Synthesis of prebiotic carbohydrates derived from cheese whey permeate by a combined process of isomerization and transgalactosylation

Running title: Synthesis of prebiotic carbohydrates from cheese whey permeate

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Abstract

BACKGROUND: Lactose from cheese whey permeate (WP) was efficiently isomerized to lactulose using egg shell, a food-grade catalyst, and the subsequent transgalactosylation reaction of this mixture with β-galactosidase from *Bacillus circulans* gave rise to a wide array of prebiotic carbohydrates derived from lactose and lactulose.

RESULTS: Lactulose, which was obtained by the efficient isomerization of the WP (16% in weight, respect to the initial amount of lactose), showed a great resistance to the hydrolytic action of β-galactosidase from *B. circulans* that preferentially hydrolyzed lactose, acting as galactosyl donor and acceptor. Lactulose had capacity as acceptor leading to the formation of lactulose-derived oligosaccharides. The enzymatic synthesis was optimized by studying reaction conditions such as pH, temperature, time, enzyme, and carbohydrate concentration. The maximum formation of galactooligosaccharides, with degree of polymerization from 2 to 4, was achieved after 5 h of reaction at pH 6.5, 50 ºC with 300 g kg^{-1} of carbohydrates and 3 U mL^{-1} of β-galactosidase.

CONCLUSION: These findings indicate that the transgalactosylation of isomerized WP with β-galactosidase from *B. circulans* could be a new and efficient method to obtain a mixture with a 50% of potentially prebiotic carbohydrates composed of lactulose, and galactooligosaccharides derived from lactose and lactulose.

Keywords: isomerization, lactulose, galactooligosaccharides, transgalactosylaction, *Bacillus circulans*, whey permeate.

INTRODUCTION

Cheese whey is the most abundant by-product of the dairy industry and its disposal in the environment causes important drawbacks because of its high biochemical oxygen demand. Consequently, it is normally spray dried and used as low-value products, such as feed for animals, or food supplement.[1] Alternatively, it is processed by ultrafiltration to yield whey protein concentrate and whey permeate (WP), the latter being an inexpensive by-product comprising mainly lactose and salts. Unlike whey proteins that find immediate food applications, the WP has so far been of little value probably due to its high salt content.[2-3] Therefore, its profitable use constitutes a relevant activity from the economic and environmental point of view.

The use of WP to produce lactose derivatives including lactulose, lactitol, lactobionic acid, tagatose and sialyllactose has long been of industrial interest.[4-5] In the last few years, an increasing interest in the consumption of prebiotic carbohydrates has been observed so that the production of new bioactive oligosaccharides is currently garnering much attention for their potential use as functional ingredients.[6] Today one of the most promising uses of WP is the synthesis of prebiotic galactooligosaccharides (GOS) from transgalactosylation of lactose catalyzed by β-galactosidases (EC 3.2.1.23) of microbial origin.[7-10] Among them, β-galactosidase from *Bacillus circulans* has shown to have the ability to produce GOS with a good yield from model systems consisting of lactose in buffered solutions.[11-13] However, scarce studies dealing with the production of GOS from cheese WP using β-galactosidase of *B. circulans* have been carried out.[14] In this sense, it is noteworthy to indicate that substantial differences, in terms of yield and oligosaccharide composition, between the production of GOS from model systems consisting of lactose in buffered solutions and from WP could be expected due to the influence of other permeate ingredients, such as mineral salts.[15-18] Furthermore,

considering the relationship between the structure and prebiotic activity of oligosaccharides,[19-20] the synthesis of new lactulose-derived oligosaccharides have recently been reported in order to find new prebiotics with improved or complementary properties.[21-26]

The aim of this work was to develop a new approach based on the combined process of isomerization of lactose present in cheese WP using a food-grade catalyst (egg shell) and the subsequent enzymatic transgalactosylation with β-galactosidase from *B. circulans* avoiding intermediate purification steps of lactulose, and contributing to the improvement of the production of a range of potential bioactive oligosaccharides. In consequence, data reported in this work could help to broaden the use of cheese WP for the efficient production of functional carbohydrates.

MATERIALS AND METHODS

Chemical and reagents

Reagents employed for chromatography analysis, including standards (glucose, galactose, fructose, lactose, lactulose, raffinose, stachyose, and β-phenyl-glucoside) were obtained from Sigma (St. Louis, USA). Acetonitrile (HPLC grade) was purchased from Lab-scan (Gliwice, Poland). All other chemicals were of analytical grade. Ultrapure water quality (18.2 MΩcm), with 1–5 ppb TOC and <0.001 EU mL^{-1} of pyrogen levels was produced in-house, using a laboratory water purification Milli-Q Synthesis A10 system (Millipore, Billerica, Massachusetts, USA) and was used throughout.

β-galactosidase from *Bacillus circulans* (Neutral Lactase) was acquired from Biocon (Barcelona, Spain). Lactase activity was 3000 U mL^{-1}, where 1 unit is the amount of enzyme required to hydrolyze 1 μmol of lactose per minute at a working

temperature of 50 °C, and a lactose concentration of 300 g kg^{-1} at pH 6.0 with 0.05 mol L^{-1} of buffer phosphate.

Egg shell powder

White egg shells were washed with tap water to remove all adhering albumen, dried at 105 ºC for 24 h and ground in a ball mill (Mixer Mill MM 200, Retsch GmbH & Co. KG, Haan, Germany) at 800 rpm (13.3 Hz), for 15 min. Resulting egg shell powder had a particle size of approximately 5 μm, and was stored in glass vials in a dry place at room temperature prior to be used.

