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Glycoprofiling Bifidobacterial Consumption of Galacto-Oligosaccharides by Mass Spectrometry Reveals Strain-Specific, Preferential Consumption of Glycans▽†

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Galacto-oligosaccharides (GOS) are versatile food ingredients that possess prebiotic properties. However, at present there is a lack of precise analytical methods to demonstrate specific GOS consumption by bifidobacteria. To better understand the role of GOS as prebiotics, purified GOS (pGOS) without disaccharides and monosaccharides was prepared and used in bacterial fermentation experiments. Growth curves showed that all bifidobacteria assayed utilized and grew on pGOS preparations. We used a novel mass spectrometry approach involving matrix-assisted laser desorption ionization–Fourier transform ion cyclotron resonance (MALDI-FTICR) to determine the composition of oligosaccharides in GOS syrup preparations. MALDI-FTICR analysis of spent fermentation media demonstrated that there was preferential consumption of selected pGOS species by different bifidobacteria. The approach described here demonstrates that MALDI-FTICR is a rapid-throughput tool for comprehensive profiling of oligosaccharides in GOS mixtures. In addition, the selective consumption of certain GOS species by different bifidobacteria suggests a means for targeting prebiotics to enrich select bifidobacterial species.

Galacto-oligosaccharides (GOS) are nondigestible carbohydrates and versatile food ingredients that possess prebiotic properties (1). In addition, other health benefits have been reported to result from consumption of these oligosaccharides, such as stimulation of intestinal mobility and mineral absorption, elimination of ammonium, and colon cancer prevention, as well as protection against certain pathogenic bacterial infections (6, 11, 19).

The physicochemical characteristics of GOS have enabled them to be incorporated in food as prebiotic ingredients. GOS have been of interest in acidic beverages and fermented milk formulations since they exhibit increased thermal stability in acidic environments compared to fructo-oligosaccharides (16, 21). Thus, in the past decade, the applications of GOS in human food products have included dairy products, sugar replacements, diet supplements, and infant formula (11).

Commercial GOS preparations are produced by enzymatic treatment of lactose with β-galactosidases from different sources, such as fungi, yeast, or bacteria, which results in a mixture of oligomers with various chain lengths (1). Thus, the basic structure of GOS includes a lactose core at the reducing end, which is typically elongated with up to six galactose residues. Structural diversity in GOS preparations is dependent on the enzyme used in the transgalactosylation reaction and the experimental conditions used, such as pH and temperature (5).

Considerable effort has been made to understand the effects of GOS in vivo, and most studies have described the impact of GOS on intestinal bacterial population shifts and production of short-chain fatty acids attributed to bacterial fermentation. While there have a been a variety of in vitro studies characterizing the growth of different gut microbes on GOS, the majority of these studies used commercially available preparations of GOS. These commercial preparations contain high concentrations of monosaccharides (i.e., galactose and glucose) and the disaccharide lactose, both of which remain in the product after the transgalactosylation reaction. However, monosaccharides are the preferred substrates for most microorganisms when they are available in a mixed-carbon source (2). Thus, to evaluate growth on GOS, removal of monosaccharides and lactose is helpful (15).

An analytical method currently used to measure GOS in food and feed products is high-pH anion-exchange chromatography (HPAEC) coupled to analysis with a pulse amperometric detector (PAD) (4). Van Laere and coworkers have used this method to monitor GOS fermentation in *Bifidobacterium adolescentis* cultures (20). However, HPAEC-PAD analysis is time-consuming and thus a low-throughput method. More importantly, due to the detector, in HPAEC-PAD analysis there is a differential response to oligosaccharides with higher degrees of polymerization (DP). Thus, new analytical approaches are needed to specifically characterize the consumption of GOS and other prebiotics by probiotic bacteria.

We have previously developed analytical methods employing high-mass-accuracy and high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometry (MS) to

