Physiology of Consumption of Human Milk Oligosaccharides by Infant Gut-associated Bifidobacteria

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The bifidogenic effect of human milk oligosaccharides (HMOs) has long been known, yet the precise mechanism underlying it remains unresolved. Recent studies show that some species/subspecies of Bifidobacterium are equipped with genetic and enzymatic sets dedicated to the utilization of HMOs, and consequently they can grow on HMOs; however, the ability to metabolize HMOs has not been directly linked to the actual metabolic behavior of the bacteria. In this report, we clarify the fate of each HMO during cultivation of infant gut-associated bifidobacteria. Bifidobacterium bifidum JCM1254, Bifidobacterium longum subsp. infantis JCM1222, Bifidobacterium longum subsp. longum JCM1217, and Bifidobacterium breve JCM1192 were selected for this purpose and were grown on HMO media containing a main neutral oligosaccharide fraction. The mono- and oligosaccharides in the spent media were labeled with 2-anthranilic acid, and their concentrations were determined at various incubation times using normal phase high performance liquid chromatography. The results reflect the metabolic abilities of the respective bifidobacteria. B. bifidum used secretory glycocidas to degrade HMOs, whereas B. longum subsp. infantis assimilated all HMOs by incorporating them in their intact forms. B. longum subsp. longum and B. breve consumed lacto-N-tetraose only. Interestingly, B. bifidum left degraded HMO metabolites outside of the cell even when the cells initiate vegetative growth, which indicates that the different species/subspecies can share the produced sugars. The predominance of type 1 chains in HMOs and the preferential use of type 1 HMO by infant gut-associated bifidobacteria suggest the coevolution of the bacteria with humans.

Human milk contains between 10 and 20 g/liter of oligosaccharides (the degree of polymerization (DP) ≥3) as the third most abundant solid component after lactose and lipids (1). These oligosaccharides are collectively termed human milk oligosaccharides (HMOs). HMOs are characterized by their complex structures. About 200 molecular species have been detected using a microfluidic chip separation coupled to mass spectrometry (2), and among them, 115 structures have been determined so far (3). HMOs have been classified into 13 core structures that consist of lactose, at the reducing end, elongated by β1–3-linked lacto-N-biose (Galβ1–3GlcNAc, LNB, type 1 chain) and/or β1–3/6-linked N-acetyllactosamine (Galβ1–4GlcNAc, LacNAc, type 2 chain) (4, 5). These core structures are frequently modified by fucose and sialic acid residues via α1–2/3/4 and α2–3/6 linkages, respectively. The unique feature of HMOs is the predominance of type 1 chains, and such a composition has not been observed in milk oligosaccharides from other mammals, including anthropoids (Ref. 3 and references therein).

HMOs are resistant to gastrointestinal digestion in host infants, and thus the majority of HMOs reach the colon (6, 7), where they may serve as prebiotics to shape a healthy gut ecosystem by stimulating the growth of beneficial microorganisms and by acting as receptor analogues to inhibit the binding of various pathogens and toxins to epithelial cells (5, 8–10). The rapid and predominant colonization of a breast-fed infant intestine by bifidobacteria has long been known in pediatrics, and the selective growth of bifidobacteria has been attributed to HMOs (11, 12). Several groups have revealed through genetic or enzymatic approaches that particular infant gut-associated bifidobacteria have dedicated pathways to assimilate HMOs (the details are described later) (13–16). However, our overall understanding of what structures of HMOs exert bifidogenic effects, and how, remains incomplete.

Ward et al. (17) and LoCascio et al. (18) examined the in vitro fermentation abilities of several bifidobacteria on HMOs and their degradation products.
analyzed the oligosaccharide compositions in spent media. The results showed that *Bifidobacterium longum* subsp. *infantis* ATCC15697 (same as JCM1222 used in this study) can grow vigorously in the presence of HMOs acting as carbon sources, whereas *Bifidobacterium bifidum* ATCC29521 (JCM1255), *Bifidobacterium breve* ATCC15700 (JCM1192), and *B. longum* subsp. *longum* ATCC15707 (JCM1217) did not grow. *B. longum* subsp. *infantis* ATCC15697 preferentially consumed HMOs with DP ≤7 during the incubation period, whereas the other species showed very limited utilization of HMOs (only a 20–30% decrease of an oligosaccharide with an *m/z* 732.3, which corresponds to lacto-N-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LNT) or lacto-N-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc, LNNT), was observed for *B. breve* and *B. longum* subsp. *longum*). More recently, Marcobal et al. (19) used a chemically synthesized medium to show that *B. longum* subsp. *infantis* ATCC15697 can utilize HMOs with DP up to 12. Sela et al. (20) demonstrated that *B. longum* subsp. *infantis* ATCC15697 can use sialylated HMOs as carbon sources. In all of these studies, matrix-assisted laser desorption/ionization-Fourier transform ion cyclotron resonance mass spectrometry was employed for analyzing the consumption of HMOs. This method is superior because of its high sensitivity and high throughput capacity; however, MALDI-Fourier transform ion cyclotron resonance MS is not suitable for the quantification of each HMO, and the technique cannot distinguish between several important isomers contained in HMOs, e.g. LNT versus LNNT and lacto-N-difucohexaose I (LNDFH I) (Fucα1-2Galβ1-3(Fucα1-4GlcNAcβ1-3Galβ1-4Glc) versus lacto-N-difucohexaose II (LNDFH II) (Galβ1-3(Fucα1-4)-GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc). In addition, the use of the matrix may interfere with the measurement of low molecular compounds, and therefore the approach may not be suitable for the detection of HMOs with DP = 3 and the degradation products (mono- and disaccharides) of HMOs during cultivation. This particular drawback is apparent in the recent reports where the presence of trisaccharides 2'-fucosyllactose (Fucα1-2Galβ1-4Glc, 2'-FL) and 3-fucosyllactose (Galβ1-4(Fucα1-3)Glc, 3-FL) are not described. 2'-FL is the most abundant HMOs unless the milk is derived from nonsecretor subjects (5, 21).

