

Variation in Consumption of Human Milk Oligosaccharides by Infant Gut-Associated Strains of *Bifidobacterium breve*

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Human milk contains a high concentration of complex oligosaccharides that influence the composition of the intestinal microbiota in breast-fed infants. Previous studies have indicated that select species such as *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium bifidum* can utilize human milk oligosaccharides (HMO) *in vitro* as the sole carbon source, while the relatively few *B. longum* subsp. *longum* and *Bifidobacterium breve* isolates tested appear less adapted to these substrates. Considering the high frequency at which *B. breve* is isolated from breast-fed infant feces, we postulated that some *B. breve* strains can more vigorously consume HMO and thus are enriched in the breast-fed infant gastrointestinal tract. To examine this, a number of *B. breve* isolates from breast-fed infant feces were characterized for the presence of different glycosyl hydrolases that participate in HMO utilization, as well as by their ability to grow on HMO or specific HMO species such as lacto-*N*-tetraose (LNT) and fucosyllactose. All *B. breve* strains showed high levels of growth on LNT and lacto-*N*-neotetraose (LNnT), and, in general, growth on total HMO was moderate for most of the strains, with several strain differences. Growth and consumption of fucosylated HMO were strain dependent, mostly in isolates possessing a glycosyl hydrolase family 29 α -fucosidase. Glycoprofiling of the spent supernatant after HMO fermentation by select strains revealed that all *B. breve* strains can utilize sialylated HMO to a certain extent, especially sialyl-lacto-*N*-tetraose. Interestingly, this specific oligosaccharide was depleted before neutral LNT by strain SC95. In aggregate, this work indicates that the HMO consumption phenotype in *B. breve* is variable; however, some strains display specific adaptations to these substrates, enabling more vigorous consumption of fucosylated and sialylated HMO. These results provide a rationale for the predominance of this species in breast-fed infant feces and contribute to a more accurate picture of the ecology of the developing infant intestinal microbiota.

In addition to essential nutrients such as lactose lipids and proteins, human milk contains a large concentration of oligosaccharides (1). Human milk oligosaccharides (HMO) are complex and diverse glycans. These molecules are composed of monomers of glucose (Glc), galactose (Gal), and *N*-acetylglucosamine (GlcNAc) and often contain fucose (Fuc) and/or *N*-acetylneuraminic acid (NeuAc), linked via several glycosidic bonds (2, 3). All HMO contain the disaccharide lactose at their reducing end. Lactose can be sialylated to form sialyllactose or fucosylated to form fucosyllactose. In more complex HMO, the lactose core is conjugated with repeats of lacto-*N*-biose I (Gal β 1-3GlcNAc; LNB; type 1 chain) or *N*-acetyllactosamine (Gal β 1-4GlcNAc; type 2 chain), producing molecules with a degree of polymerization larger than 4 (4). These core structures can be modified by fucose and sialic acid residues via different linkages, with fucosylated neutral oligosaccharides the most representative in human milk (5). Although a large number of different HMO structures have been determined, a few isomers can represent up to 70% of the total molecules (6).

Remarkably, the energetic value of HMO for the infant is minimal. Various researchers have shown that HMO are resistant to enzymatic hydrolysis from intestinal brush border membrane and pancreatic juices, and therefore the majority of these molecules transit the intestinal tract, reaching the colon intact (7–9). During their transit HMO are believed to prevent pathogen colonization by serving as decoy binding sites for epithelial glycans (10).

One of the more fascinating roles of HMO is their ability to influence the composition of the intestinal microbiota in the first

years of life. While the microbial community in breast-fed infants is often dominated by the genus *Bifidobacterium*, formula-fed infants show increased bacterial diversity (11, 12). This suggests that both pro- and antimicrobial elements in breast milk account for these differences. A conceptual basis for coevolution between bifidobacteria and milk glycans is supported by recent definition of the molecular mechanisms by which these microbes catabolize HMO. In *Bifidobacterium longum* subsp. *infantis* (*B. longum* subsp. *infantis*) ATCC 15697 these mechanisms include oligosaccharide transporters and intracellular glycosyl hydrolases (GH) such as fucosidases, hexosaminidases, and sialidases (13).

Bifidobacterium breve, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, *Bifidobacterium catenulatum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium bifidum* are the species most frequently detected in breast-fed infant feces (11, 14, 33). In general, *B. breve* and *B. longum* subsp. *infantis* are more exclusively

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found in infants, and *B. longum* subsp. *longum* and *B. bifidum* are found in both infants and adults. While several strains of *B. bifidum* and *B. longum* subsp. *infantis* have been shown to grow vigorously on HMO *in vitro* (15, 16), this phenotype has been largely unexplored for larger numbers of *B. breve* and *B. longum* subsp. *longum* isolates. Only one strain of *B. breve*, ATCC 15700, has been shown to utilize a far less diverse range of HMO isomers than *B. longum* subsp. *infantis* ATCC 15697 (17). Whether this pattern is representative of the *B. breve* species is not clear. To better address what factors explain the predominance of this species in the infant gut microbiota, we isolated and characterized a representative number of strains of *B. breve* and examined the molecular mechanisms for their consumption of milk oligosaccharides.

MATERIALS AND METHODS

Subjects. Fecal samples were collected from 40 exclusively breast-fed term infants at 3 and 4 months of age (UC Davis Lactation Study). None of the infants enrolled in this study received antibiotic treatment, infant formula, or solid food before the time of sampling. 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 dry ice and stored at -80°C before processing. The University of California 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 (identification number NCT01817127).

