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Variation in consumption of human milk oligosaccharides by infant-gut associated strains of *Bifidobacterium breve.*

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Running title: HMO utilization by *Bifidobacterium breve*.

This article contains Supplemental Figs. S1-S6 and Tables S1-S7.

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ABSTRACT

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 *B. bifidum* can utilize human milk oligosaccharides (HMO) *in vitro* as the sole carbon source, while the relatively few *B. longum* subsp. *longum* and *B. 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 HMOs 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 a high growth on lacto-*N*-tetraose 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 was 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* 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 specific 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

47 contribute to a more accurate picture of the ecology of the developing infant intestinal
48 microbiota.

49

INTRODUCTION

In addition to essential nutrients such as lactose lipids and proteins, human milk contains a large concentration of oligosaccharides (27). Human milk oligosaccharides (HMO) are complex and diverse glycans. These molecules are composed of monomers of glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), and often contain fucose (Fuc) and/or *N*-acetylneuraminic acid (NeuAc), linked via several glycosidic bonds (38-39). 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 lactose core is conjugated with repeats of lacto-*N*-biose I (Galβ1-3GlcNAc; LNB; type-1 chain) or *N*-acetyllactosamine (Galβ1-4GlcNAc; type-2 chain), producing molecules with a degree of polymerization larger than 4 (3). These core structures can be modified by fucose and sialic acid residues via different linkages, being fucosylated neutral oligosaccharides the most representative in human milk (7). Although a large number of different HMO structures have been determined, a few isomers can represent up to 70% of the total molecules (25).

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 in intact form (6, 9, 15). During their transit HMO are believed to prevent pathogen colonization, by serving as decoy binding sites for epithelial glycans (24).

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 (28, 40). This suggests that both pro- and antimicrobial elements in breast-

milk account for these differences. A conceptual basis for co-evolution 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. infantis)* ATCC 15697 these mechanisms include oligosaccharide transporters and intracellular glycosyl hydrolases (GH) such as fucosidases, hexosaminidases and sialidases (10).

Bifidobacterium breve, *B. infantis*, *B. longum* subsp. *longum (B. longum)*, and *B. bifidum* are the species most frequently detected in breast-fed infant feces (2, 28). In general, *B. breve* and *B. infantis* are more exclusively found in infants, and *B. longum* and *B. bifidum* are found in both infants and adults. While several strains of *B. bifidum* and *B. infantis* have been shown to grow vigorously on HMO *in vitro* (17, 34), this phenotype has been largely unexplored for larger numbers of *B. breve* and *B. longum* isolates. Only one strain of *B. breve*, ATCC 15700, has been evaluated for its adaptations to HMO, and it has been indicated that it utilizes a far less diverse range of HMO isomers than *B. infantis* ATCC 15697 (31). 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.

MATERIAL 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 in -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 (ID: NCT01817127).

Microbial isolations. For isolation of *Bifidobacterium*, 100 mg of each fecal sample was taken aseptically, transferred to a sterile tube, diluted tenfold 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 BSM agar (mBSM). Modified BSM agar was prepared by supplementing de Man Rogosa Sharpe (MRS) media (Becton Dickinson, Sparks, MD) with 15 g/L agar, 500 mg/L L-cysteine-HCl, 100 mg/L mupirocin, 25 mg/L kanamycin, 4.28 mg/L polymixin B, 25 mg/L iodoacetate, 20 mg/L nalidixic acid and 25 mg/mL of 2,3,5-triphenyltetrazoliumclhoride (Sigma). The plates were incubated for 48 h at 37 ºC 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 ºC 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 Microorganism (RIKEN BioResource Center, Japan), the American Type Culture Collection (Manassas, VA), and the University of California-Davis Viticulture and Enology Culture Collection (Table S1).

Identification of bifidobacteria by 16S rRNA gene sequencing. Genomic DNA was obtained from 1 ml of each culture, and centrifuged for 5 min at 2000 x *g*. The bacterial pellet was resuspended and incubated for 30 min at 37 °C with enzymatic lysis buffer 20 mM Tris-Cl pH 8.0, 2 mM sodium EDTA, 1.2% Triton X-100, and 40 mg/ml lysozyme (Sigma, MO). After enzymatic lysis, bacterial DNA was isolated from the samples using the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer instructions. DNA quality and yield was 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'-AGAGTTTGATCCTGGCTCAG and 1492R 5'-TACGGTTACCTTGTTACGA on an Applied Biosystems 2720m Thermal Cycler (Applied Biosystems, Mountain View, CA) (37). One µl of extracted DNA was added to 50 µl reaction mixture containing 50 pmol of primers, 500 mM of each dNTP, 0.1 vol of 10X PCR buffer, 2.5 mM MgCl2, and 1 U AmpliTaq gold polymerase (Applied Biosystems). Amplification mixtures were subjected to 4 min of denaturation at 94 ºC, 30 cycles of 94 ºC for 30 s, 55 ºC for 40 s, and 72 ºC for 1 min 30s, followed by a final extension period of 7 min at 72 ºC. 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, Valencia, CA). 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 7.0

137 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html), and checked by nucleotide-nucleotide
138 BLAST comparison at the NCBI database for species identification.

139 **Multilocus sequence typing (MLST) of strains.** MLST analysis of *B. breve* isolates targeted
140 intragenic regions of seven housekeeping genes *clpC*, *purF*, *gyrB*, *fusA*, *Iles*, *rplB*, *rpoB* were
141 selected based on a previous study (8). PCR amplifications were prepared as above and cycling
142 conditions were optimized for every primer set (Table S2) and consisted of an initial
143 denaturation at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, annealing at 60-67 °C
144 for 30 s, elongation at 72 °C for 60 s, final extension at 72 °C for 7 min, and holding at 4 °C. The
145 PCR products were separated and sequenced as above.

