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Isomer-specific consumption of galactooligosaccharides by bifidobacterial species

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Abstract

Prebiotics are non-digestible substrates that stimulate the growth of beneficial microbes in the human intestine. Galactooligosaccharides (GOS) are food ingredients that possess prebiotic properties, in particular, promoting the growth of bifidobacteria *in situ*. However precise mechanistic details of GOS consumption by bifidobacteria remains poorly understood. Because GOS are mixtures of polymers of different lengths and linkages, there is interest to determine which specific structures provide prebiotic effects in order to potentially create better supplements. We here present a method comprising porous graphitic carbon separation, isotopic labeling and mass spectrometry analysis for the structure specific analysis of GOS isomers and their bacterial consumption rate. Using this strategy, the differential bacterial consumption of GOS by the bifidobacteria species *B. longum* subsp. *infantis*, *B. animalis* subsp. *lactis* and *B. adolescentis* was determined, indicating that the use of specific GOS isomers in infant formula may provide enrichment of distinct species.

Keywords

galacto-oligosaccharide; Bifidobacteria; nanoHPLC; TOF mass spectrometry; prebiotics

INTRODUCTION

Human milk is often called the “perfect food”. Bioactive components in milk, such as free oligosaccharides, have been shown to benefit the infant by stimulating the immune system, mimicking epithelial binding sites for pathogenic bacteria and stimulating the growth of beneficial bacteria.[1-5] It is currently thought that the growth of these beneficial bacteria, often bifidobacteria, is the catalyst for a host of events that help safeguard the infant in the early stages of life. This notion has sparked research on understanding the consumption of human milk oligosaccharides (HMO) by different probiotic bifidobacteria species.[5-9] Interestingly, not all bifidobacterial species grow equally well on HMO. The infant-borne

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species *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) grows much faster than several other bifidobacterial strains.[5] Moreover, *B. infantis* was shown to proliferate well on smaller HMO, which are the bulk of the free oligosaccharides present in human milk.[6]

In situations where human milk (and thus HMO) may not be available, there is a need for a synthetic substitute that can bestow the same advantages as mother's milk. Because the goal is to manipulate bacterial populations, glycans being considered have to be non-digestible by humans and resistant to degradation during gastrointestinal transit in order to reach the target organisms. The prospective glycans must also, upon reaching the colon, promote the growth of the beneficial bacteria in the infant gut, and thereby partially emulate the benefits as endowed by human milk.[10-13]

Viable targets have been oligosaccharide polymers, composed from either fructose or galactose. Fructooligosaccharides (FOS), polymers of fructose connected in a (β2-1) manner, are extracted from fruits and plants, as well as enzymatically produced. Both smaller and longer chain fructans have been shown to stimulate bifidobacteria.[14] However, galactooligosaccharides (GOS) consist of a lactose core, which is elongated with galactose polymers, where the galactose residues may be linked using different glycosidic linkages (β1-3, β1-4 or β1-6). In contrast to FOS, which is a linear polymer, GOS structures may be branched, resulting in large structural heterogeneity.[15-18] Based on the natural occurrence of galactose in human milk oligosaccharides and their more branched structures, GOS is more often used as a substitute for HMO in infant formula.

The potential of GOS mixtures to stimulate bifidobacteria has been studied extensively, both *in vivo* and *in vitro*.[16, 19-22] Recently Davis et al.[23] showed that consumption of GOS resulted in a highly specific enrichment of bifidobacteria in adult subjects. While such enrichments have been observed, exact mechanistic details of GOS consumption by bifidobacteria remain elusive. Van Leare et al. initially studied the consumption of GOS in *B. adolescentis* cultures,[22] and more recently, our group[19] evaluated the fermentation of GOS in four major bifidobacterial phylotypes: *B. adolescentis*, *B. breve*, *B. infantis*, and *B. longum* subsp. *longum* (*B. longum*). In both cases differential consumption of specific DPs of GOS was observed by the various bifidobacterial species. *B. infantis* deploys three (out of the five) β-galactosidases, with different specificities, to cleave the different linkages present in GOS.[20] A recent study identified the genetic loci responsible for the transportation and consumption of GOS by B. breve and revealed the importance of an endogalactanase for the consumption of GOS with higher degrees of polymerization (DP).[24] These results provide some mechanistic insight into how GOS might differentially enrich different species and strains of bifidobacteria and help to produce a gut microbial population that emulates an infant fed human milk.[25]

