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Lactational changes in Filipino human milk oligosaccharides reveal maternal secretor status

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Abstract

Background: Breast milk contains human milk oligosaccharides (HMOs), which are the third most abundant solid component and are considered indigestible with no nutritional value to infants. However, HMOs provide protection against pathogens, support gut bacteria, and aid in brain development. This study investigated the relationship between HMOs and secretor status and tracked changes in HMO levels throughout lactation. The study employed a rapid-throughput extraction method, nano-HPLC Chip/TOF-MS analysis, and an annotated HMO structure library for oligosaccharide analysis.

Results: Results from the study revealed that 81% of Filipino mothers ($N = 155$) are secretors, while 19% are non-secretors. The total HMO abundance significantly decreased ($p = 0.0001$) throughout lactation but was significantly ($p = 0.0390$) higher in colostrum compared with mature milk. The total fucosylated HMOs had a significantly ($p = 0.0460$) lower absolute abundance, while the total sialylated HMOs showed a significantly ($p = 0.0012$) higher abundance in colostrum relative to mature milk. Non-fucosylated neutral HMOs showed no significant ($p = 0.1040$) difference in abundance between colostrum and mature milk.

Conclusion: This is the first comprehensive data on the maternal secretor status of Filipino mothers, showing the decrease in total HMO abundance throughout lactation and differences in the abundances of various oligosaccharide types between colostrum and mature milk.

KEYWORDS
HPLC Chip/TOF-MS, human milk oligosaccharides, non-secretor, secretors

INTRODUCTION

Human breast milk considered the gold standard for the nutrition of term infants is the sole food source for early infant nourishment. It contains a myriad of bioactive compounds that greatly influence growth, brain development, immune system modulation, protection against pathogens, and establishment of gut microbiota in infants.[1–3] It contains lactose as its most abundant component providing an immediate energy source, followed by lipids and proteins. It also contains oligosaccharides, the third most abundant solid component of breast milk; however, these are indigestible by infants and serve no nutritional value.[4] Interestingly, the milk oligosaccharide's concentration ranges from 20 to 24 g/L in colostrum[5,6] and 7 to 14 g/L in mature milk.[7,8]

Human milk oligosaccharides (HMOs) have various functions such as promoting a healthy infant gut, which selectively nourishes beneficial bacteria.[9] They prevent the binding of pathogenic microorganisms to gastric epithelial cells by acting as a soluble decoy and also

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modulate immune and intestinal epithelial cell responses.[10–12] It is also suggested that a sialic acid monosaccharide upregulated in the bloodstream of infants enhances brain development and memory.[13] Oligosaccharides are complex assemblies of different sugars including glucose, galactose, fucose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (a sialic acid derivative) of various sizes, charges, sequences, and abundances. They may contain between 3 and 22 monosaccharide units per molecule with different sequences, linkages, and orientations. Most oligosaccharides have a lactose core that can be elongated linearly or in a branched manner by *N*-acetylglucosamine units with fucose and sialic acid, which can be present in the terminal regions.[4,14] More than 200 HMOs have thus far been identified in human milk with 100 structures fully annotated.[14,15]

The expression of HMOs varies depending on the interaction of competing glycosyltransferases in the mother's mammary glands. The pattern of fucosylation expressed in HMOs is strongly affected by fucosyltransferases and highly dependent on the Secretor (Se) and the Lewis (Le) blood group loci. HMO variability is strongly dependent on the activity of two specific enzymes that are encoded by the Secretor (*Se*) and Lewis (*Le*) genes in the mother. *Se* and *Le* genes encode the enzymes α1-2-fucosyltransferase (FUT2) and α1-3/4-fucosyltransferase (FUT3), respectively, both affecting the biosynthesis of fucosylated HMOs.[16–18] Milk from women with inactivated FUT2 is due to a diversity of different mutations in both alleles of the *Se* gene-containing zero or traces only of the α1-2-fucosylated HMOs. Similarly, FUT3 activity can be inactivated (or severely reduced) due to a diversity of different mutations in both alleles of the *Le* gene, thus Lewis-negative women's milk contain zero or traces only of the α1-4-fucosylated HMOs (α1-3-fucosylation is encoded by several enzymes and is therefore not eliminated by FUT3 deactivation). Therefore, mothers who carry the *Se* gene express FUT2 enzyme while mothers who do not carry this gene do not express the FUT2 enzyme. The interplay of the FUT2 and FUT3 enzymes leads to two main types of Lewis antigens (Lea and Leb), and four common milk phenotypes are observed: Se+/Le(a−b+), Se-/Le(a+b−), Se+/Le(a−b−), Se−/Le(a−b−).[19–23]

For populations with comprehensive analysis of HMOs data, it is unequivocally known that oligosaccharide abundance generally decreases across the lactation period. However, to the best of our knowledge, there is still no comprehensive structural profile of free oligosaccharides in Filipino breast milk. Hence, the glycoprofiles of colostrum as well as the mature milk from Filipino mothers could be used as valuable source of information on the amount of oligosaccharides Filipino infants receive, which in turn could help in encouraging breastfeeding among the Filipino population. Overall, this study provided a comprehensive oligosaccharide profile of breast milk from Filipino donors. Specifically, the study highlights the correlation of the secretor status and oligosaccharide absolute abundance over the course of lactation.

Previous studies have shown there is high oligosaccharide concentrations in colostrum (20–25 g/L)[6,24] and mature milk (3–20 g/L).[7,8] However, we considered it important to determine the differences in the absolute abundances of different oligosaccharide types between colostrum and mature milk. Colostrum is the first breast secretion produced specifically for infant feeding during the first few days after delivery. It is a nutrient-rich milk containing immune, growth, and tissue repair factors that is produced in the mammary glands.[25] This is the reason the WHO recommended breastfeeding within the first hour of delivery. On the contrary, the late stage is marked by the secretion of mature milk, which contains reduced protein and oligosaccharide concentrations but elevated lipid and carbohydrate (lactose) concentrations.[7,8] In this study, the different oligosaccharide types present in colostrum and mature milk were analyzed in terms of relative abundance.

Materials and methods

Materials and reagents

Porous graphitized carbon (PGC) 96-well pack plates (40 µL capacity) for purification were purchased from Glygen (Columbia, MD). Variable volume (0.2, 0.8, and 1.3 mL) 96-well polypropylene plates were purchased from Sigma-Aldrich (St. Louis, MO). Solvent evaporation was done using a Minivac centrifugal evaporator (Gardiner, NY). Absolute ethanol and sodium borohydride (98%) were purchased from Sigma-Aldrich (St. Louis, MO) and Medline Industries (San Ramon, CA), respectively.

