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Structural Identification of Novel Oligosaccharides Produced by *Lactobacillus bulgaricus* and *Lactobacillus plantarum*

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Supporting Information

ABSTRACT: β-Galactosidases (β-Gal) of lactic acid bacteria produce oligosaccharides from lactose when suitable acceptor carbohydrates are present. This study aimed to elucidate the structure of oligosaccharides formed by galactosylation of *N*-acetylglucosamine (GlcNAc) and fucose. Crude cellular extract of *Lactobacillus bulgaricus* and LacLM of *Lactobacillus plantarum* were used as sources of β-Gal activity. Disaccharides obtained by galactosylation of GlcNAc were identified as Gal-β-(1→4)-GlcNAc or Gal-β-(1→6)-GlcNAc by liquid chromatography−tandem mass spectrometry (LC-MS/MS) and comparison with external standards. Trisaccharides were identified as Gal-β-(1→6)-Gal-β-(1→[4 or 6])-GlcNAc by LC-MS, analysis of the MS/MS spectra of selected in-source fragment ions, and their relative retention times. LC-MS analysis revealed the presence of five galactosylated fucosides, but their linkage type could not be identified, partly due to the lack of reference compounds. β-Gal of lactic acid bacteria may serve as suitable tools for the chemoenzymatic synthesis of therapeutic oligosaccharides.

KEYWORDS: *Lactobacillus bulgaricus, N-acetylglucosamine, fucose, hetero-oligosaccharides, β-galactosidase, LC-ESI-MS/MS*

INTRODUCTION

Galacto-oligosaccharides (GOS) are recognized as prebiotics that support digestive and immune health. β-GOS consist of one to four β-linked galactose moieties with galactose or glucose located at the reducing end. GOS other than lactose are not digested in the small intestine and have a low caloric content and prebiotic activity.[1,2] In lactose-intolerant individuals, lactose also remains undigested in the small intestine and is fermented in the colon.[3] Prebiotic activity promotes the growth of beneficial bacteria in the gut, which displaces pathogenic bacteria.[1] Dietary indigestible oligosaccharides can also prevent the adhesion of pathogens and toxins to epithelial cell walls by acting as receptor analogues.[4] The interaction between pathogen and glycans on the epithelial surface is highly specific for the carbohydrate structure. For example, both Shiga toxin 1 and Shiga toxin 2e (Stx2e) produced by *Escherichia coli* respond to globotriaosylceramide as a glycan receptor; however, only Stx2e will also recognize globotetraosylceramide.[5] Owing to the recognition of globotetraosylceramide by Stx2e, this toxin causes disease in piglets that are resistant to other Shiga-like toxins as the corresponding glycan receptor on the cell surface is lacking.[5] The inhibition of pathogen adhesion by GOS has been demonstrated both in vitro and in vivo.[6] In vivo, the oral delivery of GOS produced by *Bifidobacterium bifidum* significantly reduced colonization of *Salmonella enterica* serovar Typhimurium in mice.[7] These benefits could be relevant for human health if the amounts of GOS that are required for the prevention of pathogen adhesion do not cause gastrointestinal distress.

GOS of various chain lengths are synthesized through enzymatic transgalactosylation of lactose via β-galactosidases (β-Gal).[8,9] During lactose conversion by β-Gal, galactose is covalently linked to the active site of the enzyme and subsequently transferred to water. If lactose is present in excess, β-Gal will use lactose, galactose, or glucose as an alternative galactosyl acceptor to form GOS. Enzymatic galactosylation leads to the formation of β-(1→2)-, β-(1→3)-, β-(1→4)-, or β-(1→6)-linked GOS, and bacterial and fungal enzymes generally form more than one linkage type.[8,9] The source of β-Gal affects the type of GOS produced, as different enzymes favor the formation of specific linkages, thus creating structurally distinct GOS.[8] Transgalactosylation of acceptor carbohydrates other than glucose or galactose yields galactose-containing oligosaccharides with acceptor carbohydrates at the reducing end.[8−10] Composite oligosaccharides formed by β-Gal are referred to as hetero-oligosaccharides (HeOS). HeOS increase the variation of oligosaccharide structures and particularly may allow for novel applications to prevent pathogen adhesion. HeOS are also nondigestible oligosaccharides but are fermented by bacteria in the colon. Because HeOS remain undigested, they may also have other health benefits similar to GOS or oligosaccharides found in human milk.[11,12]

Although no single analytical method can completely structurally identify oligosaccharides present in small amounts, mass spectrometry (MS) is an effective tool offering analytical diversity and high sensitivity. Mass spectrometry or tandem mass spectrometry (MS/MS), which provides structural information through investigation of ion fragmentation, is often coupled to liquid chromatography, thereby adding an extra dimension of compound separation. Electrospray

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ionization (ESI) is a technique used in mass spectrometry to ionize biomolecules with limited fragmentation occurring in the ion source. In particular, ESI can directly ionize underivatized neutral oligosaccharides, in either negative or positive ion modes, to achieve high-sensitivity detection and the generation of informative data used for structural identification.[13,14] Moreover, ESI can ionize saccharides over a range of molecular weights or degrees of polymerization (DP). For example, picogram levels of oligosaccharides with DP 1−11 were ionized by ESI and detected by an ion trap mass spectrometer.[15] Combined ESI-MS/MS has been used to elucidate branching sequences or partial sequences of neutral oligosaccharides from human milk of DP 10 and 11.[16]

Commercial GOS production relies on microbial β-Gal used at lactose concentrations close to saturation. Food grade organisms, including bifidobacteria and lactobacilli, have also been utilized to produce GOS.[1,7,8,17] The use of food grade enzyme sources facilitates the process development for production of food ingredients.[18] However, commercial and experimental GOS preparations are a mixture of mono- and oligosaccharides and are often poorly characterized with regard to their composition and the structure of individual compounds in the preparation.[1,7] LC-MS/MS methods for oligosaccharide analysis have not been employed and validated for characterization of GOS. Particularly, the linkage type and degree of polymerization of HeOS formed by β-Gal of lactobacilli remain unknown.[19] It was therefore the aim of this study to identify the structures of galactosylated *N*-acetyl-D-glucosamine and galactosylated fucose produced by β-Gal of lactobacilli. A LC-ESI-MS/MS method was established to identify oligosaccharides. A crude cellular extract of *Lactobacillus bulgaricus* ATCC 11842 was used as biocatalyst because this has previously been shown to produce GOS and HeOS from lactose.[19] *Lactococcus lactis* MG1363 expressing LacLM of *Lactobacillus plantarum* FUA3112 was additionally employed to assess the activity of a defined enzyme.

