Synthesis and characterization of a potential prebiotic trisaccharide from cheese whey permeate and sucrose by Leuconostoc mesenteroides dextransucrase

Marina Díez-Municio[a], Antonia Montilla[a], M. Luisa Jimeno[b], Nieves Corzo[a], Agustín Olano[a], F. Javier Moreno[a*]

(a) Dpto. Bioactividad y Análisis de Alimentos. Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), CEI (UAM+CSIC), c/Nicolás Cabrera 9, 28049 Madrid, Spain.

(b) Centro Química Orgánica "Manuel Lora Tamayo" (CSIC), c/Juan de la Cierva 3, 28006 Madrid, Spain.

* Corresponding author

Tel +34 910017948; Fax +34 910017905

E-mail: javier.moreno@csic.es

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 permeates 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 broaden 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 multi-step chemical synthesis considering the high stereo- and regio-selectivity 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 by-product 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*), being its profitable use one of the biggest dairy industry challenge ahead (*8*). One of the most important uses of whey permeate is the synthesis of galactooligosaccharides (GOS) from transgalactosylation of lactose catalyzed by glycosidases (EC 3.2), being β-galactosidases (EC 3.2.1.23) of microbial origin 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 region-selectivity (*16*). Glycosyltransferases (EC 2.4) are considered as 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 (EC 2.4.99) (*16*). Within the subgroup 2.4.1 and under the term glycansucrases are considered glucansucrases or fructansucrases. This type of enzymes maintain the regiospecificity and catalyse effective synthesis of various oligosaccharides of different structural nature by using sucrose as glucosyl or fructosyl moiety donor and carbohydrates with low molecular weight 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 oligosaccharides 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.

Firstly, 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, to be then applied to the production of oligosaccharides from industrial cheese WPs.

Materials and methods

Chemical and reagents

Dextransucrase from *Leuconostoc mesenteroides* B-512F was purchased from CRITT Bio-Industries (Toulouse, France). 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 $CaCl_2$, $2H_2O$. 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). Ultra-pure water quality (18.2 MΩcm) 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 whey permeates

Two different industrial bovine cheese whey permeate powders (WPs) 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 ºC until constant weight, according to the AOAC method (*27*).

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 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 ºC 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 ºC 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. In order 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, i.e. 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 ºC. Considering the lactose content measured in both WPs, enzymatic reactions were carried out at sucrose:lactose 30%:30%, in 20mM sodium acetate buffer (pH 5.2) for 48 hours. When it was necessary, 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 1M Na_2HPO_4 and/or 20 µl of 1M 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 20mM sodium acetate buffer (pH 5.2). The enzymatic reactions were carried out as described above at 30 ºC for 48 hours.

Chromatographic determination of carbohydrates

Liquid chromatography with refractive index detector

The synthesized oligosaccharides were analysed 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 x 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. 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 intra-day and inter-day 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

In order to increase sensitivity, some of the samples were also analyzed by high performance anion exchange chromatography with pulsed amperometric detection

189 (HPAEC-PAD) on an IC 2500 Dionex System consisting of a GP50 gradient pump and
190 an ED50 electrochemical detector with a gold working electrode and Ag/AgCl reference
191 electrode. Data acquisition and processing were performed with a Chromeleon version
192 6.7 software (Dionex Corp., Sunnyvale, CA). For eluents preparation, MilliQ water,
193 50% (w:v) NaOH, and NaOAc (Panreac, Barcelona, Spain) were used. All eluents were
194 degassed by flushing with helium for 25 min. Separations were performed following the
195 method described by Spletchna et al. (*30*). Elution was at room temperature on a
196 CarboPac PA-1 column (250 × 4 mm) connected to a CarboPac PA-1 (50 × 4 mm)
197 guard column. The elution, at a flow rate of 1 mL min^{-1}, was in gradient using a
198 combination of three eluents: A (100 mM NaOH), B (100 mM NaOH and 50 mM
199 NaOAc), and C (100 mM NaOH and 1 M NaOAc). The gradient used was 100% A
200 from 0 to 20 min and 0-100% B from 20 to 70 min. After each run, the column was
201 washed for 10 min with 100% C and re-equilibrated for 15 min with the starting
202 conditions of the employed gradient.
203
204 *Gas chromatography with mass spectrometry detection*
205
206 Selected samples were also analyzed by gas chromatography with a mass
207 spectrometry detector (GC-MS) on an Agilent Technologies 7890A gas chromatograph
208 coupled to a 5975C MSD quadrupole mass detector (Agilent Technologies,
209 Wilmington, DE). The trimethylsilyloximes (TMSO), prepared as described by Sanz et
210 al. (*31*), were separated using an HP-5 MS fused-silica capillary column (30 m × 0.25
211 mm i.d. × 0.25 μm film thickness) coated with 5% phenylmethylsilicone (J&W
212 Scientific, CA). The helium flow rate was 1 mL min^{-1}. The initial oven temperature was
213 180 ºC and increased to 315 ºC at a heating rate of 3 ºC min^{-1} and held for 20 min. The

injector temperature was 280 ºC and injections were made in the split mode (1:40). 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 synthesised oligosaccharide, this trisaccharide was isolated and purified by HPLC-RID from sucrose:lactose mixtures after 24 hours of enzymatic reaction and using a semi-preparative column Kromasil® (100-NH_2) column (250 × 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 synthesised oligosaccharide were manually collected, pooled, and evaporated in a rotatory evaporator R-200 (Büchi, Switzerland) below 25 ºC for its subsequent characterization.