GOS are composed of a galactose polymer bound to a lactose core. There is a wide variety of compositional isomers in a product mixture of GOS due to variation in the core disaccharide, both in composition and linkage, along with structural variation within subsequent galactose units attached to the core, resulting in a mixture of polymer of different lengths and lingages.[26] Therefore, there is interest to determine which specific structures are most selective in order to create more targeted prebiotic applications. Characterization of the individual GOS structures is traditionally performed with high performance anion exchange chromatography (HPAEC) or NMR / GC-MS analysis and these methods have been able to deduce the structure of smaller (di/trisaccharides) GOS.[22, 26] While this combination of methods is accurate, the time required for separation and amount of sample necessary for GC/MS and NMR analysis may not always be available. More recently, a method comprising capillary electrophoresis with fluorescence detection was suggested for the analysis of galactooligosaccharides from food sources. [27] We recently introduced the use of

MALDI-FTICR-MS for monitoring the consumption of GOS polymers by bacterial strains.[19] While mass spectrometry techniques are well suited for the detection of oligosaccharides, the technology alone does not provide isomer specific information. Therefore, combination of a separation technique with mass spectrometry is necessary for the structure-specific profiling of GOS.

Porous graphitized carbon is a stationary phase that has been widely used for oligosaccharide analysis, and has been shown to provide separation of isomers.[28-34] Thus far, PGC has not been applied for the separation of galactooligosaccharides. Here we report the use of porous graphitized carbon (PGC) nHPLC-MS for the separation of galactooligosacchride isomers with degrees of polymerization (DP) from 3 to 5 for a commercially available mixture. To allow the determination of preferential consumption of individual isomers of GOS by bifidobacterial species, *B. animalis subsp. lactis*, *B. adolescentis* and *B. longum* subsp. *infantis* were with GOS as the sole carbon source. Using an isotopic labeling method[35] in combination with nLC-PGC-TOF-MS, the consumption of specific GOS isomers was quantified and differential consumption patterns were observed.

MATERIALS AND METHODS

Commercially available Vivinal GOS mixture and was obtained from FrieslandCampina Domo (Zwolle, The Netherlands). HPLC grade Acetonitrile (ACN) was purchased from Honeywell Burdick & Jackson (Morristown, NJ). 98% Sodium Borohydride ($NaBH_4$) and 98% Sodium Borodeuteride ($NaBD_4$) was purchased from Sigma-Aldrich (St Louis, MO).

Reduction and purification of GOS samples

25 μL volumes of GOS, 0.5% (w/v), were reduced with sodium borohydride by mixing an equal volume of 2M $NaBH_4$ and incubating at 65° C for 2 hours. An isotopic standard was produced by the reduction of a standard 0.5% (w/v) solution with 2M sodium borodeuteride ($NaBD_4$). This standard was mixed with the fermented GOS samples to introduce an isotopically labeled control into the tests sample. After mixing, oligosaccharides were purified by solid-phase extraction using graphitized carbon (Extract-Clean SPE Carbo Cartridges,Grace, Deerfield, IL) and eluted with 4mL of 40% aqueous ACN. All samples were then dried under vacuum and reconstituted in water to a final concentration of 2 g/uL. Samples were diluted 1:20 (100ng/uL) before injection and analysis by nLC-chip-TOF-MS.

Analysis of GOS by nLC-chip-TOF-MS

GOS samples were analyzed using an Agilent Series 6200 Series nLC-chip-TOF-MS system. The chip used consists of a 40nl enrichment column and a 43 × .75 mm ID analytical column both packed with 5 μm particle sized porous graphitized carbon. The sample of interest (1 μL) was loaded onto the enrichment column isocratically via a capillary pump at flow rates of 4.0 μL/min and then eluted using a nanolitre pump running at 0.3 μL/min. GOS separation was performed using a binary gradient consisting of an aqueous solvent A (3% acetonitrile/water (v/v) in 0.1% formic acid solution) and an organic solvent B (90% acetonitrile/water (v/v) in 0.1% formic acid solution) The gradient profile ramped from 0 – 16% B over 20 minutes, 16 – 44% B for 10 minutes, and 44-100% B over 15 minutes. The column was then conditioned for 10 minutes at 100% B and then equilibrated back to 0% B over a 20 minute period. Samples were detected over an *m/z* range of 300-3000 in the positive ionization mode. The data was analyzed using Agilent Mass Hunter Qualitative Analysis software, version B.03.01.