Microbial isolations. For isolation of *Bifidobacterium*, 100 mg of each fecal sample was taken aseptically, transferred to a sterile tube, diluted 10-fold with 1% peptone water (Becton, Dickinson, Sparks, MD), and homogenized by vortexing. Ten-fold dilutions were prepared with 1% peptone water and inoculated on modified basal salts medium (BSM) agar (mBSM). Modified BSM agar was prepared by supplementing de Man-Rogosa-Sharpe (MRS) medium (Becton, Dickinson, Sparks, MD) with 15 g/liter agar, 500 mg/liter L-cysteine-HCl, 100 mg/liter mupirocin, 25 mg/liter kanamycin, 4.28 mg/liter polymyxin B, 25 mg/liter iodoacetate, 20 mg/liter nalidixic acid, and 25 mg/ml of 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis, MO). The plates were incubated for 48 h at 37°C in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) in an atmosphere containing 5% carbon dioxide, 5% hydrogen, and 90% nitrogen. Resulting colonies were streaked onto mBSM agar, and after two passages they were grown in MRS broth supplemented with 0.05% L-cysteine-HCl and stored at -80°C in 25% glycerol. Prior to each assay all bacteria strains were subcultured twice in MRS broth supplemented with 0.05% L-cysteine-HCl and incubated at 37°C for 18 h in an anaerobic chamber.

Additional *B. breve* strains were obtained from the Japanese Collection of Microorganisms (RIKEN BioResource Center, Japan), the American Type Culture Collection (Manassas, VA), and the University of California—Davis Viticulture and Enology Culture Collection (see Table S1 in the supplemental material).

Identification of bifidobacteria by 16S rRNA gene sequencing. Genomic DNA was obtained from 1 ml of each culture and centrifuged for 5 min at $2,000 \times g$. The bacterial pellet was resuspended and incubated for 30 min at 37°C with enzymatic lysis buffer consisting of 20 mM Tris-Cl, pH 8.0, 2 mM sodium EDTA, 1.2% Triton X-100, and 40 mg/ml lysozyme (Sigma). After enzymatic lysis, bacterial DNA was isolated from the samples using a DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA quality and yield were checked using a Nanodrop spectrophotometer (Wilmington, DE); the DNA was then stored at -20°C until further use. To identify the isolates at species level, the 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-AGAGTTGATCCTGGCTCAG) and 1492R (5'-TACGGTTACCTT GTTACGA) on an Applied Biosystems 2720m Thermal Cycler (Applied Biosystems, Mountain View, CA) (18). One microliter of extracted DNA

was added to a 50- μl reaction mixture containing 50 pmol of primers, 500 μM each deoxynucleoside triphosphate (dNTP), 0.1 volume of $10\times$ PCR buffer, 2.5 mM MgCl_2 , and 1 U of AmpliTaq Gold polymerase (Applied Biosystems). Amplification mixtures were subjected to 4 min of denaturation at 94°C , followed by 30 cycles of 94°C for 30 s, 55°C for 40 s, and 72°C for 1 min 30s, with a final extension period of 7 min at 72°C . The resulting amplicons were separated on a 1% agarose gel, followed by GelRed staining (Phenix Research Products, Candler, NC) and purification using a QIAquick PCR Purification Kit (Qiagen). Sequencing was performed on an ABI 3730 Capillary Electrophoresis Genetic Analyzer using BigDye Terminator chemistries at the University of California—Davis DNA Sequencing Facility. The sequences were analyzed using BioEdit, version 7.0 (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>), and checked by nucleotide-nucleotide BLAST comparison at the NCBI database for species identification.

MLST of strains. Multilocus sequence typing (MLST) analysis of *B. breve* isolates targeted intragenic regions of seven housekeeping genes (*clpC*, *purF*, *gyrB*, *fusA*, *ileS*, *rplB*, and *rpoB*) selected based on a previous study (19). PCR amplifications were prepared as described above, and cycling conditions were optimized for every primer set (see Table S2 in the supplemental material) and consisted of an initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, annealing at 60 to 67°C for 30 s, elongation at 72°C for 60 s, with a final extension at 72°C for 7 min and holding at 4°C . The PCR products were separated and sequenced as described above.

Sequencing data for all loci were edited using BioEdit, version 7.0, and aligned using CLUSTAL W (20). Phylogenetic analysis and concatenations of the sequenced loci were performed using the Molecular Evolutionary Genetic Analysis (MEGA) software, version 5 (<http://megasoftware.net>). Descriptive evolutionary analysis including mol% G+C content, number of polymorphic sites, nucleotide diversity (π /site), and average number of nucleotide differences (k) was performed using DnaSP, version 5.10 (see Table S3 in the supplemental material). Allelic sequences were assigned as described previously (21) (see Table S4). A minimum evolution tree of the concatenated loci was calculated using MEGA, version 5.0 (see Fig. S1).

Glycosyl hydrolase gene sequencing. α -Fucosidases (Blon_2336, Blon_2335, Blon_0248/0426, and Blon_0346), α -sialidases (Blon_2348, and Blon_0646), and the β -hexosaminidase Blon_0459 protein sequences from *B. longum* subsp. *infantis* ATCC 15697 were aligned with homologous sequences from the GenBank database (accession numbers are shown in Table S5 in the supplemental material) using Bioedit, version 7.0, and degenerate primers were designed to amplify conserved regions (see Table S6). To further differentiate between Blon_0248 and Blon_0426, strains that were PCR positive for the fucosidase Blon_0248/0426 were also amplified with the primers designed previously (22) to amplify the complete gene in *B. longum* subsp. *infantis* ATCC 15697 (see Table S6). PCR amplifications were prepared as described above with 200 pmol of primers, and cycling conditions were optimized for every primer set (see Table S6); the PCR program consisted of an initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, annealing at 45 to 55°C for 30 s, elongation at 72°C for 60 s, with a final extension at 72°C for 7 min and holding at 4°C . The resulting amplicons were separated and sequenced as described above. *B. longum* subsp. *infantis* ATCC 15697 and *Bifidobacterium animalis* subsp. *lactis* JCM 10602 were used as positive- and negative-control strains, respectively.

