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Synthesis and Characterization of a Potential Prebiotic Trisaccharide from Cheese Whey Permeate and Sucrose by *Leuconostoc mesenteroides* Dextransucrase

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Supporting Information

ABSTRACT: The production of new bioactive oligosaccharides is currently garnering much attention for their potential use as functional ingredients. This work addresses the enzymatic synthesis and NMR structural characterization of 2-α-D-glucopyranosyl-lactose derived from sucrose:lactose and sucrose:cheese whey permeate mixtures by using a *Leuconostoc mesenteroides* B-512F dextransucrase. The effect of synthesis conditions, including concentration of substrates, molar ratio of donor/acceptor, enzyme concentration, reaction time, and temperature, on the formation of transfer products is evaluated. Results indicated that cheese whey permeate is a suitable material for the synthesis of 2-α-D-glucopyranosyl-lactose, giving rise to yields around 50% (in weight respect to the initial amount of lactose) under the optimum reaction conditions. According to its structure, this trisaccharide is an excellent candidate for a new prebiotic ingredient, due to the reported high resistance of α-(1→2) linkages to the digestive enzymes in humans and animals, as well as to its potential selective stimulation of beneficial bacteria in the large intestine mainly attributed to the two linked glucose units located at the reducing end that reflects the disaccharide kojibiose (2-α-D-glucopyranosyl-D-glucose). These findings could contribute to broadening the use of important agricultural raw materials, such as sucrose or cheese whey permeates, as renewable substrates for enzymatic synthesis of oligosaccharides of nutritional interest.

KEYWORDS: *cheese whey permeate, Leuconostoc mesenteroides B-512F dextransucrase, glucosyl-lactose, transglucosylation, kojioligosaccharides*

INTRODUCTION

Development of new strategies for the synthesis of oligosaccharides with functional properties is currently in great demand in the food, pharmaceutical, feed, and cosmetic industries, since carbohydrates may find immediate applications as stabilizers, bulking compounds, immunomodulating agents, or prebiotic compounds.[1] In this context, the use of enzyme catalysts is a preferred approach to multistep chemical synthesis considering the high stereo- and regioselectivity of enzymes.[2] Likewise, there is an increasing interest in alternative uses of agricultural and livestock materials, such as sucrose or whey permeates (WPs), as renewable substrates for enzymatic synthesis of valuable saccharides.[3,4] Thus, sucrose is an abundant and low-cost substrate with a great potential for its use as a sustainable raw material for the development of new food ingredients[5] and, particularly, for the synthesis of prebiotic oligosaccharides using glycosyltransferases.[6]

In addition, WP is an important byproduct of the dairy industry containing mainly lactose and salts, which is obtained when cheese or casein whey are subjected to a process of ultrafiltration membrane to concentrate whey proteins. Unlike whey proteins that find immediate food applications, the WP has so far been of little value,[7] its profitable use being one of the biggest dairy industry challenges ahead.[8] One of the most important uses of WP is the synthesis of galactooligosaccharides (GOS) from transgalactosylation of lactose catalyzed by glycosidases (EC 3.2), β-galactosidases (EC 3.2.1.23) of microbial origin being the most frequently used.[4,9−11] In this sense, it is remarkable to mention that notable differences, in terms of yield and oligosaccharide composition, have been reported between the production of GOS from model systems consisting of lactose in buffered solutions and from WPs. These dissimilarities can be attributed to the influence of other permeate ingredients, such as mineral salts, on the enzymatic reaction.[12−15]

Despite the broad specificity of glycosidases, their application is often limited by low yields and poor regioselectivity.[16] Glycosyltransferases (EC 2.4) are considered an interesting alternative to glycosidases because they include enzymes capable of transferring glycosyl groups, after hydrolysis of the donor, to water or other acceptors catalyzing, thus, oligosaccharide synthesis.[2] Glycosyltransferases are further subdivided, according to the nature of the sugar residue being transferred, into hexosyltransferases (EC 2.4.1), pentosyltransferases (EC 2.4.2), and those transferring other glycosyl groups

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(EC 2.4.99).[16] Within the subgroup 2.4.1 and under, the term glycansucrases is considered glucansucrases or fructansucrases. This type of enzyme maintains the regiospecificity and catalyzes effective synthesis of various oligosaccharides of different structural nature by using sucrose as the glucosyl or fructosyl moiety donor and carbohydrates with low molecular weights acting as acceptors.[17,18]

Concretely, dextransucrase (EC 2.4.1.5) is a glucansucrase produced by various species of *Leuconostoc*, *Lactobacillus*, and *Streptococcus*, which catalyzes the synthesis of dextran from sucrose and also the transfer of glucose from sucrose (donor) to other carbohydrates (acceptor) by linking mainly an α-(1→6)-glucosyl bond (transglycosylation reaction).[19,20] Maltose is recognized as the best acceptor providing the synthesis of a series of potential bioactive oligosaccharide acceptor products such as panose (6-α-D-glucopyranosylmaltose) and other isomaltooligosaccharides.[21,22] When lactose is the acceptor, only one acceptor product has been reported to be formed, 2-α-D-glucopyranosyl-lactose.[22−24] According to its structure, this trisaccharide is an excellent candidate for a new prebiotic ingredient, due to the high resistance of α-(1→2) linkages to the digestive enzymes in human beings and animals and their selective stimulation of bacteria that are beneficial to the large intestine.[25,26] Nevertheless, to the best of our knowledge, there are no data concerning the production of oligosaccharides from WPs and sucrose by glycosyltransferases.

First, this study will address the production and structural characterization by NMR of potentially bioactive oligosaccharides derived from sucrose and lactose catalyzed by dextransucrase of *Leuconostoc mesenteroides* B-512F, then to be applied to the production of oligosaccharides from industrial cheese WPs.