Breast milk collection

A total of 225 samples of breast milk were collected from 155 healthy term donors in Los Banos, Laguna, Philippines, the morning after infant feeding at different stages of lactation (1, 2, 3, 4, 5, and 6 months of lactation). Of the 225 milk samples, 30 were colostrum samples and 195 were mature milk samples. All the samples were stored in a −80°C freezer prior to analysis. The University of San Agustin Iloilo Health Ethics Review Board approved the study protocol, and informed consent was obtained from all the donors prior to their participation. Though all the mother donors are all Filipinos and do not belong to any of the minor ethnic groups, this study did not include the data on the minor ethnicity (ethnic groups) of the mother donors. Furthermore, an inclusion and exclusion criteria were also used in order to select donors regardless of parity. A previous study revealed that parity and age were not significant sources of variation in HMO abundance in this study.[26]

Oligosaccharide extraction, reduction, and purification

Twenty-five (25) µL of breast milk sample was placed in a 0.2-mL 96-well plate, added with an equal volume of nanopure water, and centrifuged at 4000 rpm for 30 min at 4°C. The aqueous layer between the skimmed lipids and protein/cell detritus layers was then collected to which two volumes of ethanol were added. This was then placed in a −80°C freezer for 30 min. The resulting supernatant

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was reduced by adding equal volumes of 2.0 M sodium borohydride ($NaBH_4$) and the reaction done at 65°C for 1.5 h. This was immediately purified using a 96-well pre-conditioned porous graphitized carbon (PGC) packed plates. After the samples were loaded onto the 96-well PGC plate, the salts were removed by washing the samples with two volumes of nanopure water five times. The free oligosaccharides were eluted with two volumes each of 20% ACN in 0.01% TFA and 40% ACN in 0.01% TFA. The eluted oligosaccharides were then dried in a centrifugal evaporator for 4–8 h and reconstituted with nanopure water before mass spectrometric analysis.

Evaluation of instrument reproducibility and stability

A reference HMO pool randomly collected within the sample sets was used to check the stability and reproducibility of the instrument over a long period of analysis. The pooled milk sample was repeatedly injected every 12 samples for three sets of analysis. The mean and % RSD from the reference HMO pool were calculated, providing the basis for reproducibility and stability as well as the retention shifts of some common abundant oligosaccharides.

Maternal secretor status determination

The secretor status of the donors was determined by quantifying the absolute peak areas of five secretor HMO markers found in breastmilk following an established protocol.[25] The secretor HMO markers that we found to have a significant amount of α (1, 2) fucosylation in their structures include 2′-fucosyllactose (2′FL), lactodifucotetraose (LDFT), trifucosyl-paralacto-*N*-hexaose (TFLNH), difucosyllacto-*N*-hexaose a (DFLNHa), and fucosyllacto-*N*-hexaose I (MFLNH I). The total oligosaccharide abundances were calculated using the absolute peak areas (area under the curve). The peak areas of all 5 secretor HMO markers were then summed and normalized to the total oligosaccharide abundance. Samples with ≥6% abundance (optimized percentage for determining secretor status) were determined and used to classify the donors as secretors, while those with <6% abundance as non-secretors.[25,28] The secretor status was only determined for the donors ($N = 155$) who donated their breastmilk. No blood testing was used in the determination of the Lewis gene.

HPLC Chip/TOF-MS analysis of oligosaccharides

The free oligosaccharides were analyzed using an Agilent 6200 Nano-HPLC Chip/TOF-MS instrument (Agilent Technologies, Santa Clara, CA). The instrument is equipped with a capillary pump for sample loading and enrichment, a nanopump as the analytical pump for analyte separation, a microwell-plate autosampler maintained at 4°C by a thermostat, an HPLC Chip cube as the interface, and an Agilent 6210 TOF-MS system. The column used was a microfluidic chip (Agilent Technologies) consisting of a 40 nL enrichment column and a 43 mm × 0.075 mm i.d. analytical column, both packed with graphitized carbon material of 5 µm pore size. Both pumps used a binary solvent: solution A1, 3.0% ACN/water in 0.1% formic acid (v/v); solution B1, 90% ACN/water in 0.1% formic acid (v/v). A flow rate of 3 µL/min for solution A1 was used for sample loading, with a 1 µL sample injection volume. A 60-min gradient optimized for glycan separation and delivered by a nanoflow pump with a flow rate of 0.3 µL/min was used for separation: 2.5–20.0 min, 0–16% B; 20.0–30.0 min, 16–44% B; 30.0–35.0 min, 44–99% B; 35.0–45.0 min, 99% B; and a 20 min equilibration time at 1% B. Electrospray was used as the ionization source, and data were collected in the positive mode calibrated by a dual nebulizer electrospray source with internal calibrant ions ranging from *m/z* 118.086 to 2721.895. A single calibrant ion (*m/z* 1221.991) was used for reference during analysis.

Data processing

The resulting data were collected using Agilent Mass Hunter Acquisition software and analyzed using Agilent Mass Hunter Qualitative Software B.07.00. The oligosaccharide peaks were identified using the software's "Find by Molecular Feature" algorithm. Human milk oligosaccharides were identified by matching the retention times and accurate masses against a compiled, in-house annotated HMO library developed in the Lebrilla research laboratory. The maternal secretor status of each donor was determined by quantifying the absolute peak area of five secretor HMO markers found in their breast milk. These secretor HMO markers include 2′-fucosyllactose (2′FL), lactodifucotetraose (LDFT), trifucosyl-paralacto-*N*-hexaose (TFLNH), difucosyllacto-*N*-hexaose a (DFLNHa), and I monofucosyllacto-*N*-hexaose I (MFLNH I).[25] The absolute peak areas of the five secretor HMO markers were summed and normalized to each donor's total oligosaccharide abundance (Supplementary Table S2). Specifically, the absolute abundance of the HMO structures selected for use as secretor markers was normalized to another consistent peak in the spectrum to yield a relative abundance that could be compared between samples. HMO abundance was also analyzed by oligosaccharide type (i.e., total fucosylated, total sialylated, or non-fucosylated neutral HMO) using the Python Algorithm software script developed in-house in the Lebrilla laboratory for peak alignment and correct retention time deviations.[15,27,29] The in-house Python-based program script was developed previously for rapid peak alignment and to automatically correct for retention time deviations.[25] The in-house Python-based scripts were used to rapidly calculate the summed oligosaccharide signal (total HMO abundance) and sum the peak intensities of all fucosylated OS, sialylated OS, and non-fucosylated neutral OS based on the accumulated in-house library of HMOs.[15,27,29]

Statistical analysis

The mean and standard deviation of oligosaccharide intensities were reported in both relative and absolute abundances. The Student *t*-test

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was used to compare the mean abundance of oligosaccharide expression in breast milk between secretors and non-secretors. One-way analysis of variance (ANOVA) was used to test variations in absolute and relative abundances of oligosaccharides at different stages of lactation, with Tukey–Kramer test used to compare all pair means. All statistical analyses were done using JMP program version 14.0. The statistical figures reported in this manuscript were generated in the same program.

RESULTS AND DISCUSSION

HPLC chip/TOF-MS analysis of oligosaccharides

Oligosaccharide analysis of cohort breast milk samples

The majority of the ionized oligosaccharides detected in the positive ion mode were singly-protonated $[M + H]^+$ or doubly-protonated $[M + 2H]^{2+}$ molecular ions. The oligosaccharides were one unit higher (if singly- protonated) in mass owing to an additional hydrogen ion from protonation. Between 100 and 200 oligosaccharides were extracted and identified for each breast milk sample, with masses ranging from 400 to 3700 Da based on the Lebrilla laboratory's in-house HMO library.[10] These HMOs were previously identified by the Lebrilla laboratory using glycosidases and MALDI-TOF experiments for structure elucidation.[15]

Supplementary Table S1 shows all the unduplicated HMOs for all the breast milk samples analyzed in the LC–MS positive mode, which includes both neutral and acidic species. The table includes the average retention time and average masses of unduplicated HMOs across all samples.