Structure elucidation of the purified oligosaccharide was accomplished by Nuclear Magnetic Resonance spectroscopy (NMR). 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 ºC) of *Leuconostoc 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: 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 condition (at pH 5.2 and 30ºC for 48 h). Results obtained with 0.4 U mL^{-1} of enzyme showed that sucrose was not totally consumed after 48 hours 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 hours with sucrose:lactose mixtures at concentrations of 45%:20% and 30%:30% are shown in **Figures 1A and 1B**. 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 hours of reaction four new peaks clearly appeared (peaks 1, 2, 4 and 7), whilst 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 hours of reaction by the hydrolytic action of the dextransucrase (**Figure 1B**). At 24 hours 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, that 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-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*). In order to confirm this fact, the purification and exhaustive characterization by NMR of this compound will be discussed in the next section.

Figures 2A and **2B** illustrate 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 hours 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 hours 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 (**Figures 2A** and **2B**). By comparing both set of reaction mixtures, it can be inferred that whilst the maximum amount of formed glucosyl-lactose was fairly similar in both cases (120-130 mg mL^{-1}), the formation of leucrose (which could be considered as an unwanted by-product) was two-fold lower in the presence of 30% of sucrose and 30% of lactose than when 45% sucrose and 20% lactose mixture was used (i.e., 27 and 54 mg mL^{-1} of leucrose, respectively, after 32 hours of reaction). This result can be explained by the fact that the formation of leucrose by action of dextransucrase is favoured at high fructose concentration (*16*). This means that the higher the concentration of sucrose, the higher the concentration of fructose in the reaction medium and, consequently, the greater the formation of leucrose.

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, being this value 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 hours of enzymatic reaction in the presence of sucrose:lactose mixtures at

339 30%:30% and 45%:20% were analysed by HPAEC-PAD in order to find other minor
340 oligosaccharides not detected by HPLC-RID. In addition to the compounds previously
341 detected by HPLC-RID, HPAEC-PAD chromatograms showed a series of peaks eluting
342 from 28 to 68 min which could correspond to other minor acceptor-reaction products of
343 degree of polymerization equal to or above 3 (**Figure 3**). The formation of minor
344 acceptor-reaction products explained that the loss of moles of lactose was slightly
345 higher than the production of moles of the main synthesized oligosaccharide (glucosyl-
346 lactose). As an example, at 24 hours of reaction 0.32 mol L^{-1} of lactose were lost
347 whereas 0.24 mol L^{-1} of glucosyl-lactose were produced.
348
349 *NMR characterization of glucosyl-lactose*
350
351 In order to elucidate the complete structure of the main synthesized
352 oligosaccharide, the enzymatic reaction mixture at 24 hours (sucrose:lactose, 30%:30%)
353 was repeatedly separated by HPLC-RID using a semi-preparative column and the
354 corresponding chromatographic peak was manually collected to be further analysed by
355 NMR. Then, unequivocal structural elucidation of this compound was carried out by the
356 combined use of 1D and 2D [^{1}H, ^{1}H] and [^{1}H-^{13}C] NMR experiments (gCOSY,
357 TOCSY, multiplicity-edited gHSQC and gHMBC). Experiments were carried out at
358 313K to avoid the superposition of HDO and one of the anomeric protons. ^{1}H and ^{13}C
359 NMR chemical shifts are given in **Table 1**. To the best of our knowledge, this is the first
360 report of ^{1}H and ^{13}C-NMR full assignments for this compound and full set of spectra are
361 collected in Supporting Information.
362 ^{1}H NMR spectrum of the trisaccharide showed six doublets in the anomeric
363 region. The TOCSY experiment revealed the ^{1}H signals of four glucose and two

galactose residues (**Figure 4**). These results are compatible with an anomeric mixture at one of glucose residues. In addition, ^{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 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) 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 in order to simplify the trisaccharide nomenclature.

389 *Production of 2-α-D-glucopyranosyl-lactose from cheese whey permeates*

390

391 In order to study the enzymatic synthesis of 2-α-D-glucopyranosyl-lactose from
392 WPs, two different industrial cheese WPs (WP1 and WP2) were used. A preliminary
393 physical-chemical characterization of both permeates was carried out in order to explain
394 possible dissimilarities in their behaviour to the enzymatic synthesis (**Table 2**). Both
395 cheese WPs had lactose contents within the range established for typical composition of
396 whey permeate (*34*), whilst the main identified metal ions were those derived from the
397 most abundant mineral salts detected in whey permeates such as calcium, magnesium,
398 sodium, potassium and phosphorus. Nevertheless, there were notable differences in the
399 total mineral content which were principally attributed to the significantly high levels of
400 sodium and, especially, phosphorus and potassium present in WP2 (**Table 2**). Lastly,
401 permeates also contained trace minerals including boron, zinc, bromine, rubidium,
402 strontium, iodine, titanium and barium. In addition, differences in the pH values
403 measured for both WPs could suggest that different cheese technological processes were
404 employed.