In this report, we have conducted refined HPLC profiling of HMO consumption by the infant gut-associated bifidobacteria, in which the concentrations of the main fractions of HMOs (DP of 3–6) and the degraded intermediates (mono- and disaccharides) were determined. These results were then interpreted with respect to the metabolic functionalities of the bifidobacteria. The physiology of HMO metabolism in each bifidobacterial strain is also discussed in detail. This is the first study that unequivocally reveals the fate of each tested HMO during bacterial fermentation and the results indicate that bifidobacterial species/subspecies share the degraded products.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**The standard human milk oligosaccharides 2'-FL, 3'-FL, LNT, LNNT, lacto-N-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LNFPI), LNFPII (Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc), LNFPIII (Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc), LNDFH I, and LNDFH II were purchased from Dextra Laboratory (Reading, UK). Lactodifucotetraose (Fucα1-2Galβ1-4(Fucα1-3)Glc, LDFT) was synthesized by introducing an α1-3-fucosyl linkage into the Glc residue of 2'-FL using 1,3,4,6-tetra-β-D-glucopyranosyl fluoride. LNB was synthesized as described previously (22). Lacto-N-triose II (GlcNAcβ1-3Galβ1-4Glc) was prepared by hydrolyzing LNaNT by β-galactosidase (23), and the triose was subsequently purified using Toyopearl HW-40F (Tosoh, Tokyo, Japan). Isomaltopentaose (IG5) was obtained from Seikagaku Kogyo (Tokyo, Japan). Lactose monohydrate (Galβ1-4Glc, Lac) was from Kanto Chemical (Tokyo, Japan). L-Fucose (Fuc) and N-acetylgalcosamine (GlcNAc) were from Nacalai Tesque (Kyoto, Japan), and glucose (Glc) and boric acid were from Kishida Kagaku (Osaka, Japan). Galactose (Gal), 2-anthraniolic acid, and sodium acetate were purchased from Wako Pure Chemicals (Osaka, Japan). LacNac, Lewis a/x tri-saccharides (Galβ1-3/4(Fucα1-4/3)GlcNAc) and sodium cyannoborohydride (grade, 95%) were from Sigma. The purities of the mono- and oligosaccharides other than LNB, lacto-N-triote II, LDFT, and LNFPIII were certified to be >95% by the manufacturers. The purities of LNB, lacto-N-triote II, LDFT, and LNFPIII were checked (>95%) using an HPLC equipped with a charged aerosol detector (Corona CAD, MA). All other reagents were analytical grade.

**Preparation of Oligosaccharides from Human Milk—**Fifty seven healthy Japanese mothers who had not taken any antibiotics for 1 month were recruited from several midwife clinics in Kyoto Prefecture between July and September 2008. They were 32.7 ± 4.3 years old (mean ± S.D.), with a range of 25–40 years of age, and had given birth to infants at term (gestational age of 36–41 weeks). The mothers and their babies had no health issues. Breast milk samples were collected in sterile polypropylene tubes at 74.9 ± 27.2 days after delivery with a range of 30–120 days. The samples (900 ml in total) were combined and mixed with equal volumes of chloroform and methanol and stirred vigorously for 1 h at 25 °C to remove proteins and lipids. After standing for 16 h at 25 °C, the upper layer (~1.6 liters) was collected and concentrated to ~800 ml by evaporation. The solution was centrifuged, and the supernatant was lyophilized to give 59.6 g of solid materials that contained mainly carbohydrates. The crude carbohydrate preparation was dissolved in 400 ml of distilled water, and aliquots (20 ml) were applied every 60 min for a total of 20 loadings to a Toyopearl HW-40F gel filtration column (inner diameter, 5 × 80 cm) (Tosoh). The elution was carried out using water at a flow rate of 10 ml/min. Fractions containing neutral oligosaccharides were collected and combined as a pool of human milk oligosaccharides. In some cases, fractions contained both lactose and trisaccharides. These fractions were separately collected, concentrated by lyophilization, and rechromatographed to remove the lactose. During the steps, almost all lactose (>99.5%) was removed. The purified fractions were combined with the pool and then lyophilized to give a final preparation of human milk oligosaccharides (4.74 g). Note that the sialyl oligosaccharides were eluted near
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the void fractions as polymeric compounds (24) and therefore were not contained in this preparation, as confirmed by the periodate-resorcinol method (25). The structures of the neutral oligosaccharides analyzed in this study are listed in Table 1.