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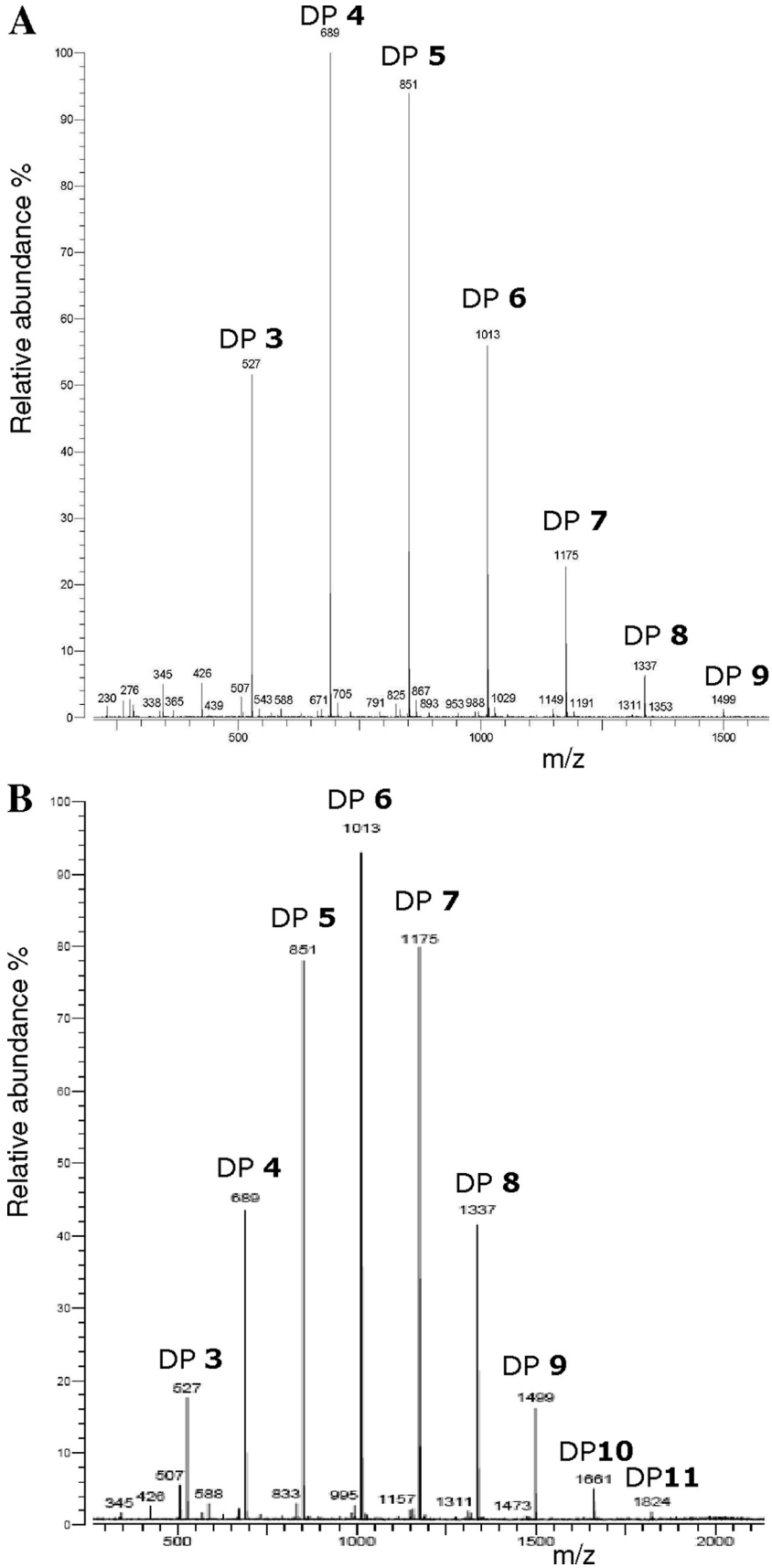

FIG. 1. Positive-mode MALDI-FTICR spectra of GOS syrup obtained under two different experimental conditions. The voltage of the MALDI-FTICR quadruple ion guide was adjusted to detect small (A) or larger (B) oligosaccharides. Major peaks at *m/z* 527, 689, 851, 1013, 1175, 1337, 1499, 1662, and 1824 represent sodium-coordinated ($[M+Na]^+$) GOS with DP ranging from 3 to 11. Minor signals observed at 18 mass units less could correspond to B-type fragments.