MATERIALS AND METHODS

Chemical and Reagents. Dextransucrase from *L. mesenteroides* B-512F was purchased from CRITT Bio-Industries (Toulouse, France). The specific activity was 0.4 U mg^{-1}, where 1 unit is the amount of enzyme required to perform the transfer of 1 μmol of glucose per minute at a working temperature of 30 °C, a sucrose concentration of 100 g L^{-1} at pH 5.2 with 10 mg L^{-1} of $CaCl_2$ and $2H_2O$. Sucrose was purchased from Panreac (Barcelona, Spain), and fructose, glucose, raffinose, lactose, and leucrose were from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (HPLC grade) was obtained from Lab-scan (Gliwice, Poland). Ultrapure water quality (18.2 MΩ cm) with 1−5 ppb total organic carbon (TOC) and <0.001 EU mL^{-1} pyrogen levels was produced in-house using a laboratory water purification Milli-Q Synthesis A10 system from Millipore (Billerica, MA). All other chemicals were of analytical grade.

Physical−Chemical Characterization of Cheese WPs. Two different industrial bovine cheese WP powders were kindly supplied by the dairy industries García Baquero (Alcázar de San Juan, Spain) and Reny Picot (Navia, Spain). The pH of both WPs was measured using a pH meter (MP 230, Mettler-Toledo, Barcelona, Spain) at a concentration of 50 mg mL^{-1}. The dry matter (DM) content of WPs was gravimetrically determined by drying the samples in a conventional oven at 102 °C until constant weight, according to the AOAC method.[27]

The ion composition of the WPs was determined using an ICP-MS ELAN 6000 Perkin-Elmer Sciex instrument at the Servicio Interdepartamental de Investigación (SIdI-UAM) of Madrid. Either a semiquantitative analysis or a quantitative analysis of the elements of interest using the external calibration method and internal standards to correct instrumental drift were carried out.[28] The total protein content was determined in WPs aqueous dissolutions (300 mg mL^{-1}) according to Bradford's dye-binding method, using bovine serum albumin (BSA) as the standard.[29]

Oligosaccharide Synthesis. Oligosaccharide synthesis in the presence of sucrose (donor) and lactose (acceptor) was carried out by incubating 1 or 2 mg (0.4 or 0.8 U, respectively) of dextransucrase per mL at 30 °C in 20 mM sodium acetate buffer with 0.34 mM $CaCl_2$ at pH 5.2. Product formation was investigated by taking aliquots from the reaction mixture at suitable time intervals up to 48 h. The enzyme was inactivated by heating at 100 °C for 5 min, and inactivated samples were then diluted with acetonitrile:water (50:50, v:v), filtered using a 0.45 μm syringe filter (Symta, Madrid, Spain), and analyzed by two different LC systems as described below. To investigate the influence of synthetic conditions on the formation of the oligosaccharide of interest, the reactions were done at two different concentrations of substrates, that is, sucrose:lactose 45:20 and 30:30, expressed in g/100 mL, leading to two different molar ratios of donor/acceptor (2.25:1 and 1:1).

Once factors such as enzyme charge and sucrose:lactose ratio were studied, the enzymatic reactions were performed with sucrose:cheese WPs mixtures at 30 and 40 °C. Considering the lactose content measured in both WPs, enzymatic reactions were carried out at sucrose:lactose 30%:30%, in 20 mM sodium acetate buffer with 0.34 mM $CaCl_2$ (pH 5.2) for 48 h. When it was necessary, the pH was adjusted to 5.2 with acetic acid.

Moreover, the effect of the major ions present in the WPs on the *L. mesenteroides* B-512F dextransucrase activity was evaluated. Thus, 8 μL of 1 M Na_2HPO_4 and/or 20 μL of 1 M K_2HPO_5 (both solutions with a pH value adjusted to 5.2) were added to 1 mL of sucrose:lactose 30%:30% previously dissolved in 20 mM sodium acetate buffer with 0.34 mM $CaCl_2$ (pH 5.2). The enzymatic reactions were carried out as described above at 30 °C for 48 h.

Chromatographic Determination of Carbohydrates. *Liquid Chromatography with Refractive Index Detector.* The synthesized oligosaccharides were analyzed by liquid chromatography with refractive index detector (HPLC-RID) on an Agilent Technologies 1220 Infinity LC System −1260 RID (Boeblingen, Germany). The separation of carbohydrates was carried out with a Kromasil (100-NH_2) column (250 mm × 4.6 mm, 5 μm particle size) (Akzo Nobel, Brewster, NY) using acetonitrile:water (75:25, v:v) as the mobile phase and eluted in isocratic mode at a flow rate of 1.0 mL min^{-1} for 50 min. The injection volume was 50 μL (800 μg of total carbohydrates). Data acquisition and processing were performed using the Agilent ChemStation software (Agilent Technologies, Boeblingen, Germany).

Carbohydrates in the reaction mixtures were initially identified by comparing the retention times (t_R) with those of standard sugars. Quantitative analysis was performed by the external standard method, using calibration curves in the range 0.01−10 mg for fructose (quantification of monosaccharides), sucrose, lactose and leucrose (disaccharides), and raffinose (quantification of trisaccharides). All analyses were carried out in triplicate. Determination coefficients obtained from these calibration curves, which were linear over the range studied, were high ($R^2 > 0.999$). Reproducibility of the method was estimated on the basis of the intraday and interday precision, calculated as the relative standard deviation (RSD) of concentrations of oligosaccharide standards obtained in $n \geq 5$ independent measurements, obtaining RSD values below 10% in all cases.

High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection. To increase sensitivity, some of the samples were also analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS 2500 Dionex System consisting of a GP50 gradient pump and an ED50 electrochemical detector with a gold working electrode and Ag/AgCl reference electrode. Data acquisition and processing were performed with a Chromeleon version 6.7 software (Dionex Corp., Sunnyvale, CA). For eluents preparation, Milli-Q water, 50% (w:v) NaOH, and NaOAc (Panreac, Barcelona, Spain) were used. All eluents were degassed by flushing with helium for 25 min. Separations were performed following the method described by Spletchna et al.[30] Elution was at room temperature on a CarboPac PA-1 column (250 mm × 4 mm) connected to a CarboPac PA-1 (50 mm × 4 mm) guard

column. The elution, at a flow rate of 1 mL min^{-1}, was in gradient using a combination of three eluents: A (100 mM NaOH), B (100 mM NaOH and 50 mM NaOAc), and C (100 mM NaOH and 1 M NaOAc). The gradient used was 100% A from 0 to 20 min and 0−100% B from 20 to 70 min. After each run, the column was washed for 10 min with 100% C and re-equilibrated for 15 min with the starting conditions of the employed gradient.