Bacterial Strains and Growth Conditions—Bacterial strains used in this study were B. bifidum JCM1254, JCM1255\(^1\), and JCM7004, B. breve JCM1192\(^1\), B. longum subsp. infantis JCM1221\(^1\), and B. longum subsp. longum JCM1217\(^1\). All strains were purchased from the Japan Collection of Microorganisms.

Bididobacteria were anaerobically grown at 37 °C in GAM medium (Nissui Pharmaceutical, Tokyo, Japan) or in basal medium consisting of 0.2% yeast extract, 1.0% peptone, 0.5% sodium acetate, 0.2% diammonium citrate, 0.02% magnesium sulfate, 0.2% potassium hydrogen phosphate, 0.1% cysteine hydrochloride, and 1% sugar (Fuc, Gal, Glc, GlcN, Lac, LNB, or HMOs). The basal medium was supplemented with 4% reducing reagent (2% cysteine hydrochloride and 11% sodium carbonate) prior to the inoculation of the bacteria. The medium was equilibrated before the next sample injection.

For the analysis of HMO consumption, the samples (0.5 ml) were mixed with 100 \(\mu\)l of the labeling reagent and 30 \(\mu\)l of the bacterial culture supernatant from 0 to 100% B for 90 min and then 0% B for 15 min to allow the column before the next sample injection. Labeled oligosaccharides were detected with excitation and emission wavelengths of 360 and 425 nm, respectively. The standard curve for each oligosaccharide was created in triplicate experiments using a solution containing both the internal standard (1 mg/ml) and an oligosaccharide with varying concentrations (0.125, 0.25, and 0.5 mg/ml for LNDFH II, 0.25, 0.5, and 1 mg/ml for the other saccharides). The peak areas of the saccharides were standardized by comparing them with that of the internal standard, and the corrected values were used for the calculations. The data represent means of two independent determinations.

Molecular Cloning of the \(gltA\) (GLBP) Genes from B. bifidum JCM1254, JCM1255, and JCM7004—The \(gltA\) genes encoding galacto-N-biose (GBN)/lacto-N-biose I (LNB)-binding proteins (GLBPs) were amplified by high fidelity PCR involving PrimeStar MAX DNA polymerase (Takara Bio, Shiga, Japan) using the genomic DNAs of B. bifidum JCM1254, JCM1255, and JCM7004 as the templates. The primers used were 5’-CACGACGAGGGGACAACTTTCC-3’ and 5’-GTGGTTGGGATGAGGAGAAA-3’. The amplified fragments were inserted into pMW118 (Nippon Gene, Tokyo, Japan) for sequencing. PCR was done in two separate tubes for each \(gltA\) gene, and the Sequences of the two independently amplified fragments were determined. The nucleotide sequences of the \(gltA\) genes from JCM1254, JCM1255, and JCM7004 were deposited in GenBank under the accession numbers of JF332149, JF332150, and JF332151, respectively.

Immunoblotting—Anti-GLBP antibodies were prepared by immunization of rabbits with the purified recombinant GLBP (29). The immunoglobulin G fraction was purified by protein A-Sepharose CL-6B column chromatography (GE Healthcare). Immunoblotting was performed as described previously (30) with slight modifications. In brief, the bifidobacterial cells were grown in the basal media containing lactose as a carbon source, harvested by centrifugation, suspended in 100 mM sodium phosphate buffer (pH 7), and disrupted by sonication. The cell-free extracts (10 \(\mu\)g) were subjected to SDS-PAGE, and subsequently electroblotted to polyvinylidene fluoride membrane (Millipore). Anti-GLBP antibodies and anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibodies (from donkey) (GE Healthcare) were used in 3,000- and 2,000-fold dilutions, respectively. The ECL chemiluminescent detection agent (GE Healthcare) was used to visualize the cross-reaction.

Enzyme Assay—HMO degrading activities were examined using 2’-FL, Lewis a/x, Lac, LNT, LNR, and lacto-N-tetraose II as the substrates to detect the presence of 1,2-α-L-fucosidase, 1,3-1,4-α-L-fucosidase, β-galactosidase (lactase), lacto-N-biosidase, β-galactosidase (specific for type 2 chains), and β-N-acetyltetraosaminidase, respectively. The bifidobacterial cells were grown in basal medium containing lactose as a carbon source, harvested by centrifugation, suspended in 50 mM sodium phosphate buffer (pH 6.5), and disrupted by sonication. The cell-free extracts were ultracentrifuged at 100,000 \(\times\) g for 60 min to separate the cytoplasmic fractions from the cell wall fractions. Each fraction (250 \(\mu\)g) was incubated with 2 mM substrates in a total volume of 10 \(\mu\)l at 37 °C for 30 min (B. bifidum and B. longum subsp. infantis) or 3 h (B. longum subsp. longum...
and *B. breve*) to determine the localization of the enzymes. Lacto-N-biosidase activity of *B. longum* subsp. *longum* was lost when the cells were disrupted by sonication, and therefore, the intact cell suspension was regarded as the cell wall fraction. The reaction products were analyzed by thin layer chromatography (TLC) using a silica gel 60 aluminum sheet (Merck). The sugars were visualized as described previously (23).

**Ethical Consideration**—This study was reviewed and approved by the Ethics Committee of the University of Shiga Prefecture, and it followed the Declaration of Helsinki. Informed consent was obtained from all donors.

