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Glycoprofiling of Bifidobacterial Consumption of Human Milk Oligosaccharides Demonstrates Strain Specific, Preferential Consumption of Small Chain Glycans Secreted in Early Human Lactation

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The molecular basis by which human breast milk supports the development of a protective intestinal microbiome in infants is unknown. After lactose and lipids, human milk oligosaccharides (HMOs) are quantitatively the third largest and most diverse component of breast milk. In this work, glycomic profiling of HMO consumption by bifidobacteria using Fourier transform ion cyclotron resonance mass spectrometry reveals that one species, *Bifidobacterium longum* biovar *infantis* ATCC 15697, an isolate from the infant gut, preferentially consumes small mass oligosaccharides, representing 63.9% of the total HMOs available. These HMOs were detected in human breast milk at the onset and constantly through the first month of lactation by use of high performance liquid chromatography-chip time-of-flight mass spectrometry. Further characterization revealed that strain ATCC 15697 possesses both fucosidase and sialidase activities not present in the other tested strains. This work provides evidence that these small mass HMOs are selectively metabolized by select bifidobacterial strains and represent a potential new class of bioactive molecules functioning as prebiotics to facilitate a protective gut colonization in breast-fed newborns.

KEYWORDS: Bifidobacteria; human milk oligosaccharides; HPLC-Chip TOF; FTICR-MS; fucosidase; sialidase; prebiotic; probiotic

INTRODUCTION

The constant selective pressure on milk as the sole source of nourishment for mammalian infants is a remarkable model for how diet affects all aspects of development and health. Breast-feeding is a balance between the infant's nutritional needs and a large metabolic effort by the mother to ensure the offspring's survival and to maximize her reproductive potential. In a recent report the World Health Organization (WHO) referred to breast-feeding as the "biological norm" and to the breast-fed infant as a "normative model for optimal growth and development" (*1*), yet the bioactive, health-promoting components of human milk and their modes of action are little understood. An extraordinary aspect exclusively noted in breast-feeding infants is the selective nourishment and support of a protective, co-evolved gut microbiota (*2*), dominated by bifidobacteria (*3*). Numerous studies have shown that certain bifidobacterial species, such as *Bifidobacterium breve* and *Bifidobacterium longum* biovar *infantis*, are found predominately in infant feces, while *Bifidobacterium adolescentis* is typically only found in adult feces; other strains, such as *Bifidobacterium bifidum* and *Bifidobacterium longum*, have been isolated from both environments (*4*).

Human milk/colostrum contains between 3 and 20 g/L (*5*) of HMOs composed of lactose reducing ends elongated with up to 25 *N*-acetyllactosamine units, which can be extensively fucosylated and/or sialylated through α-glycosidic linkages (*6*). Fucosylated HMOs protect infants against diarrhea and are an important element of an innate immune system transferred through and developed by breast milk (*7*). Infants cannot digest HMOs which arrive intact in the large intestine; while having no apparent direct nutritional role, they modulate the establishment of a protective microbiota, enriched in bifidobacteria and exclusively characteristic of breast-fed infants (*8*). Despite the importance of these bioactive components for infant health and the significance of gastrointestinal tract (GIT) microbiome composition on host health and its energy absorption (*9*), a direct

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link between specific HMO species and bifidobacteria remains to be established (*10*). Recently, we profiled with high resolution over 200 different HMO structures differing in their size, charge, and sequence, (*6*) and we developed a deuterium labeling scheme to precisely quantify bacterial consumption of HMOs (*11*). To understand the molecular basis underlying HMO metabolism by infant-gut related bacteria, we determined consumption glycoprofiles for selected bifidobacteria and report which individual milk oligosaccharide species are consumed by these microorganisms. This work has overcome the analytical complexity previously hindering the study of these oligosaccharide structures and has enabled an understanding of the relationship between specific HMOs and bifidobacteria.

MATERIALS AND METHODS

HMO Purification. HMOs were separated from pooled human breast milk samples as described in Ward et al. (*12*). Pooled milk was provided by the Mother's Milk Bank of San Jose, CA, and the Mother's Milk Bank of Austin, TX.

Bacterial Growths. *B. longum* bv. *infantis* ATCC 15697 and *B. breve* ATCC 15700 were obtained from the American type Culture Collection (Manassas, VA), and *B. longum* bv. *longum* DJO10A was obtained from D. O'Sullivan, University of Minnesota. Cultures were initially propagated in De Man–Rogosa–Sharpe (MRS) broth (Becton Dickinson, Sparks, MD) and then grown twice on a semi-synthetic MRS medium, devoid of a carbohydrate source (*13*). This medium was supplemented with 2.0% (w/v) sterile filtered (Millex-GV, 0.22 μm, Millipore, Billerica, MA) HMO as the only carbohydrate source. There was no detectable growth on this medium (optical density, OD, of the media at 600 nm, $OD_{600\ nm} < 0.2$) without the addition of a carbohydrate source. All media were supplemented with 1% (w/v) L-cysteine, and all incubations were carried out at 37 °C in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Cell growth was measured by assessing OD at 600 nm with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Samples were grown in triplicate, and controls consisted of inoculated medium lacking HMOs and uninoculated medium containing HMOs. Error bars are represented as standard deviation of the means. Samples were collected at 0, 28, 94, and 148 h for *B. longum* bv. *infantis*, 0 and 45 h for *B. breve* and *B. longum* bv. *longum*, respectively, and stored at −80 °C. When *B. longum* bv. *infantis* was grown at 0.8% (w/v) HMO, samples were collected at 0, 25, and 50 h. Prior to oligosaccharide isolation and purification, all samples were boiled in a water bath for 5 min and centrifuged at 13500 rpm for 20 min, and the resulting supernatant was collected, filtered with a 0.22 μm sterile Millex-GV (Millipore, MA), and stored at −20 °C until further use.

