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# Hydrolysis of milk gangliosides by infant-gut associated bifidobacteria determined by microfluidic chips and high-resolution mass spectrometry

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## Abstract

Gangliosides are receiving considerable attention because they participate in diverse biological processes. Milk gangliosides appear to block pathogen adhesion and modify the intestinal ecology of newborns. However, the interaction of milk gangliosides with gut bifidobacteria has been little investigated. The digestion products of a mixture of gangliosides isolated from milk following incubation with six strains of bifidobacteria were studied using nanoHPLC ChipQ-TOF MS. To understand ganglioside catabolism *in vitro*, the two major milk gangliosides—GM3 and GD3—remaining in the media after incubation with bifidobacteria were quantified. Individual gangliosides were identified through post-processing precursor ion scans, and quantitated with the "find by molecular feature" algorithm of MassHunter Qualitative Analysis software. *B. infantis* and *B. bifidum* substantially degraded the GM3 and GD3, whereas *B. longum* subsp. *longum* and *B. animalis* subsp. *lactis* only showed moderate degradation. MALDI FTICR MS analysis enabled a deeper investigation of the degradation and identified ganglioside degradation specifically at the outer portions of the glycan molecules. These results indicate that certain infant gut-associated bifidobacteria have the ability to degrade milk gangliosides releasing sialic acid, and that these glycolipids could play a prebiotic role in the infant gut.

### Keywords

Bifidobacteria; Gangliosides; Mass spectrometry; Nano liquid chromatography

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## 1 Introduction

The oligosaccharides in milk and their glycoconjugates have gained considerable attention because of their biological importance, including prebiotic activity [1, 2]. These heterogeneous glycans consist of various monosaccharides, e.g., glucose, galactose, *N*-acetylglucosamine, and frequently, fucose and/or *N*-acetylneuraminic acid (Neu5Ac or sialic acid) residues assembled via glycosidic linkages. Milk glycans are minimally affected by gastric enzymes as they transit through the stomach and small intestine, but instead they can deploy pathogen binding to the intestinal epithelium, and be utilized as a carbon source by commensal bacteria in the gut [3, 4]. These glycans can be divided into two groups, neutral and anionic, depending on the existence of anionic sialic acid residues in the molecule. Both neutral and anionic milk oligosaccharides play an important role in the enrichment of gut bifidobacteria in breast-fed infants [5–7].

Although the high concentrations of free oligosaccharides in milk can explain a substantial part of their prebiotic effect, glyconjugates such as glycolipids and glycoproteins might contribute to this function due to their carbohydrate moieties[8]. Acidic glycolipids (gangliosides) are typically elongated from a lactose core (Galβ1-4Glc) and contain at least one sialic acid. Ganglioside synthesis occurs in a stepwise fashion, with an glucose and galactose added to ceramide lipid and then subsequent sugars transferred by glycosyltransferases from nucleotide sugar donors. The most abundant gangliosides in human and bovine milk are monosialoganglioside GM3 (Neu5Acα2-3Galβ1-4GlcβCer) and disialoganglioside GD3 (Neu5Acα2-8Neu5Acα2-3Galβ1-4GlcβCer) [9]. Milk gangliosides exist in the milk fat globule membranes, which are degraded by diverse lipases in the gastrointestinal tract, releasing lipids that are readily absorbed into the cells that line the small intestine. However, only a small proportion of gangliosides seem to be absorbed after oral ingestion [10]. In contrast, Park *et al.* [11] reported that a ganglioside-enriched diet increased the content of total gangliosides in rat intestinal mucosa, plasma, and brains. However, the degree of ganglioside bioavailability has not been well investigated. A recent study showed that the bioaccessibility of gangliosides from human milk is rather low [12]. Therefore, their presence in human milk seems to correlate with a biological role in the gastrointestinal tract [13]. Therefore, the interaction between gangliosides and gut bacteria would seem to be biologically important.

A few studies showed the interaction between glycolipids and infant intestinal microbiota. Larson et al. [14] first reported glycolipid excretion in the feces of newborn and young children who were fed breast milk. They subsequently showed that extracellular glycosidases from several gut bacteria degraded intestinal glycolipids [15, 16]. Rueda et al. [17] showed in clinical studies that the addition of gangliosides, in concentrations similar to those in human milk, to an adapted milk formula altered the microbial composition of feces from preterm, newborn infants. These data suggest that fortification of infant formula with gangliosides results in a growth-promoting effect on bifidobacteria.

However, to our knowledge, the specific ability of bifidobacteria to catabolize gangliosides from milk has not been explored, and the fate of digested milk gangliosides is not well understood. This is probably due to the lack of accurate analytical methods for quantitation

of these molecules. Because of the amphipathic nature and structural complexity of gangliosides, classical analytical methods for their analysis use multiple chromatographic steps along with extensive sample preparation, which generally requires large volumes of samples. Moreover, the analytical methods often fail to detect both the ceramide and glycan moieties simultaneously. Recently, mass spectrometry (MS) analyses of gangliosides were conducted via soft ionization techniques, such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). Often, upfront chromatographic separation of a mixture of gangliosides makes it possible to detect more ions by minimizing ion suppression by other polar lipids [9, 18].