Bacterial growth using GOS

Strains representing three bifidobacterial species, *B. longum* subsp. *infantis* (ATCC15697), *B. adolescentis* (ATCC15703) and *B. animalis* subsp. *lactis* (UCD316) were obtained from UCD Viticulture and Enology Culture Collection. Two μl of each resulting overnight culture were used to inoculate 150 μl of modified MRS (mMRS) medium supplemented with 0.5% (w/v) GOS 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 °C 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. When growth reached stationary phase a 25 μL aliquot of the supernatant containing residual GOS was taken. The remaining GOS were then purified using SPE and subsequently analyzed with nLC-chip-TOF-MS. Each experiment was performed in triplicate.

Data analysis

For each chromatogram obtained, the H/D ratio was calculated by using in house software LC/MS Searcher.[35] In brief, the H/D ratio is calculated for Agilent data exported to an mzXML format. A library file that specifies the number of isomers, their retention times, and the theoretical relative intensity of the first isotope for that mass to the isotopic mass is provided to the program before processing. The the apex of each peak is then searched within an area around the retention time given and the H/D ratio is determined for the closest spectra. After extracting the H/D ratio for each isomer, average, standard deviation and CV were determined using excel (Microsoft office excel 2007). Using these values Single-Factor ANOVA were performed with an F value of .05 (Confidence level 95%). Fischer's least significant difference method was applied to determine if consumption between species could be considered significant on an individual isomer basis.

RESULTS AND DISCUSSION

Separation of GOS with nLC-chip-TOF-MS using PGC

Porous graphitized carbon (PGC) is widely recognized for its ability to separate oligosaccharide isomers. In this work, PGC is for the first time employed for the separation of isomers of GOS. The GOS were reduced to avoid separation of anomers before separation with PGC liquid chromatography. The resulting total ion chromatograms (TIC) for three instrument replicates is depicted in Figure 1 and shows the overall GOS separation, which is efficiently achieved within 20 minutes.

Separation of the total ion chromatogram into individual extracted ion chromatograms reveals that each individual degree of polymerization (DP) observed shows a characteristic elution profile (Figure 2). GOS polymers are observed for DP3 [$M + H^+ = 507.19$], DP4 [$M + H^+ = 669.24$], DP5 [$M + H^+ = 831.297611$] and DP6 [$M + H^+ = 993.35$], however, the major signals correspond to DP3, DP4 and DP5, consistent with previously reported experiments.[19] As depicted in Figure 2, 14 DP3 isomers elute between 4 and 14 minutes, 12 isomers of DP4 elute between 9 and 17 minutes and 7 isomers of DP5 elute between 10 and 15 minutes. For each DP, the respective chromatogram displays a characteristic elution profile, which highlights the structural heterogeneity in the oligosaccharide polymer.

The separation and analysis of the GOS polymer mixture is highly reproducible. This is shown in Figure 1, where an overlay is depicted of three instrument replicates of the

separation of GOS using PGCTOF-MS. Previous reports by our group, focusing on the stability of the nLC-PGC-chip-TOF method for the profiling of N-glycans for the development of biomarkers, has already indicated that the technology is highly stable.[36] Stability of the method is desirable, as multiple samples need to be compared.

Since PGC interacts with the oligosaccharides by interacting with three-dimensional cross sections of the molecule, it can be seen to lose the ability to effectively separate GOS polymers as they become longer and the unique structural characteristics average out. This can be seen by looking at the windows of elution for each of the DPs described above. For example DP3 isomers spanned a 10 minute window while DP4 spanned 8 minutes and DP5 5 minutes. This results in larger coalescence of peaks seen at the higher DPs suggesting that PGC may be less effective for the separation of very large GOS isomers.