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

412 HPLC-RID profiles of enzymatic reactions with both WPs were similar to those
413 obtained for the model systems (sucrose:lactose, 30%:30%). Thus, the main detected

carbohydrates after 24 hours 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.

Figures 2C and **2D** 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 hours, and 5.3% at 32 hours 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 hours of reaction, and then it remained constant to the end of the reaction whilst lactose content also exhibited a gradual decrease for the first 24 hours 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 transgalactosylation activity of a β-galactosidase from bacterial origin (*36*). Moreover,

438 the yield of 2-α-D-glucopyranosyl-lactose produced from WP2 was also higher than
439 that previously obtained from the sucrose:lactose (30%:30%) model system.

440 Taking into account the above results, the effect of the major ions, that were
441 present in higher amounts in the WP2 than in the WP1 (i.e., sodium, potassium and
442 phosphorus, **Table 2**), on the *L. mesenteroides* B-512F dextransucrase activity was
443 evaluated. These studies were carried out using sucrose and lactose mixtures
444 (30%:30%) and adding the major ions at the equivalent concentration as they were
445 present in the WP2. Results showed that either the combined or the unique presence of
446 sodium, phosphorous and/or potassium increased the sucrose hydrolysis rate but the
447 content of 2-α-D-glucopyranosyl-lactose was not significantly modified (data not
448 shown). In this sense, it should not be ruled out that the trace minerals contained in the
449 studied WPs (**Table 2**) might also have an effect on the transfer rate of the
450 dextransucrase and, therefore, on the content of 2-α-D-glucopyranosyl-lactose.

451 Furthermore, the enzymatic reaction was also performed at 40 ºC in addition to
452 30 ºC in order to attempt a reduction in the reaction time. However, at 40 ºC, there were
453 not significant increases in yield of 2-α-D-glucopyranosyl-lactose nor the reaction time
454 was shortened (data not shown), probably due to 40 ºC exceeds the optimum
455 temperature range of dextransucrase from *L. mesenteroides* (*32, 33*).
456
457 Given that colonic microbiota has an extraordinary contribution to well-being
458 and health of the host, there is a growing interest in identifying functional dietary
459 compounds capable of modulating the metabolic activities and composition of the
460 intestinal microbiota (*37*). An alternative to probiotics is the use of prebiotics, which
461 have been defined as "non-digestible ingredients that are selectively fermented and
462 allows specific changes, both in the composition and/or activity in the gastrointestinal

463 microflora that confers benefits upon host wellbeing and health" (*38*). The trisaccharide,
464 2-α-D-glucopyranosyl-lactose, synthesised and characterized in this work is an
465 excellent candidate for a new prebiotic ingredient since, on the one hand, it has been
466 shown the high resistance of α-(1→2) linkages to *in vitro* and *in vivo* gastrointestinal
467 digestion (*25, 39, 40*). On the other hand, this trisaccharide is an oligosaccharide
468 derived from kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) as it contains this
469 disaccharide at the reducing end of the structure (**Table 1** and **Figure 4**). Thus, previous
470 studies demonstrated the growth of 6 strains belonging to *Bifidobacterium*,
471 *Lactobacillus* and *Eubacterium* during the incubation of kojibiose with pure cultures.
472 Likewise, kojioligosaccharides with a degree of polymerization of 3 and 4 were utilized
473 by fewer strains but more selectively than kojibiose (*40*). In this context, Sanz et al. (*41*)
474 studied the influence of glycosidic linkages and monosaccharide composition on the
475 selectivity of microbial fermentation in a wide range of disaccharides following their *in*
476 *vitro* fermentation by using human fecal batch cultures; strikingly, kojibiose showed the
477 greatest prebiotic index of the 20 assayed disaccharides, identifying this disaccharide
478 and its potential derived-oligosaccharides as one of the most promising novel
479 carbohydrates that could be beneficial to gut health.
480
481 To conclude, in this study, it has been shown the capacity of dextransucrase (EC
482 2.4.1.5) from *Leuconostoc mesenteroides* B-512F to catalyze the high-yield synthesis of
483 potentially bioactive 2-α-D-glucopyranosyl-lactose, characterized by NMR, in the
484 presence of sucrose and cheese whey permeates. These findings could contribute to
485 broaden the use of these important agricultural raw materials as renewable substrates for
486 enzymatic synthesis of oligosaccharides of nutritional interest.
487

Acknowledgements

This work has been financed by projects POII10-0178-4685 from Junta de Comunidades de Castilla-La Mancha and European regional development fund (ERDF), and projects AGL2011-27884 and Consolider Ingenio 2010 FUN-C-FOOD CSD2007-00063 both from Ministerio de Ciencia e Innovación. M. Díez-Municio thanks CSIC for a JAE predoctoral fellowship. We thank Francisco José Redondo and Ricardo González from García Baquero and Reny Picot, respectively, for kindly providing us with cheese whey permeates. We also thank Inmaculada Rivas and Zaida Gil of the ICP-MS service of SIdI-UAM for help in the physical-chemical characterization of cheese whey permeates.