Oligosaccharide Isolation and Purification. Oligosaccharides recovered from the supernatant (100 μL) were reduced using 100 μL of 2.0 M sodium borohydride and incubated at 42 °C for 16 h. Thirty microliters of deuterated milk oligosaccharides were added as internal standards. The oligosaccharides were desalted and purified by solid phase extraction using a 4 mL cartridge volume of 150 mg of bed weight of nonporous graphitized carbon black-solid phase extraction (Alltech, Deerfield, IL). Prior to use, the cartridge was washed with 3 vol of 80% acetonitrile in 0.10% aqueous trifluoroacetic acid (TFA) (v/v) and with another 3 vol of deionized water. The oligosaccharides were loaded onto the cartridge and subsequently washed with 7 vol of deionized water, eluted with 2 vol of 20% acetonitrile in water (v/v), dried *in vacuo*, reconstituted with 50 μL of deionized water, and diluted 2000 times with 50% acetonitrile in 0.1% formic acid (v/v) prior to MS analysis.

MS Analysis. All mass spectra were acquired on a HiResESI QFT-hybrid Fourier transform ion cyclotron resonance mass spectrometer with a 9.4 Tesla shielded superconducting magnet (IonSpec Corp., Irvine, CA). Samples were introduced using a Micromass Z-spray source interfaced to a nano-ESI external ion source (New Objective, Woburn, MA). The mobile phase of 1:1 water/acetonitrile was delivered at a flow rate of 250 nL/min using a binary nanoLC pump (Eksigent Technologies, Livermore, CA). Ion excitation was performed by arbitrary waveform with an amplitude of 125.0 V (base-to-peak) and detected by direct broadband mode at a rate of 1 MHz and with 512K data points. Each spectrum was recorded with one acquisition scan in the positive ion mode.

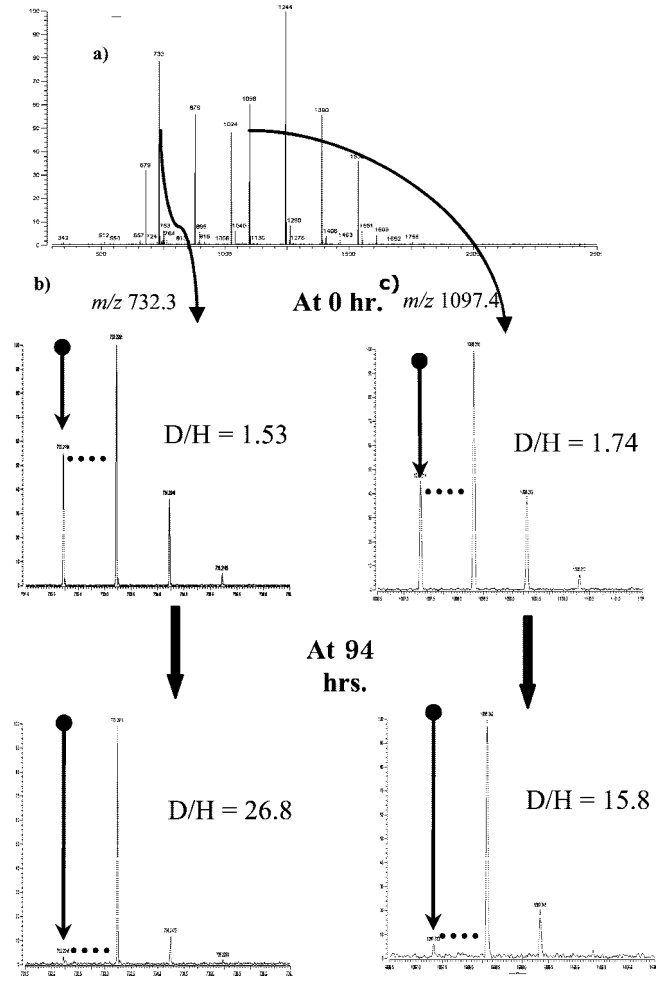

Figure 1. FTICR-MS glycoprofiling of bacterial fermentations. (**a**) FTICR-MS output with HMOs present upon inoculation with *B. longum* bv. *infantis* ($T = 0$ h), full mass spectra. (**b**, **c**) Difference in oligosaccharide peak height corresponds to bacterial consumption between 0 and 94 h, zoom-in on selected peaks (see text for details on D/H methods).

