Structural Comparison of Different Galacto-oligosaccharide Mixtures Formed by β-Galactosidases from Lactic Acid Bacteria and Bifidobacteria

Suwapat Kittibunchakul, Sander S. van Leeuwen, Lubbert Dijkhuizen, Dietmar Haltrich, and Thu-Ha Nguyen*

ABSTRACT: The LacLM-type β-galactosidase from Lactobacillus helveticus DSM 20075 expressed in both Escherichia coli (EcoBl2L1Hβ-gal) and Lactobacillus plantarum (Lp609Hβ-gal) was tested for their potential to form galacto-oligosaccharides (GOS) from lactose. The Lh-GOS mixture formed by β-galactosidase from L. helveticus, together with three GOS mixtures produced using β-galactosidases of both the LacLM and the LacZ type from other lactic acid bacteria, namely, L. reuteri (Lr-GOS), L. bulgaricus (Lb-GOS), and Streptococcus thermophilus (St-GOS), as well as two GOS mixtures (Br-GOS1 and Br-GOS2) produced using β-galactosidases (β-gal I and β-gal II) from Bifidobacterium breve, was analyzed and structurally compared with commercial GOS mixtures analyzed in previous work (Vivinal GOS, GOS I, GOS III, and GOS V) using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), high-performance size-exclusion chromatography with a refractive index (RI) detector (HPSEC-RI), and one-dimensional 1H NMR spectroscopy. β-Galactosidases from lactic acid bacteria and B. breve displayed a preference to form β-(1→6)- and β-(1→3)-linked GOS. The GOS mixtures produced by these enzymes consisted of mainly DP2 and DP3 oligosaccharides, accounting for ~90% of all GOS components. GOS mixtures obtained with β-galactosidases from lactic acid bacteria and B. breve were quite similar to the commercial GOS III mixture in terms of product spectrum and showed a broader product spectrum than the commercial GOS V mixture. These GOS mixtures also contained a number of GOS components that were absent in the commercial Vivinal GOS (V-GOS).

KEYWORDS: β-galactosidase, galacto-oligosaccharides, prebiotic, lactic acid bacteria, bifidobacteria

INTRODUCTION

β-Galactosidases (lactases; EC 3.2.1.23), which catalyze the hydrolysis of the β-1,4-d-glycosidic linkage of milk sugar lactose and its structurally related compounds to yield the monosaccharides glucose and galactose, are of importance to several biotechnological processes in the food industry. The well-known applications of β-galactosidases in lactose hydrolysis include development of low-lactose and lactose-free dairy products for lactose-intolerant consumers, improvement of technological and sensory properties of foods containing lactose, and utilization of cheese whey to diminish economic and environmental problems. In addition to their hydrolytic activity, β-galactosidases possess transgalactosylation activity to form galacto-oligosaccharides (GOS) from lactose. The mechanism of these enzymes involves two steps of which the cleavage of lactose and the formation of a covalently linked galactosyl–enzyme intermediate while releasing glucose occurs in the first step, followed by the transfer of galactosyl moieties from the donor sugar lactose to an acceptor, which can be water, lactose, or any of the sugar products in the reaction mixture. In the trans-galactosylation mode, galactosyl moieties of lactose are transferred to other saccharides instead of water to yield GOS with different degrees of polymerization as reaction products.

GOS are known as a mixture of carbohydrates consisting of non-lactose disaccharides, various trisaccharides, and higher oligosaccharides with the degree of polymerization up to eight (DP8). Sugar units in GOS typically join together with β-(1→4) and β-(1→6) linkages, but β-(1→2) and β-(1→3) linkages are also reported. Currently, GOS are considered as dominant functional food ingredients fulfilling the criteria of “prebiotics” as reported in the literature on their modulatory effects on gut microbiota including stimulation of beneficial bacteria such as bifidobacteria and lactobacilli as well as inhibition of “undesirable” bacteria, maintenance of gut health, beneficially affecting the bowel functions, and colitis prevention. In addition to these confirmed beneficial effects, postulated physiological benefits of GOS include suppression of intestinal disturbances and colorectal cancer, lowering serum cholesterol levels, reducing risk of cardiovascular diseases, increasing absorption and retention of diverent...
minerals, and promoting immune response. Although GOS are not a significant element in human breast milk, they were shown to have a similar bifidogenic effect and therefore are used as key fortificants in infant formulas.

A number of β-galactosidases from microbial sources have been isolated and purified for the production of GOS. Among the β-galactosidase-producing microorganisms, some species of lactic acid bacteria, predominantly lactobacilli, and bifidobacteria have attracted great attention and have been studied extensively because of their probiotic potential and their generally recognized as safe (GRAS) status. It is anticipated that GOS synthesized by lactobacilli and bifidobacterial β-galactosidases would have structural characteristics, which will be preferentially utilized by this group of beneficial intestinal microorganism that facilitates the growth and metabolic activity of gut microbiota.