146 Sequencing data for all loci was edited using BioEdit 7.0 and aligned using CLUSTAL W (32).
147 Phylogenetic analysis and concatenations of the sequenced loci were performed using the
148 Molecular Evolutionary Genetic Analysis (MEGA) software version 5 (http://megasoftware.net).
149 Descriptive evolutionary analysis including mol % G + C content, number of polymorphic sites,
150 nucleotide diversity π/site, average number of nucleotide differences k were calculated using
151 DnaSP version 5.10 (Table S3). Allelic sequences were assigned as described previously (5)
152 (Table S4). A minimum evolution tree of the concatenated loci was calculated using MEGA 5.0
153 (Fig. S1).

154 **Glycosyl hydrolase gene sequencing.** α-fucosidases (Blon_2336, Blon_2335, Blon_0248/0426,
155 Blon_0346), α-sialidases (Blon_2348, Blon_0646), and β-hexosaminidase Blon_0459 protein
156 sequences from *B infantis* ATCC 15697 were aligned with homologous sequences from the
157 GeneBank database (Accession numbers showed in Table S5) using Bioedit 7.0 and degenerated
158 primers were designed to amplify conserved regions (Table S6). To further differentiate between
159 Blon_0248 and Blon_0426, strains PCR positive for fucosidase Blon_0248/0426 were also

amplified with the primers designed previously (29) to amplify the complete gene in *B. infantis* ATCC 15697 (Table S6). PCR amplifications were prepared as above with 200 pmol of primers and cycling conditions were optimized for every primer set (Table S6), and consisted of an initial denaturation at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, annealing at 45-55 °C for 30 s, elongation at 72 °C for 60 s, final extension at 72 °C for 7 min; and holding at 4 °C. The resulting amplicons were separated and sequenced as above. *B. infantis* ATCC 15697 and *B. animalis* JCM 10602 were used as positive and negative control strains, respectively.

Bifidobacterial growth *in vitro* on HMO. The 24 *B. breve* strains in Table S1 were tested for growth in the presence of seven different substrates: HMO (36), LNT, lacto-*N*-neotetraose (LNnT), 2'-fucosyllactose (2FL), 3'-fucosyllactose (3FL) (Glycom, Denmark), 3'-sialyllactose (3SL), and 6'-sialyllactose (6SL) (GenChem Inc. Korean). *B. infantis* ATCC 15697, and *B. animalis* JCM 10602 were included as positive and negative control for growth in HMO, respectively. Two µl of each resulting overnight culture were used to inoculate 200 µl of modified MRS medium (mMRS), devoid of glucose and supplemented with 2% (w/v) of each sterile-filtered substrate as the sole carbohydrate source, and another 2 µl inoculated into mMRS without added sugar. The media was supplemented with 0.05% (w/v) 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 ºC in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Cell growth was monitored in real time by assessing optical density (OD) at 600 nm using a BioTek PowerWave 340 plate reader (BioTek, Winoosky, VT) every 30 min preceded by 15 seconds 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

183 respective standard deviation. These calculations were performed as previously described (4).
184 The OD obtained for each strain grown on the different substrates, was compared with the OD
185 obtained in the absence of a sugar source. This difference in OD (ΔOD) was used as a parameter
186 to evaluate each strain's ability for growing on the different substrates.

187 **Glycoprofiling.** Bacterial cultures in mMRS with 2% HMO were collected at the end of the
188 exponential phase and centrifuged at 12000 × *g* for 30 min. In the case of *B. breve* SC95, the
189 samples were collected at three different points in the growth curve, approximately OD_{600nm} =
190 0.2, 0.5 and 0.8. At least two biological replicates were performed in triplicates. Supernatants
191 were filtered using a multiscreen 96-well filtration plate 0.22 µm (Millipore, Billerica, MA) prior
192 to storage at -80ºC. Remaining oligosaccharides were recovered from the supernatants (25 µl)
193 and reduced to their alditol forms with 1M NaBH4 at 65 ºC for 1.5 h. Each replicate was
194 desalted by solid-phase extraction on graphitized carbon cartridges. Salts were removed with 6
195 mL of deionized water and oligosaccharides were eluted with 20% acetonitrile in water (v/v) and
196 with 40% acetonitrile in 0.01% trifluoroacetic acid (v/v). SPE fractions were combined and
197 dried under vacuum. Samples were reconstituted in 100 µL of deionized water and diluted 50-
198 fold for LC-MS analysis.

199 An Agilent high performance liquid chromatography chip time of flight (HPLC-Chip/TOF) mass
200 spectrometer equipped with a capillary pump for sample loading and a nano pump for
201 chromatographic separation was used for HMO analysis. Separation was performed on a
202 microfluidic chip equipped with an enrichment and nano-LC analytical column, both packed
203 with porous graphitized carbon, using a previously described method (33, 39). Briefly, HMO
204 were separated by a 65 min gradient using a binary solvent system consisting of 3%
205 acetonitrile/water in 0.1% formic acid (v/v) solvent A and 90% acetonitrile/water in 0.1% formic

206 acid (v/v) solvent B. HMO were analyzed in positive ion mode, with a mass range between 300-
207 2000 m/z. Agilent's Masshunter software was used for data acquisition and data analysis version
208 B.03.01.

209 HMO monosaccharide composition was determined using accurate mass within ± 20 ppm mass
210 error of theoretically calculated masses. Specific structures were assigned to HMO peaks by
211 matching the reproducible retention time to that reported in previously annotated HMO libraries
212 (33, 38). Percent consumption was calculated using a label-free method, employing the
213 uninoculated HMO pool as an external standard. Total HMO consumption was calculated with
214 respect to the uninoculated control by normalizing the summed abundance of all identified
215 oligosaccharide spectra in ion counts in the bacterial supernatant to that of the control using the
216 following equation:

217 $$\left[1 - \left(\frac{\sum_{i=1}^{n} API\ bacteria\ sample}{\sum_{i=1}^{n} API\ un-incoluated\ control}\right)\right] x\ 100$$

218 where API is absolute peak intensity and n is the number of identified HMO. The consumption
219 of individual HMO species was quantitated in the same manner, in which the absolute peak
220 intensity of a specific HMO structure was normalized to the peak intensity of the corresponding
221 structure in the un-inoculated control.

