**SELECTIVE BIOTECHNOLOGICAL FRACTIONATION OF GOAT MILK
CARBOHYDRATES**

Andrea Martín-Ortiz¹, Francisco Javier Moreno², Ana Isabel Ruiz-Matute^{1*}, María Luz Sanz¹

¹Instituto de Química Orgánica General (CSIC). Juan de la Cierva, 3 28006 Madrid, (Spain)

²Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), C/ Nicolás Cabrera, 9, Campus de Cantoblanco - Universidad Autónoma de Madrid, 28049 Madrid, Spain

*Corresponding author:

Email: ana.ruiz@csic.es

Abstract

Goat colostrum is a rich source of carbohydrates, mainly constituted by lactose, although several minor bioactive oligosaccharides are also present. Analysis of these caprine milk oligosaccharides (COS) is not straightforward, and usually requires a previous fractionation step to remove lactose. In this work, a biotechnological fractionation methodology based on the use of a β -galactosidase from *Kluyveromyces lactis* has been optimized (pH, incubation time, goat milk:enzyme volume ratio) to hydrolyze lactose, preserving the COS profile. Best results were obtained after 15 min of enzymatic treatment using 0.68 U.mL^{-1} of enzyme at 37°C and pH 7. Efficient removal of resulting monosaccharides was finally carried out by the incubation of these samples with *Saccharomyces cerevisiae* (37°C , 24 h). Fractionation of these carbohydrates could help to better determine COS structures and to expand the applications of the purified COS in the food and pharmaceutical industries.

Keywords: goat milk oligosaccharides, fractionation, lactose, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*

1. Introduction

Currently, goat milk is the world's third most produced variety of milk (FAOStat, 2018). Its consumption is gaining great relevance considering the numerous beneficial properties associated to its constituents (Verruck, Dantas & Prudencio, 2019), such as anti-inflammatory effects (Daddaoua, et al., 2006), prebiotic activity (Oliveira, Wilbey, Grandison, Duarte & Roseiro, 2012) or expression and development of brain and central nervous system functions (Mehra & Kelly, 2006), among others. Apart from lipids and proteins, goat milk is a rich source of carbohydrates, being lactose (galactosyl- β -D-(1 $\rightarrow$ 4)-glucose) the most abundant one (around 44 g L⁻¹) (Martínez-Ferez et al., 2006). Moreover, several neutral and acidic minor oligosaccharides (OS), some of them similar to those found in human milk, are present (Meyrand et al., 2013).

As previously reported, caprine milk oligosaccharides (COS) have complex structures with different glycosidic linkages and degrees of polymerization (between 2 and 7) (Meyrand et al., 2013; Martín-Ortiz et al., 2016). Monomeric units of glucose (Glc), galactose (Gal), *N*-acetylglucosamine or *N*-acetylgalactosamine (HexNAc) and fucose (Fuc) linked to a lactose core make up neutral COS, whereas these monomers plus *N*-acetylneuraminic (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) form acidic OS. These OS are found in goat milk at higher concentrations than in milks from other farm mammals (Martínez-Ferez et al., 2006), especially in goat colostrum, in which they are more abundant (Martín-Ortiz et al., 2017; Mehra & Kelly, 2006). Different bioactive properties have been recently attributed to COS, such as potential prebiotic activity (Oliveira, Wilbey, Grandison, Duarte & Roseiro, 2012), reduction of intestinal inflammation (Lara-Villoslada et al., 2006), and improvement of barrier function of epithelial cell co-cultures (Barnett, Roy, McNabb & Cookson, 2016). Therefore, conveniently isolated COS could be a potentially promising functional food ingredient

for improving intestinal health which could be used in different foodstuffs (e.g., infant milks).

