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Daily Variations in Oligosaccharides of Human Milk Determined by Microfluidic Chips and Mass Spectrometry

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Human milk is a complex biological fluid that provides not only primary nourishment for infants but also protection against pathogens and influences their metabolic, immunologic, and even cognitive development. The presence of oligosaccharides in remarkable abundance in human milk has been associated to provide diverse biological functions including directing the development of an infant's intestinal microflora and immune system. Recent advances in analytical tools offer invaluable insights in understanding the specific functions and health benefits these biomolecules impart to infants. Oligosaccharides in human milk samples obtained from five different individual donors over the course of a 3 month lactation period were isolated and analyzed using HPLC-Chip/TOF-MS technology. The levels and compositions of oligosaccharides in human milk were investigated from five individual donors. Comparison of HPLC-Chip/TOF-MS oligosaccharides profiles revealed heterogeneity among multiple individuals with no significant variations at different stages of lactation within individual donors.

KEYWORDS: Oligosaccharides; human milk; HPLC-Chip/MS Technology; lactation; prebiotics; bifidobacteria; microfluidic; mass spectrometry

INTRODUCTION

The factual link of food to health and the ability to have it guide desired health results is a timely and relevant topic in nutrition research. The connection between human breast milk and infants' growth, development, and health exemplifies this link. Human milk components do not solely nourish the infant; in addition, they provide bioactive functions for the infant that may influence the growth, stimulation and modulation of the immune system, cognitive development, establishment of the intestinal microflora, and prevention of pathogenic diseases (*1–3*). Although considerable effort has been made toward understanding the composition of human milk and its effects on infant (*4*), the overall complexity of specific milk bioactive components has hindered the elucidation of actual structure–function relationships. The presence and abundance of oligosaccharides in human milk as the third largest solid component are thought to provide biological, physiological, and protective functions (*2, 5–7*). However, the detailed structural basis of these important functions is not yet understood. Human milk oligosaccharides (HMOs) have been demonstrated to selectively nourish the growth of highly specific strains of bifidobacteria thus establishing the foundations to guide the development of a unique gut microbiota in breast milk fed infants (*3, 8–10*). Certain oligosaccharides derived from the mammalian epithelial cells of the mother also share common epitopes on the infant's intestinal epithelia known to be receptors for pathogens. Such structures imply that their presence in milk provides a defensive strategy acting as decoys to prevent binding of pathogens to epithelial cells, thereby protecting infants from diseases (*11*). Consistent with these multiple functions, human milk is comprised of a complex mixture of oligosaccharides that differ in size, charge, and abundance (*12*). Human milk oligosaccha-

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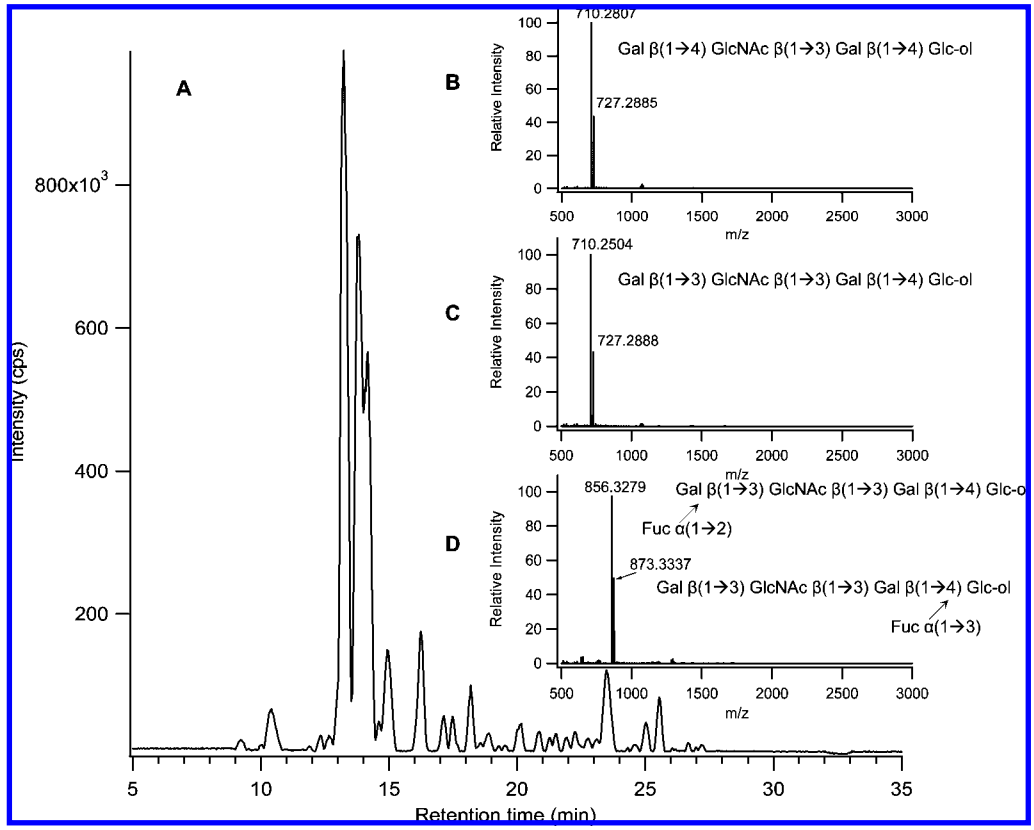

Figure 1. A representative HPLC-Chip TOF/MS profile (base peak chromatogram) of oligosaccharides obtained from a single donor (donor 5) on day 3.

rides are composed of both neutral and anionic components with building blocks of five monosaccharides: D-glucose, D-galactose, *N*-acetylglucosamine, L-fucose, and *N*-acetylneuraminic acid. The basic structure of HMOs includes a lactose core at the reducing end and elongation by *N*-acetyllactosamine units, with greater structural diversity provided by extensive fucosylation and/or sialylation wherein fucose and sialic acid residues are added at the terminal positions (*11*). The ability to understand the diversity of biological functions of HMOs has been hindered to date in part because of the lack of detailed structural knowledge of the overall complexity of HMOs in breast milk. At present, about 200 molecular species have been identified in a pooled human milk sample consisting of mostly neutral and fucosylated oligosaccharides (*12*).