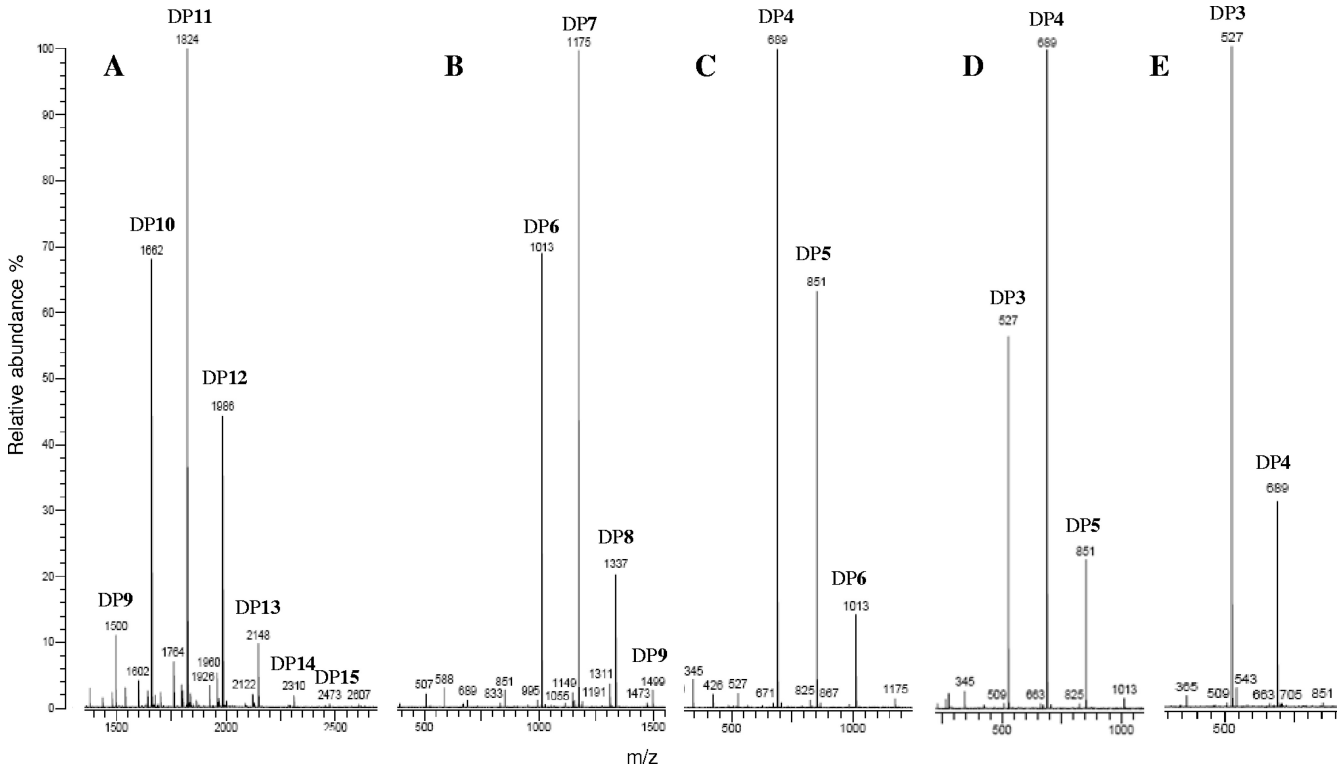

FIG. 2. Positive-mode MALDI-FTICR spectra of GOS Bio-Gel P-2 fractions. (A) Forty-five-milliliter fraction; (B) 56-ml fraction; (C) 67-ml fraction; (D) 74-ml fraction; (E) 82-ml fraction. Signals at *m/z* 527, 689, 851, 1013, 1175, 1337, 1499, 1662, 1824, 1966, 2148, 2310, and 2473 represent sodium-coordinated GOS with DP ranging from 3 to 15.

characterize bacterial consumption of human milk oligosaccharides and fructo-oligosaccharides (9, 10, 14, 17). The matrix-assisted laser desorption ionization (MALDI)–FTICR method is a sensitive and robust analytical method with high-performance capability, and it allows rapid and unambiguous assignment of oligosaccharide signals.

The aims of the present study were to investigate the oligosaccharide composition of GOS syrup preparations using MALDI-FTICR MS, to test lactose-free purified GOS (pGOS) as a sole carbon source in bifidobacterial fermentation experiments, and to determine the pGOS consumption profile by MALDI-FTICR MS. Four major bifidobacterial phylotypes, *B. adolescentis*, *Bifidobacterium breve*, *Bifidobacterium longum* subsp. *infantis*, and *Bifidobacterium longum* subsp. *longum*, were used, and our results demonstrate that there is differential consumption of individual GOS species by various bifidobacteria, which provides a conceptual basis for targeted enrichment of specific bifidobacterial strains using specific GOS fractions.

MATERIALS AND METHODS

Bacterial strains. *B. adolescentis* ATCC 15703, *B. breve* ATCC 15700, and *B. longum* subsp. *infantis* ATCC 15697 were obtained from the American type Culture Collection (Manassas, VA). *B. longum* subsp. *longum* DJO10A was a gift from D. O'Sullivan, University of Minnesota. Analysis of the ATCC 15703, ATCC 15697, and DJO10A genomes was performed with the DOE-JGI Integrated Microbial Genomes platform (12, 13).

GOS purification. pGOS was obtained by purification of Vivinal GOS (Domo Friesland Food). Sugars with DP of less than 2 (including lactose, glucose, and galactose) were removed by Bio-Gel P-2 gel size exclusion chromatography (110 by 2.6 cm; 200/400 mesh; Bio-Rad, Hercules, CA) at room temperature using water as the eluent and a flow rate of 0.16 ml/min. One-milliliter fractions were collected and analyzed by MALDI-FTICR MS. Fractions containing oligosaccharides with DP of ≥3 were pooled for bacterial fermentation experiments. Thin-layer chromatography was performed to confirm that lactose-free pGOS was obtained with a solvent mixture containing acetonitrile and water (8:2, vol/vol). The plate was developed twice at room temperature, dried, and visualized using 0.3% (wt/vol) *N*-(1-naphthyl)-ethylenediamine and 5% (vol/vol) H_2SO_4 in methanol, followed by heating at 110°C for 10 min (8).