222 **Gene expression analysis.** The full nucleotide sequences of the genes encoding a GH95 and a
223 GH29 α-fucosidase in the strain *B. breve* SC95 generated were used to design qPCR primers
224 using the primer-BLAST tool at NCBI (Table S6). For relative quantification, the *rnpA* gene
225 protein component of ribonuclease P complex was used as in (26). *B. breve* SC95 was grown as
226 described above in mMRS supplemented with either 2% lactose, 2% HMO or 2% 2FL in a
227 microplate reader, and cultures were taken at mid-exponential phase OD 0.6-1.0. Samples were

228 immediately pelleted at 12000 x g for 1 min and stored in RNA later Ambion. RNA extraction,
229 cDNA conversion and qPCR were performed as described in (14).
230

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 and six colonies of the higher dilution were isolated. We obtained around 500 isolates that were identified at 16S level, and a total of 461 isolates were identified as *Bifidobacterium.* Seven species of bifidobacteria were detected, and the species *longum* was found to be more represented, followed by *B. breve* with 77 strains (Table 1).

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

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. infantis* ATCC 15697. No homology was found to the same

glycosyl hydrolases in *B. bifidum* genomes (data not shown). Based on this, we used degenerate primers to look for genes encoding these GH in the assembled *B. breve* strains (Table 2). All of the *B. breve* strains possessed a gene homologous to β-hexosaminidase Blon_0459 in *B. infantis* (14), an α-fucosidase similar to Blon_2335 in *B. infantis* ATCC 15697 (29) and all strains excepting JCM 7020 possessed an α-sialidase, related to Blon_0646 in *B. infantis* (30). Moreover, five strains possessed a second α-fucosidase, homologous to Blon_0248 in *B. infantis* ATCC 15697 (29) belonging to GH family 29 (Table 2 and Fig. S2).

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 (36), or on individual milk oligosaccharides that represent the chemical diversity of HMO such as neutral non-fucosylated HMO (LNT and LNnT), fucosylated HMO (2FL and 3FL), and sialylated HMO (3SL and 6SL). Growth behavior on HMO (shown in Fig. S3) and maximum OD values obtained were parameters to arbitrarily classify this panel in three groups (Table 2 and Table S7).

In general, a moderate growth on HMO was witnessed for all the strains (Table 2, Fig. S3 and Table S7), 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 growth rate relative to *B. infantis* ATCC 15697 (Fig. 1A and Table S7).

All *B. breve* strains grew on LNT and LNnT to high cell densities and at levels comparable to *B. infantis* ATCC 15697 (Table 2 and Fig. S4, Fig. S5). 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, to a similar extent than *B. infantis* ATCC 15697. Using 3FL as the sole carbon source, only

277 the strains SC95 and JCM 7019 showed minimal growth (Table 2 and Fig. S6). Finally, growth
278 on 3SL and 6SL was only observed for one strain, KA179 (Table 2).

279 **Glycoprofiling of the HMO consumption by *B. breve* isolates.** Based on their growth
280 phenotypes and ability to utilize certain glycans, six strains of *B. breve* were selected to examine
281 the consumption of 22 different oligosaccharides during growth on total HMO. This included
282 strains SC95, SC154, SC568, SC580, ATCC15701, and JCM7019, as well as *B. infantis* ATCC
283 15697 and *B. breve* ATCC 15700 as positive and negative controls respectively. The supernatant
284 was collected at the end of the exponential phase during growth on HMO, and remaining
285 oligosaccharides were purified and reduced, and later detected and quantified by nano
286 HPLC/CHIP TOF MS. Identification of specific oligosaccharide and isomers in this study was
287 aided by two oligosaccharide structures libraries (38-39). The oligosaccharides quantified
288 include the most abundant neutral and sialylated HMO (25), and Table 3 shows their names,
289 masses, chemical structure, and degree of polymerization (DP).

290 Among the six strains selected, total consumption of HMO ranged between 23 and 42%. These
291 values are lower compared to *B. infantis* ATCC 15697 (64% consumption) but clearly higher
292 than *B. breve* ATCC 15700 (Fig. 2A), the only *B. breve* strain glycoprofiled to date ((18-19, 31).
293 Figure 3B presents the consumption of neutral non-fucosylated HMO in more detail showing
294 consumption patterns among the *B. breve* strains were similar. All strains were able to deplete
295 LNT/LNnT from the culture media to a high extent as previously witnessed (31). Among three
296 major hexaoses found in HMO, a preference for lacto-*N*-neohexaose (LNnH) was observed, over
297 lacto-*N*-hexaose (LNH) and para-lacto-*N*-hexaose (p-LNH).

298 In general, the ability of *B. breve* to metabolize fucosylated HMO was lower compared to *B.*
299 *infantis*, which showed high consumption levels for all the HMO tested (Fig. 3A). However,

300 strains SC95, SC154 and SC568 showed a significant consumption of monofucosylated 2FL and
301 LNFPII and difucosylated DFpLNHII and DFLNH-A HMO (Fig. 3A). Interestingly, even
302 though 2FL cannot support the growth *in vitro* of strain SC154 (Table 2), this strain utilizes
303 larger fucosylated HMO.