Calculation of the Ratio of Deuterated and Undeuterated Species (D/H). The D/H ratios were calculated using Formula 1 adapted from Xie et al. (*14*). The expected intensities for each signal were determined using the IonSpec exact mass calculator.

$$D/H = (n - mq/p)/m \quad (1)$$

where $m = A$; $n = A + 1$; $p = A$; $q = A + 1$; m, n = relative intensities; and p, q = expected intensities.

HMO Profiles through Lactation. Human milk samples were donated by four healthy mothers (M1–3, M5) of Reno, NV; for the first week, samples were collected daily from the first day of lactation and every other day for up to 10 weeks thereafter, depending on the ability of each mother to produce milk. All milk samples were stored at −80 °C prior to extraction.

Oligosaccharides were extracted, reduced, and purified according to a recently published procedure (*6*). Briefly, each milk sample (0.5 mL) was centrifuged at 3500 rpm at 4 °C for 30 min and was extracted with 10 mL (2:1) of a chloroform–methanol solution (v/v) and 2 mL of deionized water. The emulsion was centrifuged at 3500 rpm at 4 °C for 30 min, and the lower chloroform layer was discarded. The upper layer was collected; the proteins were precipitated with 7 mL of cold ethanol at 4 °C for 16 h. The solution was centrifuged at 4 °C for 30 min, and the supernatant was recovered, dried, and used for oligosac-