MATERIALS AND METHODS

Chemicals and Standards. Oligosaccharide standards lacto-*N*-biose (Galβ-(1→3)-GlcNAc), *N*-acetyl-D-lactosamine (Galβ-(1→4)-GlcNAc), and β1−6 galactosyl-*N*-acetyl glucosamine (Galβ-(1→6)-GlcNAc) were purchased from Dextra Laboratories (Reading, U.K.). Lactose, *N*-acetylglucosamine, and L-fucose with >99% purity were supplied by Sigma (Oakville, Canada). Fisher Scientific (Ottawa, Canada) supplied microbiological media, HPLC grade acetonitrile, methanol, ammonium acetate, and crystalline sodium chloride. All other solvents were of analytical grade unless specified otherwise.

Sample Production. *L. bulgaricus* ATCC11842 and *L. plantarum* FUA3112 were cultivated under microaerophilic conditions (1% O_2, balance N_2) at 37 °C in modified DeMan−Rogosa−Sharpe (mMRS) broth containing 5% w/v lactose with a pH of 6.2.[19] *Lc. lactis* MG1363 harboring pAMJ586 with LacLM from *L. plantarum*, as the sole source of β-Gal activity,[20] was grown in M17 with the addition of 0.5% glucose and 5 mg L^{-1} erythromycin at 30 °C. Cells were cultured for 12 h, harvested, and washed twice before suspension in 1 mL of 50 mM sodium phosphate buffer (pH 6.8) with 20% glycerol and 1 mM dithiothreitol. The cell suspension was transferred to screw-cap tubes with 0.5 mL of zirconia/silica beads (0.1 mm) and disrupted in a Mini Beadbeater-8 (model 693, BioSpec, Bartlesville, OK, USA) for two passes of 1.5 min and chilled in ice between passes. The disrupted cells were centrifuged at 15300*g* for 20 min at 4 °C. The supernatant, designated crude cellular extract (CCE), was used for GOS synthesis. The protein content of CCE was determined using the Bio-Rad protein assay (Bio-Rad); the specific activity (enzyme activity level relative to cell mass) was determined with *o*-nitrophenylgalactoside (Sigma) as described.[20] In keeping with previous investigations,[19,20] the β-Gal activities of CCE of *L. bulgaricus*, *L. plantarum*, and *Lc. lactis* expressing LacLM from *L. plantarum* ranged from 30 to 60 units (min × mg protein)$^{-1}$.

To analyze transgalactosylation with CCE of *L. bulgaricus*, carbohydrate solutions were prepared with 23% w/v lactose in a 50 mM sodium phosphate buffer (pH 6.8) with 100 mM KCl and 2 mM $MgCl_2$ (PB). An acceptor carbohydrate of either *N*-acetyl-D-glucosamine (GlcNAc) or L-fucose was added in a concentration of 12% w/v to each of the solutions of lactose. Solutions were filter sterilized prior to the addition of 20% v/v CCE. Enzymatic reactions were conducted at 37 °C for 24 h and terminated by heating to 95 °C for 15 min.

To analyze transgalactosylation with CCE from *L. plantarum* or *Lc. lactis* expressing LacLM of *L. plantarum*, 20 μL of CCE was mixed with 80 μL of PB, 100 mM KCl, and 1 mM $MgCl_2$ containing 0.5 M each of lactose and GlcNAc. Reactions were incubated at 45 °C for 16 h and terminated by heating at 95 °C.

High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). HPAEC analyses were performed using an ICS-3000 system consisting of an AS50 autosampler, and a pulsed electrochemical detector with a gold electrode, and an Ag/AgCl reference electrode (Dionex Corp., Sunnyvale, CA, USA). Sample oligosaccharide solutions were injected in 10 μL aliquots onto a CarboPac PA-20 Dionex carbohydrates column (3 × 150 mm) paired with a CarboPac PA-20 guard column (3 × 30 mm) and were separated at a flow rate of 0.25 mL min^{-1} for 42 min at 25 °C. Eluents A (water), B (0.2 M sodium hydroxide), and C (1 M sodium acetate) were used in the following gradient: 0 min, 30.4% B, 1.3% C; 25 min, 30.4% B, 15% C; 28 min, 0%B, 50% C; 31 min, 73% B, 17% C; 31.1 min, 30.4% B, 36.3% C; 34 min, 73% B, 17% C; 37 min, 73% B, 17% C; 40 min, 30.4% B, 1.3% C.

Combined Liquid Chromatography−Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS). Underivatized oligosaccharide fractions were analyzed by LC-ESI-MS/MS. Separations were conducted on an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA, USA) at 25 °C using a Supelcosil LC-NH_2 column (250 mm × 4.6 mm i.d., 5 μm) (Sigma Aldrich, Oakville, Canada). Each solution analyzed contained a final concentration of 0.1 g L^{-1} oligosaccharide in water. When the positive ion mode was used, 10 mM NaCl was supplemented to aid in ionization. Twenty-five microliters of each solution was injected onto the column using an isocratic flow of acetonitrile/water 80:20 (v/v) at a rate of 1 mL min^{-1}. The effluent from the column was split at a ratio of 1:4 (v/v), so that the flow rate to the mass spectrometer was 0.2 mL min^{-1} with the remainder going to waste. A postcolumn addition of ammonium acetate (40 mM in methanol) was delivered at a rate of 0.02 mL min^{-1} by an Agilent 1200 series isocratic pump to the flow entering the ESI source.