Current analytical methods to characterize oligosaccharides in human milk include high-performance liquid chromatography (HPLC), high pH anion exchange chromatography (HPAEC), capillary electrophoresis (CE), and mass spectrometry (MS) (*13–21*). These methods are technically cumbersome, and as a result there is little information on the diversity of HMO either between different lactating humans or in changes in oligosaccharide compositions and abundances during the course of lactation. Considering the various functions associated with the diverse HMO structures, the details of variations in compositions and abundances of oligosaccharides among humans and during lactation need to be understood. It was reported previously that the total concentration of oligosaccharides decreased from the first weeks postpartum to about half the concentration only after 1 year. The concentrations of HMOs between individual donors and the different stages of lactation varied significantly (*22*), while a few sialyloligosaccharides isolated from Spanish mothers milk and bovine milk were analyzed and found to decrease across the 32-day lactation period and 10-month period, respectively (*23*). A more recent analysis on the level of major neutral oligosaccharides for 3 consecutive days in human milk colostrums from 12 Japanese women was also reported. The concentrations of 2′-fucosylactose (2′-FL) and lactodifucotetraose (LDFT) on day 1 were found to be substantially higher than those on day 2 and day 3, while lacto-*N*-tetraose (LNT) concentration has increased from day 1 to day 3 (*24*). Whether the presence and quantity of the specific oligosaccharides are important for infant's nutritional demands or immunomodulatory developments remains unclear.

As reported in previous articles, an analytical strategy was undertaken by this group to routinely profile oligosaccharides in human milk using HPLC-Chip/TOF-MS technology (*12, 25*). This analytical technique employs an integrated microfluidic chip coupled with a high mass accuracy time-of-flight mass analyzer. With this analytical platform, profiles of oligosaccharides in human milk samples on a daily basis during first week postpartum and every other day thereafter were determined for five different individual donors. The levels of milk oligosaccharides and their heterogeneity were investigated both within individual donor and among multiple donors at different stages of lactation. This approach is designed to provide the basic knowledge of HMOs in normal humans as the key compositional basis to understand the relationship between the levels of milk oligosaccharides and the specific functions these biomolecules contribute to maternal and infant health and development.

MATERIALS AND METHODS

Materials and Reagents. Nonporous graphitized carbon cartridges (150 mg bed weight, 4 mL tube size) for solid phase extraction (SPE) were purchased from Alltech (Deerfield, IL). Solid phase extraction was accomplished using a SPE vacuum manifold, 12-port model from

Table 1. Average and Standard Deviation of Retenttion Time, Peak Area, and Peak Height of 10 Selected Peaks ($n = 9$).

peak	RT (min)	area (counts)	height (counts s^{-1})
1	11.73 ± 0.15	10656311 ± 9451157	452312 ± 192344
2	12.31 ± 0.02	26381889 ± 6321334	1868121 ± 143990
3	13.00 ± 0.02	903431111 ±35825472	17175301 ± 448433
4	14.55 ± 0.03	70427556 ± 5013773	3435901 ± 131624
5	15.25 ± 0.02	10709678 ± 3073516	829457 ± 125278
6	15.80 ± 0.01	161007778 ± 4880786	8047142 ± 277059
7	16.43 ± 0.02	28656111 ± 2298772	1711482 ± 129021
8	16.92 ± 0.01	65749778 ± 2555840	4065882 ± 174604
9	17.31 ± 0.02	99824000 ± 2981867	5779642 ± 183219
10	18.39 ± 0.02	92638667 ± 4788713	5316922 ± 191760

Supelco (St. Louis, MO). Evaporation of solvents was performed using a Centrivap Concentrator (Labconco Corp., Kansas City, MO). Sodium borohydride (98%) was purchased from Sigma-Aldrich (St. Louis, MO). Nanoliquid chromatography solvents were obtained from Burdick and Jackson (Muskegon, MI). All other reagents were of analytical grade or better and were used without further purification.

Milk Samples. Human milk samples were donated by five healthy women from Reno, NV, and were collected from the first day of lactation. Samples were obtained daily for the first week; samples were acquired every other day for up to 10 weeks of lactation thereafter, depending on the ability of each mother to produce milk. All milk samples were stored at −80 °C prior to extraction.

Oligosaccharide Extraction and Purification. Oligosaccharides were extracted, reduced, and purified according to a recent published procedure (*12*). Briefly, each milk sample (0.5 mL) was centrifuged at 3500 rpm at 4 °C for 30 min and was extracted with 10 mL (2:1) (v/v) of a chloroform–methanol solution and 2 mL of deionized water. The emulsion was centrifuged at 3500 rpm at 4 °C for 30 min, and the lower chloroform layer was discarded. The upper layer was collected; the proteins were precipitated with 7 mL of cold ethanol and left at 4 °C for 16 h. The solution was centrifuged at 4 °C for 30 min, the supernatant was recovered, dried, and used for oligosaccharide analysis. Each oligosaccharide-rich fraction was redissolved in 500 μL of deionized water, reduced using 500 μL of 2.0 M sodium borohydride in deionized water, and incubated at 42 °C for 16 h. A nonporous graphitized carbon solid phase extraction (GCC-SPE) procedure was used to remove borate salts and purify and fractionate the solution. Prior to use, the GCC-SPE cartridge was washed three times with 3 mL of 0.1% trifluoroacetic acid (TFA) in 80% (v/v) acetonitrile/water followed by 3 mL of deionized water three times. After the oligosaccharide mixture was loaded onto a cartridge, 3 mL of deionized water was eluted ten times. The oligosaccharides were eluted with 6 mL of 20% (v/v) acetonitrile/water and were evaporated in vacuo. The dried oligosaccharide-rich fraction was reconstituted with 50 μL of deionized water and diluted 500 times with 0.1% formic acid in 50% (v/v) acetonitrile/water prior to HPLC-Chip/TOF-MS analysis.

