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Identification of Oligosaccharides in Feces of Breast-fed Infants and Their Correlation with the Gut Microbial Community*[S]

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Glycans in breast milk are abundant and found as either free oligosaccharides or conjugated to proteins and lipids. Free human milk oligosaccharides (HMOs) function as prebiotics by stimulating the growth of beneficial bacteria while preventing the binding of harmful bacteria to intestinal epithelial cells. Bacteria have adapted to the glycan-rich environment of the gut by developing enzymes that catabolize glycans. The decrease in HMOs and the increase in glycan digestion products give indications of the active enzymes in the microbial population. In this study, we quantitated the disappearance of intact HMOs and characterized the glycan digestion products in the gut that are produced by the action of microbial enzymes on HMOs and glycoconjugates from breast milk. Oligosaccharides from fecal samples of exclusively breast-fed infants were extracted and profiled using nanoLC-MS. Intact HMOs were found in the fecal samples, additionally, other oligosaccharides were found corresponding to degraded HMOs and non-HMO based compounds. The latter compounds were fragments of N-glycans released through the cleavage of the linkage to the asparagine residue and through cleavage of the chitobiose core of the N-glycan. Marker gene sequencing of the fecal samples revealed bifidobacteria as the dominant inhabitants of the infant gastrointestinal tracts. A glycosidase from *Bifidobacterium longum* subsp. *longum* was then expressed to digest HMOs *in vitro*, which showed that the digested oligosaccharides in feces corresponded to the action of glycosidases on HMOs. Similar expression of endoglycosidases also showed that N-glycans were released by bacterial enzymes. Although bifidobacteria may dominate the gut, it is possible that specific minority species are also responsible for the major products observed in feces. Nonetheless, the enzymatic activity correlated well with the known glycosidases in the respective bacteria, suggesting a direct relationship between microbial abundances and catabolic activity. ***Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.060665, 2987–3002, 2016.***

Breast milk is composed of lactose, lipids, free oligosaccharides, and proteins, many of which are highly glycosylated (1–4). Human milk oligosaccharides (HMOs)[1] and glycoproteins are vital components of breast milk and protect the infant from bacterial infection (5). HMOs play a role in deflecting pathogen binding to gut epithelial cells via molecular mimicry (6). With over 200 identified structures, HMOs are a diverse group of oligosaccharides (7–10). There are five common monosaccharides that make up HMOs: glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid, N-acetylneuraminic acid (Neu5Ac). Most HMOs contain a lactose core (Gal(β1–4)Glc) and are extended by glycosyltransferases that add GlcNAc residues in β(1–3/6) linkages and Gal in β(1–3/4) linkages (5). These chains can then be decorated by Fuc in various linkages, depending on the specific enzymes of the mother. Fuc added in α(1–3/4)

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[1] The abbreviations used are: Bif-TRFLP, Bifidobacterium-specific terminal restriction fragment length polymorphism; BLIR, *Bifidobacterium longum/infantis* ratio; ECC, extracted compound chromatogram; FA, formic acid; Fuc, Fucose; Gal, Galactose; GCC, graphitized carbon cartridge; Glc, Glucose; GlcNAc, N-acetylglucosamine; Hex, Hexose; HexNAc, N-acetylhexosamine; HMO, human milk oligosaccharide; IFLNH I, isomer 1 fucosyl-paralacto-*N*-hexaose; IFLNH III, isomer 3 fucosyl-paralacto-*N*-hexaose; LNH, lacto-*N*-hexaose; LNnH, lacto-*N*-neohexaose; LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose; MFLNH I, monofucosyllacto-*N*-hexaose I; Neu5Ac, N-acetylneuraminic acid; p-LNH, para-lacto-*N*-hexaose; 2′FL, 2′-fucosyllactose.

linkages are controlled by the Lewis gene to both Glc and GlcNAc residues, and the secretor gene (FUT2) codes for the α(1–2) fucosyltransferase, which adds Fuc in an α(1–2) linkage on a terminal Gal residue (11). Neu5Ac can also be added via α(2–3/6) linkages to GlcNAc or terminal Gal residues (1, 12, 13).

The role of milk in developing the gastrointestinal microbiome appears to be critical in infant development. HMOs are not catabolized directly by the infant, but instead pass undigested through the upper digestive tract to the large intestine where they function as prebiotics for the distal gut microbiota (4, 14–16). HMOs help promote a healthy infant gastrointestinal tract in a number of ways: as anti-adhesives preventing viruses from binding to epithelial cell surface glycans, as antimicrobials acting as decoy receptors to pathogenic bacteria, and as energy sources for beneficial bacteria which in turn produce short-chain fatty acids for the infant (4, 6, 17, 18). Commensal bacteria such as *Bifidobacterium* and *Bacteroides* are equipped with a suite of enzymes capable of breaking down HMOs (19–21). For this reason, bifidobacteria can make up to 90% of the microbial community in the gut of breast-fed infants (5, 22). Select bifidobacterial species grow well on HMOs *in vitro* (21, 23). *Bifidobacterium longum* subsp. *infantis* has been shown to be particularly good at utilizing HMOs, displaying preferential consumption of smaller and fucosylated HMOs (21, 23, 24). Different microbial species and strains in the infant gastrointestinal tract have their own mechanisms for HMO catabolism leading to potentially diverse digested products in the feces. These digestion products are generally unknown, but characterizing these glycan species may reveal information regarding the repertoire of specific bacterial enzymes at work and indicate the active members in the communities inhabiting the infant gastrointestinal tract.

The *in vivo* catabolism of HMOs can provide important phenotypic information regarding the state of the gut microbiota. Along with the degraded HMO, intact HMOs have also been discovered in the feces of breast-fed infants (15, 16, 25, 26). Computational, genomic, and *in vitro* methods have been combined to study bacterial consumption of HMOs and other carbohydrates. Eilam *et al.* created a computational program using genomic data of microbial enzymes in order to predict degraded glycan products (27). Asakuma *et al.* grew several bifidobacteria strains on select HMO-enriched media and discovered various mechanisms for producing digestion products from the *in vitro* bacterial growth (28). Further studies found important distinctions for the feeding habits among specific bacterial species. *B. longum* subsp. *infantis* catabolizes the entire HMO internally, whereas *B. bifidum* secretes extracellular glycosidases for cleaving HMOs, leading to digested HMO products in the intestinal lumen (29, 30). These studies show that degraded glycans are created through the activity of key glycosyl hydrolases and can link relevant glycosidases with specific bacteria, thus providing insight into possible gut microbiota degradation pathways witnessed in feces.

In this study, we extracted and characterized intact oligosaccharides and digestion products from the feces of breast-fed infants to obtain the most comprehensive oligosaccharide analysis of feces. We compare the fecal oligosaccharides with matched maternal breast milk, and gained insight into the active glycosidases in the intestinal microbiome. The fecal glycans act as a phenotypic signature indicative of the enzymes that are active in the microbial communities in the infant gut. Genomic profiling of the types of microbial species present in infants' guts was used to infer those glycosidases. Intact and degraded oligosaccharides from milk glycoconjugates were identified and a fecal glycan library was created for the rapid and comprehensive analysis of postpartum alterations to the fecal glycome.

EXPERIMENTAL PROCEDURES

Breast Milk and Infant Fecal Samples—Milk samples were obtained from healthy women enrolled in the Foods for Health Institute Lactation Study at the University of California, Davis. Sample collection and subject details have been previously reported (31, 32). The UC Davis Institutional Review Board approved all aspects of the study, and informed consent was obtained from all subjects. This trial was registered on clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT01817127). For this study, a subset of one mother/infant pair and six infant fecal samples were analyzed. Briefly, milk samples were collected in the morning on day 6, 21/24, 71, and/or 120 postpartum. Subjects fully pumped one breast into a bottle, inverted six times, transferred 12 ml into a 15 ml polypropylene tube, and subsequently froze the sample in their kitchen freezers (−20 °C). Samples were collected from the subjects' freezers by study personnel biweekly, transported to the lab on dry ice, and stored at −80 °C until processing. Infant fecal samples were collected at 6/7, 21/24, 71, and/or 120 days of life. Parents transferred their infant fecal samples into sterile plastic tubes and were instructed to immediately store the samples at −20 °C until transported by study personnel. Fecal samples were transported to the laboratory on ice packs and stored at −80 °C before processing.

Oligosaccharide Extraction—Free oligosaccharides were extracted from breast milk and infant feces following previously reported methods, with slight modifications (8, 26, 33). Fecal samples were weighed, diluted with nanopure water to 1 mg/10 μl, and homogenized in the shaker overnight at 4 °C. The samples were then centrifuged at 4000 × *g* for 30 min, and 100 μl of supernatant was taken and placed on a 96-well plate. For the milk samples, 50 μl were aliquoted on a 96-well plate and defatted via centrifugation. The skimmed milk and fecal supernatants then underwent similar methods. Proteins were removed by ethanol precipitation at −80 °C for 1.5 h, and after 30 min of centrifugation the supernatant was evaporated to dryness. The resulting glycans were reduced to their alditol form with 1.0 M $NaBH_4$ at 65 °C for 1.5 h. Desalting and purification was achieved using solid phase extraction on both C-8 and graphitized carbon cartridges (GCCs) (Glygen Corp, Columbia, MD) for the fecal samples but only GCCs for milk. After the glycans were loaded onto preconditioned C8 cartridges, the flow-through was collected and the glycans were loaded onto preconditioned GCCs. The purified glycans were eluted with 20% acetonitrile (ACN)/water (v/v) and 40% ACN/water (v/v) in 0.05% trifluoroacetic acid, eluent solvent then evaporated to dryness, and reconstituted and diluted in nanopure water before analysis.

Nano-High Performance Liquid Chromatography-Chip/Time-of-Flight Mass Spectrometry (nanoLC-MS)—Milk and fecal glycans were analyzed on a nano-high performance liquid chromatography (HPLC)-chip/time-of-flight (TOF) mass spectrometry system. The HPLC system used was an Agilent 1200 series unit with a microfluidic chip, which was coupled to an Agilent 6220 series TOF mass spectrometer via chip cube interface. The capillary pump on the chromatography unit loads the sample onto the 40 nL enrichment column of the chip at a flow rate of 4.0 μl/min with a 1 μl injection volume. The nano pump is used for analyte separation on the analytical column of the chip, which is 75 μl x 43 mm and packed with porous graphitized carbon. Separation is accomplished using a binary gradient of aqueous solvent A (3% acetonitrile (ACN)/water (v/v) in 0.1% formic acid (FA)) and organic solvent B (90% ACN/water (v/v) in 0.1% FA) with a previously developed method for HMO separation (7, 8). The sample is then introduced into the TOF mass spectrometer via electrospray ionization, which was tuned and calibrated using a dual nebulizer electrospray source with calibrant ions ranging from *m/z* 118.086 to 2721.895, and data was collected in the positive mode.