In this study, we explored the ability of six representative bifidobacterial species to digest milk gangliosides *in vitro*. Following incubation with the bacteria, the major milk gangliosides—GM3 and GD3—and their subspecies were extracted from growth media and quantified using nanoHPLC Chip quadrupole-time of flight (Q-TOF) MS. Furthermore, matrix-assisted laser desorption/ionization (MALDI) Fourier transform ion cyclotron resonance (FTICR) MS was used to profile the degradation products. This is the first report to show catabolism of milk gangliosides, other than free oligosaccharides, by various *Bifidobacterium* species. The findings are suggestive that milk gangliosides may have a prebiotic effect on these microorganisms in humans, especially in breast-fed infants.

## 2 Materials and methods

### 2.1 Chemicals and materials

HPLC grade methanol, isopropanol, and 2,5-dihydroxybenzoic acid were purchased from Sigma (St. Louis, MA). Ammonium acetate and acetic acid were of analytical reagent grade and from Merck (Darmstadt, Germany). The C8 and aminopropyl ($NH_2$) silica gel cartridges were obtained from Supelco (Bellefonte, PA).

### 2.2 Preparation of milk gangliosides

Bovine milk gangliosides were purified as previously described[9], but adapted for large-scale purification. The distribution of the main bovine milk gangliosides and their subspecies was previously reported [9].

### 2.3 Microorganisms and media

The *Bifidobacterium* strains used in this study were *B. breve* SC139, *B. longum* subsp. *infantis* ATCC15697, *B. bifidum* SC555, *B. longum* subsp. *longum* SC596, *B. adolescentis* UCD318, and *B. animalis* subsp. *lactis* JCM 10602. The strains were obtained from the American Type Culture Collection (Manassas, VA), and the University of California, Davis Viticulture and Enology Culture Collection (Davis, CA). Bacteria were routinely grown on De Man, Rogosa, and Sharpe (MRS) broth supplemented with 0.05 % w/v L-cysteine (Sigma-Aldrich, St. Louis, MO) under anaerobic conditions (Coy Laboratory Products, Grass Lake, MI) at 37°C in an atmosphere consisting of 5% carbon dioxide, 5% hydrogen, and 90% nitrogen.

### 2.4 Bifidobacterial incubation with milk gangliosides

To test if bifidobacteria could use or interact with milk glycolipids, 15 mg of milk gangliosides extracted as above were first suspended in 3 mL of modified MRS (mMRS), containing 0.05% L-cysteine. Each of the six *Bifidobacterium* species mentioned above was incubated with four concentrations of the milk ganglioside preparation (0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, and 2.0 mg/mL). Two μl of each overnight culture grown on regular MRS with 0.05% cysteine were used to inoculate 200 μl of mMRS supplemented 0.05% cysteine and with each sterile-filtered glycolipid concentration as the sole carbohydrate source. Reactions were carried out in triplicate, and performed in 96-microwell plates. Optical density was monitored in a PowerWavemicroplate spectrophotometer (BioTek Instruments, Winoosky, VT) at 37° C for 60 h, reading culture absorbance at 600 nm. Finally, cells were centrifuged at 10,000 × *g* for 2 min, and supernatants were recovered and stored at −80° C. Controls with no carbon source and no bacteria were included, as well as tests for parallel growth of each strain on 2% lactose as the sole carbon source in the same conditions. The OD reads values from the control with no carbon source were considered as background and withdrawn from experimental OD reads.

### 2.5 Ganglioside purification

The gangliosides were recovered from the bacterial supernatant (2.0 mg/mL of gangliosides) through two consecutive chloroform-methanol extractions. Briefly, a 50-μL aliquot of sample was mixed with 200 μL methanol, 100 μL chloroform, and 25 μL water, and the mixture shaken vigorously for 5 sec. After centrifugation at 3,000 × *g* for 5 min, 30 μL of water were added. The supernatant was collected, the pellet re-extracted with 300 μL of 3:4:8 water/chloroform/methanol (v:v:v), and the supernatants were pooled. The ganglioside fractions were further purified through C8 solid phase extraction (SPE). Prior to use, the cartridge was washed with 3 vol of methanol:isopropanol (1:1, v/v) and conditioned with another 3 vol of methanol:water (1:1, v/v). The ganglioside samples were loaded into the SPE cartridge and washed with 6 vol of methanol:water (1:1, v/v), eluted with 2 vol of methanol:isopropanol (1:1, v/v), and dried *in vacuo*.

### 2.6 Neutral glycolipid extraction

Lipids were recovered from the incubation supernatant through Folch extraction. A 50-μL aliquot of a sample was mixed with 82.5 μLof methanol and 165 μL of chloroform, and the bottom layer was collected and dried. Neutral glycolipids were enriched through aminopropyl ($NH_2$) SPE [19]. The cartridge was washed with 3 vol of acetone:methanol (9:1.35, v/v) and conditioned with another 3 vol of ethyl acetate:hexane (15:85, v/v). The lipid fractions were loaded into the $NH_2$ cartridge and washed with 6 vol of ethyl acetate:hexane (15:85, v/v). The neutral glycolipids were eluted with 2 vol of chloroform:methanol (23:1, v/v) and 2 vol of acetone:methanol (9:1.35, v/v). These two fractions were combined and dried *in vacuo*.