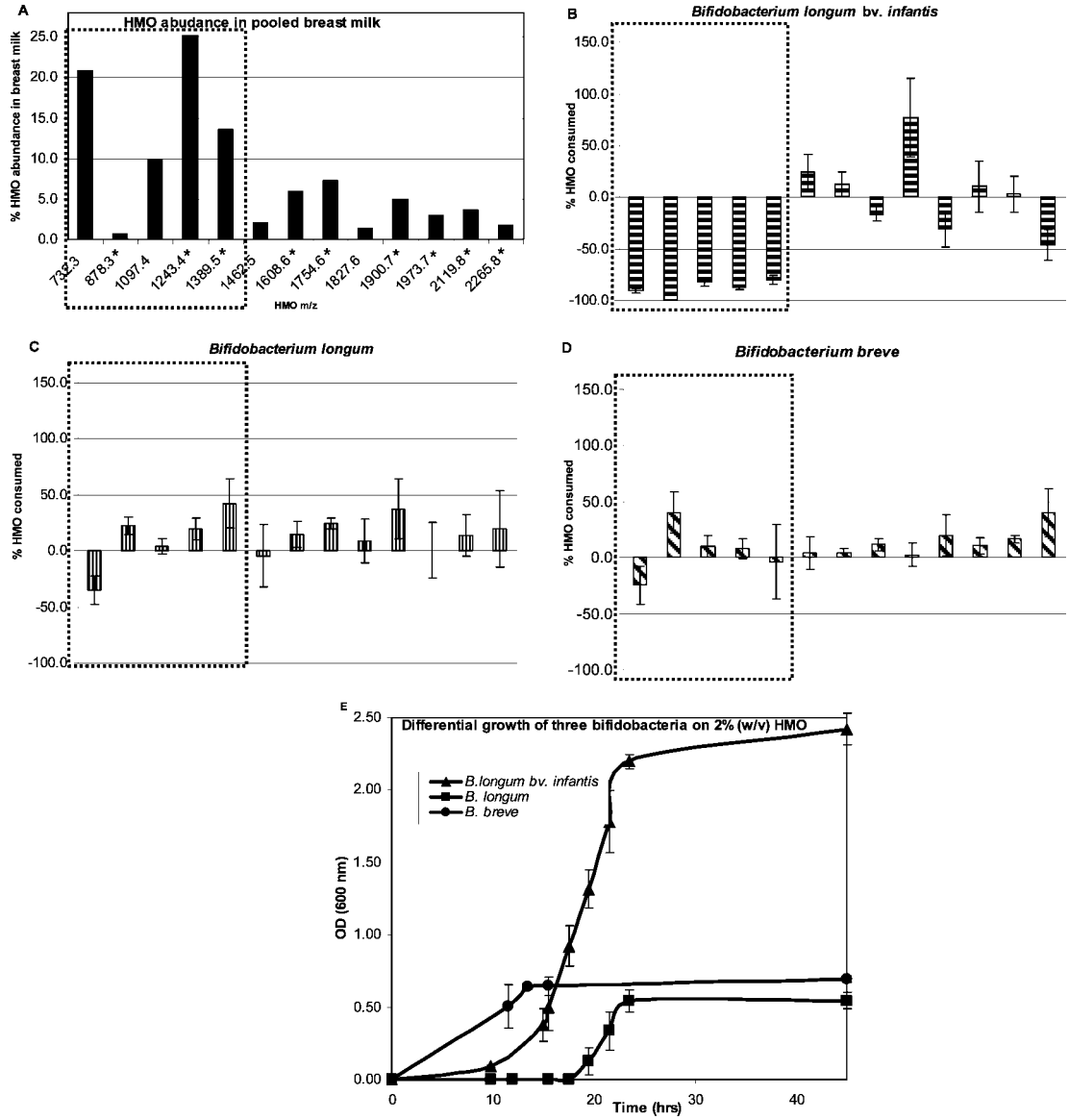

Figure 2. (**A**–**E**) HMO consumption glycoprofiling for *B. longum* bv. *infantis* ATCC 15697, *B. longum* bv. *longum* DJO10A, and *B. breve* ATCC15700. (**A**) Individual HMO abundance represented as the normalized percent contribution of each isomeric oligosaccharide species in breast milk, as measured in Ninonuevo et al. (*6*). Boxed bars are the four most abundant oligosaccharides species in the total pool of HMOs analyzed, representing 70% of the overall detectable HMO pool. * = fucosylated HMOs (see Supporting Information, Table S1, for detailed HMO compositions). NanoESI-FT-ICR (+) MS analysis of *B. longum* bv. *infantis* (**B**), *B. longum* bv. *longum* (**C**), and *B. breve* (**D**), grown on a media initially supplemented with 2.0% (w/v) HMO. This is represented as a percent difference of HMO species abundance in the media and at the end of fermentation, corresponding to $T = 0$ h and $T = 94$ h for *B. longum* bv. *infantis* and $T = 45$ h for *B. longum* bv. *longum* and *B. breve* (See Supporting Information for additional data). Measurements are triplicates of individual biological and technical replicates. (**E**) Growth curves of *B. longum* bv. *infantis* (▲), *B. longum* bv. *longum* (■), *B. breve* (●) on a semi-synthetic MRS medium supplemented with 2% (w/v) HMO. Growth was measured as OD of the media at 600 nm. Fermentations were carried out in triplicate; controls consisted of inoculated medium lacking HMO and un-inoculated medium containing HMO which was also used as a blank for OD measurements. Error bars are standard deviations of the mean for each available time point.

charide analysis. Each oligosaccharide-rich fraction was redissolved in 500 μL of deionized water, reduced using 500 μL of 2.0 M sodium borohydride in deionized water, and incubated at 42 °C for 16 h. The oligosaccharide solution was desalted and purified using the above-mentioned procedure. The dried oligosaccharide-rich fraction was reconstituted with 50 μL of deionized water and was diluted 500 times with 50% acetonitrile/water in 0.1% formic acid prior to HPLC-Chip/TOF MS analysis.

HPLC-Chip TOF MS Analysis. Oligosaccharides were analyzed using an Agilent 6200 series HPLC-Chip/TOF MS system (Santa Clara, CA) equipped with a chip consisting of a 40 nL enrichment column and a 43 × 0.75 mm i.d. analytical column, both packed with a porous graphitized carbon 5 μm stationary phase. A nanoliter pump gradient was delivered at 0.3 μL/min consisting of (A) 3.0% acetonitrile/water in 0.1% formic acid and (B) 90% acetonitrile/water in 0.1% formic acid. A 45 min LC gradient was run from 0 to 16% B, 2.5–20.0 min, 16–44% B, 20.0–30.0 min, 44–100% B, and 30.0–35.0 min with equilibration time of 20 min at 0% B. Data was acquired in the positive ionization mode with a mass range of *m*/*z* 500 to *m*/*z* 3000. Data analysis was performed using the Analyst QS 1.1 software, and the deconvoluted lists of masses were generated using the Mass Hunter (Molecular Feature Extraction) software. Oligosaccharides were identified using a Glycan Finder program (in-house) written using the Igor Pro version 5.04B software (WaveMetrics, Portland, OR).

Glycosyl Hydrolase Assays. Assayed cells were incubated anaerobically at 37 °C on semi-synthetic MRS medium (*13*), with 1% (w/v) sterile filtered lactose or HMOs as the sole carbon source. Early stationary phase cells were harvested, and 200 mg of 0.1 mm diameter

glass beads (BioSpec Products, Bartlesville, OK) were added to the suspensions which were subjected to two cycles of 45 s at a power of 6.0 m s^{-1} on a FastPrep FP120 cell disruptor (Qbiogene, Morgan Irvine, CA). The beads and cell debris were removed by centrifugation. Cell-free extracts were collected and kept temporarily on ice until the start of the enzyme assays. Protein concentrations were determined according to the Bradford method using bovine serum albumin as the standard (*15*). Activity of α-L-fucosidase (EC 3.2.1.51) and sialidase (EC 3.2.1.18) was assayed with the fluorogenic substrates, 4-methylumbelliferyl α-L-fucopyranoside, and 2′-(4-methylumbelliferyl) α-D-*N*-acetylneuraminic acid, respectively. Fucosidase activity was assayed with a fluorogenic substrate concentration of 1 mM in 0.2 M phosphate–citrate buffer (pH 5.2), while the sialidase assay employed 0.5 mM of substrate mixed in equal volume with 50 μL of 0.25 M sodium acetate–acetic acid buffer (pH 4.3). The reaction mixture containing 100 μL of cell-free extract and 100 μL of substrate solution was incubated for 1 h at 37 °C, and the reaction was stopped with 1.3 mL of 0.17 M glycine–carbonate buffer, pH 9.8. The fluorescence of enzymatically liberated 4-methylumbelliferone was determined in a Bio-Rad VersaFluor fluorometer (Eureka, CA) by excitation at 360 nm and emission measured at 460 nm. Serially diluted 4-methylumbelliferone was used to calibrate relative fluorescence units with substrate concentration. The specific activity of the glycosyl hydrolase was expressed as nanomoles of hydrolyzed substrate per milligram of protein per hour. Assays were performed in duplicates, and the standard error was reported.