Physical-chemical characterization of cheese whey permeate

An industrial bovine cheese WP powder with a lactose content of 810 g kg^{-1} was kindly supplied by the dairy industry Reny Picot (Navia, Spain). The pH of reconstituted WP was measured using a pH meter (MP 230, Mettler-Toledo, Barcelona, Spain) at a concentration of 300 g kg^{-1}.

Isomerization reaction

The isomerization reaction was performed as previously reported by Montilla *et al.*[27] with some modifications. A permeate powder solution at a concentration of 300 g kg^{-1} lactose was prepared with Milli-Q water. Sample was stirred at 750 rpm, 60 ºC for 30 min, and then, it was cooled down at room temperature and the pH adjusted to 6.8 by adding 2 mol L^{-1} NaOH. Afterwards, 100 g of this sample was placed in a 250 mL round-bottom flask provided with an additional necked sampling inlet and 3 g of egg shell powder was added. The flask was immersed in a glycerol bath at 120 ºC, stirred at 300 rpm and refluxed at 98 ºC for 180 min. Boiling start (5 min) was considered as zero

time of reaction. Samples (30 mL) were taken at 0, 60, 90, 120, 150 and 180 min. Reaction was stopped by cooling down with an ice-water bath. Egg shell was removed by centrifugation at 5000*g* and 20 ºC for 10 min. Supernatant was collected, lyophilized, and stored at –18 ºC until further analysis. Isomerization reaction was carried out in duplicate and analyses were performed twice for each isomerization treatment.

Oligosaccharide synthesis

Enzymatic synthesis of oligosaccharides from isomerized whey permeate (IWP) using β-galactosidase from *B. circulans* was carried out under different reaction conditions such as pH (5.5, 6.5, and 7.4), temperature (40, 50, and 60 ºC), enzyme concentration (1.5, 3, and 6 U mL^{-1}), carbohydrate concentration (100, 300, and 500 g kg^{-1} reconstituted in milli-Q water), and time (1, 3, 5, 8 and 24 h). Reactions were performed at a final volume of 1.5 mL in microtubes incubated in an orbital shaker at 300 rpm. Aliquots (250 μL) were withdrawn from the reaction mixture at the different times and immediately immersed in boiling water for 5 min to inactivate the enzyme. Samples were stored at -18 ºC for subsequent analysis. Besides, another assay using WP or lactulose (300 g kg^{-1}) as substrate, at 50 ºC, pH 6.5 and enzyme concentration 3 U mL^{-1} was carried out. Enzymatic reactions were made in duplicate and analyses were performed twice for each enzymatic treatment.

Chromatographic determination of carbohydrates

GC analysis

Sample preparation

200 μL of sample was made up to 2 mL with water in a volumetric flask and was filtered using a 0.45 μm syringe filter (Symta, Madrid, Spain). 0.2 mg phenyl-β-D-

glucoside was added to 100 μL of filtrate as internal standard and the mixture was dried at 38–40 ºC in a rotary evaporator. These samples were analyzed by two different GC systems as described below.

Gas chromatography with FID detection (GC-FID)

The dried mixtures were treated with 100 μl of N-trimethylsilylimidazole to silylate the carbohydrates; the reaction was completed in 30 min at 70 ºC. Silylated carbohydrates were extracted with 0.3 mL of hexane and 0.3 mL of water. Volume of 1 μl of the organic phase containing silyl derivatives were injected into the column.

The trimethylsilyl ethers of carbohydrates were analyzed as has been previously described using an Agilent Technologies 7890A gas chromatograph equipped with a commercial fused silica capillary column SPB–17, bonded, crosslinked phase (50% diphenyl/50% dimethylsiloxane; 30 m × 0.32 mm inside diameter × 0.5 μm film) (Supelco, North Harrison Road, Bellefonte, PA, USA).[28] Separation was performed at 235 ºC for 9 min, followed by an increase of up to 280 ºC at a rate of 15 ºC min^{-1} and keeping this temperature for 30 min. Injector and detector temperatures were 280 ºC. Injections were carried out in split mode (1:30), using 1 mL min^{-1} of nitrogen as carrier gas. Data acquisition and integration were performed using Agilent Chem-Station Rev. B.03.01 software (Wilmington, DE).

To study the response factor relative to the internal standard, solutions containing glucose, galactose, lactose and lactulose were prepared over the expected concentration range in samples. The identities of carbohydrates were confirmed by comparison with relative retention times of standard samples. The amount of remaining lactose, lactulose, glucose and galactose in the isomerization and transgalactosylation mixtures were expressed as g kg^{-1}.

Gas chromatography-mass spectrometry (GC-MS)

Selected samples of isomerized and/or transgalactosylated permeate were also analyzed by GC-MS. An Agilent Technologies 7890A gas chromatograph coupled to a 5975C MSD quadrupole mass detector (Agilent Technologies, Wilmington, DE, USA) was employed. The trimethylsilyl oxime, prepared as described by Cardelle-Cobas *et al.*,[29] were separated using an HP-5 MS fused-silica capillary column (30m × 0.25 mm internal diameter × 0.25 μm film thickness) coated with 5% phenylmethylsilicone (J&W Scientific, CA, USA). The helium flow rate was 1 mL min^{-1}. The initial oven temperature was 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. Injections were made in the split mode (1:40). The mass spectrometer was operated in EI mode at 70 eV. Mass spectra were acquired using Agilent ChemStation MSD software (Wilmington, DE, USA).