Maternal secretor status of Filipino donors

Filipino donors were classified as secretor or non-secretor based solely on their HMO marker profiles regardless of Lewis blood type (Supplementary Table S2). No effort was made to determine the Lewis blood type of the mothers using other bodily fluids such as saliva and blood. This is not the first time that this method on maternal secretor status was used in identifying secretor status using human breast milk alone.[24,28,30] Future efforts will include blood sample collection in addition to breast milk from volunteer mothers to allow us to obtain the Lewis gene classification.

Previous studies have reported the quantitative oligosaccharide composition and secretor status from different races using different methods such as HPLC with UV detection, high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and nuclear magnetic resonance (NMR).[31–40] This study, on the contrary, focuses on the characterization, profiling, and semi-quantitative HMO composition (absolute and relative abundance) obtained through a rapid-throughput extraction method, combined with a nano-HPLC Chip/TOF-MS analysis, based on an optimized method and in-house established library.[25,28]

To determine the secretor status of lactating mothers, a previously reported protocol was followed using five (5) of the most prevalent HMOs known to have a(1–2)-fucosylation.[25] The HMO markers allowed for a quick differentiation between secretors and non-secretors but did not necessarily distinguish between Lewis blood types. As seen in Figure 1, the HMO markers with high a(1–2)-fucosylated abundances were 2′FL, LDFT, MFLNH I, DFLNHa, and TFLNH and represented the most prevalent secretor markers, accounting for 10%–68% of the total oligosaccharides across all samples.

Previous studies have shown that a higher abundance of 2′FL, which confers huge benefits in terms of prebiotic activity in the neonate's gut, is characteristic of secretor mothers.[41–43] In addition, 2′FL was even observed to be present 1 year after the mother's delivery.[44] As expected, 2′FL was found to be not completely absent in non-secretors, as it was still observed at either low or almost negligible abundance (Supplementary Figure S1). Thus, the variation in the ion abundance of 2′FL makes 2′FL an ideal marker of secretor status, corroborating the report of Totten et al. (2012). On the contrary, 3-FL was found to be present in both secretors and non-secretors, but at significantly lower absolute abundance than 2′FL. Though lower in abundance compared with 2′FL, the 3-FL concentration is consistent throughout lactation.[44] Moreover, 3-FL showed no significant difference in absolute abundance between secretors and non-secretors, demonstrating that 3-FL is not an ideal determinant of secretor status.[28] By contrast, 3-FL was shown to increase in concentration during lactation as reported in several studies.[30,45] Furthermore, the variations reported in the reported studies may also be due to the differences in the methodologies used for the extraction and analysis of HMOs.

Analysis of the breast milk samples collected (time independent across lactation stage) from the 155 different donors revealed that, of the mothers enrolled in this study, 81% were secretors and 19% were non-secretors (Figure 2a). Though a previous study (Erney et al. 2000)[30] was able to determine the secretor status of Filipino donors, our study is the first comprehensive HMO data from Filipino mothers using a rapid-throughput method.[28] The areas where the breast milk collection was done were in the different barangays (villages/districts, (within a 5-km radius)) in the town of Los Baños, Laguna, Philippines. This is a town around 65 km south of Manila, Philippines. Though all the mother donors are all Filipinos and do not belong to any of the minor ethnic groups, this study did not include the data on the minor ethnicity (ethnic groups) of the mother donors. The milk samples from secretor mothers had higher though not significantly ($p = 0.0652$) different total oligosaccharide abundance (mean concentration of 6.3 g/L) than the non-secretor mothers (mean concentration of 5.5 g/L). The secretor and non-secretor milk samples also differed in oligosaccharide type with the secretor milk showing more diverse oligosaccharide types (Figure 2b).

The secretor milk samples were richer in oligosaccharides, particularly having a higher amount of 2′FL, than the non-secretor samples. Furthermore, the secretor samples had a significantly higher LNFP I abundance than the non-secretor samples. This was expected since LNFP I shows α (1, 2) fucosylation in its structure, a characteristic of secretor HMOs. However, the non-secretor samples had a

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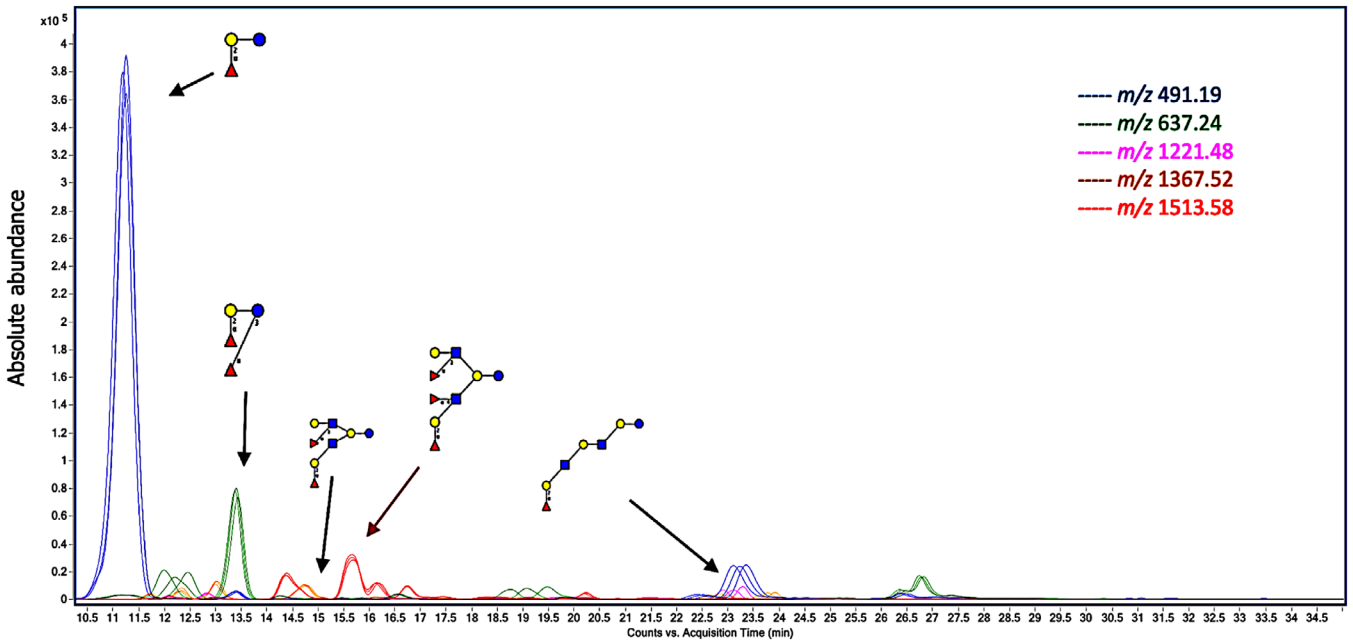


FIGURE 1 Overlaid extracted ion chromatogram (EIC) of the five HMO markers 2′FL (*m/z* 491.19), LDFT (*m/z* 636.24), MFLNH I (*m/z* 1221.48), DFLNH a (*m/z* 1367.52) and TFLNH (*m/z* 1513.58) used to determine the secretor status of Filipino mothers.