Literature cited

1. André, I.; Potocki-Véronèse, G.; Morel, S.; Monsan, P.; Remaud-Siméon, M. Sucrose-utilizing transglucosidases for biocatalysis. *Top. Curr. Chem.* **2010**, 294, 25–48.

2. Monsan, P.; Paul, F. Enzymatic synthesis of oligosaccharides. *FEMS Microbiol. Rev.* **1995**, *16,* 187-192.

3. Baciu, I. E. Extracted sugar-beet pulp and sucrose, two renewable materials as "hot" substrates for enzymatic synthesis of valuable saccharides. PhD Dissertation; *Technical University Braunschweig.* **2005**.

4. Adamczak, M.; Charubin, D.; Bednarski, W. Influence of reaction medium composition on enzymatic synthesis of galactooligosaccharides and lactulose from lactose concentrates prepared from whey permeate. *Chemical Papers.* **2009**, *63,* 111-116.

5. Khan, R. Sucrose: Its potential as a raw material for food ingredients and for chemicals. In *Sucrose; properties and applications*. Mathlouthi, M., Reiser, P., Eds.; Blackie A&P: London, U.K., 1995; pp. 264-278.

6. Monsan, P.F.; Ouarné, F. Oligosaccharides derived from sucrose. In *Prebiotics and probiotics science and technology.* Charalampopolus, D., Rastall, R.A, Eds.; Springer-Verlag: New York, NY, 2009; Vol.1, pp. 293-336.

7. Barile, D.; Tao, N.; Lebrilla, C.B.; Coisson, J.-D.; Arlorio, M.; German, J.B. Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. *Int. Dairy J.* **2009**, *19,* 524-530.

8. 3A Business Consulting. *Global Opportunities for Whey and Lactose Ingredients 2010-2014.* 2010. Available from: http://www.3abc.dk/Report%20information%20-

523 %20Global%20Opportunities%20for%20Whey%20and%20Lactose%20Ingredients%2
524 02010-2014.pdf
525 9. Rustom, I.Y.S.; Foda, M.I.; Lopez-Leiva, M. Formation of oligosaccharides from
526 whey UF-permeate by enzymatic hydrolysis: analysis of factors. *Food Chem.* **1998**, *62,*
527 141-147.
528 10. Goulas, A.; Tzortzis, G.; Gibson, G.R. Development of a process for the production
529 and purification of α- and β-galactooligosaccharides from *Bifidobacterium bifidum*
530 NCIMB 41171. *Int. Dairy J.* **2007**, *17,* 648-656.
531 11. Splechtna, B.; Nguyen, T.H.; Haltrich, D. Comparison between discontinuous and
532 continuous lactose conversion processes for the production of prebiotic galacto-
533 oligosaccharides using β-galactosidase from *Lactobacillus reuteri*. *J. Agric. Food*
534 *Chem.* **2007**, *55,* 6772-6777.
535 12. Mozaffar, Z.; Nakanishi, K.; Matsuno, R. Formation of oligosaccharides during
536 hydrolysis of lactose in milk using β-Galactosidase from *Bacillus circulans*. *J. Food Sci.*
537 **1985**, *50,* 1602-1606.
538 13. Hellerová, K.; Čurda, L. Influence of type of substrate and enzyme concentration on
539 formation of galacto-oligosaccharides. *Czech J. Food Sci.* **2009**, *27,* 372-374.
540 14. Gosling, A.; Alftrén, J.; Stevens, G.W.; Barber, A.R.; Kentish, S.E.; Gras, S.L.
541 Facile pretreatment of *Bacillus circulans* β-galactosidase increases the yield of
542 galactosyl oligosaccharides in milk and lactose reaction systems. *J. Agric. Food Chem.*
543 **2010**, *57,* 11570-11574.
544 15. Pocedicová, K.; Curda, L.; Misún, D.; Dryáková, A.; Diblíková, L. Preparation of
545 galacto-oligosaccharides using membrane reactor. *J. Food Eng.* **2010**, *99,* 479-484.

16. Plou, F.J.; Martín, M.T.; de Segura, A.G.; Alcalde, M.; Ballesteros, A. Glucosyltransferases acting on starch or sucrose for the synthesis of oligosaccharides. *Can. J. Chem.* **2002**, *80,* 743-752.

17. Monchois, V.; Willemot, R.M.; Monsan, P. Glucansucrases: mechanism of action and structure–function relationships. *FEMS Microbiol. Rev.* **1999**, *23,* 131-151.

18. Monsan, P.; Remaud-Siméon, M.; André, I. Transglucosidases as efficient tools for oligosaccharide and glucoconjugate synthesis. *Curr. Opin. Microbiol.* **2010**, *13,* 293-300.

19. Robyt, J.F.; Eklund, S.H. Stereochemistry involved in the mechanism of action of dextransucrase in the synthesis of dextran and the formation of acceptor products. *Bioorg. Chem.* **1982**, *11,* 115-132.