Bifidobacterial growth *in vitro* on HMO. The 24 *B. breve* strains listed in Table S1 in the supplemental material were tested for growth in the presence of seven different substrates: HMO (23), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), 2'-fucosyllactose (2FL), 3'-fucosyllactose (3FL) (Glycom, Denmark), 3'-sialyllactose (3SL), and 6'-sialyllactose (6SL) (GenChem Co., South Korea). *B. longum* subsp. *infantis* ATCC 15697 and *B. animalis* subsp. *lactis* JCM 10602 were included as positive and negative controls for growth on HMO, respectively. Two microliters of each resulting overnight culture was used to inoculate 200

μl of modified MRS medium (mMRS), devoid of glucose and supplemented with 2% (wt/vol) of each sterile-filtered substrate as the sole carbohydrate source, and another 2 μl was inoculated into mMRS medium without added sugar. The medium was supplemented with 0.05% (wt/vol) L-cysteine, and in all the cases the cultures in the wells of the microtiter plates were covered with 30 μl of sterile mineral oil to avoid evaporation. The incubations were carried out at 37°C in an anaerobic chamber (Coy Laboratory Products). Cell growth was monitored in real time by assessing optical density at 600 nm (OD_{600}) using a BioTek PowerWave 340 plate reader (BioTek, Winoosky, VT) every 30 min, preceded by 15 s of shaking at variable speed. Two biological replicates and three technical replicates each were performed for every studied strain. Maximum OD and growth rates were calculated and expressed as the mean of all replicates with the respective standard deviation. These calculations were performed as previously described (24). The OD obtained for each strain grown on the different substrates was compared with the OD obtained in the absence of a sugar source. This difference in OD (ΔOD) was used as a parameter to evaluate each strain's ability for growth on the different substrates.

Glycoprofiling. Bacterial cultures in mMRS medium with 2% HMO were collected at the end of the exponential phase and centrifuged at $12,000 \times g$ for 30 min. In the case of *B. breve* SC95, the samples were collected at three different points in the growth curve, i.e., OD_{600} values of approximately 0.2, 0.5, and 0.8. At least two biological replicates were performed in triplicates. Supernatants were filtered using a multiscreen 96-well, 0.22- μm -pore-size filtration plate (Millipore, Billerica, MA) prior to storage at -80°C . Remaining oligosaccharides were recovered from the supernatants (25 μl) and reduced to their alditol forms with 1 M NaBH_4 at 65°C for 1.5 h. Each replicate was desalted by solid-phase extraction (SPE) on graphitized carbon cartridges. Salts were removed with 6 ml of deionized water, and oligosaccharides were eluted with 20% (vol/vol) acetonitrile in water and with 40% acetonitrile in 0.01% trifluoroacetic acid (vol/vol). SPE fractions were combined and dried under vacuum. Samples were reconstituted in 100 μl of deionized water and diluted 50-fold for liquid chromatography-mass spectrometry (LC-MS) analysis.

An Agilent high-performance liquid chromatography–chip–time of flight (HPLC-chip/TOF) mass spectrometer equipped with a capillary pump for sample loading and a nano-pump for chromatographic separation was used for HMO analysis. Separation was performed on a microfluidic chip equipped with enrichment and nano-LC analytical columns, both packed with porous graphitized carbon, using a previously described method (3, 25). Briefly, HMO were separated by a 65-min gradient using a binary solvent system consisting of 3% acetonitrile–water in 0.1% formic acid (vol/vol) (solvent A) and 90% acetonitrile–water in 0.1% formic acid (vol/vol) (solvent B). HMO were analyzed in positive ion mode, with a mass range between 300 and 2,000 m/z . Agilent's Masshunter software was used for data acquisition and data analysis (version B.03.01).

HMO monosaccharide composition was determined using accurate mass within a ± 20 ppm mass error of theoretically calculated masses. Specific structures were assigned to HMO peaks by matching the reproducible retention times to those reported in previously annotated HMO libraries (2, 25). Percent consumption was calculated using a label-free method, employing the uninoculated HMO pool as an external standard. Total HMO consumption was calculated with respect to the uninoculated control by normalizing the summed abundance of all identified oligosaccharide spectra in ion counts in the bacterial supernatant to that of the control using the following equation:

$$\left[1 - \left(\frac{\sum_{i=1}^n \text{API bacteria sample}}{\sum_{i=1}^n \text{API uninoculated control}} \right) \right] \times 100 \quad (1)$$

where API is absolute peak intensity and n is the number of identified HMO. The consumption of individual HMO species was quantified in the same manner, in which the absolute peak intensity of a specific HMO structure was normalized to the peak intensity of the corresponding structure in the uninoculated control.

TABLE 1 Distribution of isolates of bifidobacteria from breast-fed infants identified by 16S rRNA gene sequencing

Species	No. of isolates identified
<i>B. longum</i>	297
<i>B. breve</i>	77
<i>B. pseudocatenulatum</i>	45
<i>B. bifidum</i>	22
<i>B. dentium</i>	8
<i>B. adolescentis</i>	7
<i>B. animalis</i>	5

Gene expression analysis. The full nucleotide sequences of the genes encoding a GH95 and a GH29 α -fucosidase in the strain *B. breve* SC95 generated were used to design qPCR primers using the primer-BLAST tool at NCBI (see Table S6 in the supplemental material). For relative quantification, the *rnpA* gene was used as described by O'Connell Motherway et al. (26). *B. breve* SC95 was grown as described above in mMRS medium supplemented with either 2% lactose, 2% HMO, or 2% 2FL in a microplate reader, and cultures were taken at mid-exponential phase (OD of 0.6 to 1.0). Samples were immediately pelleted at $12,000 \times g$ for 1 min and stored in RNA Later (Ambion). RNA extraction, cDNA conversion, and quantitative PCR (qPCR) were performed as described by Garrido et al. (27).

RESULTS

Isolation and identification of *Bifidobacterium* from breast-fed infant feces. In order to obtain a representative number of *B. breve* strains, we collected fecal samples from 40 breast-fed term infants. Diluted fecal samples were grown on mBSM medium, and six colonies from the highest dilution were isolated. We obtained around 500 isolates that were identified by 16S rRNA gene sequence analysis, and a total of 461 isolates were identified as *Bifidobacterium*. Seven species of bifidobacteria were detected, and the species *B. longum* was the most represented (297 isolates), followed by *B. breve* with 77 isolates (Table 1).