Nano-HPLC-Chip/Quadrupole-TOF MS—Structural elucidation of fecal glycans was performed on a nano-HPLC-chip/quadrupole-TOF (Q-TOF) mass spectrometer, Agilent LC 1200 series and Q-TOF 6520 series. The chromatographic separation method on the Q-TOF was the same as that for the nano-HPLC-chip/TOF. Tandem mass spectrometry was also performed in the positive mode with collision induced dissociation for fragmentation. The following voltages were optimized for oligosaccharide fragmentation: fragmentor 175 V, skimmer 60 V, and octopole 1 RF 750 V, and nitrogen drying gas was used at a flow rate of 5 L/min at 325 °C. Auto MS/MS was used with 0.63 spectra/second for both MS 1 and MS/MS. Precursor ions were selected based on abundance, with doubly charged ions given first priority, singly charged ions second, triply charged third, then last, other multiply charged ions. All calibrant ions were excluded from precursor selection with a 4 *m/z* isolation window. Collision energy (CE) was based on the ion's mass-to-charge (*m/z*) ratio with higher energy for larger ions, according to the following equation

$$\mathrm{CE(V)} = \frac{m/z}{100(\mathrm{Da})} \times 1.3 - 3.5 \qquad \text{(Eq. 1)}$$

where 1.3 is the slope and −3.5 is the *y*-intercept. The equation was empirically determined by the manufacturer (7).

Oligosaccharide Data Analysis—Data was collected using Agilent MassHunter Work station Data Acquisition version B.02.01 on the nanoHPLC-chip/TOF and version B.05.00 on the nanoHPLC-chip/Q-TOF and then analyzed with Agilent MassHunter Qualitative Analysis software versions B.03.01 and B.06.00. Glycans were identified using the *Find Compounds by Molecular Feature* function to within 20 ppm of theoretical masses. Compound abundances were extracted as volume in ion counts, which was directly correlated to absolute abundances of the compounds present in each sample. Known structures were identified by matching accurate mass and retention time to previously developed annotated HMO libraries which contains 102 composition entries (7, 8). Peak alignment for correcting for retention time shifts was performed using in-house software (33). Tandem MS data were then annotated by hand and in-house software. The MS data have been deposited to the Proteome Xchange Consortium via the PRIDE partner repository with the data set identifier PXD004434 (34).

Cloning and Protein Expression of BLNG_00015 from B. longum subsp. *longum* SC596—BLNG_00015 (β-galactosidase) was cloned using the pEco-T7-cHis cloning kit (Gentarget Inc., San Diego, CA). The Qiagen DNeasy Blood & Tissue Kit was used to purify genomic DNA from *B.longum* subsp. *longum* SC596 culture following the manufacturer's guidelines for purification of Gram-positive bacterial DNA. BLNG_00015 was amplified by PCR. 225 μl of PCR product was gel purified and cloned into a pEco-T7-cHis (Gentarget Inc) vector. Sequencing of the insert of the recombinant BL21 Star clone was carried out to confirm the sequence of the entire glycosyl hydrolase insert. Protein expression was carried out using 700 ml of LB with 100 μg/ml carbenicillin. Cells were grown until an OD of 0.5 was reached and induced overnight using a final concentration of 0.5 mM isopropyl-β-d-thiogalactopyranoside. The culture was centrifuged using an Eppendorf 5804 centrifuge (Hauppauge, NY) for 20 min at 4 °C and frozen at −80 °C. Bugbuster protein extraction reagent (EMD chemicals, Gibbstown, NJ) was used to resuspend culture. 60 μl of DNase I (Roche, Basel, Switzerland) from a 10 U/μl stock and 120 μl of lysozyme from a 50 mg/ml stock were added per 20 ml of Bugbuster suspension. After a 5 min incubation, the Bugbuster suspension was then centrifuged for 30 min at 13,200 rpm at 4 °C. For purification a 1 ml Bio-Scale Mini Profinity immobilized-metal affinity chromatography column attached to a EP-1 Econo Pump (Bio-Rad, Hercules, CA) was used. Following purification, imidazole was exchanged with PBS using 10 kDa cutoff Amicon Ultra-15 centrifugal unit (Millipore, Billerica, CA). Purity and size of the recombinant protein were confirmed using a 4–15% SDS-PAGE gel.

In vitro Enzyme Digestion—Expressed enzymes were stored at −80 °C until analysis. A pool of HMOs (1.5 μl, 5 mg/ml) reduced to the alditol was combined with 3 μl of enzyme BLNG_00015 and 5.5 μl of 0.1 M ammonium acetate buffer at pH 6.0. The solution was incubated for 1 h at 55 °C followed by C18 zip-tip (Agilent Technologies, Santa Clara, CA) clean up. The samples were then dried and reconstituted in 50 μl nanopure water for analysis. This experiment was performed in triplicate. An undigested HMO pool (1.5 μl in 50 μl nanopure water) was run with the digested pool on the nano-HPLC-chip/TOF mass spectrometer.

Fecal DNA Extraction—DNA was extracted from 150 mg of stool sample using the ZR Fecal DNA MiniPrep kit (ZYMO, Irvine, CA) in accordance with the manufacturer's instructions, which included a bead-beating step using a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA) for 2 min at 25 °C at a speed of 6.5 m/s.

Bifidobacterium-specific Terminal Restriction Fragment Length Polymorphism (Bif-TRFLP)—The method of Lewis *et al.* was used to perform the *Bifidobacterium*-specific terminal restriction fragment length polymorphism assay (35). Briefly, DNA from feces was amplified in triplicate by PCR using primers NBIF389 (5′-[HEX]-GCCTTCGGGTTGTAAAC) and NBIF1018 REV (GACCATGCACCACCTGTG). DNA was purified using the Qiagen QIAquick PCR purification kit and then cut with restriction enzymes AluI and HaeIII. The resulting fragments were analyzed on an ABI 3100 genetic analyzer, and sizes were compared against the published database for species identification.

Bifidobacterium longum/infantis Ratio (BLIR)—A PCR-based assay, BLIR, was used to determine which subspecies of *B. longum* were present in each sample and to gain an estimate of their relative abundance to each other (36). Briefly, PCR was performed using three primers (FWD_BL_BI (5′-[HEX]-AAAACGTCCATCCATCACA), REV_BL (5′-ACGACCAGGTTCCACTTGAT), and REV_BI (5′-CGCCTCAGTTCTTTAATGT)) targeting a conserved portion of the genome (between Blon_0424 and Blon_0425) shared by both subspecies, but generating different amplicon lengths for each. Amplicons were analyzed by capillary electrophoresis on an ABI 3100 genetic analyzer (Applied Biosystems, Carlsbad, CA) and interpreted with PeakScanner 2.0 software (Applied Biosystems) to estimate relative abundances of each subspecies.

Marker Gene Sequencing—DNA samples were prepared for 16S rRNA marker gene sequencing as previously described (31, 37) with the following modifications. Universal barcoded primers with Illumina sequencing adapters (Illumina, San Diego, CA) (adapters are italicized

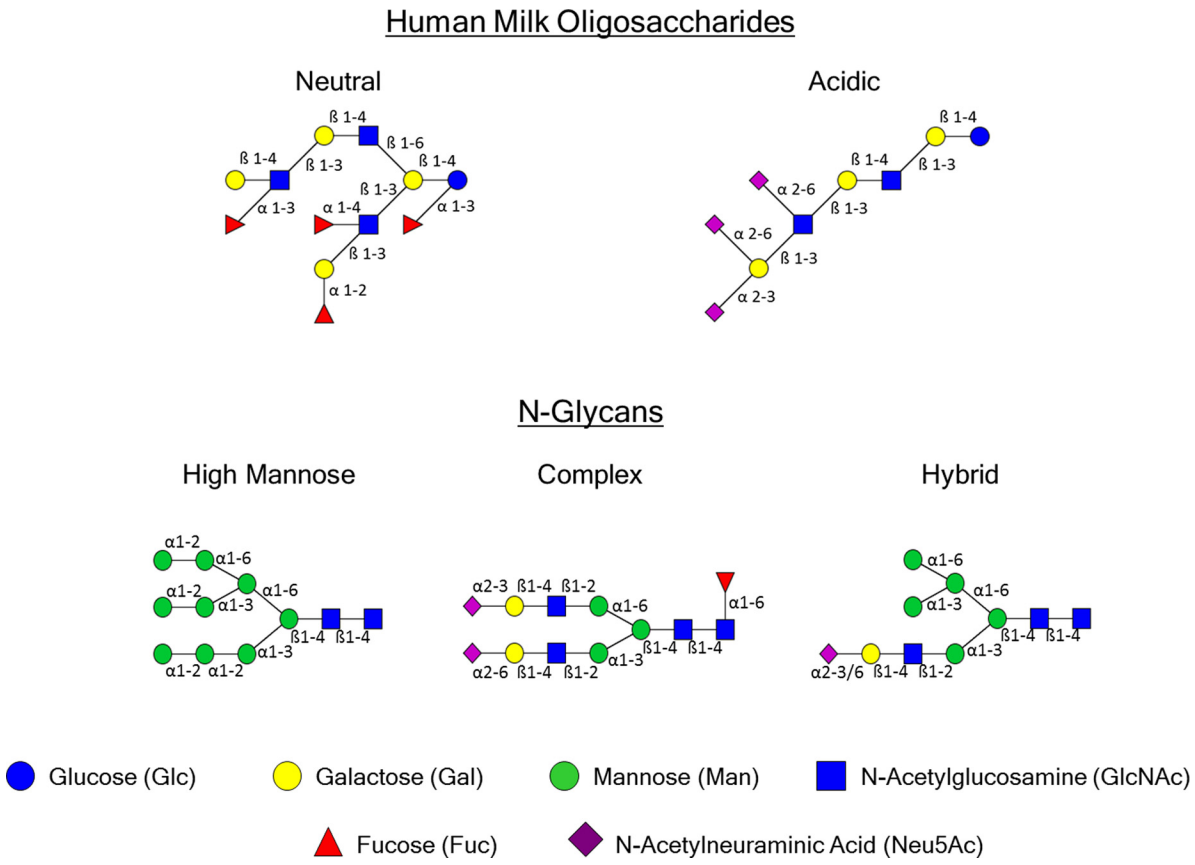

FIG. 1. **Names and graphical representations of the different monosaccharides that compose human milk oligosaccharides (HMOs) and N-glycans.** Representative branched and linear neutral and acidic HMOs and the different subclasses of N-glycans, along with their potential linkages are also depicted.

and the barcode is highlighted in bold) V4F (5′-*AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT* **NNNNNNNN**GTGTGCCAGCMGCCGCGGTAA-3′) and V4Rev (5′-*CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT*CCGGACTACHVGGGTWTCTAAT-3′) were used to PCR amplify the V4 region of the 16S rRNA gene in each sample (37). A pooled library was sequenced at the University of California DNA Technologies Core Facility on an Illumina MiSeq platform (Illumina) (150 bp single read).

