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Article

Combining HPAEC-PAD, PGC-LC−MS, and 1D ^{1}H NMR to Investigate Metabolic Fates of Human Milk Oligosaccharides in 1-Month-Old Infants: a Pilot Study

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ABSTRACT: A solid-phase extraction procedure was optimized to extract 3-fucosyllactose and other human milk oligosaccharides (HMOs) from human milk samples separately, followed by absolute quantitation using high-performance anion-exchange chromatography-pulsed amperometric detection and porous graphitized carbon-liquid chromatography−mass spectrometry, respectively. The approach developed was applied on a pilot sample set of 20 human milk samples and paired infant feces collected at around 1 month postpartum. One-dimensional ^{1}H nuclear magnetic resonance spectroscopy was employed on the same samples to determine the relative levels of fucosylated epitopes and sialylated (Neu5Ac) structural elements. Based on different HMO consumption patterns in the gastrointestinal tract, the infants were assigned to three clusters as follows: complete consumption; specific consumption of non-fucosylated HMOs; and, considerable levels of HMOs still present with consumption showing no specific preference. The consumption of HMOs by infant microbiota also showed structure specificity, with HMO core structures and Neu5Ac(α2-3)-decorated HMOs being most prone to degradation. The degree and position of fucosylation impacted HMO metabolization differently.

KEYWORDS: metabolization, infant nutrition, milk groups, consumption pattern, breastfeeding, 3FL

INTRODUCTION

Exclusive breastfeeding is recommended by the World Health Organization for infants under 6 months of age, making human milk the preferred sole source of nutrition in the first months of life.[1] As the third most abundant component in human milk, human milk oligosaccharides (HMOs) have been examined in increasing number of studies on the myriad of health benefits it brought to infants. After ingestion by infants, a majority of HMOs in human milk escape the digestion of stomach and upper small intestine, reaching the large intestine of infants,[2,3] where they can be assimilated by beneficial microbes, mostly *bifidobacteria*.[4,5] HMOs are also known to play a role in the immune system of infants,[6] as well as to prevent infectious diseases among infants by inhibiting the adherence of enteric pathogens.[7−10] Specific HMO structures, including 2′-fucosyllactose (2′FL) and 3/6′-sialyllactose (3′SL; 6′SL), are considered beneficial for the cognitive development of individuals by, for example, improving learning and memory.[11−14] HMOs consist of five monosaccharides: glucose, galactose, *N*-acetylglucosamine, fucose, and *N*-acetylneuraminic acid (also called sialic acid). With a lactose at the reducing end, a number of lacto-*N*-biose or *N*-acetyllactosamine repeats can be added via (β1-3) or (β1-6) linkages to form the so-called core structures.[15] Core structures can be further decorated by variable amounts of fucoses via (α1-2)-, (α1-3)-, or (α1-4)-linkages, and/or sialic acids via (α2-3)- or (α2-6)-linkages, contributing to the complexity of HMO structures.[15] Heretofore, over 240 HMOs have been

recognized, with approximately 160 structures being fully characterized.[16−19] It should be noted that the HMO composition in human milk (both the amount and presence of specific structures) differs between mothers, depending on their genetic profiles. One key factor that determines the fucosylated HMO profiles is the secretor status and Lewis blood types of mothers.[20] The Lewis-positive mothers have the *Le* gene which encodes the enzyme α1,-3/4-fucosyltransferase (FUT3), while the secretor mothers have the *Se* gene that corresponds to α1,-2-fucosyltransferase (FUT2). Depending on different combinations of FUT2 and FUT3 activities, different ensembles of HMOs are produced in four groups of human milk.[21] Lewis-positive secretors produce (α1-2)-, (α1-3)-, and (α1-4)-linked fucosylated HMOs, which contain Le^a/Le^b/(pseudo-) Le^x/(pseudo-) Le^y/H epitopes. Lewis-negative secretors lack (α1-4)-linked fucosylated HMOs and contained only (pseudo-) Le^x/(pseudo-) Le^y/H epitopes. Lewis-positive non-secretors produce only Le^a/(pseudo-) Le^x and lack (α1-4)-linked structures. Only (α1-3)-linked structures are observed in Lewis-negative non-secretors, corresponding to (pseudo-) Le^x epitopes.[21] In a Vietnamese cohort study,
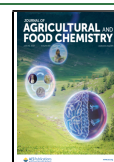
individuals have been observed who are Lewis-negative and are unable to produce (pseudo-)Le^x epitopes,[22] a feature also observed in an early Japanese study.[23]

Besides the Lewis secretor status, there are many other factors reported to influence the HMO composition of human milk, for example, lactation stages,[24,25] geographic origins,[26,27] and gestational ages.[20,28] A systematic review by Thurl et al. summarized the concentration ranges and mean values of 33 HMOs present in human milk from 21 eligible studies.[29] Large interlaboratory variations in the HMO concentrations were summarized in the review, partly due to numerous analytical methods employed, aside from other determinants.[29] The major techniques for HMO analysis include high-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD),[30,31] capillary electrophoresis with laser-induced fluorescence detection (CE-LIF),[32,33] matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS),[34,35] nuclear magnetic resonance (NMR) spectroscopy,[21] and liquid chromatography (LC) either by derivatization with a fluorescent label at the reducing end[36] or by reduction to alditols prior to porous graphitized carbon (PGC) separation combined with mass spectrometry (MS).[17,18,37] A review by van Leeuwen critically evaluated the pros and cons of these techniques, as well as the sample preparation steps.[38] One important step of sample preparation is extracting HMOs from other interfering fractions in human milk, which is often achieved by solid-phase extraction (SPE). The PGC material has commonly been used in SPE to remove salt, monosaccharides, and lactose.[20,32,39] However, van Leeuwen,[38] as well as Xu et al.,[40] pointed out the loss of 3-fucosyllactose (3FL) during the clean-up step of PGC-SPE, which was attributed to a lower affinity of 3FL to the column material. Xu et al. skipped the SPE step and directly injected the samples containing an overload of lactose directly into the UPLC to prevent the loss of 3FL.[40] However, mature human milk contains ±70 g/L of lactose;[15] therefore, lactose removal by SPE is usually done to avoid interference such as the co-elution or ion suppression of HMOs.[38] In this context, a new clean-up method should be developed to include 3FL in the HMO analysis in order to avoid underestimation. Beyond human milk, HMO analysis has also been performed in other biological fluids ranging from infant feces[32,41] to urine[42,43] and from plasma to amniotic fluid,[44−46] in order to understand the fate of HMOs after ingestion. Regarding the metabolization of HMOs by infant gut microbiota, most studies either took only HMO profiles of infant feces into consideration without matching the corresponding human milk[47] or were using only small sample numbers.[48,49] Therefore, the full picture of the metabolic fate of HMOs during gut transit, as well as inter-individual differences, is not yet clear.

The aim of the present study is to develop a new approach to quantitate HMOs including 3FL from human milk, and to combine three analytical methods, HPAEC-PAD, PGC-LC–MS, and one-dimensional ^{1}H NMR (1D ^{1}H NMR), to profile the major HMOs present in human milk and infant feces. This approach was then applied on a pilot sample set of 20 maternal milk and 20 paired infant fecal samples, collected one month postpartum, to determine the Lewis secretor phenotype of the mothers and to investigate the consumption patterns of HMOs by the gut microbiota of one-month-old infants. Preferences to different HMO structures (groups) by the infant's microbiota have been examined by comparing the HMO profiles of mother−infant dyads.

■ MATERIALS AND METHODS

Materials. Milk oligosaccharide standards, 3FL, lacto-N-neo-tetraose (LNnT), lacto-N-hexaose (LNH), lacto-N-neohexaose (LNnH), lacto-N-fucopentaose I (LNFP I), lacto-N-fucopentaose II (LNFP II), lacto-N-fucopentaose III (LNFP III), lacto-N-difucohex-aose I (LNDFH I), sialyl-lacto-N-tetraose a (LST a), sialyl-lacto-N-tetraose b (LST b), and sialyl-lacto-N-tetraose c (LST c) were purchased from Dextra Laboratories (Reading, UK). As to the other milk oligosaccharide standards, 2′FL and lacto-N-tetraose (LNT) were purchased from Carbosynth Ltd (Compton, UK); difucosyllac-tose (DFL) was purchased from ELICITYL (Crolles, France); and lacto-N-fucopentaose V (LNFP V), 3′SL, and 6′SL were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Twenty human milk and infant fecal samples were analyzed in the current study, from 20 mother−infant dyads that were included in the KOALA (acronym in Dutch for Child, Parent, and Health: Lifestyle and Genetic constitution) birth cohort study (N = 2834). The KOALA study was conducted in the south of the Netherlands and approved by the medical ethics committee of the Maastricht University/University Hospital of Maastricht, with informed consent signed by all participants.[50] Design of the KOALA study, including the baseline characteristic registration of mother−infant dyads and collection procedures of human milk and infant feces, have been published previously.[37,50,51] The 20 infants included in the current study were born from November 2002 to August 2003, at home or hospital by vaginal delivery or Caesarean section. The human milk and infant feces samples were collected at around 1 month postpartum on the same day. All the infants were exclusively breastfed till the time of sampling, without antibiotic administration. The milk and feces were collected in sterile tubes and sent to the lab within 1 day. As to the collection of feces, a diaper and a spoon were used, with a sanitary napkin to prevent absorption. Before storage, fecal samples were 10-fold diluted in peptone glycerol solution, and milk samples were centrifuged at 4 °C to remove lipid fraction. The processed samples were stored at −80 °C before further analysis.