Considering the high concentrations of lactose and the similar structure of the minor OS, the isolation of COS is challenging and not straightforward. Several methods for the selective fractionation of these carbohydrates have been proposed mainly based on the removal of monosaccharides by membrane-based techniques (Martínez-Ferez et al., 2009; Martínez-Ferez, Guadix, Zapata-Montoya & Guadix, 2008) or on the use of size exclusion chromatography (SEC) to fractionate OS according to their degree of polymerization (Martín-Ortiz et al., 2017; Martín-Ortiz et al., 2016). Among these techniques, SEC provides relatively good yields of OS (81-92%) with high degree of purity, although it has several disadvantages such as it is a time-consuming technique, the permeation gel used is expensive and the sample obtained is relatively diluted (Hernández, Ruiz-Matute, Olano, Moreno & Sanz (2009).

The use of microbiological treatments for carbohydrate fractionation has also been proposed. Among them, the use of *Saccharomyces cerevisiae* has been proposed as a clean and easily scalable biotechnological procedure to be applied for the removal of interfering low molecular weight carbohydrates (LMWC) from inositols-enriched legume extracts (Ruiz-Aceituno et al., 2013), honeys (Ruiz-Matute et al, 2007; Sanz, González, Lorenzo, Sanz & Martínez-Castro, 2005) and prebiotic oligosaccharide mixtures such as galactooligosaccharides (GOS) (Hernández-Hernández, Calvillo, Lebrón-Aguilar, Moreno & Sanz, 2011; Goulas, Tzortis & Gibson, 2007) and fructooligosaccharides (Nobre et al., 2016). However, lactose is not metabolized by this particular yeast (Yoon, Mukerjea & Robyt, 2003); therefore, it has not directly been applied to milk samples. On the contrary, *Kluyveromyces lactis* has been traditionally used as industrial yeast in dairy products, as it is generally recognized as a safe (GRAS)

microorganism and has a good growth yield and a higher β -galactosidase activity as compared to other yeasts (Zolnere & Ciprovica, 2017). The employment of enzymes as β -galactosidases (EC 3.2.1.23) is an attractive alternative to the carbohydrate fractionation and different applications of commercial β -galactosidases [e.g. Maxilact® (DSM, Netherlands), HA-lactase® (Christian-Hansen, Denmark)] have been efficient for the elaboration of some foods free of lactose such as ice-creams, milk drinks or dairy preparations designed for elderly people (Ruiz-Matute et al., 2012; Montilla et al., 2015). Remarkably, there have been efforts to transfer genes from *K. lactis* to *S. cerevisiae* to generate genetically modified yeast strain(s) with the ability to consume lactose (Rubio-Teixeira et al., 2000). Nevertheless, most of those strains exhibited unsuitable characteristics, such as genetic instability of the Lac phenotype or diauxic growth. In this context, the approach based on the combination of a β -galactosidase enzyme exerting high hydrolytic activity with a yeast strain efficient in removing released monosaccharides could be a successful strategy for the fractionation of carbohydrate mixtures which are vastly dominated by lactose. De Moura Bell et al. (2016) have proposed the use of a β -galactosidase from *A. oryzae* to hydrolyse the lactose from bovine colostrum whey permeate and improve the selectivity and efficiency of membrane processes in the recovery of bioactive oligosaccharides. However, to the best of our knowledge, applications of microbiological treatments to the removal of LMWC from goat milks are scarce. Some studies have evaluated the use of commercial β -galactosidases (from *Kluyveromyces lactis* and/or from *Aspergillus oryzae*) to promote the formation of new GOS (allolactose, 6'galactobiose and 6'galactosyl-lactose) in reconstituted goat milk, however, the content and preservation of COS was not evaluated and these studies were not carried out for fractionation purposes (Zhu, Prosser, Zhu, Otter & Hemar, 2018; Pruksasri & Supee, 2013). Only

Aquino et al. (2017) have recently proposed a novel approach based on the use of a β -galactosidase from *A. oryzae*, followed by the treatment with *S. cerevisiae* and nanofiltration, for the purification at a large scale of oligosaccharides from goat milk. However, during these treatments special care should be taken with the degradation of the bioactive OS and the occurrence of transgalactosylation reactions, which can give to new OS of different composition. Aquino et al. (2017) stated that the β -galactosidase from *A. oryzae* did not degrade the 3'sialyllactose, 6'sialyllactose and 6'sialyllactosamine, as previously assayed (De Moura Bell et al., 2016); however, the evolution of other OS and the potential production of new OS was not evaluated. As far as we know, the use of these treatments for the removal of lactose from goat colostrum, has not yet been carried out.