Bacterial fermentations. Bifidobacterial cultures were initially propagated on a modified MRS medium supplemented with 1% L-cysteine and 1.5% (wt/vol) lactose as a carbon source as described previously (9). Cultures were inoculated (1%) into medium containing 0.5 to 2% (wt/vol) pGOS as the sole carbon source. Growth studies were carried out in clear, nontreated, 96-well plates (Nalgene Nunc International, Rochester, NY) containing 100 μl of medium per well, and each well was covered with 40 μl of sterilized mineral oil. Incubation was carried out at 37°C, and cell growth was measured by assessing the optical density at 600 nm (OD_{600}) with an automated PowerWave microplate spectrophotometer (BioTek Instruments, Inc., Winoosky, VT) placed inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Each fermentation experiment was performed using biological triplicates. The controls consisted of inoculated medium lacking pGOS and uninoculated medium containing pGOS.

Oligosaccharide purification after fermentation. After cell growth, the residual pGOS was recovered from culture supernatants and purified. Samples (100 μl) were collected 72 h postinoculation and centrifuged at 4,000 × *g* for 10 min. The resulting supernatants were transferred into new tubes, heated at 95°C for 5 min, sterilized with a 0.22-μm Millex-GV filter (Millipore, MA), and stored at −80°C. Oligosaccharides were purified from the supernatants using microcolumns containing 100 μl of Dowex 50WX8 H^+ (Supelco, Bellefonte, PA) on the bottom and 100 μl of C_{18} resins (taken from disposable C_{18} cartridges [Waters, Milford, MA]) on the top. Resins were packed into empty columns (MicroBio-Spin columns; Bio-Rad) with deionized water. Supernatant samples were applied, and pGOS was eluted with 0.3 ml water, dried in a vacuum, and stored at −80°C. Samples were then reconstituted with deionized water to obtain the initial concentration before MS analyses.

MALDI-FTICR MS analysis. All mass analyses were carried out with a ProMALDI-FTICR MS instrument with an external MALDI source, a 355-nm pulsed Nd:YAG laser, a hexapole accumulation cell, a quadrupole ion guide, and a 7.0-T superconducting magnet (Varian/IonSpec, Lake Forest, CA). Tandem MS was performed by using the infrared multiphoton dissociation (IRMPD) method, and a CO_2 laser (10.6 μm; maximum power, 20 W; Parallax, Waltham, MA) was added to the instrument in order to provide infrared photons for the experiments. Malto-oligosaccharides derived from beer were used to calibrate the instrument and as a molecular reference standard for oligosaccharides consisting of hexose residues. One microliter of analyte (GOS and pGOS samples) was spotted onto a 100-well stainless steel sample plate (Applied Biosystems, Foster City, CA) using the "thin-layer" method, and this was followed by addition of 0.2 μl of 0.1 mM NaCl as a dopant and 1 μl of 0.4 M 2,5-dihydroxybenzoic acid (in acetonitrile-water [50%, vol/vol]) as the matrix. The spots were dried under a stream of air before analysis. MALDI-FTICR MS analysis was performed in the positive-ion mode in the *m/z* scan range from 220 to 4500. The ions accumulated in the hexapole and then were transferred to the ion cyclotron resonance cell via the ion guide for excitation and detection. Four acquisitions were recorded for each biological replicate, the values were averaged, and the standard deviations were calculated for each signal. In tandem IRMPD experiments, selected precursor ions were isolated in the ion cyclotron resonance cell and irradiated with photons for 500 ms.

RESULTS

MALDI-FTICR analysis of GOS syrup. To determine the DP of GOS in GOS syrup preparations, samples were diluted and analyzed by MALDI-FTICR MS. Both glucose and galactose, the monomer components of GOS, have an exact residue molecular mass of 162.0528 Da. Exact molecular mass measurement was used to determine the DP of GOS, and the quasimolecular ions were assigned with less than 5-ppm difference between the theoretical and calculated molecular masses. The positive-ion-mode MALDI-FTICR spectrum obtained contained signals at *m/z* 527.16, 689.21, 851.26, 1013.32, 1175.37, 1337.42, and 1499.47, indicating that GOS syrup contains oligosaccharides with DP ranging from 3 to 9 (Fig. 1A). Figure 1B shows the spectrum obtained for the same sample but under slightly different conditions; the voltage of the MALDI-FTICR quadruple ion guide, which works like a molecular mass filter, was adjusted in order to detect oligosaccharides with higher molecular masses. As a result, there was a reduction in the relative abundance of oligosaccharides with lower molecular masses, and signals corresponding to GOS with DP of 10 and 11 (*m/z* 1661.52 and 1823.58) were observed. In addition, when GOS syrup preparations were fractionated in a size exclusion chromatography column, MALDI-FTICR analysis of Bio-Gel P-2 excluded fractions showed that GOS mixtures contained oligomers with DP up to 15 (*m/z* 1985.63, 2147.68, 2309.73, and 2471.79) (Fig. 2A).