Sample Preparation. Milk or fecal samples were thawed at 4 °C overnight. A volume of 0.6 mL of each sample was taken and diluted with an equal volume of Milli-Q water, followed by centrifugation at 21,000 × g (4 °C, 15 min). Exactly 1.0 mL of the clear liquid fraction of milk or fecal solution was taken and applied on a Carbograph SPE cartridge (Grace, Breda, the Netherlands, 300 mg bed volume for milk samples and 150 mg bed volume for feces). The SPE procedure was based on Albrecht et al.,[32] with modifications. The cartridges were activated with 3 × 1.5 mL of 80% (v/v) acetonitrile (ACN) containing 0.1% (v/v) trifluoroacetic acid (TFA), thereafter rinsed with 3 × 1.5 mL of Milli-Q water. After sample loading, the cartridges were first washed with 3 × 1.5 mL Milli-Q water to remove salts and monomers. Subsequently, the fraction of 3FL and lactose was collected by applying 3 × 1.5 mL of 3% ACN in water and then, the fraction of other milk oligosaccharides was collected with 3 × 1.5 mL of 40% ACN containing 0.05% TFA in scintillation vials. Both fractions were evaporated under a nitrogen (N$_2$) stream to remove ACN and eventually lyophilized. The step of washing with 3% ACN was omitted for fecal samples.

HPAEC-PAD Quantitation. The 3% ACN fraction of human milk samples were further analyzed by HPAEC-PAD. The lyophilized human milk was rehydrated and diluted with Milli-Q water, reaching a final dilution factor of 100. 10 μL of each sample was injected to an ICS 5000 system (Dionex, Sunnyvale, CA) equipped with a CarboPac PA-1 column (250 mm × 2 mm ID) was employed, which is preceded by a CarboPac PA guard column (25 mm × 2 mm ID). The column temperature was 20 °C, and the flow rate was 0.3 mL/min. Mobile phases A and B were 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH, respectively, with a gradient elution of B from 0 to 10% in the first 10 min. Following the gradient elution, 100% B was used to wash the column for 5 min, and 15 min 0% B to equilibrate to starting conditions. A pulsed amperometric detector (Dionex ICS-5000 ED) monitored the oligosaccharide signals, which were subsequently
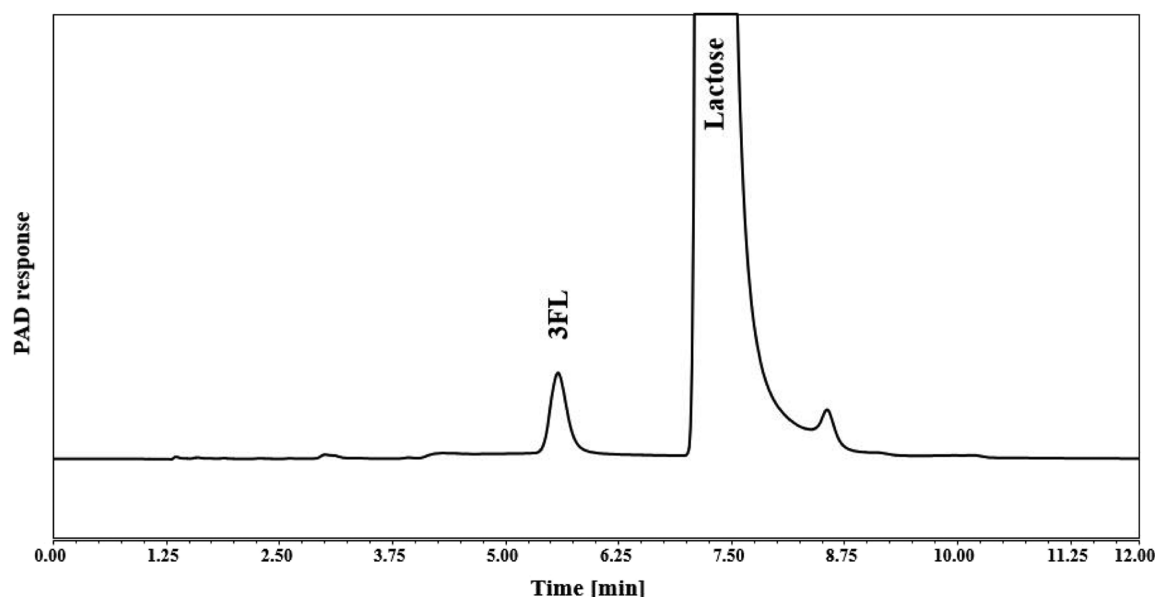
Figure 1. HPAEC-PAD elution of 3FL in the 3% ACN fraction of PGC-SPE of a human milk sample.

processed by using a Chromeleon 7.1 (Dionex). A 3FL calibration curve (0.4–20 μg/mL Milli-Q water) was used for quantification.

HMO Reduction and PGC-LC−MS Quantitation. HMOs present in the 40% ACN SPE fraction of human milk samples and infant feces were further chemically reduced at their reducing end sugar residue before being analyzed by PGC-LC−MS. The reduction of HMOs was performed to avoid split peaks of α- and β-anomers, caused by the strong separation ability of PGC column, as well as to achieve higher response factors in MS.[52] The lyophilized samples were rehydrated with Milli-Q water to make a 10-fold dilution of human milk, and 2-fold dilution of infant feces. A volume of 200 μL of the rehydrated samples was mixed with 200 μL of freshly prepared 0.5 M sodium borohydride (NaBH$_4$), subsequently left overnight at room temperature, to reduce the HMOs into alditols. A stock solution of HMO standards containing 40 μg/mL of 3FL, 2′FL, LNT, LNnT, LNH, LNnH, LNFP I, LNFP II, LNFP III, LNDFH I, 3′SL, 6′SL, LST a, LST b, and LST c, respectively, as well as 20 μg/mL of DFL were given the same treatment. The reduced samples and standards were purified on Carbograph SPE cartridges: first, 1.5 mL of 80:20 (v/v) ACN/water containing 0.1% (v/v) TFA, followed by 1.5 mL of Milli-Q water to activate the cartridges; then the 400 μL of reduced samples were loaded and washed with 4 × 1.5 mL of Milli-Q water to remove salts; and the HMO fraction was eluted with 1.5 mL of 40% ACN containing 0.05% TFA. The eluted HMO fractions were dried under N$_2$ stream and lyophilized.

The lyophilized human milk and infant feces were rehydrated and diluted with Milli-Q water to reach the final dilution factors of 20 and 4, respectively. HMO standard solutions of different concentrations ranging from around 0.40 to 20 μg/mL (0.20 to 10 μg/mL for DFL) were prepared from serial dilutions of the reduced stock solution. A volume of 5 μL of reduced samples was injected onto an Accela ultra-high-pressure liquid chromatography (HPLC) system (Thermo Scientific, Waltham, MA, USA), which was equipped with a Thermo Hypercarb column (100 × 2.1 mm, 3 μm particle size) preceded by a Hypercarb guard column (10 × 2.1 mm, 3 μm particle size). The elution gradient, using 1% (v/v) ACN in water containing 0.1% (v/v) formic acid as eluent A and ACN containing 0.1% (v/v) formic acid as eluent B, was set as published previously:[37] isocratic, 3% B in the first 5 min; 5−22 min, 3 to 20% B; 22−32 min, 20 to 40% B; and washing step with 100% B for 10 min and an equilibration step with 3% B for 21 min. The column oven was set at 25 °C and sample tray at 10 °C. The flow rate was 200 μL/min, except for the washing step and the first 10 min of equilibration, where the flow rate was 300 μL/min. A Velos Pro mass spectrometer (Thermo Scientific) with an

electrospray ionization probe was operated in negative-ion mode over a mass-to-charge ratios (m/z) range of 300−2000. Samples composed of a mixture of HMO standards were included both in the beginning and the end of sample sequences to verify the stability of the MS signal during the analysis. HMOs were identified by the same retention times with the specific standards, as well as checking the m/z values and confirming with the literature.[17,18] Xcalibur 4.3 (Thermo Scientific) was used for data processing. The quantitation of each identified HMOs was based on the integrated peak area from MS signals and corresponding calibration curves. The limit of quantitation (LOQ) of a given HMO structure was determined as the lowest concentration at which the signal/noise ratio ≥6. If needed, individual samples were 10× further diluted to have the concentrations of certain HMOs within the corresponding linear ranges.