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

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

316 Finally, to elucidate possible substrate preferences in a *B. breve* strain with high HMO
317 consumption, we monitored the consumption of nine representative oligosaccharides at different
318 points during the fermentation of HMO by strain SC95 (Fig. 5). These HMO correspond to
319 neutral and acid HMO that were consumed at levels higher than 40%. Remarkably,
320 glycoprofiling of early exponential growth revealed that acidic HMO disappeared from the
321 media first, together with LNnH. In contrast, LNT seems to be metabolized first during the mid-
322 exponential phase, and as observed previously is majorly depleted at the end of the growth on

HMO. We also observed that while monofucosylated HMO as 2FL and LNFP II are depleted from the media at the mid-exponential phase and not later, difucosylated HMO appear to be steadily consumed during the three growth points (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 (13). 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 gut community has been supported by several studies, especially in breast-fed infants (2, 28, 40), where this species together with *B. longum* and *B. infantis* can largely outnumber other microorganisms (22, 35). 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, contribute to intestinal colonization of the infant. (21). Some strains of this species are currently studied by their probiotic properties, as for example in the production of conjugated linoleic acid or important immunomodulatory activities. (16, 21, 26).

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. infantis* and *B. bifidum* (19, 34). 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

(18-19, 31). 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 compared to the type strain ATCC 15700. The HMO consumption in *B. breve* is however moderate compared to *B. infantis* ATCC 15697 (Fig. 2A).

Mass spectrometry-based analysis of the HMO remaining after growth provides a detailed representation of the preferences of these strains for different oligosaccharide subsets present in the HMO pool. For example, all strains showed a vigorous growth on LNT and LNnT, and the molecular mass representing both species (709) was the most consumed in pooled HMO. The utilization of LNnT is interesting since this oligosaccharide is not readily fermented by all species of *Bifidobacterium* found in the infant gut (23). Moreover growth on LNnT was shown to enable *B. infantis* to outcompete *Bacteroides fragilis* in a mouse model (20).

HMO with mass 1074 represent approximately 10% of the total (25), and includes three neutral isomers, LNH, LNnH and p-LNH (Table 3). Interestingly, LNnH is the most abundant of the three isomers (39) 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 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.*

373 *infantis* 2FL and fucosylated larger HMO are likely imported by different solute binding proteins
374 (12).

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

379 *B. infantis* ATCC 15697 is currently a model to study genetic adaptations to growth on human
380 milk glycans (11). Several observations here indicate that the mechanisms of HMO consumption
381 in *B. breve* are very similar to *B. infantis*, with a preference to import intact oligosaccharides
382 followed by intracellular degradation, rather than the extracellular degradation observed by *B.*
383 *bifidum* (10). For example, *B. breve* strain ATCC 15700 can quickly deplete LNT from the spent
384 media and the absence of intermediate monosaccharides indicating that this strain imports this
385 substrate (1). Moreover, the GH genes identified in this study lacked signal peptides, suggesting
386 intracellular localization. Finally, the GH gene sequences obtained are homologous to previously
387 characterized enzymes in *B. infantis*, including β-hexosaminidases Blon_0459, two α-
388 fucosidases Blon_2335 and Blon_0248 and an α-sialidase Blon_0646, suggesting a common
389 origin. Further studies will address in more detail the molecular mechanisms that *B. breve*
390 deploys to utilize HMO.

391 In conclusion, these results provide a rationale for the predominance of *B. breve* in the infant
392 intestinal microbiota, improving our understanding about the ecology of this unique
393 environment. The genetic variation of these strains and the strain-dependent character of the
394 HMO utilization, are factors to consider in probiotic and prebiotic studies. Finally, we consider
395 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.

Acknowledgements

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542 **FIGURE LEGENDS**

543 **FIGURE 1**. Growth of *B. breve* on HMO. *B. breve* isolates were inoculated on mMRS medium
544 supplemented with 2% w/v HMO (A) and 2FL (B). *B. infantis* ATCC 15697 was included as a
545 high growth control. Fermentations were carried out in triplicate.

546 **FIGURE 2.** Glycoprofiling of the HMO consumption by selected *B. breve* strains. (A) Total
547 utilization of HMO. Consumption is calculated as the percent difference in HMO between the
548 start and the end of exponential phase. (B) Glycoprofiles of the consumption of neutral non-
549 fucosylated HMO by seven *B. breve* strains. *B. infantis* ATCC 15697 was included as positive
550 control.

551 **FIGURE 3.** Glycoprofiling of the consumption of fucosylated and acidic HMO by select *B.*
552 *breve* strains. Consumption of eight fucosylated HMO (A), and eleven sialylated HMO (B) was
553 calculated. *B. infantis* ATCC 15697 was included as positive control. HMO consumption is
554 represented as the percent difference in HMO between the start and the end of exponential phase.

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

558 **FIGURE 5.** Temporal glycoprofile of the consumption of select neutral and acidic HMO by
559 *Bifidobacterium breve* SC95 at different stages in the exponential phase.

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564 **TABLE 1:** Distribution of isolates of bifidobacteria from breast-fed infants identified by 16S rRNA gene
565 sequencing.

Species	Number of isolates identified
B. longum	297
B. breve	77
B. pseudocatenulatum	45
B. bifidum	22
B. dentium	8
B. adolescentis	7
B. animalis	5

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

	Glycosyl hydrolases[a]				Bacterial growth						
	α-fucosidase		α-sialidase	β-hexosaminidase							
Strain	GH95	GH29	GH33	GH20	HMO[b]	LNT	LNnT	2FL	3FL	3SL	6SL
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	-	-	-	-	-	-	-	-	-	-	-

579 [a] Positive amplification + indicates that the sequence of the PCR product is > 55% homologous at the
580 aminoacid level to the respective GH gene in *B. infantis* ATCC15697.

581 [b]Level of growth was classified as Negative -: Max. OD 600nm <0.200; Low +: OD 0.200-0.550;
582 Moderate ++: OD 0.550-0.750; High +++: OD > 0.750

TABLE 3: Names, structures and masses of human milk oligosaccharides glycoprofiled, adapted from ((38, 39)).

