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In Vitro Fermentation of Breast Milk Oligosaccharides by *Bifidobacterium infantis* and *Lactobacillus gasseri*

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It has been proposed that human milk oligosaccharides (HMO) function as a prebiotic for bifidobacteria, yet this activity has not been adequately investigated. In this study, *Bifidobacterium infantis* was shown to ferment purified HMO as a sole carbon source, while another gut commensal, *Lactobacillus gasseri*, did not ferment HMO. Our results support the hypothesis that HMO selectively amplify bacterial populations in the infant intestine.

Human milk is unique because of the high concentration and diversity of human milk oligosaccharides (HMO). HMO are the third most abundant component of human milk (9), and at least 130 different masses have been identified (14). In vitro, HMO are resistant to catabolism by host hydrolases (3, 4), and based on the mass balance between consumption and excretion, Chaturvedi et al. (1) calculated that 97% of HMO pass through infants undigested, while Coppa et al. (2) estimated that 40 to 50% of HMO pass through infants undigested.

A prebiotic function was attributed to HMO based on studies done in the 1950s with *Bifidobacterium bifidus* subsp. *pennsylvannicus* (6, 7), yet the measurements were based on growth enhancement, not fermentability, as the media contained lactose. Direct fermentation of HMO by *Bifidobacterium* spp. and/or *Lactobacillus* spp. has not been demonstrated yet, and there are many questions about the metabolic fate of these molecules. Do subpopulations mediate specific functions, such as pathogen binding or prebiotic activity? Are specific bacterial species required for catabolism in the gut? The goals of this investigation were to determine the fermentability of HMO by two representative species of breast-fed infant microbiota, *Bifidobacterium infantis* and *Lactobacillus gasseri* (8, 10) and to characterize the changes in the HMO after bacterial growth. Determination of the biological basis underlying the HMO abundance in human milk should be of general interest in human nutrition (15).

Pooled milk was provided by the Mother's Milk Bank of San Jose, CA, and the Mother's Milk Bank of Austin, TX. Oligosaccharides were extracted as described by Gnoth et al. (5), with modifications. One liter of milk was centrifuged at 5,000 × *g* for 30 min at 4°C, and the fat was removed. Ethanol (2 liters) was added, and the solution was incubated overnight at 4°C. The precipitate was removed by centrifugation at 5,000 × *g* for 30 min at 4°C, and the solvent was removed by rotary evaporation. The concentration of the solution was adjusted to 0.05 M with phosphate buffer (pH 6.8), 3,000 U β-galactosidase (*Kluyveromyces fragilis*) was added, and the solution was incubated for 1 h at 37°C. Then the solution was extracted with 4 volumes of chloroform-methanol (2:1, vol/vol), and the aqueous layer was collected.

As described by Redmond and Packer (12), monosaccharides and disaccharides were removed by selective adsorption of HMO, using solid-phase extraction with graphitized nonporous graphitized carbon cartridges (Supelco Inc., Bellefonte, PA). The oligosaccharides that were retained were eluted with water-acetonitrile (60:40) containing 0.01% trifluouroacetic acid. The residual lactose and glucose contents in the eluant were determined enzymatically (R-Biopharm, South Marshall, MI).

The bacteria used were *B. infantis* ATCC 15697 and *L. gasseri* ATCC 33323. The medium used was L-cysteine-supplemented MRS with either 2% (wt/vol) glucose, 2% (wt/vol) inulin, or 2% (wt/vol) HMO carbohydrate. Bacteria were grown in triplicate cultures in 7.5 ml of broth in screw-cap culture tubes (13 by 100 mm) at 37°C. Sterile filtered carbohydrate was added to autoclaved media, and tubes were inoculated with a sterile loop from frozen stock preparations. The negative controls included inoculated medium lacking carbohydrate and uninoculated medium. Optical density was measured with a Klett-Summerson colorimeter (Klett Manufacturing Co., Inc., New York, NY) using a no. 45 (green) filter. After growth, tubes were centrifuged at 2,000 × *g* for 30 min. The supernatant was collected and used for analysis.