Sequence Analysis—The QIIME software package (version 1.7.0) was used to analyze the results of the Illumina sequencing run. Illumina V4 16S rRNA gene sequences (Illumina) were demultiplexed and quality filtered using the QIIME 1.7.0 software package with default settings unless otherwise specified (38). Reads were truncated after a maximum number of three consecutive low quality scores. The minimum number of consecutive high-quality base calls to include a read (per single end read) as a fraction of the input read length was 0.75. The minimum acceptable Phred quality score was set at 20. Similar sequences were clustered into operational taxonomic units (OTUs) using open reference OTU picking with UCLUST software (39). Taxonomy was assigned to each OTU with the Ribosomal Database Project (RDP) classifier (40) and the RDP taxonomic nomenclature (41). OTU representatives were aligned against the Greengenes core set (42) with PyNAST software (43).

RESULTS

Glycomic Profile of Feces from Breast-fed Infants—The considerations of potential sources of oligosaccharides in feces include HMOs, digested HMOs, and the digestion of other milk glycoconjugates including glycoproteins and glycolipids (Fig. 1). Fig. 2*A* shows the extracted compound chromatogram (ECC) of intact HMOs from a breast milk sample from one mother 24 days postpartum, and the ECC of a fecal sample from her offspring 24 days postnatal (mother/infant pair 1028) (Fig. 2*B*). The direct comparison of the nanoLC-MS glycan profile of the milk to that of infant feces yielded considerable overlap because of undigested HMO in feces. Nonetheless, even a casual inspection showed significant differences (Fig. 2). There were peaks in feces that appeared and peaks that disappeared compared with the milk sample. For example, peaks from 25–26 min found in the milk sample were diminished in the fecal sample, whereas a peak at 8.5 min appeared in the fecal chromatogram that was absent in the milk (Fig. 2).

To confirm the identity of the intact HMOs, a previously optimized method was used to identify structures in human milk through the use of accurate masses, tandem MS, reproducible retention times, and an annotated structure library (7, 8). For example, the peak at 10.3 min (Fig. 2*A*) with *m/z* 491.18 corresponds to 2′-fucosyllactose (2′FL - structure inset). The same peak was found in feces at 10.0 min with *m/z* 491.19

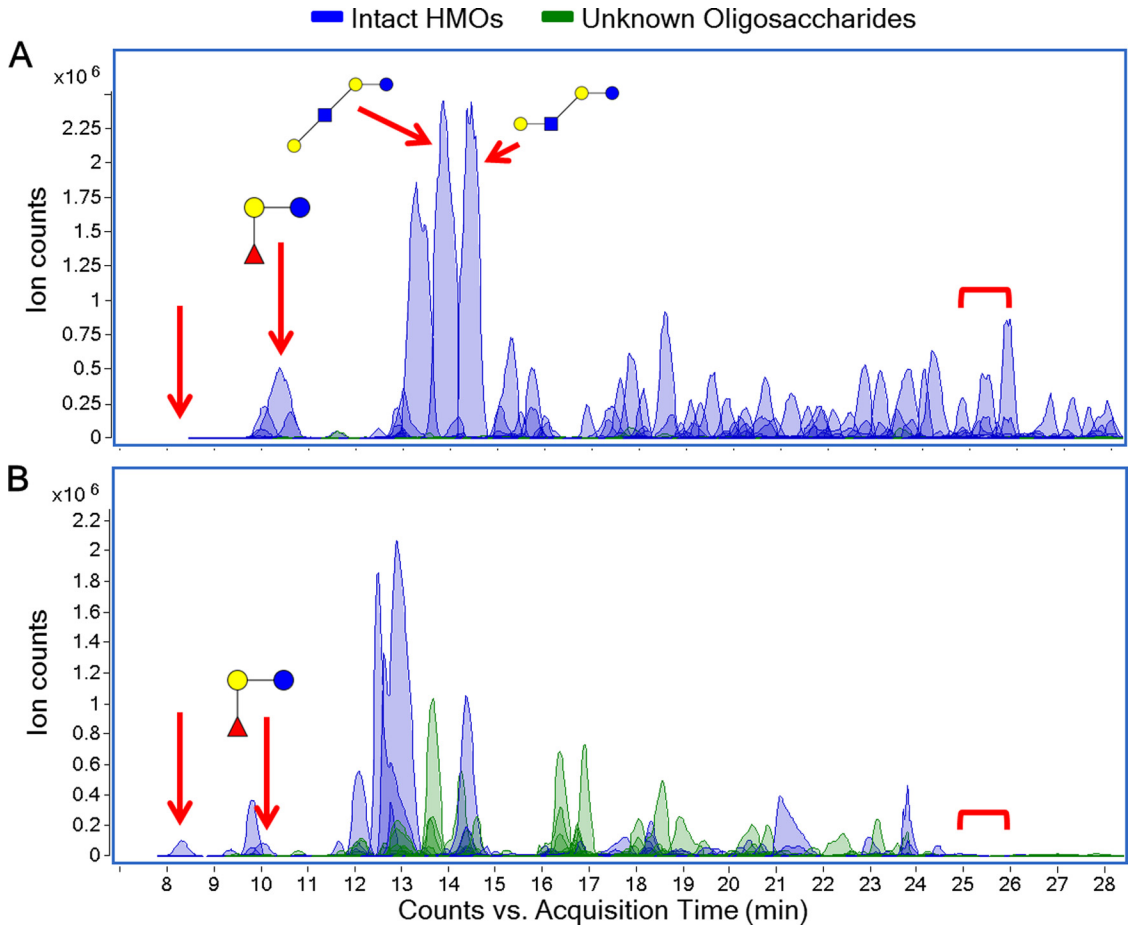

FIG. 2. **(A) Extracted compound chromatograms of a mother's breast milk oligosaccharide profile at day 24 postpartum compared with (B) her infant's fecal glycan profile at day 24 postnatal.** The structure found in (*A*) at retention time 10.3 min is 2′FL, 13.8 min is LNT, and 14.4 min is LNnT with monosaccharides glucose (●), galactose (●), and fucose (▲), all having significantly lower (2′FL) or no abundance (LNT and LNnT) in (*B*). Sialylated structures found in (*A*) from 25–26 min show significant decrease in (*B*) at the same retention time.

(Fig. 2*B*). Another notable change was the lack of isomers lacto-*N*-tetraose (LNT, 13.8 min) and lacto-*N*-neotetraose (LNnT, 14.4 min) in the fecal sample (Fig. 2). The intact HMOs identified in breast milk and infant feces are listed in Table I, with a complete list of intact HMOs found in milk and feces in supplemental Table S1. In general, we found that many of the species transit through the gut, albeit in depleted amounts at least early in the lactation period (day 24).

The HMO profile of feces displays preferential consumption of select glycan species. For example, fewer sialylated compounds were identified in feces that eluted from 25 to 26 min, compared with the milk (Fig. 2). There are 30% fewer sialylated HMO structures in feces compared with milk (supplemental Table S1). However, the largest drop in the number of HMO compounds was for the undecorated structures (containing neither Fuc nor Neu5Ac), which decreased by 60% in feces (supplemental Table S1).

Determination of Unidentified HMO and Non-HMO Species—The non-HMO compounds present in the chromatogram were further probed to determine if they were indeed oligosaccharides. To determine these new structures, their compositions were first determined by accurate masses and confirmed by tandem MS. For example, a compound in Fig. 2*B* with retention time 13.6 min was found to have a neutral molecular mass of 547.21 Da, corresponding to a composition of 2Hex:1HexNAc. The tandem MS spectrum confirmed the composition (supplemental Fig. S1). Two peaks with *m/z* 204.09 and 366.16 were observed corresponding to HexNAc and 1Hex:1HexNAc, respectively. Also observed was a neutral loss of 162.06, because of the loss of one Hex, and 182.07, a Hex reduced to the alditol. Reduction of the glycans is incorporated in the extraction method to eliminate peak splitting during chromatographic separation because of the presence of anomers and to identify the reducing end saccharide in the tandem MS.

Another example is illustrated with the compound found at 17.2 min (Fig. 2*B*). The MS yields a neutral molecular mass of 693.26 Da, which corresponds to the composition 2Hex:1HexNAc:1Fuc. The tandem MS spectrum is consistent with an oligosaccharide but was not consistent with an

TABLE I

Intact human milk oligosaccharides (HMOs) identified in breast milk at day 24 postpartum and her infant's feces at day 24 postnatal. The neutral mass, HMO composition given as Hex_HexNAc_Fuc_Neu5Ac, name of the HMO, volumes, and retention time are provided for the extracted HMOs based on nanoLC-MS analysis

Present in Mother's Milk					Present in Infant's Feces	
Neutral Mass	Composition	Name	Volume	Retention Time (min)	Identified	Volume
490.19	2_0_1_0	3FL	39951	1.1	X	56044
490.19	2_0_1_0	2′FL	15635419	10.4	X	1831235
855.322	3_1_1_0	LNFP II	3546858	10.6	X	5618447
636.248	2_0_2_0	LDFT	6371802	13.0		
1366.512	4_2_2_0	DFpLNH II	900714	13.0		
855.322	3_1_1_0	LNFP I+III	52395292	13.3	X	36476336
709.264	3_1_0_0	LNT	63089936	13.8		
1512.57	4_2_3_0	TFLNH	2693264	14.1	X	3840508
709.264	3_1_0_0	LNnT	55102424	14.4		
635.227	2_0_0_1	6′S;L	70111	14.8	X	101752
1220.454	4_2_1_0	MFpLNH IV	3272666	15.0		
1220.454	4_2_1_0	4120a	2488854	15.5		
1366.512	4_2_2_0	DFLNHa	8704108	15.7	X	63718448
1220.454	4_2_1_0	MFLNH I+III	102094	16.6		
1220.454	4_2_1_0	IFLNH III	6249002	17.6	X	11315122
1074.396	4_2_0_0	LNH	11563610	17.8	X	3005247
1074.396	4_2_0_0	LNnH	16168102	18.5		
1512.57	4_2_3_0	4320a	2112660	19.1	X	1550701
1366.512	4_2_2_0	DFLNHc	2765833	20.1		
1220.454	4_2_1_0	IFLNH I	2926815	20.2		
1074.396	4_2_0_0	p-LNH	3687914	21.6	X	543930
635.227	2_0_0_1	3′S;L	2067527	21.7	X	209245
1146.417	3_1_1_1	F-LSTc	2189294	22.5	X	550042
1000.359	3_1_0_1	LSTc	11694818	23.7		
1000.359	3_1_0_1	LSTb	12852529	24.2	X	357439
1365.492	4_2_0_1	S-LNH	944179	24.8	X	1208673
1365.492	4_2_0_1	4021a+S-LNnH II	16969513	25.8	X	909254
1000.359	3_1_0_1	LSTa	4658526	27.2	X	10931904

HMO as there were no fragments indicative of the lactose (Gal(β1–4)Glc) core, but a neutral loss of 182.08 (a reduced Hex) was present (supplemental Fig. S2). The results of these analyses are summarized in Table II with a list of the compound compositions and their respective retention times. Also listed are the potential sources of the oligosaccharides, which are either HMOs or glycoproteins as discussed below.

