home were collected within 2 hours of the study visit. They were immediately placed into the provided insulated cooler bag and refrigerated. Frozen ice packs were added to the cooler bag before transport to the study visit. Samples were stored frozen at the clinic sites (−20°C) before being transported to the central laboratory on dry ice.

### Urine Samples

At the DOL 28 and 84 visits, parents who consented to the optional biological sampling were given urine sample collection kits, and samples were collected during the DOL 42 and 119 visits. Parents were instructed to place a urine collection pad in front of a clean diaper within 1 hour of their scheduled study visit. During the visit, study staff extracted 2 individual samples of urine, each a minimum of 1 mL, by placing a syringe tip into a wet area of the pad and withdrawing the plunger; samples were then transferred to a vial. Urine samples contaminated by feces were not collected. Samples were immediately frozen and stored at −20°C before being shipped to the central laboratory on dry ice. The parents were asked if their infant had received any alternate feedings within the previous 48 hours. If they had, parents were told to continue the assigned study feeding, and the sample collection was rescheduled within the study window or within 3 days, whichever was greater. At the time of the rescheduled sample collection, parents were again asked whether the infant had received any alternate feedings; if they had, the sample was not collected.

### Blood Samples

At the DOL 42 and 119 visits, parents who consented to the optional biological sampling were asked whether their infant had received any alternate feedings or oral nonsteroidal anti-inflammatory medications within the previous 48 hours or had a present respiratory tract infection. If they had, parents were told to continue the assigned study feeding, and the sample collection was rescheduled within the study window or within 3 days, whichever was greater. In addition, mothers of HM-fed infants were not to have used nonsteroidal anti-inflammatory drugs within 48 hours of the blood collection. During the visit, 2 to 3 mL of nonfasting venous blood was drawn by a trained nurse into sodium heparin vacutainer tubes. Blood samples were shipped at ambient temperature to the laboratory and received within 24 hours of collection. Plasma was obtained by standard centrifugation procedure, dispensed into small plastic vials, and stored at −80°C until analysis.

### Stool Samples

At the DOL 42 and 119 visits, parents who consented to the optional biological sampling also provided stool samples from their infants. This data will be used to examine the concentration of IgA, characterization of microbiota, and characterization of biological factors influential to GI health. This data will be presented in a subsequent publication.

### 2′FL Analyses

The plasma, urine, and HM samples were stored at less than −20°C and shipped frozen for analysis to Metabolon, Inc (Durham, NC). No more than 1 sample per subject per time point was analyzed for plasma, urine, and HM. HM samples were first diluted 1:500 in water. All of the samples were spiked with an internal standard and subjected to protein precipitation with methanol. Following centrifugation, supernatant was removed. Plasma supernatant was further evaporated to dryness and reconstituted in methanol:water (75:25, vol/vol). Aliquots of urine and HM supernatant, reconstituted plasma extract, and freshly prepared calibration standards were injected onto an Agilent 1290/AB Sciex QTrap 5500 liquid chromatography with tandem mass spectrometry system (AB Sciex, Framingham, MA) equipped with a BEH Amide UHPLC column (Waters Corporation, Milford, MA). Data were acquired using electrospray ionization in negative ionization mode. 2′FL concentrations were calculated based on the area ratios of 2′FL and internal standard peaks using a weighted (1/×) least squares regression analysis generated from external calibration standards included in each run.

### 2′FL Uptake

The relative absorption of 2′FL from the diet was estimated by dividing the concentration of 2′FL in the plasma by the concentration of 2′FL in the formula or HM. Relative excretion was estimated by dividing the concentration of 2′FL in the urine by the concentration of 2′FL in the feed (10).

## Statistical Analysis

The sample size was calculated by using the software package nQuery Advisor 5.0 (Statistical Solutions Ltd, Cork, Ireland). The study hypothesis was that growth would be similar between the control and the 2 experimental feeding groups. The trial was designed to show noninferiority instead of superiority. Therefore, in calculating sample size and power, no adjustment was made for the number of groups (multiple comparisons) that were studied. A sample size of 64 subjects in each formula feeding group has 80% power to detect a difference in means of ≥3 g/day assuming that the common standard deviation is 6 g/day, using a 2-group *t* test with a 0.05 2-sided significance level. With an assumed attrition rate of 30%, the target enrollment was ~92 subjects per formula feeding group. In addition, a HM reference group was enrolled with approximately the same number of subjects as each of the formula feeding groups. Therefore, the targeted number of subjects in the study was 368. Subjects were added to the study to obtain the targeted number of laboratory samples.