20. Robyt, J.F. Mechanisms in the glucansucrase synthesis of polysaccharides and oligosaccharides from sucrose. *Adv. Carbohydr. Chem. Biochem.* **1995**, *51,* 133-168.

21. Robyt, J.F.; Walseth, T.F. The mechanism of acceptor reactions of *Leuconostoc mesenteroides* B-512F dextransucrase. *Carbohydr. Res.* **1978**, *61,* 433-445.

22. Robyt, J.F.; Eklund, S.H. Relative, quantitative effects of acceptors in the reaction of *Leuconostoc mesenteroides* B-512F dextransucrase. *Carbohydr. Res.* **1983**, *121,* 279-286.

23. Bourne, E.J.; Hartigan, J.; Weigel, H. 469. Mechanism of the enzymic synthesis of a branched trisaccharide containing the α-1:2-glucosidic linkage. *J. Chem. Soc.* **1959**, 2332-2337.

24. Bailey, R.W.; Barker, S.A.; Bourne, E.J.; Stacey, M. Enzymic Synthesis of a 'Branched' Trisaccharide. *Nature.* **1955**, *176,* 1164-1165.

569 25. Remaud-Siméon, M.; Willemot, R.M.; Sarçabal, P.; Potocki de Montalk, G.;
570 Monsan, P. Glucansucrases: molecular engineering and oligosaccharide synthesis. *J.*
571 *Molec. Catal. B: Enzym.* **2000**, *10,* 117-128.
572 26. Sanz, M.L.; Côté, G.L.; Gibson, G.R.; Rastall, R.A. Influence of glycosidic linkages
573 and molecular weight on the fermentation of maltose-based oligosaccharides by human
574 gut bacteria. *J. Agric. Food Chem.* **2006**, *54,* 9779-9784.
575 27. Association of Official Analytical Chemists; by Helrich, K. AOAC method 950.01,
576 Official methods of analysis of AOAC; Arlington, VA,: 1990; Vol.1 pp. 684.
577 28. Zuluaga, J.; Rodríguez, N.; Rivas-Ramirez, I.; de la Fuente, V.; Rufo, L.; Amils, R.
578 An improved semiquantitative method for elemental analysis of plants using inductive
579 coupled plasma mass spectrometry. *Biol. Trace Elem. Res.* **2011**, 1-16.
580 29. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram
581 quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**,
582 *72,* 248-254.
583 30. Splechtna, B.; Nguyen, T.; Steinböck, M.; Kulbe, K.D.; Lorenz, W.; Haltrich, D.
584 Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases
585 from *Lactobacillus reuteri*. *J. Agric. Food Chem.* **2006**, *54,* 4999-5006.
586 31. Sanz, M.L.; Sanz, J.; Martinez-Castro, I. Gas chromatographic-mass spectrometric
587 method for the qualitative and quantitative determination of disaccharides and
588 trisaccharides in honey. *J. Chromatogr., A.* **2004**, *1059,* 143-148.
589 32. Kim, D.; Robyt, J.F. Production and selection of mutants of *Leuconostoc*
590 *mesenteroides* constitutive for glucansucrases. *Enzyme Microb. Technol.* **1994**, *16,* 659-
591 664.
592 33. Kim, M.; Day, D.F. Optimization of oligosaccharide synthesis from cellobiose by
593 dextransucrase. *Appl. Biochem. Biotechnol.* **2008**, 707-716.

34. U.S. Dairy Export Council. *Reference Manual for U.S. Whey and Lactose Products.* 2004; Available from: http://usdec.files.cmsplus.com/PDFs/2008ReferenceManuals/Whey_Lactose_Reference_Manual_Complete2_Optimized.pdf.

35. Flores, M.V.; Ertola, R.J.; Voget, C.E. Effect of monovalent cations on the stability and activity of *Kluyveromyces lactis* β-galactosidase. *Lebensm. -Wiss. u. -Technol.* **1996**, *29,* 503-506.

36. Garman, J.; Coolbear, T.; Smart, J. The effect of cations on the hydrolysis of lactose and the transferase reactions catalysed by β-galactosidase from six strains of lactic acid bacteria. *Appl. Microbiol. Biotechnol.* **1996**, *46,* 22-27.

37. Rastall, R. Functional oligosaccharides: application and manufacture. *Annu. Rev. Food Sci. Technol.* **2010**, *1,* 305-339.

38. Gibson, G.R.; Probert, H.M.; Loo, J.V.; Rastall, R.A.; Roberfroid, M.B. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* **2004**, *17,* 259-275.

39. Valette, P.; Pelenc, V.; Djouzi, Z.; Andrieux, C.; Paul, F.; Monsan, P.; Szylit, O. Bioavailability of new synthesised glucooligosaccharides in the intestinal tract of gnotobiotic rats. *J. Sci. Food Agric.* **1993**, *62,* 121-127.