RESULTS

The development of analytical methods employing a high mass accuracy and high resolution Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) in combination with deuterium-labeled HMOs as internal standards has enabled the detailed structural analysis of HMOs (*11*). This MS-based technique has been applied to monitor the changes in absolute quantities of individual neutral and fucosylated oligosaccharide species (see Supporting Information, Table S1). The mass to charge ratio (*m*/*z*) correlates to chain lengths and types of sugars present; the intensity of a particular mass indicates the extent of specific HMO consumption by the bacteria (**Figure 1**). *B. longum* bv. *longum*, *B. longum* bv. *infantis*, and *B. breve*, representative of bifidobacterial species isolated from the gut in infant and adults, were profiled for their selectivity in fucosylated and neutral HMO consumption. Glycoprofiling of *B. longum* bv. *infantis*, a predominant bifidobacterial species in the breast-fed infant colon (*3*), revealed that oligosaccharides preferably consumed by the bacteria have a degree of polymerization (DP) ≤ 7 (*m*/*z* 1389 and below) and represent the most abundant species of HMO isomers in pooled human milk—indicating a selective correspondence between what the mother secretes in milk and what this strain consumes (**Figures 2A**–**D** and **3**). Additionally, some HMOs with a DP ≥ 10 were partially consumed, in particular oligosaccharide species with *m*/*z* 1754.6 (−16.2%), 1900.7 (−30.8%), and 2265.8 (−46.2%). Overall, *B. longum* bv. *infantis* consumed 63.9% of the total HMO pool and most of these glycans were fucosylated (**Figure 2B**). Conversely, the other strains tested, *B. longum* bv. *longum* DJO10A and *B. breve* ATCC 15700, only consumed 35.2% and 24.4%, respectively, of a single, nonfucosylated/nonsialylated HMO species, lacto-*N*-tetraose (LNT) (**Figure 2C**,**D**, *m*/*z* = 732.3). While LNT is the second most abundant HMO in breast milk, its metabolism by *B. longum* bv. *longum* and *B. breve* represents only a 7.3% and 5.1% consumption of the overall detectable HMO pool, respectively.

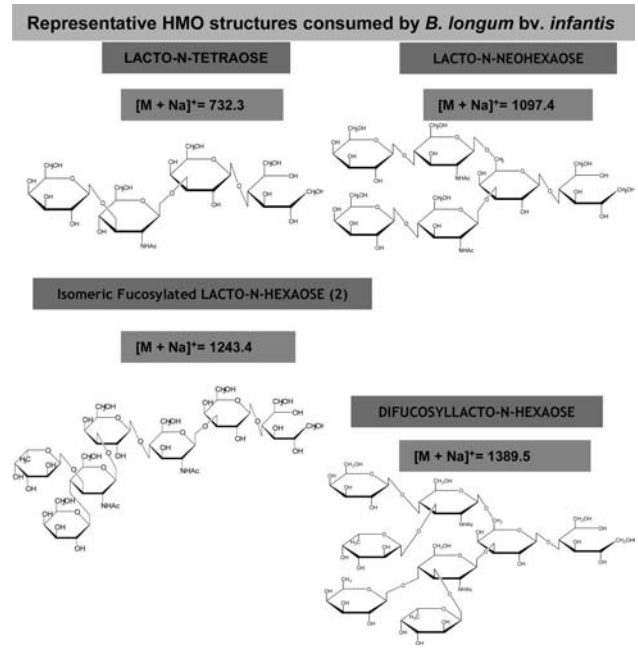

Figure 3. Structures of the most abundant HMOs consumed by *B. longum* bv. *infantis* ATCC 15697. Only selected isomers known to be present in human breast milk are represented; other isomers are listed in the Supporting Information (supplementary text and Figures 1SI–10SI).

To test whether *B. longum* bv. *infantis* preferentially consumed only HMO species with a DP ≤ 7, the consumption glycoprofiles were repeated under carbohydrate limiting conditions. This was achieved by performing growth studies in which HMO concentrations in the media were lowered to 1.6, 0.8, and 0.4% (w/v), in the latter of which only minimal growth occurred (**Figure 4a**). Upon limiting the availability of HMOs in the growth media, *B. longum* bv. *infantis* consumed almost all the available carbohydrate substrate including glycans with a DP > 7 (**Figure 4b**), which were previously only partially consumed (**Figure 2B**). This indicates that *B. longum* bv. *infantis* preferentially consumed small mass HMOs (DP ≤ 7), but it is nonetheless capable of metabolizing all of the available oligosaccharide substrates.