In the present study, we describe the formation of GOS from lactose using the recombinant LacLM-type β-galactosidase from the industrially important lactic acid bacterium Lactobacillus helveticus DSM 20075. Individual GOS components of the resulting GOS mixtures were identified and structurally compared with different GOS mixtures formed by β-galactosidases from several lactic acid bacteria and bifidobacteria, including Lactobacillus reuteri, Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus salivarius subsp. thermophilus, and Bifidobacterium breve, in comparison with some commercial GOS products studied previously. Significantly, the information on the GOS profiles obtained from this study will provide deeper insights into lactic acid bacterial and bifidobacterial β-galactosidases with respect to the spectrum of their GOS products.

### MATERIALS AND METHODS

#### Chemicals

All chemicals and analytical standards of D-glucose, D-galactose, and lactose used in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise and were of the highest purity available. Vivinal GOS (V-GOS; 59% GOS, 21% lactose, 19% glucose, and 1% galactose) and the GOS mixtures, GOS I, GOS III, and GOS V are the commercial GOS standards from previous work. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Roth (Karlsruhe, Germany). Peptide pheromone IP-NP was measured at 420 nm using a spectrophotometer (Beckman, Silver Spring, MD). The purified EcoliBL21Lh-gal-gal and L. plantarum TGL02 (Lp609Lh-gal) were collected and stored at −20 °C. EcoliBL21Lh-gal was then purified to apparent homogeneity. The purification was carried out by immobilized metal affinity chromatography (IMAC) using a prepacked 1 mL HisTrap HP column with Ni-Sepharose resin (GE Healthcare, Uppsala, Sweden) as described previously. Active fractions were collected, desalted, and concentrated by ultrafiltration using an Amicon ultra centrifugal filter unit with a 30 kDa cutoff membrane (Millipore, MA, USA). The purified EcoliBL21Lh-gal-gal was kept in 50 mM NaPB (pH 6.5) at 4 °C.

#### GOS Formation by Recombinant β-Galactosidases from L. helveticus

Batch conversions of lactose for the formation of GOS were carried out using recombinant β-galactosidases from L. helveticus, crude enzyme Lp609Lh-gal, and purified enzyme EcoliBL21Lh-gal. The reactions were performed based on the enzyme properties obtained from our previous study. Reaction conditions were 205 g/L initial lactose concentration in 50 mM NaPB (pH 6.5) containing 1 mM MgCl₂, β-galactosidase (1.5 U/l/mL of reaction mixture), and constant agitation (300 rpm). Different reaction temperatures varying from 37–50 °C were applied for crude enzyme Lp609Lh-gal, while those varying from 30–50 °C were applied for purified enzyme EcoliBL21Lh-gal. Samples were withdrawn periodically to monitor their residual enzyme activities with oNPG as the substrate. Sugar compositions and size distribution of the GOS mixtures were analyzed by HPAEC-PAD, HPSEC-RI, and one-dimensional 1H NMR spectroscopy.

#### Other GOS Preparations

The purified GOS mixtures Lr-GOS, free from monosaccharides and unconverted lactose, produced using recombinant LacLM-type β-galactosidase from L. reuteri L103 were prepared as previously described by Maicherberger et al. Preparation of the GOS mixtures St-GOS, using LacZ-type β-galactosidases from L. delbrueckii subsp. bulgaricus DSM 20014 and S. salivarius subsp. thermophilus DSM 20259, respectively, and Br-GOS1 and Br-GOS2 using purified LacZ-type β-galactosidases β-gal and β-gal II from B. breve DSM 20213, were carried out under the conditions that yielded the highest GOS for each enzyme as reported previously. The GOS mixtures were analyzed by HPAEC-PAD, HPSEC-RI, and one-dimensional 1H NMR spectroscopy.

#### High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Carbohydrate contents in the reaction mixtures were analyzed by HPAEC-PAD at the Food Biotechnology Lab (BOKU; Vienna). HPAEC-PAD analyses were performed on a Dionex DX-500 system (Dionex Corp., Sunnyvale, CA) as previously described in detail. The GOS yield (%) was defined as the percentage of GOS produced in the samples compared to initial lactose.