NMR Spectroscopy. The 40% ACN fraction of human milk samples as well as infant feces were further analyzed by 1D ^{1}H NMR. Based on published methods,[21,22] the lyophilized samples were exchanged with 99.9%$_{atom}$ D$_2$O (Cambridge Isotope Laboratories, Andover, MA, USA), followed by another lyophilization and exchange with D$_2$O. Subsequently, 650 μL of D$_2$O containing acetone as the internal standard (δ_H 2.225) was used to dissolve the sample, before analysis on a Varian Inova 600 spectrometer (Groningen Biomolecular Sciences and Biotechnology Institute, NMR center, University of Groningen, The Netherlands). With probe temperatures set at 300 K, a spectral width of 4800 Hz, as well as zero filled to 32k, 1D ^{1}H NMR spectra were recorded at 16k complex points. The suppression of the HOD signal was performed by applying a pre-saturation Wet1D pulse. MestReNova 10.2 (Mestrelab Research SL, Santiago de Compostela, Spain) was used for spectra processing, employing a Whittaker smoother baseline correction with a median filter 20 and smooth factor 100. The interpretation of the structural-reporter-group signals, and integration of specific spectral regions were described in previous studies.[21,22]

Data Analysis. Hierarchical cluster analysis (HCA) was performed for a subset of samples that fulfilled two criteria that human milk belonged to the Le$^+$Se$^+$ group and total HMO concentrations in fecal samples exceeded 100 μg/mL as the diluted solution, which equals 1 μg/mg of fresh feces. HCA was conducted in R (Version 3.4.0) using the factoextra package, based on the relative compositional changes of the 17 HMOs from milk to feces. First, initial relative compositional changes were calculated by subtracting the relative abundance (%) of individual HMO in milk from the corresponding relative abundance (%) in paired feces, then divided by the former value. Then, all the positive values were normalized to 0−
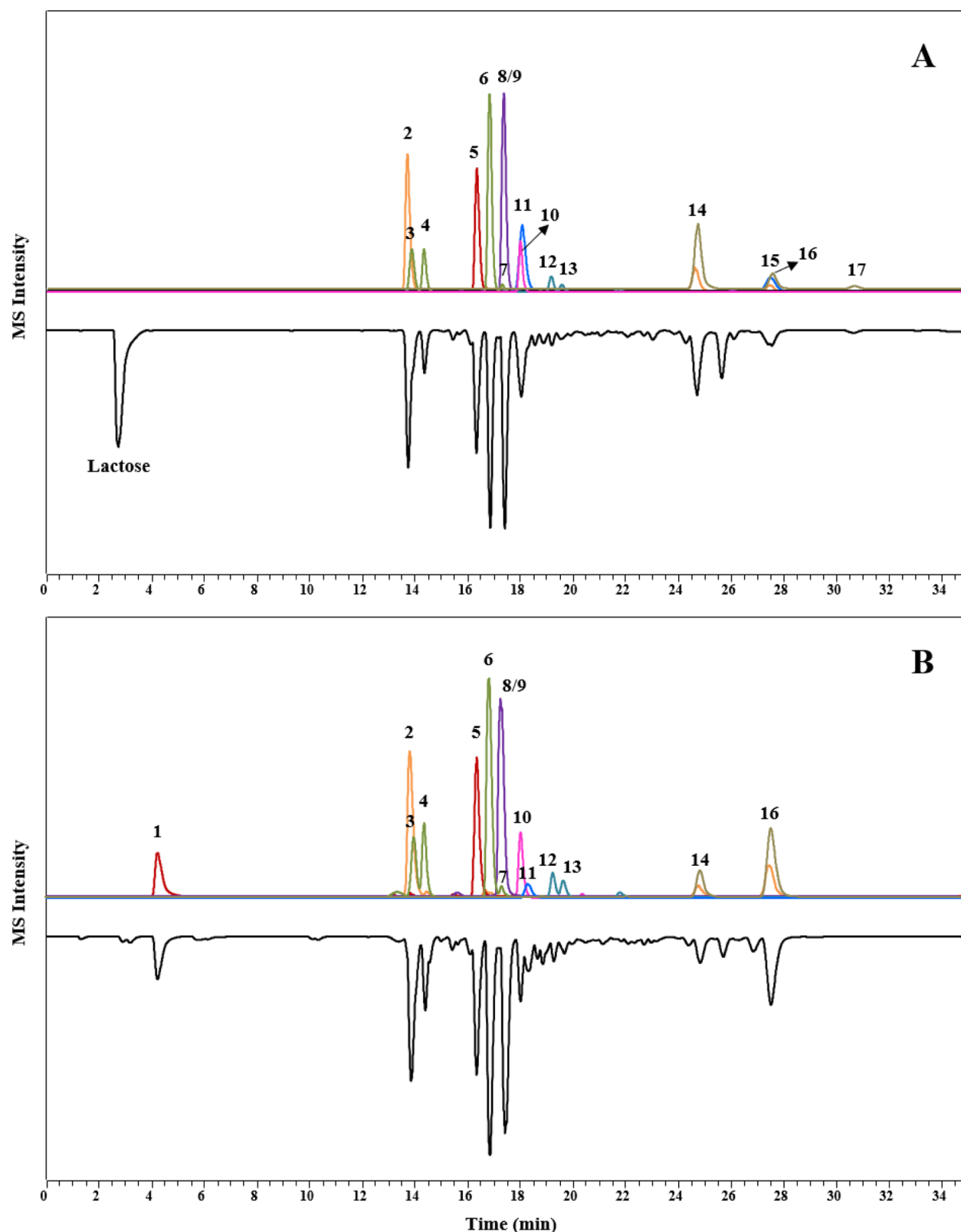
Figure 2. PGC-LC−MS elution patterns of a representative mother−infant pair samples: (A) human milk and (B) infant feces, in an extracted ion spectrum (colored lines, overlaid) and full spectrum (black lines, mirrored view). Names and structures of the identified HMO peaks 1−17 are listed in Table 1.

100%, by defining the initial maximum positive values in each sample as 100%. The relative compositional changes were visualized as heat map, using color scale function in Microsoft Excel 2019. When the total HMO concentration in a fecal sample was below 1 μg/mg, it was

considered as complete degradation. Other statistical analyses, including Kruskal−Wallis test and Pearson's chi-squared test for evaluating the baseline data of study subjects among different groups, as well as Wilcoxon signed-rank test for comparing proportions of

Table 1. Overview of the HMOs Quantitated in the Current Method by PGC-LC−MS[a]

Peak No.	RT (min)	m/z	Name	Abbreviation	Structure	Se/Le[#]
1	4.24	489.4, 535.4	3-Fucosyllactose	3FL		Ps-Le[x]
2	13.8	1000.5, 1046.5	Lacto-*N*-difucohexaose I	LNDFH I		Le[b]
3	13.9	854.4, 900.4	Lacto-*N*-fucopentaose III	LNFP III		Le[x]
4	14.4	854.4, 900.3	Lacto-*N*-fucopentaose II	LNFP II		Le[a]
5	16.3	489.3, 535.2	2′-Fucosyllactose	2′FL		Ps-H
6	16.9	854.4, 900.4	Lacto-*N*-fucopentaose I	LNFP I		H
7	17.4	854.4, 900.4	Lacto-*N*-fucopentaose V	LNFP V		Ps-Le[x]
8	17.4	708.4, 754.3	Lacto-*N*-tetraose	LNT		NA
9	17.4	708.4, 754.3	Lacto-*N*-neotetraose	LNnT		NA
10	18.0	681.3	Difucosyllactose	DFL		Ps- Le[y]
11	18.1	634.3	6′-Sialyllactose	6′SL		NA
12	19.2	1119.5, 1073.5	Lacto-*N*-hexaose	LNH		NA
13	19.6	1119.4, 1073.4	Lacto-*N*-neohexaose	LNnH		NA
14	24.7	999.5	Sialyl-lacto-*N*-tetraose c	LST c		NA
15	27.4	634.3	3′-Sialyllactose	3′SL		NA
16	27.6	999.5	Sialyl-lacto-*N*-tetraose b	LST b		NA
17	30.7	999.5	Sialyl-lacto-*N*-tetraose a	LST a		NA

[a]HMO structures were designed by using GlycoWorkbench[53] and kept consistent with previous studies:[17,18] blue circle—glucose; yellow circle—galactose; blue square—*N*-acetylglucosamine; red triangle—fucose; and purple diamond—*N*-acetylneuraminic acid. The linkages, if not stated in the table, are β linkages indicated following the rule of [structure symbol]. [b]Secretor- and Lewis-based histo-blood groups are assigned according to van Leeuwen et al.;[22] NA means not applicable.

different HMO structure groups between human milk and infant feces, were conducted in SPSS Statistics Version 26 (IBM Corp., Armonk, NY, USA).

■ RESULTS

HMO Analysis of Human Milk Samples. Huge variations exist among studies in terms of HMO concentrations present in human milk, and one major HMO, 3FL, has been often undetected or underestimated according to previous reports.[38,40] Therefore, in the current study, we developed a separate extraction and quantitation method of 3FL from the starting HMO pool. The PGC-SPE method was adapted from a previous report[32] to collect 3FL in the 3% ACN fraction

along with the major fraction of lactose before eluting the total HMO fraction. Subsequently, the 3% ACN fraction is analyzed by HPAEC-PAD, as shown in Figure 1, with the 3FL peak being identified by the same retention time as a commercial standard run concurrently. No interference with the huge amount of lactose was observed. The quantitation of 3FL was performed by using a calibration curve ($y = 1.6929x − 0.0823$) generated on a serial dilution of 3FL standards, with linear range covering 0.40−19.94 μg/mL and linear correlation (R^2) being 0.999.