Oligosaccharides are not all derived from HMOs. There are structures that are consistent with N-glycans based on their fragmentation patterns. Fig. 3*A* shows the tandem MS spectrum of a doubly charged glycan with *m/z* 631.74, which corresponds to the composition 3Hex:3HexNAc:1Fuc. The loss of 111.53 correlates to a doubly charged reduced HexNAc, and the singly charged reduced HexNAc can be observed at *m/z* 224.10, both indicative of a reduced GlcNAc from an N-glycan core (Fig. 3*A*). The fragmentation pattern further suggests a fucosylated species with losses corresponding to Hex and Fuc (Fig. 3*A*).

Tandem MS was used to differentiate between oligosaccharides derived from either HMOs or N-glycans. One example is a compound with composition 5Hex:3HexNAc at *m/z* 1440.52, corresponding either to an HMO or a hybrid-type N-glycan. The neutral loss of 344.11 in the tandem MS spectrum indicates a reduced lactose core of an HMO (Fig. 3*B*). The repeated neutral loss of 365.14 was also suggestive of the repeating lactosamine (Gal(β1–3/4)GlcNAc) units that make up the backbone of HMOs. The lack of *m/z* 224.11, which would have indicated a reduced GlcNAc from the chitobiose N-glycan core, was another indication that this oligosaccharide was an HMO and not an N-glycan. Fragmentation of an antenna or the trimannosyl portion of the N-glycan core would lead to subsequent neutral losses of 162.05, but neither were present.

A more difficult example is the compound with composition 3Hex:2HexNAc (*m/z* 913.35), which appears to be an N-glycan core. The fragmentation showed neutral losses of 344.12, which corresponds to a reduced lactose core of an HMO (Fig. 3*C*). Also observed were neutral losses of 182.05, the mass of a reduced Hex, which corresponds to the reduced Glc of the lactose core (Fig. 3*C*). Neutral losses of 223.10 or a peak at *m/z* 224.11 would have been indicative of a reduced HexNAc and evidence of the N-glycan core, however these were not observed. Because these diagnostic markers were not found in the MS spectra, composition 3Hex:2HexNAc was assigned as a degraded HMO (Table II).

There are compositions whose origins could not be differentiated between HMOs and N-glycans, even when their frag-

TABLE II

Extracted oligosaccharides from infant feces. Neutral mass, composition given as Hex_HexNAc_Fuc_Neu5Ac, oligosaccharide type, volume, and retention time are provided based on nanoLC-MS and nanoLC-MS/MS analysis

Neutral mass	Composition	Oligosaccharide type	Volume	Retention time (min)
750.291	2_2_0_0	HMO digest	279610	9.5
3377.240	6_6_4_2	Fucosylated and sialylated complex	196226	9.8
1829.690	4_5_1_0	Fucosylated complex/hybrid	37904	10.0
1464.560	3_4_1_0	Fucosylated complex	138251	10.1
1261.480	3_3_1_0	N-glycan digest	549487	10.8
1407.540	3_3_2_0	Fucosylated complex	137259	10.8
1407.540	3_3_2_0	Fucosylated complex	153055	11.5
1204.460	3_2_2_0	HMO digest	502558	11.7
896.3490	2_2_1_0	HMO digest	182085	11.8
1407.540	3_3_2_0	Fucosylated complex	49544	11.8
1439.530	5_3_0_0	Intact HMO	184152	12.0
1058.400	3_2_1_0	HMO digest	574538	12.1
1204.460	3_2_2_0	HMO digest	2103312	12.1
1220.450	4_2_1_0	Intact HMO	2381382	12.1
1772.670	4_4_2_0	Fucosylated complex/Hybrid	57362	12.1
2354.820	11_2_1_0	Fucosylated high mannose	212239	12.2
1569.590	4_3_2_0	Fucosylated complex/hybrid	200417	12.3
1843.630	10_1_0_0	N-glycan digest	106806	12.4
1521.580	3_5_0_0	Complex	100672	12.5
1115.420	3_3_0_0	HMO digest or complex/hybrid	985914	12.6
1261.480	3_3_1_0	N-glycan digest	231816	12.6
912.343	3_2_0_0	HMO digest	1891751	12.9
1058.400	3_2_1_0	HMO digest	4549122	12.9
1204.460	3_2_2_0	HMO digest	5749952	12.9
896.3490	2_2_1_0	HMO digest	123440	13.0
2178.830	4_6_2_0	Fucosylated complex	595334	13.0
3579.300	6_7_2_3	Fucosylated and sialylated complex	1059568	13.0
1667.640	3_5_1_0	Fucosylated complex	94540	13.2
1382.510	5_2_1_0	Intact HMO or fucosylated complex/hybrid	47879	13.3
896.349	2_2_1_0	HMO digest	596870	13.4
1261.480	3_3_1_0	N-glycan digest	240843	13.4
1407.540	3_3_2_0	Fucosylated complex	175285	13.4
547.211	2_1_0_0	HMO digest	881324	13.6
1058.400	3_2_1_0	HMO digest	3824270	13.6
3465.260	6_5_4_3	Fucosylated and sialylated complex	124225	13.6
1220.450	4_2_1_0	Intact HMO	17185486	13.7
1115.420	3_3_0_0	HMO digest or complex/hybrid	947184	14.3
1261.480	3_3_1_0	N-glycan digest	9057561	14.3
1058.400	3_2_1_0	HMO digest	3292608	14.4
1204.460	3_2_2_0	HMO digest	1285079	14.4
1569.590	4_3_2_0	Fucosylated complex/hybrid	314997	14.5
2428.900	5_5_2_1	Fucosylated and sialylated complex/hybrid	273097	14.5
547.211	2_1_0_0	HMO digest	884078	14.6
912.343	3_2_0_0	HMO digest	71884	14.6
1382.510	5_2_1_0	Intact HMO or fucosylated complex/hybrid	3820702	14.6
1991.740	5_5_1_0	Fucosylated complex/hybrid	83669	15.0
2210.820	6_6_0_0	Complex	209486	15.0
2194.820	5_6_1_0	Fucosylated complex	708236	15.2
1204.460	3_2_2_0	HMO digest	455992	15.9
1893.700	6_3_2_0	Fucosylated hybrid	114767	15.9
1204.460	3_2_2_0	HMO digest	615505	16.1
1934.720	5_4_2_0	Fucosylated complex/hybrid	157273	16.1
1569.590	4_3_2_0	Fucosylated complex/hybrid	6479052	16.4
1731.640	5_3_2_0	Intact HMO	250306	16.4
2908.100	4_6_5_1	Fucosylated and sialylated complex	614794	16.4
1626.610	4_4_1_0	Fucosylated complex/hybrid	989645	16.5
1261.480	3_3_1_0	N-glycan digest	758468	16.6
912.343	3_2_0_0	HMO digest	108600	16.7
1115.420	3_3_0_0	HMO digest or complex/hybrid	1471326	16.7

TABLE II—*continued*

Neutral mass	Composition	Oligosaccharide type	Volume	Retention time (min)
1480.550	4_4_0_0	Complex/Hybrid	963689	16.7
1843.630	10_1_0_0	N-glycan digest	321012	16.7
2073.800	3_7_1_0	Fucosylated complex	2077952	16.7
1261.480	3_3_1_0	N-glycan digest	535548	16.8
1756.670	3_4_3_0	Fucosylated complex	109769	16.8
1423.530	4_3_1_0	Intact HMO	9728194	16.9
1569.590	4_3_2_0	Fucosylated complex/hybrid	779149	16.9
1731.640	5_3_2_0	Intact HMO	628018	16.9
1893.700	6_3_2_0	Fucosylated hybrid	152929	17.0
693.269	2_1_1_0	HMO Digest	170774	17.2
4267.542	7_6_5_4	Fucosylated and sialylated complex	708277	17.2
4121.480	7_6_4_4	Fucosylated and sialylated complex	491182	17.3
1724.660	3_6_0_0	Complex	175583	17.6
1714.630	4_3_1_1	Fucosylated and sialylated complex	705623	17.7
2908.100	4_6_5_1	Fucosylated and sialylated complex	344617	17.8
2997.110	6_7_2_1	Fucosylated and sialylated complex	106195	17.8
1115.420	3_3_0_0	HMO digest or complex/hybrid	1280814	17.9
1626.610	4_4_1_0	Fucosylated complex/hybrid	137941	18.0
1731.640	5_3_2_0	Intact HMO	2250499	18.0
3306.240	7_7_5_0	Fucosylated complex	4112155	18.0
1519.530	8_1_0_0	N-glycan digest	133033	18.1
2046.720	10_2_0_0	High mannose	104831	18.4
1439.530	5_3_0_0	Intact HMO	1206590	18.5
1585.590	5_3_1_0	Intact HMO	9652349	18.5
1683.630	4_5_0_0	Complex/Hybrid	900577	18.5
1074.400	4_2_0_0	Intact HMO	736506	18.6
547.211	2_1_0_0	HMO digest	295271	18.8
1058.400	3_2_1_0	HMO digest	285071	18.8
2354.820	11_2_1_0	Fucosylated high mannose	733722	18.8
1731.640	5_3_2_0	Intact HMO	6499774	19.0
1626.610	4_4_1_0	Fucosylated complex/hybrid	317167	19.2
2063.770	4_4_2_1	Fucosylated and sialylated complex	82428	19.2
2080.780	5_4_3_0	Fucosylated complex/hybrid	221900	19.4
2242.830	6_4_3_0	Intact HMO	1995646	19.4
1033.370	5_1_0_0	N-glycan digest	65705	19.6
2354.820	11_2_1_0	Fucosylated high mannose	253745	19.8
1074.400	4_2_0_0	Intact HMO	133408	20.0
1115.420	3_3_0_0	HMO Digest or complex/hybrid	863030	20.0
1569.590	4_3_2_0	Fucosylated complex/hybrid	522844	20.1
2242.830	6_4_3_0	Intact HMO	596267	20.1
2137.800	5_5_2_0	Fucosylated complex/hybrid	354307	20.3
1407.540	3_3_2_0	Fucosylated complex	352279	20.4
1439.530	5_3_0_0	Intact HMO	4727694	20.5
1585.590	5_3_1_0	Intact HMO	1417829	20.5
2354.820	11_2_1_0	Fucosylated high mannose	186081	20.7
2980.090	5_7_1_2	Fucosylated and sialylated complex	58089	20.7
750.291	2_2_0_0	HMO digest	514796	20.8
1115.420	3_3_0_0	HMO digest or complex/hybrid	348468	20.8
1261.480	3_3_1_0	N-glycan digest	3131834	20.8
1318.500	3_4_0_0	Complex	272553	20.8
1480.550	4_4_0_0	Complex/hybrid	430320	20.8
1382.510	5_2_1_0	Intact HMO	351419	20.9
1934.720	5_4_2_0	Fucosylated complex/hybrid	356331	20.9
1714.630	4_3_1_1	Fucosylated and sialylated complex	757691	21.0
1788.670	5_4_1_0	HMO digest or fucosylated complex/hybrid	80219	21.0
2957.100	7_6_4_0	Fucosylated complex	152530	21.1
1480.550	4_4_0_0	Complex/Hybrid	673670	21.2
1917.710	4_4_1_1	Fucosylated and sialylated complex	67225	21.2
1439.530	5_3_0_0	Intact HMO	373534	21.4
1480.550	4_4_0_0	Complex/Hybrid	1074868	21.8
1568.570	4_3_0_1	Sulfated complex	124551	21.8