Catabolic capacity of these bacteria towards HMO metabolism can be also measured by monitoring the sialidase and fucosidase activities required to deconstruct these complex glycan structures. Enzymatic assays showed that *B. longum* bv. *infantis* has a 16.6- and 33.7-fold higher sialidase activity when grown on lactose as compared to *B. longum* and *B. breve*, respectively. Even though we cannot exclude a minimal/nonspecific sialidase activity in *B. longum* and *B. breve*, this data suggests that *B. longum* bv. *infantis* has an inherent and constitutive ability to process sialylated compounds. Furthermore, among the three strains tested, fucosidase activity was only present in *B. longum* bv. *infantis* and was only detected upon growth on HMO (Supporting Information, Table S2). The marked differences in the ability to consume HMO correlate with the enzymatic activities observed for the three strains, and these are clearly reflected in their growth patterns (**Figure 2E**) which demonstrate that *B. longum* bv. *infantis* has an underlying genetic component enabling its rapid growth on HMOs. Noticeable increases in the abundance of glycans with higher DP (**Figure 2B**,**C**,**D**) were interpreted to be generated by bifidobacterial neuraminidases acting on sialylated HMOs. This is supported by evidence that among the three strains tested, only *B. longum* bv. *infantis* is capable of releasing sialic acids bound to HMO (data not shown) because of its sialidase activity

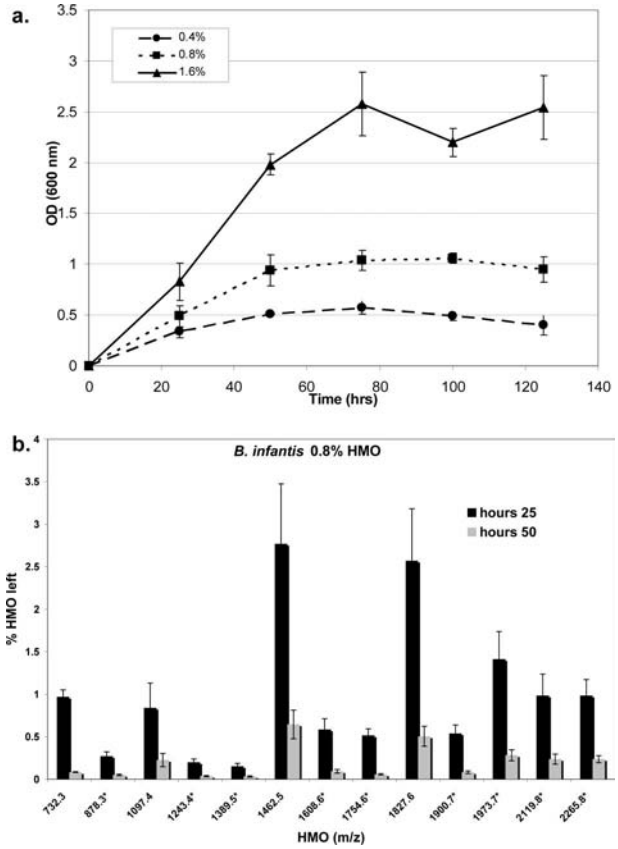

Figure 4. HMO consumption glycoprofiling for *B. longum* bv. *infantis* ATCC 15697 under substrate limiting conditions. (**a**) Growth curves of *B. longum* bv. *infantis* on a semi-synthetic MRS medium supplemented with 1.6% (▲), 0.8% (■), and 0.4% (●) (w/v) HMO. Growth was measured as OD of the media at 600 nm. Fermentations were carried out in triplicate; controls consisted of inoculated medium lacking HMO and un-inoculated medium containing HMO which was also used as a blank for OD measurements. Error bars are standard deviations of the mean for each available time point. (**b**) NanoESI-FT-ICR (+) MS analysis of *B. longum* bv. *infantis* grown on a media initially supplemented with 0.8% (w/v) HMO.

(Supporting Information, Table S2). Although there is a limited body of literature available on the possible activity of bifidobacterial glycosidases (*16*, *17*), it cannot be excluded that these enzymes can also reverse their function (*17*), thus assembling larger oligosaccharides from smaller ones (**Figure 2B**,**C**,**D**).

If short HMOs (DP ≤ 7) are the factor responsible for seeding the bifidobacteria microbiota that becomes characteristic of breast-fed infants in the first month postpartum (*18*), then these glycans would be expected to be present in breast milk throughout this period. For this purpose, we surveyed the presence of the most abundant HMOs in the breast milk of four lactating women using HPLC-Chip TOF. This employed a nanoLC separation MS technique using porous graphitized carbon as a separating column to separate structural HMO isomers (*19*). As shown in **Figure 5**, four of the HMOs consumed by *B. longum* bv. *infantis* are present immediately from the commencement and well throughout the first month of lactation. The presence of these oligosaccharides through this period of lactation and infant development thus provides the infant gut with the necessary prebiotic substrates needed to establish, maintain, and select a specific microbiota. Although fluctuations among different mothers in specific glycans were detected, generally a constant flux of small molecular weight HMOs from mother to infant is maintained.