HMO		Name		Mass	Structure	DP
Neutral	non fucosylated	Lacto-*N*-tetraose/Lacto-*N*-neotetraose	LNT/LNnT	709	Galβ1-3GlcNacβ1-3Galβ1-4Glc/Galβ1-4GlcNacβ1-3Galβ1-4Glc	4
		Lacto-*N*-hexaose	LNH	1074	Galβ1-3GlcNacβ1-3[Galβ1-4GlcNacβ1-6]Galβ1-4Glc	6
		Lacto-*N*-neohexaose	LNnH	1074	Galβ1-4GlcNacβ1-3[Galβ1-4GlcNacβ1-6]Galβ1-4Glc	6
		para-Lacto-*N*-hexaose	p-LNH	1074	Galβ1-3GlcNacβ1-3Galβ1-4GlcNacβ1-3Galβ1-4Glc	6
	fucosylated	2'-Fucosyllactose	2FL	490	Fucα1-2Galβ1-4Glc	3
		Lacto-*N*-fucopentaose	LNFPI	855	Fucα1-2Galβ1-3GlcNacβ1-3Galβ1-4Glc	5
			LNFPII	855	Galβ1-3[Fucα1-4]GlcNacβ1-3Galβ1-4Glc	5
		Monofucosyllacto-*N*-hexaose	MFLNHI	1220	Fucα1-2Galβ1-3GlcNacβ1-3[Galβ1-4GlcNacβ1-6]Galβ1-4Glc	7
		Difucosyllacto-*N*-hexaose	DFLNHa	1366	Fucα1-2Galβ1-3GlcNacβ1-3[Galβ1-4[Fucα1-4]GlcNacβ1-6]Galβ1-4Glc	8
			DFLNHb	1366	Galβ1-3[Fucα1-4]GlcNacβ1-3[Galβ1-4[Fucα1-3]GlcNacβ1-6]Galβ1-4Glc	8
			DFLNHc	1366	Fucα1-2Galβ1-3[Fucα1-4]GlcNacβ1-3[Galβ1-4GlcNacβ1-6]Galβ1-4Glc	8
			DFpLNHII	1366	Galβ1-3[Fucα1-4]GlcNacβ1-3Galβ1-4[Fucα1-3]GlcNacβ1-3Galβ1-4Glc	8
Acidic	Sialylated	3-Sialylactose	3SL	635	NeuAcα2-3Galβ1-4Glc	3
		Monosialyllacto-*N*-tetraose	LSTa	1000	NeuAcα2-3Galβ1-3GlcNacβ1-3Galβ1-4Glc	5
			LSTb	1000	Galβ1-3[NeuAcα2-6]GlcNacβ1-3Galβ1-4Glc	5
		Monosialyllacto-*N*-neotetraose	LSTc	1000	NeuAcα2-6Galβ1-4GlcNacβ1-3Galβ1-4Glc	5
		Monofucosylmonosialyllacto-*N*-tetraose	F-LSTc	1146	NeuAcα2-6Galβ1-4GlcNacβ1-3Galβ1-4[Fucα1-3]Glc	5
		Monosialyllacto-*N*-hexaose	S-LNH	1365	Galβ1-3GlcNacβ1-3[[NeuAcα2-6]Galβ1-4GlcNacβ1-6]Galβ1-4Glc	7
			4021a	1365	Galβ1-3[NeuAcα2-6]GlcNacβ1-3[Galβ1-4GlcNacβ1-6]Galβ1-4Glc	7
		Monosialyllacto-*N*-neohexaose	S-LNnHII	1365	[NeuAcα2-6]Galβ1-4GlcNacβ1-3[Galβ1-4GlcNacβ1-6]Galβ1-4Glc	7
		Monofucosylmonosialyllacto-*N*-hexaose	4121a/ 4121b	1511	[NeuAcα2-6]Galβ1-4[Fucα1-3]GlcNacβ1-3Galβ1-4GlcNacβ1-3Galβ1-4Glc/ [NeuAcα2-6]Galβ1-4GlcNacβ1-3Galβ1-4[Fucα1-3]GlcNacβ1-3Galβ1-4Glc	8
			FS-LNHIII	1511	Galβ1-3[Fucα1-4]GlcNacβ1-3[[NeuAcα2-6]Galβ1-4GlcNacβ1-6]Galβ1-4Glc	8
			4121 unknown	1511	Not defined	8

DP: degree of polymerization.