Three sets of models were fitted for most of the variables (model 1 CF vs EF1, model 2 CF vs EF2, and model 3 all 4 study groups). Analysis of variance (ANOVA) was used in baseline comparisons of continuous variables, whereas Cochran-Mantel-Haenszel test statistics were used for baseline comparisons of categorical variables. Anthropometric and intake data were compared among treatment groups using ANOVA techniques, including analysis of covariance and repeated measures analyses. If there was an overall significant treatment group effect (or significant treatment group interaction), then least squares means were compared between each pair of treatment groups and adjusted for multiple comparisons by using the step-down Bonferroni adjustment. Questionnaire and adverse event data were compared among groups using categorical analyses such as Cochran-Mantel-Haenszel and Fisher exact test.

2′FL concentrations and relative absorption for infant plasma were compared among treatment groups using ANOVA. 2′FL concentrations for infant urine and relative excretion for infant urine were compared among treatment groups using nonparametric methods (Kruskal-Wallis test and Wilcoxon rank-sum test). Change

in 2′FL concentrations from 42 to 119 days in infant plasma and urine were calculated using paired *t* tests separately for each treatment group. SAS version 9.2 (SAS Institute, Cary, NC) was used to perform the statistical analyses.

## RESULTS

Of the 424 infants enrolled, 420 were included in the intent-to-treat analysis (101 CF, 104 EF1, 109 EF2, and 106 HM); 4 subjects were excluded from the intent-to-treat group because they never received any study product. A total of 338 infants completed the study (84 CF, 81 EF1, 83 EF2, and 90 HM), 304 of whom completed the study on the assigned feeding or HM (79 CF, 70 EF1, 72 EF2, and 83 HM). The number of premature discontinuations of the study formulas was not different among the formula-fed groups. There were no significant differences among feeding groups for age at enrollment, sex, weight, length, or head circumference at birth, and mode of delivery except a significant difference between CF and HM for age at enrollment ($P = 0.020$). There was a significant difference between the EF2 and HM groups with respect to race, with HM having more infants that were white and EF2 having more infants that were black or of other races (Table 2). The remaining study results (Tables 3 and 4 and Figs. 1 and 2) are based on infants who completed the study per protocol and whose data were included in the evaluable analyses.

### Growth

There were no significant differences (sex-specific or sex-combined) in mean weight, length, or head circumference among feeding groups during the study, and no significant differences among feeding groups in mean gains in these measures from DOL 14 to 119 (Table 3). Secondary analyses of the sex-combined data for several shorter time periods revealed that from DOL 14 to 28, the HM group gained significantly more weight than the EF1 group ($P = 0.016$), and from DOL 84 to 119 the EF2 group gained significantly more weight than the HM group ($P = 0.022$) (Table 3). Sex-specific weight-, length-, and head circumference-for-age percentiles are shown in Figure 1.

### Intake

The mean daily volume of study formula consumed from enrollment to DOL 28 and for the 3-day periods before the DOL 42, 84, and 119 visits was similar between the CF, EF1, and EF2 groups (data not shown), with the exception of the period from enrollment to DOL 28, during which the CF group consumed significantly more formula than the EF1 group ($661 \pm 17$ vs $614 \pm 18$ mL/day [least squares means ± standard error of the mean], respectively, $P = 0.024$).

### Tolerance

The mean number of stools per day was significantly greater for the HM group versus the CF, EF1, and EF2 groups from enrollment to DOL 28 ($4.9 \pm 0.2$, $2.0 \pm 0.1$, $2.2 \pm 0.2$, and $2.5 \pm 0.2$, respectively, $P < 0.0001$) and for the 3-day periods before DOL 42 ($3.8 \pm 0.2$, $1.4 \pm 0.1$, $1.4 \pm 0.1$, and $1.5 \pm 0.1$, respectively, $P < 0.0001$) and DOL 84 ($2.6 \pm 0.2$, $1.4 \pm 0.1$, $1.4 \pm 0.1$, and $1.4 \pm 0.1$, respectively, $P = 0.004$), and it was higher for the HM group versus the CF group for the 3-day period before DOL 119 ($2.0 \pm 0.2$ and $1.2 \pm 0.1$, respectively, $P = 0.008$). The percent of feedings with spitting up or vomit within 1 hour of feeding was quite variable among groups, but from the enrollment to DOL 28 it was significantly higher in the CF, EF1, and EF2 groups versus the HM group ($17.5 \pm 2.6$, $21.5 \pm 2.9$, $18.0 \pm 2.5$, and $10.5 \pm 1.6$, respectively, $P \leq 0.05$). There were no differences among all of the groups after DOL 28. The mean rank stool consistency (MRSC) (1 = watery, 5 = hard) was significantly greater for the EF2 ($2.26 \pm 0.05$) versus HM group ($2.04 \pm 0.05$) from enrollment to DOL 28 ($P = 0.021$). Repeated measure analysis during the DOL