Quantification of GOS using H/D ratios

Reductive isotopic labeling was used for the quantitation of the changes in relative abundance of GOS isomers. The procedure is outlined in Figure 3. The consumed GOS sample, after being used for the growth of bifidobacterial strains, would be reduced with sodium borohydride, while a standard sample of the original GOS mixture would be reduced with sodium borodeuteride and thus provide an isotopically labeled control that can be spiked into the test sample (Figure 3A). Mixing the two samples together provides a unique isotopic distribution (Figures 3B and C) that can be used to quantify the consumption of the GOS by different bacterial strains, as was previously shown for HMO consumption.[35]

To evaluate the accuracy of the relative quantitation of the GOS isomers, a calibration curve was generated. Equal amounts and concentrations of a standard GOS solution were reduced with either sodium borohydride or sodium borodeuteride. First, labeled and unlabeled samples were mixed in a 1:1 ratio followed by a 10% decrease in the unlabeled fraction for each subsequent mixture. The different mixtures were then analyzed using nHPLC Chip-TOF-MS. For each of the observed isomers from DP3 to DP5, the spectra were extracted from the optimum of the signal, and using in house software, the isotopic distribution was corrected for the original C13 contribution from our test sample and subsequently used to determine the relative ratio of sample consumed in comparison to the standard. The relative ratios were then plotted against the actual percentage to provide a linear response curve (Figure 3D). A linear equation was fitted and was shown to be linear with a coefficient of determination value of 0.98, indicating a good linear range and high accuracy of the method down to 10% of the original sample.

Application to bacterial consumption

To evaluate the potential of the proposed method, it was applied toward the determination of the consumption of GOS by three bifidobacterial strains: *B. infantis* (ATCC15697), *B. adolescentis* (ATCC15703) and *B. lactis* (UCD316). These strains were chosen because they can all grow on GOS but grow differentially on HMO; growth curves of the three bifidobacteria are shown in Figure 4. *B. infantis* ATCC15697 grows vigorously on HMO[7] and contains a number of transport systems for various glycan substrates.[37] Conversely, *B. adolescentis* ATCC15703 and *B. lactis* UCD316 do not grow on HMO[7, 38] and are rarely found in the breast fed infant gut. The bacteria were grown in a media where the GOS mixture of interest was the sole source of carbon and each bifidobacterial strain was grown in biological triplicate. Growth was allowed to continue until the end of the exponential growth phase before the supernatant was extracted. Subsequently, the samples were reduced and mixed with an isotopically labeled standard (Figure 3) before analysis by nLC-chip-TOF-MS.

For each DP, the consumption of the individual isomers was determined according to the method described above and subsequently compared between the three species. For DP3, these results are shown in Figure 4. Interestingly, several of the DP3 structures were significantly consumed (e.g. 1 and 6), while other DP3 structures were only marginally consumed (e.g. 10 and 13). A single-factor ANOVA (F = .05) was used to determine if a significant difference existed in GOS consumption between the species. All isomers of DP3 except for 1, 3 and 6 were shown to differ significantly in their consumption between the three species. Differences among the consumption patterns of individual isomers between the three bifidobacteria species were then identified using least significant difference testing. Table 1 shows a summary of all the results for each of the DP. Statistical results are recorded as either a “yes” for a statistical significance or a “no” for no statistical significance. Interestingly, only three of the DP3 isomers, none of the DP4 and two of the DP5 isomer structures did not show differences in consumption between the three species, indicating that the bifidobacteria each clearly have different preferences with regard to GOS consumption. While different consumption patterns were observed for each of the strains, the consumption pattern of *B. lactis* seems more different compared to *B. infantis* and *B. adolescentis*. Some of the structures were clearly more consumed by *B. lactis* compared to the two other species (DP3-7, DP4-2 and DP4-6), while other structures were clearly less consumed (DP3-2, DP3-5DP3-12, DP3-14, DP4-1, DP4-7, DP4-9, DP5-1, DP5-2 and DP5-7).