HPLC-Chip/TOF-MS Analysis. Oligosaccharide fractions were analyzed using an Agilent 6200 series HPLC-Chip/ TOF-MS system equipped with a microwell-plate autosampler (maintained at 20. 0 °C), capillary sample loading pump, nanopump, HPLC-Chip interface, and an Agilent 6210 TOF LC/MS. The chip used consisted of a 40 nL enrichment column and 43 × 0.75 mm i.d. analytical column, both packed with porous graphitized carbon 5 μm stationary phase. For the sample loading, the capillary pump delivered isocratically 0.1% formic acid in 3.0% (v/v) acetonitrile/water at 4 μL/min. Injection volume was 0.5 μL. A nanoliter pump gradient was delivered at 0.3 μL/min consisting of (A) 0.1% formic acid in 3.0% acetonitrile/water (v/v) and (B) 0.1% formic acid in 90% (v/v) acetonitrile/water. A 45-min nanoLC gradient was run from 0 to 16% B, 2.5–20.0 min, 16–44% B, 20.0–30.0 min, and 44–100% B, 30.0–35.0 min with equilibration time of 20 min at 0% B. The drying gas temperature was set at 325 °C with a flow of 4.0 L/min (2 L of filtered nitrogen gas and 2 L of filtered dry grade compressed air). Data were acquired in the positive ionization mode within a mass range of *m/z* 500–3000. Mass correction was enabled using a reference mass of *m/z* 519.139.

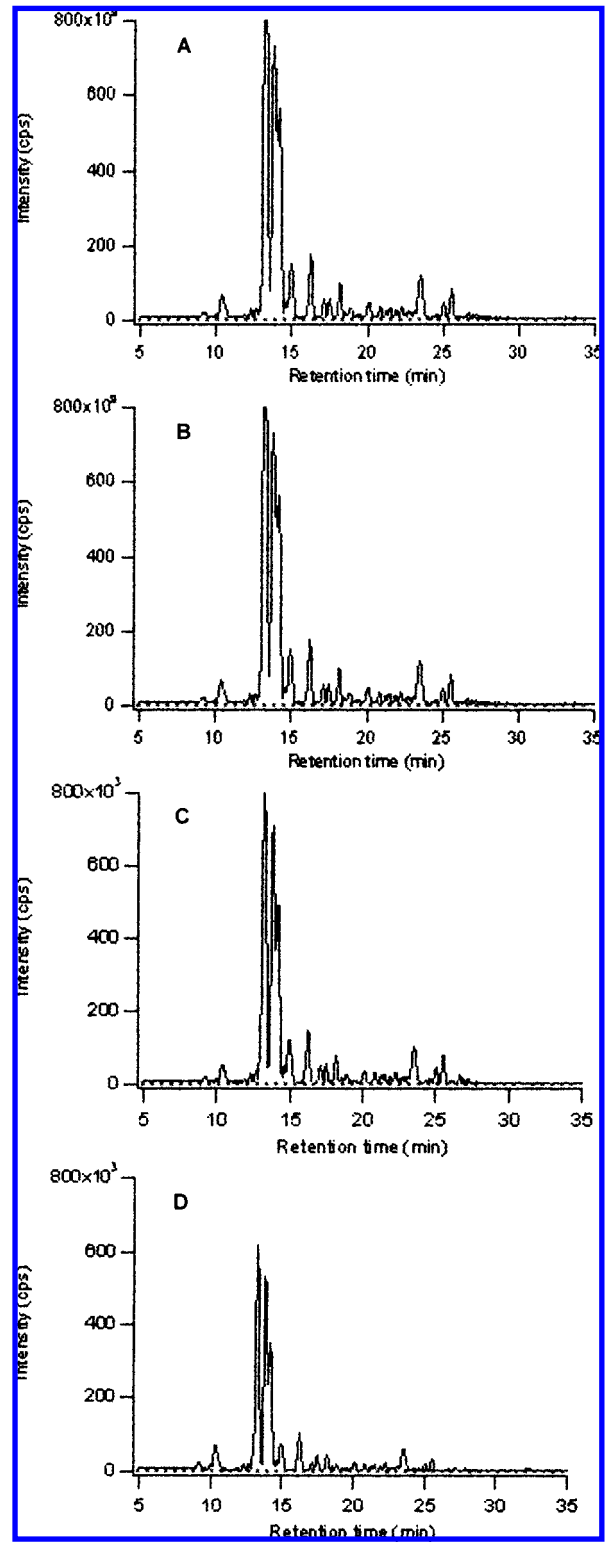

Figure 2. Base peak chromatograms of human milk oligosaccharides isolated from donor 5 at the early stages of lactation: day 2 (**A**); day 3 (**B**); day 4 (**C**); day 5 (**D**).