We further investigated the identity of the *B. breve* isolates at the strain level by MLST, as described elsewhere (19). Our analysis also included nine strains from culture collections (see Table S1 in the supplemental material). A total of 172 single nucleotide polymorphisms (SNPs) were found in seven loci, and they generated between 8 *rplB* and 65 *purF* polymorphic sites (see Table S3). Twenty different allelic profiles were identified in the 86 *B. breve* isolates analyzed (see Table S4). Taking a conservative approach, strains isolated from the unrelated infants that shared identical MLST profiles were considered separate isolates in our study. This resulted in a library of 24 strains of *B. breve* (see Table S1), for which a consensus phylogenetic tree of the concatenated MLST data is shown in Fig. S1 in the supplemental material.

Glycosyl hydrolase genes in *B. breve* isolates. In order to study the possible adaptations of the *B. breve* isolates for growth on HMO, we first determined the presence of three key GH classes required to cleave HMO into its constituent monosaccharides. The β -galactosidase activity was not examined because it is widespread in the *Bifidobacterium* genus. The genome of *B. breve* UCC2003 (26) contains an α -fucosidase, an α -sialidase, and a β -hexosaminidase with significant homology to cognate enzymes in *B. longum* subsp. *infantis* ATCC 15697. No homology was found to the same glycosyl hydrolases in *B. bifidum* genomes. Based on this, we used degenerate primers to look for genes encoding these GH in the assembled *B. breve* strains (Table 2). All of

TABLE 2 Presence of glycosyl hydrolases and growth on different HMO by *B. breve* strains

Strain	Glycosyl hydrolase and family ^a				Bacterial growth on ^b :						
	α -Fucosidase		α -Sialidase (GH33)	β -Hexosaminidase (GH20)	HMO	LNT	LNnT	2FL	3FL	3SL	6SL
	GH95	GH29									
UCC2003	+	-	+	+	+	+++	+++	-	+	-	-
ATCC15700	+	-	+	+	+	+++	+++	-	-	-	-
ATCC15698	+	-	+	+	++	+++	+++	-	-	-	-
ATCC15701	+	-	+	+	+++	+++	+++	-	-	-	-
JCM7017	+	-	+	+	++	+++	+++	-	-	-	-
JCM7019	+	-	+	+	++	+++	+++	+	+	-	-
JCM7020	+	+	-	+	++	+++	+++	-	-	-	-
S-17c	+	-	+	+	+	+++	+++	-	+	-	-
S-46	+	-	+	+	++	+++	+++	-	+	-	-
SC81	+	-	+	+	++	+++	+++	-	-	-	-
SC95	+	+	+	+	+++	+++	+++	+++	+	-	-
SC139	+	-	+	+	++	+++	+++	-	-	-	-
SC154	+	+	+	+	+++	+++	+++	-	-	-	-
SC500	+	-	+	+	++	+++	+++	-	-	-	-
SC506	+	+	+	+	++	+++	+++	-	-	-	-
SC508	+	-	+	+	+	+++	+++	-	-	-	-
SC522	+	-	+	+	++	+++	+++	-	+	-	-
SC559	+	-	+	+	++	+++	+++	-	-	-	-
SC567	+	-	+	+	++	+++	+++	-	-	-	-
SC568	+	+	+	+	++	+++	+++	+++	+	-	-
SC573	+	-	+	+	+	+++	+++	-	+	-	-
SC580	+	-	+	+	++	+++	+++	-	+	-	-
SC670	+	-	+	+	+	+++	+++	-	-	-	-
KA179	+	-	+	+	++	+++	+++	+	-	+	+
ATCC15697	+	+	+	+	+++	+++	+++	+++	+++	+++	+++
JCM10602	-	-	-	-	-	-	-	-	-	-	-

^a Positive amplification (+) indicates that the sequence of the PCR product is >55% homologous at the amino acid level to the respective GH gene in *B. longum* subsp. *infantis* ATCC 15697.

^b Level of growth was classified as follows: -, negative (maximum OD₆₀₀ of <0.200); +, low (OD₆₀₀ of 0.200 to 0.550); ++, moderate (OD₆₀₀ of 0.550 to 0.750); +++, high (OD₆₀₀ of >0.750).

the *B. breve* strains possessed a gene homologous to β -hexosaminidase Blon_0459 in *B. longum* subsp. *infantis* (27) and an α -fucosidase similar to Blon_2335 in *B. longum* subsp. *infantis* ATCC 15697 (22), and all strains excepting JCM 7020 possessed an α -sialidase, related to Blon_0646 in *B. longum* subsp. *infantis* (28). Moreover, five strains possessed a second α -fucosidase, homologous to Blon_0248 in *B. longum* subsp. *infantis* ATCC 15697 (22), belonging to GH family 29 (GH29) (Table 2; see also Fig. S2 in the supplemental material).

Growth of *B. breve* on pooled and individual HMO. The panel of *B. breve* strains was examined for growth *in vitro* on total HMO isolated from breast milk as the sole carbon source (23) or on individual milk oligosaccharides that represent the chemical diversity of HMO, such as neutral nonfucosylated HMO (LNT and LNnT), fucosylated HMO (2FL and 3FL), and sialylated HMO (3SL and 6SL). Growth behavior on HMO (shown in Fig. S3 in the supplemental material) and maximum OD values obtained were parameters to arbitrarily classify this panel in three groups (Table 2; see also Table S7).

In general, moderate growth on HMO was witnessed for all the strains (Table 2; see also Fig. S3 and Table S7 in the supplemental material), with strain-level differences among the isolates to utilize HMO (Table 2). Interestingly, three strains (SC95, SC154, and ATCC 15701) exhibited remarkable growth on HMO compared to the type strain *B. breve* ATCC 15700 but still lower overall

growth and a lower growth rate than *B. longum* subsp. *infantis* ATCC 15697 (Fig. 1A; see also Table S7).