Therefore, in this work, a biotechnological procedure using a β -galactosidase from *K. lactis* (Lactozym[®] pure 6500L) has been optimized to hydrolyze lactose from a pooled goat colostrum sample, keeping COS and minimizing the formation of other OS by transgalactosylation reactions. The approach was completed by the use of *S. cerevisiae* to remove the released galactose and glucose.

2. Materials and methods

2.1. Goat colostrum samples

For this study, colostrum from twelve individual Murciano-Granadina goats reared at Hermanos Archiduque farm (Granada, Spain) were kindly provided by Dr. A. Clemente (EEZ, CSIC; Spain). Samples were collected, pooled, aliquoted and immediately frozen at -80 °C until further analysis. Animals were cared and handled in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 on the protection of animals used for experimentation or other scientific purposes) in line of

corresponding European Directive (2010/63/EU). An experimental protocol was approved by the Ethics Committee for Animal Research from the Animal Nutrition Unit.

2.2. Enzyme and yeast

A commercial preparation β -galactosidase from *K. lactis* (Lactozym[®] Pure 6500L HP G) was kindly supplied by Novozymes (Bagsvaerd, Denmark). The β -galactosidase activity as determined by the manufacturer was 6,500 LAU.g⁻¹.

S. cerevisiae type II from Sigma Chemical Co. (St. Louis, MO, US) was activated with 1.5 mL of Milli-Q water at the selected incubation temperature (37°C) for 30 min under stirring (160 rpm).

2.3 Carbohydrate standards

Glucose, lactose and 4'-galactosyl-lactose were acquired from Sigma-Aldrich. Galactooligosaccharides (GOS) synthesized according to Martínez-Villaluenga, Cardelle-Cobas, Corzo, Olano & Villamiel, (2008) by using Lactozym 3000L (Novozymes) were used as reference.

2.4. Fat and protein removal

Fat and proteins were removed from the samples following the methodology described by Martínez-Ferez, Guadix & Guadix, (2006) with small modifications. Briefly, samples were defatted by centrifugation at 6500 $\times$ g for 15 min at 5 °C, then kept in an ice bath for 30 min and filtrated through Whatman N° 1 filter paper to remove the supernatant lipid layer, which was discarded.

The total protein fraction was precipitated by adding two volumes of cold ethanol to the skimmed colostrum samples and shaking for 2 h in an ice bath. The solution was then

centrifuged at $6500 \times g$ for 30 min at 5 °C and supernatant was carefully collected. Ethanol was evaporated from the sample in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at 37 °C and the remaining aqueous solution containing the carbohydrate fraction was frozen and lyophilized.

2.5. Enzymatic treatment

2.5.1. Effect of pH

To select the optimal conditions for lactose hydrolysis, 350 mg of lactose were dissolved in 5 mL of different buffers: (i) 0.1 M sodium acetate at pH 4.5, (ii) 0.1 M sodium phosphate at pH 7 and (iii) 0.1 M tris-(hydroxymethyl)-aminomethane-HCl (Tris-HCl) at pH 8. These solutions (995 µL) were mixed with 5 µL, corresponding to 0.34 U.mL⁻¹, of β-galactosidase and incubated at 37 °C for 1 h under constant stirring using a Thermomixer at 600 rpm. Samples were taken at 0 and 1h. After these times, the reaction was stopped at 100°C for 1 min and samples were immediately cooled in ice.