Gas Chromatography with Mass Spectrometry Detection. Selected samples were also analyzed by gas chromatography with a mass spectrometry detector (GC-MS) on an Agilent Technologies 7890A gas chromatograph coupled to a 5975C MSD quadrupole mass detector (Agilent Technologies, Wilmington, DE). The trimethylsilyloximes (TMSO), prepared as described by Sanz et al.,[31] were separated using an HP-5 MS fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) coated with 5% phenylmethylsilicone (J&W Scientific, CA). The helium flow rate was 1 mL min^{-1}. The initial oven temperature was 180 °C and increased to 315 °C at a heating rate of 3 °C min^{-1} and held for 20 min. The injector temperature was 280 °C, and injections were made in the split mode (1:40). The mass spectrometer was operated in electronic impact mode at 70 eV. Mass spectra were acquired using Agilent ChemStation MSD software (Wilmington, DE). Identification of TMSO derivatives of carbohydrates was carried out by comparison of their retention indices and mass spectra with those of standard compounds previously derivatized.

Purification and Structural Characterization of the Main Acceptor Product by Nuclear Magnetic Resonance. Considering the absence of commercially available standard for the main synthesized oligosaccharide, this trisaccharide was isolated and purified by HPLC-RID from sucrose:lactose mixtures after 24 h of enzymatic reaction and using a semipreparative column Kromasil (100-NH_2) column (250 mm × 10 mm, 5 μm particle size) (Akzo Nobel, Brewster, NY). Thus, 500 μL of reaction mixtures (15 mg of total carbohydrates) was repeatedly eluted with acetonitrile:water (75:25, v:v) as the mobile phase at a flow rate of 5 mL min^{-1}, and fractions corresponding to the main synthesized oligosaccharide were manually collected, pooled, and evaporated in a rotatory evaporator R-200 (Büchi, Switzerland) below 25 °C for its subsequent characterization.

Structure elucidation of the purified oligosaccharide was accomplished by nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were recorded at 298 and 313 K, using D_2O as the solvent, on a Varian SYSTEM 500 NMR spectrometer (^1H 500 MHz, ^{13}C 125 MHz) equipped with a 5 mm HCN cold probe. Chemical shifts of ^1H (δ_H) and ^{13}C (δ_C) in ppm were determined relative to an external standard of sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)-propanoate in D_2O (δ_H 0.00 ppm) and 1,4-dioxane (δ_C 67.40 ppm) in D_2O, respectively. One-dimensional NMR experiments (^1H and ^{13}C) were performed using standard Varian pulse sequences. Two-dimensional [^1H, ^1H] NMR experiments (gCOSY and TOCSY) were carried out with the following parameters: a delay time of 1 s, a spectral width of 1675.6 Hz in both dimensions, 4096 complex points in t2 and 4 transients for each of 128 time increments, and linear prediction to 256. The data were zero-filled to 4096 × 4096 real points. Two-dimensional [^1H−^{13}C] NMR experiments (gHSQC and gHMBC) used the same ^1H spectral window, a ^{13}C spectral windows of 30165 Hz, 1 s of relaxation delay, 1024 data points, and 128 time increments, with a linear prediction to 256. The data were zero-filled to 4096 × 4096 real points. Typical numbers of transients per increment were 4 and 16, respectively.

RESULTS AND DISCUSSION

Optimization of Enzymatic Synthesis Conditions by Using Model Systems Based on Sucrose:Lactose Mixtures. Given that the optimum pH (5.2) and temperature (30 °C) of *L. mesenteroides* B-512F dextransucrase for glucansucrase activity have been previously well established,[32,33] the production of oligosaccharides was studied as a function of the ratio of sucrose to lactose and the concentration of enzyme as described below.

A sucrose concentration of 45% and a lactose concentration of 20%, expressed in g/100 mL, were initially employed. These values were based on the following: (i) the higher the concentration of substrates, the greater the inhibition of formation of dextran and the greater the formation of acceptor-reaction products;[20] (ii) previous studies described an initial sucrose concentration of 45% to be optimal for glucooligosaccharides production from sucrose:maltose mixtures.[25] Furthermore, taking into account that an increase in the ratio of acceptor to sucrose leads to a dramatic decrease in the amount of dextran with a concomitant increase in the amount of acceptor products,[20] enzymatic reactions with 30% of sucrose and 30% of lactose, expressed in g/100 mL, were also evaluated. Finally, both sets of samples were treated with two different concentrations of dextransucrase (0.4 and 0.8 U mL^{-1}) under standard reaction conditions (at pH 5.2 and 30 °C for 48 h). Results obtained with 0.4 U mL^{-1} of enzyme showed that sucrose was not totally consumed after 48 h of reaction, and lower levels of oligosaccharides were formed (data not shown) as compared to the equivalent reaction mixtures treated with 0.8 U mL^{-1} of dextransucrase. These results are in good agreement with data reported by Robyt,[20] who indicated that keeping a constant 1:1 ratio of maltose to sucrose, an increase in the concentration of enzyme gave rise to a decrease in dextran and to an increase in the amount of acceptor-reaction products. Thus, all results shown in this work correspond to a concentration of enzyme of 0.8 U mL^{-1}.