All *B. breve* strains grew on LNT and LNnT to high cell densities and at levels comparable to those of *B. longum* subsp. *infantis* ATCC 15697 (Table 2; see also Fig. S4 and S5 in the supplemental material). Interestingly, a few strains were able to grow on fucosylated HMO (Fig. 1B and Table 2). The isolates SC95 and SC568 grew well on 2FL and to a similar extent as *B. longum* subsp. *infantis* ATCC 15697. Using 3FL as the sole carbon source, only the strains SC95 and JCM 7019 grew, albeit minimally (Table 2; see also Fig. S6). Finally, growth on 3SL and 6SL was only observed for one strain, KA179 (Table 2).

Glycoprofiling of the HMO consumption by *B. breve* isolates. Based on their growth phenotypes and ability to utilize certain glycans, six strains of *B. breve* were selected to examine the consumption of 22 different oligosaccharides during growth on total HMO. This included strains SC95, SC154, SC568, SC580, ATCC 15701, and JCM7019, as well as *B. longum* subsp. *infantis* ATCC 15697 and *B. breve* ATCC 15700 as positive and negative controls, respectively. The supernatant was collected at the end of the exponential phase during growth on HMO, and remaining oligosaccharides were purified and reduced and later detected and quantified by nano-HPLC-chip/TOF MS. Identification of specific oligosaccharides and isomers in this study was aided by two oligosaccharide structure libraries (2, 3). The oligosaccharides

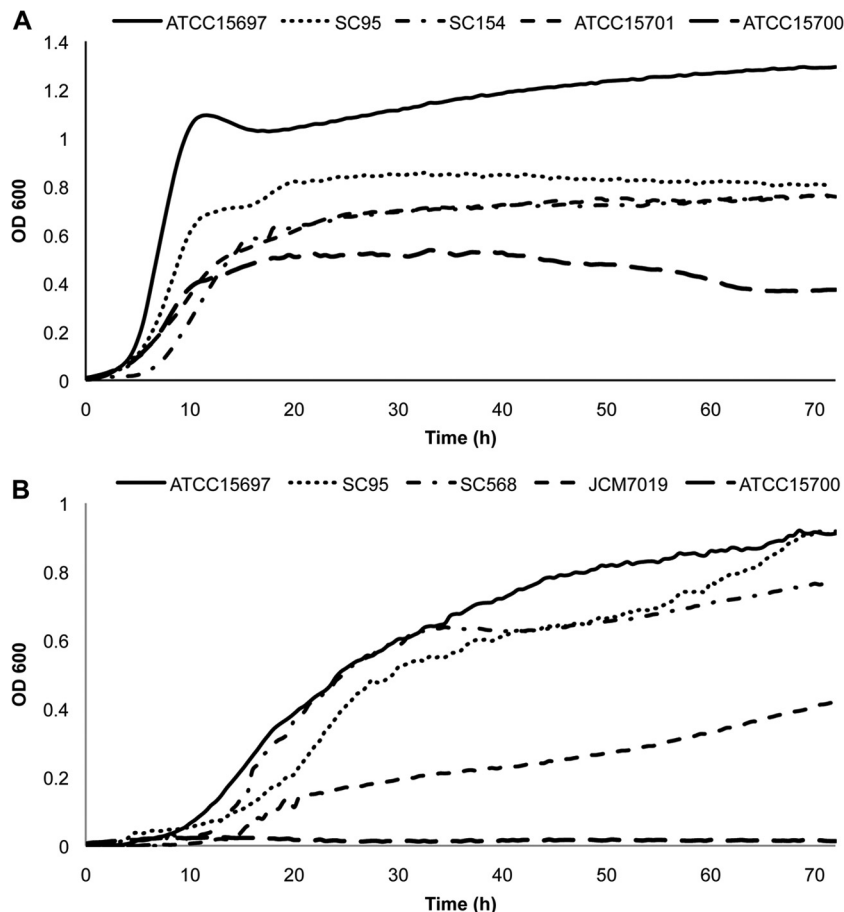


FIG 1 Growth of *B. breve* on HMO. *B. breve* isolates were inoculated on mMRS medium supplemented with 2% (wt/vol) HMO (A) and 2FL (B). *B. longum* subsp. *infantis* ATCC 15697 was included as a high-growth control. Fermentations were carried out in triplicate.

quantified include the most abundant neutral and sialylated HMO (6), and Table 3 shows their names, masses, chemical structures, and degree of polymerization (DP).

Among the six strains selected, total consumption of HMO ranged between 23 and 42%. These values are lower than for *B. longum* subsp. *infantis* ATCC 15697 (64% consumption) but clearly higher than for *B. breve* ATCC 15700 (Fig. 2A), the only *B. breve* strain glycoprofiled to date (17, 29, 30). Figure 3B presents the consumption of neutral nonfucosylated HMO in more detail, showing that consumption patterns among the *B. breve* strains were similar. All strains were able to deplete LNT/LNnT from the culture medium to a high extent as previously witnessed (17). Among three major hexaoses found in HMO, a preference for lacto-*N*-neo-hexaose (LNnH) was observed over lacto-*N*-hexaose (LNH) and *para*-lacto-*N*-hexaose (p-LNH).

In general, the ability of *B. breve* to metabolize fucosylated HMO was lower than that of *B. longum* subsp. *infantis*, which showed high consumption levels for all the HMO tested (Fig. 3A). However, strains SC95, SC154, and SC568 showed a significant consumption of monofucosylated 2FL and lacto-*N*-fucopentaose (LNFPII) and difucosyllacto-*N*-hexaoses (DF-pLNHII and DFLNH-A) (Fig. 3A and Table 3). Interestingly, even though 2FL cannot support the growth *in vitro* of strain SC154 (Table 2), this strain utilizes larger fucosylated HMO.

We observed that growth on fucosylated HMO was more

prominent in strains possessing an additional GH29 α -fucosidase (Fig. 3A and Table 2). To determine if this gene is involved, we evaluated the relative gene expression of the fucosidases in strain SC95. Growth on 2FL as the sole carbon source upregulated 40-fold the expression of the GH29 fucosidase gene (Fig. 4), while expression of the GH95 fucosidase was not altered, suggesting that the presence of the GH29 fucosidase endows these strains with the ability to consume fucosylated oligosaccharides. In contrast, growth on total HMO did not affect the expression of these genes.