Identification of trimethylsilyloximes derivatives of carbohydrates was carried out by comparison of their relative retention times and mass spectra with those of standard compounds previously derivatized.

Liquid chromatography with refraction index detector (HPLC-RID)

Samples of isomerized and transgalactosylated permeate were diluted with acetonitrile:water (50:50%, v:v), filtered using a 0.45 μm syringe filter (Symta), and analyzed on an Agilent Technologies 1260 series HPLC system (Boeblingen, Germany). The separation of carbohydrates was carried out with a Kromasil® column (100-NH_2; Akzo Nobel, Brewster, NY) (250 mm x 4.6 mm, 5 μm particle size) (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, 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 glucose (quantification of monosaccharides), lactose (disaccharides), raffinose (trisaccharides) and stachyose (tetrasaccharides). All analyses were performed in duplicate, obtaining relative standard deviation RSD values below 10% in all cases. Amount of different carbohydrates present in the reaction mixtures were expressed as weight percentage of the total carbohydrate content.

RESULTS AND DISCUSSION

Isomerization of whey permeate using egg shell as catalyst

In order to carry out the isomerization reaction, an industrial cheese WP was used and egg shell was chosen as catalyst instead of chemical reagents such as borates, sodium aluminate or hydroxides, due to its multiple advantages, i.e., lower quantity of required catalyst, easy removal of the egg shell by centrifugation or filtration as compared to homogeneous catalysts, lower formation of products derived from side-reactions, and relatively good yields of isomeric disaccharides. For that purpose, 3 g of egg shell were added to 100 g of reconstituted cheese WP (equivalent to 30 g of lactose) and the mixture was kept under reflux following the previous studies reported by Montilla *et al.*[27]. According to GC-FID analyses, an optimal production of lactulose was reached within 150 min of reaction. The carbohydrate composition of reaction mixture after isomerization was: galactose 7.0 g kg^{-1}, glucose 1.0 g kg^{-1}, *epi*-lactose 1.1 g kg^{-1}, lactulose 48.2 g kg^{-1} and lactose 209.1 g kg^{-1}, thus, a 16.1% of lactulose with respect to

the initial amount of lactose was obtained under the assayed conditions. Similar yield (18% of lactulose with respect to the initial amount of lactose) was obtained by Montilla *et al*.[27] using milk permeate concentrated.

Transgalactosylation of isomerized cheese whey permeate using β-galactosidase from *Bacillus circulans*

The effect of pH, temperature and enzyme concentration on formation of GOS was studied for initial carbohydrate concentration of 300 g kg^{-1}. The effect of substrate concentration, from 100 to 500 g kg^{-1}, was also assayed.

Effect of pH

Three values within the optimum pH range given by the manufacturer (i.e. pH 7.4, the value of permeate after the isomerization reaction, 6.5 and 5.5) were assayed at 50 ºC with 3 U mL^{-1} of β-galactosidase. Formation of GOS from IWP was monitored by HPLC-RID as it is shown in Fig. 1. As expected, the order of elution was according to the degree of polymerization of carbohydrates. Thus, monosaccharides eluted at 7-10 min, disaccharides at 11-20 min, trisaccharides around 22-33 min and tetrasaccharides above 33 min. Additionally, it cannot be ruled out the presence of pentasaccharides in minor amounts since the β-galactosidase from *B. circulans* has shown the capacity of producing pentasaccharides.[30] Glucose (Glc, peak 1), galactose (Gal, peak 2), lactulose (Lu, peak 3) and lactose (Lac, peak 5), were identified by comparison of their retention times from those of commercial standards; β-D-Gal*p*-(1→6)-Glu (allolactose), was identified by comparison with the standard previously isolated in our laboratory.[31] Formed disaccharides could tentatively be assigned to galactosyl-disaccharides with links β-(1→2), β-(1→3), according to previous studies on transgalactosylation of

lactose by β-galactosidase from *B. circulans.*[12] While the principal trisaccharide, β-D-Gal*p*-(1→4)-Lac (peak 6), was identified by comparison with the standard previously synthesized in our laboratory.[31]

Fig. 2 shows the time-course of β-galactosidase-catalyzed reaction at pH 5.5, 6.5 and 7.4. The lactose concentration quickly decreased from the start of the reaction to 24 h (Fig. 2a), being this decrease more slowed down at pH 7.4. Hydrolysis of lactose was very efficient (from 79% at the initial time to 17% after 24 h at pH values 6.5 and 5.5) and gave rise to the formation of glucose and a smaller quantity of galactose, regardless the studied pH value (Fig. 2b), which is indicative of the transfer of galactose to form GOS. Although the lactose hydrolysis rate was similar at pH 5.5 and 6.5, the amount of free galactose at pH 6.5 was lower and, consequently, the GOS formation (tri- and tetrasaccharides) was higher and faster than at pH 5.5 (Fig. 2c). Moreover, during the first five hours of reaction, the trisaccharides were the most abundant carbohydrates formed, followed by the disaccharides and the tetrasaccharides, respectively. Nevertheless, after 24 h of reaction, the disaccharides were the predominant saccharides formed, presumably due to the partial degradation of the tri- and tetrasaccharides, as well as to the continuous synthesis of disaccharides (Fig. 2c). Thus, the maximum formation of GOS, which led to a 40% of total sugars (w:w), was achieved after 5 h of reaction at pH 6.5, whilst it was needed 8 h of reaction to the maximum formation of GOS, i.e. 38% of total sugars (w:w), at pH 5.5 (Fig. 2a). Other studies on transgalactosylation of whey permeate solutions by β-galactosidase from *B. circulans* reported yields ranging from 12 to 31%.[15, 32]. Cheng *et al.*[3] using similar reaction conditions to the reported in this work, but with lactose solutions, obtained 34% of GOS yield. Other studies carried out with lactose solution and β-galactosidase from *B.*