TABLE II—*continued*

Neutral mass	Composition	Oligosaccharide type	Volume	Retention time (min)
1788.670	5_4_1_0	HMO digest or fucosylated complex/hybrid	154531	21.8
1934.720	5_4_2_0	Fucosylated complex/hybrid	132694	22.3
2096.780	6_4_2_0	Intact HMO or fucosylated hybrid	4336232	22.4
2137.800	5_5_2_0	Fucosylated complex/hybrid	489632	22.6
2615.980	4_6_3_1	Fucosylated and sialylated complex	49307	22.9
1480.550	4_4_0_0	Complex/Hybrid	189839	23.1
1845.690	5_5_0_0	Complex/Hybrid	429557	23.1
1950.720	6_4_1_0	Intact HMO	3551248	23.2
2022.740	5_3_2_1	Intact HMO	329446	23.3
1439.530	5_3_0_0	Intact HMO	152536	23.4
1714.630	4_3_1_1	Fucosylated and sialylated complex	382781	23.4
2210.820	6_6_0_0	Complex	194164	23.4
2323.870	4_6_1_1	Fucosylated and sialylated complex	41426	23.5
1585.590	5_3_1_0	Intact HMO	945054	23.6
1480.550	4_4_0_0	Complex/Hybrid	1275312	23.8
1804.660	6_4_0_0	Intact HMO	188455	23.8
3272.210	5_7_3_2	Fucosylated and sialylated complex	91951	23.8
2251.840	5_7_0_0	Complex	83628	24.1
1569.590	4_3_2_0	Fucosylated complex/hybrid	403151	24.2
2022.740	5_3_2_1	Intact HMO	51901	24.2
1845.690	5_5_0_0	Complex/Hybrid	352433	24.3
1876.680	5_3_1_1	Intact HMO	115578	24.3
2251.840	5_7_0_0	Complex	199570	24.3
1569.590	4_3_2_0	Fucosylated complex/hybrid	81166	24.6
1318.500	3_4_0_0	Complex	62543	25.2
1934.720	5_4_2_0	Fucosylated complex/hybrid	95705	26.1
1876.680	5_3_1_1	Intact HMO	168198	26.5
1730.620	5_3_0_1	Intact HMO or sialylated hybrid	497318	26.8
2095.760	6_4_0_1	Intact HMO or sialylated hybrid	481884	27.1
2219.860	3_7_2_0	Fucosylated complex	380620	27.8
1204.460	3_2_2_0	HMO digest	46764	28.0

mentation patterns are known. This is because of their low abundances, which made tandem MS difficult to obtain. The masses of these overlapping compositions were added to the Fecal Glycan Library, with both oligosaccharide sources listed (Table II).

Selection and Expression of Microbial Enzymes—Previous analysis on the feces of these infants showed that the infants were dominated by types of bifidobacteria that are capable of catabolyzing HMOs (supplemental Fig. S3) (24, 31). For this reason, glycosidases from bifidobacterial strains were expressed to determine whether the enzymes can act on HMOs and produce the structures present in the infant feces. For this work, we selected an exogalactosidase and endoglycosidase from bifidobacteria to determine whether these acted on milk glycoconjugates. An example is illustrated in Fig. 4 with a β-galactosidase from *B. longum* subsp. *longum* SC596, BLNG_00015. For these experiments, pooled HMOs were treated with the enzyme (a galactosidase, pH 6.0 at 55 °C for 1 h). The abundances of HMOs in the digested pool were compared with those in an undigested HMO pool. The chromatograms in Fig. 4*A* show digestion of select structures with *m/z* 611.24 (4Hex:2HexNAc:1Fuc). Two isomers - monofucosyllacto-*N*-hexaose I (MFLNH I, retention time 14.5 min, inset structure 4) and isomer 3 fucosyl-paralacto-*N*-hexaose (IFLNH III, retention time 15.2 min, inset structure 5) - were noticeably lower in abundances in the digested HMO pool compared with the undigested HMO pool (Fig. 4*A*). Isomer 1 fucosyl-paralacto-*N*-hexaose (IFLNH I, retention time 18.3 min, inset structure 6), however, has no terminal Gal residue and therefore the exogalactosidase was unable to digest this structure as shown by the overlapping chromatograms (Fig. 4*A*). The exogalactosidase was also able to degrade select undecorated intact HMOs with *m/z* 1075.41 (4Hex:2HexNAc) (Fig. 4B). Lacto-*N*-hexaose (LNH, retention time 15.6 min, inset structure 7) and lacto-*N*-neohexaose (LNnH, retention time 16.2 min, inset structure 8) decreased in abundance, but para-lacto-*N*-hexaose (p-LNH, retention time 19.5 min, inset structure 9) went undigested (Fig. 4*B*).

After analyzing intact HMO digestion, we turned our attention to the digestion products that were formed by the exogalactosidase activity. If LNH and LNnH (4Hex:2HexNAc) were to lose a terminal Gal residue the digestion products would have the composition 3Hex:2HexNAc. This composition was seen in high abundance in the digested sample (Fig. 4*C*). Further digestion would lead to the loss of another terminal Gal and composition 2Hex:2HexNAc (Fig. 4*D*). The abundance in the undigested pool was at baseline for these

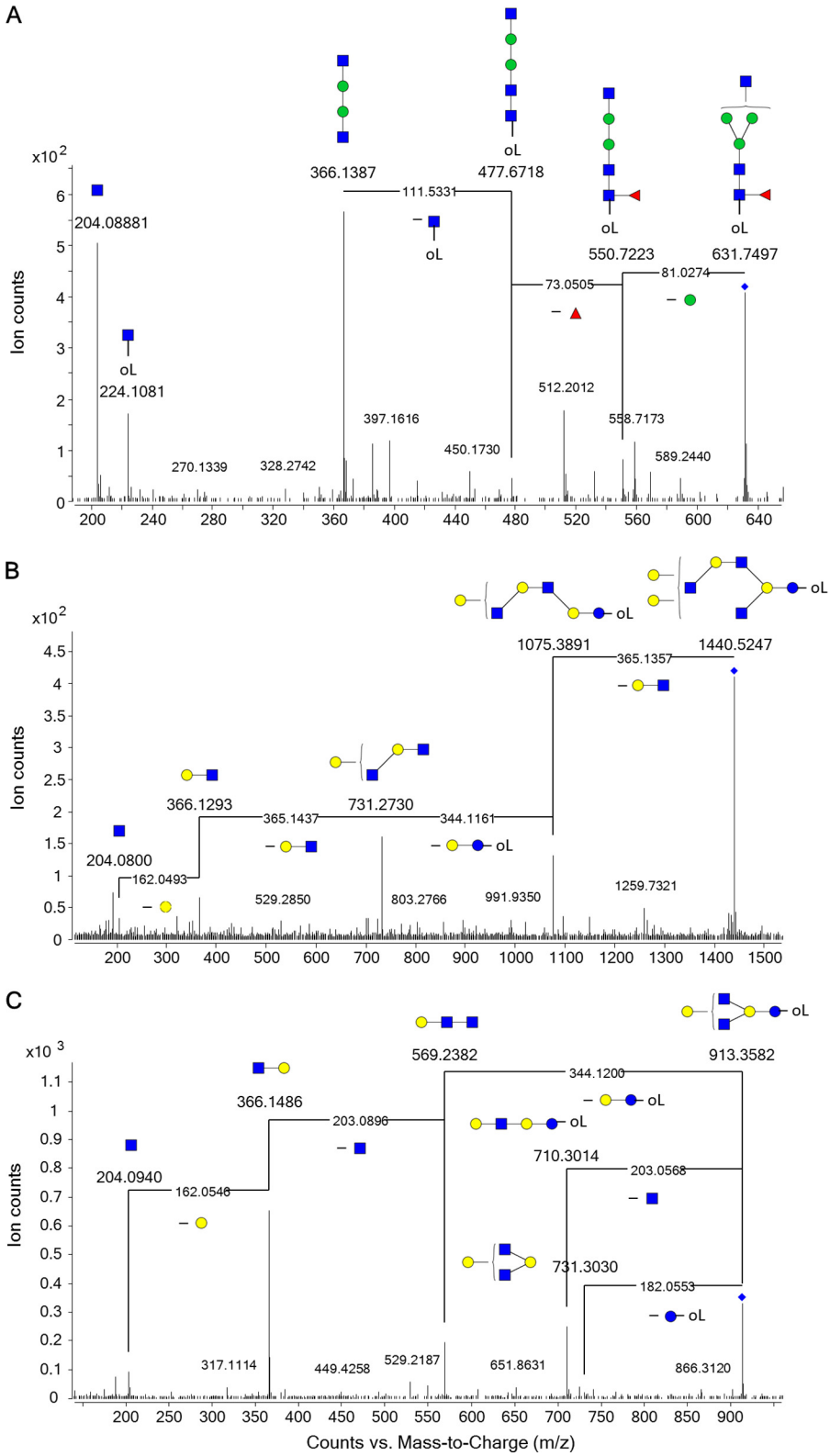

FIG. 3. **Tandem mass spectra of oligosaccharides extracted from infant feces reveals they are of human milk oligosaccharide (HMO) and glycoprotein origins.** Monosaccharides represented as glucose (●), galactose (●), mannose (●), N-acetylglucosamine (■), and fucose (▲). *A*, Doubly charged precursor ion with *m/z* 631.74 shows neutral losses of monosaccharides consistent with an intact N-glycan, including the reduced GlcNAc core. *B*, Precursor ion with *m/z* 1440.52 shows neutral losses of mono- and disaccharides consistent with an HMO structure. *C*, Precursor ion with *m/z* 913.35 has tandem mass spectrum pattern consistent with an HMO structure, including the lactose core.