Detailed structural analyses of the GOS mixtures were also performed using HPAEC-PAD at the laboratory of Groningen Biomolecular Sciences and Biotechnology Institute (University of Groningen). GOS syrup samples were diluted by pipetting 20.2 ± 0.4 µL of GOS syrup up to 1.990 ± 0.007 mL containing 1.01 ± 0.02 mM fucose as an internal standard. Reference GOS samples, used to identify peaks, were diluted ~400 times first by diluting a volume of thick GOS syrup 1:1 with Milli-Q and then diluting it 200 times by adding ~10 µL in 1.990 ±0.007 mL of Milli-Q.
Q water containing a 1.01 ± 0.02 mM fucose internal standard. GOS samples (10 μL, ∼1.0 mg/mL) were profiled on a Dionex ICS-3000 workstation (Dionex, Amsterdam, The Netherlands) equipped with a CarboPac PA-1 column (250 × 2 mm, Dionex) and an ICS-3000 ED pulsed amperometric detector (PAD). Oligosaccharides were eluted using a complex gradient of A: 100 mM NaOH, B: 600 mM NaOAc in 100 mM NaOH, C: Milli-Q water, and D: 50 mM NaOAc. The fractionations were performed at 0.25 mL/min with 10% A, 85% C, and 5% D in 25 min to 40% A, 10% C, and 50% D followed by a 35 min gradient to 75% A and 25% B directly followed by 5 min washing with 100% B and reconditioning for 7 min with 10% A, 85% B, and 5% D.23,28,29

High-Performance Size-Exclusion Chromatography with a Refractive Index Detector (HPSEC-RI). Size distribution analysis of GOS samples (10 μL injections of ∼10 mg/mL GOS) was performed on a Rezex RSO-01 oligosaccharide Ag+ (4%) column (200 × 10 mm; Phenomenex, Utrecht, The Netherlands) using a Waters 2690XE Alliance HPLC system (Waters, Etten-Leur, The Netherlands) equipped with a Waters 2410 RI detector. Elutions were carried out with Milli-Q water at a flow rate of 0.3 mL/min. The peaks were identified and quantified in relation to a calibration curve (0.05–20 mg/mL) of Glc, Gal, Lac, and isolated GOS fractions up to DPs.

NMR Spectroscopy. Samples of ∼10 mg of GOS were exchanged with 0.99 atom % D2O with intermediate lyophilization and finally dissolved in 650 μL of D2O containing 25 ppm acetone as an internal standard (δH 2.225). One-dimensional 1H NMR spectra were recorded at a probe temperature of 25 °C on a Varian Inova 500 spectrometer (NMR Department, University of Groningen, The Netherlands). Spectra were recorded with a 4500 Hz spectral width at 16,000 complex data points using a WET1D pulse to suppress the HOD signal. All spectra were processed using MestReNova 12 (Mestrelabs Research SL, Santiago de Compostela, Spain), applying manual phase corrections and Whittaker Smoother baseline corrections.

Statistical Analysis. All experiments and measurements were conducted at least in duplicate, and the standard deviation (SD) never exceeded 5%. The data are expressed as the mean ± SD with significant digits when appropriate.

RESULTS AND DISCUSSION

Lactose Conversion and GOS Formation by Recombinant β-Galactosidases from L. helveticus. The thermophilic lactic acid bacterium L. helveticus is extensively used as starter cultures for various milk fermentation processes. The strain is capable of utilizing lactose by exhibiting intracellular β-galactosidase activity.30,31 It was shown previously that two recombinant β-galactosidases from L. helveticus DSM 20075, EcoliBL21Lhβ-gal and Lp609Lhβ-gal, were able to convert lactose using a batch mode of conversion and were found to be promising candidates for the production of prebiotic GOS.25 It was also shown in our previous study that both enzymes are stable in the presence of high concentrations of lactose, retaining more than 75 and 60% of the initial activity, respectively, after 24 h of incubation at 50 °C.28 Therefore, there is great potential for application of these recombinant β-galactosidases in lactose conversion processes at temperatures up to 50 °C.