circulans, but different reaction conditions, obtained considerable lower yields ranging from 6 to 26%,[9,11,12]

Moreover, lactulose concentration moderately decreased only during the first 3 h of reaction, (from 18% at 0 h to 10-11% at 3 h), and then remained fairly constant (10% at 24h), indicating that β-galactosidase from *B. circulans*, in the presence of both disaccharides, is prone to hydrolyze lactose instead of lactulose; the GC analyses of these samples confirmed the scarce presence of fructose (<0.5% at 8 h). Thereby, the slight decrease of lactulose could be mainly attributed to the formation of lactulose derived oligosaccharides. This fact was corroborated by comparing the GC-MS profiles of GOS obtained from WP and IWP treated with β-galactosidase of *B. circulans*, where an additional trisaccharide probably corresponding to a galactosyl-lactulose derivate was detected in the latter. This trisaccharide was also detected following transgalactosylation of purified lactulose with β-galactosidase of *B. circulans* (chromatogram not shown), and was identified by comparison with the standard β-D-Gal*p*-(1→4)-Lu also previously synthesized in our laboratory;[31] this compound also appeared in Fig.1 labeled as peak 7 and coeluted with β-D-Gal*p*-(1→4)-Lac. The MS spectrum of the main lactulose-derived trisaccharide was characterized by the following *m/z* ions in decreasing order of abundance: 204, 73, 361, 217, 205, 147, 191, 103, 129, 169, 321, 319, 271, 305, and 448. Whilst, the *m/z* ions from lactose-derived trisaccharide (β-D-Gal*p*-(1→4)-Lac) were: 204, 361, 73, 217, 205, 147, 191, 129, 103, 169, 271, 319, 451, 331, and 305. This means that ions *m/z* 321 and 448 were characteristic for lactulose-derived trisaccharide, while ions *m/z* 451 and 331 were for β-D-Gal*p*-(1→4)-Lac.

Effect of temperature

In addition to 50 ºC, reactions at 40 and 60 ºC, pH 6.5 with 3 U mL^{-1} of β-galactosidase were carried out. Lactose hydrolysis was accelerated at 50 and 60 ºC in comparison to 40 ºC (Fig. 3a), which is in concordance with the higher levels of glucose detected throughout the reaction at 50 and 60 ºC (Fig. 3b). Nevertheless, the levels of galactose were higher at 40 ºC than at 50 and 60 ºC (Fig. 3b), which is in good agreement with the fact that the formation of total GOS was higher and faster (maximum formation at 5 h) at 50 ºC (39.5 ± 1.5%) and 60 ºC (37.5 ± 2.0%) than at 40 ºC (35.6 ± 2.0%) where the maximum levels of GOS were obtained after 8 h of reaction (Fig. 3a). In consequence, the commercial enzyme used in these assays seems to be more thermo-resistant than that used by Boon *et al.*[30], who observed an inactivation on lactose hydrolysis after 90 min at 60 ºC.

In addition, the different degrees of polymerization of GOS were also studied as it is shown in Fig. 3c. Similar amounts of disaccharides were formed at the end of the reaction carried out at 50 and 60 ºC (24-25% of total sugars, w:w respectively), whilst 19% of disaccharides were found at 40 ºC. The maximum levels of trisaccharides were reached after 3 h of reaction at the three assayed temperatures, and then, a gradual decrease with time was observed. A maximum of 21-22% of trisaccharides were quantified at 40º and 50 ºC, whilst only 15% were found at 60 ºC. Similar quantities of tetrasaccharides were obtained for the three temperatures (3-4% of total sugars), although the maximum levels were achieved faster when the reaction was carried at 50 ºC and 60 ºC (3 h) than at 40 ºC (5 h).

Although similar levels of total GOS were obtained at 50 ºC and 60 ºC (Fig. 3a), the temperature selected for the following analyses was 50 ºC because higher amounts of tri- and tetrasaccharides were obtained. Mozaffar *et al.*[11] reported an optimum

temperature of 60 ºC for two isoforms of β-galactosidase from *B. circulans*, although these authors provided data of total GOS and no differentiation of degree of polymerization was carried out.