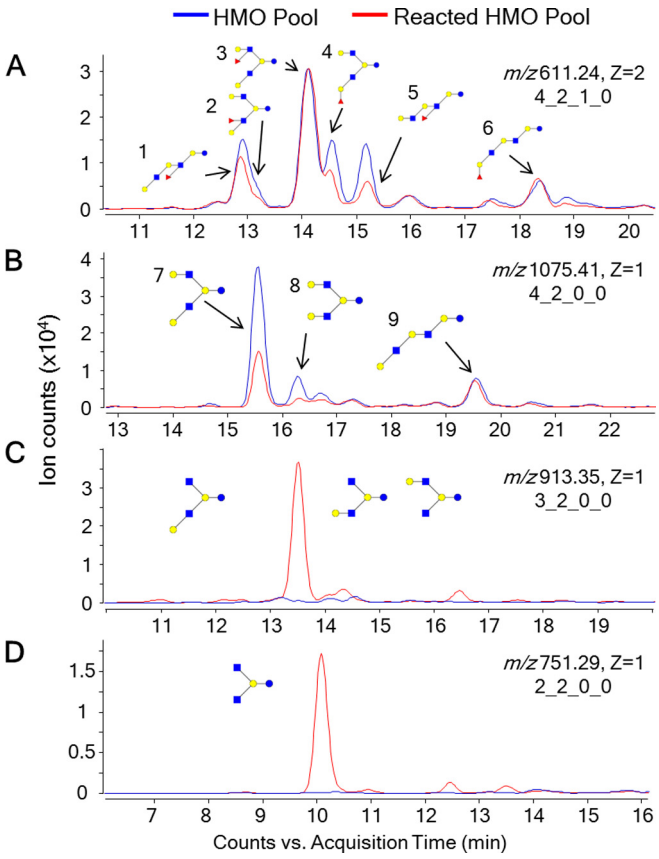

FIG. 4. **Overlaid extracted ion chromatograms (EICs) of β-galactosidase BLNG_00015 digestion of a human milk oligosaccharide (HMO) pool (–) compared with an unreacted HMO pool (–).** Monosaccharide composition of structures given as Hex_HexNAc_Fuc_Neu5Ac and represented as glucose (●), galactose (●), N-acetylglucosamine (■), and fucose (▲). *A*, EICs of doubly charged *m/z* 611.24 shows decreased abundance of specific fucosylated isomers in the reacted HMO pool (1: MFpLNH IV, 2: 4120a, 3: MFLNH III, 4: MFLNH I, 5: IFLNH III, 6: IFLNH I). *B*, EICs of *m/z* 1075.41 shows decreased abundance of specific undecorated isomers in the reacted HMO pool (7: LNH, 8: LNnH, 9: p-LNH). *C*, EICs of *m/z* 913.35 shows increased abundance of potential digestion products in the reacted HMO pool. *D*, EICs of *m/z* 751.29 shows increased abundance of the potential digestion product in the reacted HMO pool.

digestion products, revealing that they are not found as intact HMO structures in the pooled breast milk but were only observed after performing enzymatic digestion reactions. These new digested HMO products were added to the Fecal Glycan Library (supplemental Table S2).

An endoglycosidase from *B. longum* subsp. *infantis* was previously expressed to determine its activity. Instead of degrading HMOs, this enzyme (EndoBI-1) was active on N-glycans, demonstrating preferential cleavage between the two GlcNAcs of the chitobiose core (44). These types of degraded N-glycans were observed in feces. For example, the *m/z* 1034.37 peak detected in feces corresponds to an oligosaccharide with composition 5Hex:1HexNAc (fragmentation shown in Fig. 5). This composition is not consistent with an intact or digested HMO for several reasons. Large HMO structures have repeating lactosamine units, and this compound only has one HexNAc. The tandem MS yields a peak at *m/z* 224.11, consistent with a reduced HexNAc, which is a rare occurrence in HMOs (Fig. 5). All the neutral mass losses that led up to that peak were 162, indicative of Hex losses, most likely mannose residues (Fig. 5). Because of the reduced GlcNAc and the five successive Hex, we deduced that this oligosaccharide is indeed a high mannose type N-glycan cleaved by an endoglycosidase. Both intact and digested N-glycans, their masses, and corresponding compositions were also added to the Fecal Glycan Library (supplemental Table S2).

Observation of Infant Fecal Glycome and Microbiome Profile Changes Postnatal—The compilation of fecal glycans (the Fecal Glycan Library) with their retention times and accurate masses were used to determine the fecal glycome profiles of infants over lactation. Fecal samples for three of the infants from the Davis Lactation Study were analyzed at several postnatal time points. For all three infants, there was a drastic decrease in the abundance of intact HMO from the first week of life (days 6 and 7) to week 17 (day 120) (Fig. 6). The absolute abundance dropped 75.3% on average, also observed was an opposite trend for digested HMO over lactation, which increased 124.0% at week 17 (Fig. 6). The bacterial profiling revealed that bifidobacteria, known to consume HMOs comprised on average 68.0% of all bacteria in the infant gut at week 17 (Supplemental Fig. 3) (20, 21). For two of the infants, (1004 and 1054) the abundance of digested N-glycans also increased from week 1 to week 17 (Fig. 6). *B. longum* subsp. *longum* comprised 9.8% of all bacteria in the infant 1054 fecal microbiota at week 17 (supplemental Table S3). Schell *et al.* showed that *B. longum* contains a genomic cluster with an endoglycosidase for cleaving GlcNAcs which explains our observance of digested N-glycans (45). *B. longum* subsp. *infantis,* known to catabolize the entire HMO internally, was found to comprise 6.7% and 4.2% of all bacteria in infant 1040 at weeks 3 and 17, respectively (supplemental Fig. S3) (29, 30). This correlated with lower amounts of digested HMOs at those time points (Fig. 6). This trend is consistent with the gut microbiota becoming established, niches being filled, and more of the oligosaccharides being degraded later in lactation.

DISCUSSION

Human breast milk contains both free and bound oligosaccharides. As the infant consumes breast milk, the contents pass through the digestive tract through the intestine, where they encounter the gut microbiota. The bacteria are equipped with glycosidases that cleave HMOs and other glycoconjugates, potentially leaving digestion products and free oligosaccharides to pass through the gut and exit as feces. Glycoconjugates such as N-glycans may be degraded while still linked to proteins, but they can also be enzymatically released

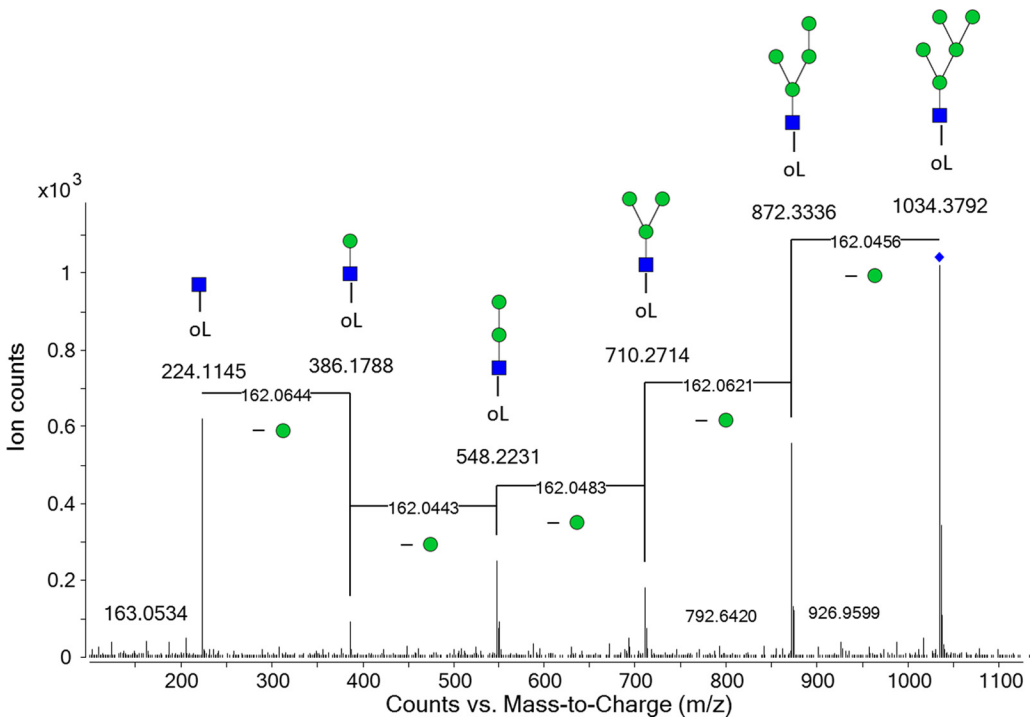

FIG. 5. **Oligosaccharide extracted in infant feces was determined to be a degraded N-glycan based on tandem mass spectra.** Monosaccharides represented as mannose (●) and N-acetylglucosamine (GlcNAc) (■). Precursor ion with *m/z* 1034.37 shows neutral subsequent losses of hexose residues consistent with a high mannose type N-glycan. Only one GlcNAc present in the core is indicative of endoglycosidase activity on the glycoprotein this structure was derived from.

from glycoproteins to form new free oligosaccharides. After their release, free N-glycans can be further degraded by exoglycosidases to form additional digestion products. Our oligosaccharide enrichment method may also extract O-glycans. *In vitro* studies have shown bacterial growth on, binding to, and degradation of mucus O-glycans, yet no glycosidases have been characterized that can release large O-glycans (larger than disaccharides) from glycoproteins (46–49). Furthermore, O-glycans are in much lower abundances in milk compared with HMOs and N-glycans and are therefore not expected to be present in appreciable abundances (50, 51). Free oligosaccharides may also be released from glycolipids (52), yet much like O-glycans, glycolipids are far less abundant compared with HMOs and N-glycosylated proteins and are not likely to be observed. In order to identify structures that are digestion products of HMOs, enzymatic i*n vitro* experiments were performed. One involved the use of an exogalactosidase (BLNG_00015) from *B. longum* subsp. *longum* SC596, as this species was found in high abundance in the infant fecal samples. Digestion of intact HMOs showed that the enzyme was capable of degrading structures with a terminal galactose residue, but more interesting were the structures not degraded. The lack of digestion for p-LNH is explained by the preference for β(1–4)Gal linkages of this specific enzyme. Both LNH and LNnH have at least one terminal β(1–4)Gal so those isomers were digested, but because p-LNH only has a terminal β(1–3)Gal linkage, the hydrolase was unable to cleave the linkage leaving the structure intact. This specificity was also observed in the fucosylated structures MFLNH I and IFLNH III, which both contain a terminal β(1–4)Gal residue so the exogalactosidase was capable of degrading those structures. Having a Fuc present on the same GlcNAc as a β(1–4)Gal appeared to hinder the enzyme from hydrolyzing that linkage. This type of analysis reveals the specificity of microbial enzymes. Observance of certain isomers, along with the absence of others, can indicate the preferences of those enzymes present. These experiments also confirmed that degraded oligosaccharides obtained in infant feces can be produced under *in vitro* conditions with the appropriate enzymes.