The 40% ACN fraction of human milk eluted from SPE, which contained all other HMOs, was analyzed by PGC-LC−MS. Elution pattern of a representative human milk sample is
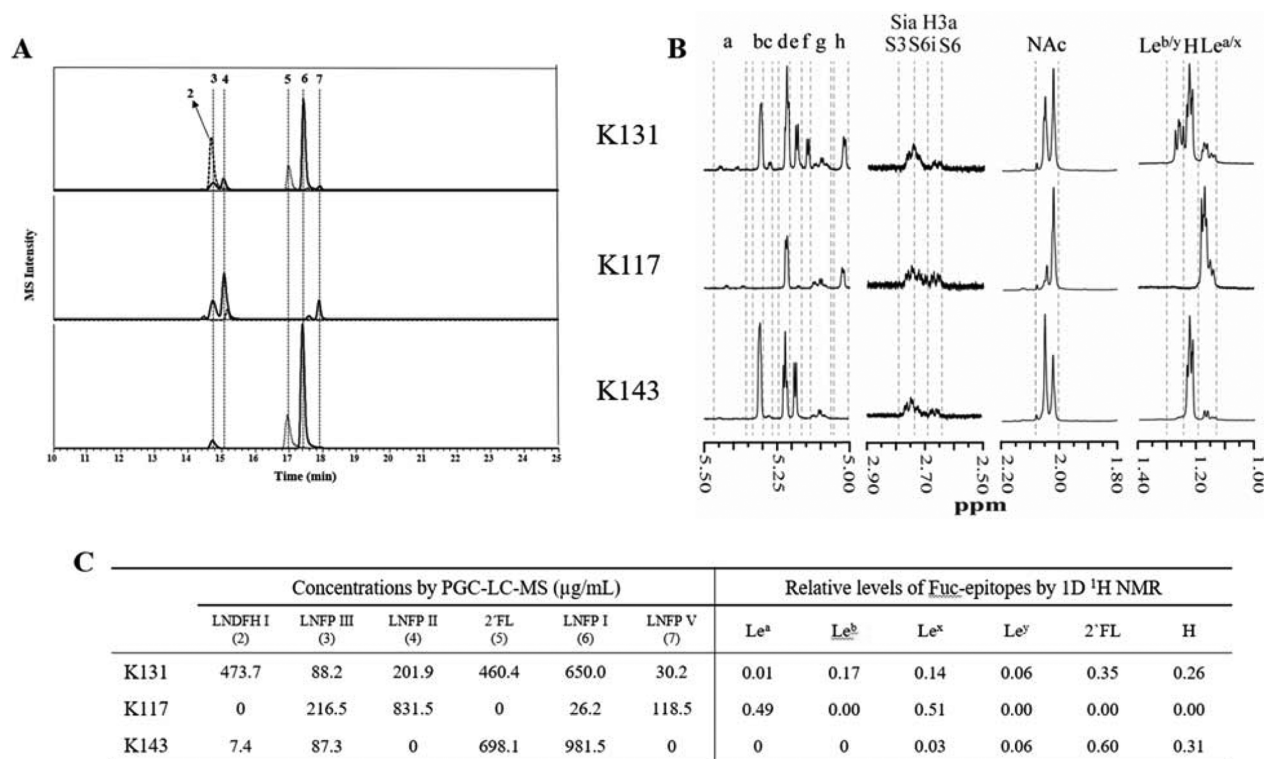
Figure 3. (A) Extracted and overlaid ion spectra of PGC-LC−MS chromatograms of three human milk samples: K131 (Le⁺Se⁺), K117 (Le⁺Se⁻), and K143 (Le⁻Se⁺). Ion spectra extracted with different m/z are shown in lines of different formats: solid (LNFP), square dot (LNDFL), and round dot (FL). (B) Relevant parts (δ 5.00−5.50, δ 2.50−2.90, δ 1.80−2.20, and δ 1.10−1.30 ppm) of 1D ^{1}H NMR spectra of the same human milk samples as in (A). Bracket identities of the anomeric region δ 5.00−5.50: (a) Fuc-(α1-3)-H-1 in pseudo-Le^x (3FL) and pseudo-Le^y (DFL) epitopes, (b) Fuc-(α1-2)-H-1 in 2′FL, (c) Fuc-(α1-2)-H-1 in Le^y epitopes, (d) α-D-Glc$p$ H-1, (e) Fuc-(α1-2)-H-1 in the H-antigen epitopes and α-D-Glc$p$ H-1 in 3FL and DFL, (f) Fuc-(α1-2)-H-1 in Le^b epitopes, (g) Fuc-(α1-3)-H-1 in Le^x and Le^y epitopes and (h) Fuc-(α1-4)-in Le^a and Le^b epitopes. Neu5Ac region δ 2.50−2.90: S3. Neu5Ac-(α2-3)-H-3e, S6. Neu5Ac-(α2-6)-Gal H-3e and S6i. Neu5Ac-(α2-6)-GlcNAc H-3e. Fuc CH$_3$ region δ 1.00−1.40: Le$^{b/y}$. CH$_3$ signals of Fuc residues in Le^b and Le^y epitopes, H. CH$_3$ signals of Fuc residues in H-antigen epitopes, Le$^{a/x}$. CH$_3$ signals of Fuc residues in Le^a and Le^x epitopes. Note: the Fuc anomeric region is not scaled in the same proportion as the Neu5Ac H-3e, NAc, and Fuc CH$_3$ regions. (C) Names and concentrations of each annotated peaks of (A) in the three human milk samples, as well as their relative levels of Fuc-epitopes derived from the 1D ^{1}H NMR integration of anomeric region sections a−h in (B).

given in Figure 2A, with names, structures, and retention times of the annotated peaks listed in Table 1. Most of the neutral HMOs were detected in both [M − H]⁻ and [M + FA-H]⁻, with [M + FA-H]⁻ being predominant; the sialylated HMOs were detected only as [M − H]⁻. As shown in Figure 2A, the extracted ion spectra of the HMOs listed in Table 1 constitutes over 90% of the full mass spectrum, with a few minor peaks excluded in the present study. A small amount of lactose could still be detected, indicating its incomplete removal; nevertheless, its minor presence did not interfere with HMO analysis. The peak of 3FL was absent in the LC chromatograms of the 40% ACN elution fraction, confirming its full elution into the 3% ACN fraction. Other identified HMOs eluted during 13−31 min, with neutral HMO peaks being eluted first within this window, followed by sialylated HMO peaks (23−28 min), except for 6′SL that was eluted at around 18.1 min.

Integrated peak areas from MS signals were used to quantitate each identified HMOs, based on calibration curves which were generated on serial dilutions of corresponding standards. Calibration curves of the 17 HMOs quantitated in the current study are given in the Supporting Information Table 1, with linear ranges, LOQ and R^2 of each curve evaluated. All the 17 calibration curves showed good linearity with R^2 > 0.99, covering concentrations mostly from 0.39 to 20

μg/mL (except for DFL, 0.078−10 μg/mL; LNnH, LST c, 3′SL and LST a, 0.078−20 μg/mL; and LNH and LST b, 0.156−20 μg/mL). Due to the fact that LNT was eluted at the same time as its isomer LNnT, which has a rather similar MS spectrum, it is hardly possible to differentiate between the two components, consistent with previous report.[54] Considering the similar response factors of their calibration curves, LNT and LNnT in human milk samples were integrated as one peak and quantitated using the calibration curve of LNT. Despite the co-elution of LNT and LNnT, we are able to separate and quantitate the absolute concentrations of almost all major HMOs present in human milk samples with the PGC-LC−MS method.

For comparison, a previously developed system of a 1D ^{1}H NMR structural reporter group signals was applied on the same set of samples for the annotation of Fuc- and Neu5Ac-containing HMOs.[21,22] Based on this system, structure-dependent regions are determined for the α-anomeric region (δ 5.00−5.50), NAc CH$_3$ region (δ 1.80−2.20), Fuc CH$_3$ region (δ 1.00−1.40), and Neu5Ac H3a region (δ 2.50−2.90), as shown in Figure 3B. In the Fuc CH$_3$ region (δ 1.00−1.40), three structure regions are observed. The bracket δ 1.24−1.30 contains CH$_3$ signals for Fuc(α1-2), Fuc(α1-3), and Fuc(α1-4) in case of two neighboring residues are fucosylated, that is, in Le^b or Le^y epitopes. The δ 1.19−1.24 bracket contains CH$_3$

signals belonging to Fuc-(α1-2) residues in an H-antigen epitope and the δ 1.14−1.19 bracket contains the CH_3 signals for Fuc(α1-4) and Fuc(α1-3) in Le^a and Le^x epitopes.[21] Beside these brackets the α-anomeric region shows a fingerprint of the Fuc-epitopes in 8 brackets **a−h**. Using these fingerprint brackets, together with the Fuc CH_3 brackets it is possible to distinguish Le^b from Le^y and Le^a from Le^x.[21] Also, a separate peak is observed for Fuc-(α1-2)-in 2′FL (bracket **b**), allowing a distinction of this HMO structure from other H-antigen structures (e.g., LNFP I). An estimation of the relative levels of Le^a, Le^b, Le^x, and Le^y can be made based on the peak intensity of these regions, as well as a differentiation between 2′FL-derived H-antigen and other H-antigen epitopes.