Acidic HMO represent approximately 15% of total HMO, and in this study we also screened the consumption of 11 sialylated HMO in the spent supernatants of these strains during growth on total HMO (Fig. 3B). The levels of consumption were very similar among the strains tested and comparable to those of *B. longum* subsp. *infantis*. In particular sialyl-LNT b (LSTb), sialyl-lacto-*N*-hexaose (S-LNH), and mass 4121a/4121b were preferentially utilized by *B. breve* (Fig. 3B).

Finally, to elucidate possible substrate preferences in a *B. breve* strain with high HMO consumption, we monitored the consumption of nine representative oligosaccharides at different points during the fermentation of HMO by strain SC95 (Fig. 5). These nine HMO correspond to neutral and acidic HMO that were consumed at levels higher than 40%. Remarkably, glycoprofiling of early exponential growth revealed that acidic HMO disappeared from the medium first, together with LNnH. In contrast, LNT

TABLE 3 Names, structures, and masses of human milk oligosaccharides glycoprofiled^a

HMO type	Name	Abbreviation or designation	Mass (m/z)	Structure	DP ^b	
Neutral	Nonfucosylated	Lacto- <i>N</i> -tetraose/lacto- <i>N</i> -neotetraose	LNT/LnNT	709	Galβ1-3GlcNAcβ1-3Galβ1-4Glc/Galβ1-4GlcNAcβ1-3Galβ1-4Glc	4
		Lacto- <i>N</i> -hexose	LNH	1,074	Galβ1-3GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4Glc	6
	Lacto- <i>N</i> -neohexose	LNnH	1,074	Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4Glc	6	
	<i>para</i> -Lacto- <i>N</i> -hexose	p-LNH	1,074	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	6	
	Fucosylated	2'-Fucosyllactose	2FL	490	Fucα1-2Galβ1-4Glc	3
		Lacto- <i>N</i> -fucopentaose	LNFP1	855	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	5
			LNFP2	855	Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc	5
			MFLNHI	1,220	Fucα1-2Galβ1-3GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4Glc	7
			DFLNNH-A	1,366	Fucα1-2Galβ1-3GlcNAcβ1-3[Galβ1-4]Fucα1-4[GlcNAcβ1-6]Galβ1-4Glc	8
			DFLNNH-B	1,366	Galβ1-3[Fucα1-4]GlcNAcβ1-3[Galβ1-4]Fucα1-3[GlcNAcβ1-6]Galβ1-4Glc	8
		DFLNNH-C	1,366	Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4Glc	8	
		DFpLNHHI	1,366	Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc	8	
Acidic sialylated	3-Sialyllactose	3SL	635	NeuAcα2-3Galβ1-4Glc	3	
		LSTa	1,000	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	5	
	Monosialyllacto- <i>N</i> -tetraose	LSTb	1,000	Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc	5	
	Monosialyllacto- <i>N</i> -neotetraose	LSTc	1,000	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	5	
	Monofucosylmonosialyllacto- <i>N</i> -tetraose	F-LSTc	1,146	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4[Fucα1-3]Glc	5	
	Monosialyllacto- <i>N</i> -hexaose	S-LNH	1,365	Galβ1-3GlcNAcβ1-3[[NeuAcα2-6]Galβ1-4GlcNAcβ1-6]Galβ1-4Glc	7	
		4021a	1,365	Galβ1-3[NeuAcα2-6]GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4Glc	7	
	Monosialyllacto- <i>N</i> -neohexaose	S-LNnHI	1,365	[NeuAcα2-6]Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc/ [NeuAcα2-6]Galβ1-4GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc	8	
	Monofucosylmonosialyllacto- <i>N</i> -hexaose	4121a/4121b	1,511	[NeuAcα2-6]Galβ1-4GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc/ Galβ1-3[Fucα1-4]GlcNAcβ1-3[[NeuAcα2-6]Galβ1-4GlcNAcβ1-6]Galβ1-4Glc	8	
		FS-LNHHI	1,511	Not defined	8	
	4121 unknown	1,511	Not defined	8		

^a Adapted with permission from references 2 and 3.^b DP, degree of polymerization.