### 2.7 nanoHPLC Chip Q-TOF analysis of gangliosides

Gangliosides in the supernatants were analyzed using a nanoHPLC Chip Q-TOF system equipped with an Agilent 1200 series microwell-plate autosampler, capillary pump, nano

pump, HPLC-Chip interface, and an Agilent 6520 Q-TOF MS (Agilent Technologies, Inc., Santa Clara, CA). The chip consisted of a 40-nL enrichment column and a 43 × 0.075-mm ID analytical column. Both columns were packed with a ZORBAX C18 (5-μm pore size) stationary phase. The mobile phases were water (solvent A) and 15% isopropanol in methanol (v/v) (solvent B), both containing 20 mM ammonium acetate and 0.1% acetic acid. For the loading of the sample onto the enrichment column, the capillary pump was operated at 3 μL/min with 70% solvent B. A gradient-based chromatographic separation was performed on the analytical column, driven by the nanoliter pump running at 300 nL/min. The gradient used for this separation was as follows: 70% of B until 1 min after sample injection (1 μL), followed by a linear increase to 80% B after 3 min. The gradient further increased in a linear manner over 40 min to 100% B and was kept at 100% B for 5 min. At that point, the amount of solvent B was decreased to 70% and maintained at this level until completion of the run.

The Agilent 6520 Q-TOF MS was operated in the negative-ion mode for MS scans and MS/MS. The drying gas temperature and gas flow were 325°C and 4 L/min, respectively. Recorded mass ranges were *m/z* 500–2500 for MS only and *m/z* 50–1500 for MS/MS. Acquisition rates were 1 spectrum/s for MS and 3 spectra/s for MS/MS. All mass spectra were internally calibrated with reference masses at *m/z* 680.036 and 1279.995. Data-dependent MS/MS analysis was performed, with collision energies set at 40V. The precursor ions yielding 1000 counts/s ion intensity or more were automatically selected for MS/MS.

Data analyses were performed with MassHunter Qualitative Analysis software ver. B.04.01 (Agilent Technologies, Inc., Santa Clara, CA). Molecular Feature Extraction was performed through the “Find by Molecular Feature” function for the non-targeted approach. The software was capable of generating a peak list (m/z, retention time and peak area), taking into account all ions exceeding 1000 counts. The *m/z* correlated to the gangliosides; the intensity of a particular mass indicated the relative amount of specific gangliosides remaining after incubation with bacteria. A focused post-processing precursor ion-scan analysis was performed through the “Find by Auto MS/MS” to detect gangliosides specifically. In the negative-ion mode, Neu5Ac ion (*m/z* 290.095) was the fragment ion chosen to determine the Q1 masses representing gangliosides. Due to the high mass accuracy and resolution of the Q-TOF mass analyzer, it was possible to extract an exact compound chromatogram with a narrow mass window for gangliosides, thus excluding the false positives.

Relative GM3 and GD3 abundances were calculated with respect to the uninoculated control by normalizing the summed abundance of each GM3 and GD3 spectra in ion counts in the supernatant from the gangliosides incubated with bacteria (bacteria sample) to that of the control using the following equation:

$$\text{Relative intensity}\ (\%)\ \text{for GM3} = \left(\frac{\sum_{i=1}^{n}\text{GM3 API bacteria sample}}{\sum_{i=1}^{n}\text{GM3 API uninoculated control}}\right)\times 100 \quad \text{Eqn. 1}$$

$$\text{Relative intensity}\,(\%)\,\text{for GD3}=\left(\frac{\sum_{i=1}^{n}\text{GD3 API bacteria sample}}{\sum_{i=1}^{n}\text{GD3 API uninoculated control}}\right)\times 100 \quad \text{Eqn. 2}$$

where API is absolute peak intensity and *n* is the number of identified gangliosides.

### 2.8 MALDI-FTICR MS analysis

Neutral glycolipids were profiled via a HiRes MALDI FTICR MS instrument with an external MALDI source, a 355-nm pulsed Nd:YAG laser, a quadrupole ion guide, and a 7 Tesla superconducting magnet (IonSpec, Irvine, CA). A 1-μL aliquot of each sample (in 50% chloroform in methanol) was spotted on a stainless steel probe, followed by the addition of 0.5 μL of 0.01M NaCl and 1μL of the 2,5-dihydroxybenzoic acid matrix. The samples were mixed on the probe surface and dried under vacuum prior to analysis. Ions were desorbed from the sample target plate by laser shots. In the ion cyclotron resonance cell, the ions were excited and detected. Acquisition scans were performed on each sample in the positive mode. Transients were acquired with IonSpec OMEGA software.

## 3 Results and discussion

Mothers' milk and humans' intestines contain high contents of gangliosides, and they may have a key role in neonatal health as they are involved in modification of intestinal microbiota and the promotion of intestinal immunity development in the neonate [13, 20]. Disialoganglioside GD3 and monosialolganglioside GM3 are the major gangliosides in human milk. GD3 is the predominant form in colostrum and it decreases during lactation, whereas GM3 is the main ganglioside in milk during late lactation [21, 22]. In normal physiological processes, changes occur in the molecular compositions of the gangliosides. The GD3 increases in cells during development and cell differentiation [23, 24]. Changes of the gangliosides depend on the balance between the activities of the enzymes in the biosynthetic and degradative pathways. To ensure proper functioning, cells regulate the amounts of gangliosides in the body by intracellular compartmentalization, rapid turnover, and chemical modification [25]. Because their biological functions depend on their structures, GM3 and GD3 have different roles in physiological processes. Ganglioside GM3 is an enterocyte receptor analogue for pathogens such as enterotoxigenic and enteropathogenic *Escherichia coli*, and it seems to have a protective role relative to infections, whereas GD3 is involved in modulating immunity [26–29]. Therefore, we assume that gut bacteria may contribute to the chemical modulation of ingested gangliosides and ultimately change their biological functions.