Tandem MS analysis of select oligosaccharides was carried out using the IRMPD method. The IRMPD mass spectra of GOS with DP of 5, 4, and 3 are shown in Fig. 3A to C. Tandem MS analysis yielded a mixture of fragments ions corresponding to glycosidic bond cleavages (losses of 162 Da) that allowed verification of the oligosaccharide composition. In addition, fragment ions corresponding to cross-ring cleavages, shifted in 60, 90, and 120 Da, from all parent ions were also abundant.

GOS purification. To better understand bifidobacterial growth behavior, GOS syrup was fractionated, and the monosaccharides (glucose and galactose) and disaccharides (including lactose and GOS with a DP of 2) were separated from the larger oligosaccharides by size exclusion chromatography. Fractions were collected and analyzed by using MALDI-FTICR, and the DP of oligomers that eluted in each fraction were displayed (Fig. 2A to E). Disaccharide- and monosaccharide-free fractions were confirmed by thin-layer chromatography (data not shown) and pooled based on the desired DP. Figure 4 shows a MALDI-FTICR mass spectrum of pGOS

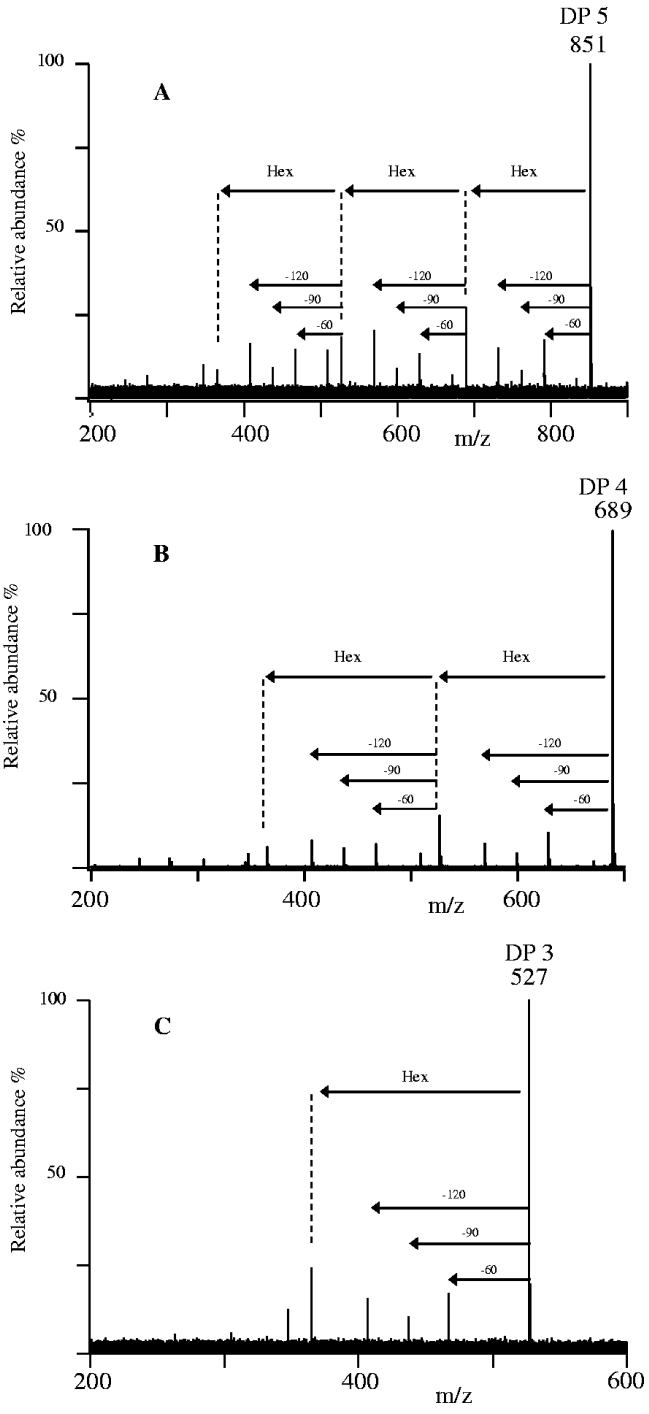

FIG. 3. IRMPD MALDI-FTICR spectra of GOS. (A) GOS with a DP of 5; (B) GOS with a DP of 4; (C) GOS with a DP of 3. Fragment ions corresponding to glycosidic bond cleavages (Hex) and cross-ring cleavages (60, 90, and 120) were obtained.