HPLC-RID chromatograms of the enzymatic reactions mixtures at 0 and 24 h with sucrose:lactose mixtures at concentrations of 45%:20% and 30%:30% are shown in Figure 1A,B. As expected, at the initial time, only two well-resolved peaks corresponding to sucrose (peak 3, t_R = 9.7 min) and lactose (peak 5, t_R = 12.5 min) were detected. Nevertheless, after 24 h of reaction, four new peaks clearly appeared (peaks 1, 2, 4, and 7), while the peak corresponding to lactose substantially decreased, which is indicative of its capacity as acceptor. Likewise, at sucrose:lactose mixtures of 30%:30%, the peak corresponding to sucrose was detected at trace levels, showing that sucrose was readily consumed after 24 h of reaction by the hydrolytic action of the dextransucrase (Figure 1B). At 24 h of reaction, the less retained peaks (1 and 2) corresponded to fructose and glucose (t_R = 6.9 and 7.8 min, respectively), and they were derived from the hydrolysis of sucrose. Furthermore, fructose was much more abundant than glucose, indicating that glucose was efficiently transferred. The other two new peaks (named 4 and 7, t_R = 10.7 and 20.3 min) were detected in the eluting area of di- and trisaccharides, respectively, suggesting that both of them were acceptor-reaction products. Finally, a minor set of peaks (termed 6) was also detected around 16 min, which might correspond to minor acceptor-reaction products (Figure 1).

Prior to quantification, peak 4 corresponding to a disaccharide (t_R = 10.7 min, Figure 1) was identified as leucrose (5-*O*-α-D-glucopyranosyl-D-fructopyranose) by GC-MS analysis of its corresponding trimethylsilyloxime (TMSO) and comparison with a standard previously derivatized (spectrum not shown). The mass spectrum of leucrose was characterized by the *m*/*z* ions (by decreasing order of abundance): 361, 204, 217, 538, 271, and 243. The formation of leucrose proves that fructose can also act as acceptor in the dextransucrase-catalyzed reactions.[16] Likewise, the major acceptor-reaction product eluting on the trisaccharide area (peak 7, t_R = 20.3, Figure 1) could correspond to glucosyl-

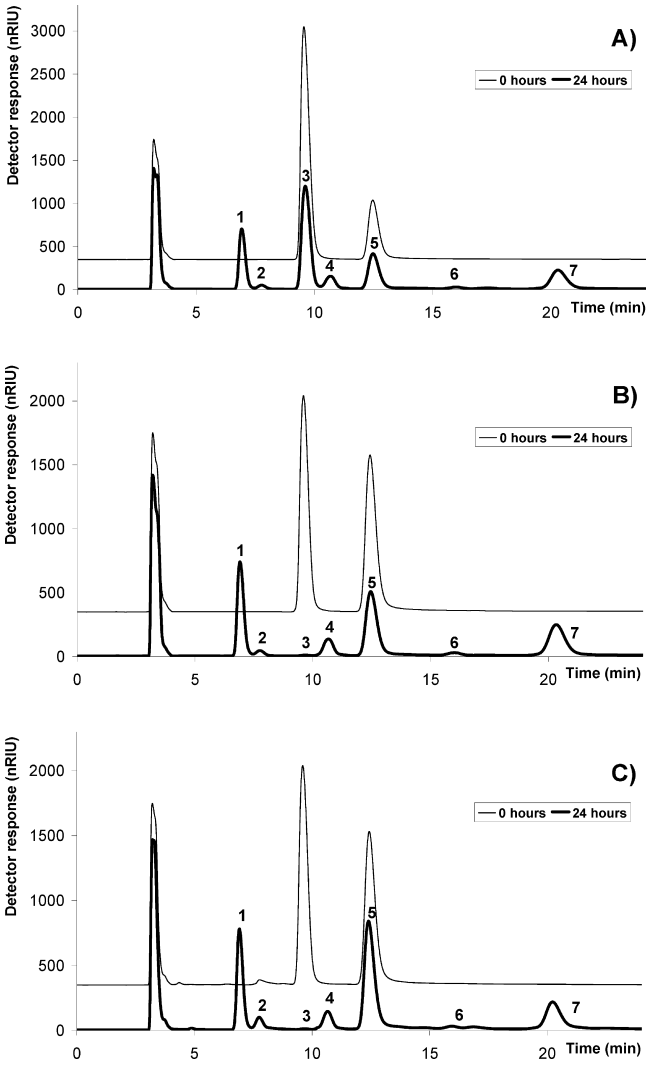

Figure 1. HPLC-RID profiles of transglycosylation reactions based on sucrose:lactose and sucrose:WP mixtures catalyzed by dextransucrase from *L. mesenteroides* B-512F (0.8 U mL^{-1}) at 30 °C, in 20 mM sodium acetate buffer at pH 5.2, for 0 and 24 h. (A) Sucrose:lactose 45%:20%, (B) sucrose:lactose 30%:30%, and (C) sucrose:WP 2 (equivalent to a concentration of sucrose:lactose of 30%:30%). Labeled peaks are as follows: 1, fructose; 2, glucose; 3, sucrose; 4, leucrose; 5, lactose; 6, minor acceptor-reaction products; and 7, 2-α-D-glucopyranosyl-lactose.