We evaluated the ability of six species of intestinal bifidobacteria to grow in the presence of purified bovine milk gangliosides *in vitro*. *Bifidobacterium* is the most abundant genus in the infant gut microbiota in breast-fed infants [30–33]. *B. breve*, *B. longum* subsp. *infantis*, *B. bifidum*, and *B. longum* subsp. *longum* are the dominant bifidobacteria isolated from breast-fed infants, whereas bifidobacteria species in feces from bottle-fed infants are more diverse and include *B. adolescentis*, which is often found in feces of adults [30, 33–35]. The representative bifidobacteria species were incubated in ganglioside-containing media, and growth curves showed that gangliosides stimulated the growth of some bifidobacteria. Some

of the strains, especially *B. bifidum*, *B. breve*, and *B. longum* subsp. *infantis*, grew to varying extents with gangliosides as their sole carbon source, as shown by low-to-medium optical density (OD) values during growth over a 60-h time course (Figure 1). The other bifidobacterial strains did not seem to grow on any of the concentrations tested (data not shown). The results indicated that the three bifidobacterial species may cleave the glycan moiety of these lipids and hypothetically use the glycan portion as a carbon source. Human milk oligosaccharides have been reported to be utilized by *B. longum* subsp. *infantis*, *B. bifidum*, and to a lesser extent, strains of *B. breve* [6, 36]. The utilization of milk oligosaccharides was explained to result from enzymatic processing of these molecules by glycosylhydrolases, including sialidases [1, 7, 37]. Therefore, it is probable that species of bifidobacteria utilize milk gangliosides as carbon sources by cleaving the glycan parts of the molecule with glycosylhydrolases.

Mass spectrometry analysis of the milk gangliosides remaining after incubation with *Bifidobacterium* species provided a detailed representation of the consumption of milk GM3 and GD3 gangliosides. Recently, glycoprofiling of free milk oligosaccharides after bifidobacteria digestion was conducted by nanoHPLC Chip Q-TOF MS [38]. Here we developed a nanoHPLC method for milk ganglioside profiling. The nanoLC separations of gangliosides using microchip-based columns were successfully achieved. Mixtures of gangliosides extracted from media after incubation with different bifidobacteria species were separated on a C18 stationary phase. The nanoLC-MS with high mass accuracy and high retention time reproducibility, along with MS/MS capabilities, provided accurate assignment and quantitative results.

Figure 2 shows the extracted compound chromatograms (ECCs) of the recovered gangliosides from the uninoculated ganglioside control (Fig. 2A), the mMRS media containing *B. breve* (Fig. 2B), *B. longum* subsp. *infantis* (Fig. 2C), and *B. bifidum* (Fig. 2D). The separation of milk gangliosides was performed effectively with the C18 stationary phase. The stationary phase separated the native gangliosides on the basis of the chain length of the ceramides and the degree of saturation, as shown previously [18]. Thirteen GM3 (peak shaded in blue) and 15 GD3 (peak shaded in pink) peaks were found by the “Find by Auto MS/MS” algorithm. No gangliosides other than GM3 and GD3 were found. Peak assignments with retention times are presented in Supporting Information Table 1S. The ECCs indicated that *B. longum* subsp. *infantis* and *B. bifidum* significantly depleted the initial amounts of milk gangliosides.

The data obtained from quantitative analysis of three *Bifidobacterium* species are shown in Figure 3 and in Supporting Information Table 1S. Some bifidobacteria species depleted ganglioside GD3 and its subspecies from the culture medium. Specifically, *B. longum* subsp. *infantis* and *B. bifidum* degraded most of the GD3. There was less GM3 in gangliosides incubated with *B. longum* subsp. *infantis* and *B. bifidum* than in the control without bacteria. Interestingly, *B. bifidum* depleted most of the GD3, and only two GM3 subspecies were detected. Regarding the structures of the ceramides, GD3 with d34:1 and d41:1 were the most abundant subspecies in the non-inoculated milk ganglioside control. It is worth noting that there was not much difference in degree of catabolism among the gangliosides with

different ceramide chains, implying that the bacteria accessed the ganglioside molecules according to their sugar groups rather than their ceramide chains.

Figure 4 shows the sum of the ion intensities of GM3 and GD3 subspecies. *B. longum* subsp. *infantis* and *B. bifidum* consumed 63% (from 100% to 37%) and 100% (from 100% to 0%) of GD3, respectively, whereas *B. longum* subsp. *longum*, *B. adolescentis*, and *B. animalis* subsp. *lactis* degraded moderate amounts of GD3, i.e., 30% (from 100 to 70%), 28% (from 100 to 72%), and 48% (from 100 to 52), respectively. The catabolic capacity of the bacteria towards sialylated oligosaccharides in human milk was previously determined by monitoring the sialidase activities. *B. longum* subsp. *infantis* and *B. bifidum* exhibited high sialidase activities, which may explain the high degradation of gangliosides by these two strains [6, 7, 37]. *B. breve* is known to have sialidases, which preferentially consume select acidic oligosaccharides in human milk [38]; however, this strain was not able to utilize the sialic acids in gangliosides. The ion intensity of GM3 was increased 123% for *B. breve* and 108% for *B. longum* subsp. *longum*, but it was decreased by 42% (from 100 to 58%) for *B. longum* subsp. *infantis* and by 99% (from 100 to 1%) for *B. bifidum*. In the case of gangliosides incubated with *B. breve* and *B. longum* subsp. *longum*, the increased GM3 could be the result of sialidase hydrolysis products from GD3. *B. longum* subsp. *infantis* and *B. bifidum* might exhibit strong α2-3 sialidase activity, which would even degrade GM3 [7].