Effect of enzyme and substrate concentration

To determine the effect of the enzyme concentration on GOS production, in addition to 3 U mL^{-1} of β-galactosidase, 1.5 or 6 U mL^{-1} were also assayed at 50 ºC, pH 6.5 and 300 g kg^{-1} of carbohydrates. Fig. 4a illustrates the remaining lactose content during the time course of reaction. Results showed that the lowest assayed concentration of enzyme (1.5 U mL^{-1}) led to the lowest hydrolysis of lactose (27% of remaining lactose after 24 h of reaction) and the subsequent lowest formation of monosaccharides (Fig. 4b). However, no differences on the lactose hydrolysis rate were found between 3 and 6 U mL^{-1} of enzyme. Fig. 4c shows di, tri and tetrasaccharides yields throughout the enzymatic reaction. Although the highest amount of formed trisaccharides was similar for all enzyme concentration assayed (20-21%), the lowest trisaccharides formation rate was observed for 1.5 U mL^{-1} enzyme concentration. Moreover, the hydrolysis rate of oligosaccharides increased with the enzyme concentration assayed. Formation of disaccharides constantly increased with the reaction time for the three enzyme concentrations assayed. The highest disaccharide content (27%) was found after 24 h when the synthesis was performed with 6 U mL^{-1} of enzyme. Generally, in enzyme-catalyzed reactions, the reaction rate is directly proportional to the enzyme concentration until a certain amount which loses that proportionality. The same effect was obtained by Das *et al.*[14] who reported that beyond the dose of 0.5% of a β-galactosidase from *B. circulans*, no further effect on GOS yield was observed. The same effect was observed by other authors for different enzymes and substrated[33,34] In our

assays, since the yields differences between the reactions with 3 and 6 U mL^{-1} of enzyme were negligible, the minor amount of enzyme was chosen to reduce the cost of operation.

The last factor studied was the initial concentration of substrate; reactions with 100, 300 and 500 g kg^{-1} of carbohydrates at 50 ºC, pH 6.5, with 3 U mL^{-1} of enzyme were carried out. At the lowest substrate concentration, the reaction was too fast, and after 5 h of reaction the remaining lactose was 14%. In this condition, the highest amount of trisaccharides formed was 14% after 1 h of reaction, to be then quickly hydrolyzed. Nevertheless, when the highest concentration of carbohydrates (500 g kg^{-1}) was used, the lactose hardly was hydrolyzed after 24 h of reaction. In assays performed with 6 U mL^{-1} and 500 g kg^{-1} of enzyme and carbohydrate concentration, respectively, no improvement in GOS yields were obtained as compared to those reported by using 300 g kg^{-1} of starting carbohydrate (data not shown).

CONCLUSIONS

To summarize and according to the obtained results, the maximum formation of GOS was achieved after 5 h of reaction carried out at pH 6.5 and 50 ºC with 300 g kg^{-1} of carbohydrates and 3 U mL^{-1} of β-galactosidase, giving rise to 24% monosaccharides, 25% lactose, 11% lactulose, and 40% GOS with DP 2-4 (and whose distribution was 16% formed disaccharides, 21% trisaccharides and 3% tetrasaccharides). These results highlight the formation of oligosaccharides with a different structure and, thus, with potentially different prebiotic properties.

Several papers have demonstrated that glycosidic linkages, monosaccharide composition and degree of polymerization of GOS contribute toward the selectivity of fermentation by beneficial bacteria.[19-20] In this context, the production of a mixture of

prebiotics with a wide diversity of structural features might provide a value-added functional ingredient since it could broaden its positive effects on the modulation of gut microbiota. Likewise, the presence of lactulose, in addition to GOS, could provide an additional value to the final product since lactulose has shown to exert a series of biological activities, such as prebiotic action[35], improvement of the intestinal transit time[36], as well as other beneficial physiological actions, such as the treatment of chronic constipation, hepatic encephalopathy, or inflammatory bowel disease.[37]

In conclusion, our results could contribute to the diversification of synthesized oligosaccharides, indicating that a novel approach, based on the combined process of isomerization of lactose from cheese WP using a food-grade catalyst (egg shell) and, subsequent, enzymatic transgalactosylation with β-galactosidase from *B. circulans*, was useful to produce a mixture composed of a 50% of potentially prebiotic carbohydrates formed by lactulose, and GOS derived from lactose and lactulose. Both type of GOS have proven to be an excellent alternative to monosaccharides to support growth of probiotic and improve their survival through the gastrointestinal tract.[26]

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Figure 1. HPLC-RID profile of carbohydrate mixture obtained by transgalactosylation reaction of isomerizated cheese whey permeate at pH 6.5, 50 ºC for 5 h with β-galactosidase from *B. circulans* (3 U ml^{-1}) and initial carbohydrate concentration of 300 mg mL^{-1}. Identified peaks: glucose (Glc) (1); galactose (Gal) (2); lactulose (Lu) (3); lactose (Lac) (4), β-D-Gal*p*-(1→6)-D-Glc (allolactose) (5); β-D-Gal*p*-(1→4)-Lac (6); β-D-Gal*p*-(1→4)-Lu (7).