2.5.2. Effect of incubation time and enzyme concentration

Colostrum carbohydrate fraction (375 mg) and lactose (350 mg) were independently dissolved in 5 mL of phosphate buffer at pH 7. These amounts were chosen according to previous assays which indicated that this lactose concentration was high enough to increase the cost-efficiency of the hydrolysis process without saturating the enzyme β-galactosidase (Diez-Municio et al., 2014). Of these solutions 985-997 µL were mixed with enzyme (3, 5, 10 and 15 µL, corresponding to 0.20, 0.34, 0.68 and 1.02 U.mL⁻¹, respectively) to get a final volume of 1 mL. Incubations were carried out at 37 °C and under constant stirring. Aliquots of 100 µL were taken at 0, 1, 3, 5 and 8 h and the reaction was stopped as indicated before. Evolution of OS during enzymatic treatment was also studied at shorter incubation times using 5, 10 and 15 µL of enzyme (0.34,

0.68 and 1.02 U.mL⁻¹), taking aliquots at 0, 15, 30 and 45 min. All samples were kept at -18°C for further analyses. All assays were carried out in triplicate.

2.6. Yeast treatment

Yeast treatment was carried out as described by Ruiz-Aceituno et al., (2013). Goat colostrum and lactose control samples, obtained after incubation with β -galactosidase (0.68 U.mL⁻¹) for 15 min, were treated with 1% (w/v) *S. cerevisiae* (Sigma-Aldrich) at 37 °C under stirring for 30 h. All assays were done in triplicate. Aliquots were taken just before yeast addition (time 0) and at 10 min, 2, 4, 6, 24 and 30 h of treatment. Then, they were centrifuged at 4,400 × g at 10 °C for 10 min and filtered through Whatman No. 4 filters to remove yeast, and kept at -20 °C until analysis.

2.7. Analysis by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Prior to HPAEC-PAD lactose determination, samples were diluted 1/20 (v:v) using Milli-Q water. For OS analyses, samples were analysed without dilution. A ICS-2500 Dionex system (Dionex Corp., Sunnyvale, CA, USA) consisting of a GP50 gradient pump, an automatic injector and an ED50 electrochemical detector with a gold working electrode and a Ag/AgCl reference electrode was used. Separation of carbohydrates was carried out on a CarboPac PA 1 guard column (50 x 4 mm) and a CarboPac PA-1 anion-exchange column (250x 4 mm) (Dionex Corp.). Different linear gradients using 150 mM sodium hydroxide (VWR, Barcelona, Spain) as solvent A and 50 mM sodium acetate (Merck, Madrid, Spain) in 150 mM sodium hydroxide as solvent B were tested. Optimal conditions for lactose analysis (ramp 1) were as follows: Eluent B was kept at 5 % for 1 min, changed to 10 % in 1 min, kept at these conditions for 3 min, changed to 40% in 1 min and kept constant for 5 min. After each run, the column was washed with

100% of eluent C (150 mM sodium hydroxide and 1 M sodium acetate) and re-equilibrated for 10 min at initial conditions; 20 μ L of testing samples were injected. Optimal chromatographic conditions for the analysis of OS (ramp 2) were: Eluent B was kept at 5 % for 1 min, changed to 10 % in 1 min, kept at these conditions for 18 min, changed to 40% in 1 min and kept constant for 5 min. After each run, the column was washed with 100% of eluent C (150 mM sodium hydroxide and 1 M sodium acetate) and then, re-equilibrated for 10 min at initial conditions; several injection volumes (between 50 and 100 μ L) of testing samples were assayed.

All eluents were degassed by flushing helium. In both cases, the flow rate was 1.0 mL min^{-1} and the column temperature 25 $^{\circ}\text{C}$. Carbohydrates were detected using triple pulsed amperometry with the following potentials and durations: E1= +0.15 V (t_1 = 400 ms), E2= +0.75 V (t_2 = 200 ms), E3= 0.6 V and E4= -0.8 V (t_3 = 200 ms). Acquisition and processing of data were carried out using the Chromeleon software version 6.7 (Dionex Corp., Sunnyvale, CA).