*B. longum* subsp. *infantis* and *B. bifidum* had strong α2-8 sialidase activity, which degraded most of the GD3. It was previously shown that *B. longum* subsp. *infantis* and *B. bifidum* utilized the oligosaccharides in human milk, and the genome sequence of the species provided an explanation for this phenotype [39–41]. These two strains possess a number of genes that are involved in the consumption of complex carbohydrates, including α2-3 and α2-6 sialidase genes [7, 42]. However, the two species are known to have different strategies [43, 44]. *B. longum* subsp. *infantis* transfers oligosaccharides via binding protein, and they are degraded by intracellular glycosidases; whereas *B. bifidum* utilizes and degrades human milk oligosaccharides at their extracellular surface. Therefore, it is likely that the two species utilize gangliosides in different ways. For example, *B. bifidum* may cleave sialic acids in the gangliosides' terminal positions and transport them into the cell. Regarding the behavior of *B. longum* subsp. *infantis* towards gangliosides, we have observed that this strain is able to degrade GM3 and GD3. Interestingly, most of the glycosylhydrolases in this bacterium are intracellular, which suggest that the gangliosides are processed inside the bacterium. Although *B. breve* and *B. longum* subsp. *longum* have weak glycosidase activities, a recent study showed that *B. breve* seems to use α-sialidase in the preferential consumption of selected acidic oligosaccharides in human milk [38, 43]. In the present study, *B. longum* subsp. *longum* consumed GD3 to a lesser extent, and no gangliosides were utilized by *B. breve*.

To elucidate the glycolipid remaining after their incubation with bifidobacteria, we profiled the neutral glycolipid and ceramide fractions using MALDI FTICR MS (Figure 5). Whereas no neutral glycolipids were found in the uninoculated ganglioside control, distinct lactosylceramides were observed in most of the media incubated with bifidobacteria. For example, spectra of the media containing *B. longum* subsp. *infantis*, displayed several peaks at *m/z* of 884.607, 954.688, 968.705, 982.717, and 996.734, which corresponded to LacCer

(d34:1), LacCer (d39:1), LacCer (d40:1), LacCer (d41:1), and LacCer (d42:1), respectively (Supporting Information Table 2S). The structures of their oligosaccharides were confirmed by tandem MS (data not shown). This high-resolution MS analysis showed the degradation of specific gangliosides at the outer points of the glycan, leaving lactosylceramide. It is known that gangliosides and neutral glycolipids are excreted in the feces of infants during the first months of life[14]. GM3 is the dominant fecal ganglioside in the feces of breast-fed infants, and the glycolipids are not fully degraded in infants until 2–3 months after birth, at which time the microbiota are dominated by bifidobacteria. Therefore, probably the growth of gut bifidobacteria and the digestion of gangliosides result in the appearance of lactosylceramide in feces at the time of weaning [14].

### 3.1 Concluding Remarks

In view of the many biological activities (e.g. as inhibitors of bacterial toxins and enteric pathogens) of gangliosides, their degradation by subsets of the microbiota in infant feces would seem to be biologically important. Investigation of the degradation of gangliosides *in vitro* by six *Bifidobacterium* species showed that specific species actively hydrolyzed sialic acid moieties in gangliosides leaving behind lactosylceramides. The growth of certain *Bifidobacterium* species may be explained, at least in part, by their utilization of sialic acid. Ganglioside catabolism results in the release of free sialic acids in the intestinal tract that may change the glycolipid profile in the gut's intestinal epithelial cells and feces. The results lead the authors to speculate that the gangliosides in milk, or their degradation products such as sialic acids, may exert a selective, prebiotic activity on specific bifidobacteria species in infants.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **MS** | mass spectrometry |
| **FTICR** | Fourier transform ion cyclotron resonance |
| **MRS** | De Man, Rogosa, and Sharpe medium |
| **Neu5Ac** | *N*-acetylneuraminic acid |

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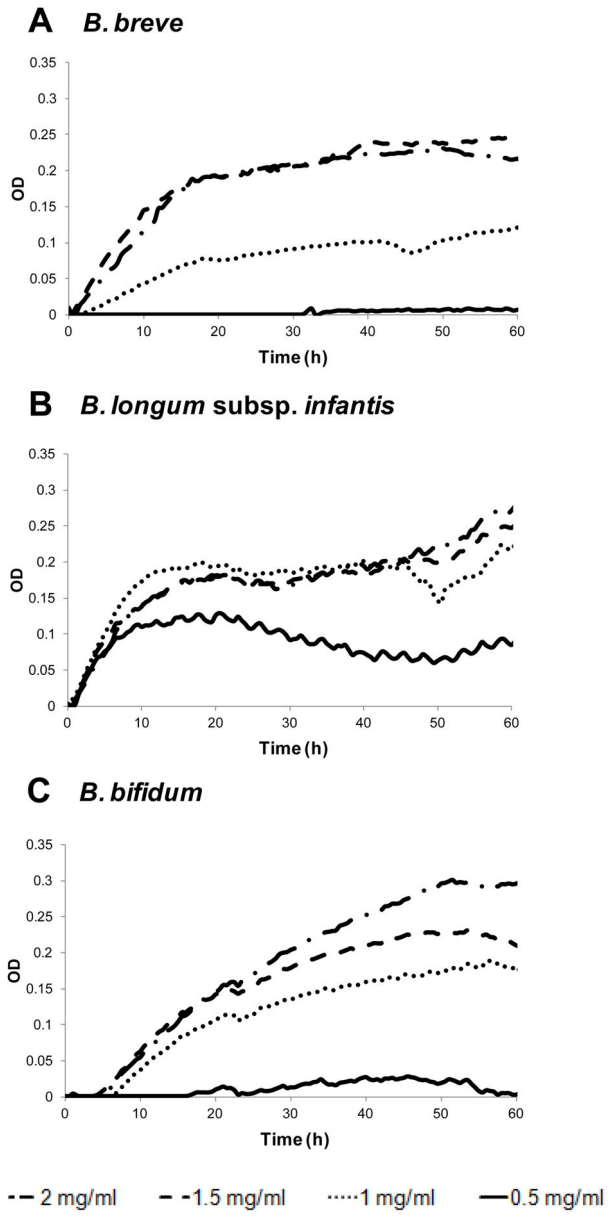