Mass spectrometry (MS) was performed with a HiRes matrix-assisted laser desorption ionization—Fourier transform MS (MALDI-FTMS) instrument (IonSpec Corp., Irvine, CA). Media (100 μl) containing HMO were concentrated using nonporous graphitized carbon cartridge solid-phase extraction columns and were eluted with 20% acetonitrile in water. The eluants were dried and reconstituted in 40 μl deionized water. For analysis, 2 μl of a sample was spotted with 2 μl of 0.4 M 2,5-dihydroxybenzoic acid and 1 μl of 0.01 M NaCl.

Separation of HMO from lactose and monosaccharides was confirmed by thin-layer chromatography (data not shown). From 1 liter of milk, the yield was 2.5 g of solids. Glucose and lactose accounted for <2% of the total carbohydrate. The protein and fat contents were not measured.

The growth assay was suitable for fermentation studies as

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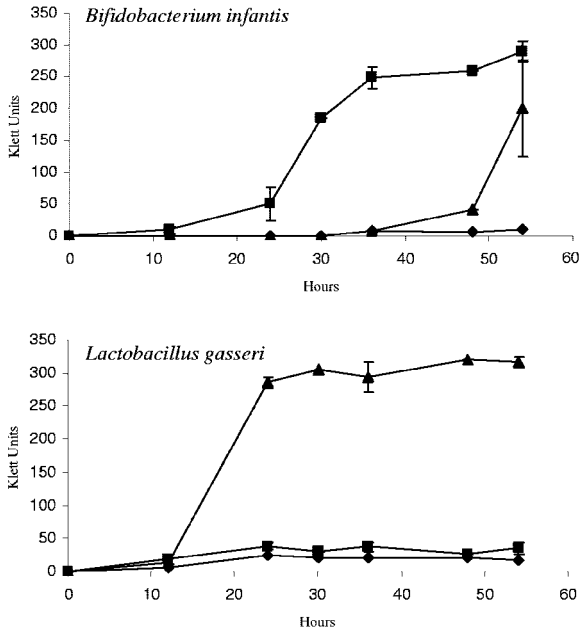
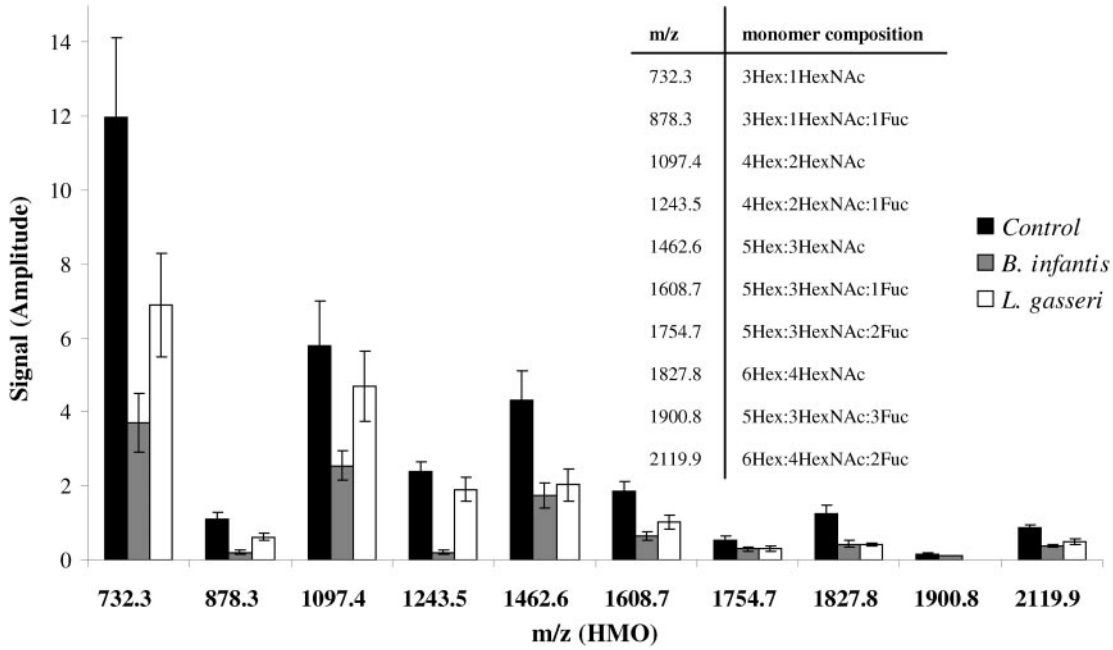