Positive and negative ion ESI-MS and collision-induced dissociation tandem mass spectrometry (CID-MS/MS) were performed on a QStar Elite hybrid orthogonal Q-TOF mass spectrometer coupled to a TurboIon Spray source with Analyst QS 2.0 software (Applied Biosystems/MDS Sciex, Concord, Canada). In negative ion mode, the source conditions were as follows: nebulizer gas, 50 (arbitrary units); ion spray voltage, −4500 V; curtain gas, 25; declustering potential, −45 V; focusing potential, −170 V; and declustering potential 2, −20 V, scanning over a mass range of *m*/*z* 50−600. Fragmentation was achieved using nitrogen as a collision gas at a collision energy that varied between −10 and −25 eV, optimized for each saccharide. Similarly, conditions for the optimal formation and analysis of positive ions were as follows: nebulizer gas, 45; ion spray voltage, 5000 V; curtain gas, 25; declustering potential, 45 V; focusing potential, 170 V; declustering potential 2, 20 V, with a scan range of *m*/*z* 50−1100. Collision energy for positive ions was optimal between 25 and 40 eV depending on the analyte. Auxiliary gas flow was optimized at 60 arbitrary units and a source temperature of 400 °C for both negative and positive ion modes. Quasi MS^3 spectra were obtained from the MS/MS spectra of in-source fragment ions formed by increasing the declustering potential by an additional 30 V. Estimation of the amounts of galactosylated GlcNAc compounds present in samples was

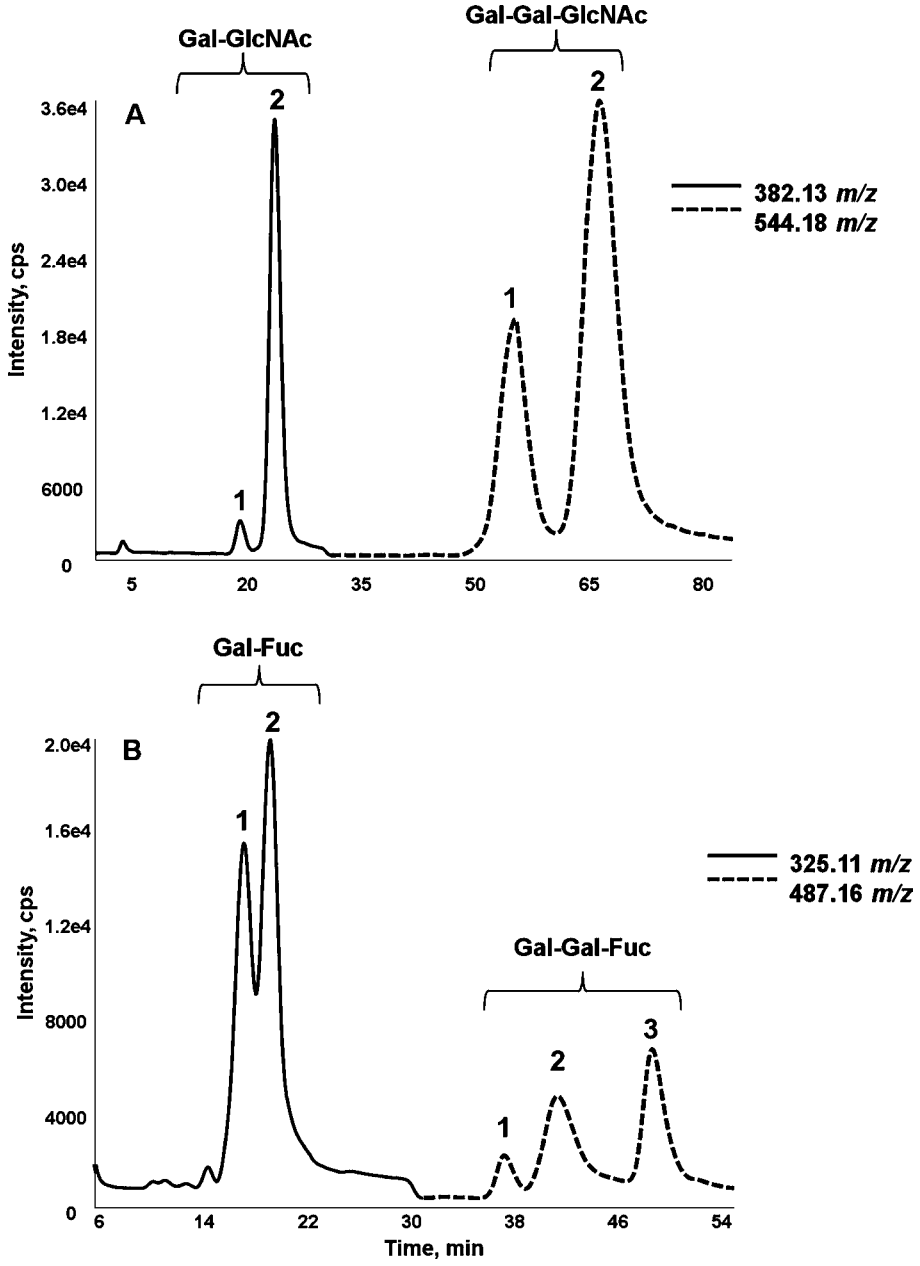

Figure 1. LC-ESI-MS extracted ion chromatogram (XIC) overlays of (A) the $[M - H]^-$ ions of Gal-GlcNAc m/z 382.13 and Gal-Gal-GlcNAc m/z 544.18 and (B) the $[M - H]^-$ ions of Gal-Fuc m/z 325.11 and Gal-Gal-Fuc m/z 487.16.

Table 1. Mass Accuracy (of Sodiated Adducts and Deprotonated Molecules) and Retention Times of All Possible HeOS Formed between Samples with either GlcNAc or Fucose Added as Acceptor Carbohydrate

			$[M + Na]^+$			$[M - H]^-$		
acceptor CHO	compound	retention time (min)	measured mass (Da)	exact mass (Da)	error (mDa)	measured mass (Da)	exact mass (Da)	error (mDa)
+ GlcNAc	Gal-GlcNAc	18.5; 23.2	406.1323	406.1319	0.3	382.1364	382.1354	0.9
	Gal-Gal-GlcNAc	55.2; 67.3	568.1850	568.1848	0.3	544.1883	544.1883	0.0
+ Fuc	Gal-Fuc	17.0; 19.1	349.1101	349.1105	−0.4	325.1150	325.1140	1.0
	Gal-Gal-Fuc	37.1; 41.0; 48.2	511.1635	511.1633	0.2	487.1694	487.1668	2.5

achieved using calibration curves for the external standards, Galβ-(1→4)-GlcNAc and Galβ-(1→6)-GlcNAc, with correction for the response of fucose added as an internal standard.