**Figure 1.**

Growth curves over a 60-h time course for three *Bifidobacterium* species grown in MRS medium supplemented with 0.5mg/mL, 1.0mg/mL, 1.5mg/mL and 2.0mg/mL milk gangliosides. (A) *B. breve*, (B) *B. longum* subsp. *infantis*, and (C) *B. bifidum*. Growth was measured as optical density (OD) of the media at 600nm. Experiments were carried out in triplicate. Controls consisted of uninoculated medium with milk gangliosides.

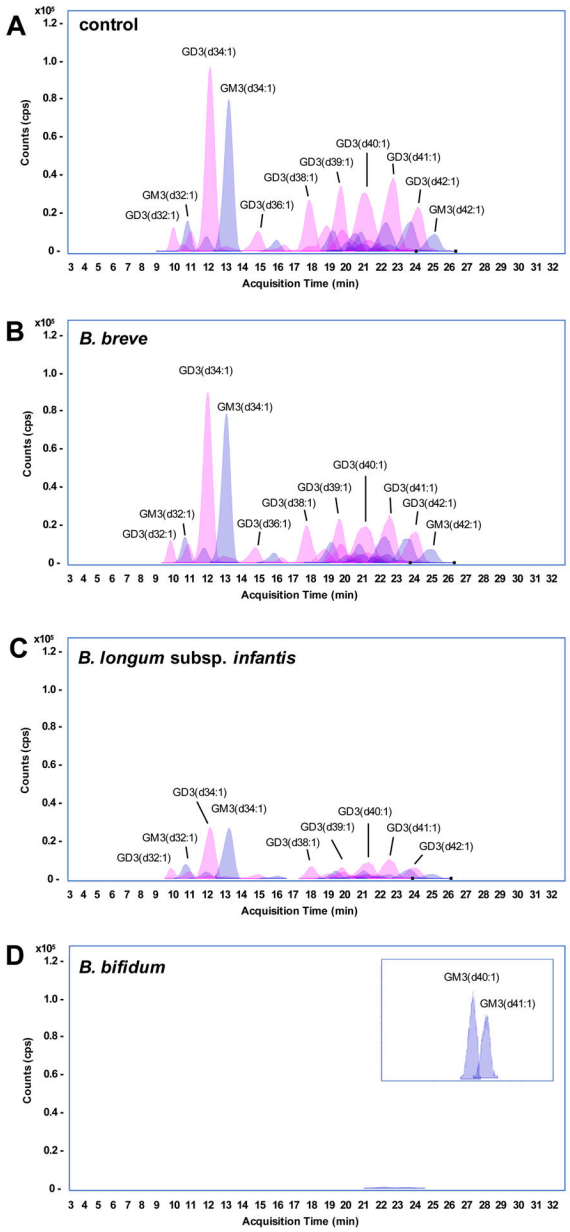


**Figure 2.**

Extracted compound chromatograms (ECCs) of GD3 and GM3 gangliosides remaining in media after bacterial fermentation.(A) Uninoculated control**,** (B) *B. breve*, (C) *B. longum* subsp. *infantis*, and (D) *B. bifidum*. ECCs show the elution profile of gangliosides via nano-LC/MS.