**RESULTS**

**Preparation and Analysis of HMOs**

As mentioned above, HMOs are classified into 13 core structures, and the core structures are frequently modified by fucose and sialic acid via α1–2/3/4 and α2–3/6 linkages, respectively. The final HMOs prepared in this study contained neutral oligosaccharides with DP between 3 and 8 and appeared not to contain larger oligosaccharides and acidic (sialylated) oligosaccharides that are known to represent minor components (~10% of the total HMOs) (31). The purified HMOs were added to the medium at a concentration of 1%, and their consumptions by bifidobacteria were monitored using normal phase HPLC. Fig. 1 shows the HPLC profiles of mono- and oligosaccharides in the spent media of *B. bifidum* JCM 1254 at different times. The main HMOs were successfully separated under the conditions employed, with the exception of LNFP II and III. At 0 h, monosaccharides (Fuc, Gal, and Glc), Lac (small contaminants derived from the HMO preparation step) and oligosaccharides with DP of 7 and 8 (not identified) were also detected as minor peaks. Table 1 presents the list of oligosaccharides analyzed in this study and also shows their initial concentrations in the culture. The total oligosaccharide concentration was estimated to be 10.66 g/liter, which is consistent with the medium composition (1% carbon source). The concentrations of the respective oligosaccharides and the ratios between them were not significantly different from those reported previously (31) and may reflect the lactation periods of the donors (74.9 ± 27.2 days) (24, 32, 33).

**Strain Description**

In the genus *Bifidobacterium*, *B. bifidum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. breve* are frequently isolated from the feces of breast-fed infants (11, 34). We used *B. bifidum* JCM1254, *B. longum* subsp. *infantis* JCM1222, *B. longum* subsp. *longum* JCM1217, and *B. breve* JCM1192 strains to evaluate how these infant gut-associated bifidobacteria consume HMOs. They are the type strains of their respective species/subspecies except for *B. bifidum*. The type strain of *B. bifidum* (JCM1255) was found not to possess the functional GNB/LNB transporter (described later), whereas all of the other bifidobacterial strains examined had the transporters, as shown by Western blot analysis using anti-GLBP antibodies (Table 2).

![Image](image-url)

**TABLE 1**

**Structures of oligosaccharides and their initial concentrations in the culture medium**

| Oligosaccharide | Structure | g/liter | Concentrationa | unit |
|-----------------|----------|---------|----------------|------|
| *Lac* | Galβ1–4Glc | 0.45 ± 0.04 | 1.25 ± 0.11 |
| 2′-FL | Fucβ1–2Galβ1–4Glc | 1.48 ± 0.15 | 3.03 ± 0.31 |
| 3-FL | Galβ1–4(Fucβ1–3)Glc | 1.40 ± 0.15 | 2.86 ± 0.30 |
| LDFT | Fucβ1–2Galβ1–4(Fucβ1–3)Glc | 0.80 ± 0.11 | 1.27 ± 0.17 |
| LNT | Galβ1–3GlcNAcβ1–3Galβ1–4Glc | 1.11 ± 0.13 | 1.57 ± 0.18 |
| LNOrT | Galβ1–4GlcNAcβ1–3Galβ1–4Glc | 0.32 ± 0.06 | 0.45 ± 0.08 |
| LNFP I | Fucβ1–2Galβ1–3GlcNAcβ1–3Galβ1–4Glc | 0.67 ± 0.07 | 0.78 ± 0.08 |
| LNFP II | Fucβ1–2Galβ1–3(Fucβ1–4)GlcNAcβ1–3Galβ1–4Glc | 1.78 ± 0.19 | 2.09 ± 0.22 |
| LNFP II + III | Fucβ1–2Galβ1–3(Fucβ1–4)GlcNAcβ1–3Galβ1–4Glc (LNFP II) | 2.53 ± 0.25 | 2.53 ± 0.25 |
| LNDFH I | Fucβ1–2Galβ1–3(Fucβ1–4)GlcNAcβ1–3Galβ1–4(Fucβ1–3)Glc | 0.12 ± 0.01 | 0.12 ± 0.01 |
| LNDFH II | Galβ1–3(Fucβ1–4)GlcNAcβ1–3Galβ1–4(Fucβ1–3)Glc | 10.66 ± 0.81 |

*The abbreviations used is as follows: Lac, lactose.
* Values represent mean ± S.D. of the data obtained from duplicate measurements of the four different bacterial cultures at 0 h.*
and supplemental Fig. S1A). The sequence analysis revealed that the dysfunction of GLBP in B. bifidum JCM1255 is because of a frameshift mutation within the C-terminal region, which was caused by the duplication of a 17-bp sequence in its gene (supplemental Fig. S1B). Given the results, strain JCM1254 was employed in this study as the representative of B. bifidum. The intact GLBP genes (gltA) are also found in B. bifidum JCM7004, S17, and PRL2010 (supplemental Fig. S1) (16, 35).