The larger oligosaccharides, after being consumed, may contribute to the lower mass oligosaccharides signal and potentially skew the results. To further assess this, the average consumption was calculated for all DP. 62% of the DP3 isomers were consumed on average in comparison to only 35% of the DP5 structures, thus indicating that smaller oligosaccharides are preferentially consumed over the larger ones. This is in agreement with previous publications.[19]

Galactooligosacchride isomers have been shown to be quickly and reproducibly separated using nHPLC with a PGC stationary phase. A quantitative method using isotopic labeling was used to determine the fermentation of individual galactooligosacchride isomers, and was shown to provide accurate results. The method was applied to three bifidobacterial species and significant differences in the consumption of particular isomers was determined through ANOVA and least significant difference testing. It is anticipated that the methodology described here will further expedite progress toward understanding the GOS structural moieties responsible for the resulting prebiotic effect.

The gut microbiota of breast-fed infants is often dominated by specific infant-borne bifidobacterial species, while formula-fed infants often have a more diverse adult-like bifidobacterial composition.[14] In this study, it is demonstrated that the individual GOS structures are consumed at different rates by the different bifidobacteria and the consumption of specific oligosaccharides will likely result in the presence of specific bifidobacterial species in the infants gut. Therefore, our results provide a conceptual basis for production of tailored GOS products targeted towards the preferences of specific bifidobacterial species or even strains. Further structural elucidation of the different GOS isomers may therefore reveal structural features that will provide guidance for specific enrichment of certain bifidobacterial species in the infant gut.

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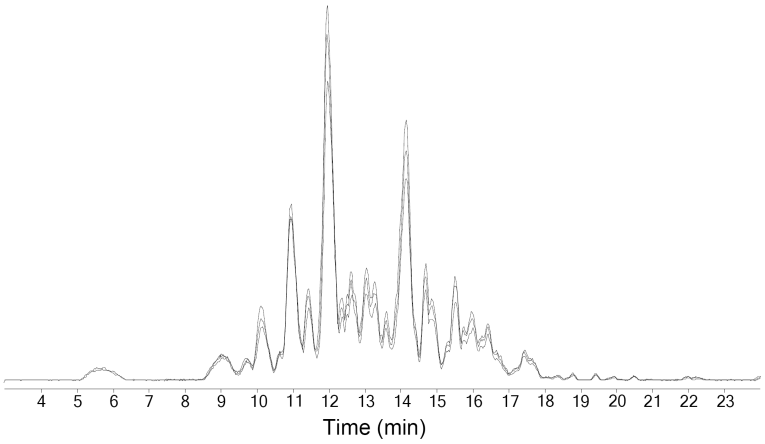

Figure 1. Overlay of three total ion chromatograms obtained from analysis of GOS by nLC-chip-TOF-MS

Signal mainly corresponds to isomers with lengths from DP 3 to 5.