KOALA Birth Cohort Study Pilot Samples. The three analytical methods of HMOs, HPAEC-PAD, PGC-LC−MS, and 1D ^{1}H NMR, were applied on a subset of participants who enrolled in the KOALA birth cohort study. A total of 20 human milk samples from 20 mothers are analyzed in the current study, with the overall baseline data of these study subjects being summarized in Table 2. Sample collection was

Table 2. Baseline Data of the Study Subjects, Overall, and from Different Designated Consumption Pattern Clusters (Only Lewis-Positive Secretor Milk Group)[a]

variables	Overall n = 20	cluster A n = 3	cluster B n = 9	cluster C n = 5
age postpartum, day, mean (SD)	32 (4)	32 (6)	32 (3)	32 (4)
gestational age, week, mean (SD)	40 (1)	39 (0)	40 (1)	40 (1)
birthweight, g, mean (SD)	3657 (395)	3780 (505)	3643 (376)	3611 (394)
gender, male, n (%)	15 (75)	2 (67)	7 (78)	3 (60)
delivery mode, vaginal, n (%)	19 (95)	3 (100)	8 (89)	5 (100)
delivery place, home, n (%)	15 (75)	1 (33)	6 (67)	5 (100)

[a]The values of each variable were evaluated among different clusters using Kruskal−Wallis test, or Pearson's chi-squared test, yet no significant differences were found (data not shown).

conducted at 32 days postpartum on average (SD = 4), with all infants born in the full term and of normal birthweight, as well as exclusively breastfed till samples were collected. Out of the 20 mothers, only one mother delivered via Caesarean section, 75% of the mothers delivered at home. Concentrations of 3FL and the other 16 HMOs in the 20 human milk samples were quantitated by HPAEC-PAD and PGC-LC−MS, respectively, with the results of each individual sample given in the Supporting Information Table 2. Relative levels of Fuc-epitopes, namely, Le^a, Le^b, Le^x, Le^y, 2′FL, and non-2′FL H-antigen, derived from 1D ^{1}H NMR for the 20 milk samples are presented in the Supporting Information Table 4. Results of the selected samples will be described in the following sections.

Lewis/Secretor Milk Group Identification. In the present study, secretor status and Lewis blood groups of the 20 mothers were identified by examining HMO concentrations as quantitated by PGC-UPLC−MS. Results of Fuc-epitope levels as integrated from 1D ^{1}H NMR spectroscopy led to exactly the same grouping of mothers. Out of 20 human milk samples, 18 samples fit the profile of Le$^+$Se$^+$ (90%), one sample (K117) fits with the pattern of Le$^+$Se$^-$ (5%), and one (K143) fits Le$^-$Se$^+$ (5%). LC−MS chromatograms and 1D ^{1}H NMR

spectra of one Le$^+$Se$^+$ milk K131 are given in Figure 3A,B, respectively, together with that of K117 and K143. Extracted ion spectra of FL, LNFP, and LNDFH, showing their absolute intensities, are presented in Figure 3A, as 2′FL, LNFP I (H-antigen), LNFP II (Le^a), and LNDFH I (Le^b) are major and typical components for Lewis and secretor determination. Presence of the other two annotated peaks, LNFP III (Le^x) and LNFP V (pseudo-Le^x), are also important fucosylated structures, although independent from Lewis and secretor status of mothers. Concentrations of each annotated peak of the three milk samples, as well as the relative levels of Fuc-epitopes, are listed in Figure 3C.

Similar to the K131 milk sample, 18 milk samples fitting the Le$^+$Se$^+$ profile contained significant levels of Le$^{b/y}$, H-antigen and Le$^{a/x}$ CH_3 peaks (Supporting Information Table 4), in consistency with the high abundances of 2′FL, LNFP I, LNFP II, and LNDFH I present in the milk samples (Supporting Information Table 2). The relative levels of four Le-epitopes and H-antigen showed different balances among these Le$^+$Se$^+$ milks; likewise, large variations existed in concentrations of 2′FL, LNFP I, LNFP II, and LNDFH I presented in these samples, as measured by PGC-LC−MS. As shown in Figure 3, the milk sample from Le$^-$Se$^+$ mother K143 contained a trace amount of LNDFH I (7.4 μg/mL); however, the value is less than 2% of that in K131 milk. Moreover, K143 showed a strong H-antigen peak (e.g., 2′FL, LNFP I), despite the hardly detected Le$^{b/y}$ epitopes (e.g., LNDFH I), which was most likely hidden in the background noise. With a minor peak in the Le$^{a/x}$ region belonging only to Le^x epitopes (e.g., LNFP III), as evidenced by the absence of a Fuc(α1-4) anomeric signal (e.g., LNFP II), K143 was confirmed as the Le$^-$Se$^+$ milk group. As to milk sample K117, no peaks in Le$^{b/y}$ nor H-antigen Fuc CH_3 regions were detected, while anomeric peaks fitting Le^a as well as Le^x structures are observed, implying the mother's phenotype being Le$^+$Se$^-$. This is confirmed by high abundances of LNFP II, LNFP III, and LNFP V in LC−MS, whereas LNDFH I was hardly detected, and LNFP I was found only in trace amounts.

HMO Analysis of Infant Fecal Samples. Together with human milk samples, 20 infant feces were collected around the same day postpartum from the 20 mother−infant dyads. The collected infant feces were processed to extract HMOs and then analyzed by PGC-LC−MS and 1D ^{1}H NMR. Unlike human milk, lactose content significantly decreases during the transit through infants' gastro-intestinal (GI) tract, ending up in almost complete absence.[31] Therefore, the step of washing with 3% ACN was omitted for feces during SPE, which led to the elution of 3FL in the 40% ACN fraction of fecal samples, as well as analysis of 3FL by PGC-LC−MS together with other HMOs. Figure 2B presents chromatograms of the infant fecal sample corresponding to the human milk, as shown in Figure 2A. 3FL eluted after 4.2 min (Figure 2B; Table 1), much earlier than the other HMOs, but close to the retention time of lactose (2.6 min), as seen in Figure 2A, which was consistent with the loss of 3FL in the lactose removal step during PGC-SPE. Similar to the other 16 HMOs, the concentration of 3FL in fecal samples was quantitated based on its corresponding calibration curve, as shown in the Supporting Information Table 1. Three infant fecal samples (K109, K117, and K143) were only analyzed by 1D ^{1}H NMR, and PGC-LC−MS results were not available. Quantitation results of the 17 HMOs (only sum of LNT and LNnT were given) detected in each of the other 17 infant fecal samples are given in Supporting

Information Table 3. Relative levels of Le[a], Le[b], Le[x], Le[y], 2′FL, and non-2′FL H-antigen in the 20 infant feces, based on 1D ^{1}H NMR, are listed in the Supporting Information Table 4.

HMO Concentrations of the Le[+]Se[+] Group in Both Milk and Feces. To further study the 18 mothers whose milk samples are assigned to the Le[+]Se[+] milk group, the means and standard deviations of each quantitated HMO in the milk and paired infant feces samples, as determined by HPAEC-PAD and PGC-LC−MS, are summarized in Table 3. In the human

Table 3. Concentrations of 17 HMOs (μg/mL; Only the Sum of LNT and LNnT Was Given) and Proportions of Different HMO Structure Groups in Human Milks (N = 18) and in Paired Infant Feces in 10-Fold Diluted Solutions (N = 17) from the Lewis-Positive Secretor Milk Group by Using HPAEC-PAD or PGC-LC−MS[a]

	human milk	infant feces
compound	n = 18	n = 17
*fucosylated**	*61.0% (5.19%)*	*75.3% (15.7%)*
3FL	146 (54.7)	74.4 (76.7)
2′FL	561 (117)	91.3 (94.6)
LNFP I	536 (165)	132 (139)
LNFP II	290 (158)	108 (108)
LNFP III	134 (46.3)	40.1 (37.3)
LNFP V	40.6 (26.1)	1.70 (4.84)
DFL	84.8 (89.8)	116 (122)
LNDFH I	505 (142)	189 (115)
*non-fucosylated core***	*25.5% (5.23%)*	*8.60% (6.30%)*
LNT + LNnT	869 (367)	45.7 (52.4)
LNH	66.7 (41.5)	0.694 (1.54)
LNnH	71.6 (69.1)	1.21 (2.27)
sialylated, %	*13.6% (2.12%)*	*16.0% (13.4%)*
3′SL	94.3 (24.0)	1.01 (1.64)
LST a	20.9 (7.18)	1.52 (3.73)
6′SL	74.3 (30.0)	16.1 (27.6)
LST c	134 (58.9)	37.2 (64.0)
LST b	194 (100)	131 (281)
total	3822 (853)	986 (880)

[a]The results are shown as means (standard deviations). Significant differences were evaluated for proportions of different HMO structure groups between human milk and infant feces by Wilcoxon signed-rank test, with results noted as *p < 0.05, **p < 0.001.

milk samples, the sum of the 17 HMOs ranged from 2.1 to 5.6 mg/mL, with an average of 3.8 mg/mL. Fucosylated HMOs accounting for the largest proportion (60%), followed by non-fucosylated core structures (26%), and sialylated structures (14%). The HMOs having the highest average concentrations are LNT and LNnT, which were quantitated together. Other abundantly present HMOs include 2′FL, LNFP I, and LNDFH I, while LST b is the most abundant sialylated HMO on average. As for the fecal samples, the variation of single HMO concentrations between individuals is quite large and hardly any solid generic conclusions on individual HMO concentrations could be made. Average proportions of the total fucosylated, total non-fucosylated core, and total sialylated HMOs in fecal samples were compared to those in human milk, with a significant increase found in relative amounts of fucosylated structures, and highly significant reduction in relative amounts of non-fucosylated core structures. This added new observations concerning the consumption preference by infant colonic microbiota to specific HMO structural

elements and allowed us to look in more detail to the metabolization of HMOs ingested.