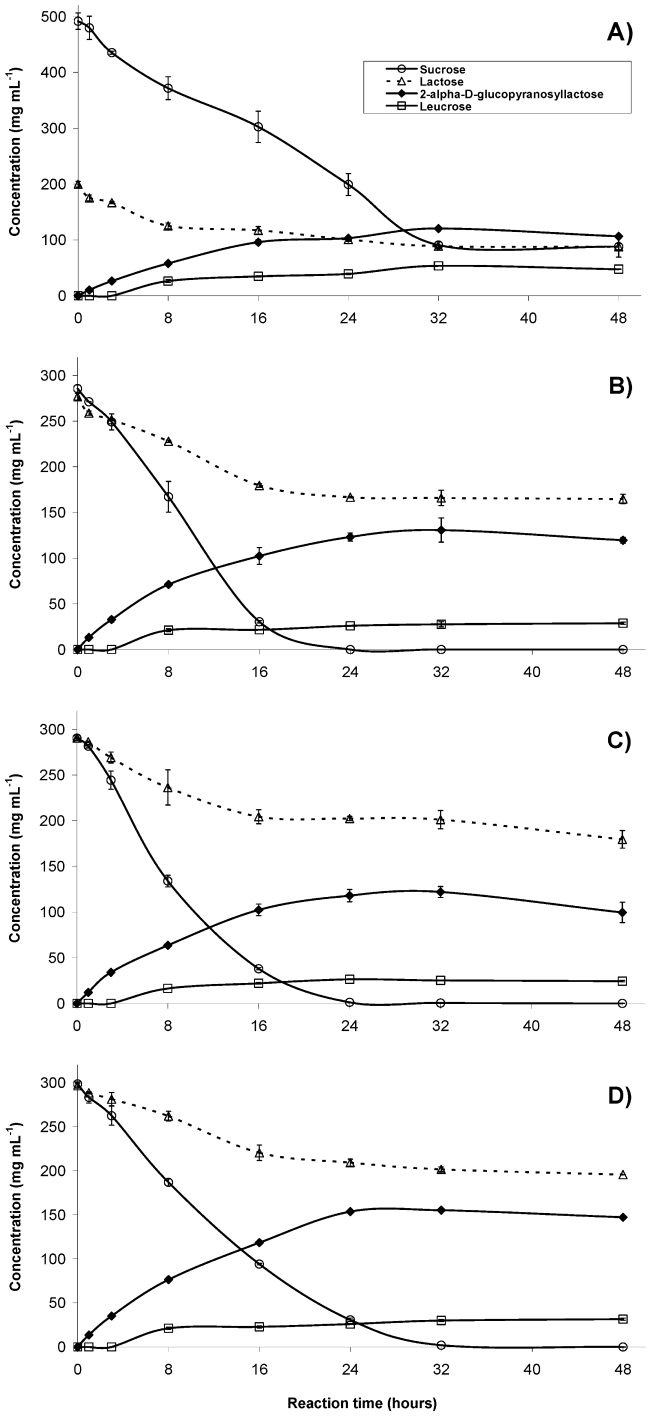

Figure 2. Concentrations of sucrose, lactose, leucrose, and 2-α-D-glucopyranosyl-lactose upon transglycosylation reactions based on sucrose:lactose and sucrose:WP mixtures catalyzed by dextransucrase from *L. mesenteroides* B-512F (0.8 U mL^{-1}) at 30 °C in 20 mM sodium acetate buffer at pH 5.2. (A) Sucrose:lactose 45%:20%, (B) sucrose:lactose 30%:30%, (C) sucrose:WP 1 (equivalent to a concentration of sucrose:lactose of 30%:30%), and (D) sucrose:WP 2 (equivalent to a concentration of sucrose:lactose of 30%:30%). Key: sucrose (○), lactose (△), leucrose (□), and 2-α-D-glucopyranosyl-lactose (◆). Vertical bars represent standard deviations (n = 3).

lactose as it has been previously reported in studies on the enzymatic synthesis of oligosaccharides in a lactose-sucrose medium either inoculated with *Betacoccus arabinosaceous* cultures[23,24] or after addition of dextransucrase from *L. mesenteroides* B-512F.[22] To confirm this fact, the purification and exhaustive characterization by NMR of this compound will be discussed in the next section.

Figure 2A,B illustrates the concentration of sucrose, lactose, leucrose, and glucosyl-lactose upon the enzymatic reaction time at sucrose:lactose ratios 45%:20% and 30%:30%, respectively. The maximum formation of the main acceptor-reaction product was achieved at 24−32 h and then remained practically constant to the end of the enzymatic reaction. This increase and subsequent plateau coincided with the gradual decrease of lactose observed up to 24 h of reaction (loss of 40−49% of lactose in weight respect to the initial amount) and the posterior plateau, confirming that lactose was the acceptor molecule for the formation of the main trisaccharide (Figure 2A,B). By comparing both set of reaction mixtures, it can be inferred that while the maximum amount of formed glucosyl-lactose was fairly similar in both cases (120−130 mg mL^{-1}), the

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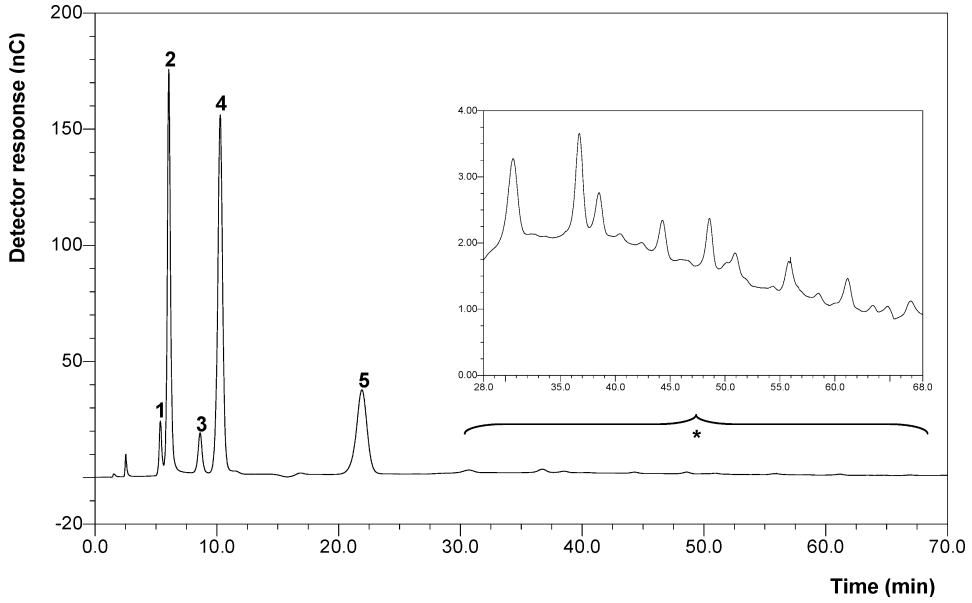

Figure 3. HPAEC-PAD profile of transglycosylation reaction based on sucrose:lactose (30%:30%) catalyzed by dextransucrase from *L. mesenteroides* B-512F (0.8 U mL^{-1}) at 30 °C, in 20 mM sodium acetate buffer at pH 5.2, for 24 h. Labeled peaks are as follows: 1, glucose; 2, fructose; 3, leucrose; 4, lactose; and 5 (2-α-D-glucopyranosyl-lactose. *The inset shows a zoom area of the eluted minor acceptor-reaction products of degree of polymerization above 3.

formation of leucrose (which could be considered as an unwanted byproduct) was 2-fold lower in the presence of 30% of sucrose and 30% of lactose than when 45% sucrose and 20% lactose mixture were used (i.e., 27 and 54 mg mL^{-1} of leucrose, respectively, after 32 h of reaction). This result can be explained by the fact that the formation of leucrose by action of dextransucrase is favored at high fructose concentration.[16] This means that the higher the concentration of sucrose is, the higher the concentration of fructose in the reaction medium is, and consequently, the greater the formation of leucrose is.