RESULTS

Separation of GOS and HeOS. HPAEC-PAD was first used to confirm the presence of HeOS formed by trans-galactosylation of fucose and GlcNAc. A comparison between

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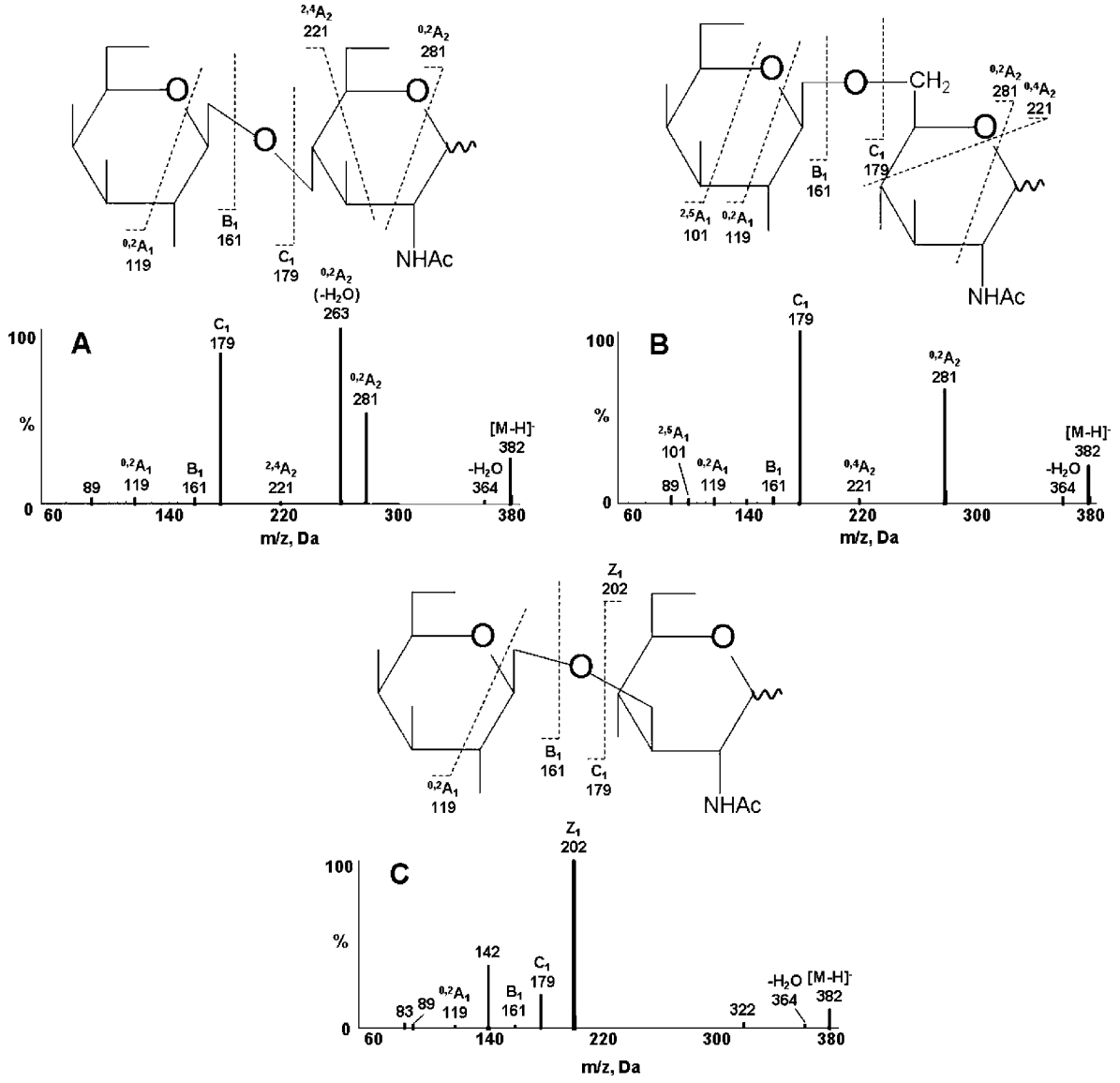

Figure 2. ESI-MS/MS spectra of $[M - H]^-$ ions representative of Gal-GlcNAc isomers: (A) Galβ-(1→4)-GlcNAc standard; (B) Galβ-(1→6)-GlcNAc standard; (C) Galβ-(1→3)-GlcNAc standard.

the chromatograms of the CCE enzymatic reactions carried out in the presence of GlcNAc or fucose with those of a control reaction carried out in the presence of lactose only confirmed transfer of galactose to either fucose or GlcNAc, as previously observed[10] (as well as data not shown). To obtain structural information of HeOS, LC-MS data were obtained to detect molecular species arising from HeOS produced by transgalactosylation of GlcNAc or fucose. A total of four HeOS formed by transgalactosylation of GlcNAc were observed in addition to five HeOS formed by transgalactosylation of fucose. In reactions containing GlcNAc as an acceptor, two disaccharides and two trisaccharides were identified (Figure 1A). Additionally, two disaccharide and three trisaccharide isomers were detected in reactions containing fucose as an acceptor (Figure 1B). The composition of the HeOS species was confirmed by exact mass measurements in both positive and negative ion modes (Table 1). The low mass accuracy for the fucose−HeOS molecular ions in negative ionization mode was likely due to the low signal-to-noise ratio. However, the exact masses for the molecular ions of all compounds of interest were all better than 5 ppm (Table 1).

Structural Identification of Oligosaccharides with Electrospray−Mass Spectrometry. Tandem mass spectrometry was then employed to elucidate monomer sequence and glycosidic linkage information. Three external standards (Galβ-(1→3)-GlcNAc, Galβ-(1→4)-GlcNAc, and Galβ-(1→6)-GlcNAc) were available as references for GlcNAc-derived HeOS, but none were obtained for fucose-derived HeOS. The fragments that were observed from MS/MS experiments were labeled according to the nomenclature of Domon and Costello.[21] In this nomenclature, A-, B-, and C-type fragments are those retaining the charge at the nonreducing end of the oligosaccharide, whereas X-, Y-, and Z-type fragments retain the charge at the terminal unit. A and X indicate cross-ring fragmentation; B, C, Y, and Z signify fragmentation of glycosidic linkages.