In this study, we looked in more detail at the lactose conversions catalyzed by these two recombinant β-galactosidases from L. helveticus DSM 20075 at various process temperatures and performed a detailed analysis of the GOS mixtures formed. As shown in Figure 1A,B, lactose conversions catalyzed by the crude enzyme Lp609Lhβ-gal and the purified enzyme EcoliBL21Lhβ-gal approached completion (>98%) within 12 h at the examined process temperatures varying from 30 to 50 °C. The rate of lactose conversion considerably increased with increasing reaction temperature. The conversions at 50 °C occurred most rapidly and achieved ~99% lactose conversion after 6 and 4 h with the crude enzyme Lp609Lhβ-gal and the purified enzyme EcoliBL21Lhβ-gal,
| peak | GOS component | Dp | V-GOS | GOS I | GOS III | GOS V | Lh-GOS | Lr-GOS | Lh-GOS | St-GOS | Br-GOS1 | Br-GOS2 |
|------|---------------|----|-------|-------|---------|-------|--------|--------|--------|--------|---------|---------|
| 1    | β-D-Galp (β-D-galactose) |    |       |       |         |       |        |        |        |        |         |         |
| 2    | β-D-GlcP (β-D-glucose) |    |       |       |         |       |        |        |        |        |         |         |
| 3    | β-D-Galp-1(→2)-D-Galp | 2  |       |       |         |       |        |        |        |        |         |         |
| 4    | β-D-Galp-1(→6)-D-GlcP (α(altrose) | 2  |       |       |         |       |        |        |        |        |         |         |
| 5    | β-D-Galp-1(→4)-D-GlcP (lactose) |    |       |       |         |       |        |        |        |        |         |         |
| 6a   | β-D-Galp-1(→4)-β-D-Galp-1(→6)-D-GlcP | 3  |       |       |         |       |        |        |        |        |         |         |
| 6b   | β-D-Galp-1(→6)-β-D-Galp-1(→4)-D-GlcP |    |       |       |         |       |        |        |        |        |         |         |
| 7    | β-D-Galp-1(→4)-D-Galp |    |       |       |         |       |        |        |        |        |         |         |
| 8a   | β-D-Galp-1(→2)-D-GlcP |    |       |       |         |       |        |        |        |        |         |         |
| 8b   | β-D-Galp-1(→3)-D-GlcP |    |       |       |         |       |        |        |        |        |         |         |
| 9    | β-D-Galp-1(→2)-β-D-Galp-1(→4)-D-GlcP | 3  |       |       |         |       |        |        |        |        |         |         |
| 10a  | β-D-Galp-1(→2)-β-D-Galp-1(→6)-D-GlcP |    |       |       |         |       |        |        |        |        |         |         |
| 10b  | β-D-Galp-1(→3)-β-D-Galp-1(→6)-D-GlcP |    |       |       |         |       |        |        |        |        |         |         |
| 11   | β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP |    |       |       |         |       |        |        |        |        |         |         |
| 12   | β-D-Galp-1(→3)-β-D-Galp-1(→4)-D-GlcP |    |       |       |         |       |        |        |        |        |         |         |
| 13a  | β-D-Galp-1(→4)-β-D-Galp-1(→2)-D-GlcP |    |       |       |         |       |        |        |        |        |         |         |
| 13b  | β-D-Galp-1(→4)-β-D-Galp-1(→3)-D-GlcP |    |       |       |         |       |        |        |        |        |         |         |
| 14a  | β-D-Galp-1(→4)-β-D-Galp-1(→6)-β-D-Galp-1(→4)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 14b  | β-D-Galp-1(→4)-β-D-Galp-1(→6)-β-D-Galp-1(→6)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 15a  | β-D-Galp-1(→4)-β-D-Galp-1(→2)-β-D-Galp-1(→4)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 15b  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→2)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 16a  | β-D-Galp-1(→4)-β-D-Galp-1(→2)-β-D-Galp-1(→6)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 16b  | β-D-Galp-1(→4)-β-D-Galp-1(→6)-β-D-Galp-1(→6)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 16c  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→6)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 17   | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 18a  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→2)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 18b  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→3)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
| 19a  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→6)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 19b  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 19c  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 20a  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 20b  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 20c  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→2)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 21a  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→6)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 21b  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→2)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 21c  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→1)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 22   | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 23a  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 23b  | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→3)-D-GlcP | 5  |       |       |         |       |        |        |        |        |         |         |
| 24   | β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP | 6  |       |       |         |       |        |        |        |        |         |         |
| 25   | β-D-Galp-1(→4)-β-D-Galp-1(→3)-D-GlcP | 3  |       |       |         |       |        |        |        |        |         |         |
| 26   | β-D-Galp-1(→6)-β-D-Galp-1(→4)-β-D-Galp-1(→4)-D-GlcP | 4  |       |       |         |       |        |        |        |        |         |         |
respectively. The conversion of lactose was thus faster when using the purified enzyme EcoliBL21Lhβ-gal than when using the crude enzyme Lp609Lhβ-gal under similar process conditions. The suppressed catalytic efficiency of the crude enzyme may be caused by the interference of the impurities, such as other proteins and substances in the cell-free extract.28

The highest total GOS yield was ∼26% (∼53 g/L) at approximately 90% lactose conversion at 37, 42, and 50 °C (Figure 2A) when the crude enzyme Lp609Lhβ-gal was used in batch conversions with an initial lactose concentration of 205 g/L. The time needed to obtain 90% lactose conversion decreased from a reaction time of 7 h at 37 °C to 5 h at 42 °C and 4 h at 50 °C (Figure 1A). Trans-galactosylation may thus become more pronounced than hydrolysis at higher reaction temperatures29 although the increased temperature hardly affected the maximum GOS yield.63,53 Interestingly, the maximum GOS yield increased to ∼33% (∼68 g/L) when using the purified enzyme EcoliBL21Lhβ-gal under reaction conditions similar to those used for the crude enzyme Lp609Lhβ-gal. This highest GOS yield was reached when approximately 90% of the initial lactose was converted after 6 and 4 h of reaction at 30 and 37 °C, respectively (Figures 1B and 2B). The maximum GOS yield obtained with the purified enzyme EcoliBL21Lhβ-gal was achieved in a much shorter time at elevated reaction temperatures after 2 h at 42 °C (∼70% lactose conversion) and only after 1 h at 50 °C (∼60% lactose conversion) (Figures 1B and 2B). The highest GOS yield of approximately 33% obtained with the purified enzyme EcoliBL21Lhβ-gal (Figure 2B) is comparable to the reported GOS yields for other lactobacillal β-galactosidases, for instance 31% for L. pentosus β-galactosidase,54 38% for L. reuteri β-galactosidase,27 and 41% for L. sakei β-galactosidase.13