Overall, the maximum yields of glucosyl-lactose and leucrose were 23.3 and 5.1%, based on total carbohydrates, respectively, when 30% of sucrose and 30% of lactose were initially present in the enzymatic reaction. This means that a yield of approximately 47% of glucosyl-lactose (in weight respect to the initial amount of lactose) was attained, this value being markedly higher than those previously reported.[22,23] This can be attributed to the fact that in the present study the initial substrates (sucrose and lactose) were much more concentrated than those employed by those authors.

Considering that electrochemical detectors such as pulsed-amperometric detector (PAD) have a higher sensitivity that the refractive index detector (RID), samples taken after 24 h of enzymatic reaction in the presence of sucrose:lactose mixtures at 30%:30% and 45%:20% were analyzed by HPAEC-PAD to find other minor oligosaccharides not detected by HPLC-RID. In addition to the compounds previously detected by HPLC-RID, HPAEC-PAD chromatograms showed a series of peaks eluting from 28 to 68 min, which could correspond to other minor acceptor-reaction products of degree of polymerization equal to or above 3 (Figure 3). The formation of minor acceptor-reaction products explained that the loss of moles of lactose was slightly higher than the production of moles of the main synthesized oligosaccharide (glucosyl-lactose). As an example, at 24 h of reaction, 0.32 mol L^{-1} of lactose was lost, whereas 0.24 mol L^{-1} of glucosyl-lactose was produced.

NMR Characterization of Glucosyl-lactose. To elucidate the complete structure of the main synthesized oligosaccharide, the enzymatic reaction mixture at 24 h (sucrose:lactose, 30%:30%) was repeatedly separated by HPLC-RID using a semipreparative column, and the corresponding chromatographic peak was manually collected to be further analyzed by NMR. Then, unequivocal structural elucidation of this compound was carried out by the combined use of 1D and 2D [^{1}H, ^{1}H] and [^{1}H–^{13}C] NMR experiments (gCOSY, TOCSY, multiplicity-edited gHSQC and gHMBC). Experiments were carried out at 313 K to avoid the superposition of HDO and one of the anomeric protons. ^{1}H and ^{13}C NMR chemical shifts are given in Table 1. To the best of our

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectral Data for 2-α-D-Glucopyranosyl-lactose[a]

Isomer	Position	Gal		Glu		Glu'	
		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
β anomer	1	4.33 (6.3)	103.99	4.70 (8.0)	97.07	5.24 (3.9)	98.81
	2	3.43	72.04	3.31	79.40	3.43	72.50
	3	3.54	73.62	3.63	74.12	3.64	73.89
	4	3.81	69.62	3.56	79.73	3.35	70.33
	5	3.60	76.37	3.48	75.67	3.95	72.58
	6	3.65	62.07	3.70-3.84	61.35	3.68	61.24
α anomer	1	4.35 (6.1)	103.90	5.32 (3.5)	90.23	4.98 (3.8)	97.46
	2	3.45	72.04	3.58	76.64	3.43	72.44
	3	3.55	73.62	3.84	70.90	3.68	73.84
	4	3.81	69.62	3.59	79.43	3.34	70.43
	5	3.60	76.37	3.86	71.03	3.86	72.78
	6	3.65	62.07	3.70-3.84	61.04	3.86	61.24

[a]Chemical shift (δ, ppm) and coupling constants (J in Hz, in parentheses).

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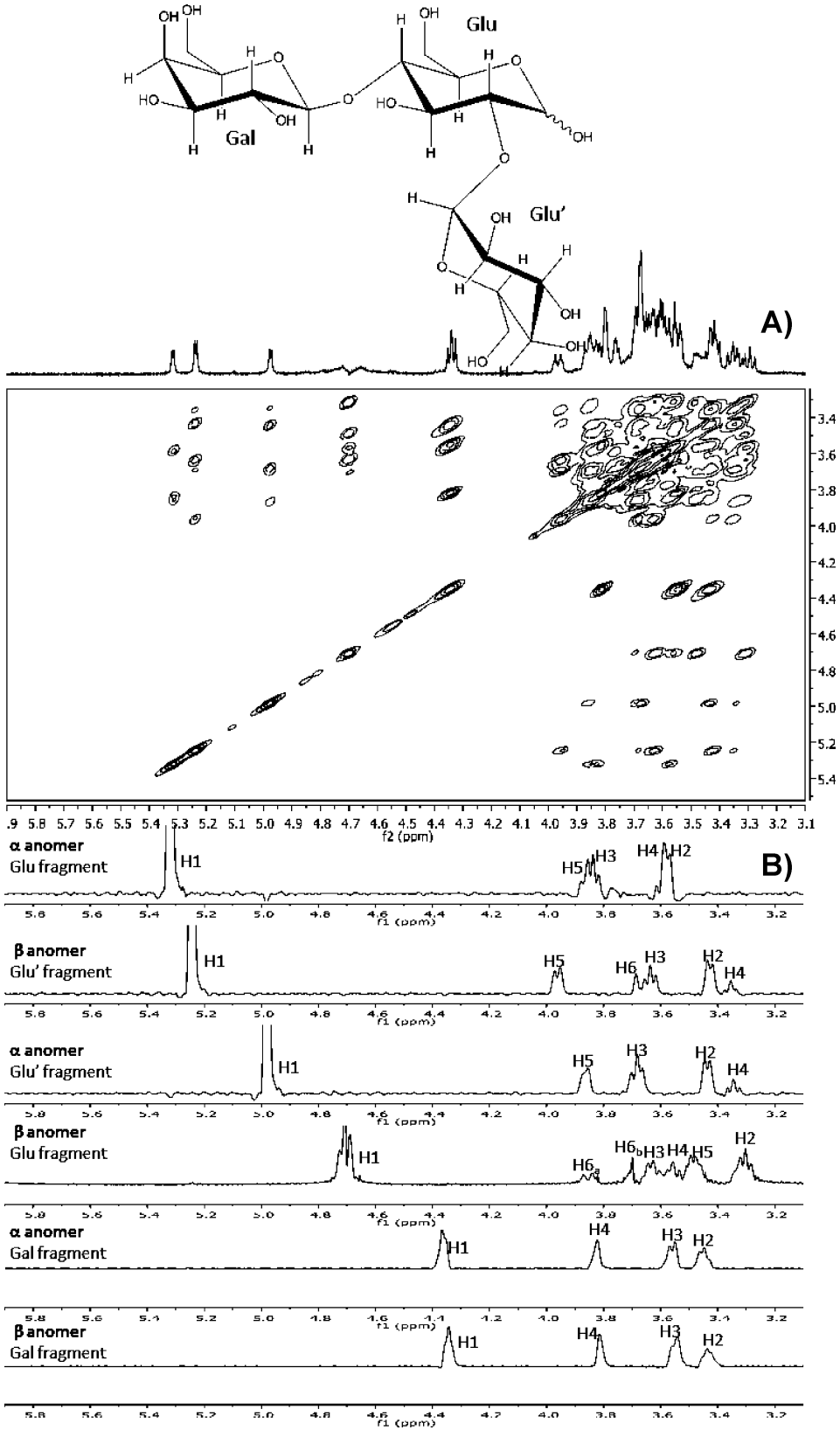