Quantitative analysis of monosaccharides (glucose+galactose), disaccharides (lactose, allolactose and trehalose) and oligosaccharides (GOS and COS) in colostrum samples was performed in triplicate using external standard calibration curves of glucose, lactose and 4'-galactosyl-lactose, respectively, within the range 0.001–2.0 mg.mL^{-1} . Calibration curves were: glucose, $y=638.43x+2.9128$ ($R^2=0.9932$); lactose, $y=348.05x+10.78$ ($R^2=0.9874$); 4'-galactosyl-lactose, $y=232.86x-0.1037$ ($R^2=0.9970$). Results were expressed in mg.g^{-1} of defatted and deproteinized colostrum sample.

2.8. Statistical analysis

For statistical analysis the Univariate analysis of variance (ANOVA) and Tukey test (SPSS software) were used. Differences were considered to be significant at $P < 0.05$.

3. Results and Discussion

3.1. Analysis of goat colostrum

Lactose and oligosaccharides from pooled goat colostrum could not be analyzed in a single run, considering the differences in their respective concentrations. Therefore, two chromatographic methods (different dilutions of the samples, injection volumes and chromatographic conditions) were used as indicated in the Materials and Methods section. Figure 1A shows the HPAEC-PAD profile of the carbohydrate fraction of pooled goat colostrum eluted using ramp 2 and 75 μ L of injection volume. Under these chromatographic conditions, glucose + galactose (peak 1) and lactose (peak 2) were separated from COS (peaks 3 and 4), allowing the quantitation of these minor carbohydrates. All detected peaks were satisfactorily resolved with the exception of a coelution between lactose and a peak eluting at 3.8 min (labelled as 3a). By comparing this chromatographic profile with that of the reference GOS (obtained under conditions previously reported by Martínez-Villaluenga, Cardelle-Cobas, Corzo, Olano & Villamiel, 2008) and with those thoroughly described in the literature (Yin et al., 2017; Rodríguez-Colinas et al., 2011), this peak was tentatively assigned as 6'galactosyl-lactose (β -D-galactosyl- β -D-(1 $\rightarrow$ 6)-lactose). Unfortunately, this carbohydrate could not be quantified without interferences at goat colostrum. Presence of 6'galactosyl-lactose had been previously detected in goat milks at low concentrations (Meyrand et al, 2013). Other peaks eluting between 5 and 7.5 min (peaks 3) were identified as trisaccharides, constituted by galactose and glucose units by comparison with the reference GOS sample and according to those reported in the literature (Zolnere & Ciprovica, 2017; Cardelle-Cobas et al., 2009). Considering that 3'galactosyl-lactose is the most abundant trisaccharide previously detected in goat milks (Meyrand et al., 2013, Mehra & Kelly, 2006), peak 3b was tentatively assigned to this trisaccharide. These carbohydrates

(peaks 3) were identified in this work as COSg. Peaks eluting between 8.8 and 11 min and between 16 and 21 min, only present in pooled goat colostrum, were assigned as COSc (peaks 4). A lactose standard solution (control) was also analyzed under these conditions for comparative purposes (Figure 1B).

3.2. Effect of pH conditions on lactose hydrolysis

Table 1 shows the percentages of resulting carbohydrates from the hydrolysis of lactose standard (75 mg mL⁻¹) by the action of β -galactosidase from *K. lactis* (0.34 U mL⁻¹) for 1 h at 37 °C using different buffer solutions (pH 4.5, 7 and 8). While lactose could hardly be hydrolyzed at pH 4.5 and pH 8 (98.2% and 94.1% of lactose remaining), this reaction was successful at pH 7 (1.6% of lactose remaining). Minor amounts of different GOS were produced under these conditions. Therefore, 0.1 M sodium phosphate at pH 7 was selected for further studies. Martínez-Villaluenga, Cardelle-Cobas, Corzo, Olano & Villamiel, (2008) found that inactivation of β -galactosidase from *K. lactis* was at pH 5.5 and the optimal pH conditions for the hydrolysis of lactose were between 6.5 and 7.5. However, these conditions were also optimal for the production of GOS. Then, as one of the aims of this work was to minimize GOS production, evaluation of incubation time and enzyme concentration was further carried out.