Data Analysis. Data analysis was performed using Analyst QS 1.1 software, and the lists of deconvoluted masses were generated using Agilent Mass Hunter (Molecular Feature Extraction) software. Oligosaccharide compositions were assigned using an in-house Glycan Finder program written in Igor Pro version 5.04B software from WaveMetrics, Inc. (Portland, OR). The algorithm was designed to examine a list of experimentally measured masses and search for all possible monosaccharide combinations matching the experimental mass

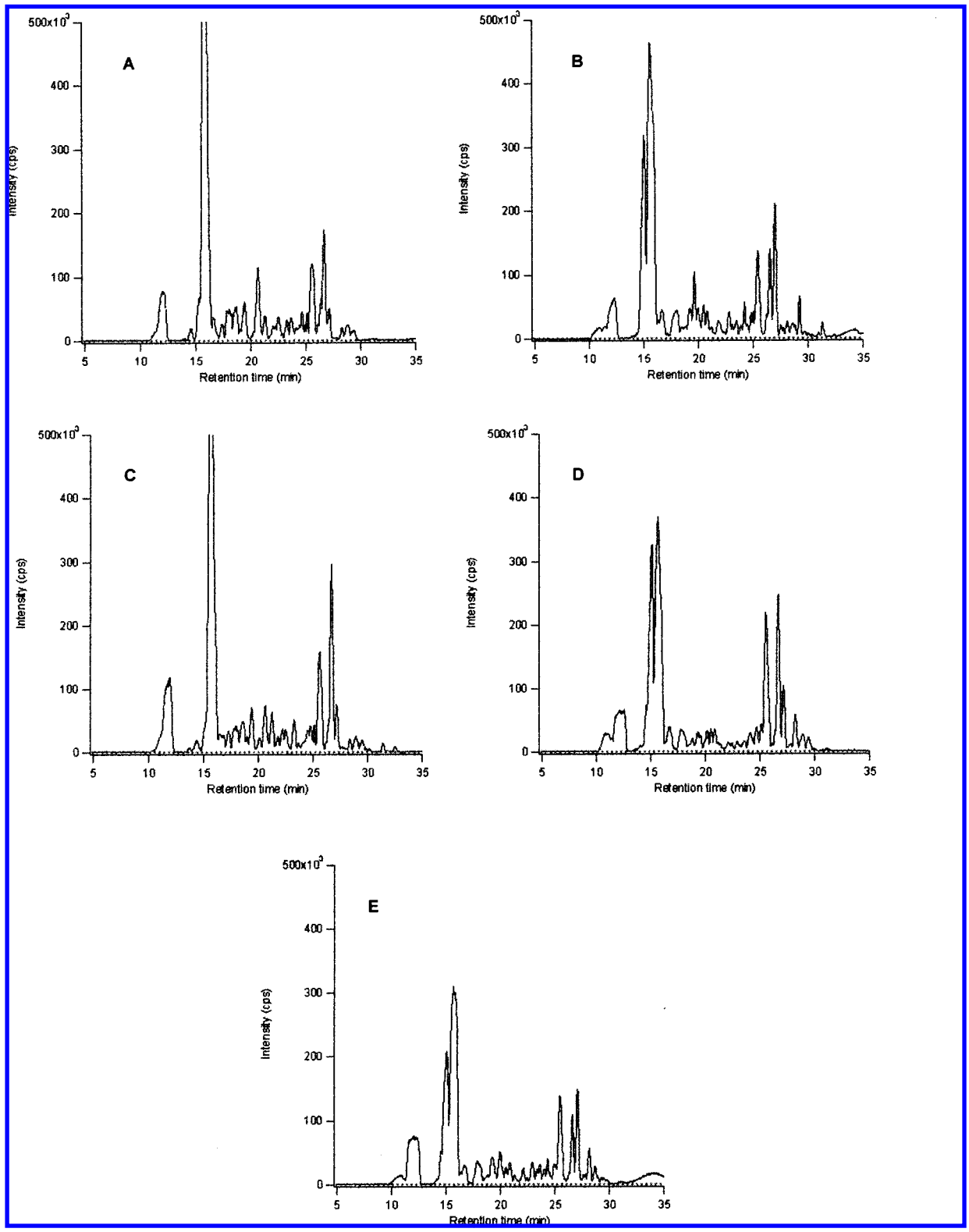

Figure 3. Base peak chromatograms of human milk oligosaccharides obtained from five different individual donors on day 6: donor 1 (**A**); donor 2 (**B**); donor 3 (**C**); donor 4 (**D**); donor 5 (**E**).

within a specified tolerance level (mass error). The program output also sorts each measured mass based upon its retention time and relative intensity. The Glycan Finder program outputs were resorted based on the mass error (on the average of less than 9 ppm) and the possible combinations of oligosaccharide compositions based on known basic structure of milk oligosaccharides (i.e., containing the lactose core group as minimum requirement). Agilent GeneSpring MS 1.0.1 software was also used to determine differential abundance of mass entities using a one-way ANOVA test.

RESULTS AND DISCUSSION

NanoLC Separation of Human Milk Oligosaccharides. The basic approach for annotating the human milk glycome employ-

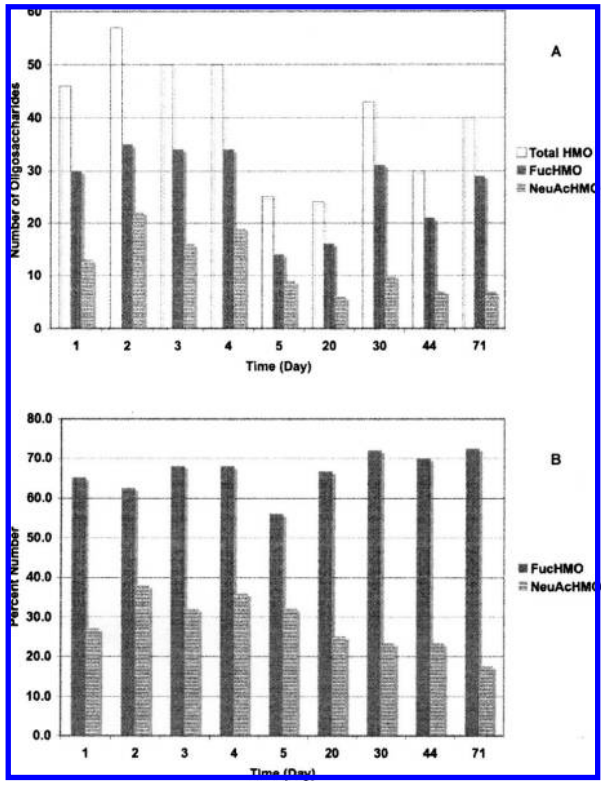

Figure 4. Numbers of total, fucosylated, and sialylated oligosaccharides identified from donor 1 during lactation in absolute number (**A**) and percentage (**B**).