Figure 4. Two-dimensional TOCSY NMR spectrum with water suppression corresponding to 2-α-D-glucopyranosyl-lactose obtained at 500 MHz in D_2O. (A) Contour plot of the sugar region. (B) Horizontal traces corresponding to the resonances of anomeric protons of galacto- and gluco-residues.

knowledge, this is the first report of ^{1}H and ^{13}C NMR full assignments for this compound, and the full set of spectra are collected in the Supporting Information.

The ^{1}H NMR spectrum of the trisaccharide showed six doublets in the anomeric region. The TOCSY experiment revealed the ^{1}H signals of four glucose and two galactose

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residues (Figure 4). These results are compatible with an anomeric mixture at one of glucose residues. In addition, the ^{13}C NMR spectrum displayed two sets of 18 resonances. The major set of resonances, corresponding to the most populated isomer, contained three anomeric carbons at δ 103.99, 98.81, and 97.07. The minor set of resonances also contained three anomeric carbons at δ 103.90, 97.46, and 90.23. A multiplicity-edited gHSQC spectrum was used to link the carbon signals to the corresponding proton resonances. So, taking the anomeric carbons as a starting point, one residue of β-galactose [Gal, J(H1,H2) = 6.3 Hz], one residue of β-glucose [Glu, J(H1,H2) = 8.0 Hz], and one residue of α-glucose [Glu′, J(H1,H2) = 3.9 Hz] were identified for the major isomer, and one residue of β-galactose [Gal, J(H1,H2) = 6.1 Hz], one residue of α-glucose [Glu, J(H1,H2) = 3.5 Hz], and one residue of α-glucose [Glu′, J(H1,H2) = 3.8 Hz] were identified for the minor isomer.

The position of glycosidic linkages was analyzed as follows. For the major isomer, gHMBC showed correlations between the Gal-H1 anomeric proton (4.33 ppm) and the Glu-C4 carbon (79.73 ppm) and between the Glu′-C1 anomeric carbon (98.81 ppm) and the Glu-H2 proton (3.31 ppm). For the minor isomer, gHMBC showed correlations between the Gal-H1 anomeric proton (4.35 ppm) and the Glu-C4 carbon (79.43 ppm) and between the Glu′-C1 anomeric carbon (97.46 ppm) and the Glu-H2 proton (3.58 ppm). So, the major compound was identified as *O*-β-D-galactopyranosyl-(1→4)-*O*-[α-D-glucopyranosyl-(1→2)]-β-D-glucopyranose and the minor isomer as *O*-β-D-galactopyranosyl-(1→4)-*O*-[α-D-glucopyranosyl-(1→2)]-α-D-glucopyranose. The anomeric mixture was 58% (β-anomer) and 42% (α-anomer) by integration of the anomeric protons. Both anomers can be defined as 2-α-D-glucopyranosyl-lactose to simplify the trisaccharide nomenclature.

Production of 2-α-D-Glucopyranosyl-lactose from Cheese WPs. To study the enzymatic synthesis of 2-α-D-glucopyranosyl-lactose from WPs, two different industrial cheese WPs (WP1 and WP2) were used. A preliminary physical−chemical characterization of both permeates was carried out to explain possible dissimilarities in their behavior to the enzymatic synthesis (Table 2). Both cheese WPs had lactose contents within the range established for typical composition of WP,[34] while the main identified metal ions were those derived from the most abundant mineral salts detected in WPs such as calcium, magnesium, sodium, potassium, and phosphorus. Nevertheless, there were notable differences in the total mineral contents, which were principally attributed to the significantly high levels of sodium and, especially, phosphorus and potassium present in WP2 (Table 2). Lastly, permeates also contained trace minerals including boron, zinc, bromine, rubidium, strontium, iodine, titanium, and barium. In addition, differences in the pH values measured for both WPs could suggest that different cheese technological processes were employed.

For the enzymatic synthesis of 2-α-D-glucopyranosyl-lactose, sucrose was added in both WPs as donor at a 1:1 weight ratio considering the concentration of lactose previously determined (Table 2). Thus, the enzymatic reaction started with 30% of sucrose and 30% of lactose, since these conditions gave the best result for higher production of the oligosaccharide of interest with the model systems. Likewise, the pH of both WPs was adjusted to a value of 5.2 (optimum pH for the activity of dextransucrase).