Both $[M + Na]^+$ and $[M - H]^-$ ions were detected in relatively high intensity for HeOS under either positive or negative ionization polarity, respectively. In general, the mass

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spectra of $[M - H]^-$ species gave more informative MS/MS spectra than did $[M + Na]^+$ ions. Hence, although the MS/MS spectra of the $[M + Na]^+$ ions from HeOS samples did in some cases give some complementary fragmentation to that seen in negative ion mode, the more informative negative ion MS/MS analysis is the focus of the data interpretation presented below.

Disaccharides Formed in the Presence of an *N*-Acetylglucosamine Galactosyl Acceptor. A total of two isomers could be resolved by HPLC separation: Gal-GlcNAc peaks 1 and 2 (Figure 1A). Tandem mass spectrometry data for the two peaks present different fragmentation patterns, indicating that Gal-GlcNAc isomers in peaks 1 and 2 have different glycosidic linkages (Supporting Information, Supplemental Figure 1A,B). Interglycosidic B_1 and C_1 fragments at m/z 179 and 161, respectively, present in both spectra, are consistent with the monosaccharide sequence of Gal-GlcNAc. The retention time of Gal-GlcNAc peak 1 was congruent with the retention time of a Galβ-(1→4)-GlcNAc standard, and the retention time of Gal-GlcNAc peak 2 was also congruent with the standard Galβ-(1→6)-GlcNAc. Compounds eluting at retention times corresponding to the Galβ-(1→4)-GlcNAc and Galβ-(1→6)-GlcNAc standards were also identified in the HPAED-PAD analysis of total oligosaccharides (data not shown). To gain further information, the three available isomeric standards of Gal-GlcNAc were utilized under identical chromatographic and MS/MS conditions as those of compounds Gal-GlcNAc peaks 1 and 2. Panels A, B, and C of Figure 2 show the MS/MS spectra of $[M - H]^-$ ions from the Galβ-(1→4)-GlcNAc, Galβ-(1→6)-GlcNAc, and Galβ-(1→3)-GlcNAc standards; sodiated spectra are shown in Supplemental Figure 2 of the Supporting Information. The MS/MS spectra of these isomers are distinctly different (Figure 2). Fragment ions seen in the MS/MS spectrum of the Galβ-(1→4)-GlcNAc standard match, in terms of both the ions present and their relative intensities, those seen Gal-GlcNAc peak 1 (Figure 2A and Supplemental Figure 1A of the Supporting Information). Thus, Gal-GlcNAc peak 1 was identified as being Galβ-(1→4)-GlcNAc. Similarly, fragment ions in the MS/MS spectra of the Galβ-(1→6)-GlcNAc standard closely matched those from Gal-GlcNAc peak 2 (Figure 2B and Supplemental Figure 1B of the Supporting Information), thereby confirming the identity of the latter. In addition, both the retention time and the MS/MS spectra of standard Galβ-(1→3)-GlcNAc differed from those of the Gal-GlcNAc peaks in samples containing HeOS.

It was noted that the spectra for the Galβ-(1→6)-GlcNAc standard was not entirely consistent with literature reports for other 1,6-linked oligosaccharides. In earlier papers on the MS/MS spectra of oligosaccharide anions, it has been indicated that 1,3 linkages display no $^{0,2}A$ cross-ring fragmentation; the presence of m/z 263 and 281 and the absence of m/z 251 ions indicate a 1,4 linkage, and the presence of m/z 251 and 281 but the absence of m/z 263 indicates a 1,6 linkage.[22−24] In this work, the MS^2 spectrum of Galβ-(1→4)-GlcNAc exhibited a $^{0,2}A_2$ cross-ring fragment of m/z 281 and a water loss of m/z 263, indicating a 1,4 linkage and consistent with the above rules. However, the MS/MS spectrum of Galβ-(1→6)-GlcNAc presents the $^{0,2}A_2$ cross-ring fragment of m/z 281 with the absence of m/z 263 as expected, but in addition m/z 251 ions were not detected. This inconsistency in the spectrum of Galβ-(1→6)-GlcNAc compared to literature data for 1,6-linked oligosaccharides is likely due to the effect of the *N*-acetyl group on the fragmentation pattern compared to hexose moieties without this functional group. Furthermore, this same fragmentation pattern was observed using an authentic standard as reported in Figure 2B and Supplemental Figure 1B of the Supporting Information.

Trisaccharides Formed in the Presence of an *N*-Acetylglucosamine Galactosyl Acceptor. The two distinct peaks seen in the extracted ion chromatogram of the $[M - H]^-$ ion of $(Gal)_2GlcNAc$ indicate the presence of two isomeric species (peaks 1 and 2 in Figure 1A). The MS/MS spectra of each of these isomers were found to have quite different fragmentation patterns (Figures 3A and 4A; Supporting Information, Supplemental Figure 4). However, both isomers display the fragment ions C_1 at m/z 179, C_2 at m/z 341, and the corresponding B_1 and B_2 fragment ions at 18 m/z units lower. These confirm that the monosaccharide sequence of both peaks 1 and 2 is Gal-Gal-GlcNAc. Due to the lack of authentic standards for the trisaccharide, a quasi MS^3 experiment was implemented to obtain additional linkage information. This was accomplished on a Q-TOF type instrument by increasing the ESI cone voltage to induce in-source fragmentation; the resulting fragment ions were then