ing a combination of analytical strategies including the use of a prototype microfluidic chip packed with porous graphitized carbon and coupled with an accurate mass time-of-flight (TOF) MS analyzer for high mass accuracy measurements was described previously as the detailed analysis of oligosaccharide compositions in human milk from a pooled sample (*12*). For the current study, a recently commercially available HPLC-Chip/TOF-MS was used to obtain nearly daily profiles of oligosaccharides in human milk for a period of nearly 3 months across early lactation. The HPLC-Chip/MS system replaces the traditional column and regular fittings in standard LC/MS systems with an integrated microfluidic chip providing effective separation with significantly improved sensitivity and reproducibility. A representative glycan profile (base peak chromatogram) from the HPLC-Chip/TOF-MS run for an individual (donor 5) on day 3 is shown in **Figure 1A**. There are three components of significant abundances making up over 55% of the total. On the basis of the mass spectra (**Figure 1B–D**), the most abundant species were identified to be lacto-*N*-neotetraose (LNnT, **Figure 1B**), lacto-*N*-tetraose (LNT, **Figure 1C**) with corresponding protonated ion of *m*/*z* 710.3 $[M + H]^+$ and ammonium adduct ion of *m*/*z* 727.3 $[M + NH_4]^+$, and lacto-*N*-fucopentaose I/V (LNFP I/LNFP V, **Figure 1D**) with ions corresponding to *m*/*z* 856.3 $[M + H]^+$ and *m*/*z* 873.4 $[M + NH_4]^+$. Reference standards, examined independently, confirmed the identities of these glycans (data not shown). This finding is also in agreement with published work in which the same oligosaccharides were observed as the major components of term and preterm milk with a concentration of about 0.5–2.0 g/L (*6*). Additionally, there are no other signals observed in the mass spectra whose presence would indicate the coelution of other endogenous compounds or background ions. The results further demonstrate the high efficiency of the separation.

One of the primary requirements for this type of profiling is a reproducible nanoLC separation. The chromatographic reproducibility of the HPLC-Chip/TOF-MS platform was examined by monitoring the retention time (RT) shift, the peak area, and peak height deviation of ten randomly selected peaks. The oligosaccharide sample was injected nine times consecutively over a single period, and 10 peaks were monitored to calculate for the average and standard deviation of the retention time, peak area (counts), and peak height (counts per second) (**Table 1**). The reproducibility of the retention times for 10 selected peaks was high, which were typically within 0.1% relative standard deviation. The shifts in retention time ranged from 0.01 to 0.15 min. This performance is superior compared to standard and other nanoLC chromatographic techniques and can be attributed to the integrated preconcentration and analytical column on the microfluidic chip (*25*).

Larger retention time shifts of about 1.5 min were observed over a period of several days on a single chip and between different chips. An alignment algorithm developed as part of the software (Agilent GeneSpring MS) compensates for the larger shifts in retention time. For greater consistency, all oligosaccharide samples for each individual donor were examined on the same day using the same microfluidic chip.

The reproducibility of MS signal intensity was also investigated by calculating the percent relative standard deviations (%RSDs) of peak areas and peak heights for the 10 selected peaks. The peak areas and peak heights vary from 3 to 29% and from 3 to 15% RSDs, respectively. It is noticeable that %RSDs are smaller for the most prominent peaks compared to the weaker abundant peaks. Therefore, the quantification of the more abundant peaks between individuals is more accurate than those significantly less abundant peaks.

Daily Variations in Oligosaccharide among Individual Donors and between Donors. Figure 2 demonstrates glycan profiles of human milk from several days of donor 5 at the early stages of lactation. As shown by these profiles (base peak chromatogram), the oligosaccharides during the early lactation period (colostrums, days 2, 3, 4, and 5) showed little significant variation. The numbers of peaks are approximately the same with only minor differences in peak abundances. The three major peaks, as before, were LNnT, LNT, and LNFP I and/or LNFP V. At this time, it is difficult to differentiate between LNFP I and LNFP V. The two isomers differ by the position and the linkage of the fucose and do not separate well with HPLC. This behavior is supported by the analyses of standard mixtures of the compound where they yield nearly identical retention times. These isomers can only be distinguished by employing specific exoglycosidases for α-1,2 and α-1,3-linked fucose compounds. Nonetheless, little variation is observed in the oligosaccharide content during the first five days of the lactation period, consistent with a previous study that reported the total oligosaccharide concentrations do not vary in the first week postpartum (*22*). Therefore, neither the total oligosaccharide content nor the individual components vary significantly during the first week of lactation.