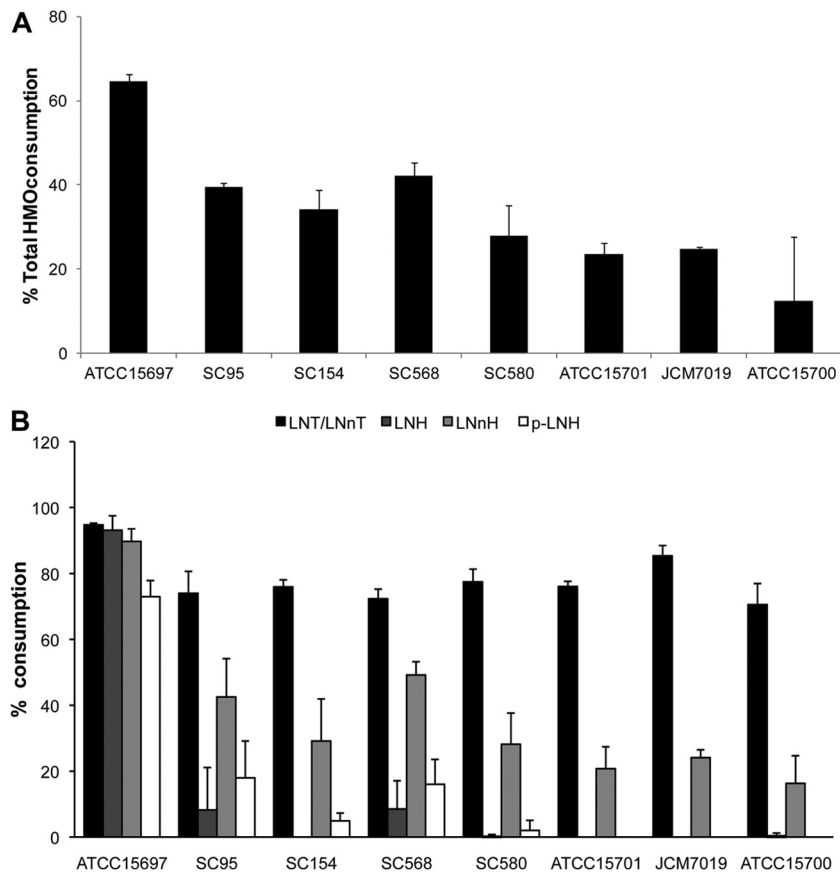


FIG 2 Glycoprofiling of HMO consumption by select *B. breve* strains. (A) Total utilization of HMO. Consumption is calculated as the percent difference in HMO between the start and the end of exponential phase. (B) Glycoprofiles of the consumption of neutral nonfucosylated HMO by seven *B. breve* strains. *B. longum* subsp. *infantis* ATCC 15697 was included as a positive control.

seems to be metabolized during the mid-exponential phase and, as observed previously, is substantially depleted at the end of the growth on HMO. We also observed that while monofucosylated HMO such as 2FL and LNFPII are depleted from the medium at the mid-exponential phase and not later, difucosylated HMO appear to be steadily consumed during the three growth phases (Fig. 5).

DISCUSSION

B. breve is one of the most representative species of bifidobacteria found in the infant intestinal microbiota. Unfortunately, the factors that contribute to this persistence in infants are not well understood. We have previously determined that some *B. breve* strains are equipped with enzymes that release *N*-glycans from host glycoproteins, and some strains can use *N*-glycosylated proteins as the sole carbon source (31). This suggested that glycoproteins could play a prebiotic role for *B. breve*. In order to determine whether free HMO also contribute to the persistence of *B. breve* in the infant intestinal microbiota, we evaluated a significant number of *B. breve* strains for growth on and consumption of HMO.

The dominance of *B. breve* in the gut community has been supported by several studies, especially in breast-fed infants (11, 14), where this species together with *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* can largely outnumber other microorganisms (32, 33). It can be considered that breast milk itself is also

another habitat for this species, which, in addition to the vaginal and fecal microbiota of the mother, contributes to intestinal colonization of the infant (34). Some strains of this species are currently studied for their probiotic properties, such as production of conjugated linoleic acids or important immunomodulatory activities (26, 35).

Since the predominance of bifidobacteria in breast-fed infants can be attributed in part to milk components such as HMO, the utilization of these substrates *in vitro* and *in vivo* is an important reflection of the adaptations of intestinal microorganisms to the environmental conditions prevalent in the infant gut. HMO utilization has been shown for a few *Bacteroides* species and certain infant gut-associated bifidobacteria, especially strains of *B. longum* subsp. *infantis* and *B. bifidum* (16, 30). Previous studies have addressed this phenotype only for the type strain of *B. breve*, ATCC 15700 (JCM 1192), indicating that this microorganism has a limited ability to consume HMO (17, 29, 30). In this study, we have expanded these observations and concluded that several infant-associated strains of *B. breve* can readily utilize HMO, consuming larger amounts of total HMO than the type strain ATCC 15700. The HMO consumption in *B. breve* is, however, moderate compared to that of *B. longum* subsp. *infantis* ATCC 15697 (Fig. 2A).

Mass spectrometry-based analysis of the HMO remaining after growth provides a detailed representation of the preferences of

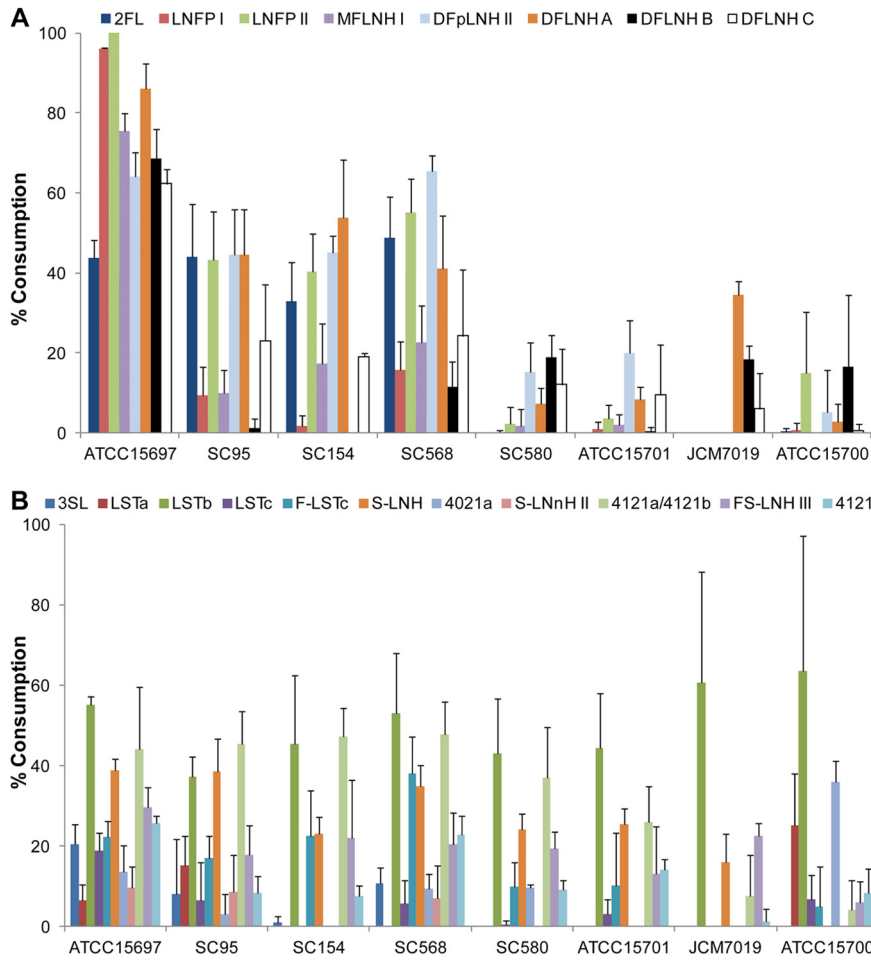


FIG 3 Glycoprofiling of the consumption of fucosylated and acidic HMO by select *B. breve* strains. Consumption of eight fucosylated HMO (A) and 11 sialylated HMO (B) was calculated. *B. longum* subsp. *infantis* ATCC 15697 was included as a positive control. HMO consumption is represented as the percent difference in HMO between the start and the end of the exponential phase.