40. Nakada, T.; Nishimoto, T.; Chaen, H.; Fukuda, S. Kojioligosaccharides: Application of kojibiose phosphorylase on the formation of various kojioligosaccharides. Oligosaccharides in Food and Agriculture. In *Oligosaccharides in Food and Agriculture. ACS Symposium Series*; Eggleston, G.; Côté, G. L., Eds.; American Chemical Society: Washington, DC, 2003; Vol. 849, pp. 104-117.

41. Sanz, M.L.; Gibson, G.R.; Rastall, R.A. Influence of disaccharide structure on prebiotic selectivity in vitro. *J. Agric. Food Chem.* **2005**, *53,* 5192-5199.

Figure captions

Figure 1. HPLC-RID profiles of transglycosylation reactions based on sucrose:lactose and sucrose:whey permeate mixtures catalyzed by dextransucrase from *Leuconostoc 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 hours. **A)** sucrose:lactose 45%:20%; **B)** sucrose:lactose 30%:30%; **C)** sucrose:whey permeate 2 (equivalent to a concentration of sucrose:lactose of 30%:30%).

Labelled peaks are as follows: 1 (fructose); 2 (glucose); 3 (sucrose); 4 (leucrose); 5 (lactose); 6 (minor acceptor-reaction products); 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:whey permeate mixtures catalyzed by dextransucrase from *Leuconostoc 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:whey permeate 1 (equivalent to a concentration of sucrose:lactose of 30%:30%); **D)** sucrose:whey permeate 2 (equivalent to a concentration of sucrose:lactose of 30%:30%). Sucrose (—○—), lactose (- -△- -), leucrose (—□—), 2-α-D-glucopyranosyl-lactose (—◆—). Vertical bars represent standard deviations (n = 3).

Figure 3. HPAEC-PAD profile of transglycosylation reaction based on sucrose:lactose (30%:30%) catalyzed by dextransucrase from *Leuconostoc mesenteroides* B-512F (0.8 U mL^{-1}) at 30 °C, in 20 mM sodium acetate buffer at pH 5.2, for 24 hours. Labelled peaks are as follows: 1 (glucose), 2 (fructose), 3 (leucrose), 4 (lactose), 5 (2-α-D-

644 glucopyranosyl-lactose). (*) The inset shows a zoom area of the eluted minor acceptor-
645 reaction products of degree of polymerization above 3.
646
647 **Figure 4.** 2D TOCSY NMR spectrum with water suppression corresponding to 2-α-D-
648 glucopyranosyl-lactose obtained at 500 MHz in D2O. **A)** Contour plot of the sugar
649 region. **B)** Horizontal traces corresponding to the resonances of anomeric protons of
650 galacto- and gluco-residues.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data for 2-α-D-glucopyranosyl-lactose. Chemical shift (δ, ppm) and coupling constants (J in Hz, in parenthesis).

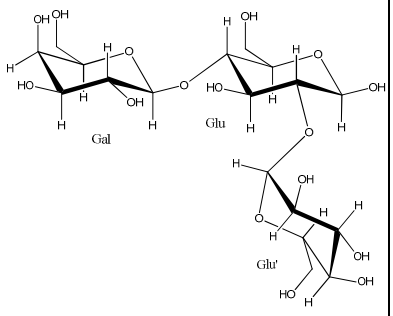
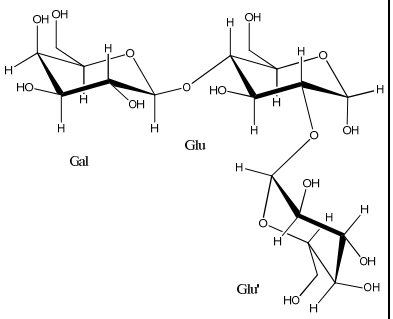
Isomer	Position	Gal		Glu		Glu'	
		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
β anomer 	1	4.33 (6.3)	103.99	4.70 (8.0)	97.07	5.24 (3.9)	98.81
	2	3.43	72.04	3.31	79.40	3.43	72.50
	3	3.54	73.62	3.63	74.12	3.64	73.89
	4	3.81	69.62	3.56	79.73	3.35	70.33
	5	3.60	76.37	3.48	75.67	3.95	72.58
	6	3.65	62.07	3.70-3.84	61.35	3.68	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

Table 2. Physical-chemical characterization of industrial cheese whey permeates (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*
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*	3
Titanium	n.d.**	6
Barium	n.d.**	9
pH	5.4	6.6