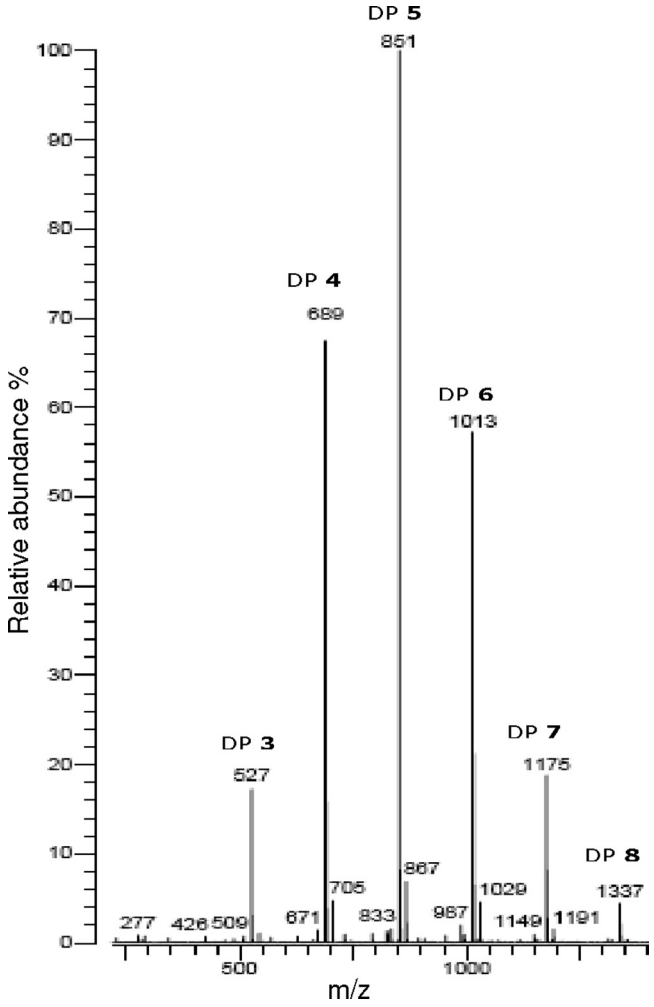

FIG. 4. Positive-mode MALDI-FTICR spectrum of pGOS with selected DP used in bifidobacterial fermentation experiments.

preparations used in fermentation experiments indicating the presence of oligosaccharides with DP ranging from 3 to 8.

pGOS fermentations. We recently developed a microplate protocol for growing bifidobacteria on human milk oligosaccharide, followed by MALDI-FTICR analysis to determine the specific oligosaccharide species consumed (10). In this work we used a similar approach to examine pGOS fermentation with cells grown anaerobically using a 96-well plate format. Four bifidobacterial strains were tested: *B. breve* ATCC 15700 and *B. longum* subsp. *infantis* ATCC 15697, which were isolated from infant feces, and *B. adolescentis* ATCC 15703 and *B. longum* subsp. *longum* DJO10A, which were isolated from adult feces. All bifidobacteria assayed were able to utilize and grow on pGOS at the four concentrations tested (0.5%, 1%, 1.5%, and 2%, vol/vol) (Fig. 5A to D; see Fig. S1 in the supplemental material); however, different pGOS growth phenotypes were observed for the various strains assayed. *B. longum* subsp. *infantis* ATCC 15697 produced the highest cell density with all four pGOS concentrations tested (OD_{600}, 1.2). *B. longum* subsp. *longum* DJO10A produced the lowest biomass with 0.5% pGOS (OD_{600}, 0.4); however, the biomass