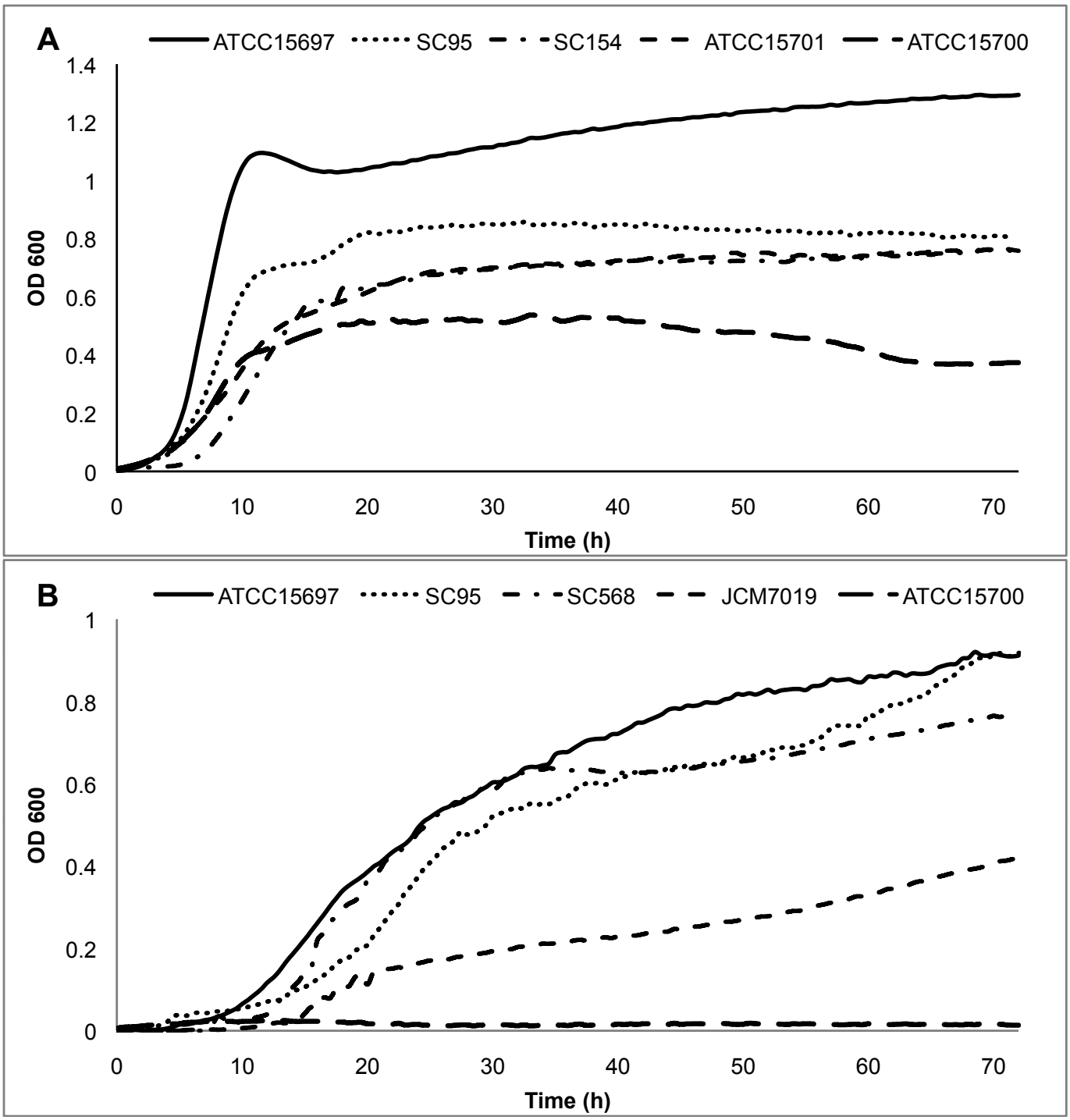
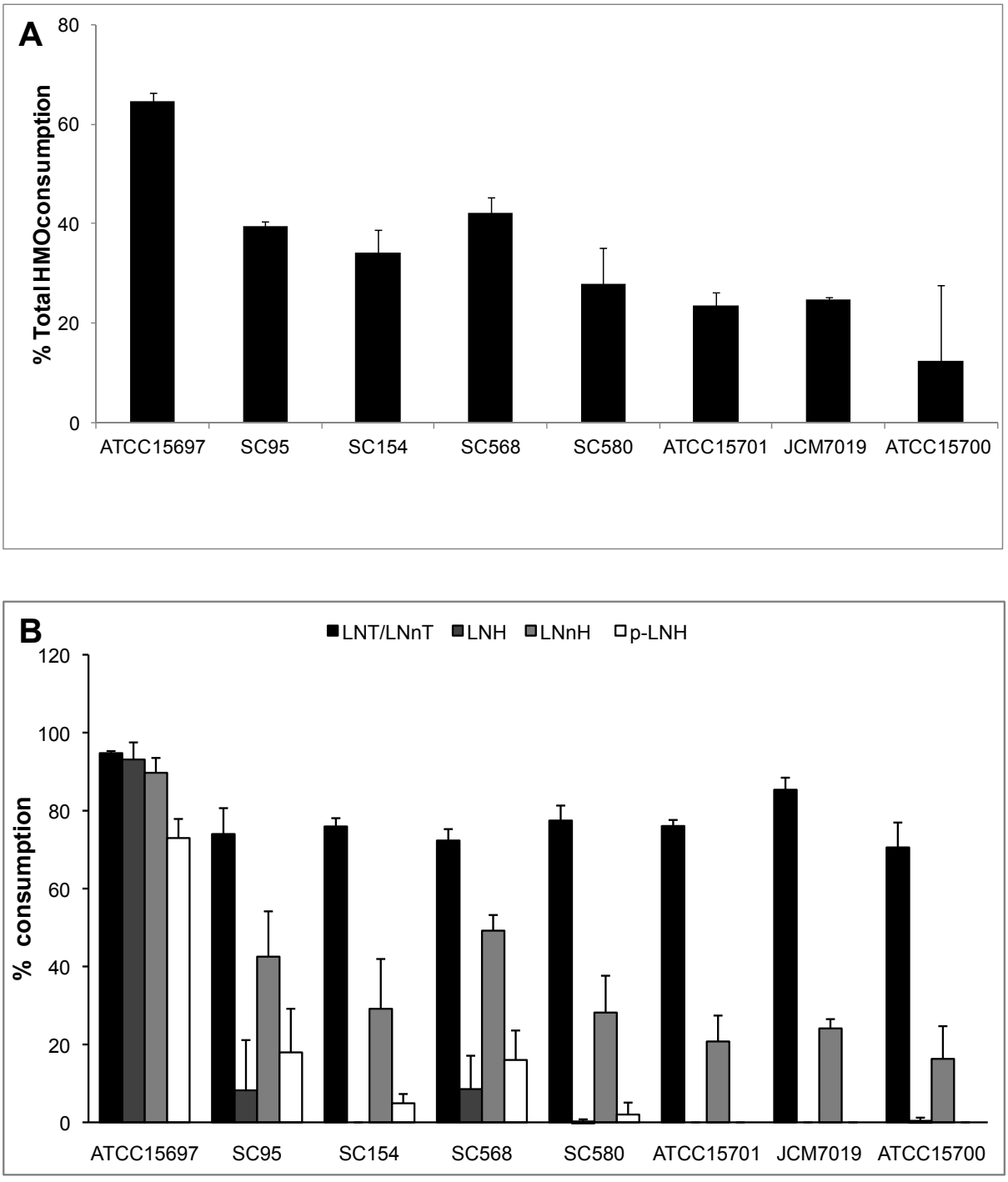


FIGURE 1. Growth of *B. breve* on HMO. *B. breve* isolates were inoculated on mMRS medium supplemented with 2% w/v HMO (A) and 2FL (B). *B. infantis* ATCC 15697 was included as a high growth control. Fermentations were carried out in triplicate.