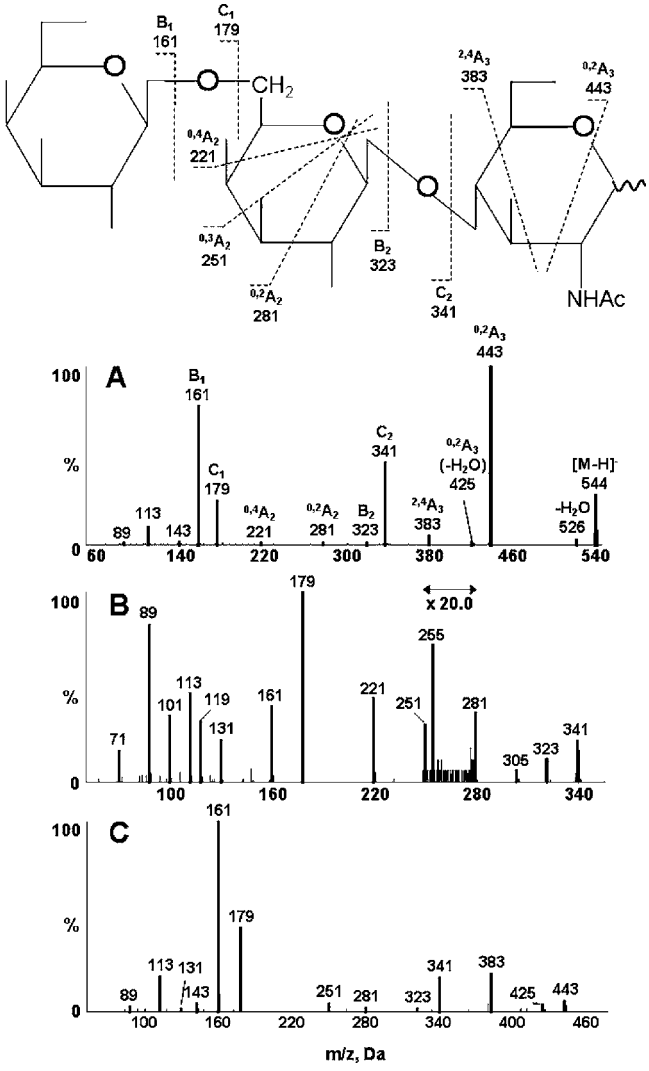

Figure 3. ESI-MS/MS spectra of sample compound Gal-Gal-GlcNAc peak 1: (A) $[M - H]^-$ ion at m/z 544; (B) in-source fragment ion C_2 at m/z 341; (C) in-source fragment ion $^{0,2}A_3$ at m/z 443.

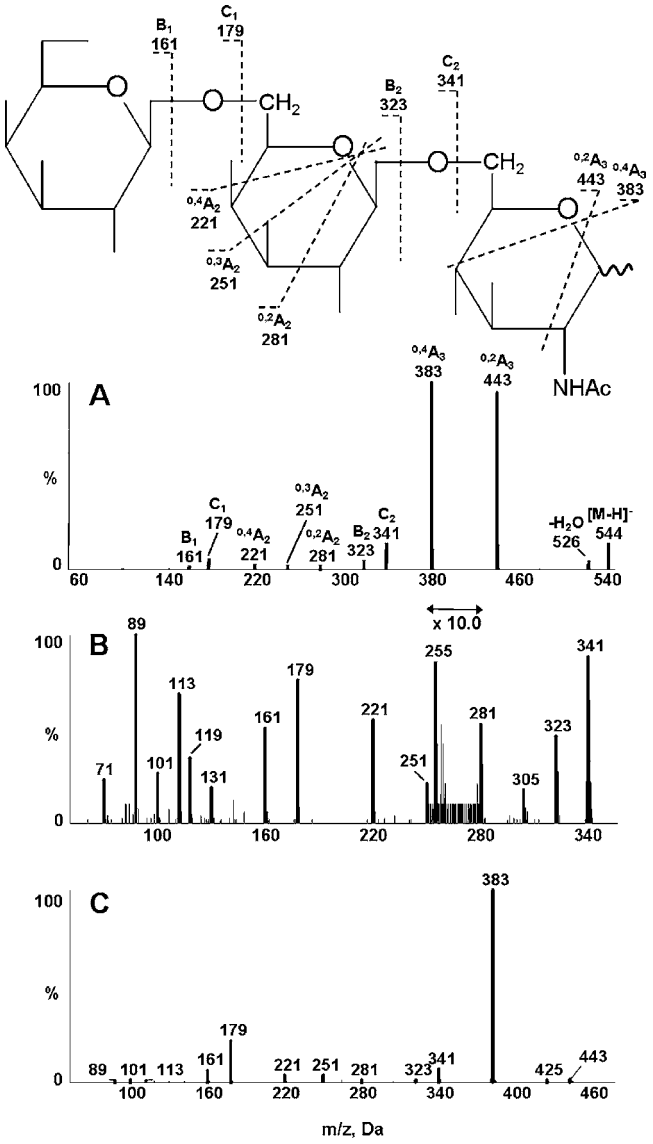

Figure 4. ESI-MS/MS spectra of sample compound Gal-Gal-GlcNAc peak 2: (A) $[M - H]^-$ at m/z 544; (B) in-source fragment ion C_2 at m/z 341; (C) in-source fragment ion $^{0,2}A_3$ at m/z 443.

selected and subjected to collision-induced dissociation. In this way, the MS/MS spectra of the C_2 fragment ion at m/z 341 from Gal-Gal-GlcNAc were obtained for both peaks 1 and 2. The C_2 fragment ion was specifically selected so that the galactose backbone and the first glycosidic linkage of the Gal-Gal-GlcNAc molecule would remain intact, eliminating the *N*-acetyl glucosamine group. It was found that the MS/MS spectra of these C_2 ions from both Gal-Gal-GlcNAc peaks 1 and 2 are identical (Figures 3B and 4B), indicating that the first glycosidic linkage in each compound is the same. In these MS/MS spectra the presence of small but significant ion peaks of m/z 281 and 251 is indicative of a 1,6 linkage, so that the structure of the two isomers can be deduced to be Galβ-(1→6)-Galβ-(1→X)-GlcNAc.

Next, MS/MS experiments of the $^{0,2}A_3$ fragment ion at m/z 443 were obtained (Figures 3C and 4C). Because the $^{0,2}A_3$ fragment ion arises through fragmentation excluding the *N*-acetyl glucosamine group, both the first and second glycosidic linkages remain intact, and hence the MS/MS spectrum of this ion is indicative of both linkages. The MS/MS spectra of the $^{0,2}A_3$ fragment ions arising from Gal-Gal-GlcNAc peaks 1 and 2 are different, but the A-type fragments observed do not provide unambiguous information on the second glycosidic linkage. However, it is believed that the presence of the cross-ring fragment $^{0,2}A_3$ through the terminal sugar excludes the possibility of an oligosaccharide with the structure Galβ-(1→6)-Galβ-(1→3)-GlcNAc[25] because the blocked hydroxyl group of a 3-linked saccharide prevents the anomeric ring from opening and undergoing retro-enol rearrangement.[26] This explanation is well demonstrated in Figure 2C by the complete absence of an $^{0,2}A$ cleavage from the Galβ-(1→3)-GlcNAc standard. Hence, a 1,3 linkage between Gal and GlcNAc would not be consistent with the data. On the other hand, during the enzymatic synthesis of HeOS by transgalactosylation of GlcNAc, digalactosylated GlcNAc must originate from transgalactosylation of disaccharides. Thus, under the experimental conditions employed in this study, trisaccharides are formed from Galβ-(1→4)-GlcNAc or Galβ-(1→6)-GlcNAc as described above. This gives rise to corresponding structures Galβ-(1→6)-Galβ-(1→4)-GlcNAc and Galβ-(1→6)-Galβ-(1→6)-GlcNAc as the only possibility that is consistent with the disaccharides produced in the enzymatic reactions. In addition, under the chromatographic conditions used, linkage positioning