*Tr: Traces
**n.d: not detected

Figure 1. ***Díez-Municio et al.***

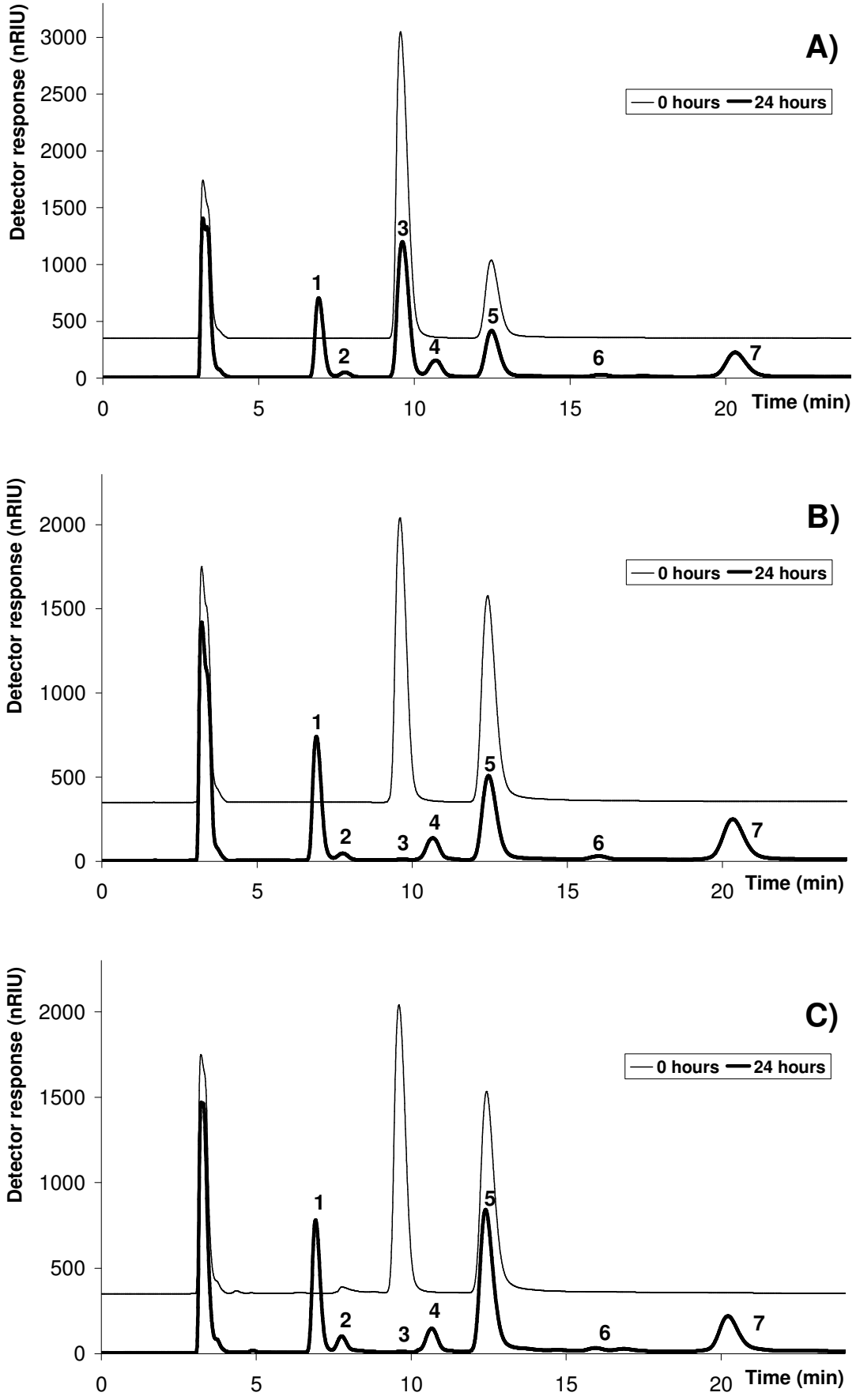


Figure 2. ***Díez-Municio et al.***