The occurrence and localization of HMO-degrading enzymes in each bifidobacterial strain are summarized in Table 2 and supplemental Fig. S2. B. bifidum JCM1254 secretes 1,2-α-L-fucosidase and 1,3-1,4-α-L-fucosidase to unmask the core structures of HMOs. The core structure with the type 1 chain, e.g. LNT, is hydrolyzed by an extracellular lacto-N-biosidase to produce LNB and Lac (supplemental Fig. S2). The liberated LNB is subsequently incorporated into cells by the GNB/LNB transporter and enters the GNB/LNB pathway (described below) (22, 36). Lac may be hydrolyzed by an extracellular β-galactosidase into Gal and Glc (23) or directly transported into the cells by a yet unidentified Lac transporter. The type 2 core structure is considered to be sequentially hydrolyzed by extracellular β-galactosidase and β-N-acetylhexosaminidase. For example, LNnT is first degraded into Gal and lacto-N-triose II, and the latter is subsequently cleaved to GlcNAc and Lac (supplemental Fig. S2) (23). In contrast to B. bifidum, which has a repertoire of extracellular enzymes, B. longum subsp. infantis JCM1222 is equipped with intracellular enzymes that degrade HMOs (14). The organism possesses a large gene cluster (43 kb), the so-called HMO cluster-1, in its genome, and the cluster contains the homologous genes of 1,2-α-L-fucosidase (Blon_2335), 1,3-1,4-α-L-fucosidase (Blon_2336), β-galactosidase (Blon_2334), and β-N-acetylhexosaminidase (Blon_2335). These genes encode enzymes that do not have signal peptides at the N termini and anchoring motifs at the C termini, and to reflect these sequential motifs, all enzymatic activities were detected in the cytoplasmic fraction (supplemental Fig. S2). The cluster also contains various ABC transporter genes that are thought to be involved in importing HMOs (14). In addition, B. longum subsp. infantis JCM1222 has a GNB/LNB transporter (supplemental Fig. S1A) at a locus different from the HMO cluster-1 (14). The strain does not possess a homolog of lacto-N-biosidase, and accordingly, the activity was not detected. B. longum subsp. longum JCM1217 and B. breve JCM1192 did not exhibit α-L-fucosidase activities; however, the activities of β-galactosidase, which can liberate Gal from both LNT and LNnT, and β-N-acetylhexosaminidase, which liberates GlcNAc from lacto-N-triose II, were detected in their cytoplasmic fractions. Both of the strains could express the functional GNB/LNB transporter, as their cell-free extracts specifically cross-reacted with the anti-GLBP antibodies (supplemental Fig. S1A). B. longum subsp. longum JCM1217 expressed a secretory lacto-N-biosidase, whereas B. breve JCM1192 did not show this activity (supplemental Fig. S2) (15).

**Growth of Bifidobacteria on HMOs**

Fig. 2 shows the growth curves of the bifidobacteria in the presence of HMOs as the carbon sources. B. lactis subsp. infantis JCM1222 grew fast, and the cell density reached a maximum (A600 > 2) within 10 h of incubation. B. bifidum JCM1254 also exhibited significant growth and entered a stationary phase after 15 h. In contrast, B. longum subsp. longum JCM1217 and B. breve JCM1192 did not show considerable growth, and the A600 of their cultures did not exceed 0.3; however a slight increase of the A600 was observed for both strains. Note that all of the strains grew well when Glc was added as a carbon source (supplemental Table S1), although no growth was observed in the absence of sugars.

**Utilization of HMOs by Each Bifidobacterial Strain**

Samples of each bifidobacterial strain grown on HMOs were withdrawn at different times, and the mono- and oligosaccharide concentrations in the supernatants were determined. The
saccharide concentrations were plotted as a function of the incubation period for *B. bifidum* JCM1254 (Figs. 1 and 3), *B. longum* subsp. *infantis* JCM1222 (Fig. 4 and supplemental Fig. S3), *B. longum* subsp. *longum* JCM1217 (Fig. 5 and supplemental Fig. S4), and *B. breve* JCM1192 (Fig. 6 and supplemental Fig. S5).

*B. bifidum* JCM1254—The concentrations of 2'-FL as well as LNT rapidly decreased during the early logarithmic phase (5 h) with a concomitant increase of LNB and Lac. 2'-FL, LNB, and LNT had disappeared by the mid-exponential phase (10 h), although trace amounts of Lac remained. The concentrations of LDFT, LN\(_r\)T, LNFP I, LNFP II + III, LNDFH I, and LNDFH II gradually decreased during the early to mid-exponential phase periods. The concentration of 3-FL slightly increased by the mid-logarithmic phase and then decreased. All oligosaccharides were completely consumed after 25 h of incubation. At this time, the cells had ceased growing. The concentrations of Fuc and Gal increased and remained unchanged during the growth, whereas Glc was found to increase by the mid-exponential phase and then decreased by the end of the growth period. GlcNAc was not detected throughout the culturing period.