TABLE 2. Demographic and clinical characteristics of infants fed CF, EF with different levels of GOS and 2′-fucosyllactose (EF1 or EF2), or HM

| Characteristic | CF n = 101 | EF1 n = 104 | EF2 n = 109 | HM n = 106 |
|---|---|---|---|---|
| Age at enrollment, days* | 3.8 ± 0.1 | 3.5 ± 0.1 | 3.7 ± 0.1 | 3.4 ± 0.1 |
| Males, n (%) | 51 (50) | 51 (49) | 54 (50) | 57 (54) |
| Gestational age, wk | 39.3 ± 0.1 | 39.2 ± 0.1 | 39.2 ± 0.1 | 39.3 ± 0.1 |
| Birth weight, g | | | | |
| Males | 3453 ± 60 | 3306 ± 61 | 3344 ± 53 | 3480 ± 62 |
| Females | 3327 ± 66 | 3311 ± 66 | 3240 ± 55 | 3397 ± 59 |
| Birth length, cm | | | | |
| Males | 50.9 ± 0.3 | 50.8 ± 0.3 | 51.2 ± 0.3 | 51.5 ± 0.3 |
| Females | 50.7 ± 0.3 | 50.1 ± 0.3 | 50.1 ± 0.3 | 50.8 ± 0.4 |
| Birth head circumference, cm | | | | |
| Males | 34.9 ± 0.3 | 34.3 ± 0.3 | 34.4 ± 0.2 | 34.8 ± 0.3 |
| Females | 34.0 ± 0.4 | 33.7 ± 0.3 | 33.6 ± 0.3 | 34.0 ± 0.3 |
| Race, n (%)† | | | | |
| White | 71 (70) | 71 (68) | 62 (57) | 83 (78) |
| Black | 23 (23) | 21 (20) | 31 (28) | 15 (14) |
| Other | 7 (7) | 12 (12) | 16 (15) | 8 (8) |
| Mode of delivery, n (%) | | | | |
| Vaginal | 65 (64) | 74 (71) | 80 (73) | 80 (75) |
| Cesarean section | 36 (36) | 30 (29) | 29 (27) | 26 (25) |

Data represent the mean ± SEM unless otherwise indicated. CF = control formula; EF = experimental formula; GOS = galactooligosaccharides; HM = human milk.

*Significant difference between CF and HM, $P = 0.020$.

† Significant difference between EF2 and HM, $P = 0.029$.

TABLE 3. Gains in weight, length, and head circumference from DOL 14 to 119 in infants fed CF, EF (EF1 or EF2), or HM

| Characteristic | CF | EF1 | EF2 | HM |
|---|---|---|---|---|
| Weight gain, g/day* | | | | |
| Males (n) | 30.4 ± 1.2 (34) | 30.2 ± 1.3 (29) | 31.4 ± 1.0 (30) | 30.9 ± 1.1 (33) |
| Females (n) | 26.8 ± 0.8 (34) | 25.8 ± 1.1 (33) | 26.5 ± 1.2 (29) | 25.0 ± 1.0 (32) |
| Length gain, cm/day | | | | |
| Males (n) | 0.106 ± 0.003 (32) | 0.107 ± 0.003 (28) | 0.109 ± 0.002 (30) | 0.102 ± 0.004 (32) |
| Females (n) | 0.097 ± 0.003 (34) | 0.102 ± 0.003 (32) | 0.101 ± 0.004 (29) | 0.095 ± 0.002 (32) |
| Head circumference gain, cm/day | | | | |
| Males (n) | 0.061 ± 0.001 (31) | 0.059 ± 0.002 (28) | 0.061 ± 0.002 (30) | 0.057 ± 0.001 (32) |
| Females (n) | 0.055 ± 0.001 (33) | 0.056 ± 0.002 (31) | 0.053 ± 0.003 (29) | 0.049 ± 0.001 (31) |

Data represent the mean ± SEM. Sex-combined differences in gains during different time intervals are shown in footnotes. CF = control formula; DOL = day of life; EF = experimental formula; HM = human milk; LSM = least squared means.

* From DOL 14 to 28, HM > EF1 (43.6 ± 1.8 vs 36.7 ± 1.9 g/day [LSM ± SE]; $P = 0.016$). From DOL 84 to 119, EF2 > HM (25.0 ± 1.2 vs 20.5 ± 1.2 g/day [LSM ± SE]; $P = 0.022$).

42, 84, and 119 visits revealed that the MRSC was significantly greater for the formula groups versus the HM group (CF > HM, $P = 0.004$; EF1 > HM, $P = 0.001$; EF2 > HM, $P = 0.009$). MRSC was not significantly different among the 3 formulas groups. The range of MRSC from enrollment to DOL 28 to 119 for the formula groups was as follows: CF 2.2 ± 0.06 to 2.4 ± 0.09, EF1 2.21 ± 0.06 to 2.38 ± 0.09, and EF2 2.26 ± 0.05 to 2.404 ± 0.11.