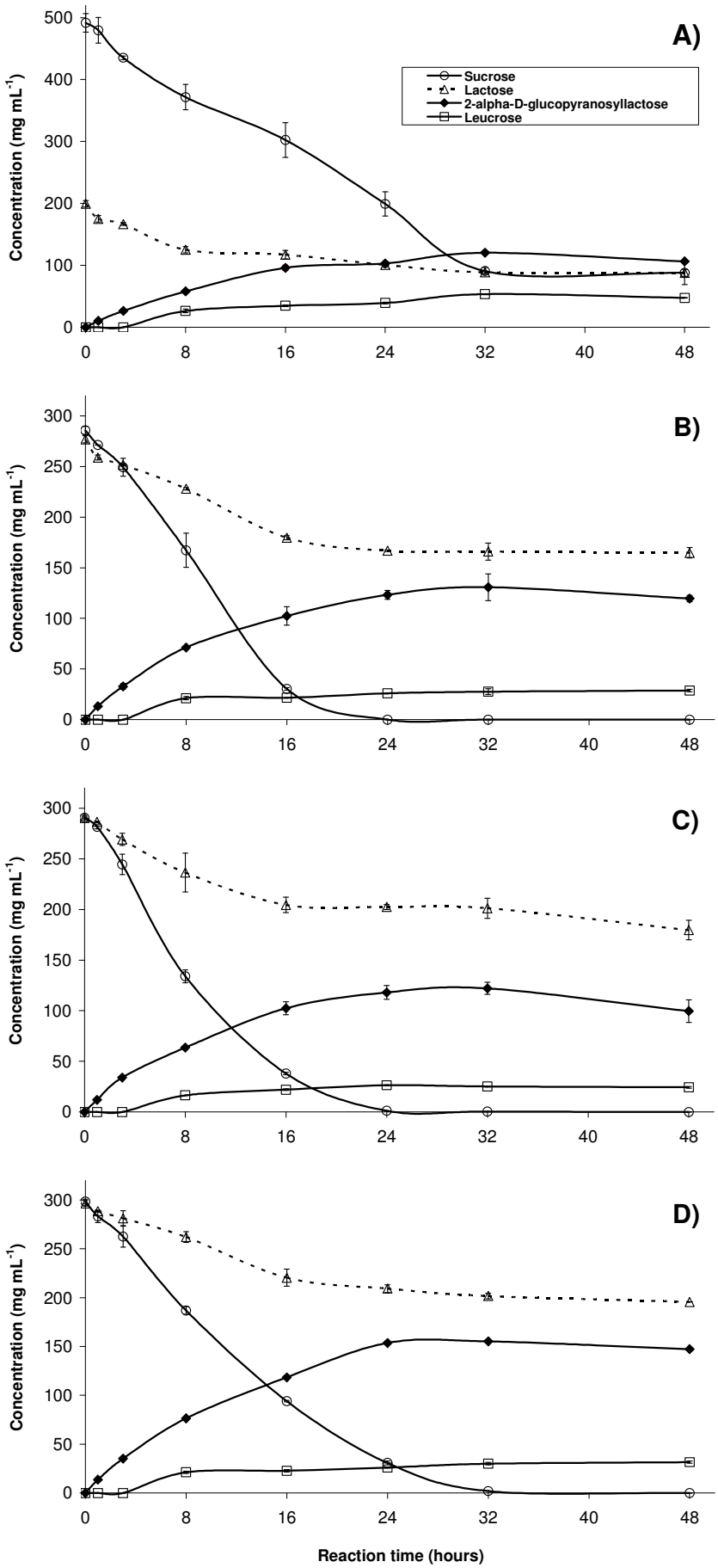


Figure 3. ***Díez-Municio et al.***

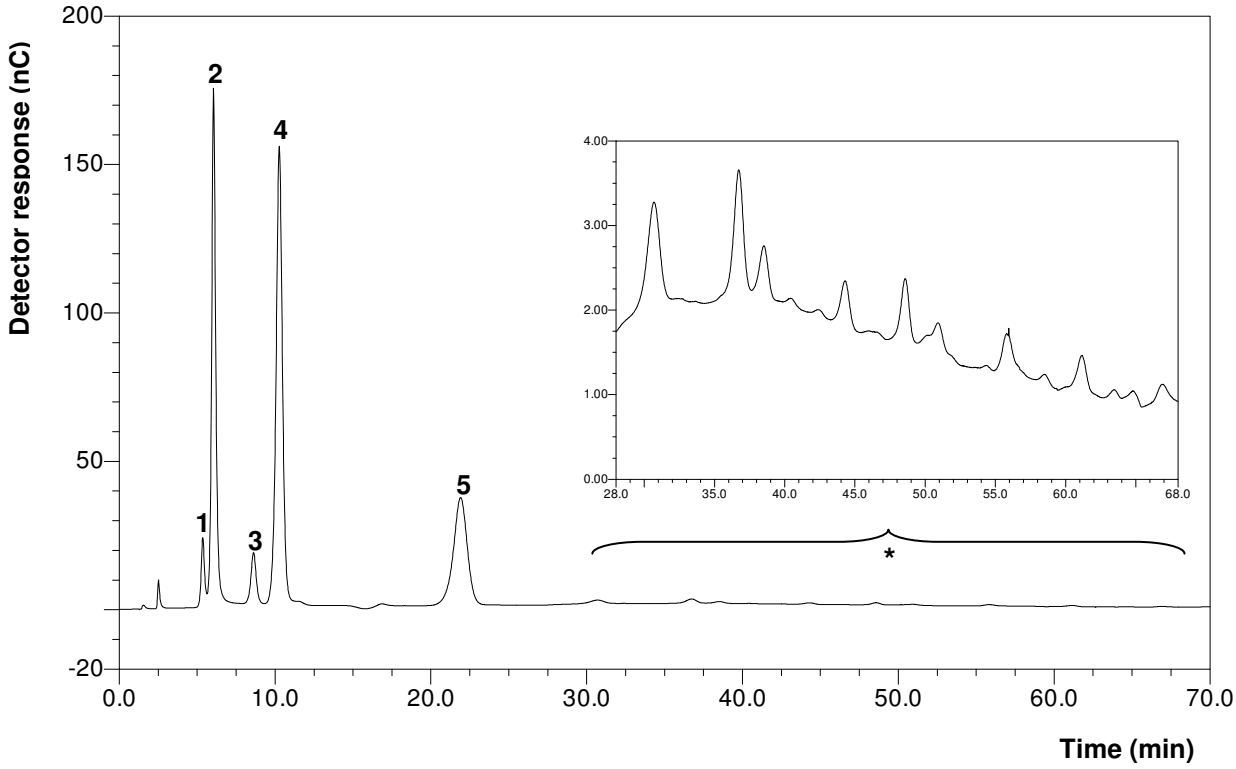


Figure 4. ***Díez-Municio et al.***