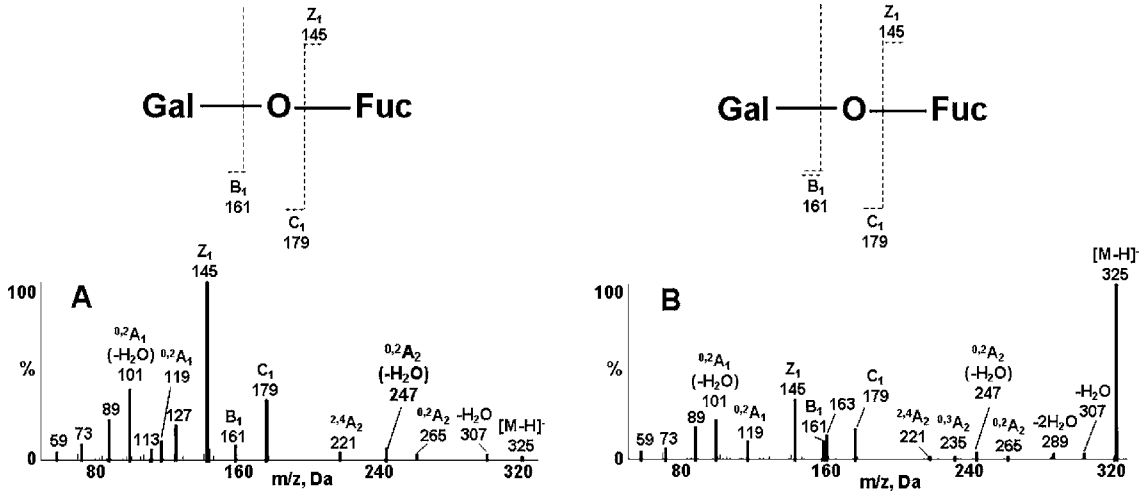

Figure 5. ESI-MS/MS spectra of the $[M - H]^-$ ions of sample compound (A) Gal-Fuc peak 1 and (B) Gal-Fuc peak 2.

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alone influenced elution times such that Galβ-(1→6)-GlcNAc eluted after Galβ-(1→3)-GlcNAc or Galβ-(1→4)-GlcNAc (Figure 1 and data not shown). By analogy, it is concluded that the trisaccharide Galβ-(1→6)-Galβ-(1→6)-GlcNAc will elute after the trisaccharide Galβ-(1→6)-Galβ-(1→4)-GlcNAc, which differs only by the linkage at the reducing sugar. Hence, in Figure 1A, the trisaccharide Gal-Gal-GlcNAc peak 1 is believed to be Galβ-(1→6)-Galβ-(1→4)-GlcNAc, whereas peak 2 is Galβ-(1→6)-Galβ-(1→6)-GlcNAc.

Fucosylated Heterooligosaccharides. Five new compounds were separated by LC-MS after transgalactosylation of fucose with CCE of *L. bulgaricus* (Figure 1B). Isomers of the disaccharide and trisaccharide HeOS were analyzed via MS/MS spectra of their $[M - H]^-$ ions, resulting in complementary B-, C-, and Z-type glycosidic fragments to determine the monosaccharide sequence. Sodiated spectra are shown in Supplemental Figure 5A,B of the Supporting Information. Gal-Fuc peaks 1 and 2 (Figure 5) both displayed m/z 161 (B_1), m/z 179 (C_1), and m/z 145 (Z_1), confirming that the sequence is indeed Gal-Fuc. However, both isomers of Gal-Fuc displayed different fragment ion patterns and relative intensities in the MS/MS spectra, which is indicative of linkage variation because all other structural aspects correspond. MS/MS spectra analysis was also performed on the $[M - H]^-$ ions of the $(Gal)_2Fuc$ peaks 1−3 (Figure 6), indicating the monosaccharide sequence

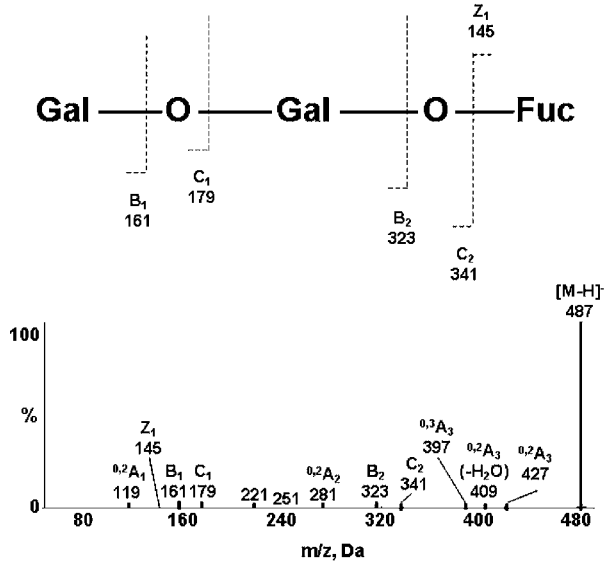

Figure 6. ESI-MS/MS spectra of $[M - H]^-$ ions representative of sample compound Gal-Gal-Fuc peaks 1−3.

to be Gal-Gal-Fuc. However, there was no discernible difference between the three spectra; therefore, Figure 6 is presented as representative of all three isomers. The intensities of $(Gal)_2Fuc$ fragments under optimized collision energies in MS/MS in both positive (Supporting Information, Supplemental Figure 5C) and negative ion modes were poor. This could be due to a low concentration of fucosylated HeOS formed in solution, inadequate ionization, or the orientation of the glycosidic linkage. Standards of galactosylated fucose oligosaccharides were not available for comparison.