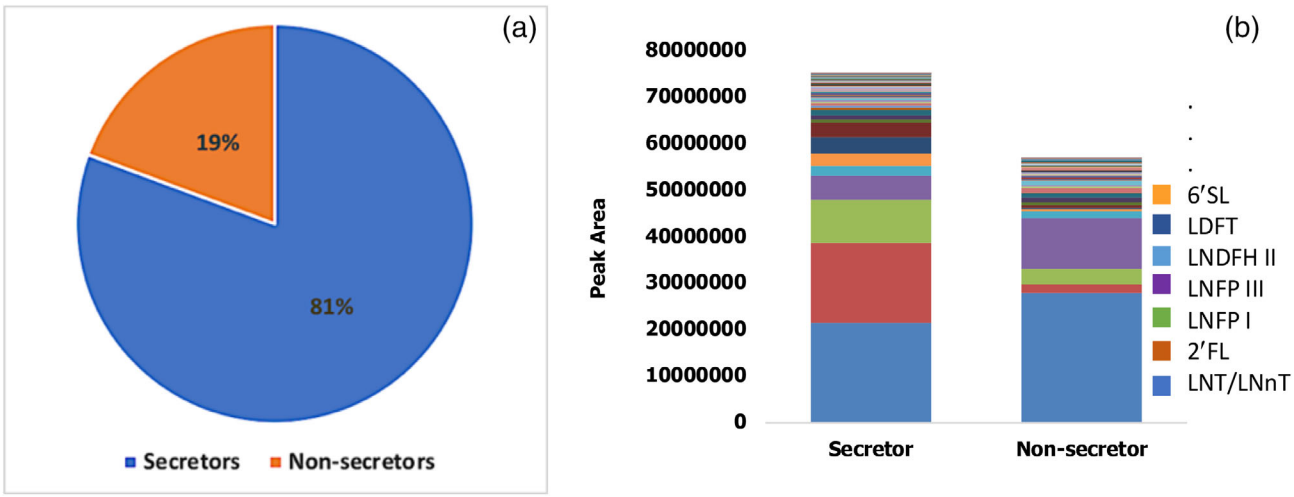


FIGURE 2 (a) Secretor ($N = 125$) and non-secretor ($N = 30$) distributions of mothers based on their breast milk samples. (b) Distribution of different HMOs between secretors and non-secretors.

significantly higher LNFP III abundance than the secretor samples. Again, this was expected since LNFP III shows (1–3) fucosylation in its structure, characteristic of non-secretor HMOs, consistent with the findings of Xu et al. (2017).[46]

The difference in oligosaccharide abundance affects the anti-pathogenic and prebiotic capacities of milk.[9,47] This is evident if one considers that a higher abundance of fucosylated oligosaccharides is typical in secretor populations,[30,45] which is similar to the secretor status of Asian populations (Japan, Korea, Samoa, Bangladesh) as previously reported.[48–50] A known secretor marker, 2′FL, is two orders of magnitude greater in secretors than in non-secretors. In our results, secretor women have a significantly ($p < 0.0001$) higher ion abundance of 2′FL in their breast milk than non-secretor women, as shown in the boxplot of the peak areas of 2′FL (Figure 3). As reported in previous studies, HMO structures containing (α1–2)-linked fucose (the H-determinant) are present in significantly lower abundance if not absent from non-secretor milk.[24,45,51–54] Previous studies have also shown that non-secretors expressed 2′FL in very small abundance.[30,46,55]

Figure 4 shows the EICs of LNT and LNnT with the same precursor mass of 710.23. These HMO isomers were not baseline-separated and appeared to be abundant in all the samples, with LNT being the

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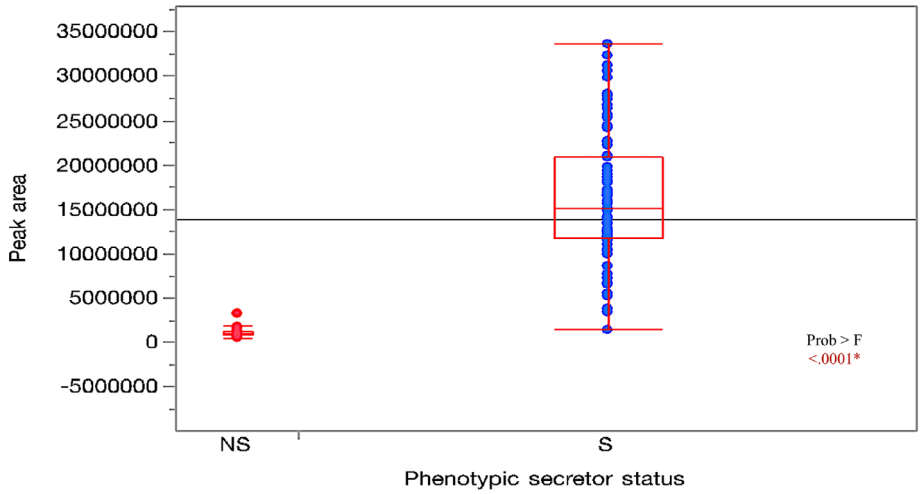


FIGURE 3 Peak areas of 2′FL from secretors (blue) and non-secretors (red). The plot was generated using Tukey–Kramer test and analyzed using JMP statistical software v.14.

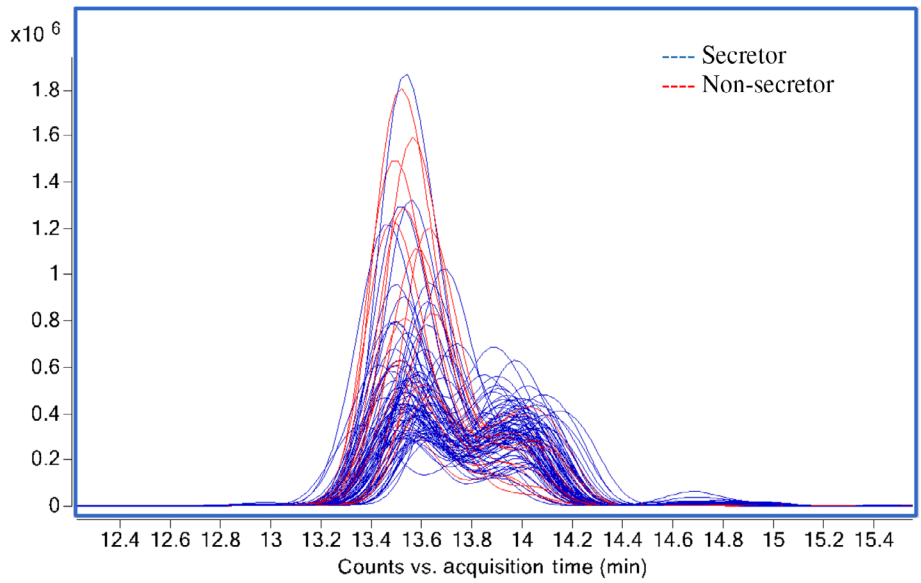


FIGURE 4 EIC of LNT and LNnT with *m/z* 710.23 from all breast milk samples.

most abundant and consistent with the previous results in determining the markers for secretor status.[25] When considering the higher abundance of LNT together with the secretor status, it was observed that non-secretor mothers (red chromatogram, Figure 4) have a significantly ($p = 0.0040$) higher LNT abundance than secretor mothers (blue chromatogram, Figure 4). This higher absolute abundance of non-fucosylated neutral oligosaccharide in non-secretors than in secretors may reflect the insufficiency of fucose transfer in the machinery of fucosyltransferase activity of the mothers. This result suggests the lack of or modified activity of FUT2 and FUT3 fucosyltransferases, which correlates with previously reported data.[25] Note the higher abundances of non-fucosylated neutral HMOs (i.e., HMO containing higher LNT) were previously observed in non-secretors than in secretors.[25]