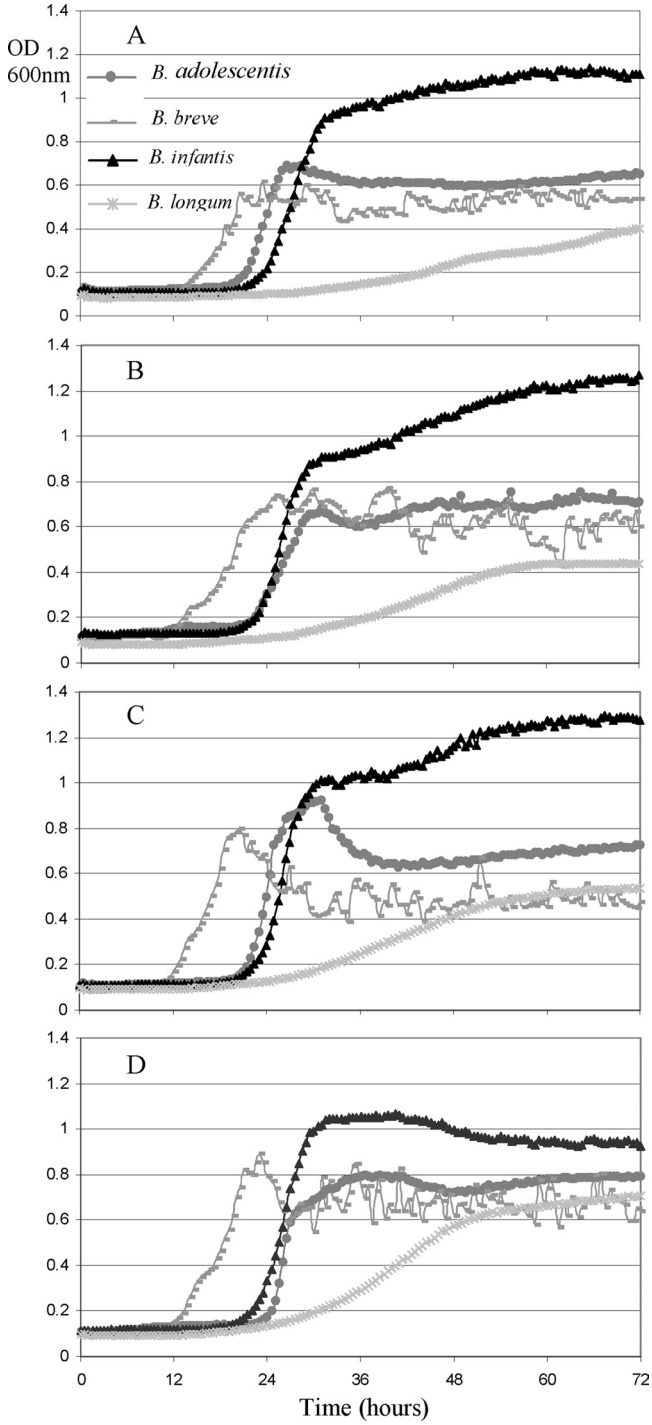

FIG. 5. Growth curves of *B. adolescentis* ATCC 15703, *B. breve* ATCC 15700, *B. longum* subsp. *infantis* ATCC 15697, and *B. longum* subsp. *longum* DJO10A for growth on modified MRS containing (A) 0.5% (wt/vol) pGOS, (B) 1% (wt/vol) pGOS, (C) 1.5% (wt/vol) pGOS, and (D) 2% (wt/vol) pGOS. Growth studies were carried out in triplicate, and a representative data set is shown. Erratic curves for *B. breve* were observed when it began to aggregate.

obtained with higher pGOS concentrations was similar to the biomass obtained for the *B. adolescentis* and *B. breve* strains. There were also lag phase differences between the strains; however, we have found that the level of biomass formed

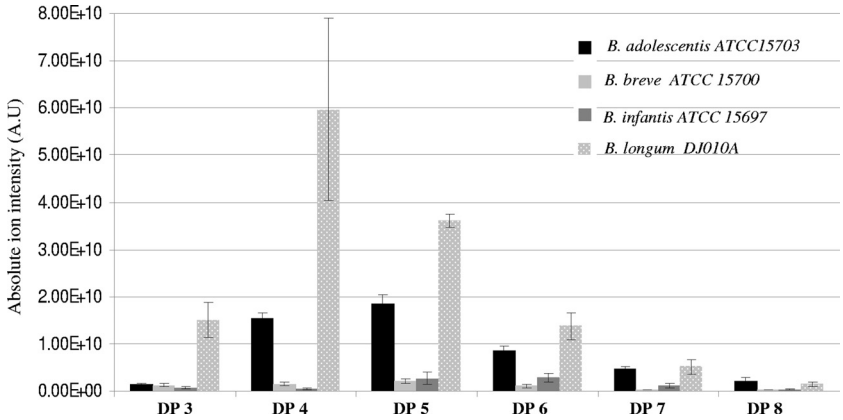

FIG. 6. Absolute ion intensities of pGOS remaining after bacterial fermentation obtained from MALDI-FTICR mass spectra. Four spectra were acquired and averaged for each biological replicate. The error bars indicate standard deviations. A.U, arbitrary units.

(OD_{600}) is a more reliable indicator of fermentation efficiency, and lag phase differences between replicates can be more variable, most likely due to the physiological status of the inoculum (data not shown).