Metabolization of HMO in Infants. To further investigate the different metabolic fates of HMOs by infant colonic microbiota, relative compositional changes of the 17 HMOs between milk and paired feces were calculated for 14 selected mother−infant pairs. The 14 samples fulfilled two criteria that human milk belonged to the Le[+]Se[+] group, and total HMO concentrations in fecal samples exceeded 100 μg/mL as the diluted solution, which equals 1 μg/mg fresh feces. Concentrations of individual HMOs of fecal sample K109 was unavailable. Feces K026, K028, and K090 were excluded because of the limited presence of HMOs in the infant feces. These three fecal samples were designated as cluster A of the consumption pattern in the present study, which indicated the (almost) complete consumption of HMOs by infant colonic microbiota.

The relative changes of the HMO composition between milk and feces of the 14 included mother−infant pairs were visualized with a heat map, with hierarchical clustering performed to show similarities between these subjects (Figure 4; calculated values in Supporting Information Table 5). There are two main clusters, with nine subjects in the one cluster (designated as cluster B) and five in the other (designated as cluster C). Clear differences could be observed between the two clusters; subjects in cluster B showed increased relative levels of most fucosylated HMOs, while cluster C showed considerable reductions of those. As for core structures and sialylated structures, both clusters showed comparable reductions of relative levels in feces. This observation implies a specific consumption of non-fucosylated structures by cluster B infants; conversely, cluster C infants utilized HMOs from all three structural groups at considerable levels without obvious preferences. It is also noted that all infants from cluster B excreted higher concentrations of total HMOs (839.9−3065.3 μg/mL) compared to those from cluster C (120.9−641.7 μg/mL). To explore any maternal or infant factors associated with these consumption patterns, baseline variables of subjects from the three clusters are evaluated and summarized in Table 2; however, no significant differences are found among the clusters. When comparing the relative compositional changes of individual HMO structures in both cluster B and cluster C, it is noted that LNT, LNnT, LNH, and LNnH, which are core structures, as well as 3′SL and LST a, which are Neu5Ac(α2-3) structures, were the heavily degraded HMOs in most cases. Compared to Neu5Ac(α2-3) structures, Neu5Ac(α2-6) structures, including 6′SL, LST b, and LST c, were degraded to a lesser degree, as well as in fewer cases. This shows general preferences of colonic microbiota for different isomeric HMO structures. As for Fuc-containing structures, it is noted that DFL and LNDFH I were in general excreted in higher amounts in infant feces than structures with only one fucose residue, implying that a higher degree of fucosylation results in a lower degree of enzymatic degradation by colonic bacteria. The three mother−infant pairs grouped in cluster A showed almost no HMO peaks in the LC−MS chromatograms of their fecal samples (results not shown), indicating high consumption activities of gut microbiota which fully utilized the ingested HMOs.

To compare with the observations from the heat map and hierarchical clustering, 1D ^{1}H NMR spectra of one representative mother−infant pair from each of the three clusters are shown in Figure 5. All three milk samples showed
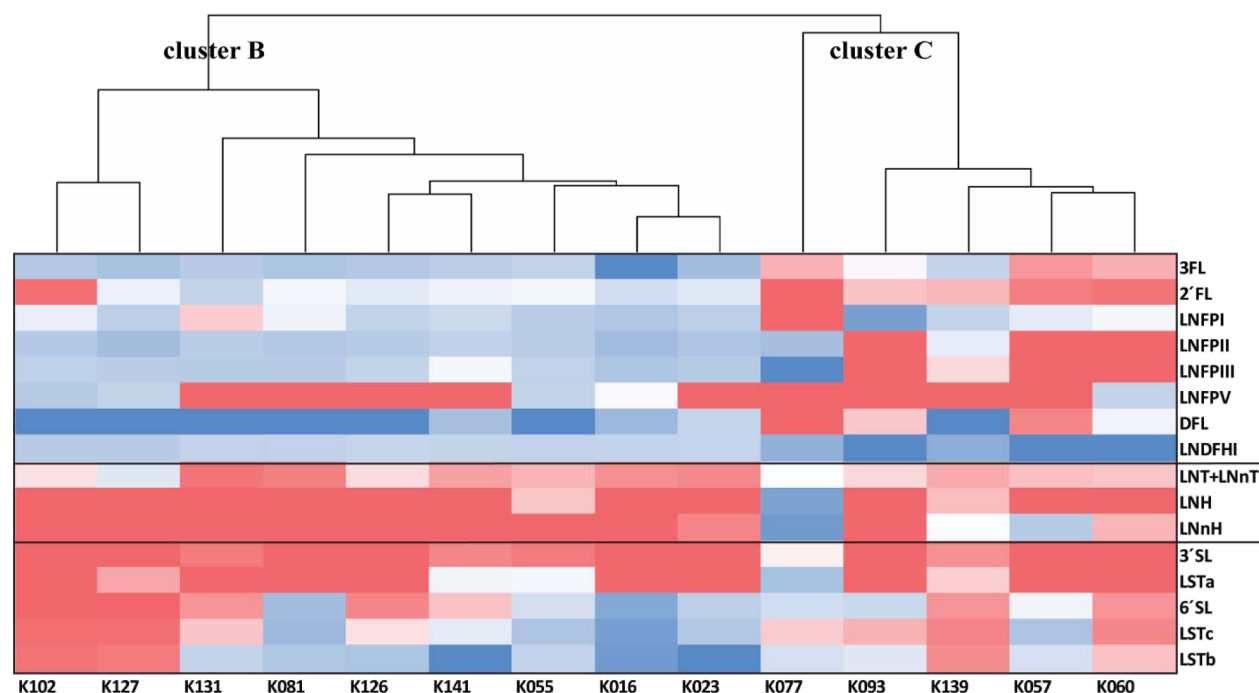
Figure 4. Dendrogram and heatmap of relative compositional changes of HMOs between human milk samples and the corresponding infant feces. The colors range from red (relative reduction in feces) to blue (relative increment in feces). Hierarchical clustering results of the 14 mother−infant pairs, whose human milk samples were classified into the Lewis-positive secretor milk group and were not completely degraded in the colon of the corresponding infants. Cluster A consisting of three mother−infant pairs is not shown here because of the full utilization of HMOs.

patterns with occurrence of all Fuc-containing epitopes and H-antigen structural elements, as well as the presence of Neu5Ac(α2-3) and Neu5Ac(α2-6) structures (Figure 5 cluster A−C). As for infant feces, K090 from cluster A showed no presence of HMO-like structures, confirming the proposed full consumption of HMOs from the milk with no structural bias. K023 from cluster B showed reduction in 2′FL, combined with a minor increase in Le^a, Le^b, and Le^x. Here, the Neu5Ac(α2-3) was consumed, while Neu5Ac(α2-6) showed a relative reduction in levels. Referring to Supporting Information Table 5, which provides concentrations of individual HMOs as quantitated by LC−MS, 2′FL relatively decreased by 28%, where LNFP II and LNFP III relatively increased by 20 and 13%, respectively. Although LNDFH I remained relatively unchanged, the relative composition of 3FL showed 31% of increment. Full consumption of 3′SL and LST a is consistent with NMR spectra. However, levels of 6′SL, LST b, and LST c relatively increased in feces compared to milk, which was different from the observation of NMR spectra. Therefore, examining relative changes of individual HMOs could indicate slightly different utilization levels compared with what was expected from the NMR structural groups. The difference was mainly caused by the presence of other HMOs with the same fucosylated structural elements in the same sample, which also contributed to the overall signal of NMR spectra. It is important for researchers to be aware of the limitations of each technique applied in their study, in order to draw objective conclusions. As to K077 from cluster C, a nearly complete disappearance of H-antigen structures and Le^y specific anomeric peaks is observed, with relatively reduced Le^b epitopes, whereas Le^x epitopes are still relatively abundant. Likewise, 2′FL and DFL were completely absent in K077 feces, while LNFP III increased the most in relative levels compared to LNDFH I, as quantitated by LC−MS (Supporting

Information Table 5). Nevertheless, it is noted that even a relative increase of an HMOs structural element in feces when compared to the paired milk could still mean that consumption by gut microbiota has taken place.