Since a similar trend of decreasing oligosaccharide abundance was observed throughout lactation for both secretors and non-secretors, the ion abundances of different oligosaccharide types were combined into one inclusive group. Figure 5a shows that the secretor samples are richer in fucosylated HMOs ($p = 0.0124$) than the non-secretor samples. Breast milk from the secretors yielded a higher mean relative abundance (64.1% ± 0.8%) of fucosylated HMOs compared with the non-secretors (60.0% ± 1.4%). As expected, 2′FL (*m/z* 491.19), the most abundant fucosylated oligosaccharide, had a significantly ($p < 0.0001$) higher (14-fold) abundance in the secretor samples than in the non-secretor samples, clearly indicating the considerable expression of 2′FL in secretors. The 2′FL is also found to be abundant in milk samples from secretors.[24,40,56] Its isomer 3FL, although present in both secretors and non-secretor samples, is not abundant in breast

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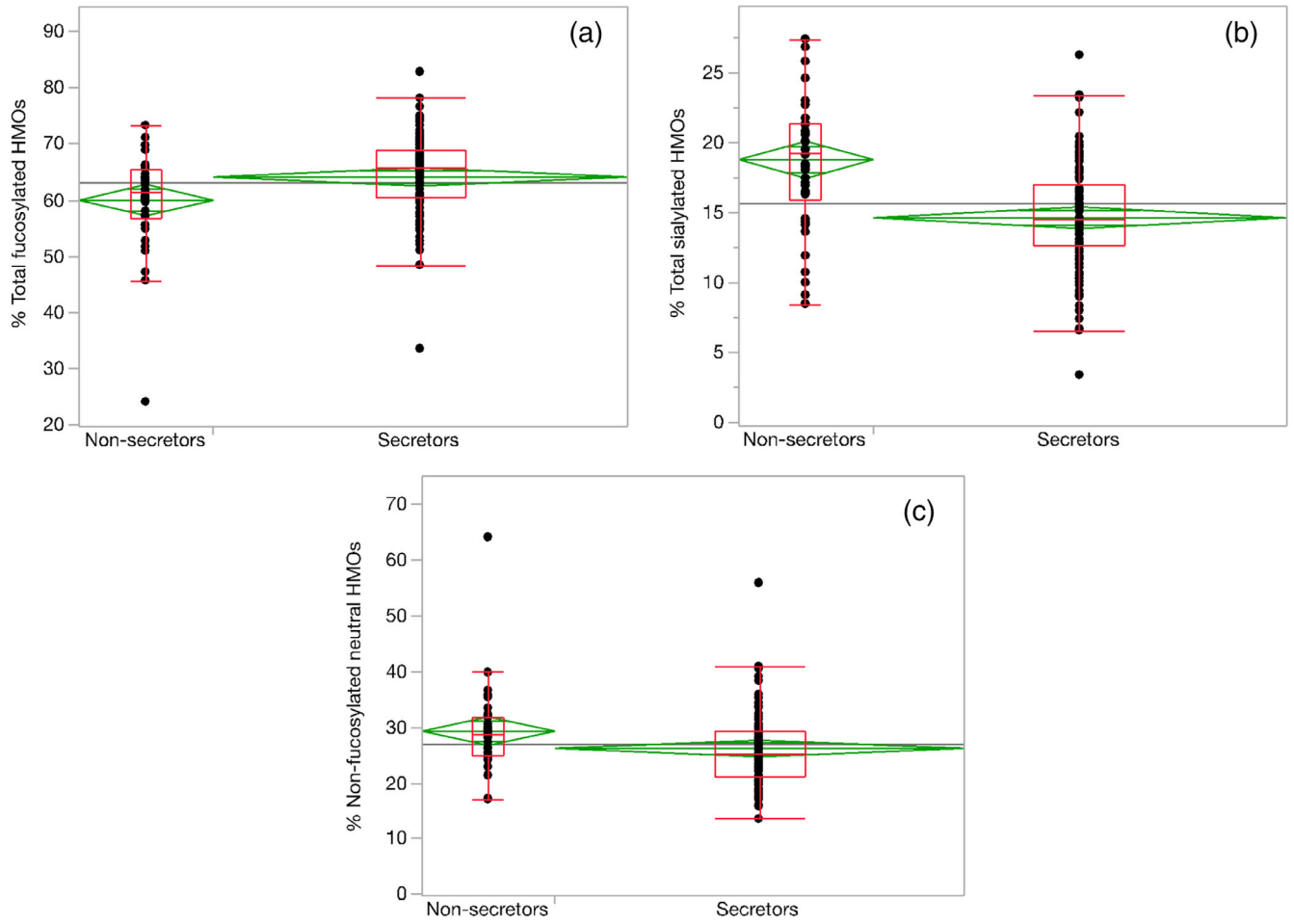


FIGURE 5 Relative abundances of different HMO types between secretors ($N = 125$) and non-secretors ($N = 30$). (a) Total fucosylated HMOs; (b) total sialylated HMOs; and (c) non-fucosylated neutral HMOs. The plots were generated using Tukey–Kramer test and analyzed using JMP statistical software v.14.

milk and therefore could not compensate for the total fucosylation in the non-secretor samples.[40,45] The increased fucosylation observed in the secretor samples has also been observed in several studies of American and African mothers.[25,29,45,57] This significantly lower abundance of fucosylated HMOs in non-secretor breast milk is a consistent feature of different populations worldwide.[45,51]

The relatively low abundances of total sialylated oligosaccharides (Figure 5b) showed a significant ($p < 0.0001$) difference between the secretor (14.6% ± 0.4%) and non-secretor (18.9% ± 0.7%) samples. Sialylated HMOs such as 3′SL and 6′SL are associated with protection against *Streptococcus pneumoniae* and reduced influenza infection in cell culture assays.[9] In addition, sialyted HMOs have been shown to help in the prevention of allergies such as peanut allergy.[58–60]

The sialylated HMO 3′SL (m/z 636.23) showed a 5-fold higher abundance ($p < 0.0001$) in the secretor samples than in the non-secretor samples while its isomer, 6′SL, also showed a significant ($p = 0.0251$) higher abundance in the secretor samples. On the contrary, LSTa and LSTb (m/z 1001.35) showed significantly ($p = 0.0107$ and $p = 0.0151$, respectively) higher abundances in the non-secretor samples.