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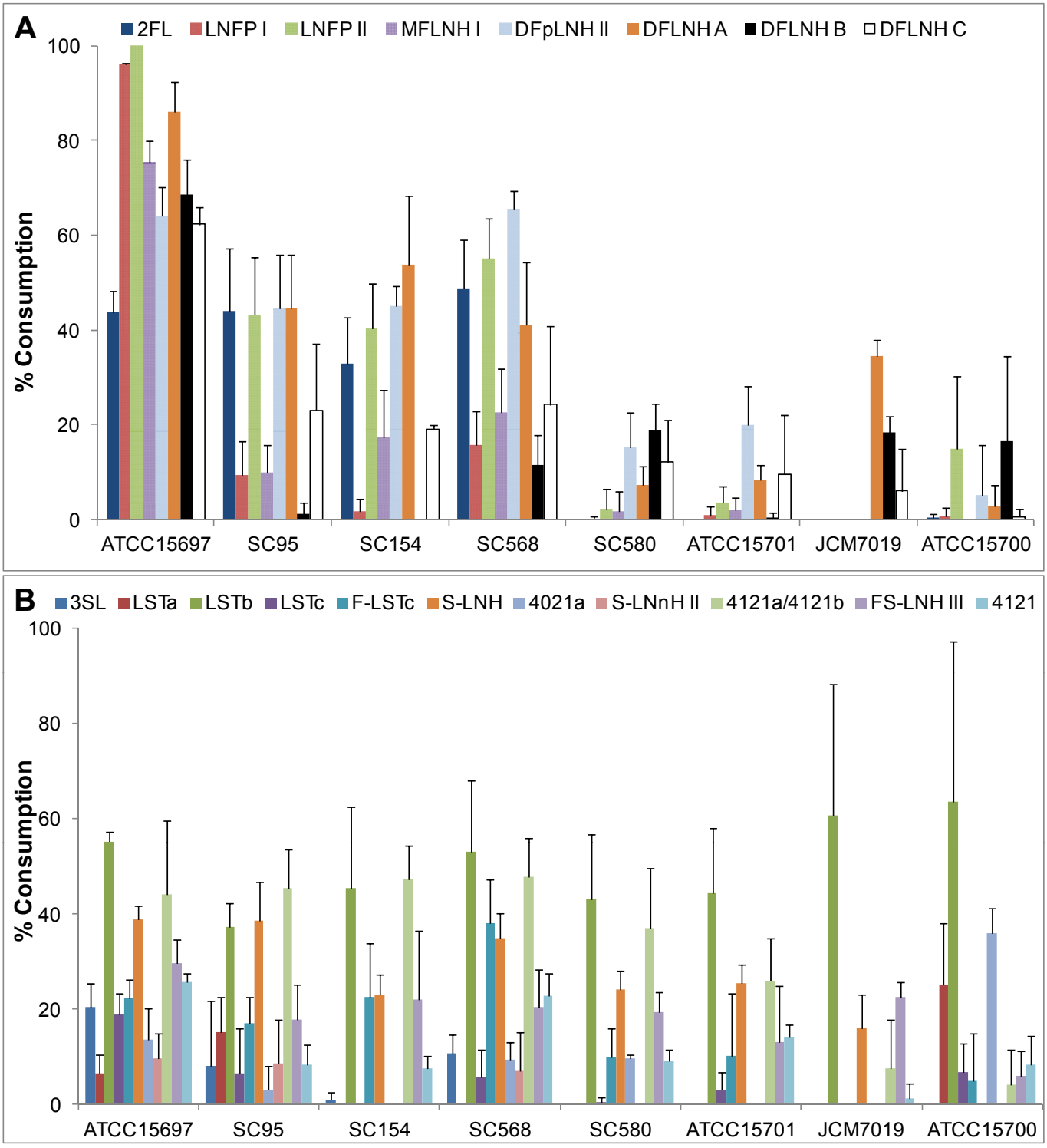