## Safety

There were no significant differences in the overall percentage of subjects with adverse events or serious adverse events in the CF versus the EF1 or EF2 groups. The CF and EF2 groups had significantly more subjects with reported adverse events in the ''infections and infestations'' category compared with the EF1 group ($P < 0.05$) with 28 and 38, respectively, compared with 11 in the EF1 group. The types of adverse events were similar among groups with a high proportion of upper respiratory tract symptoms, otitis media, viral infections, and oral candidiasis. Within this category, however, there were no significant differences among study groups for any specific preferred term. The CF group had a significantly higher percentage of subjects (n = 5) with reported eczema compared with the EF1 and EF2 groups who had zero ($P < 0.05$). Overall, there were no safety concerns noted with either of the EFs (EF1 and EF2).

## 2′FL Uptake

Levels of 2′FL in the DOL 42 and 119 plasma samples of infants fed CF were below the limit of detection; therefore, relative absorption and excretion data were not calculable. The mean plasma concentrations of 2′FL at DOL 42 were significantly different for each pair of treatment groups (HM > EF2 > EF1) and reflected the amounts in the feeds; however, relative absorption of 2′FL was similar (0.07%, 0.05%, and 0.05% for EF1, EF2, and HM, respectively; not significant) (Table 4; Fig. 2A). Mean urine concentrations were significantly different for each pair of treatment groups (HM, EF2 > EF1 > CF), with the exception of the EF2 and HM groups. Relative excretion was similar among the groups fed 2′FL (1.50%, 1.26%, and 1.35% for the EF1, EF2, and HM groups, respectively; not significant).

HM was not collected at DOL 119; therefore, relative absorption and excretion values for 2′FL were not calculated for the HM group. In contrast to DOL 42, mean plasma concentrations at DOL 119 were not significantly different for each pair of treatment groups. Relative absorption was similar between the EF1 and EF2 groups (0.02% and 0.03%, respectively; not significant). Mean urine concentrations were significantly different for each pair of treatment groups (HM, EF2 > EF1 > CF), with the exception of the EF2 and HM groups. Relative excretion was

TABLE 4. 2′FL concentrations in feeds, plasma and urine, and relative absorption and excretion of 2′FL

| DOL | Characteristic | Units | CF | EF1 | EF2 | HM |
|---|---|---|---|---|---|---|
| 42 | Feed (n) | g/L | 0* | 0.20* | 1.00* | 1.98 ± 0.17 (76) |
| | Plasma (n) | mg/L | <0.03 ± 0.00 (36) | $0.13^a$ ± 0.02 (32) | $0.52^b$ ± 0.07 (33) | $1.00^c$ ± 0.17 (36) |
| | Relative absorption† | % | NC | 0.07 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 |
| | Urine (n) | mg/L | $0.08^a$ ± 0.01 (59) | $3.00^b$ ± 0.33 (54) | $12.60^c$ ± 1.92 (61) | $35.55^c$ ± 6.89 (58) |
| | Relative excretion† | % | NC | 1.50 ± 0.17 | 1.26 ± 0.19 | 1.35 ± 0.23 |
| 119 | Feed (n) | g/L | 0* | 0.20* | 1.00* | NT |
| | Plasma (n) | mg/L | <0.03 ± 0.00 (12) | 0.05 ± 0.01 (12) | 0.29 ± 0.09 (14) | 0.43 ± 0.17 (11) |
| | Relative absorption† | % | NC | 0.02 ± 0.01 | 0.03 ± 0.01 | NC |
| | Urine (n) | mg/L | $0.09^a$ ± 0.01 (53) | $2.88^b$ ± 0.71 (45) | $11.18^c$ ± 1.95 (45) | $19.52^c$ ± 4.51 (54) |
| | Relative excretion† | % | NC | 1.44 ± 0.35 | 1.12 ± 0.19 | NC |

Groups were fed CF, EF with different levels of GOS and 2′FL (EF1 or EF2), or HM. Data represent the mean ± SEM unless otherwise indicated. DOL 119 breast milk sample not collected. Means with different superscripts are significantly different from each other, ($P < 0.05$). 2′FL = 2′-fucosyllactose; CF = control formula; DOL = day of life; EF = experimental formula; GOS = galactooligosaccharides; HM = human milk; n = number of samples tested (1 sample per subject per time point for each variable); NC = not calculable; NT = not tested.

* Concentration of 2′FL in formula, g/L.

† Concentration of 2′FL in plasma or urine relative to concentration in feed.