For each individual, there appeared small variations in the oligosaccharides throughout the first 2–3 months (see below). A more comprehensive investigation on the oligosaccharides profiling data was also accomplished using Agilent GeneSpring MS software, which is capable of assessing large amounts of LC/MS data. The software allows a more extensive analysis of all components and tracks each individual component through each sample. Initial analysis of the data was performed using the Mass Hunter software

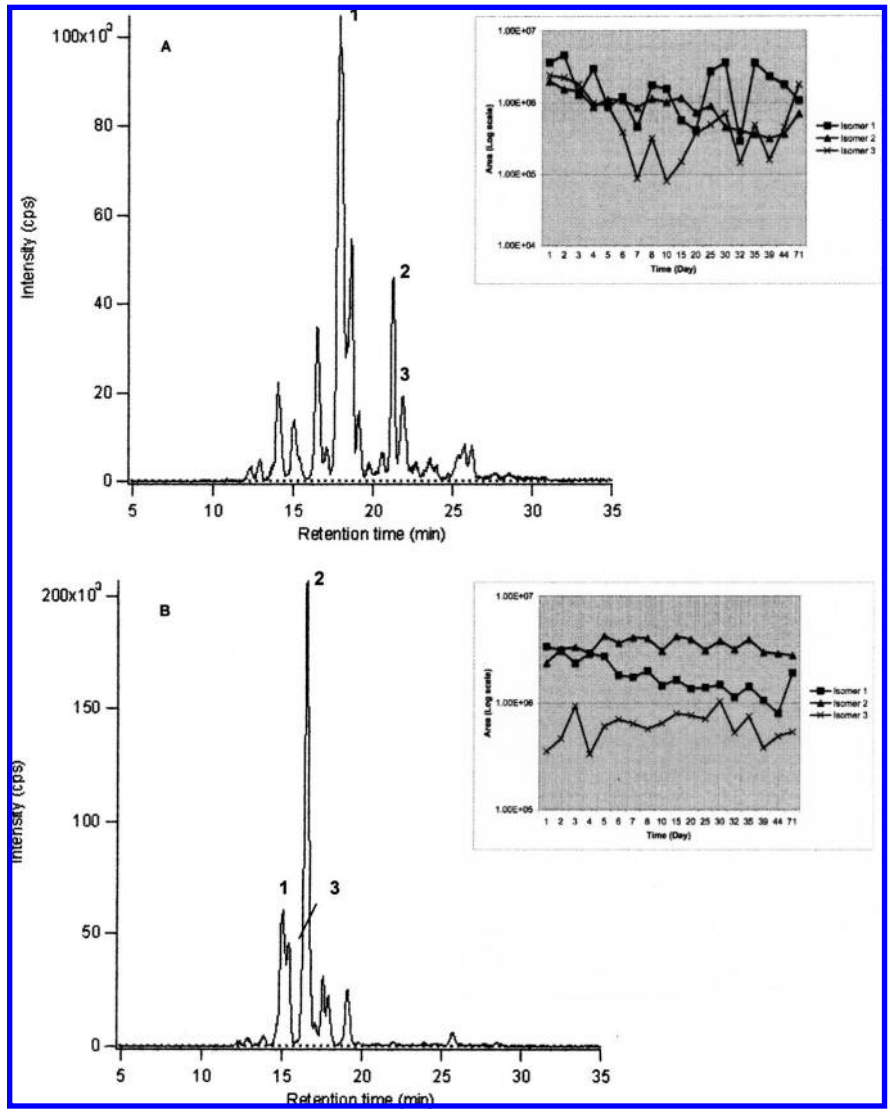

Figure 5. (**A**) A typical extracted ion chromatogram of *m*/*z* 1075.4 (4 Hex; 2HexNAc) from HPLC-Chip/TOF-MS run of oligosaccharides from donor 1 on day 20. **Inset:** Abundances of *m*/*z* 1075.4 over 71 day lactation period: isomer 1 (17.96 min); isomer 2 (21.22 min); isomer 3 (21.84 min). (**B**) A typical extracted ion chromatogram of *m*/*z* 1221.4 (4Hex; 2HexNAc; 1Fuc) from HPLC-Chip/TOF-MS run of oligosaccharides from donor 1 on day 20. **Inset:** Abundances of *m*/*z* 1221.4 over 71 day lactation period: isomer 1 (15.02 min); isomer 2 (15.41 min); isomer 3 (16.52 min).

(Molecular Feature Extraction) where all the compounds with the same features (retention times and MS) were grouped together. The program removed persistent background masses, resolved coelutions, identified and reduced isotopic clusters to their monoisotopic peaks, identified related adducts (H^+, Na^+, K^+, NH_4^+) of a compound and reduced them to a single neutral accurate mass, and the corresponding abundance, and reported a list of neutral masses with their corresponding retention times and abundances (molecular features). The files generated were then imported and further analyzed using the Agilent GeneSpring MS 1.0.1 software. The workflow for the analysis consists of alignment and normalization of features (to account for shifts in retention times), followed by sample clustering or grouping. Finally, the group or compound was then examined using "one-way analysis of variance" (ANOVA) to track the variation in abundances of each individual compound. The imported data from Agilent Mass Hunter were combined as follows: colostrums (days 1–7), transitional (days 8–27), and mature (days 28–72) milk. For example, for donor 1 there are seven samples for colostrum 1, six samples for transitional, and eight samples for mature milk according to this classification. For donor 2, the corresponding numbers are six, nine, and five; four, six, and five for donor 3; five, six, and zero for donor 4; and six, six, and zero for donor 5. After the normalization steps, one-way ANOVA was performed to identify mass entities with statistically differential abundances within individual donors. Values of $p < 0.05$ were considered significant. As a result of this statistical test, there were only minor differential differences found in oligosaccharides abundances. For example with donor 2, only two components with minor abundances (<1% of total) and neutral masses corresponding to 1731.6 Da (5Hex; 3HexNAc; 2Fuc) and 709.3 Da (3Hex; 1HexNAc) with retention times 20.2 and 18.3 min, respectively, yielded the greatest variation across the lactation period. In the same way, minor components (<1%) with mass 1074.4 Da (4Hex; 2HexNAc) at RT 25.5 min for donor 3 and mass 1058.4 Da (3Hex; 2HexNAc; 1Fuc) at RT 16.4 min for donor 5 varied significantly across the lactation period. With each individual, the oligosaccharide profile