Synthesis of GOS by β-galactosidases is kinetically controlled as a consequence of the competition between hydrolysis and trans-galactosylation, and the GOS formed via trans-galactosylation are also potential substrates for hydrolysis. The total amount of GOS decreased dramatically after reaching their maximum yields as a result of the degradation of GOS by crude Lp609Lhβ-gal and purified EcoliBL21Lhβ-gal (Figure 2A,B, respectively), and consequently, hydrolysis prevailed over trans-galactosylation when the substrate lactose was depleted. This phenomenon was well observed and reported in the literature.15,35

**Identification of Individual GOS Components Formed Using Recombinant β-Galactosidases from L. helveticus.** The main products of the GOS mixture (Lh-GOS) formed via trans-galactosylation of lactose using recombinant β-galactosidases from L. helveticus were identified to be β-D-Galp-(1→6)-D-Galp (peak 3), β-D-Galp-(1→6)-D-Glc (allo-lactose) (peak 4), β-D-Galp-(1→6)-D-Lac (peak 5), β-D-Galp-(1→3)-D-Glc (peak 6), β-D-Galp-(1→3)-D-Lac (peak 12), β-D-Galp-(1→3)-D-Glc (peak 8b), and β-D-Galp-(1→3)-D-Galp (peak 38) (Table 1, Figure 3). The enzyme thus showed a strong preference for the formation of β-(1→3)- and β-(1→6)- linkages in accordance with other lactobacillal β-galactosidases reported previously.13–15,34

The composition of the GOS mixture formed in batch conversion at 30 °C with an initial lactose concentration of 205 g/L using the purified enzyme EcoliBL21Lhβ-gal was determined to be 19.3% disaccharides (DP2), 11.5% trisaccharides (DP3), and 1.2% tetrasaccharides (DP4) (Table 2).

**Structural Comparison of Different GOS Mixtures.** GOS mixtures produced using β-galactosidases from different lactic acid bacteria (Lh-GOS, Lr-GOS, Lb-GOS, and St-GOS) and B. breve (Br-GOS1 and Br-GOS2) were analyzed and structurally compared with previously studied commercial GOS of known compositions (V-GOS, GOS I, GOS III, and GOS V).23 All the GOS samples, except Lr-GOS, contain the mono saccharides (galactose, peak 1 and glucose, peak 2) and unconverted lactose (peak 5) (Figure 3). The Lr-GOS mixture was removed after the conversion to remove the mono saccharides and unconverted lactose.14 A major component found in all GOS samples is the disaccharide allolactose (peak 4, Figure 3). Trans-galactosylation is known to involve intermolecular and intramolecular reactions of which the latter reaction is the result of direct galactosyl transfer to D-glucose to

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**Table 1. continued**

| peak | GOS component | DP | V-GOS | GOS I | GOS III | GOS V | Lh-GOS | Lr-GOS | Lb-GOS | St-GOS | Br-GOS1 | Br-GOS2 |
|------|---------------|----|-------|-------|---------|-------|--------|--------|--------|--------|---------|---------|
| 27   | β-D-Galp-(1→6)-β-D-Galp-(1→4)-β-D-Galp-(1→3)-D-Glc | 4  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 28   | β-D-Galp-(1→3)-β-D-Galp-(1→6)-D-Glc | 3  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 29   | β-D-Galp-(1→3)-β-D-Galp-(1→3)-D-Glc | 3  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 30   | β-D-Galp-(1→3)-β-D-Galp-(1→2)-D-Glc | 3  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 31   | β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glc | 4  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 32   | β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→3)-D-Glc | 4  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 33   | β-D-Galp-(1→3)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glc | 4  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 34   | β-D-Galp-(1→3)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glc | 4  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 35   | β-D-Galp-(1→3)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glc | 4  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 36   | β-D-Galp-(1→3)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glc | 4  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 37   | β-D-Galp-(1→6)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glc | 4  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| 38   | β-D-Galp-(1→3)-D-Galp | 2  | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |
| X    | UNK | UNK | o     | o     | o       | o     | o      | o      | o      | o      | o       | o       |