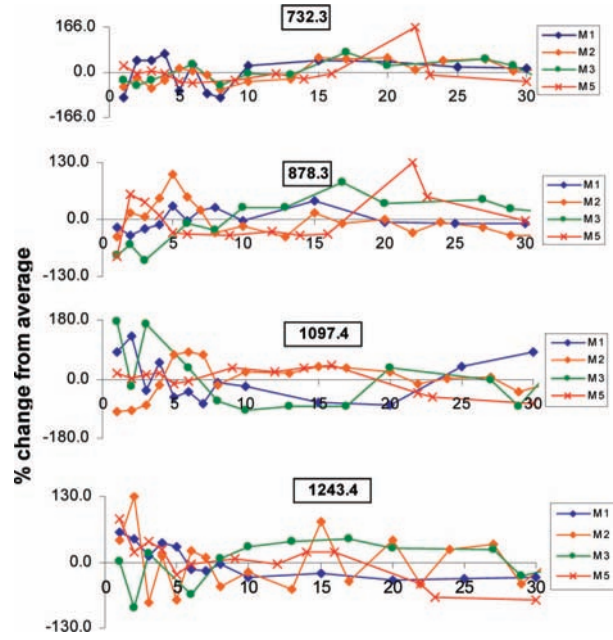

Figure 5. Flux of selected HMOs throughout one month postpartum. Abundance data was normalized with respect to the mean abundance for each HMO monitored throughout the first month of lactation in each of four respective mothers. Data is shown as the percent difference from mean value. HMOs monitored *m*/*z*: 732.3, 878.3, 1097.4, 1243.4.

DISCUSSION

The differential glycoprofiles and growth on HMOs observed for these representative bifidobacteria species (**Figure 2B**,**C**,**D**) document that growth on HMOs is not a property of all bifidobacteria. These results provide molecular and mechanistic evidence that specific strains of bifidobacteria consume selected classes of HMOs, suggesting that through this mechanism human milk and bifidobacteria have co-evolved to their mutual advantage. That is, the diversity of specific oligosaccharide structures found within human milk has evolved not to provide direct nutrition to the infant but to act as specific substrates for sustaining growth of selected beneficial bacteria that aid in the development and protection of the newborn. In the case of *B. longum* bv. *infantis*, this strain has the ability to preferentially consume the most abundant HMOs. Modern infant formulas are increasingly supplemented with plant oligosaccharides that elicit an unspecific bifidogenic response (*20*), lack the complexity and diversity of HMOs, and are therefore unlikely to successfully mimic the structure-specific effects of HMOs. Revealing the role of this key bioactive component of human breast milk will advance our understanding of the biological function of breast-feeding for sustaining infant health and potentially for providing long term benefits to human health. Defining and linking specific oligosaccharide structures to specific bacteria provides a scientific path for targeting infant health by establishing protective microbial communities, beneficial to their hosts and potentially applicable to different stages of human life and health states.

ABBREVIATIONS USED

HMO, human milk oligosaccharide; GIT, gastrointestinal tract; HPLC-Chip TOF MS, high performance liquid chromatography-chip time-of-flight.

Supporting Information Available: Complete list of HMO isomers consumed by *B. longum* bv. *infantis*, not represented

in Figure 2 (Table S1). List of HMOs detected in human breast milk. (Table S2) Enzymatic assays. (Figure 1SI–10SI) Structures of all known HMOs from pooled human milk, as profiled by nanoESI-FT-ICR (+) MS. (Figure 11SI) HMO consumption glycoprofiling for *B. longum* bv. *infantis* ATCC 15697 as measured at $T = 28$ h and 148 h. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Onis, M.; Onyango, A.; Borghi, E.; Siyam, A.; Pinol, A.; Garza, C.; De Onis, M.; Martines, J.; Martorell, R.; Victora, C. G.; Bhan, M. K.; de Onis, M.; Martines, J.; Onyango, A.; Pinol, A.; Victora, C. G.; Araujo, C. L.; Lartey, A.; Owusu, W. B.; Bhan, M. K.; Bhandari, N.; Norum, K. R.; Aa, G. E.; Mohamed, A. J.; Dewey, K. G.; Garza, C.; Belbase, K.; Black, M.; Chumles, W. C.; Cole, T.; Frongillo, E.; Grummer-Strawn, L.; Martorell, R.; Shrimpton, R.; Van den Broeck, J.; Araujo, C. L.; Victora, C. G.; Albernaz, E.; Tomasi, E.; da Silveira, R. D. F.; Nader, G.; Lartey, A.; Owusu, W. B.; Sagoe-Moses, I.; Gomez, V.; Sagoe-Moses, C.; Bhandari, N.; Bhan, M. K.; Taneja, S.; Rongsen, T.; Chetia, J.; Sharma, P.; Bahl, R.; Bjoerneboe, G. E. A.; Baerug, A.; Tufte, E.; Norum, K. R.; Rudvin, K.; Nysaether, H.; Mohamed, A. J.; Alasfoor, D.; Prakash, N. S.; Mabry, R. M.; Al Rajab, H. J.; Helmi, S. A.; Dewey, K. G.; Nommsen-Rivers, L. A.; Cohen, R. J.; Heinig, M. J. Enrolment and baseline characteristics in the WHO Multicentre Growth Reference Study. *Acta Paediatr.* **2006**, *95*, 7–15.