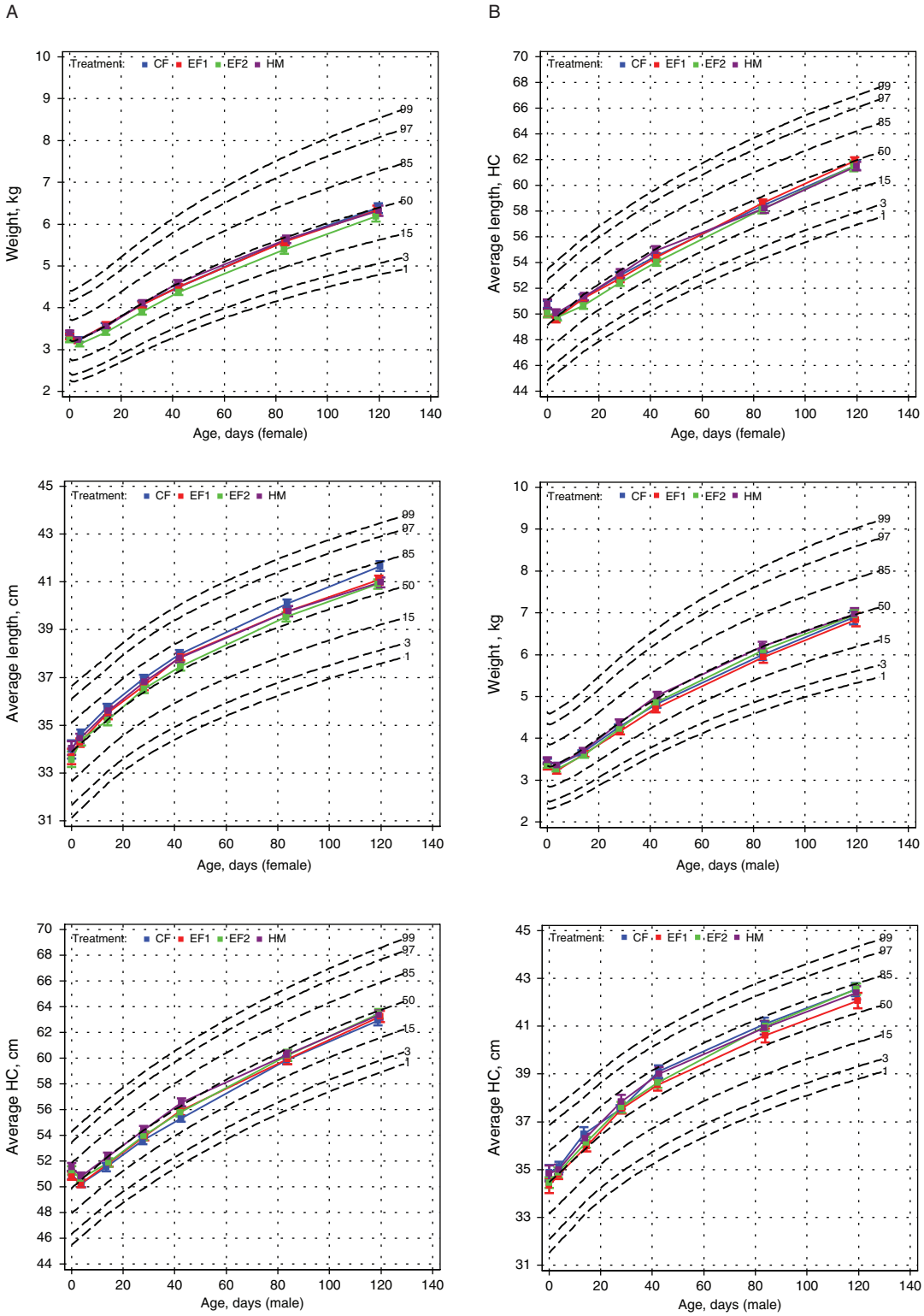


**FIGURE 1.** A and B, Weight, length, and head circumference growth of female (A) and male (B) infants plotted on WHO growth charts. CF = control formula; EF = experimental formula; HC = head circumference; HM = human milk; WHO = World Health Organization.