FIG. 1. Growth of *B. infantis* ATCC 15697 and *L. gasseri* ATCC 33323 on glucose (▲), HMO (■), and inulin (◆). The initial pHs of the MRS broth media were 6.13 (HMO), 6.44 (inulin), and 6.47 (glucose). The final pHs for *B. infantis* were 4.63 (HMO), 5.05 (glucose), and 6.46 (inulin). The final pHs for *L. gasseri* were 5.83 (HMO), 3.94 (glucose), and 6.13 (inulin).

there was very little growth without added carbohydrate. Figure 1 shows growth curves for both bacterial species on all three substrates. The final pHs are indicated in the figure legend and in general are consistent with the change in optical density resulting from fermentation. *B. infantis* (Fig. 1, top panel) grew best on the HMO and began exponential growth on glucose only after 40 h. *L. gasseri* (Fig. 1, bottom panel) grew only on glucose. It was surprising that neither bacterium fermented inulin, as this compound is a well-documented substrate for bifidobacteria (13). However, the degree of polymerization of the inulin used in this study was about 30, and Perrin et al. (11) found that *B. infantis* metabolized <5% of the inulin when the degree of polymerization was >27.

Figure 2 shows the mass spectrometry data for both bacteria and the control. These data indicate that the reduction in HMO with *B. infantis* was more pronounced than the reduction in HMO with *L. gasseri*. The change in the pH of the HMO-containing media after *L. gasseri* growth was slight, and the low cell density observed indicated that this strain did not ferment much of the HMO. However, the analytes with *m*/*z* 1462 and *m*/*z* 1827 were clearly different in the control and *L. gasseri* media. At least two explanations could account for this observation. First, MALDI-FTMS detects intact masses, and it is possible that *L. gasseri* partially catabolized these analytes without fermentation of the resulting monosaccharides. Alternatively, it is also possible that these analytes were selectively adsorbed to the *L. gasseri* cells that were present in the media, which were then removed prior to analysis.

HMO polymers are composed of five monosaccharides linked by at least 12 different glycosidic bonds, and the degree of polymerization ranges from 3 to 32. Consequently, complete catabolism of these molecules should require an extensive set of glycosidases and membrane transporters. The increase in the cell density of *B. infantis*, coupled with the reduction in the pH and the disappearance of HMO species in the spent media, indicates some of the HMO were fermented. Based on the results of this work, it appears that *B. infantis* has at least some



m/z	monomer composition
732.3	3Hex:1HexNAc
878.3	3Hex:1HexNAc:1Fuc
1097.4	4Hex:2HexNAc
1243.5	4Hex:2HexNAc:1Fuc
1462.6	5Hex:3HexNAc
1608.7	5Hex:3HexNAc:1Fuc
1754.7	5Hex:3HexNAc:2Fuc
1827.8	6Hex:4HexNAc
1900.8	5Hex:3HexNAc:3Fuc
2119.9	6Hex:4HexNAc:2Fuc

FIG. 2. Results of MALDI-FTMS analysis of HMO in control and spent media. The inset shows the constituent monosaccharides for the HMO species detected. Hex, hexose; HexNAc, hexosamine; Fuc, fucose.

of the enzymes necessary for catabolism of HMO. While our results support the hypothesis that HMO may function as a prebiotic, validation of this hypothesis requires demonstration that these compounds are not metabolized by other genera. If this is the case, the differential detection of HMO in the feces of breast-fed infants may be a function of the composition of the infant microflora.

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