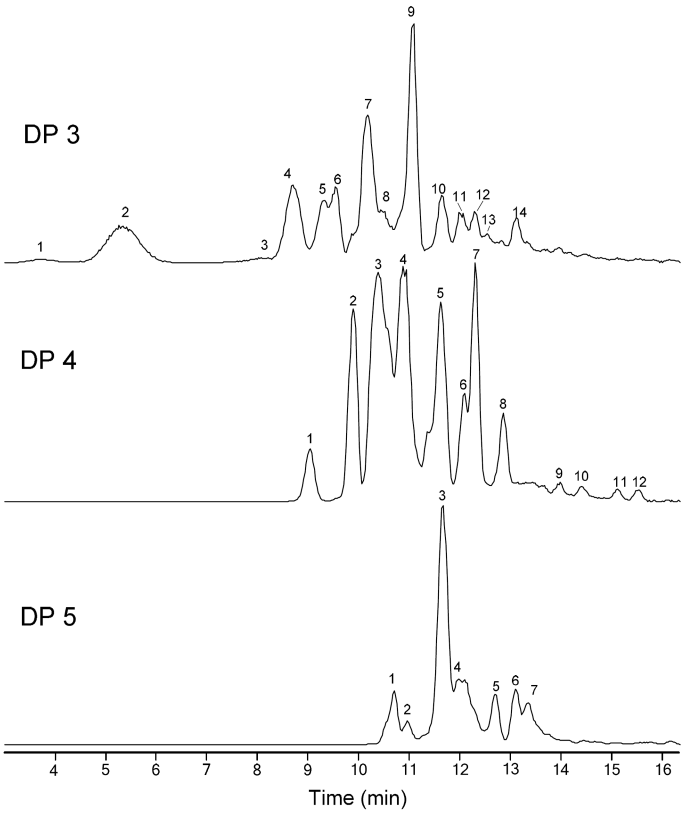

Figure 2. Separation of GOS isomers using PGC stationary phase

Extracted ion chromatograms of DP 3 – 5 using TOF detection in the positive ionization mode. Separation of the isomers occurs with different efficacies: DP3 isomers span a 10 minute window while DP4 span 8 minutes and DP5 5 minutes.

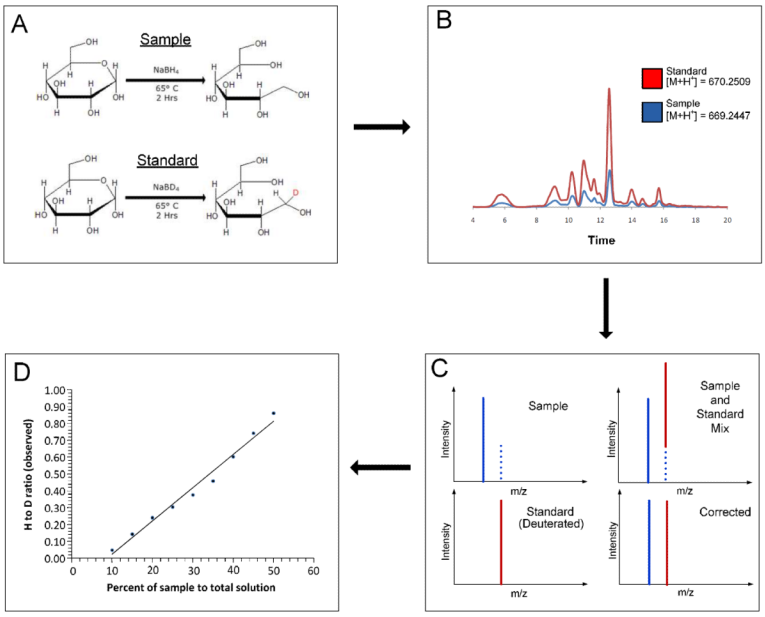

Figure 3. Systematic overview of the H/D Ratio strategy

(A) First, consumed samples are reduced using sodium borohydride while a standard sample is reduced with sodium borodeuteride. (B) Equal concentrations of sample/standard are mixed and analyzed by nLC-PGC-chip-TOF-MS. (C) The resulting spectra are corrected for the samples' isotopic distribution. The resulting intensities of the monoisotopic and first isotopic peak of the given sugar mass then represent the consumed sample and deuterated standard concentrations respectively. (D) By comparing the intensity of the sample to the standard, a calibration curve was generated, which as used for calibration of the procedure.

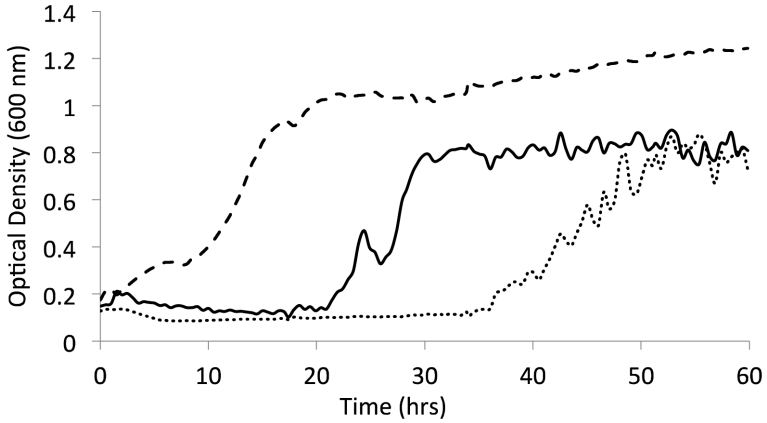

Figure 4. Growth curves of the bifidobacteria used in this study

Growth of *B. longum* subsp. *infantis* ATCC15697 (dashed line), *B. adolescentis* ATCC15703 (continuous line) and *B. animales* subsp. *lactis* UCD316 (dotted line) on GOS (0.05% w/v) in modified MRS medium. Samples for glycomic analyses were taken at early stationary phase for each of the three cultures.