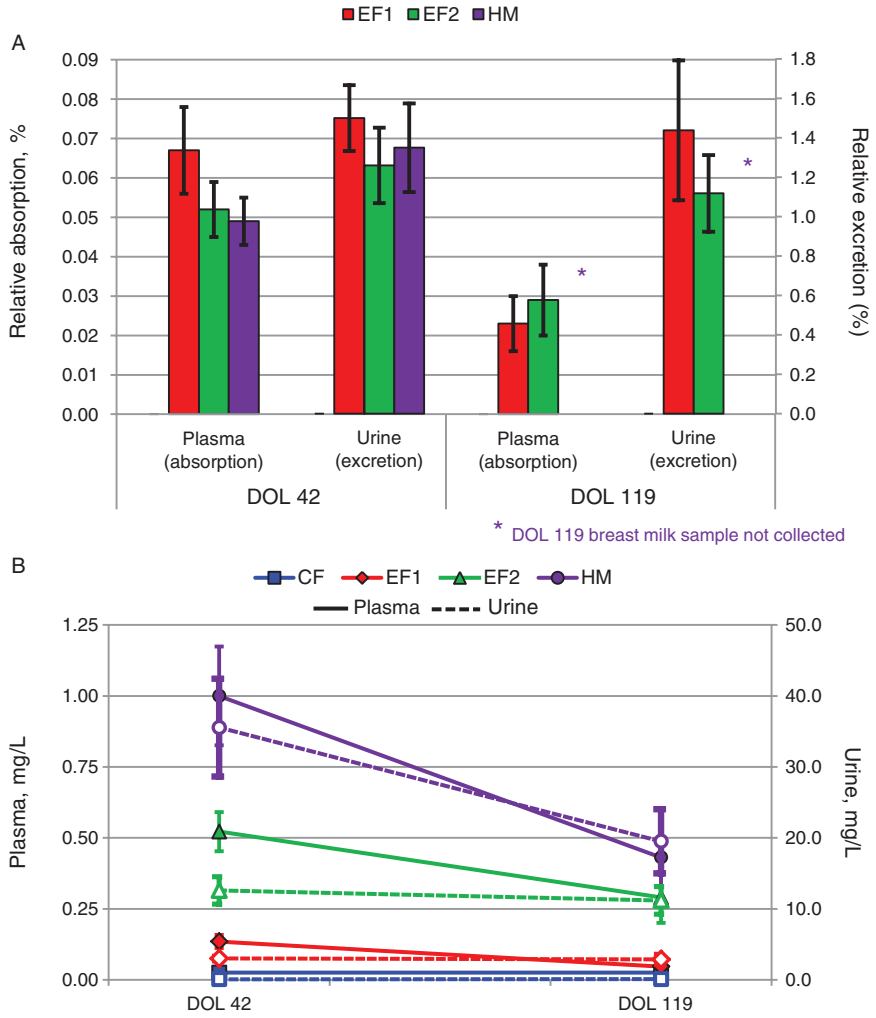


**FIGURE 2.** A and B, The relative absorption and excretion of 2′FL at DOL 42 and 119 (A) and the levels of 2′FL in plasma (solid line) and urine (dotted line) at DOL 42 and 119 (B). 2′FL = 2′-fucosyllactose; CF = control formula; DOL = day of life; EF = experimental formula; HM = human milk.

similar between the EF1 and EF2 groups, 1.44% for EF1 and 1.12% for EF2.

From DOL 42 to 119, plasma concentrations of 2′FL decreased significantly for the EF1, EF2, and HM groups ($P = 0.017$, 0.008, and 0.015, respectively). Urine concentrations decreased significantly for the HM group ($P = 0.018$) but did not change significantly for the EF1 and EF2 groups (Fig. 2B).

## DISCUSSION

To our knowledge, this is the first growth and tolerance study of infant formulas with a caloric density similar to that of HM. There were no significant differences in weight, length, and head circumference between infants fed HM or the 64.3 kcal/dL formulas during the 4-month study period. Each of the 3 formulas contained GOS and 2 contained 2′FL. All of the formulas were well tolerated, and the relative absorption and excretion of 2′FL were similar to those of HM-fed infants.

There are few published studies that report the feeding of infant formulas with caloric densities lower than standard formulas. Foman et al (28,29) published 2 studies nearly 40 years ago in which infants were fed diets with caloric densities much lower than that of HM; thus, their relevance to the present study is limited. More recently, Timby et al (30) randomly assigned infants <2 months of age to be fed a standard 66 kcal/dL formula or an experimental 60 kcal/dL formula until 6 months of age; the EF also contained bovine milk fat globule and had a lower protein content. Infants fed the EF ingested larger volumes of formula; however, there were no significant differences between groups in linear growth, weight gain, body mass index, percentage of body fat, or head circumference. In our study, the formula with lower caloric density was not associated with higher volumes of intake, compared with previous study data, which may be because of the more modest, 5% decrease in caloric density.

In a recent systematic review, we showed that in the first 2 weeks of life, infants fed standard formulas have a 1.2- to 9.5-fold greater energy intake and a 1.2- to 4.8-fold greater protein intake than breast-fed infants (24). Numerous studies have shown that formula-fed infants grow at a faster rate than HM-fed infants during early life (31–36). The slower growth rate of HM-fed infants may explain, in part, the long-term advantages of breast-feeding (37,38). Greater weight gain during early life has been associated with

adverse outcomes, including higher risk of obesity (34,39–41), hypertension (42), diabetes (43,44), and cardiovascular disease (38,45). The risk for these outcomes is reportedly higher in formula versus breast-fed infants (34,43,46–48). Despite accumulating data supporting a connection between higher weight gain early in life and later adverse outcomes, there is a paucity of data showing that formulas with a caloric density more similar to HM are safe and support adequate growth. In the present study, the growth patterns of infants fed the 64.3 kcal/dL formula were similar to those of HM-fed infants, indicating that formula with a caloric density similar to HM is safe and supports growth patterns similar to those of HM-fed infants.