these strains for different oligosaccharide subsets present in the HMO pool. For example, all strains showed vigorous growth on LNT and LNnT, and the molecular mass representing both species (709 *m/z*) was the most consumed in pooled HMO. The utiliza-

tion of LNnT is interesting since this oligosaccharide is not readily fermented by all species of *Bifidobacterium* found in the infant gut (36). Moreover, growth on LNnT was shown to enable *B. longum* subsp. *infantis* to outcompete *Bacteroides fragilis* in a mouse model (37).

HMO with a mass of 1,074 *m/z* represent approximately 10%

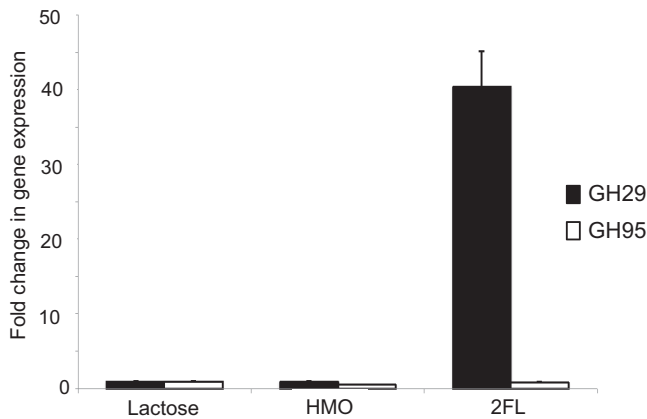


FIG 4 Fold in change expression for genes encoding α -fucosidases from GH families 95 and 29 in *B. breve* SC95 during mid-exponential-phase growth on HMO and 2FL. Growth on lactose was used as a control.

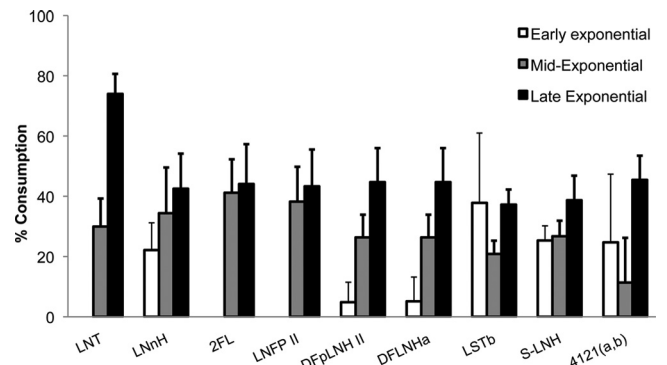


FIG 5 Temporal glycoprofile of the consumption of select neutral and acidic HMO by *B. breve* SC95 at different stages in the exponential phase.

of the total (6) and includes three neutral isomers, LNH, LNnH, and p-LNH (Table 3). Interestingly, LNnH is the most abundant of the three isomers (3), and it was selectively consumed by *B. breve* compared to the other two isomers. This indicates structure-based preferences in HMO consumption in *B. breve* (Fig. 2B and Table 3).

Strain-dependent differences were more evident in the growth of *B. breve* on fucosylated HMO. Fucosidase activity has not been described previously in *B. breve*, and while all the strains studied possessed a gene encoding a GH95 α -fucosidase, we observed that the presence of a second α -fucosidase from GH29 in isolates SC95, SC568, and SC154 correlated with their consistent growth and consumption of fucosylated HMO (Fig. 3A and Table 2). Some strains with this additional GH29 α -fucosidase, however, did not grow on 2FL and 3FL. We hypothesize that these smaller HMO are imported by different transport mechanisms. For example, in *B. longum* subsp. *infantis*, 2FL and larger fucosylated HMO are likely imported by different solute binding proteins (38).

Remarkably, all *B. breve* strains seem to consume acidic HMO to a significant extent, and an α -sialidase was identified. All strains glycoprofiling showed a preferential consumption of select acidic HMO such as LSTb and monosialyllacto-*N*-hexaose (S-LNH), but not smaller HMO, which might additionally explain why growth on 3SL and 6SL was negligible (Table 2).

B. longum subsp. *infantis* ATCC 15697 is currently a model to study genetic adaptations to growth on human milk glycans (39). Several observations here indicate that the mechanisms of HMO consumption in *B. breve* are very similar to those of *B. longum* subsp. *infantis*, with a preference to import intact oligosaccharides followed by intracellular degradation, rather than the extracellular degradation observed in *B. bifidum* (13). For example, *B. breve* strain ATCC 15700 can quickly deplete LNT from the spent medium, and the absence of intermediate monosaccharides indicates that this strain imports this substrate (40). Moreover, the GH genes identified in this study lacked signal peptides, suggesting intracellular localization. Finally, the GH gene sequences obtained are homologous to previously characterized enzymes in *B. longum* subsp. *infantis*, including β -hexosaminidase Blon_0459, two α -fucosidases (Blon_2335 and Blon_0248), and an α -sialidase (Blon_0646), suggesting a common origin. Further studies will address in more detail the molecular mechanisms that *B. breve* deploys to utilize HMO.

In conclusion, these results provide a rationale for the predominance of *B. breve* in the infant intestinal microbiota, improving our understanding about the ecology of this unique environment. The genetic variation of these strains and the strain-dependent character of the HMO utilization are factors to consider in probiotic and prebiotic studies. Finally, we consider that better characterization of the diversity and physiology of beneficial strains of bifidobacteria and more selective substrates that allow their implantation in the intestine could be useful in the near future to design more selective synbiotic preparations.

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