pGOS consumption determined by MALDI-FTICR MS. To further understand the bifidobacterial pGOS fermentations, the remaining oligosaccharides in culture supernatants were recovered 72 h postinoculation, purified, and analyzed using MALDI-FTICR MS. The absolute ion intensities for the remaining pGOS from bifidobacterial cultures containing 0.5% pGOS are shown in Fig. 6. *B. breve* ATCC 15700 and *B. longum* subsp. *infantis* ATCC 15697 were found to be the most efficient strains for pGOS consumption (Fig. 6; see Fig. S2 in the supplemental material). Although the results were slightly different, the *m*/*z* 689, 851, 1013, 1175, and 1337 signals were strongly reduced in both samples, indicating that there was significant consumption of pGOS with DP ranging from 4 to 8. Remarkably, the *m*/*z* 689 signal, corresponding to tetrasaccharides, was almost absent following fermentation by *B. longum* subsp. *infantis*, demonstrating that there was preferential consumption of pGOS with a DP of 4 by this strain. Like *B. breve*, *B. longum* subsp. *infantis* ATCC 15697 also showed an important reduction in the signal corresponding to oligosaccharides with a DP of 3. Similarly, *B. adolescentis* ATCC 15703 showed a significant decrease in the *m*/*z* 527 signal, indicating that there was consumption of GOS with a DP of 3. Although for *B. adolescentis* the signals corresponding to longer oligosaccharides were not greatly altered, some consumption of oligosaccharides with DP of 4 and 5 were evident (Fig. 6). *B. longum* subsp. *longum* DJO10A was the least efficient strain in terms of GOS utilization. Unlike the other strains tested, signals corresponding to remaining oligosaccharides coordinating K^+ were the main signals observed (see Fig. S2 in the supplemental material). The relative abundance of remaining pGOS with DP of 3 and 4 did not change, indicating that there was no consumption of these oligosaccharides. Conversely, oligosaccharides with DP of 5 and 6 were consumed to some extent, and there was a minor reduction in the relative abundance of molecular masses corresponding to DP of 7 and 8. The comparative analysis of the mass spectra obtained clearly showed that there are specific oligosaccharide preferences in the utilization of pGOS, indicating that the bifidobacteria assayed have different fermentative capacities.

DISCUSSION

GOS structures are highly variable and dependent on the enzyme and conditions used during their synthesis (5, 7, 23). Previous work suggested that the upper limit for the DP for GOS is 8 (11). However, MALDI-FTICR MS analysis of GOS syrup clearly demonstrated that there were oligosaccharides with DP of up to 15. This increase in resolution of the oligosaccharides with higher DP is likely due to the superior sensitivity and capabilities of FTICR MS compared with the high-performance liquid chromatography and nuclear magnetic resonance techniques used previously for GOS analysis (5, 7, 20). Similar results were reported for plant-derived fructo-oligosaccharides when they were analyzed by MALDI-FTICR MS (17).

The efficacy of prebiotics for promoting human health has been strongly related to their chemical structure (3). It has been shown that GOS with DP of 2 and 3 can have different isomeric structures (5, 7). Tandem MS analysis performed with oligosaccharides with DP of 3, 4, and 5 yielded cross-ring cleavage fragment ions that have been described for oligosaccharides containing β(1-6) linkages or β(1-3) linkages (22). The types of fragment ions observed in each IRMPD mass spectrum suggest that mixtures of GOS isomers with DP of 3, 4, and 5 are present and highlight the need for further studies in order to accurately identify them.

In the present work we report the in vitro growth behavior of different bifidobacterial strains on disaccharide- and monosaccharide-free fractions of GOS (pGOS). While all the strains tested were able to grow on the pGOS substrate, the specific growth rate and biomass produced varied by strain. To date, GOS consumption with specific DP preferences has been determined only for *B. adolescentis* DSM 20083 using HPAEC-PAD (20). Thus, to profile and compare pGOS consumption before and after bacterial fermentation, an MS-based approach was used. MALDI-FTICR MS analysis of conditioned media demonstrated that there were strain-specific bifidobacterial preferences for pGOS utilization and that each bi-

fidobacterial strain tested had DP-specific preferences. In general, the infant-borne isolates (*B. longum* subsp. *infantis* and *B. breve*) were able to consume the GOS species with DP ranging from 3 to 8 more efficiently, while *B. adolescentis* and *B. longum* subsp. *longum* exhibited more diffferential consumption of select DPs. While the consequences of these preferences for competitive behavior in the gut remain to be determined, this work demonstrated the utility of MALDI-FTICR MS analysis for comprehensive profiling of oligosaccharide species in GOS mixtures and identified consumption preferences for various bacterial species and strains.

The genetics underlying bifidobacterial GOS utilization are diverse and may result in the different consumption glycoprofiles observed here. GOS oligomers are degraded to galactose and glucose in a catabolic reaction that requires a β-galactosidase activity (EC 3.2.1.23). Analysis of the genome of *B. adolescentis* ATCC 15703 has revealed the presence of 10 genes with a putative β-galactosidase function (18), while *B. longum* subsp. *infantis* ATCC 15697 and *B. longum* subsp. *longum* DJO10A contain 7 and 3 such genes, respectively (see Table S1 in the supplemental material). All 20 enzymes are predicted to be intracellular as they lack transmembrane helices or signal peptides. At the moment, however, it is not clear if the different GOS consumption phenotypes are attributable to concerted activities of individual or multiple β-galactosidase isozymes in these strains.

While further studies are needed to clarify the health-promoting effect of GOS consumption in humans, the selective bacterial consumption of GOS described here highlights the potential for rational design and development of specific prebiotics which can target enrichment of select bifidobacterial phylotypes.

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