3.3. Effect of incubation time and enzyme concentration on lactose hydrolysis

Once the pH conditions were chosen, the effect of incubation time and enzyme concentration was evaluated to select the most appropriate conditions to maximize lactose hydrolysis. Figures 1C and 1D show the HPAEC-PAD profiles of the goat colostrum and the lactose standard (control), respectively, after incubation with 0.68 U.mL⁻¹ of β -galactosidase (*K. lactis*) for 45 min. As it can be observed, and comparing

with Figures 1A and 1B, lactose (peak 2) experimented a markedly decrease in these samples, while glucose + galactose (peak 1) noticeably increased.

Figure 2 shows the changes of lactose content (%) experimented in both goat colostrum (Figure 2A) and lactose control sample (Figure 2B) during enzymatic treatment using different β -galactosidase concentrations. While a concentration of 0.20 U.mL^{-1} of enzyme was not enough to hydrolyze the lactose present in the control and in the goat colostrum, even after 8 h of treatment (54% and 45% lactose remaining, respectively), better results were obtained using 0.34, 0.68 and 1.02 U.mL^{-1} of enzyme, respectively. Under these conditions, concentrations of lactose lower than 5% after 1 h of β -galactosidase treatment were detected.

The efficiency of the lactose hydrolysis was then evaluated in both goat colostrum and lactose control samples at shorter reaction times (15, 30 and 45 min) using 0.34, 0.68 and 1.02 U.mL^{-1} of enzyme, respectively (Figure 3). While only 15 min of incubation were enough to remove more than 95% of lactose using 0.68 and 1.02 U.mL^{-1} of enzyme, 60 min of treatment were required to reach these results using 0.34 U.mL^{-1} of enzyme. Therefore, 0.68 and 1.02 U.mL^{-1} of enzyme were selected for further studies.

3.4. Effect of enzymatic treatment on OS concentrations

To choose the optimal conditions is also necessary to evaluate the effect of the enzymatic treatment on COS (COSc and COSg) concentrations and GOS production to minimize the degradation of COS and the formation of GOS by transgalactosylation reactions. During enzymatic treatment, new peaks eluting between 3 and 7 min (peaks 6), were observed in both goat colostrum (Figure 1C) and lactose control sample (Figure 1D). Peak eluting at 3.4 min (peak 5) was identified as allolactose (6-O- β -D-galactosyl-D-glucose) by comparison with the GOS reference sample and with that

reported elsewhere (Zolnere & Ciprova, 2017). Moreover, an increase in 6'-galactosyl-lactose (peak 3a) was also observed. These carbohydrates were produced as a result of transgalactosylation reactions. This is in good agreement with that previously reported (Zolnere & Ciprova, 2017; Chockchaisawasdee, Athanasopoulos, Niranjana & Rastall, 2005) regarding the high production of GOS with linkages β -(1 $\rightarrow$ 6) after transgalactosylation reactions catalyzed by Lactozym[®] 3000 L HP G.

Peaks eluting between 4.4 and 7 min, corresponding to GOS trisaccharides (peaks labelled as 6), were also obtained by transgalactosylation catalyzed by the enzyme and were produced in both goat colostrum and lactose control. Moreover, during these treatments the production of new minor OS (t_R : 7.2 min; 11-14.5 min; 19-20 min) was detected only in goat colostrum. These carbohydrates could be the result of the hydrolysis of higher molecular weight COS during enzymatic treatment or due to transferase reactions with COSc acting as acceptors of galactose. These peaks were labelled in this work as COSh (peaks 7). In general, small qualitative differences were observed in COS profile of goat colostrum before (Figure 1A) and after (Figure 1C) enzymatic treatment.