2′FL has previously been found in the plasma and urine of breast-fed but not exclusively formula-fed infants (10,16). Here, we show for the first time that infants fed a formula supplemented with 2′FL exhibit uptake similar to that of HM-fed infants and at levels relative to the concentration fed. Although there were no significant differences in relative absorption between treatment groups, somewhat unexpectedly, both plasma concentrations and relative absorptions decreased from DOL 42 to 119. The urine concentrations for the HM group also decreased; however, the urine concentrations for EF1 and EF2 were consistent between time points.

It is known that both the structure and function of the GI tract mucosa are immature at birth (49). Additionally, the composition of intestinal microbiota transforms throughout infancy (5). These developmental changes may account for the decline in plasma concentrations as the gut becomes less permeable and the microbiota populations evolve to better use 2′FL.

Renal excretion mechanisms, such as glomerular filtration rate and tubular secretory pathways, are also underdeveloped at birth and steadily rise until adult values are reached by 8 to 12 months (49). This increase in renal function from DOL 42 to 119 may be sufficient to counter the decline in absorption of 2′FL and account for the lack of decrease in the 2′FL concentration in urine of formula-fed infants. The decrease in the concentration of 2′FL in the urine of the HM group may be because of the decrease in the concentration of 2′FL in the breast milk fed because levels of 2′FL in HM are known to diminish during the course of lactation (13).

Our study has a limitation. We did not include infants fed formula with the standard caloric density, therefore cannot compare growth and intake between infants fed formula with the standard versus lower caloric density. In a previous study comparing the lower energy formula and a CF (20 kcal/fl oz), there were no significant differences in the average volume of study formula intake per day or average weight gains through 28 days of life. Infants, however, fed the lower calorie formula grew at a rate similar to that of HM-fed infants—the criterion standard (Abbott Nutrition, unpublished results). A strength of our study was the statistical power and study design which assured careful monitoring of growth and tolerance.

In conclusion, the feeding of infant formula with a caloric density similar to that of HM results in growth similar to that of breast-fed infants. In addition, formulas supplemented with 2′FL are well tolerated, and 2′FL absorption profiles are similar to those of breast-fed infants.

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Abstract: The objective of this study was to evaluate gastrointestinal tolerance of infants fed infant formula supplemented with short-chain fructooligosaccharides (scFOS) and 2-fucosyllactose (2'FL). We conducted a prospective, randomized, multi-center, double-blinded, controlled 3-arm tolerance study in full term, singleton infants (birth weight ≥ 2490g) enrolled between 0 and 8 days of age. At enrollment, formula-fed infants were randomized to one of two experimental milk-based infant formulas with a caloric density of 643 kcal/L. Experimental Formula 1 (EF1) did not contain oligosaccharides (n=42) and Experimental Formula 2 (EF2) contained 2g/L scFOS and 0.2g/L 2'FL (n=46). The 2 formula groups were compared with a human milk-fed (HM) reference group (n=43). Infants were exclusively fed formula or human milk from enrollment until 35 days of age. Data related to intake, stool patterns, anthropometrics and parental questionnaires were collected. The primary outcome was average mean rank stool consistency (MRSC) from Study Day 1 to Visit 3. MRSC was calculated from stool records (1=watery, 2=loose/mushy, 3=soft, 4=formed, 5=hard). Thirty-six (86%) subjects in the EF1 group, 41 (89%) in the EF2 group and 42 (98%) in the HM group completed the study. There were no differences among groups for gender, ethnicity, race, gestational age, birth weight or age at enrollment. Reported results are from the protocol evaluable analysis. From Study Day 1 to Visit 3, there were no differences in MRSC among the three feeding groups. MRSC was 2.37 ± 0.10, 2.15 ± 0.10 and 2.06 ± 0.08 for the EF1, EF2 and HM groups respectively. There were also no differences among groups for predominant stool consistency from Study Day 1 to Visit 3. The average number of stools per day for the HM group was significantly greater than EF1 (p<0.0001) and EF2 (p<0.0001) from Study Day 1 to Visit 3. At Visit 3, there were no differences between groups for average volume of study formula intake, number of study formula feedings per day, anthropometric data or percent feedings with spit-up/vomit. An experimental formula containing 2'FL and scFOS was well tolerated in young infants as evidenced by stool consistency, formula intake, anthropometric data and percent feedings with spit-up/vomit similar to that of infants fed formula without oligosaccharides or HM.