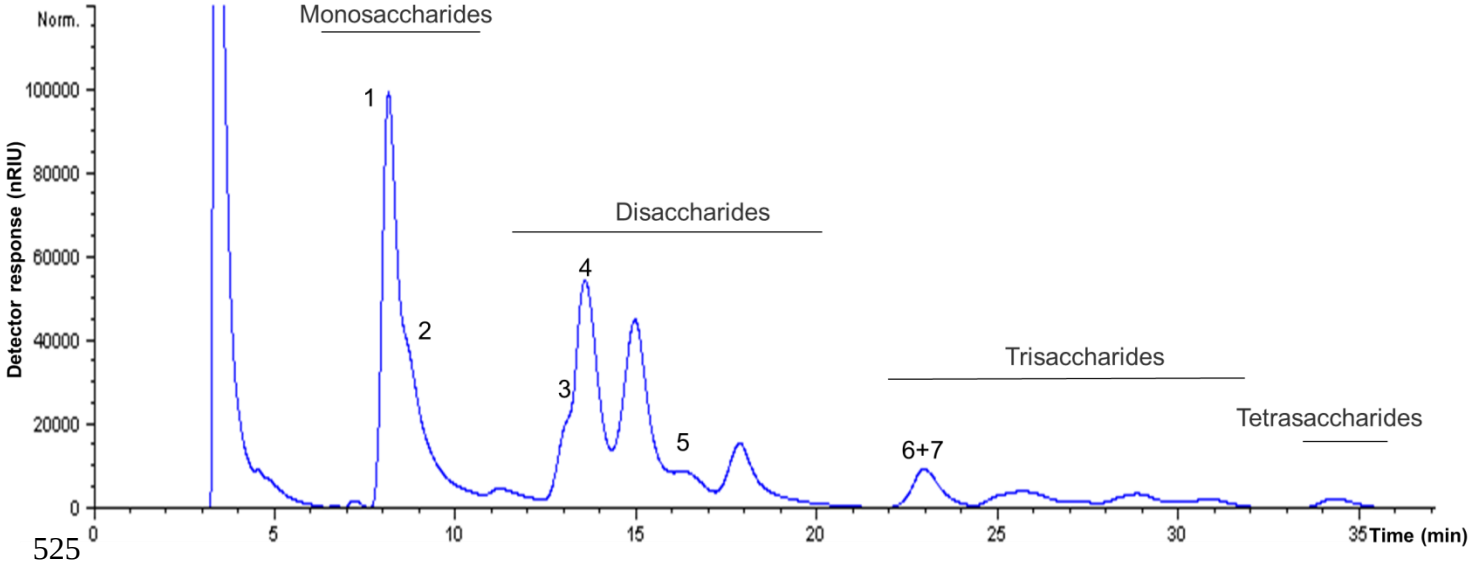

Figure 2. Effect of pH on hydrolysis of isomerized cheese whey permeate (300 mg mL^{-1} of carbohydrates) and oligosaccharide production during the enzymatic treatment with β-galactosidase from *Bacillus circulans* (3 U mL^{-1}) at 50ºC and pH (X) 7.4; (■) 6.5; (▲) 5.5. Vertical bars represent standard deviations (n = 4).