It was not surprising to find intact N-glycans in the infant feces at all sampled time points, as these molecules have been described in breast milk. The observation of degraded N-glycans *in vivo* in infant feces is, however, a novel finding. Degradation of N-glycans has been predicted before by enzymatic studies of individual microbes. For example, *Bacteroides* is known to inhabit breast-fed infant digestive tracts, was found in the feces of the infants in this study, and is known to have an enzyme capable of cleaving intact N-glycans from glycoproteins (19, 31, 53). Another enzyme, Peptide:N-glycosidase F (PNGase F), is known to cleave N-glycans at the glycosylamine linkage, but this enzyme was discovered in the pathogenic bacterium *Flavobacterium meningosepticum* (54, 55). Experiments were not performed in

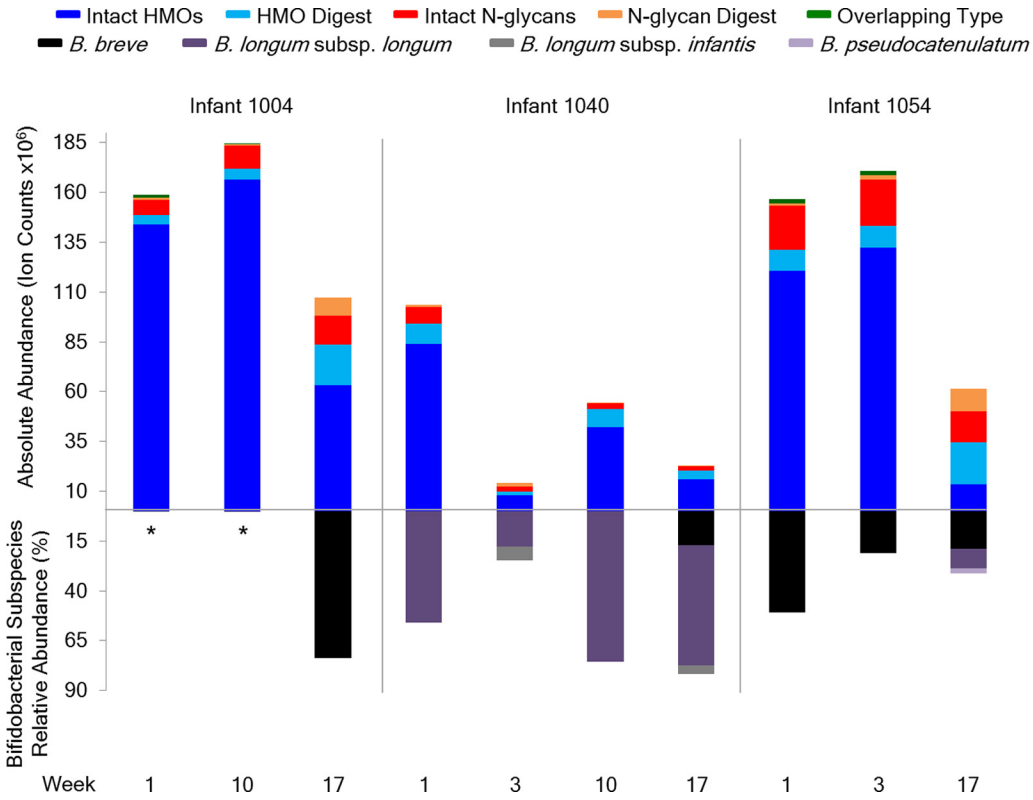

FIG. 6. **Changes in three infant fecal glycan and bifidobacteria profiles for 1, 3, 10, and/or 17 weeks postnatal.** Bar graphs represent absolute abundances of the different glycan types (intact HMOs (■), digested HMOs (■), intact N-glycans (■), digested N-glycans (■), and indistinguishable glycans with overlapping compositions (■)), as well as relative abundances of bifidobacterial subspecies (*B. breve* (■), *B. longum* subsp. *longum* (■), *B. longum* subsp. *infantis* (■), and *B. pseudocatenulatum* (■)) in relation to the entire microbiota. Large decrease in intact HMOs for all infants from week 1 to week 17, along with an increase in digested HMOs in that same time frame. Infants 1004 and 1054 also showed an increase in digested N-glycans from week 1 to 17. Intact HMOs and bifidobacterial species display inverse relationship, with increase in relative abundance of bifidobacteria from week 1 to week 17 for infants 1004 and 1040. *Relative abundances of bifidobacteria were <1% at weeks 1 and 10 for infant 1004.

this study using this specific enzyme because of its widely established activity on glycoproteins. Although *F. meningosepticum* was not specifically detected in the feces of the infants, it is a potential glycoprotein degrader and contributor to intact N-glycans in feces. Other N-glycans observed were hydrolyzed at the chitobiose core. We previously discovered an endoglycosidase, EndoBI-1, in *B. longum* subsp. *infantis* that was able to release all types of N-glycans from lactoferrin and immunoglobulins A and G by cleaving the oligosaccharide between the two core GlcNAcs, which is consistent with the product found in feces and may explain other similar digested N-glycan products (44). *B. longum* was found in all infants (*B. longum* subsp. *infantis* and *B. longum* subsp. *longum*), and one strain of *B. longum* was also found to contain genes for an endoglycosidase (45). Endoglycosidases have also been found in other bifidobacteria species, along with *Streptomyces plicatus*, and pathogenic *Enterococcus faecalis*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae* (44, 56–59). These studies employed genetic analysis and *in vitro* experiments to determine the enzyme's activity, but did not test their *in vivo* implications.

Fecal glycan profile changes were observed for infants postnatally. There was an initial increase in total oligosaccharide abundance for infants 1004 and 1054, but not for infant 1040. This initial increase with a later decrease has been observed previously (60), and has been attributed to the initial lack of saccharolytic bacteria. The HMOs accumulate until the establishment of HMO-consuming microbiota in the infant's gut. This process of maturation occurs at different rates for infants, however, the general trend is that the HMOs decrease. There is a drastic decrease in intact HMOs from week 1 to week 17 postpartum for all three infants, as well as an increase in digested HMOs. It has been shown that the abundance of HMOs in breast milk decrease over the course of lactation, which could explain the diminished abundance of observed intact HMOs in infant feces (61). However, infants consume more volume and feed more frequently as they age before solid foods are introduced. Although the mother may be producing milk with a lower concentration of oligosaccharides, the infant could still be consuming the same amount of HMOs because of the increased milk intake. We also observed an increase in bifidobacterial abundance in the infants'

gastrointestinal tracts, and bifidobacteria are known to consume HMOs (20, 21). This suggests that the decrease in intact HMOs and increase in digested HMOs is because of bacterial metabolism. An interesting correlation was observed, however, in infant 1040, whose feces contained decreased amounts of digested HMOs during weeks 3 and 17, but also a presence of *B. longum* subsp. *infantis* at those same time points. *B. longum* subsp. *infantis* is unique among saccharolytic bacteria in that it imports the entire HMO structure for complete internal catabolism. This process could explain the low amounts of HMO digestion products in feces during later weeks (29, 30). Even though the microbiota community becomes dominated by bifidobacteria over the postnatal period leading to fewer intact HMOs and more digested HMOs in infant feces, it appears that specific strains can have an impact as to the types of fecal oligosaccharides observed. Analysis of fecal oligosaccharides has the potential of becoming a noninvasive method of assaying biomarkers likely relevant to the health of an infant (62).

Analyzing which milk glycans remain intact in the feces of an infant can help provide insight into the types of bacteria present in the infant gut microbiome and the functions they provide. We observed HMO and bacterial interaction by directly comparing a mother's HMO composition to the oligosaccharide profile of her infant's feces. Many of the HMOs passed through intact in the feces, but in significantly depleted amounts. The two most abundant structures in breast milk, LNT and LNnT, were completely lacking from the feces, showing preferential consumption of those structures. Some HMOs were found in larger abundance in feces compared with milk, would could be attributed to large HMOs being digested to form smaller HMOs, adding to their abundance. These types of observations can allude to the interactions between microbiota and oligosaccharides. Large-scale functional characterization of the different types of bacteria in the infant's gastrointestinal tract is typically performed via metagenomic, proteomic, or metabolomic experiments. Here, we propose that fecal glycans are suggestive of the bacterial enzymes present in an infant's gut. This information allows greater understanding of how bacteria interact with HMOs and other glycoconjugates in the gut. Interestingly, a small number of select enzymes can account for the vast majority of the products, although the glycosidases may come from different strains and still represent different catabolic activities. Although additional work is still needed to describe all the relevant microbial enzymes and their specificities, we have shown that a glycan-centered analysis can provide information about the active glycosidases operating on human milk.

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REFERENCES

1. Ruhaak, L. R., and Lebrilla, C. B. (2012) Analysis and role of oligosaccharides in milk. *BMB Reports* **45,** 442–451
2. Nwosu, C. C., Aldredge, D. L., Lee, H., Lerno, L. A., Zivkovic, A. M., German, J. B., and Lebrilla, C. B. (2012) Comparison of the human and bovine milk N-Glycome via high-performance microfluidic chip liquid chromatography and tandem mass spectrometry. *J. Proteome Res.* **11,** 2912–2924
3. Liao, Y., Alvarado, R., Phinney, B., and Lonnerdal, B. (2011) Proteomic characterization of human milk whey proteins during a twelve-month lactation period. *J. Proteome Res.* **10,** 1746–1754
4. Bode, L. (2012) Human milk oligosaccharides: Every baby needs a sugar mama. *Glycobiology* **22,** 1147–1162
5. Kunz, C., and Rudloff, S. (1993) Biological functions of oligosaccharides in human milk. *Acta Paediatr.* **82,** 903–912
6. Newburg, D. S., Ruiz-Palacios, G., and Morrow, A. L. (2005) Human Milk Glycans Protect Infants Against Enteric Pathogens. *Annu. Rev. Nutr.* **25,** 37–58
7. Wu, S., Grimm, R., German, J. B., and Lebrilla, C. B. (2011) Annotation and structural analysis of sialylated human milk oligosaccharides. *J. Proteome Res.* **10,** 856–868
8. Wu, S., Tao, N., German, J. B., Grimm, R., and Lebrilla, C. B. (2010) Development of an annotated library of neutral human milk oligosaccharides. *J. Proteome Res.* **9,** 4138–4151
9. Marino, K., Lane, J. A., Abrahams, J. L., Struew, W. B., Harvey, D. J., Marotta, M., Hickey, R. M., and Rudd, P. M. (2011) Method for milk oligosaccharide profiling by 2-aminobenzamide labeling and hydrophilic interaction chromatography. *Glycobiology* **21,** 1317–1330
10. Kobata, A. (2010) Structures and application of oligosaccharides in human milk. *Proc. Japan Acad., Series B* **86,** 731–747
11. Thurl, S., Henker, J., Siegel, M., Tovar, K., and Sawatzki, G. (1997) Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconjugate J.* **14,** 795–799
12. Kunz, C., Rudloff, S., Baier, W., Klein, N., and Strobel, S. (2000) Oligosaccharides in Human Milk: Structural, Functional, and Metabolic Aspects. *Annu. Rev. Nutr.* **20,** 699–722
13. Urashima, T., Kitaoka, M., Asakuma, S., and Messer, M. (2009) Milk Oligosaccharides. In: McSweeney, P., and Fox, P., eds. *Advanced Dairy Chemistry: Lactose, Water, Salts, and Minor Constituents*, pp. 295–348, Springer Science, New York, NY.
14. Engfer, M. B., Stahl, B., Finke, B., Sawatzki, G., and Daniel, H. (2000) Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *Am. J. Clin. Nutrition* **71,** 1589–1596
15. Chaturvedi, P., Warren, C. D., Buescher, C. R., Pickering, L. K., and Newburg, D. S. (2001) Survival of human milk oligosaccharides in the intestine of infants. *Adv. Exp. Med. Biol.* **501,** 315–323
16. Coppa, G. V., Pierani, P., Zampini, L., Bruni, S., Carloni, I., and Gabrielli, O. (2001) Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. *Adv. Exp. Med. Biol.* **501,** 307–314
17. Arora, T., and Sharma, R. (2010) Fermentation potential of the gut microbiome: implications for energy homeostasis and weight management. *Nutrition Rev.* **69,** 99–106
18. Macfarlane, S., and Macfarlane, G. T. (2003) Regulations of short-chain fatty acid production. *The 7th International VahounyFibre Symposium*, pp. 67–72, Edinburgh, Scotland.