*B. longum* subsp. *infantis* JCM1222—The concentrations of 2'-FL, 3-FL, LDFT, LNT, LN\(_r\)T, LNFP I, LNFP II + III, LNDFH I, and LNDFH II rapidly decreased when the cells entered the logarithmic phase (3 h), and negligible levels were detected by the mid-exponential phase (6.5–7.5 h). A transient increase was observed for Lac, Fuc, Gal, and Glc at the mid-exponential phase with peak values at 5.5 h. The majority of the mono- and oligosaccharides were consumed by the end of the growth period (12.5 h). GlcNAc and LNB were not detected at any incubation times.
growth of select bifidobacteria in the gut of infants (3, 14). How-
quently, this observation indicates that HMOs support the
ess the metabolic capabilities to degrade HMOs (38). Conse-
zymatic studies indicate that particular bifidobacteria pos-
infants even thought they are included as the third most abun-
human milk. HMOs have apparently no nutritional value for
ically decrease after weaning, and the gut microflora becomes
of occupancy varies in different studies (11, 34). The population
bacteria dominate the infant gut ecosystem, although the ratio
infant, and bacteria, and it is commonly accepted that bifido-

FIGURE 6. Changes in the concentrations of mono- and oligosaccharides in the culture of B. breve JCM1192 grown in HMO medium. The samples were taken at the indicated times and analyzed. The symbols used are the same as described in the legend of Fig. 3.

B. longum subsp. longum JCM1217 and B. breve JCM1192—
In the spent media of B. longum subsp. longum JCM1217, a
rapid decrease in the concentration of LNT was observed from
0 to 11 h, with a concomitant increase in the concentration of
Lac. The other oligosaccharides remained rather constant
during the cultivation period, although a slow and slight decrease
of LNFP I occurred. GlcNAc and LNB were not detected, and
the Fuc, Gal, and Glc concentrations were at the lowest levels
throughout the cultivation period. In the culture of B. breve
JCM1192, the LNT concentration also immediately decreased
between 0 and 9 h of incubation. The concentrations of the
other oligosaccharides did not change during the cultivation
except for a decrease of the Lac concentration. Fuc, Gal, Glc,
GlcNAc, and LNB were at negligible levels throughout the
growth period.

DISCUSSION

Intestinal microflora of breast-fed infants is formed as a
result of a tripartite relationship between mother’s milk, the
infant, and bacteria, and it is commonly accepted that bifido-
bacteria dominate the infant gut ecosystem, although the ratio
of occupancy varies in different studies (11, 34). The population
of bifidobacteria in a gut ecosystem has been found to drasti-
cally decrease after weaning, and the gut microflora becomes
adult-like, indicating the presence of bifidogenic compounds in
human milk. HMOs have apparently no nutritional value for
infants even thought they are included as the third most abun-
dant component in human milk (8–10). Recent genetic and
enzymatic studies indicate that particular bifidobacteria pos-
se the metabolic capabilities to degrade HMOs (38). Conse-
sequently, this observation indicates that HMOs support the
growth of select bifidobacteria in the gut of infants (3, 14). How-
ever, the structural diversity of HMOs and the lack of a simple
and reliable method for quantifying HMOs and their metabo-
lites (mono- and disaccharides) hamper a comprehensive
understanding of the consumption of HMOs by bifidobacteria.
In this study, using a normal phase HPLC approach with a pre-
column labeling method, we have elucidated how each of the
main neutral HMO components is consumed by the infant gut-
associated bifidobacteria.

Procedure for Glycoprofiling—A method for the quantitation
of bacterial consumption of HMOs was first developed by Ninonuevo et al. (2) and LoCascio et al. (18). In that method,
HMOs in the control media and in the spent media were
reduced with NaBH₄ and NaBD₄, respectively, mixed in equal
volumes, and then subjected to MALDI-Fourier transform ion
cyclotron resonance MS analysis. Quantitation of each HMO
was carried out by comparing the deuterium/hydrogen ratio of
each peak in the mass spectrum, and the values were expressed
as the percentage consumed. This method is very simple and
robust, but it is not a suitable quantification approach and can-
not differentiate between the glycosyl linkage isomers. In addi-
tion, mono- to trisaccharides are difficult to analyze, perhaps
because of matrix interference. Conversely, we have recently
developed the quantification method of HMOs using a normal
phase HPLC method with pre-column labeling with 2-amino-
pyridine or 1-methyl-3-phenyl-5-pyrazolone (39, 40). Reverse
phase HPLC using 2-anthranilic acid as the labeling reagent is
also found to be useful in the analysis of HMOs (28). The advan-
tages of these methods are the high sensitivity toward sugars
(regardless of their sizes) and the good separation of isomers. In
this study, we selected normal phase HPLC and pre-column
labeling with 2-anthranilic acid because the fluorescence inten-
sities of the labeled standard mono- to hexasaccharides were
found to linearly increase as a function of their concentrations
(data not shown). The effectiveness of this method is validated
in the results presented in Table 1 and Fig. 1.

Physiology of HMOs Degradation in Bifidobacteria—The
presence of the metabolic pathway dedicated to the HMO
assimilation was first described on B. longum subsp. longum
JCM1217 and B. bifidum JCM1254 (13). The pathway (the
GNB/LNB pathway) involves the five proteins/enzymes
required for the uptake and subsequent degradation of LNB (a
building block of type 1 HMOs), i.e. the GNB/LNB transporter,
GNB/LNB phosphorylase, N-acetylhexosamine 1-kinase,
UDP-glucose-hexose-1-phosphate uridylyltransferase, and
UDP-galactose epimerase (37). In addition to this pathway, B.
bifidum JCM1254 possesses extracellular lacto-N-biosidase,
1,2-α-L-fucosidase, 1,3–1,4-α-L-fucosidase, sialidase, β-galac-
tosidase, and β-N-acetylhexosaminidase (Table 2 and supple-
mental Fig. S2) (15, 23, 41–43). Using these enzymes, the orga-
nism can degrade a range of HMOs. Recent reports on the
genomic sequences of the two B. bifidum strains PRL2010 and
S17 revealed that they are also equipped with the same repeto-
ire of enzymes as B. bifidum JCM1254 (16, 35), suggesting the
conservation of these enzymes in this species. The type strain
of B. bifidum JCM1255, however, lacks the functional GLBP (sup-
plemental Fig. S1). The importance of the GNB/LNB trans-
porter for the assimilation of HMOs can be inferred from the
paper of Turroni et al. (16). They showed by comparative
Glycoprofiling of HMO Degradation by Bifidobacteria