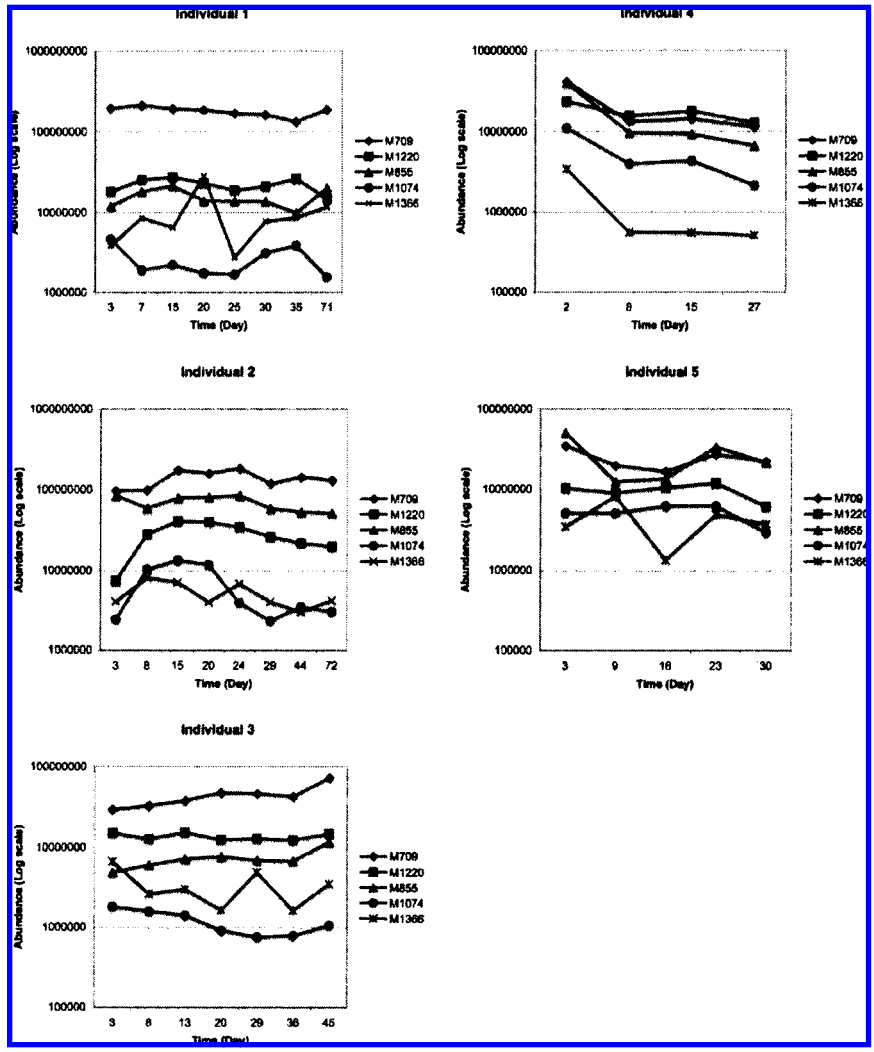

Figure 6. Abundances of selected preferentially consumed oligosaccharides by *B. longum* bv. *infantis* in five individual donors over the course of lactation.

varied little during the first 30 days with the greatest variation found only for the least abundant components.

In the previous study, large variations were reported between the oligosaccharides of five donors taken at the different points of lactation (*12*). Close to 200 oligosaccharide species were monitored in that study. These results were supported by reports from other laboratories that showed similarly large heterogeneity in milk oligosaccharides employing other methods including HPLC and HPAEC methods but monitoring less than 20 oligosaccharide components (*22, 26*). In this current report, five additional donors were examined and all detectable oligosaccharides were monitored every day in the first week and over a lactation period up to 70 days.

Although each donor was internally consistent, the differences between donors were apparent. **Figure 3** shows the base peak chromatograms of the five donors at a specific day (day 6). Each chromatogram was plotted in a fixed scale. Donors 1 and 3 made up of a single component LNT (structure inset **Figure 1**) that was off-scale and consisted of 60% of the total abundances. In a previous study that monitored the oligosaccharide consumption of three intestinal bifidobacteria, this oligosaccharide was the only one consumed by all three (*27*). *Bifidobacterium longum* bv. *infantis*, consumed this compound completely as well as several oligosaccharides with seven or less monosaccharide residues. Conversely, *B. longum* bv. *longum* and *B. breve* did not consume any other oligosaccharides from human milk (see below) (*27*). Donors 4 and 5 (**Figure 3D**,**E**) present similar glycan profiles with three major peaks at retention times ~13.2, 13.6, 14.0 min corresponding to LNT, LNnt, and LNFP I/V. Donor 2 has two major peaks at 13.6 and 14.1 min corresponding to LNT and LNFP I/V.

The five most abundant oligosaccharide components were identified in the five donors. For donors 1 and 3, the most dominant component (LNT) was 10 times more intense than the next most abundant (LNFP I/V). For donors 4 and 5, the three most abundant components (LNnT, LNT, LNFP I/V) make up over 50% of the total abundances. Among these samples, neutral oligosaccharide with neutral mass 709.3 Da (3Hex, 1HexNAc–LNT) is the most prominent that are common to all samples. This specific species is found to be preferentially consumed to some extent by several bifidobacterial strains as previously mentioned (see below) (*27*). The next most common species with relatively strong abundances consist of fucosylated oligosaccharides with masses 855.3 and 1220.4 Da (found in all five donors) and 1511.6 Da a fucosylated species with a sialic acid residue. The presence of these HMOs in great quantity may be correlated to important biological roles in maintaining a healthy gut microflora or in prevention of diseases among infants as previously reported (*8, 11, 28, 29*).