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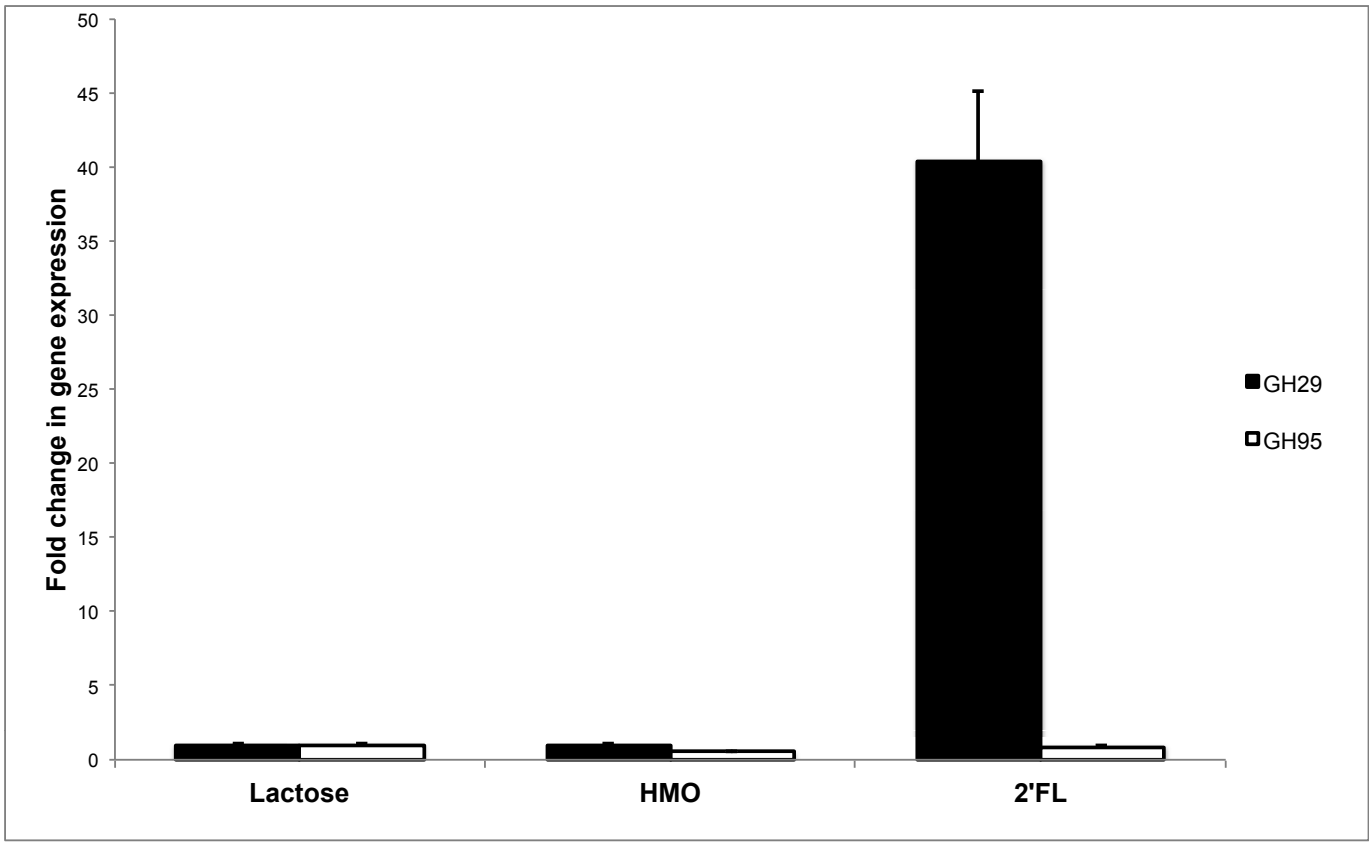

FIGURE 4. Fold in change expression for genes encoding α-fucosidases from GH families 95 and 29 in *B. breve* SC95, during mid-exponential growth on HMO and 2FL. Growth on lactose was used as a control.

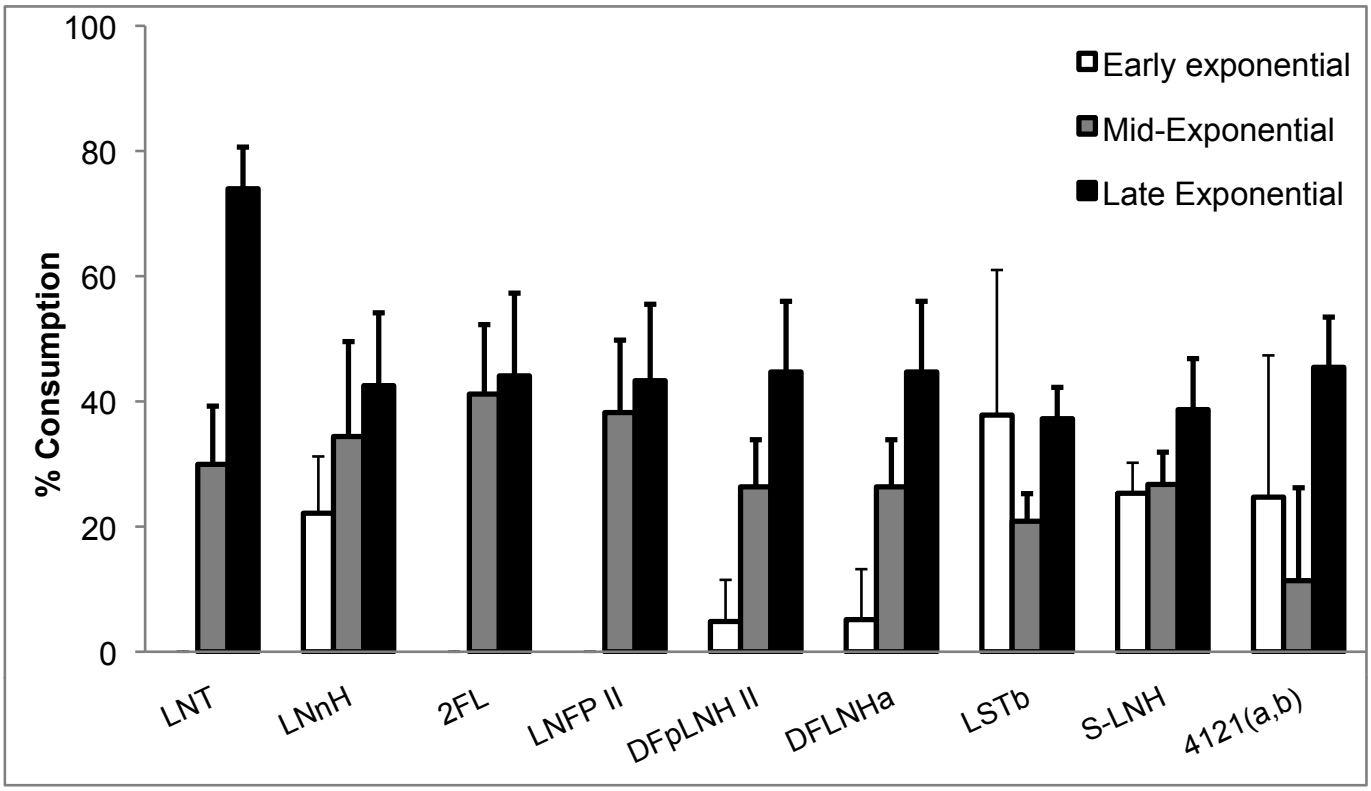

FIGURE 5. Temporal glycoprofile of the consumption of select neutral and acidic HMO by *Bifidobacterium breve* SC95 at different stages in the exponential phase.