“Graphical presentations of individual GOS structures are shown in;24 [ ] represents branched elongation.26 ○ structure present; ● structure absent; and UNK is abbreviated for unknown structure.3 Peak numbers correspond to the HPAEC-PAD chromatograms presented in Figure 3.”
yield regioisomers of lactose, such as allolactose. In intramolecular trans-galactosylation, the glycosidic bond of lactose is cleaved and immediately formed again at a different position of the glucose.27,36,37 All commercial GOS mixtures contained β-(1→4), β-(1→3), and β-(1→2) transfer products.23 The β-(1→4)-linked oligosaccharides are predominant in V-GOS produced using β-galactosidase from B. circulans, which was previously reported to produce predominantly β-

Table 2. Composition (as % Mass of Total Sugars) of the GOS Mixtures Prepared Using β-Galactosidases from Lactic Acid Bacteria and B. breve as Analyzed by HPAEC-PAD and HPSEC-RI

| GOS mixture | DP4   | DP3   | DP2 (non-lactose) | glucose | galactose | lactose |
|-------------|-------|-------|-------------------|---------|-----------|---------|
| Lh-GOS      | 1.20 ± 0.13 | 11.53 ± 0.51 | 19.28 ± 0.04 | 33.32 ± 0.28 | 26.07 ± 0.30 | 8.59 ± 0.10 |
| Lr-GOS 60%  | 5.11 ± 0.15 | 71.17 ± 0.45 | 23.36 ± 0.09 | 0.37 ± 0.09 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Lr-GOS 80%  | 9.12 ± 0.14 | 71.23 ± 0.64 | 19.07 ± 0.27 | 0.53 ± 0.44 | 0.05 ± 0.07 | 0.00 ± 0.00 |
| Lb-GOS      | 3.57 ± 0.05 | 35.11 ± 0.03 | 13.12 ± 0.37 | 23.50 ± 0.04 | 12.29 ± 0.08 | 12.41 ± 0.40 |
| St-GOS      | 7.12 ± 0.76 | 19.88 ± 0.37 | 18.22 ± 0.11 | 29.58 ± 0.13 | 16.40 ± 0.07 | 8.80 ± 0.08 |
| Br-GOS2     | 1.84 ± 0.15 | 17.05 ± 0.07 | 15.79 ± 0.79 | 23.50 ± 0.04 | 21.07 ± 0.04 | 17.18 ± 0.87 |

Lh-GOS is the GOS mixture formed with purified enzyme EcoliBL21Lhβ-gal (recombinant β-galactosidase from L. helveticus). The reactions were carried out at 30 °C under the conditions as described in Materials and Methods. Lr-GOS is the GOS mixture formed with β-galactosidase from L. reuteri, collected at 60% lactose conversion and purified to remove lactose and monosaccharides.14 Lr-GOS is the GOS mixture formed with β-galactosidase from L. reuteri, collected at 80% lactose conversion and purified to remove lactose and monosaccharides.14 Lr-GOS is the GOS mixture formed with β-galactosidase from L. bulgaricus.15 St-GOS is the GOS mixture formed with β-galactosidase from S. thermophilus.7 Br-GOS2 is the GOS mixture formed with β-galactosidase (β-gal II) from B. breve.12

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Figure 3. HPAEC-PAD profiles of different GOS mixtures. Assigned peaks are numbered and correspond with the GOS components presented in Table 1.

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GOS mixtures were also analyzed by HPSEC-RI and DP distribution data of the previously studied commercial V-GOS, GOS I, GOS III, and GOS V and were included for comparison. V-GOS provided

(1→4) transfer products. GOS mixtures were also analyzed by HPSEC-RI and DP distribution data of the previously studied commercial V-GOS, GOS I, GOS III, and GOS V and were included for comparison. V-GOS provided

Figure 4. One-dimensional 1H NMR spectra of GOS mixtures prepared using β-galactosidases from lactic acid bacteria and B. breve. Peaks of structural-reporter-group signals have been previously explained.

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the broadest spectrum of oligosaccharide components with a DP ranging from DP2 to DP6, while other commercial GOS samples (GOS I, GOS III, and GOS V) and the GOS mixtures produced using lactic acid bacterial and bifidobacterial β-galactosidases contain GOS components with the DP up to DP4. The compositions of the latter GOS mixtures in terms of DP2, DP3, and DP4 are summarized in Table 2.