Furthermore, NMR spectra of the corresponding infant fecal samples of Le$^-$Se$^+$ (K143) and Le$^+$Se$^-$ (K117) milk groups were also examined (Figure 5). The LC−MS results of fecal samples of these two pairs were not available for comparison. K143 showed almost no carbohydrate related peaks, with minor levels of H-antigen still observed. Absence of 2′FL peaks at δ 5.31 indicates only H-antigen in LNFP I-like structures remain. K117 showed only trace amounts of carbohydrate peaks, and a preferred consumption of Le^a for this sample set according to the change in relative levels of Le^a and Le^x from milk (0.49, 0.51) to feces (0.13, 0.88) as given in Supporting Information Table 4. Nevertheless, no solid conclusions could be made on these two milk groups, due to limited sample numbers.

■ DISCUSSION

Three commonly used HMO analytical techniques, HPAEC-PAD, PGC-LC−MS, and 1D ^{1}H NMR, were combined to determine HMO profiles of a set of human milk and infant fecal samples from 20 mother−infant dyads, who were enrolled in the KOALA birth cohort study. Absolute concentrations of 3FL in human milk were determined by using HPAEC-PAD, while its concentration in infant fecal samples was quantitated by PGC-LC−MS. The other 16 major HMO structures in both milk and feces were quantitated by PGC-LC−MS. Loss of 3FL in the SPE clean-up step has been occasionally mentioned previously. Such losses could explain a lower content or even the absence of 3FL in human milk samples as reported in some studies compared to others.[38] Some analytical techniques could hardly differentiate structural isomers due to co-elution,
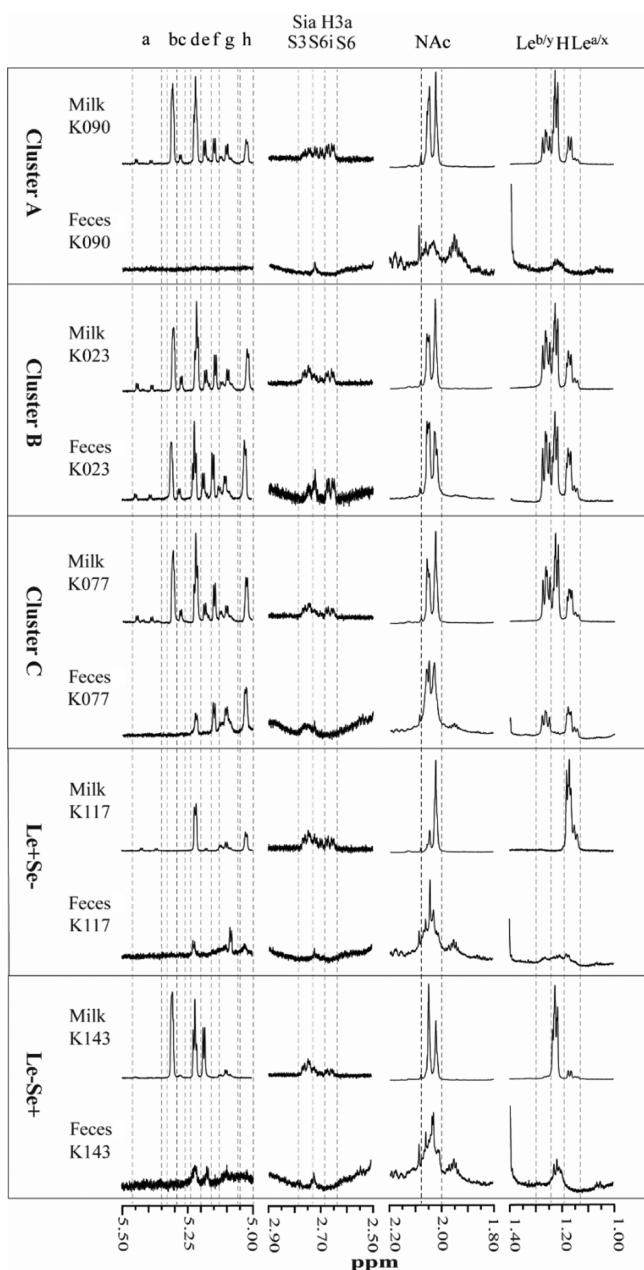
Figure 5. Relevant parts (δ 5.00−5.50, δ 2.50−2.90, δ 1.80−2.20, and δ 1.10−1.30 ppm) of 1D ^{1}H NMR spectra of representative human milk samples and their corresponding infant feces. The structure-dependent regions are annotated in Figure 3.

for example, CE-LIF or MALDI-TOF-MS, which were only able to report the combined intensity of 2′FL and 3FL. Despite the fact that 2′FL, LNnT and sialylated HMOs were the most studied structures regarding health benefits, limited studies allude to certain health potentials brought by 3FL.[55,56] The current study adapted the collection of 3FL in a separate fraction and then analyzed 3FL by HPAEC-PAD, which opens the window of including 3FL in efficacy studies in the future. Although the interaction mechanism between HMOs and PGC material is not fully clear,[38] it is observed that the presence, number, and linkage type of fucosylation and sialylation have distinct influences on the retention times in the PGC-LC−MS method, which is also true for HPAEC-PAD.[57] However, the identification and quantitation of

multiple isomeric structures by HPAEC-PAD were not optimal, as only retention time was used without further confirmation by MS.[52,58] It is noted that the total HMO concentrations present in human milk samples included in the current study (2.1−5.6 g/L) were lower than the reported value 5−20 g/L in the literature.[15] The systematic review by Thurl et al. concluded the average total concentration of neutral HMOs to be 14.78 g/L in mature milk of secretor mothers and acidic HMOs to be 2.13 g/L.[29] The values given in the review also included some larger, multi-fucosylated structures such as trifucosyllacto-*N*-hexaose,[29] which was reported to be around 2.6−3.1 g/L in only a few studies,[24,59,60] resulting in increased relative HMO levels. Despite the fact that less HMO structures were included in the calculation in the current study, concentrations of individual HMOs were all lower than the corresponding values from the review.[29] The lower concentrations might be attributed to the possible degradation of HMOs during the long storage time, considering that the samples were collected over 10 years ago in the period 2002−2003. Concentrations of relatively simple structures, such as 2′FL and 6′SL, were much lower than the more complicated structures such as LNDFH I and LST,[29] indicating that the simple HMOs were more prone to degradation during long-term storage. Furthermore, the concentrations of HMOs presented were based on the watery fraction of the maternal milk samples after lipid removal, which also contributed to the lower concentrations. Nevertheless, there are other studies that also reported lower total HMO concentrations (5−8 g/L),[61,62] which were closer to the current study. Besides other determinants that would influence HMO results, large variations between different laboratories were already noticed in the previous review, in which a proposal of performing interlaboratory tests in parallel on the same standardized human milk samples was made.[29] One limitation of the current method is the exclusion of disialyllacto-*N*-tetraose (DSLNT) from quantitation. Previous studies showed a relatively high abundance of DSLNT in human milk (0.37−0.71 g/L in mothers delivering at full term)[29] and indicated its preventive effects on necrotizing enterocolitis in preterm infants.[63,64] However, the presence of two sialic acid moieties on DSLNT required a much longer retention time than the HMOs quantitated in the current LC−MS method. We decided to continue with the current high-throughput LC−MS method which will be applied to larger sizes of sample sets and leave DSLNT for future method development studies. Furthermore, a few new peaks emerged in the LC−MS chromatograms of infant feces compared to the paired milk (Figure 2), which represented the presence of metabolites that originated from HMO degradation or endogenous sources such as intestinal glycoconjugates.[49,65] Metabolite investigation in infant feces could be another potential direction for future research.

Several previous studies have presented four milk groups with representative HMO profiles corresponding to mothers who belong to Lewis-positive secretor (Le$^+$Se$^+$), Lewis-negative secretor (Le$^-$Se$^+$), Lewis-positive non-secretor (Le$^+$Se$^-$), and Lewis-negative non-secretor (Le$^-$Se$^-$).[21,25,66,67] In the current study, we successfully assigned the mothers to different Lewis secretor milk groups using the HPAEC-PAD combined with the LC−MS method, with the results confirmed by NMR spectroscopy. Notably, the NMR spectroscopy approach identified no Fuc(α1-2)-signals in sample K117, while the LC-PGC−MS method identified 26.4 mg/L

LNFP I. The detected LNFP I concentration is significantly lower than the amount observed in the other 19 samples, together with the complete absence of 2′FL leads to a confident assignment of non-secretor (Le⁺Se⁻) for sample K117. There have been reports of the remaining Fuc(α1-2) epitopes in non-secretor individuals,[62,67] and it has been hypothesized that FUT1 can sometimes provide small amounts of Fuc(α1-2) epitopes in the milk of FUT2 deficient individuals. However, a side activity of FUT1 would most likely also result in residual amounts of other Fuc(α1-2)-containing oligosaccharides, such as 2′FL and LNDFH I, which are absent in this sample. Moreover, such remaining activity should provide detectable NMR peaks as well. Alternatively, we cannot exclude the possibility of another LNFP-type structure overlapping in the LC-PGC−MS. LNnFP V, for example, has also been detected in previous studies, which is not in the panel of compounds analyzed in the current study. Considering the concentration ratio of LNFP V and LNnFP V in the aforementioned studies, the intensity of the peak detected for K117 fits with this hypothesis. Moreover, the peak in K117 which was detected as LNFP I is slightly shifted in retention, compared with LNFP I in the other samples, for example, K131 and K143 (Figure 3).