# GASTROINTESTINAL TOLERANCE OF FORMULA SUPPLEMENTED WITH OLIGOSACCHARIDES

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## Background

There are over 100 major milk oligosaccharides in human milk (1). Mature human milk contains 12-13 g/L of oligosaccharides, representing the 3rd largest solid component, following lactose and fat, and is present at about a 20-fold higher concentration than that found in bovine milk (2,3). Levels of human milk oligosaccharides (HMOs) vary between individuals and over the course of lactation (2,4-6). The most abundant is 2-fucosyllactose (2'FL), which ranges from 0.06 to 4.65 g/L (7). Human milk oligosaccharides have been shown to act as prebiotics, selectively promoting colonization by *Bifidobacterium bifidum*, a bacterium that is especially prevalent in the intestines of human milk-fed infants (8).

In a previous growth and tolerance study (9), infants whose parents intended to feed their infants formula exclusively were randomized to be fed a control formula (CF) or one of two experimental formulas that were similar to the CF, only they contained levels of galactooligosaccharides (GOS) slightly different from those in the CF, and they contained 2'FL at 0.2 or 1.0 g/L. The total oligosaccharides was 2.4 g/L in all three formulas. No safety concerns were noted with either of the experimental formulas and stool consistencies and other measurements of tolerance were not significantly different among the three formula groups from day of life (DOL) 14-119.

Short-chain fructooligosaccharides (scFOS) are non-digestible carbohydrates that are found in several plant-based foods, including bananas. scFOS are considered a prebiotic, as defined by Roberfroid (10), because they are a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host well-being and health.

## Specific Aim

The objective of the study was to evaluate gastrointestinal tolerance of formula supplemented with 2.0 g/L (scFOS) and 0.2 g/L of 2'FL.

## Methods

- The study was a prospective, randomized, multi-center, double-blinded, controlled trial.
- Subjects were full term, singleton infants with a gestational age of 37- 42 weeks (birth weight ≥ 2490g) enrolled between 0 and 8 days of age.
- At enrollment, formula-fed infants were randomized to one of two experimental milk-based infant formulas with a caloric density of 643 kcal/L. The two formula groups were compared with a human milk-fed (HM) reference group.
- Infants were exclusively fed formula or human milk from enrollment until 35 days of age (Visit 3).
- Data related to intake, stool patterns, anthropometrics and parental questionnaires were collected.
- The primary outcome was average mean rank stool consistency (MRSC) from Study Day 1 to Visit 3.

### STUDY FORMULA

Table 1. Approximate Composition of Study Formula

| Ingredient | Experimental Formula 1 (EF1) | Experimental Formula 2 (EF2) |
|---|---|---|
| Energy, kcal/L | 643 | 643 |
| Protein, g/L | 14 | 14 |
| Fat, g/L | 35 | 35 |
| Carbohydrate, g/L | 70 | 70 |
| scFOS, g/L | 0 | 2 |
| 2'FL, g/L | 0 | 0.2 |

## Results

### Subjects

- Thirty-six (86%) subjects in the EF1 group, 41 (89%) in the EF2 group and 42 (98%) in the HM group completed the study.
- There were no differences among groups for gender, ethnicity, race, gestational age, birth weight or age at enrollment.

### MRSC

- There were no differences in MRSC among the three feeding groups, for the protocol evaluable analysis, from SDAY 1-Visit 3.
- MRSC was calculated from parent reported stool records.

Table 2. Average MRSC (1=watery, 2=loose/mushy, 3=soft, 4=formed, 5=hard)

| Time Interval | EF1 | EF2 | HM |
|---|---|---|---|
| SDAY1-DAY14 | 2.41 ± 0.09* | 2.31 ± 0.10 | 2.07 ± 0.08 |
| DAY15-Visit 3 | 2.32 ± 0.11 | 2.05 ± 0.11 | 2.05 ± 0.08 |
| **SDAY1-Visit 3** | **2.37 ± 0.10** | **2.15 ± 0.10** | **2.06 ± 0.08** |

***Avg. Mean Rank Stool Consistency (SDAY1-DAY14):** EF1>Human Milk, p=0.0409

### Study Day 1 to Visit 3

- There were also no differences among groups for predominant stool consistency
- The average number of stools per day for the HM group was significantly greater than EF1 (p<0.0001) and EF2 (p<0.0001)

### Visit 3

There were no differences between groups for:

| | |
|---|---|
| Average volume of study formula intake | Anthropometric data |
| Number of feedings per day | Percent feedings with spit-up/vomit |

## Conclusions

An experimental formula containing 2'FL and scFOS was well tolerated in young infants as evidenced by stool consistency, formula intake, anthropometric data and percent feedings with spit-up/vomit similar to that of infants fed formula without oligosaccharides or HM.

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