Interestingly, GOS mixtures obtained with β-galactosidases from lactic acid bacteria and B. breve are quite similar to the commercial GOS III in terms of the product spectrum. They contain several GOS components that are not present in V-GOS, such as β-d-Galp-(1→6)-β-d-Galp-(1→3)-d-Glcp (peak 25), β-d-Galp-(1→3)-β-d-Galp-(1→6)-d-Glcp (peak 28), β-d-Galp-(1→3)-β-d-Galp-(1→4)-d-Glcp (peak 31), and β-d-Galp-(1→6)-β-d-Galp-(1→3)-β-d-Galp-(1→4)-d-Glcp (peak 37), and clearly provided a more varied GOS composition than GOS V (Figure 3). Lactic acid bacterial and bifidobacterial β-galactosidases clearly showed a preference for β-d-Galp-(1→6) transfer to form 6′-galactosialactose (peak 3), allolactose (peak 4), 6′-galactosyllactose (peak 6), and 6′-6′-digalactosyllactose (peak 34 in the cases of Lb., Lr., Lb., and St-GOS mixtures) as well as for the formation of β-d-Galp-(1→3) structural elements including β-d-Galp-(1→3)-d-Glcp (peak 8b), 3′-galactosyllactose (peak 12), 3′-galactosylallolactose (peak 28), 3′-3′-digalactosyllactose (peak 31), and 3′-galactobiocose (peak 38) (Figure 3). Notably, two trisaccharides, 6′-galactosyllactose and 3′-galactosyllactose, which are the two main components of GOS mixtures produced using lactic acid bacterial and bifidobacterial β-galactosidases, are present in the V-GOS and GOS I samples only in minor and trace amounts, respectively. Furthermore, Lr-GOS and St-GOS mixtures also showed the presence of an unknown component (peak x, after peak 31) in the HPAEC-PAD chromatograms (Figure 3), which was not present in any commercial GOS sample.

Generally, β-(1→6) and β-(1→3) linkages were formed predominantly in all GOS mixtures prepared using β-galactosidases from lactic acid bacteria and B. breve, not only with linear elongations but also with branched elongations (Table 1). There was also no evidence of β-(1→4) elongation for oligomers formed when using these enzymes. The results obtained from HPAEC-PAD analyses were supported by one-dimensional 1H NMR spectroscopy data. A reference library of 1H NMR spectroscopy data for GOS was established previously.23,28,29 In the one-dimensional 1H NMR spectra (Figure 4, Table 3), the presence of the major components allolactose (HPAEC-PAD peak 4, Figure 3) and 6′-galactosyllactose (HPAEC-PAD peak 6, Figure 3) was confirmed by the presence of peak d (∼δ 5.22 ppm). The occurrence of Galp-(1→6)-Galp elements was reflected by the presence of the structural-reporter-group signal at ∼4.06 ppm (peak i). The presence of 6-substituted Glc was shown by the presence of peak e (δ 4.42) and peak f (δ 4.16).23,28,29 The presence of 3-substituted reducing Gal (HPAEC-PAD peak 38, Figure 3) was confirmed by the NMR peak q (δ 4.10), corresponding to the H-5 signal of a 3-substituted reducing Gal residue in α-configuration.23,28,29 The presence of 2-substituted reducing Glc was shown by the one-dimensional 1H NMR peak at δ 5.45 (peak a) and occurred only in minor levels in St-GOS, Br-GOS1, and Br-GOS2.

The structural building blocks of different GOS mixtures strongly depend on the β-galactosidases used during their synthesis. β-Galactosidases from lactic acid bacteria and B. breve show the preference to form β-(1→6)- and β-(1→3)- linked GOS. It was reported that the administration of a GOS mixture containing β-(1→3) as well as β-(1→4) and β-(1→6) linkages proved to have a better bifidogenic effect than a mixture containing GOS with β-(1→4) and β-(1→6) linkages.39 Recently, we have reported the specific growth stimulation of certain desired intestinal bacteria by one of our GOS mixtures, which contained mainly oligosaccharides of β-(1→6) and β-(1→3) glycosidic linkages, and our GOS mixture stimulated the growth of several probiotics strains more than the two commercial preparations, which contain mainly β-(1→4)-linked GOS.40 GOS mixtures produced with these enzymes consist mainly of DP2 and DP3 oligosaccharides, accounting for ~90% of all GOS components. The major disaccharides

![Table 3. 1H NMR Structural-Reporter-Group Signals Employed for the Evaluation of One-Dimensional 1H NMR Spectra of GOS Mixtures](https://dx.doi.org/10.1021/acs.jafc.9b08156)