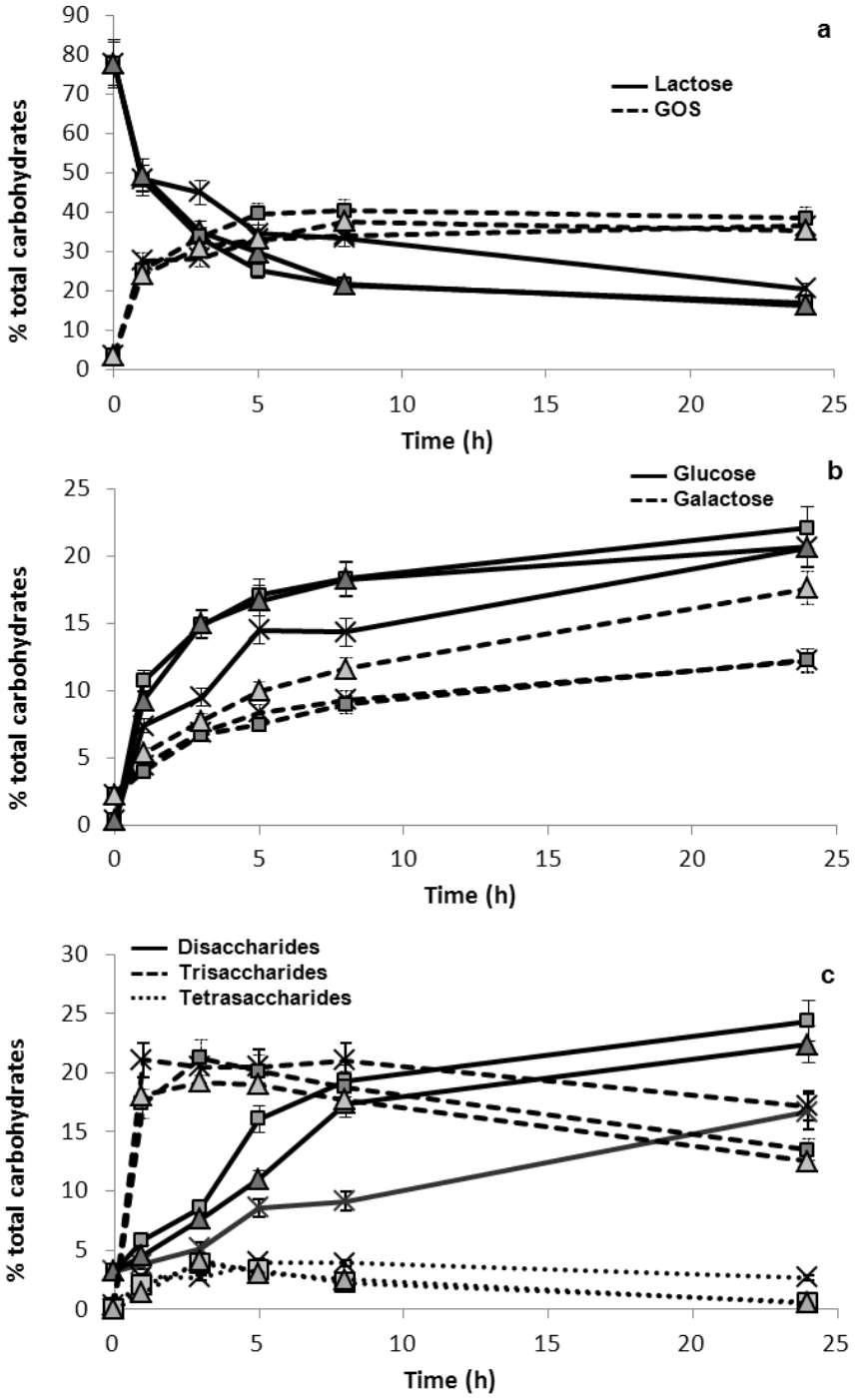

Figure 3. Effect of temperature on hydrolysis of isomerized cheese whey permeate (300 mg mL^{-1} of carbohydrates) and oligosaccharide production during the enzymatic treatment with β-galactosidase from *Bacillus circulans* (3 U mL^{-1}) at pH 6.5 and (X) 40 ºC; (■) 50 ºC; (▲) 60 ºC. Vertical bars represent standard deviations (n = 4).

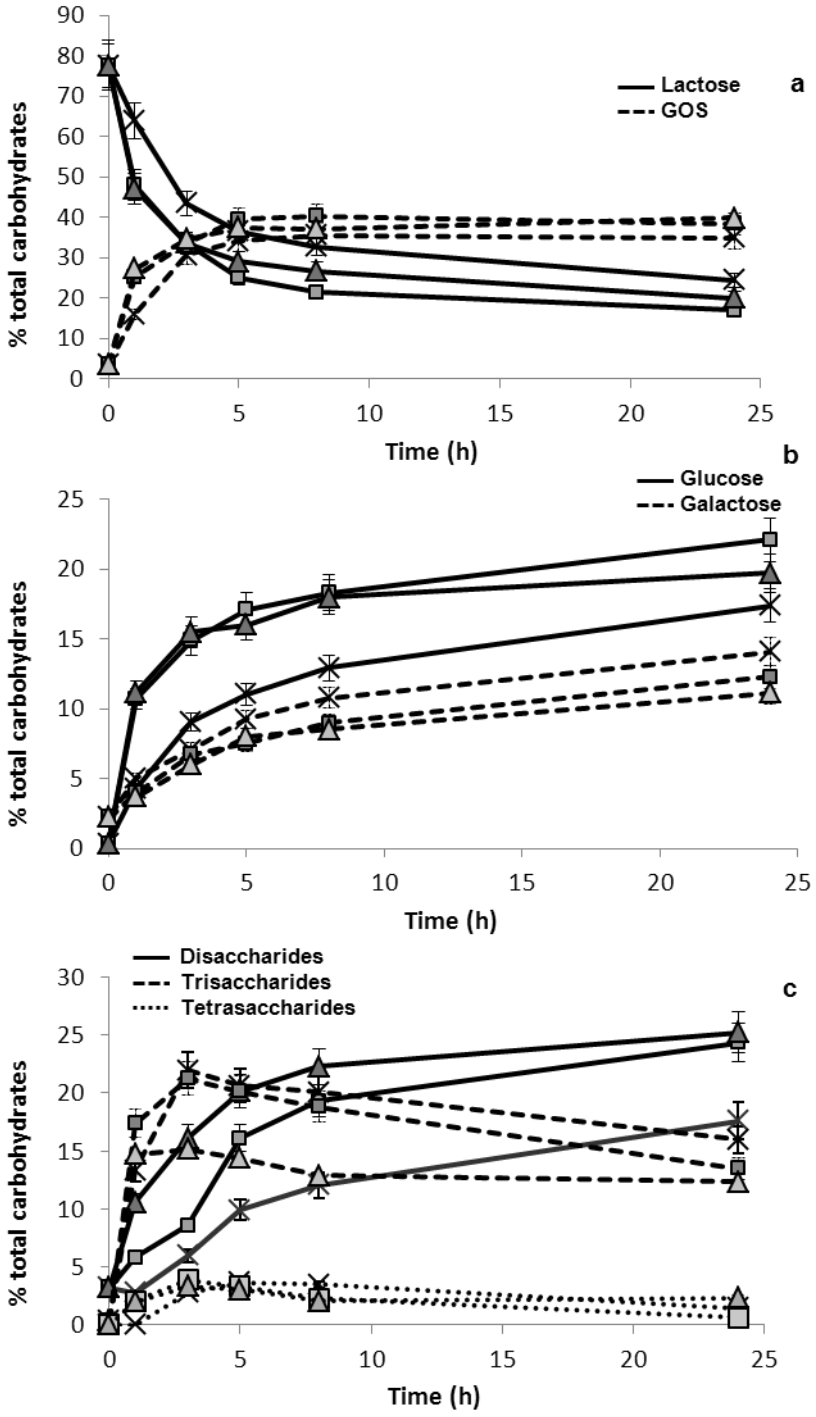

Figure 4. Effect of enzyme concentration on hydrolysis of isomerized cheese whey permeate (300 mg mL^{-1} of carbohydrates) and oligosaccharide production during the enzymatic treatment with β galactosidase from *Bacillus circulans* (X) 1.5 U mL^{-1}; (■) 3 U mL^{-1}; (▲) 6 U mL^{-1} at 50ºC pH 6.5. Vertical bars represent standard deviations (*n* = 4).