Furthermore, when the isomeric ratios of 6′SL to 3′SL in secretor and non-secretor breast milk samples were compared, a clear tendency to produce an isomer with α(2–6)-linked sialic acid residue was observed in non-secretors.[55] Sialylated HMO species have long been associated with infant brain development.[13] In general, non-secretor breast milk has a slightly higher abundance of sialylated oligosaccharides than secretor breast milk.[19]

Non-fucosylated neutral oligosaccharides (Figure 5c) are undecorated structures containing only glucose, galactose, and *N*-acetylglucosamine. The relative abundance of this oligosaccharide type is significantly ($p = 0.0419$) higher in the non-secretor samples (29.3% ± 1.3%) than in the secretor samples (26.2% ± 0.8%). This high abundance of neutral HMOs reflects how non-secretor mothers can compensate for the reduced FUT2 activity in their breast milk. The lack of fucose transfer occurring in non-secretors suggests the lack of modified activities of FUT2 and FUT3 fucosyltransferases. The levels of non-fucosylated neutral HMOs in breast milk from the Filipino donors (29% in secretors, 26% in non-secretors) are comparable with those observed in the Davis, CA cohort (25% in secretors, 22% in non-secretors) but significantly lower than those observed in the

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Gambian cohort (43% in secretors, 55% in non-secretors). It has been suggested that Gambian women may have evolved to compensate for their low fucosyltransferase activity, as evidenced by the lower degree of fucosylation in their milk oligosaccharides compared with the Davis and Filipino cohorts.[25]

Changes in oligosaccharides between colostrum and mature milk

The total oligosaccharide abundance was significantly ($p = 0.0390$) higher in colostrum (30 samples) than in mature milk (195 samples), which was expected since the total oligosaccharide abundance decreased with lactation time. However, the relative abundance of total fucosylated HMOs was significantly ($p = 0.0460$) lower in colostrum than in mature milk (Figure 6a). This lower abundance is postulated to be the effect of the lower volume but higher concentrations of other components of colostrum with bioactive effects such as antibodies and protective proteins. One particular antibody delivered to the infant through colostrum is the secretory immunoglobulin A (sIgA), which helps protect the mucous membranes in the throat, lungs, and intestines of the infant.[25] Fucosylated HMOs are associated with the stimulation of a healthy gut by selectively nourishing beneficial microorganisms such as bifidobacterial species.[25] Sela et al. (2012) revealed that *Bifidobacterium longum* subsp. *infantis* ATCC 15697 utilizes small-mass neutral HMOs, particularly fucosylated oligosaccharides.[42,55,61,62]

The abundances of sialylated HMOs were significantly ($p = 0.0012$) higher in colostrum than in mature milk (Figure 6b). Previous studies indicated that sialic acids aid in brain development.[13,63,64] On the contrary, the concentration of non-fucosylated neutral HMOs remains constant throughout lactation. Moreover, relative abundances of non-fucosylated neutral HMOs did not significantly ($p = 0.1040$) differ between colostrum and mature milk, although they were slightly higher in colostrum (Figure 6c).

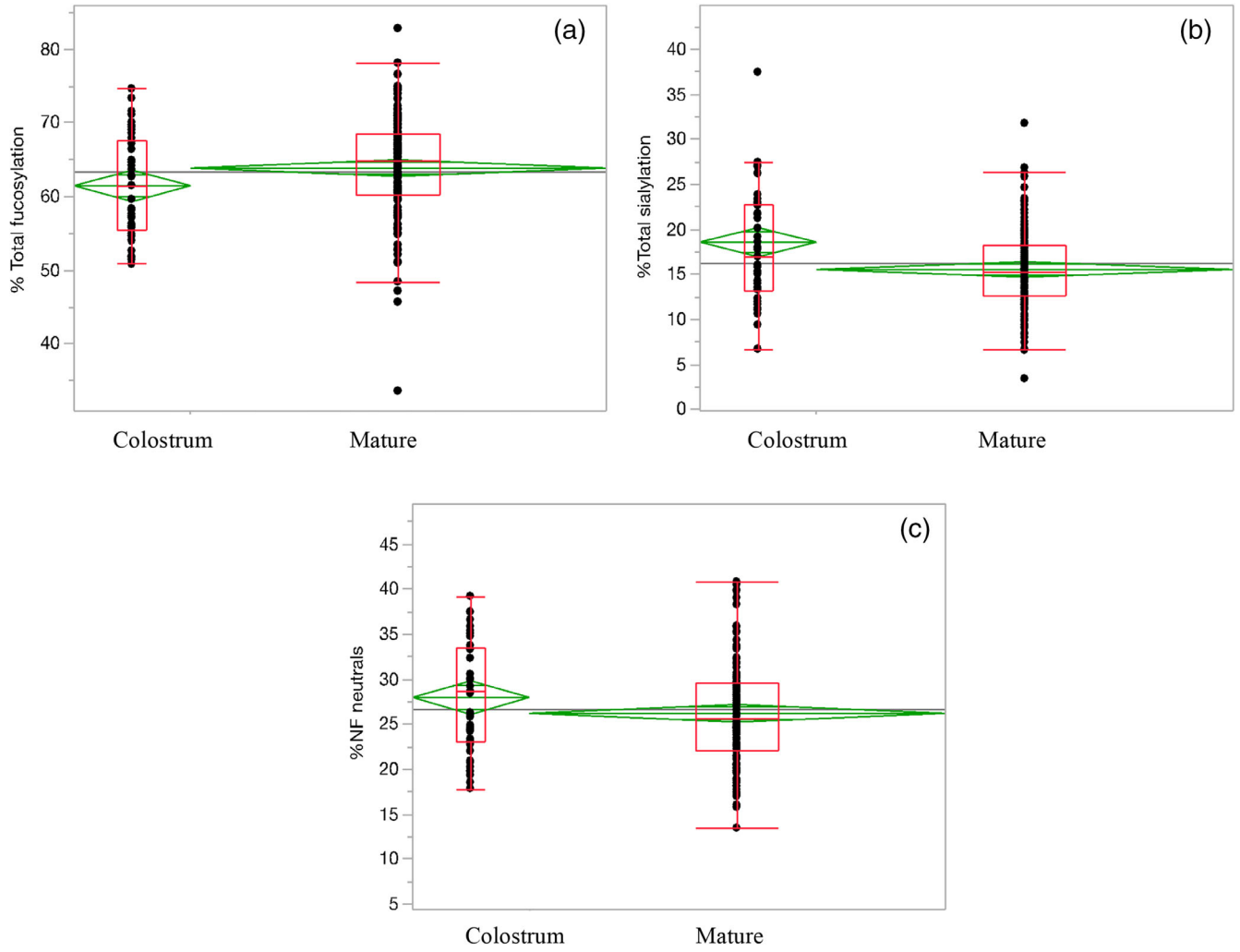


FIGURE 6 Relative abundances of oligosaccharides in early and late stages of lactation. (a) Total fucosylated oligosaccharides; (b) total sialylated oligosaccharides; and (c) non-fucosylated neutral oligosaccharides. The plots were generated using Tukey–Kramer test and analyzed using JMP statistical software v.14.

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TABLE 1 Secretor status of distribution of different populations available in the literature.

Country	Secretors (%)	Non-secretors (%)
Asia		
Philippines[a]	81	19
Bangladesh[50]	60	40
Iraq[47]	76	24
Japan[48]	77	23
Korea[65]	76	24
Vietnam[66]	70	30
China[67]	77	23
UAE[68]	74	26
Malaysia[69]	83	17
Africa		
Burkina Faso[70]	76	24
Gambia[19]	73	27
Ghana[71]	68	32
Malawi[46]	78	22
America		
Peru[71]	98	2
United States (Davis)[46]	74	26
Europe		
Finland[72]	88	12
Italy[70]	77	23
Spain[71]	76	24
Sweden[71]	79	21
Australia[73]	65	35

[a]Values shown are from the results of the present study.

The secretor status distributions of different populations from previous studies were compared with our results (Table 1). It was evident that secretor status markedly varies among different populations, that is, different populations have different secretor status distributions, which is most likely associated with genetic predisposition and geographical location.