Regarding quantitative analysis, Figure 4 shows the evolution of total OS (COSc, COSg, COSh and GOS) of goat colostrum during enzymatic treatment using 0.68 U.mL⁻¹ (Figure 4A) and 1.02 U.mL⁻¹ (Figure 4B). Individual behaviors of all OS quantified in this study for each enzyme concentration are shown in Figures S1 and S2 of Supplementary Material.

GOS concentration significantly increased up to 30 min of incubation with 0.68 U.mL⁻¹ of enzyme and 15 min with 1.02 U.mL⁻¹ of enzyme, reaching the highest values under the latter conditions (70.08 mg g⁻¹ colostrum sample), with allolactose experimenting

the highest increase (Figure S1 and Figure S2). At longer incubation times, concentration of GOS decreased, probably due to their hydrolysis. In general, lower concentrations of GOS were obtained with 0.68 U.mL⁻¹ of enzyme (maximum: 40.12 mg g⁻¹ colostrum sample at 30 min).

Regarding COSh, a significant increase of these OS was observed for both 0.68 and 1.02 U.mL⁻¹ of enzyme from 15 min of incubation; however, concentrations of these carbohydrates were tiny and lower than 2.8 mg.g⁻¹ colostrum sample. A higher number of peaks corresponding to COSh were detected when 1.02 U.mL⁻¹ of enzyme was used (12 vs. 7 using 1.02 U.mL⁻¹ and 0.68 U.mL⁻¹ of enzyme, respectively); however, none of them showed concentrations higher than 0.5 mg.g⁻¹ colostrum sample (Figures S1 and S2 of Supplementary Material). Therefore, considering that the aim of this work was to minimize GOS production and to preserve COS profile, 0.68 U.mL⁻¹ of enzyme was selected as optimal concentration of β -galactosidase treatments.

A significant increase of COSg (Figure 4A) was observed during the whole treatment, due to the production of 6'galactosyl-lactose by transgalactosylation reactions. However, the lowest concentrations of COSg were observed at 15, 45 and 60 min of incubation. Regarding COSc, no significant changes on their concentration were observed at 15 min. Therefore, 0.68 U.mL⁻¹ of enzyme, 37°C, 15 min and pH 7 were selected as optimal conditions. In this sample, 24 mg COSg, 20 mg of GOS, 30 mg of COSc and 1 mg of COSh per g of pooled goat colostrum sample were present. However, high concentrations of glucose and galactose (348.5 mg.g⁻¹ of colostrum sample) were produced. Then, the use of an additional treatment to remove these monosaccharides using yeast was evaluated.

3.5. Yeast treatment

Pooled goat colostrum sample, previously treated with 0.68 U.mL⁻¹ of enzyme for 15 min was submitted to a yeast treatment (*S. cerevisiae* at 37°C). As shown in Figure 5, the monosaccharides glucose and galactose exhibited a sharp decrease during the first hours of treatment and only low percentages (10%) remained after 6 h. On the contrary, lactose was kept constant during the whole treatment. As expected, trehalose (α -D-glucopyranosyl-(1 $\rightarrow$ 1)- α -D-glucopyranoside), produced as consequence of yeast metabolism (Jules, Guillou, Francois & Parrou, 2004), was also detected from the beginning of the treatment. This carbohydrate was totally removed at 24 h of treatment. No noticeable changes in COS were detected under these conditions; this is in good agreement with Hernández, Ruiz-Matute, Olano, Moreno & Sanz (2009), who reported that carbohydrates with β -linked galactose and glucose units could not be hydrolyzed by *S. cerevisiae* due to the absence of enzymes in this yeast able to hydrolyze this linkage. Therefore 24 h of *S. cerevisiae* incubation was selected as the optimum time.