Disaccharides Formed by LacLM of *L. plantarum* FUA3112. The formation of β-(1→4)-linked acceptor products by β-Gal of lactic acid bacteria is in apparent contrast with reports on the linkage type of GOS produced by lactobacilli and *Streptococcus thermophilus*.[8] To analyze the spectrum of disaccharides formed from a single, defined β-Gal, transglycosylation of GlcNAc was performed with CCE of *L. plantarum* and *Lc. lactis* expressing LacLM of *L. plantarum* FUA3112 as the sole source of β-Gal activity. LC-ESI-MS/MS aimed to identify the linkage type in all disaccharides, including galacto-oligosaccharides. Identification of galactosylated GlcNAc confirmed the exclusive presence of Galβ-(1→4)-GlcNAc and Galβ-(1→6)-GlcNAc (Supporting Information, Supplemental Figure 6); mass spectra of mono- and digalactosylated GlcNAc obtained with LacLM from *L. plantarum* were identical to those observed in reactions with *L. bulgaricus*. The concentrations of Galβ-(1→4)-GlcNAc and Galβ-(1→6)-GlcNAc measured in enzymatic reactions with *Lc. lactis* expressing LacLM of *L. plantarum* FUA3112 were 0.71 and 19.5 g L^{-1}, respectively, indicating a strong preference for formation of the β-(1→6) linkages. The analysis of disaccharides carrying glucose or galactose at the reducing end (GOS) revealed the presence of β-(1→6)-, β-(1→3)-, and β-(1→4)-linked GOS[22] (Supporting Information, Supplemental Figure 7). The presence of two β-(1→4)-linked disaccharides, representing Galβ-(1→4)-Gal and lactose, demonstrates that LacLM also forms β-(1→4) linkages. However, the relative quantity of β-(1→4)-linked products could not be determined because lactose is both substrate and product of the reaction.

DISCUSSION

This study determined the structure of disaccharides and trisaccharides produced by transgalactosylation of β-Gal from *L. bulgaricus* and LacLM from *L. plantarum* with lactose as galactosyl donor and GlcNAc as galactosyl acceptor. LC-ESI-MS/MS analysis and the use of external standards identified the HeOS Galβ-(1→4)-GlcNAc and Galβ-(1→6)-GlcNAc. Galβ-(1→6)-Galβ-(1→4)-GlcNAc and Galβ-(1→6)-Galβ-(1→6)-GlcNAc were identified by a combination of LC-ESI-MS/MS analysis, their retention behavior, and the identification of the precursor compounds in the enzymatic synthesis. Fucose also served as a galactosyl acceptor to form two disaccharides and three trisaccharides; however, the limited information that could be obtained from the MS/MS data, the lack of available standards, and the low concentrations of the analytes prevented identification of the linkage types within these compounds.

In keeping with previous reports of GOS and HeOS formation by lactobacilli,[19,27,28] linkage types in oligosaccharides produced by enzymes from *L. bulgaricus* and *L. plantarum* were identical. The identification of β(1→4)-linked disaccharides as products of transgalactosylation of GlcNAc and galactose by β-Gal of lactobacilli extends information from previous papers,[8,9,27,28] which identified only β(1→3)- or β(1→6)-linked GOS. Past studies identifying the linkage type of GOS, produced with β-Gal from lactobacilli, relied solely on chromatographic methods paired with the use of external standards,[27,28] which left Gal-β(1→4)-Gal undetected. The β(1→4)-linked disaccharides observed in this study were a minor product compared to β(1→6)-linked oligosaccharides, but the sensitivity of the MS/MS method nevertheless allowed their identification. The specificity of β-Gal from *L. bulgaricus* and *L. plantarum* thus differs from the β-Gal from *Bacillus circulans*.[10] Tri- and tetrasaccharides formed with GlcNAc as acceptor carbohydrate by *B. circulans* β-Gal exhibited exclusively β-(1→4) linkages.[10] This absence of Gal-β(1→4)-GlcNAc in *B. circulans* may be attributable to steric hindrance

by the *N*-acetyl moiety at the C2 position. Because the linkage type of oligosaccharides in an important determinant for their ability to prevent pathogen adhesion,[4,5] this study increased the variation of oligosaccharide structures formed in the acceptor reaction of β-Gal.

These results can be applied to the food grade conversion of whey permeate, a lactose-containing product of the ultrafiltration of whey, to produce novel oligosaccharides. GOS production and β-Gal activity in whey permeate was 1.8-fold higher when compared to a lactose solution.[18] This difference is attributable to the presence of monovalent and divalent cations in whey permeate, which enhance β-Gal activity.[29] Whey permeate is a readily available source of lactose, which can be used for GOS and HeOS production. Additionally, GlcNAc is one of the most abundant polysaccharides on earth, derived from the hydrolysis of chitin, an extracellular polymer found in invertebrates, fungi, and algae.[30]

Possible physiological functions of HeOS produced by lactobacilli are also related to structural similarities to human milk oligosaccharides (HMOS). The core molecule of HMOS consists of galactose and GlcNAc monomers $\beta(1\rightarrow3/4)$-linked repetitively with lactose at the reducing end. Additional fucosylation and sialylation of these core molecules also create other composite structures.[11,31] Different functions are attributed to individual types of HMOS, which relates to their wide variety of structures.[11,32] Currently, with their structural and compositional complexity, HMOS cannot be industrially produced, and infant formula is supplemented with simpler GOS structures to mimic the bifidogenic effect of human milk oligosaccharides.[1] *N*-Acetyllactosamine, the Galβ-(1→4)-GlcNAc core structure of HMOS, was one of the products of transgalactosylation of GlcNAc by β-Gal from lactobacilli. Enzymatic synthesis of Galβ-(1→4)-GlcNAc could thus be employed act as a step in the chemoenzymatic synthesis of HMOS. Moreover, *N*-acetyllactosamine is a preferred substrate for bifidobacteria[32] and was identified as a competitive inhibitor to enteropathogenic *E. coli*.[33] Thus, HeOS formed in food grade conversions by the β-Gal of lactobacilli could be used as food additives, particularly to supplement infant formulas.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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