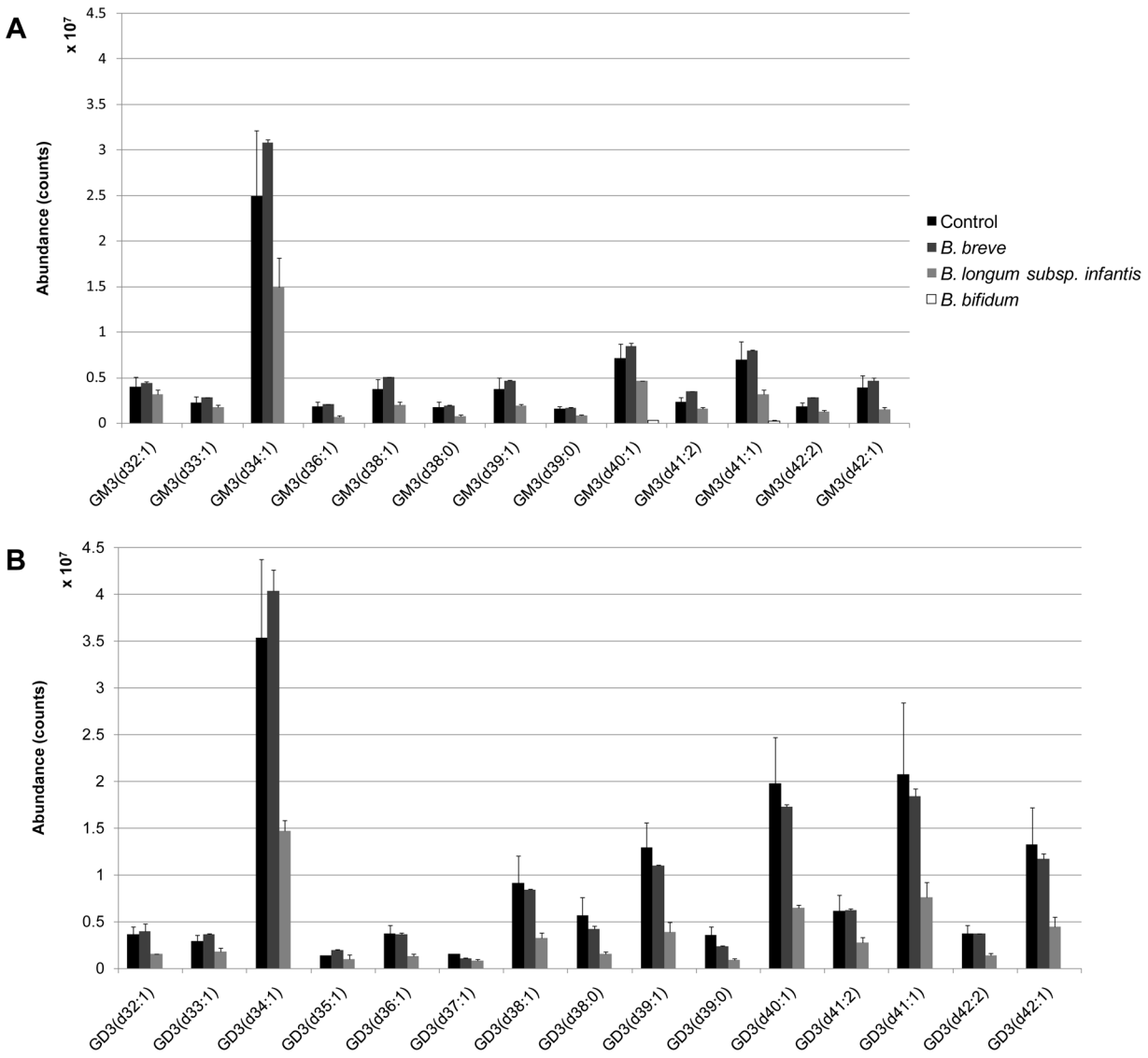


**Figure 3.**

nanoHPLCQ-TOF profiles of GM3 and GD3 gangliosides extracted from media after incubation with bifidobacteria. (A) Ganglioside GM3 and (B) ganglioside GD3.