A comparison of the secretor status of Filipino mothers with those of other populations revealed interesting variations. As an archipelago of 7,641 islands, the Philippines offers an interesting scenario where breast milk samples from different islands or regions vary depending on diet, genetic predisposition, and the environment. The breast milk samples used in this study came from mothers residing in Los Banos, Laguna, a town located around 65 km south of Manila, the capital of the country. The presence of the University of the Philippines Los Baños in the area created an environment in which a sizeable population in the area came from Philippine regions other than the southern Tagalog region to pursue higher education or work in and outside the university resulting in a diverse population.

As seen in Table 1, the variations in secretor status distribution among different populations in the world suggest a multitude of factors such as geographical location, the environment and ethnicity that affect the type of oligosaccharide produced in breast milk by mothers worldwide. In addition, geographical location and genetic predisposition play a great role in determining how oligosaccharides are expressed over time. With more research on HMOs in the breast milk of lactating women around the world, the effects of geographical location and natural genetic variations on oligosaccharide composition can be further investigated.

Changes in oligosaccharides throughout lactation

Figure 7 shows the absolute abundance of each oligosaccharide type throughout lactation (1, 2, 3, 4, 5, and 6 months of lactation). By contrast, previous studies presented the varied lactation period (i.e., 0–30 days, 0–60 days, 0–100 days, 0–>100 days) of mothers both in term and pre-term gestation.[40] The 1st month of lactation showed the highest abundances of all oligosaccharide types (compared with the 2nd to 5th months of lactation), which decreased over time. This decrease was consistent with the decrease in HMO abundance throughout lactation observed by Chaturvedi et al. (2001).[57]

A significant ($p = 0.0001$) decrease in the mean total oligosaccharide abundance was observed throughout lactation. The mean absolute abundance was significantly higher during the 1st month of lactation than in the later months (each pair stepwise Newman–Keuls test). This indicates that there was an influx of oligosaccharides during the 1st month of lactation, which subsequently tapered over time. This result correlates with those of other studies showing a decreasing total oligosaccharide abundance throughout lactation.[24,25,28,45,57] This decrease in oligosaccharide volume abundance could be linked to the decrease in breast milk volume with the progress of lactation, which is inevitable for breastfeeding mothers.[74] Breast milk volume starts to taper as the infant starts to require increased nutrients over time and transitions to weaning foods at 6 months. The slow introduction of solid food after 6 months is deemed to be important in the continuous shaping of the infant's gut intestinal microflora, and it is only after 2.5–3 years of age that an infant develops an adult-like gut microbiota.[75–78]

Although the breast milk samples used in our study came from different mothers at different time lactation stages, a general trend was still deduced. The absolute abundances of the three HMO types (fucosylated, sialylated, and non-fucosylated neutral) decreased from their corresponding initial values in the 1st month of lactation. The absolute abundance of each oligosaccharide type was transformed into relative abundance to account for individual variations in total oligosaccharide count. The relative abundances of the HMO types showed different trends: decreasing for the fucosylated and sialylated HMOs, and almost constant for the non-fucosylated neutral HMOs. Specifically, the relative abundances of the different HMO types were the total oligosaccharides across the lactation period (months 1–6). Non-fucosylated neutral HMOs were the most abundant type at 60%–65% of the mean total absolute oligosaccharide abundance (mainly due to LNT abundance). Remarkably, the abundance of fucosylated HMOs remained constant throughout lactation.

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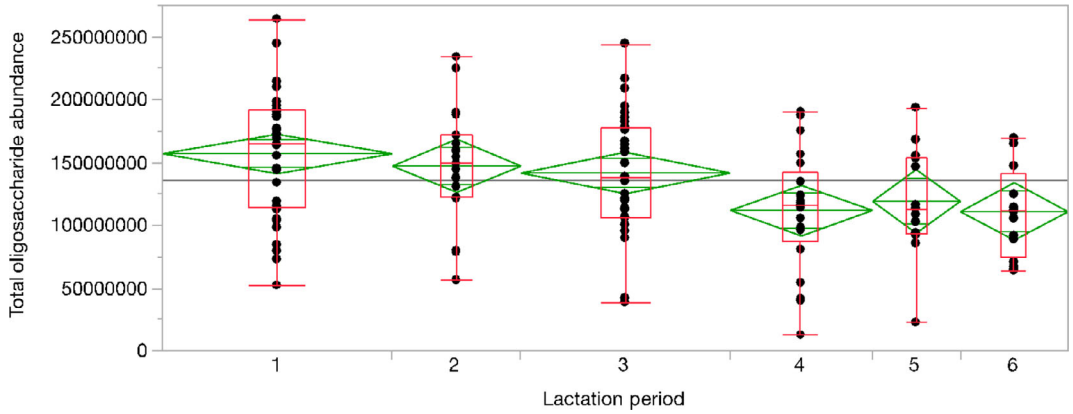


FIGURE 7 Absolute total oligosaccharide abundances over the course of lactation. The plots are generated using Tukey–Kramer test and analyzed using JMP statistical software v.14.