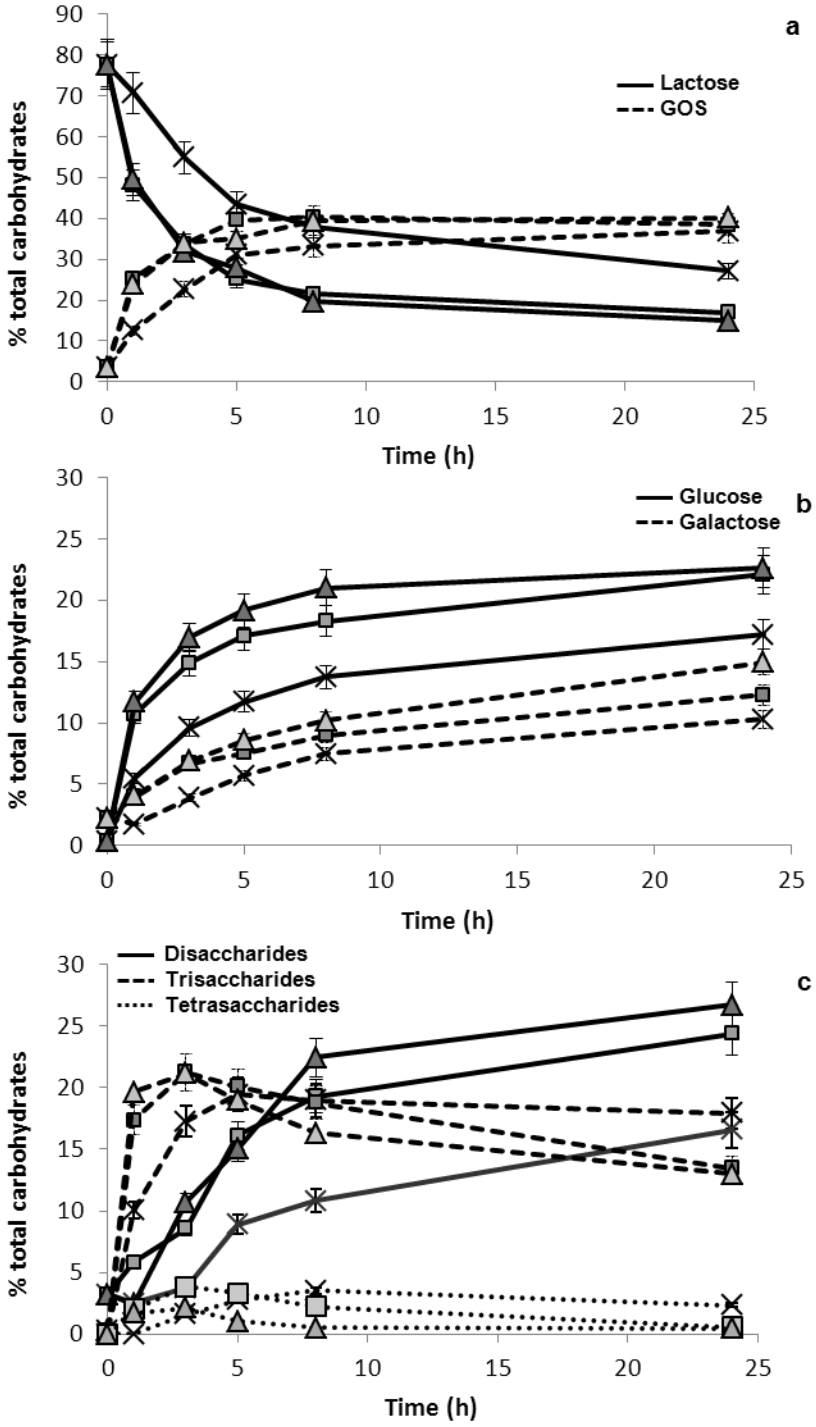

Table 1. Content of galactose, glucose, *epi*-lactose, lactulose and lactose (mean ± standard deviation, *n*=4) produced during heating at reflux of permeate powder solutions at a concentration of 300 g kg^{-1}, pH 6.8, and 30 g kg^{-1} of egg shell powder.

Time (min)	Galactose g kg^{-1}	Glucose g kg^{-1}	Epi-lactose g kg^{-1}	Lactulose g kg^{-1}	Lactose g kg^{-1}
0	0.8 ± 0.2	1.2 ± 0.2	n.d.*	n.d.	293.5 ± 5.1
60	3.0 ± 0.2	1.1 ± 0.1	0.4 ± 0.1	22.4 ± 0.1	261.1 ± 4.6
90	4.5 ± 0.5	1.0 ± 0.1	0.8 ± 0.1	32.0 ± 1.4	238.8 ± 8.0
120	6.9 ± 0.3	1.2 ± 0.1	1.0 ± 0.1	38.3 ± 0.1	232.5 ± 5.8
150	7.0 ± 0.2	1.0 ± 0.0	1.1 ± 0.0	48.2 ± 0.3	209.1 ± 1.9
180	10.2 ± 0.2	1.0 ± 0.0	1.4 ± 0.0	43.9 ± 1.8	200.6 ± 3.8

*n.d. No detected

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