In the case of K143, which was identified as non-Lewis (Le⁻Se⁺), it showed peaks for H-antigen and 2′FL in the NMR spectrum but no signals for Le^a of Le^b. The LC-PGC−MS approach identified a small amount of LNDFH I. There is only one paper describing residual Fuc(α1-4) in non-Lewis individuals,[67] whereas all other studies clearly show a complete absence of Fuc(α1-4) epitopes in non-Lewis individuals. We cannot exclude the possibility of an overlapping peak, for example, and LNnDFH-type structure, containing Le$^{x/y}$ epitopes, which are still observed in the milk of non-Lewis individuals.

The frequencies of milk groups in the pilot study were found to be 90% of Le⁺Se⁺, 5% of Le⁺Se⁻, and 5% of Le⁻Se⁺, in which the numbers slightly deviated from previously reported values (∼70:20:10%) for European mothers.[21,25,66,68] This distribution is within the expected range, considering the rather small sample size (n = 20) in the current pilot study. All three aforementioned analytical methods led to consistent results for milk group assignment, with the difference being that HPAEC-PAD and LC−MS examined concentrations of individual HMOs, whereas NMR compared the relative levels of Le-epitopes and H-antigen structural elements. With either HPAEC-PAD combined with PGC-LC−MS, or NMR method alone, it should be sufficient to correctly identify milk groups based on HMO profiles in future studies.

It is noted that when using PGC-LC−MS alone, it is quite demanding to quantitate all HMOs in biological samples (17 HMOs in our methods), considering the time and labor required. Instead, NMR mainly provides information on specific structural elements (Le-epitopes, H-antigen, and Neu5Ac structural groups) without any preceded separation of individual HMOs. In this study, NMR provided a helicopter view of all HMO structural elements present in the samples but lacked information on individual HMOs. Therefore, which techniques to use should depend on the research question.

With the developed methods, we identified three characteristic HMO consumption patterns among the infants in the present study (Figure 4), with cluster A (n = 3) showing complete consumption, cluster B (n = 9) showing specific consumption of non-fucosylated structures, and cluster C

infants (n = 5) utilizing HMOs without preference to specific structures but a considerable level of HMOs could still be detected in feces. These patterns were also recognized by a previous study of a Vietnamese cohort at 1 month postpartum using NMR spectroscopy,[22] where three profile types were proposed, namely, full consumption, presence of all structural elements, and significant consumption of specific structural elements, especially 2′FL and H-antigens. However, not all studies observed the same varying consumption patterns, for example, Chaturvedi et al. found generally similar HMO profiles between all the infant feces and paired human milk being analyzed, using reverse-phase HPLC−MS.[65] Albrecht et al. reported dominance of either neutral or acidic HMOs in infant feces in the first weeks to months, with empty fecal HMO profiles mostly occurring after 6 months postpartum.[49] Besides the nonspecific consumption patterns shown by infants from cluster C, their fecal HMO concentrations were found lower than those from cluster B. Albrecht et al. has found a gradual decrease of HMO excretion in infant feces with age as a result of ripening of gut microbiota.[49] It is likely that infants from cluster C had developed the gut microbiota composition more quickly toward a HMO-consuming microbial community compared to those from cluster B. However, it is noted that the comparisons were made based on the wet weight of infant feces, which were affected by the water content in the samples. The amount of milk consumed by the infants, and the amount of feces excreted by the infants were not available. Therefore, the observations here on infant fecal HMO quantitation were used for indications but no solid conclusions could be made yet. Despite differences in HMO composition secreted by mothers, the large variations observed in infant fecal HMO patterns were mainly caused by different microbiota compositions of infant's colon.[47] Considering some studies reporting the influence of ingested HMOs on maturity of infant gut microbiota,[69,70] the exact mechanism of how these two factors interact with each other is much more complicated and not fully understood heretofore. Isomer-specific consumption was recognized previously in a proof-of-concept study, which involved two infants, showing that certain HMOs were consumed at a much higher degree compared to their structural isomers.[48] Our results showed that non-fucosylated core structures were more likely to be degraded compared to those decorated by fucose and/or sialic acid, while fucosylated structures were more resistant to degradation and remained mostly intact till their excretion in feces. As the most abundant HMOs in human milk, LNT and LNnT were in most cases significantly reduced after transit through the infant's GI tract, this implies that these structures are more prone to degradation by infant colon microbiota compared to other structures. This observation was supported by a few studies where infant feces were found to be devoid of LNT and/or LNnT,[32,41,71] albeit one study presented the opposite conclusion.[47] HMO core structures decorated with fucose at different levels and linkage types were found to exert different influences on their metabolic fates in infant colon. Difucosylated HMO structures, despite their lower levels in human milk than monofucosylated ones, had relatively higher survival in the infant gut, which fits with the higher likelihood of anti-adhesive properties because of their multivalent interaction with glycan binding proteins from enteric pathogens.[72] As for sialylated HMOs, it is found that Neu5Ac(α2-3) linked HMOs were more readily consumed compared to the Neu5Ac(α2-6)-linked ones. Furthermore, the correlation between maternal

HMO synthesis and infant fecal microbiota composition, as well as the key microbial phylotypes responding for HMO degradation, was published elsewhere,[37] with samples from the KOALA cohort study including the current pilot sample set. The current study is limited by only one time point of sample collection, lacking information of how fecal HMO excretion patterns would progress with the maturity of infant gut microbiota composition, as shown in the literature.[47,49]

To conclude, HMOs are valuable nutrients provided by mothers to their nursing infants, which makes it important to develop analytical methods for HMO profiling in human milk as well as infant feces, in order to find out the optimal HMO composition for infant's health, as well as how different HMO structures were metabolized by infants. The extraction and quantitation of 3FL separately from the major HMOs pool provided more accurate information on this relevant HMO. With the combination of HPAEC-PAD and PGC-LC−MS, concentrations of individual HMO structures were quantitated; meanwhile, 1D ^{1}H NMR provided a clear picture showing relative levels of different structural elements. Depending on the future research questions, the most suitable method should be chosen to obtain the correct information.

In the current pilot study, interindividual variation was shown not only in mother's milk but also in the consumption of HMOs by the infant gut microbiota. Three distinct HMO consumption patterns were recognized at 1 month postpartum. The level of degradation of HMOs was different depending on the specific structures and linkage types. Due to limited sample size and only one-time sample collection, future studies involving more mother−infant pairs as well as multiple timepoints are warranted to define the maternal or infant factors that determined these variations we observed. Answers to all these questions would help with making decisions regarding infant formula development with personalized HMO combinations for individual babies.

■ ASSOCIATED CONTENT

ⓈI Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c07446.

> Linear range, linear regression equation, linear correlation and limit of quantitation of the HMOs quantitated by using the current PGC-LC−MS method; concentrations of HMOs in individual human milk samples, as analyzed by HPAEC-PAD and PGC-LC−MS; concentrations of HMOs in diluted infant fecal solutions, as analyzed by HPAEC-PAD and PGC-LC−MS; relative levels of Fuc-epitopes derived from 1D ^{1}H NMR integration of different anomeric region sections of HMOs isolated from milk and the corresponding fecal samples; relative compositional changes of the 17 quantitated HMOs between human milk samples and paired infant fecal samples (PDF)

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Notes

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■ ABBREVIATIONS

1D ^{1}H NMR, one-dimensional ^{1}H nuclear magnetic resonance; 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose; ACN, acetonitrile; CE-LIF, capillary electrophoresis with laser-induced fluorescence detection; DFL, difucosyllactose; FUT2, α1,-2-fucosyltransferase; FUT3, α1,-3/4-fucosyltransferase; HCA, hierarchical cluster analysis; HMOs, human milk oligosaccharides; HPAEC-PAD, high-performance anion exchange chromatography-pulsed amperometric detection; KOALA, Child, Parent, and Health: Lifestyle and Genetic constitution (in Dutch); LNDFH, lacto-*N*-difucohexaose; LNFP, lacto-*N*-fucopentaose; LNH, lacto-*N*-hexaose; LNnH, lacto-*N*-neohexaose; LNT, lacto-*N*-tetraose; LOQ, limit of quantitation; LST, sialyl-lacto-*N*-tetraose; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NaBH$_4$, sodium borohydride; N$_2$, nitrogen; PGC-LC−MS, porous graphitized carbon-liquid chromatography−mass spectrometry; SD, standard deviation; SPE, solid-phase extraction; TFA, trifluoroacetic acid

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