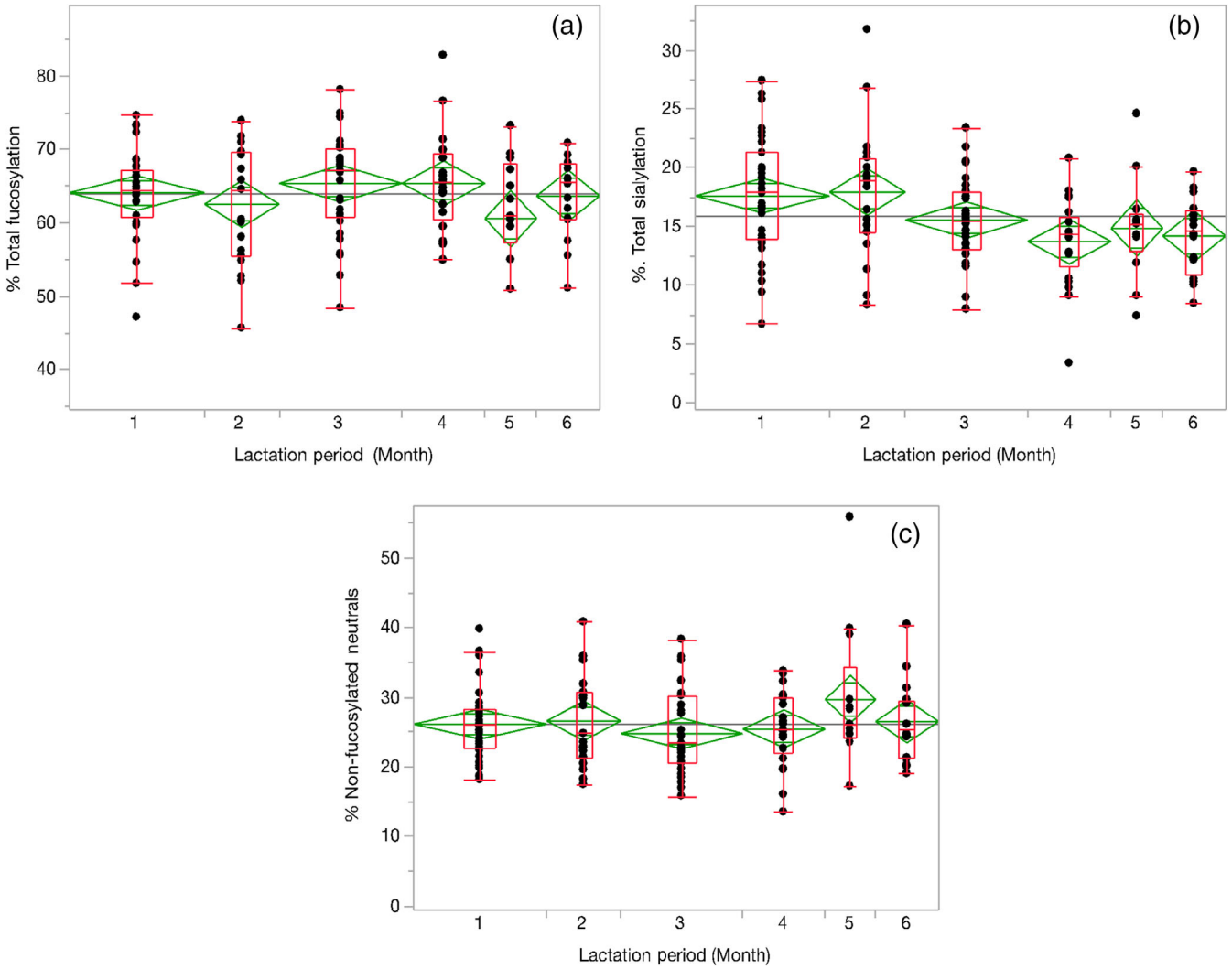


FIGURE 8 Relative abundances of different oligosaccharide types across lactation period: (a) Total fucosylated oligosaccharides; (b) total sialylated oligosaccharides; and (c) non-fucosylated neutral oligosaccharides. The plots are generated using Tukey–Kramer test and analyzed using JMP statistical software v.14.

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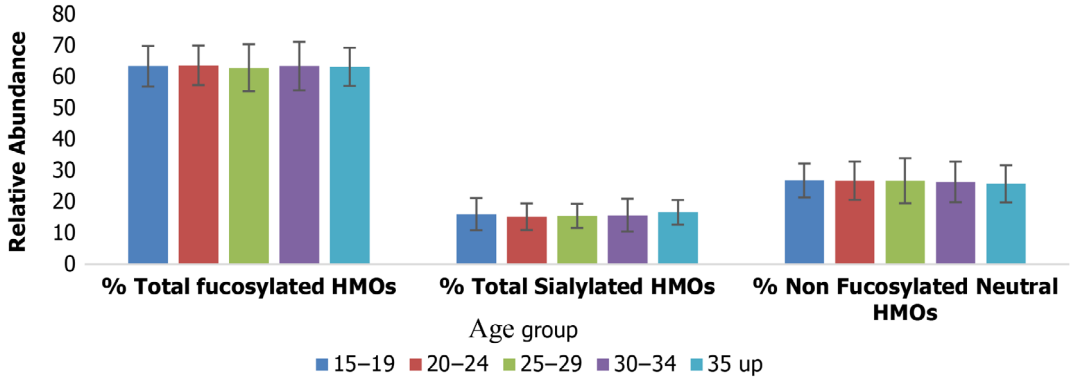


FIGURE 9 Absolute abundance of HMOs from Filipino donors at different age groups.

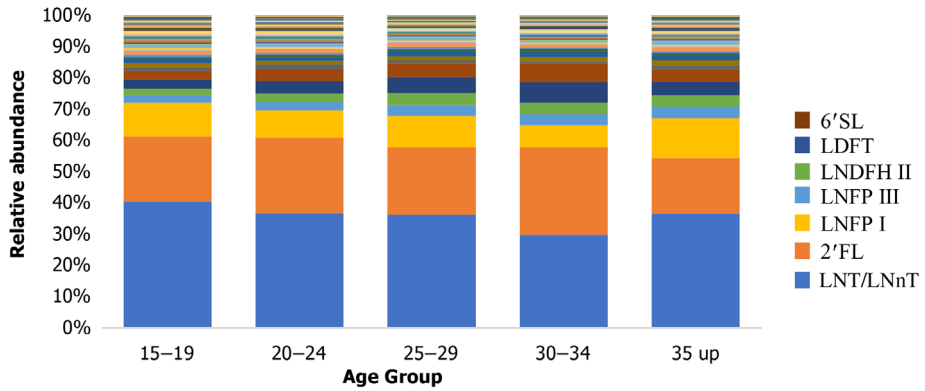


FIGURE 10 Oligosaccharide abundance of HMOs from Filipino donors at different age groups.

There was no significant ($p = 0.3235$) difference in the relative abundance of fucosylated compounds across the lactation period (Figure 8a).

According to our findings (Figure 8b), the total presence of sialylated HMOs in breast milk ranges from 13% to 18% and differs significantly ($p = 0.0038$) throughout the lactation period, which aligns with previous research.[45] Previous studies have suggested that sialylated HMOs may have a positive effect on epithelial cells, making them more resistant to uropathogenic *E. coli* (UPEC)[9], as well as disrupting *E. coli* binding in the gut.[79] It is important to note that the terminal portion of HMO structures contains both fucose and sialic acids, which play a crucial role in interactions with other substrates. These interactions can inhibit the binding of pathogens, such as bacteria and viruses, by acting as a soluble decoy.[80]

The remaining HMOs found in breast milk samples (Figure 8c), approximately 24%–30%, were fucosylated HMOs. There was no significant ($p = 0.2989$) difference observed in the mean relative abundance of fucosylated and non-fucosylated HMOs throughout lactation. The most abundant non-fucosylated neutral HMO in all breast milk samples was LNT, making up between 25% and 60% of the total oligosaccharide abundance. This finding is consistent with previous research that also showed high LNT abundance in breast milk.[25]

Changes in oligosaccharides with age

The effect of the age of a mother on the observed oligosaccharide abundance was also investigated. The ages of the breast milk donors were categorized into different age groups and tabulated according to different oligosaccharide types, as shown in Figure 9. It can be inferred from the results that a mother's age does not affect oligosaccharide abundance.

Furthermore, the levels of different oligosaccharide types such as fucosylated, sialylated, and non-fucosylated neutral were almost constant in the breast milk samples from all the donors of different ages, indicating that mothers can still provide their infants with necessary oligosaccharides regardless of age. The result also indicates that, although women produce different concentrations of oligosaccharides of the same type, this is not linked to the mother's age and that women can still provide adequate oligosaccharides.

When the abundances of individual oligosaccharides were monitored for each age group (Figure 10), it was observed that they remained constant regardless of age group and that there were no oligosaccharides unique to a specific age group. Variations in oligosaccharide abundance and type were more dependent on secretor status.

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The data presented here are preliminary results of our study on human milk oligosaccharides from Filipino donors. These results can serve as the basis or reference for subsequent studies on Filipino HMOs. We plan to conduct a follow-up study on the effect of age on HMO levels and other variables using a greater number of samples from different Filipino mother age groups.

Overall, this study provided the first comprehensive profile of HMOs present in Filipino breast milk and their variation with maternal secretor status. Furthermore, this study unequivocally confirmed that the total absolute HMO abundance of Filipino breast milk decreases throughout lactation and that colostrum and mature milk contain different oligosaccharide types.

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CONFLICT OF INTEREST STATEMENT

The author and co-authors do not have any conflict of interest to disclose.

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