| NMR signal | chemical shift (ppm) | explanation | HPAEC-PAD signal |
|------------|----------------------|-------------|-----------------|
| a          | 5.45                 | H-1 of a 2-substituted α-D-Glcp unit; [β-d-Galp-(1→2)-α-D-Glcp] | 8a, 9, 10a, 15b, 16b, 20a, 21a, 30, 33 |
| d          | 5.23–5.22            | H-1 of a 4-, 6-, and/or 1-substituted α-D-Glcp unit; [β-d-Galp-(1→4/6)-α-D-Glcp; α-D-Glcp-(1→1)-β-d-Galp] | 4, 5, 6ab, 11, 12, 14ab, 16c, 17, 19a, 22, 24, 26, 28, 31, 34, 35, 36, 37, 2 |
| e          | 4.22–4.21            | H-6a of a 6-substituted β-D-Glcp unit; [β-d-Galp-(1→6)-β-D-Glcp] | 4, 10ab, 16abc, 21abc, 28 |
| f          | 4.17–4.16            | H-6a of a 6-substituted α-D-Glcp unit; [β-d-Galp-(1→6)-α-D-Glcp] | 4, 10ab, 16abc, 21abc, 28 |
| g          | 4.21–4.17            | H-4 of a 3- and/or 4-substituted (reducing) β-D-Galp unit; [β-d-Galp-(1→3/4)-β-D-Galp-(1→1)] | 7, 11, 12, 13ab, 14ab, 15ab, 16abc, 17, 18ab, 19abc, 20abc, 21abc, 22, 23×ab, 24, 26, 27, 28, 29, 30, 31, 32, 33 |
| h          | 5.26                 | H-1 of free α-D-Galp | 1 |
| i          | 4.08–4.05            | H-6a of a 6-substituted (reducing) β-D-Galp unit; [β-d-Galp-(1→6)-β-D-Galp-(1→1)] | 3, 6b, 25, 26, 27 |
| j          | 5.27                 | H-1 of a 4- and/or 6-substituted reducing α-D-Galp unit; [β-d-Galp-(1→6)-α-D-Galp] | 3, 7 |
| m          | 5.41–5.42            | H-1 of a 3-substituted β-D-Galp-(1→4)- unit in a β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glcp sequence | 12, 31 |
| q          | 4.10                 | H-5 of a 3-substituted (reducing) α-D-Galp unit; [β-d-Galp-(1→6)-α-D-Galp] | 38 |
present in these GOS mixtures are allolactose, 6′-galactobiase, 3′-galactobiase, and 3′-galactosyl glucose. Sanz et al.41 investigated the prebiotic potential of a number of disaccharides and found that β-D-Galp-(1→6)-D-Gal (6′-galactobiase), one of the major disaccharides in our GOS mixtures, is a highly prebiotic molecule. In our recent study, we have reported that some bifidobacteria preferentially utilized DP2 GOS and some lactobacilli often utilized only the DP2 and DP3 molecules.42 Trisaccharides are the main constituents in Lr-GOS and Lb-GOS of which 6′-galactosylactose and 3′-galactosylactose are the major components. The tetrascarachide 3′-3′-digalactosyllactose is present in all GOS mixtures produced using lactic acid bacterial and bifidobacterial β-galactosidases, whereas 6′-6′-digalactosyllactose is only found in the GOS mixtures formed with lactic acid bacterial β-galactosidases (Lh-GOS, Lr-GOS, Lb-GOS, and St-GOS). The two tetrascarachides, which are the β-D-Galp-(1→3) elongation of 6′-galactosyllactose and the β-D-Galp-(1→6) elongation of 3′-galactosyllactose, are found in the GOS mixtures formed with lactic acid bacterial β-galactosidases and also in Br-GOS2. These GOS mixtures contain a number of GOS components that are not present in the commercial Vivinal GOS mixture. The information on individual GOS obtained in this study as analyzed by HPAEC-PAD and confirmed with 1H NMR spectroscopy has given more insights into the basic structural elements of the GOS mixtures produced using lactic acid bacterial and bifidobacterial β-galactosidases, and it will be of interest for studies on the correlation between individual GOS structures and their prebiotic potential.

**AUTHOR INFORMATION**

**Corresponding Author**

Thu-Ha Nguyen — Food Biotechnology Laboratory, Department of Food Science and Technology, BOKU-University of Natural Resources and Life Sciences, Vienna, A-1190 Vienna, Austria; orcid.org/0000-0002-5253-6865; Phone: 43-1-47654 75215; Email: thu-ha.nguyen@boku.ac.at; Fax: 43-1-47654 75039

**Authors**

Suwapat Kittibunchakul — Food Biotechnology Laboratory, Department of Food Science and Technology, BOKU-University of Natural Resources and Life Sciences, Vienna, A-1190 Vienna, Austria; Institute of Nutrition, Mahidol University, Nakhon Pathom 73170, Thailand

Sander S. van Leeuwen — Microbiol Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, NL-9747 AG Groningen, The Netherlands; Laboratory Medicine, Cluster Human Nutrition & Health, University Medical Center Groningen (UMCG), NL-9713 GZ Groningen, The Netherlands

Luibert Dijkhuisen — Microbiol Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, NL-9747 AG Groningen, The Netherlands; Carbohydrate Research BV, NL-9747 AN Groningen, The Netherlands; orcid.org/0000-0003-2312-7162

Dietmar Haltrich — Food Biotechnology Laboratory, Department of Food Science and Technology, BOKU-University of Natural Resources and Life Sciences, Vienna, A-1190 Vienna, Austria; orcid.org/0000-0002-8722-8176

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.9b08156

**Author Contributions**

S.K. and S.S.v.L. share first authorship.

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