19. Marcobal, A., and Sonnenburg, J. L. (2012) Human milk oligosaccharide consumption by intestinal microbiota. *Clin. Microbiol. Infect* **18,** 12–15
20. German, J. B., Freeman, S. L., Lebrilla, C. B., and Mills, D. A. (2007) Human Milk Oligosaccharides: Evolution, Structures, and Bioselectivity as Substrates for Intestinal Bacteria. In: Bier, D. M., German, J. B., and B., L., eds. *62nd Nestle Nutrition Workshop, Pediatric Program*, pp. 205–222, Helsinki, Finland.
21. Ward, R. E., Ninonuevo, M. R., Mills, D. A., Lebrilla, C. B., and German, J. B. (2006) In vitro fermentation of breast milk oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri. *Appl. Environmental Microbiol.* **72,** 4497–4499
22. Murphy, E., Murphy, C., and O'Mahony, L. (2009) Influence of the Gut Microbiota with Ageing. In: Percival, S. L., ed. *Microbiology and Aging: Clinical Manifestations*, p. 161, Springer Science+Business Media, LLC, New York, NY.
23. Garrido, D., Dallas, D. C., and Mills, D. A. (2013) Consumption of human milk glycoconjugates by infant-associated bifidobacteria: mechanisms and implications. *Microbiology* **159,** 649–664
24. LoCascio, R. G., Ninonuevo, M. R., Freeman, S. L., Sela, D. A., Grimm, R., Lebrilla, C. B., Mills, D. A., and German, J. B. (2007) Glycoprofiling of bifodobaterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *J. Agricultural Food Chem.* **55,** 8914–8919
25. Albrecht, S., Schols, H. A., van den Heuvel, E. G. H. M., Voragen, A. G. J., and Gruppen, H. (2011) Occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk. *Carbohydrate Res.* **346,** 2540–2550
26. De Leoz, M. L. A., Wu, S., Strum, J. S., Ninonuevo, M. R., Gaerlan, S. C., Mirmiran, M., German, J. B., Mills, D. A., Lebrilla, C. B., and Underwood, M. A. (2013) A quantitative and comprehensive method to analyze human milk oligosaccharide structures in the urine and feces of infants. *Anal. Bioanal. Chem.* **405,** 4089–4105
27. Eilam, O., Zarecki, R., Oberhardt, M., Ursell, L. K., Kupiec, M., Knight, R., Gophna, U., and Ruppin, E. (2014) Glycan Degradation (GlyDeR) Analysis predicts mammalian gut microbiota abundance and host diet-specific adaptations. *mBio* **5,** 1–12
28. Asakuma, S., Hatakeyama, E., Urashima, T., Yoshida, E., Katayama, T., Yamamoto, K., Kumagai, H., Ashida, H., Hirose, J., and Kitaoka, M. (2011) Physiology of consumption of human milk oligosaccharides by infant gut-associated Bifidobacteria. *J. Biol. Chem.* **286,** 34583–34592
29. LoCascio, R. G., Ninonuevo, M. R., Kronewitter, S. R., Freeman, S. L., German, J. B., Lebrilla, C. B., and Mills, D. A. (2009) A versatile and scalable strategy for glycoprofiling bifidobacterial consumption of human milk oligosaccharides. *Microb. Biotechnol.* **2,** 333–342
30. Sela, D. A., and Mills, D. A. (2010) Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. *Trends Microbiol.* **18,** 298–307
31. Lewis, Z. T., Totten, S. M., Smilowitz, J. T., Popovic, M., Parker, E., Lemay, D. G., Van Tassell, M. L., Miller, M. J., Jin, Y. S., German, J. B., Lebrilla, C. B., and Mills, D. A. (2015) Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. *Microbiome* **3,** 13
32. Ferris, A. M., and Jensen, R. G. (1984) Lipids in Human Milk: A Review. 1: Sampling, determination, and content. *J. Ped. Gastroenterol. Nutrition* **3,** 108–122
33. Totten, S. M., Wu, L. D., Parker, E. A., Davis, J. C. C., Hua, S., Stroble, C., Ruhaak, L. R., Smilowitz, J. T., German, J. B., and Lebrilla, C. B. (2014) Rapid-throughput glycomics applied to human milk oligosaccharide profiling for large human studies. *Anal. Bioanal. Chem.* **406,** 7925–7935
34. Vizcaino, J. A., SCsordas, A., del-Toro, N., Dianes, J. A., Griss, J., Lavidas, I., Mayer, G., Perez-Riverol, Y., Reisinger, F., Ternent, T., Xu, Q. W., Wang, R., and Hermjakob, H. (2016) 2016 update of the PRIDE database and related tools. *Nucleic Acids Res.* **44,** D447–D456
35. Lewis, Z. T., Bokulich, N. A., Kalanetra, K. M., Ruiz-Moyano, S., Underwood, M. A., and Mills, D. A. (2013) Use of bifidobacterial specific terminal restriction fragment length polymorphisms to complement next generation sequence profiling of infant gut communities. *Anaerobe* **19,** 62–69
36. Lewis, Z. T., Shani, G., Masarweh, C. F., Popovic, M., Frese, S. A., Sela, D. A., Underwood, M. A., and Mills, D. A. (2015) Validating bifidobacterial species and subspecies identity in commercial probiotic products. *Pediatr Res.* **79,** 445–452
37. Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., and Knight, R. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 4516–4522
38. Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., Mcdonals, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevindsky, J. R., Turnbugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010) QIIME allows analysis of high- throughput community sequencing data. *Nat. Methods* **7,** 335–336
39. Edgar, R. C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26,** 2460–2461
40. Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol*. **73,** 5261–5267
41. Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M., and Tiedje, J. M. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* **37,** D141–D145
42. DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G. L. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environmental Microbiol.* **72,** 5069–5072
43. Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010) PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26,** 266–267
44. Garrido, D., Nwosu, C., Ruiz-Moyano, S., Aldredge, D., German, J. B., Lebrilla, C. B., and Mills, D. A. (2012) Endo-β-N-acetylglucosaminidases from infant gut-associated bifidobacteria release complex N-glycans from human milk glycoproteins. *Mol. Cell Proteomics* **11,** 775–785
45. Schell, M. A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., Zwahlen M-C, Desiere, F., Bork, P., Delley, M., Pridmore, R. D., and Arigoni, F. (2002) The genome sequence of *Bifidobacterium longum* reflets its adaptation to the human gastrointestinal tract. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 14422–14427
46. Koropatkin, N. M., Cameron, E. A., and Martens, E. C. (2001) 4) How glycan metabolism shapes the human gut microbiota. *Natl. Rev. Microbiol.* **10,** 323–335
47. Martens, E. C., Chiang, H. C., and Gordon, J. I. (2008) Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe* **4,** 447–457
48. Tailford, L. E., Crost, E. H., Kavanaugh, D., and Juge, N. (2015) Mucin glycan foraging in the human gut microbiome. *Front. Genetics* **6,** 1–18
49. Marcobal, A., Southwick, A. M., Earle, K. A., and Sonnenburg, J. L. (2013) A refined palate: Bacterial consumption of host glycans in the gut. *Glycobiology* **23,** 1038–1046
50. Schulz, B. L., Packer, N. H., and Karlsson, N. G. (2002) Small-scale analysis of O-linked oligosaccharides from glycoproteins and mucins separated by gel electrophoresis. *Anal. Chem.* **74,** 6088–6097
51. Royle, L., Mattu, T. S., Hart, E., Langridge, J. I., Merry, A. H., Murphy, N., Harvey, D. J., Dwek, R. A., and Rudd, P. M. (2002) An analytical and structural database provides a strategy for sequencing O-Glycans from microgram quantities of glycoproteins. *Anal. Biochem.* **304,** 70–90
52. Lee, H., Garrido, D., Mills, D. A., and Barile, D. (2014) Hydrolysis of milk gangliosides by infant-gut associated bifidobacteria determined by microfluidic chips and high-resolution mass spectrometry. *Electrophoresis* **35,** 1742–1750
53. Macfarlane, G. T., and Gibson, G. R. (1991) Formation of glycoprotein degrding enzymes by *Bacteroides fragilis. FEMS Microbiology Letters* **77,** 289–294
54. Plummer, T. H. J., Elder, J. H., Alexander, S., Phelan, A. W., and Tarentino, A. L. (1984) Demonstration of Peptide:*N*-Glycosidase F Activity in Endo-β-*N*-acetylglucosaminidase F Preparations. *J. Biol. Chem.* **259,** 10700–10704

55. Tarentino, A. L., Gomez, C. M., and Plummer, T. H. J. (1985) Deglycosylation of asparagine-linked glycans by peptide:*N*-glycosidase F. *Biochemistry* **24,** 4665–4671
56. Muramatsu, H., Tachikui, H., Ushida, H., Song X-j, Qiu, Y., Yamamoto, S., and Muramatsu, T. (2001) Molecular cloning and expression of endo-β-N-acetylglucosaminidase D, which acts on the core structure of complex type asparagine-linked oligosaccharides. *J. Biochem.* **129,** 923–928
57. Trimble, R. B., and Maley, F. (1977) The use of endo-β-N-acetylglucosaminidase H in characterizing the structure and function of glycoproteins. *Biochem. Biophys. Res. Commun.* **78,** 935–944
58. Collin, M., and Fischetti, V. A. (2004) A novel secreted endoglycosidase from Enterococcus faecalis with activity on human immunoglobulin G and ribonuclease B. *J. Biol. Chem.* **279,** 22558–22570
59. Allhorn, M., Olin, A. I., Nimmerjahn, F., and Collin, M. (2008) Human IgG/FcγR interactions are modulated by streptococcal IgG glycan hydrolysis. *PLoS ONE* **3,** e1413
60. De Leoz, M. L., Kalanetra, K. M., Bokulich, N. A., Strum, J. S., Underwood, M. A., German, J. B., Mills, D. A., and Lebrilla, C. B. (2015) Human milk glycomics and gut microbial genomics in infant feces show a correlation between human milk oligosaccharides and gut microbiota: a proof-of-concept study. *J. Proteome Res.* **14,** 491–502
61. Coppa, G. V., Gabrielli, O., Pierani, P., Catassi, C., Carlucci, A., and Giorgi, P. (1993) Changes in carbohydrate composition in human milk over 4 months of lactation. *Pediatrics* **91,** 637–641
62. Frese, S. A., and Mills, D. A. (2015) Should infants cry over spilled milk? Fecal glycomics as an indicator of a healthy infant gut microbiome. *J.Pediatric Gastroenterol. Nutrition* **60,** 695