Levels of Fucosylated and Sialylated Oligosaccharides over the Course of Lactation. Analyses of the composition based on masses were also performed to examine the total

numbers and abundances of oligosaccharides with biologically significant residues, i.e., fucosylated and sialylated oligosaccharides. Donor 1 is illustrative of the five donors. **Figure 4** shows the fraction of fucosylated oligosaccharides in total numbers of oligosaccharides. There are slight variations in the total number of fucosylated species compared to the total amount. For donor 1, the total number of oligosaccharides with fucose residues ranged between 57 and 73% of the total number from day 1 to day 71. The total abundances of fucosylated oligosaccharides compared to the total abundances ranged between 26 and 44% for the same period (data not shown). The total number of sialylated oligosaccharides varied between 18 and 38% of the total number, while the total abundances ranged between 4 and 17%. The total number of oligosaccharides ranged between 24 and 57 for the 71 day period. This value depends naturally on the sensitivity of the technique and is affected by the presence of a few strongly abundant components. However, the total number is significantly less than the ~200 oligosaccharides previously found in pooled sample of human milk. The numbers of oligosaccharides from each donor are not necessarily large, but there is significant variability between each donor.

Monitoring Isomers of Oligosaccharides during the Lactation Period. The oligosaccharide compositions having a single mass can be made up of several isomers. For example, *m*/*z* 1075.4 is made up of several isomers as exemplified by the extracted ion chromatogram shown in **Figure 5A** of oligosaccharides from donor 1 on day 20. Similarly, **Figure 5B** shows the separation of several isomers with a single mass of *m*/*z* 1221.4. The extent and roles of oligosaccharide isomers (microheterogeneity) in milk or in any biological mixtures are still not well understood (*30*), primarily because the complexity of the mixture makes it difficult to monitor individual isomers and elucidate their functions. Two groups of isomers composed of 4Hex; 2HexNAc (*m*/*z* 1075.4) and 4Hex; 2HexNAc: 1Fuc (*m*/*z* 1221.4) were monitored for donor 1 over a 71 day lactation period. **Figure 5A** (inset) shows the abundances of three isomeric nonfucosyalted oligosaccharides with *m*/*z* 1075.4. The abundances of isomer 1 (RT, 18.0 min) vary from day to day. However, isomer 2 (RT, 21.2 min) drops from day 1 to 4, remains constant up to day 15, and drops slightly between day 15 and 40. It then increases slightly between day 44 and 71. A comparatively similar trend is observed for isomer 3.

The behavior of three fucosylated isomers varied for the same donor (**Figure 5B** (inset)). The fucosylated compounds with *m*/*z* 1221.4 show three nearly different behaviors corresponding to one that slightly increases (isomer 2), another that decreases (isomer 1), and one that stays constant throughout the lactation period (isomer 3).

Large variations in the oligosaccharide contents varied primarily with the minor components. Whether the minor daily variations are due to the mother's diet, physiology, or the feeding behavior of the infant is not known. The major components are consistently present and have been observed to be responsible for the selectivity in their stimulation of microbiome population growth and are, for example, consumed by *B. longum* bv. *infantis* (see below).

Variations in Oligosaccharides Preferably Consumed by *B. longum* bv. *infantis*. Specific oligosaccharides preferentially consumed by *B. longum* bv. *infantis* have been previously identified (*27*). These compounds correspond to the most prominent components (709.3, 855.3, 1074.4, 1220.4, and 1366.5 Da) and their isomers. The most significant component for donors 1 and 2, and 3 (709.3) belongs to this group. These oligosaccharides remain relatively constant over the course of lactation with few exceptions (**Figure 6**) For donor 1, with a single major component, that major species remained relatively constant through the 71 day lactation period with only a minor drop in abundances later in the period. For donor 3, this compound even increased over the shorter period, while all the other components remain constant. There are some slight drops observed in the major components in donors 4 and 5. Nonetheless, these oligosaccharides are consistently produced in large abundances in the monitored lactation period and likely provide a selective growth substrate for specific bifidobacteria in the establishment of an infant's healthy intestinal microbiota.

In summary, HPLC-Chip/TOF-MS profiling of oligosaccharides in human milk samples is a robust tool to analyze complex mixtures of oligosaccharides and identify exact compositions accurately with high reproducibility. With the combination of high throughput capabilities and rigorous data analysis, statistically significant variations of the profile features can be detected in each sample. With this advancement in analytical technology, automatic profiling of human milk oligosaccharides can be applied to understanding specific biological functions of these biomolecules and the values that they provide to the health and development of infants.

The high abundances and the constant production of bioselective substrates for intestinal microbes may be the primary functions of oligosaccharides in human milk. That the major components of HMO are preferentially consumed by bifidobacteria found in the feces of breast fed infants suggest that oligosaccharides achieve their microbiome sculpting functions by providing the necessary carbon source for the nutrition of the gut microflora. However, the large heterogeneity and the daily variations of some of the components may be further manifestations of their additional roles.

A curious feature of the HMO profiles is the tendency of a few donors to produce a single component in very large quantity. The implications of the production of a single dominant oligosaccharide by certain mothers are interesting and potentially important to the health of their infants and merits further research.

ABBREVIATIONS USED

HMO, human milk oligosaccharides; HPLC, high-performance liquid chromatography; HPAEC, high pH anion exchange chromatography; CE, capillary electrophoresis; MS, mass spectrometry; 2′-FL, 2′-fucosylactose; LDFT, lactodifucotetraose; LNT, lacto-*N*-tetraose; SPE, solid phase extraction; TFA, trifluoroacetic acid; TOF, time-of-flight; LNnt, lacto-*N*-neotetraose; LNFP, lacto-*N*-fucopentaose; RT, retention time; RSD, relative standard deviation; ANOVA, one-way analysis of variance; Hex, hexose; HexNAc, *N*-acetylhexosamine; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid

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