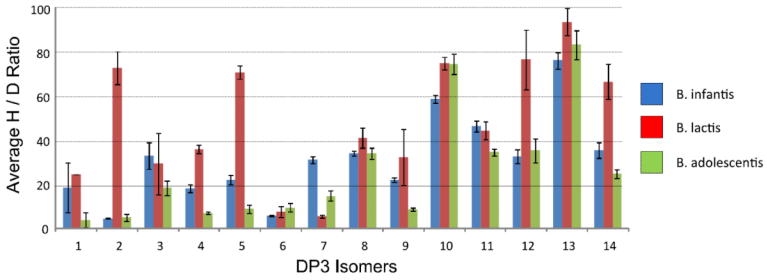

Figure 5. Bacterial consumption of DP3 as determined using nLC-chip-TOF-MS and H/D Ratio

Bargraph representing corresponding H/D ratios for each of the DP3 isomers. Bars for B. infantis are represented in blue, bars for B. lactis are represented in red and bars for B. adolescentis are represented in green. Error bars represent the standard deviation calculated over the biological triplicates. For the EIC chromatogram depicting the elution and numerical annotation of the DP3 GOS isomers see Figure 2A. Differences in the consumption of individual isomers, between one or more species, could indicate a preference the species might have with regards to that particular structure.

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Table 1

Differential consumption of GOS structures by Bifidobacteria species.

	DP3	H/D Ratios												
	Species/ Isomer Digested	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**12**	**13**	**14**
Group differences:	B. infantis	18 ± 11	4 ± 0	33±6	18 ± 2	22±2	6±0	31 ± 2	34±1	22 ± 1	59±2	32 ± 3	76 ± 4	35±4
	B. lactis	24 ± 0	73 ± 8	29 ± 14	35 ± 2	71±3	7±3	5 ± 1	41±5	32 ± 13	75±3	76 ± 14	93 ± 6	66±8
	B. adolescentis	3 ± 3	4 ± 2	18±3	7 ± 1	8±2	9±2	14 ± 2	33±2	9 ± 1	74±5	35 ± 6	83 ± 7	24±2
	Significant difference in the group	No	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Species differences:	infantis to lactis	No	Yes	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes
	infantis to adolescentis	No	No	No	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes
	lactis to adolescentis	No	Yes	No	Yes	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes

	DP4	H/D Ratios											
	Species/ Isomer Digested	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Group differences:	B. infantis	32 ± 3	70 ± 2	25 ± 1	45 ± 1	37±1	69±1	23 ± 1	68±4	44 ± 3	80±4	48 ± 8	54 ± 9
	B. lactis	96 ± 3	6 ± 0	74±3	57 ± 2	76±2	13±1	71 ± 9	57±3	106 ± 5	97±4	73 ± 7	51 ± 16
	B. adolescentis	43 ± 6	75 ± 2	49±4	49 ± 4	73±3	58±1	23 ± 3	43±2	47 ± 4	56±3	26 ± 12	1 ± 1
	Significant difference in the group	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Species differences:	infantis to lactis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
	infantis to adolescentis	Yes	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	Yes
	lactis to adolescentis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

	DP5	H/D Ratios						
	Species/ Isomer Digested	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Group differences:	B. infantis	41 ± 2	53 ± 2	64 ± 1	72 ± 1	58±3	48±1	54 ± 1
	B. lactis	84 ± 2	91 ± 3	64 ± 1	61 ± 3	88±3	56±1	89 ± 4
	B. adolescentis	58 ± 23	73 ± 12	64 ± 1	67 ± 12	75±9	45±7	52 ± 30
	Significant difference in the group	Yes	Yes	No	No	Yes	Yes	Yes
Species differences:	infantis to lactis	Yes	Yes	No	No	Yes	Yes	Yes
	infantis to adolescentis	No	Yes	No	No	Yes	No	No
	lactis to adolescentis	Yes	Yes	No	No	Yes	Yes	Yes