4. Conclusions

Selective removal of lactose, and the resulting glucose and galactose, from pooled goat colostrum has been successfully achieved using a biotechnological procedure based on the combined use of β -galactosidase from *K. lactis* (optimal conditions: 0.68 U.mL⁻¹ of enzyme, 37 °C, 15 min and pH 7) and yeast from *S. cerevisiae* (optimal conditions: 37 °C, 24 h). Under these conditions 55 mg of COS, 20 mg of GOS and 21 mg of lactose per g of pooled goat colostrum sample were remaining. This is a clean, food-grade and straightforward scalable fractionation methodology, which could be easily applied for the feasible enrichment of bioactive oligosaccharides from milk samples. The resulting high-value added product could find immediate applications in areas dealing with the development of novel nutraceuticals, functional foods and/or food supplements. Concretely, the cost-efficient and food-grade conversion of goat colostrum whey

permeate to manufacture novel hetero-oligosaccharides based on the combination of conveniently enriched endogenous bioactive oligosaccharides and enzymatically-synthesized GOS, but keeping the main COS structures, may be appealing for the dairy industry. This interest is sparked by the fact that the industrial production of human milk oligosaccharides is not currently feasible, which leads to the necessity of searching for suitable replacers as it is the case of GOS. Therefore, further scientific studies could be orientated towards the comparison of the functional properties of hetero-oligosaccharides obtained from different milk sources, most promisingly goat milk, in order to unravel their additional advantages, if any, as compared to conventional GOS.

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520

Figure captions

Figure 1. HPAEC-PAD profile of carbohydrates present in samples before enzymatic treatment [(A) pooled goat colostrum and (B) lactose standard] and after 45 min of β -galactosidase incubation (0.68 U/mL) [(C) pooled goat colostrum and (D) lactose standard]. 1) Glucose + galactose; 2) lactose; 3) COSg; 3a) 6'galactosyl-lactose; 3b) 3'galactosyl-lactose; 4) COSc; 5) allolactose; 6) GOS trisaccharides; 7) COSh.

Figure 2. Evolution of lactose content (%) during β -galactosidase treatment at long incubation times at pH 7 and using different concentrations of enzyme in pooled goat colostrum (A) and in lactose standard solution (B).

Figure 3. Evolution of lactose content (%) during β -galactosidase treatment at short incubation times at pH 7 and using different concentrations of enzyme in pooled goat colostrum (A) and in lactose standard solution (B).

Figure 4. Concentration (mg.g^{-1}) of oligosaccharide (COS, GOS y GOSc) during β -galactosidase treatment at short incubation times at pH 7 and using different concentrations of enzyme in pooled goat colostrum: 0.68 U.mL^{-1} (A) and 1.02 U.mL^{-1} (B). Different letters indicate significant differences ($P < 0.05$) among the OS concentrations.

Figure 5. Evolution of lactose, trehalose and monosaccharide contents (%) upon *Saccharomyces cerevisiae* incubation during 30 h at 37°C in pooled goat colostrum previously treated with β -galactosidase from *Kluyveromyces lactis*.

Table 1. Percentage of carbohydrates (%) obtained after incubation of lactose with β -galactosidase (*K. lactis*) for 1 h at 37°C at different pH values.

	Carbohydrates (%)		
	pH 4.5	pH 7	pH 8
Glucose+Galactose	1.8 (0.1) *	98.0 (0.1)	5.51 (0.05)
Lactose	98.2 (0.1)	1.60 (0.05)	94.1 (0.4)
Oligosaccharides	0.0	0.40 (0.03)	0.3 (0.2)

* Standard deviation in parenthesis

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Supplementary material

Figure 1S. Concentration (mg.g^{-1}) of individual oligosaccharides during β -galactosidase treatment at short incubation times at pH 7 using 0.68 U.mL^{-1} of enzyme in pooled goat colostrum.

Figure 2S. Concentration (mg.g^{-1}) of individual oligosaccharide during β -galactosidase treatment at short incubation times at pH 7 using 1.02 U.mL^{-1} of enzyme in pooled goat colostrum.

Figure 1S.

Figure 2S.