Genomic analysis that the absence of BBPR_1056 (an integral membrane protein of the GNB/LNB transporter) correlates with a reduced growth on mucin (a good source of GNB). Indeed, B. bifidum JCM1255 did not show significant growth on HMOs (17), whereas robust growth was observed for JCM1254 (Fig. 2) and PRL2010 (16). B. longum subsp. longum JCM1217 possesses extracellular lacto-N-biosidase and intracellular β-galactosidase and β-N-acetylgalactosaminidase in addition to the GNB/LNB pathway, but it does not appear to have α-1-fucosidase (Table 2 and supplemental Fig. S2). Lacto-N-biosidase activity of B. longum subsp. longum is strain-dependent as described previously (15), but intracellular β-galactosidase and β-N-acetylgalactosaminidase could be commonly present in this subspecies as B. longum subsp. longum NCC2705 and DJO10A have the corresponding genes (44, 45). Sela et al. (14) found that the genome of B. longum subsp. infantis ATCC15697 (JCM1222) contains, in addition to the genes for the GNB/LNB pathway, the genetic suite (HMO cluster-1) that apparently enables this organism to assimilate all kinds of HMOs in different ways to that of B. bifidum. The cluster is conserved among many strains of B. longum subsp. infantis, and the presence of this cluster is highly correlated with the growth ability on HMOs (46). Considering the absence of the lacto-N-biosidase in B. longum subsp. infantis JCM1217 and DSM20213 contain the genes encoding the putative intracellular 1,2-α-1-fucosidase, sialidase, β-galactosidase, and β-N-acetylgalactosaminidase, and all of the genes for the GNB/LNB pathway, but do not include the genes for lacto-N-biosidase and 1,3-1,4-α-1-fucosidase in the cell-free extracts of B. breve JCM1192, the expression of GLBP was detected, and the activities of β-galactosidase and β-N-acetylgalactosaminidase were detected in its cytoplasmic fraction; however, 1,2-α-1-fucosidase activity was not detected (Table 2 and supplemental Figs. S1 and S2).

These metabolic capabilities explain the growth properties of the respective bifidobacterial strains and reflect the fates of HMOs and their metabolites during cultivation. In B. bifidum, secretory 1,2-α-1-fucosidase and 1,3-1,4-α-1-fucosidase act on fucosylated HMOs (2'-FL, 3-FL, LDFT, LNFP I/II/III, and LNDFH I/II) to unmask the core structures (Lac, LNT, and LNnT). The rapid decrease of 2'-FL compared with LDFT and the slight increase of 3-FL during the incubation period can be explained by the facts that the specific activity of 1,2-α-1-fucosidase for 2'-FL is considerably higher than for LDFT and that the catalytic efficiency (Keq/Km) of 1,2-α-1-fucosidase is significantly higher than that of 1,3-1,4-α-1-fucosidase (41, 42), although the expression levels of two α-1-fucosidases may differ. 1,2-α-1-Fucosidase of B. bifidum is also known to prefer 2'-FL over LNFP I (41), which agrees with the faster decrease of 2'-FL than LNFP I. The combined actions of the two α-1-fucosidases resulted in an increase in the concentration of Fuc in the medium. However, B. bifidum JCM1254 could not use Fuc as a carbon source (supplemental Table 1), although the genomic sequencing of the strain PRL2010 indicated the presence of a Fuc permease (16). The rapid hydrolysis of 2'-FL and LNT caused an increase in the levels of Lac and LNB. The presence of LNB indicates that LNT was actually hydrolyzed by extracellular lacto-N-biosidase, a critical enzyme for the subsequent functioning of the GNB/LNB pathway. Interestingly, the results show that the organism leaves these intermediates outside of the cell at a time when the cells have begun to grow (Fig. 2).

Considering the initial concentrations of 2'-FL, LNT, and LNFP I (3.0, 1.6, and 0.8 mM) (Table 1), the observed increases in the concentrations of Lac (2.3 g/liter, 6.7 mM) and LNB (0.50 g/liter, 1.3 mM) at 5 h are significant. The results imply that these two metabolites are shared with different bacterial species that are not capable of fully metabolizing HMOs but can utilize Lac and LNB as carbon sources (supplemental Table S1) (47). The increase in the monosaccharide concentrations, especially Gal (0.7 g/liter, 3.9 mM at 31 h), is also interesting. The extracellular β-galactosidase from B. bifidum can liberate Gal from Lac and type 2 chains of HMOs (supplemental Fig. S2) (23), and B. bifidum is able to utilize Gal as the sole carbon source (supplemental Table S1); however, a significant amount of Gal was left unconsumed. The result also suggests that the monosaccharide is shared between bacterial species.