(2) Backhed, F.; Ley, R. E.; Sonnenburg, J. L.; Peterson, D. A.; Gordon, J. I. Host-bacterial mutualism in the human intestine. *Science* **2005**, *307* (5717), 1915–1920.

(3) Haarman, M.; Knol, J. Quantitative Real-Time PCR Assays To Identify and Quantify Fecal Bifidobacterium Species in Infants Receiving a Prebiotic Infant Formula. *Appl. Environ. Microbiol.* **2005**, *71* (5), 2318–2324.

(4) Ventura, M.; Elli, M.; Reniero, R.; Zink, R. Molecular microbial analysis of Bifidobacterium isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Microbiol. Ecol.* **2001**, *36* (2–3), 113–121.

(5) Coppa, G. V.; Gabrielli, O.; Pierani, P.; Catassi, C.; Carlucci, A.; Giorgi, P. L. Changes in Carbohydrate-Composition in Human-Milk over 4 Months of Lactation. *Pediatrics* **1993**, *91* (3), 637–641.

(6) Ninonuevo, M. R.; Park, Y.; Yin, H.; Zhang, J.; Ward, R. E.; Clowers, B. H.; German, J. B.; Freeman, S. L.; Killeen, K.; Grimm, R.; Lebrilla, C. B. A Strategy for Annotating the Human Milk Glycome. *J. Agric. Food Chem.* **2006**, *54* (20), 7471–7480.

(7) Newburg, D. S.; Ruiz-Palacios, G. M.; Altaye, M.; Chaturvedi, P.; Meinzen-Derr, J.; Guerrero, M. D.; Morrow, A. L. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology* **2004**, *14* (3), 253–263.

(8) Chaturvedi, P.; Warren, C. D.; Buescher, C. R.; Pickering, L. K.; Newburg, D. S. Survival of human milk oligosaccharides in the intestine of infants. *Bioact. Compon. Hum. Milk* **2001**, *501*, 315–323.

(9) Turnbaugh, P. J.; Ley, R. E.; Mahowald, M. A.; Magrini, V.; Mardis, E. R.; Gordon, J. I. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **2006**, *444* (7122), 1027–1031.

(10) Bode, L. Recent Advances on Structure, Metabolism, and Function of Human Milk Oligosaccharides. *J. Nutr.* **2006**, *136* (8), 2127–2130.

(11) Ninonuevo, M. R.; Ward, R. E.; LoCascio, R. G.; German, J. B.; Freeman, S. L.; Barboza, M.; Mills, D. A.; Lebrilla, C. B. Methods for the quantizations of human milk oligosaccharides in bacterial fermentation by mass spectrometry. *Anal. Biochem.* **2007**, *361* (1), 15–23.

(12) Ward, R. E.; Ninonuevo, M.; Mills, D. A.; Lebrilla, C. B.; German, J. B. In Vitro Fermentation of Breast Milk Oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri. *Appl. Environ. Microbiol.* **2006**, *72* (6), 4497–4499.

(13) Barrangou, R.; Altermann, E.; Hutkins, R.; Cano, R.; Klaenhammer, T. R. Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by Lactobacillus acidophilus. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (15), 8957–8962.

(14) Xie, Y. M.; Liu, J.; Zhang, J. H.; Hedrick, J. L.; Lebrilla, C. B. Method for the comparative glycomic analyses of O-linked, mucin-type oligosaccharides. *Anal. Chem.* **2004**, *76* (17), 5186–5197.

(15) Bradford, M. M. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72* (1–2), 248–254.

(16) Splechtna, B.; Nguyen, T. H.; Steinbock, M.; Kulbe, K. D.; Lorenz, W.; Haltrich, D. Production of prebiotic galacto-oligosaccharides from lactose using beta-galactosidases from Lactobacillus reuteri. *J. Agric. Food Chem.* **2006**, *54* (14), 4999–5006.

(17) Tzortzis, G.; Goulas, A. K.; Gibson, G. R. Synthesis of prebiotic galactooligosaccharides using whole cells of a novel strain, Bifidobacterium bifidum NCIMB 41171. *Appl. Microbiol. Biotechnol.* **2005**, *68* (3), 412–416.

(18) Favier, C. F.; Vaughan, E. E.; De Vos, W. M.; Akkermans, A. D. L. Molecular monitoring of succession of bacterial communities in human neonates. *Appl. Environ. Microbiol.* **2002**, *68* (1), 219–226.

(19) Packer, N. H.; Lawson, M. A.; Jardine, D. R.; Redmond, J. W. A general approach to desalting oligosaccharides released from glycoproteins. *Glycoconjugate J.* **1998**, *15* (8), 737–747.

(20) Fanaro, S.; Boehm, G.; Garssen, J.; Knol, J.; Mosca, F.; Stahl, B.; Vigi, V. Galacto-oligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas: A review. *Acta Paediatr.* **2005**, *94*, 22–26.

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