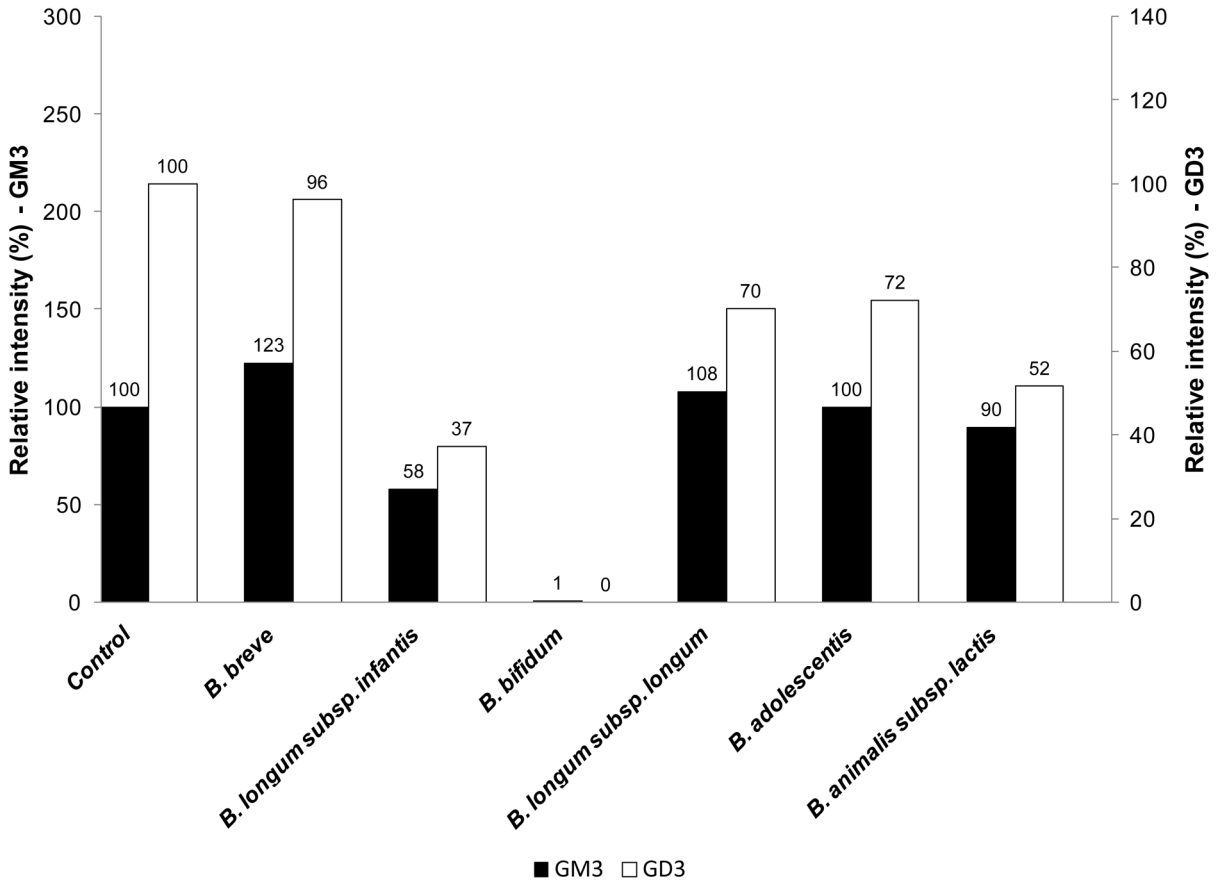


**Figure 4.**

Total GM3 and GD3 extracted from media following incubation with *Bifidobacterium* species. The control was medium containing the gangliosides incubated without bifidobacteria. Calculations were based on Equation 1 and Equation 2 for GM3 and GD3, respectively.

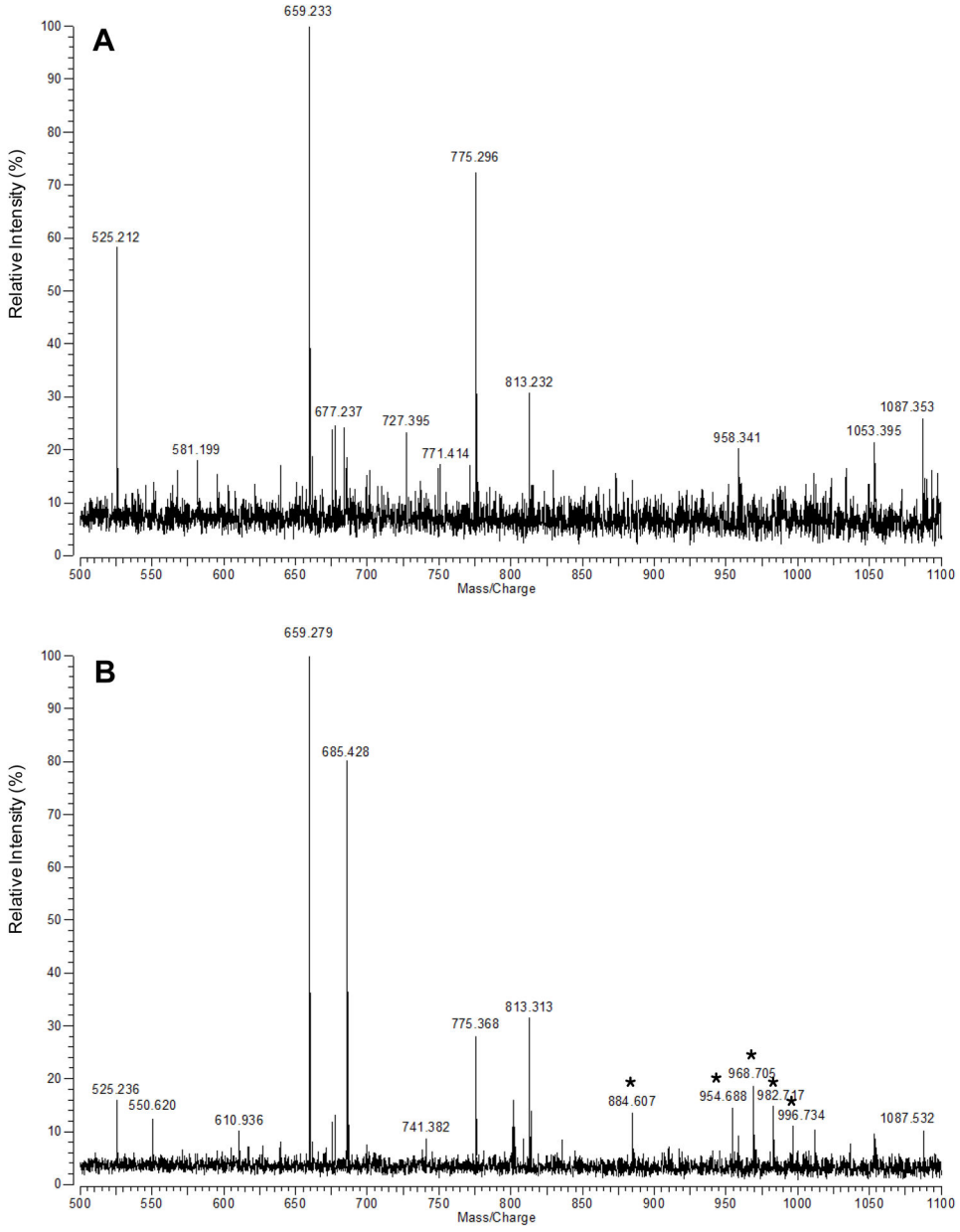


**Figure 5.**

Positive mode MALDI FTICR MS spectra of the neutral glycolipid fraction of (A) control and (B) *B. longum* subsp. *infantis*-containing medium following incubation. Asterisks (*) indicate lactosylceramides. The ions are $[M+Na]^+$ forms.