Conversely, the HMO consumption behavior of B. longum subsp. infantis JCM1222 was simple. The concentrations of all HMOs were reduced to an equal extent when the cells began to grow. The results strongly suggest that the organism incorporates HMOs in their intact forms and then successively degrades these compounds in the cytoplasm from the nonreducing ends, as deduced by Sela et al. (14). Considering the intracellular localization of the HMOs degrading glycosidases in this subspecies (Table 2 and supplemental Fig. S2), the transient increase in the levels of monosaccharides and Lac is intriguing. We do not have a clear answer to this observation, but it is likely that the cells excrete these saccharides to counter the changes in the osmotic pressure caused by the rapid incorporation of HMOs. B. longum subsp. infantis JCM1222 does not possess the fucose utilization genes and accordingly could not grow on Fuc (supplemental Table 1); however, its genome encodes a putative Fuc permease (Blon_2307) (14).

B. longum subsp. longum JCM1217 and B. breve JCM 1192 showed slight growth and consumed LNT only. The action of extracellular lacto-N-biosidase to degrade LNT in B. longum subsp. longum can be inferred by the transient increase in the concentration of Lac as observed for B. bifidum. Here, the increase in the concentration of Lac (0.5 g/liter, 1.5 mM) was consistent with a decrease in the concentration of LNT (1.6 mM) (Table 1). LNB was not detected, indicating the preferential utilization of LNB over Lac by this organism using the GNB/LNB pathway (13, 15). In contrast, B. breve appears to incorporate LNT in the intact form, because no intermediates (Gal, Lac, LNB, and lacto-N-triose II) were formed. A previous study on GLBP from B. longum subsp. longum demonstrated that the protein can bind LNT, although its affinity was significantly lower than those for GNB and LNB (37). The low affinity of GLBP from B. longum subsp. longum for LNT could be due to steric hindrance caused by lysine 83, which is located at the entrance of the cavity. Interestingly, in the GLBP gene (gltA)
encoded in the draft genome of *B. breve* JCM 1192, the corresponding residue is replaced with an asparagine (data not shown), which may enable the GLBP of this strain to bind LNT more efficiently. Examination of the complete and draft genomes of bifidobacteria indicates that this position varies among species and strains. We cannot rule out the possibility that *B. breve* JCM 1192 has the other degradation pathway for LNT; however, the absence of secretory lacto-N-biosidase, β-galactosidase, and β-N-acetylhexosaminidase (supplementary Fig. S2) suggests the direct intake of LNT by the strain.

**Concluding Remarks**—The above description indicates the preferential use of type 1 HMOs by bifidobacteria except for *B. longum* subsp. *infantis*, which equally incorporates type 1 and type 2 HMOs. The conservation of the GNB/ LNBI pathway in the four infant gut-associated bifidobacteria and the predominance of type 1 structures found in HMOs imply that bifidobacteria have coevolved with humans; the presence of these bacteria appears to be beneficial to the well being of the infants (8–10). During evolution, *B. bifidum* and *B. longum* subsp. *infantis* appear to have acquired an advanced ability to assimilate HMOs. However, significant amounts of HMO metabolites (mono- and disaccharides) remained unconsumed in the spent media of late HMOs. However, significant amounts of HMO metabolites appear to have acquired an advanced ability to assimilate HMOs, even when the cells did not attain their maximum growth. The results strongly suggest the symbiotic sharing of these metabolites between the bacteria. In this sense, it is interesting to note that LNB is able to preferentially use type 1 HMOs by bifidobacteria except for *B. longum* subsp. *infantis*, *B. breve* JCM 1192, the corresponding residue is replaced with an asparagine (data not shown), which may enable the GLBP of this strain to bind LNT more efficiently. Examination of the complete and draft genomes of bifidobacteria indicates that this position varies among species and strains. We cannot rule out the possibility that *B. breve* JCM 1192 has the other degradation pathway for LNT; however, the absence of secretory lacto-N-biosidase, β-galactosidase, and β-N-acetylhexosaminidase (supplementary Fig. S2) suggests the direct intake of LNT by the strain.

**Concluding Remarks**—The above description indicates the preferential use of type 1 HMOs by bifidobacteria except for *B. longum* subsp. *infantis*, which equally incorporates type 1 and type 2 HMOs. The conservation of the GNB/LNB pathway in the four infant gut-associated bifidobacteria and the predominance of type 1 structures found in HMOs imply that bifidobacteria have coevolved with humans; the presence of these bacteria appears to be beneficial to the well being of the infants (8–10). During evolution, *B. bifidum* and *B. longum* subsp. *infantis* appear to have acquired an advanced ability to assimilate HMOs. However, significant amounts of HMO metabolites (mono- and disaccharides) remained unconsumed in the spent media of *B. bifidum* grown on HMOs, even when the cells did not attain their maximum growth. The results strongly suggest the symbiotic sharing of these metabolites between the bacteria. In this sense, it is interesting to note that LNB is able to selectively stimulate the growth of bifidobacteria, but not the other genera such as *Clostridia*, *Enterococci*, and *Lactobacillus* (47, 48).

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