HPLC-RID profiles of enzymatic reactions with both WPs were similar to those obtained for the model systems (sucrose:lactose, 30%:30%). Thus, the main detected carbohydrates after 24 h of enzymatic reaction corresponded to (by decreasing order of abundance): lactose, 2-α-D-glucopyranosyl-lactose, fructose, leucrose, and glucose (Figure 1C). In addition, as it occurred for the model systems, a series of peaks at trace levels, probably corresponding to minor acceptor-reaction products (peak 6), were detected at 16−17 min.

Table 2. Physical−Chemical Characterization of Industrial Cheese WPs (WP1 and WP2)

	WP1	WP2
dry matter (DM)	94.2 ± 0.0	95.5 ± 0.2
lactose (% w/w DM)	89.9	81.6
protein (% w/w DM)	0.1 ± 0.0	Tr[a]
mineral (mg/g DM)	24.6	47.0
main elements (mg/g DM)		
sodium	3.2	6.8
magnesium	1.0	1.3
potassium	6.3	26.5
calcium	9.8	5.5
phosphorus	4.2	7.0
minor elements (μg/g DM)		
boron	17	19
zinc	5	1
bromine	9	35
rubidium	7	24
strontium	4	2
iodine	Tr[a]	3
titanium	ND[b]	6
barium	ND[b]	9
pH	5.4	6.6

[a]Tr, traces. [b]ND, not detected.

Figure 2C,D show the concentration of sucrose, lactose, leucrose, and 2-α-D-glucopyranosyl-lactose upon the enzymatic reaction time with both type of cheese WPs. Regarding leucrose, maximum yields were 4.5% at 24 h and 5.3% at 32 h for WP1 and WP2, respectively (in weight respect to total carbohydrates). In good agreement with the results obtained for the model systems, the maximum formation of 2-α-D-glucopyranosyl-lactose in both cheese WPs was attained at 24 h of reaction, and then, it remained constant to the end of the reaction, while lactose content also exhibited a gradual decrease for the first 24 h of reaction. However, when both WPs were compared, WP2 presented higher levels of 2-α-D-glucopyranosyl-lactose than WP1. Thus, the highest yields of 2-α-D-glucopyranosyl-lactose were 42 (122 mg mL^{-1}) and 52.4% (155 mg mL^{-1}) for WP1 and WP2, respectively (in weight respect to the initial amount of lactose). These dissimilarities in yield of oligosaccharide synthesis could be partially attributed to the substantial differences found in the mineral salts content for both assayed WP, where WP2 had higher levels of sodium, phosphorus, and potassium than WP1 (Table 2). Although diverse studies have shown that the effect of metal ions on oligosaccharide synthesis largely depends on the source from which the enzyme has been isolated[12] and on the substrate used in the assay,[35] cations, such as sodium, potassium, and magnesium, have been described to modify the trans-galactosylation activity of a β-galactosidase from bacterial origin.[36] Moreover, the yield of 2-α-D-glucopyranosyl-lactose produced from WP2 was also higher than that previously obtained from the sucrose:lactose (30%:30%) model system.

Taking into account the mentioned results, the effect of the major ions, which were present in higher amounts in the WP2 than in the WP1 (i.e., sodium, potassium, and phosphorus, Table 2), on the *L. mesenteroides* B-512F dextransucrase activity was evaluated. These studies were carried out using sucrose and lactose mixtures (30%:30%) and adding the major ions at the equivalent concentration as they were present in the WP2. Results showed that either the combined or the unique presence of sodium, phosphorus, and/or potassium increased the sucrose hydrolysis rate, but the content of 2-α-D-glucopyranosyl-lactose was not significantly modified (data not shown). In this sense, it should not be ruled out that the trace minerals contained in the studied WPs (Table 2) might also have an effect on the transfer rate of the dextransucrase and, therefore, on the content of 2-α-D-glucopyranosyl-lactose.

Furthermore, the enzymatic reaction was also performed at 40 °C in addition to 30 °C to attempt a reduction in the reaction time. However, at 40 °C, there were not significant increases in yield of 2-α-D-glucopyranosyl-lactose nor was the reaction time shortened (data not shown), probably because 40 °C exceeds the optimum temperature range of dextransucrase from *L. mesenteroides*.[32,33]

Given that colonic microbiota has an extraordinary contribution to the well-being and health of the host, there is a growing interest in identifying functional dietary compounds capable of modulating the metabolic activities and composition of the intestinal microbiota.[37] An alternative to probiotics is the use of prebiotics, which have been defined as "non-digestible ingredients that are selectively fermented and allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health".[38] The trisaccharide, 2-α-D-glucopyranosyl-lactose, synthesized and characterized in this work, is an excellent candidate for a new prebiotic ingredient since, on the one hand, it has shown high resistance of α-(1→2) linkages to in vitro and in vivo gastrointestinal digestion.[25,39,40] On the other hand, this trisaccharide is an oligosaccharide derived from kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) as it contains this disaccharide at the reducing end of the structure (Table 1 and Figure 4). Thus, previous studies demonstrated the growth of six strains belonging to *Bifidobacterium*, *Lactobacillus*, and *Eubacterium* during the incubation of kojibiose with pure cultures. Likewise, kojioligosaccharides with degrees of polymerization of 3 and 4 were utilized by fewer strains but more selectively than kojibiose.[40] In this context, Sanz et al.[41] studied the influence of glycosidic linkages and monosaccharide composition on the selectivity of microbial fermentation in a wide range of disaccharides following their in vitro fermentation by using human fecal batch cultures; strikingly, kojibiose showed the greatest prebiotic index of the 20 assayed disaccharides, identifying this disaccharide and its potential derived-oligosaccharides as one of the most promising novel carbohydrates that could be beneficial to gut health.

To conclude, in this study, the capacity of dextransucrase (EC 2.4.1.5) from *L. mesenteroides* B-512F to catalyze the high-yield synthesis of potentially bioactive 2-α-D-glucopyranosyl-lactose, characterized by NMR, in the presence of sucrose and cheese WPs has been shown. These findings could contribute to broadening the use of these important agricultural raw materials as renewable substrates for enzymatic synthesis of oligosaccharides of nutritional interest.

■ ASSOCIATED CONTENT

Supporting Information

Figures of ^{1}H and ^{13}C NMR, gCOSY, TOCSY, gHSQC, and gHMBC. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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