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Purification of caprine oligosaccharides at pilot-scale

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Abstract

The purification of caprine milk oligosaccharides (COS) by membrane filtration has been hampered by the low concentration of target COS and high concentration of lactose. In addition, their molecular weight proximity hinders the recovery of a COS fraction with high degree of purity and recovery yield. In this work, the recovery of a high purity COS concentrate was obtained by the optimization of an integrated approach including complete lactose hydrolysis, fermentation of the resulting monosaccharides and nanofiltration. All carbohydrates were quantified using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC PAD). Defatted goat whey was ultrafiltered with discontinuous diafiltrations to increase the recovery of COS in the whey permeate which was then subsequently concentrated by nanofiltration. COS recovery yields of 75% with negligible amounts of monosaccharides (0.3% of the initial amount of lactose in the whey permeate) were achieved. A final retentate containing 67.6 and 34.4% of acidic and neutral oligosaccharides respectively was obtained from caprine milk.

Keywords

Bioactive oligosaccharides; lactose hydrolysis; Microfiltration; Ultrafiltration; Fermentation; Nanofiltration

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Chemical compounds studied in this article:

3′-sialyllactose (PubChem CID: 123914)

6′-sialyllactose (PubChem CID: 643987)

6′-sialyl-*N*-acetyllactosamine (PubChem CID: 16212424)

Lacto-*N*-difucohexaose (PubChem CID: 3082109)

1. Introduction

Milk oligosaccharides (OS[4]) are carbohydrates found in all mammalian species that possess diverse biological activities. The interest in milk oligosaccharides has grown substantially over the past few decades following observations that the carbohydrate fraction of milk is likely responsible for the development of a protective *Bifidobacterium*-rich microbiota in breastfed children (Kunz et al., 2000). The composition of human milk is unique, in part due to its complex OS profile, wherein OS are the third most abundant component after lactose and lipids with a concentration ranging from 7 to 30 g L^{-1} depending upon the lactation stage (Coulet et al., 2013; Hickey, 2012; Stahl et al., 2007). Important biological functions of milk oligosaccharides for the developing neonate include prebiotic action to stimulate the growth of beneficial bifidobacteria in the gut (Gopal and Gill, 2000; Zivkovic and Barile, 2011). Additionally, OS can prevent infection by acting as soluble receptor decoys thus inhibiting the adhesion of pathogens to intestinal epithelial cells and subsequent invasion (Daddaoua et al., 2006; Lara-Villoslada et al., 2006; Ninonuevo et al., 2006).

Although not used directly as nutrients for the infant, OS perform a wide variety of functions, which appear to be related to their unique structural features. Indeed, OS have complex structures which distinguish them from other milk sugars like lactose, glucose, and galactose. Whereas the latter are all digested and absorbed in the gastro-intestinal tract, OS contain a variety of monosaccharides joined together by indigestible linkages; therefore they reach the lower intestine intact and are utilized as carbon source by select bacterial species that possess the appropriate enzymes to break down the otherwise inaccessible sugars. The lactose core of OS is generally elongated by key monosaccharides such as *N*-acetylglucosamine, fucose and/or sialic acid, yielding a constellation of structures that can be classified as neutral or acidic (based on the absence/presence of the sialic acid).

Considering the limited supply of human milk as a commercial source of oligosaccharides, alternative sources of human milk-like oligosaccharides have been identified, including bovine and caprine milks. Compared with human milk or colostrum, the levels of OS in the milk of domestic mammals (cows, sheep, goats and horses) are much lower than the ones in human milk, typically less than 1.0 g L^{-1} (Urashima et al., 2001).

Innovative strategies attempting to reproduce the structural diversity of human milk OS include the use of metabolically engineered bacteria, transgenic animals, chemical and enzymatic synthesis (Bridiau and Maugard, 2011; Chen, 2015; Endo and Koizumi, 2000; Prieto, 2012; Watanabe et al., 2008). While the use metabolically engineered bacteria has the potential to produce grams to kilograms quantities of OS (Endo and Koizumi, 2000) the use of transgenic animals has not proved as effective since usually results in either the animal's or its offspring's death (Prieto, 2012; Watanabe et al., 2008). Chemical synthesis allows for the production of a wide array of OS, however this technique remains expensive and time

[4]OS: Oligosaccharides; COS: Caprine milk oligosaccharides; CF: Concentration factor; TMP: Transmembrane pressure; HPAE-PAD: High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection; UHT/HTST: Ultra High Temperature/High temperature short time; SPES: sulfonated poly-ethersulfone; 3′-SL: 3′-sialyllactose; 6′-SL: 6′-sialyllactose; 6′-SLN: 6′-sialyl-*N*-acetyllactosamine; LNDFH I: lacto-*N*-difucohexaose; LNFP I: lacto-*N*-fucopentaose I; 2′-FL: 2′-fucosyllactose; LNnH: lacto-*N*-neohexaose; LDFT: lactodifucotetraose.

consuming, and extensive purification techniques are required to eliminate the chemicals used in the production (Chen, 2015). Enzymatic methods have overcome some of the limitations encountered by the chemical synthesis (reaction time and cost) but reduced enzyme availability and poor region selectivity have resulted in low production yields of OS (Bridiau and Maugard, 2011; Lu et al., 2012; Michalak et al., 2014; Rodríguez-Díaz et al., 2013; Sandoval et al., 2012). Although some of the above described techniques have the potential to produce diverse OS in adequate quantities in the foreseeable future, the recovery of existing complex OS from many food streams represents a more attractive opportunity from an environmental and economic perspective for the production of grams quantities and simultaneously capture a pool of diverse OS structures.

Goat whey is a co-product of goat cheese manufacturing and is primarily composed of lactose (74%), proteins (18.9%), and fat (1.2%) on a solid basis. Whey is also a source of caprine milk oligosaccharides (COS), but the high lactose concentration and its molecular-weight proximity with most oligosaccharides represents a challenge for the production of lactose-free COS by membrane filtration (Martinez-Ferez et al., 2006; Moreno-Indias et al., 2009). The recovery of COS from goat whey would enable the production of high-value nutritional products while mitigating financial and sustainability issues associated with the underutilization and disposal of this co-product. The prebiotic activity of COS recovered from caprine whey has already been validated by several *in vitro* studies, where substantial growth of *Bifidobacterium* spp. was observed on isolated COS (Oliveira et al., 2012).

Current technological approaches to recover COS utilize membrane filtration and diafiltration to improve the purity of recovered oligosaccharides. Usually, high recovery yields of COS has been achieved at the expense of COS purity. The combination of ultrafiltration (50 kDa) and nanofiltration (1 kDa) of caprine milk yielded 80% of the original oligosaccharides, but the remaining 5% of original lactose significantly compromised the overall oligosaccharide purity, which was as low as 8% (Martinez-Ferez et al., 2006). Oliveira et al. (2012) have used a similar approach where a 25 kDa UF membrane followed by the use of a tighter 1 kDa UF membrane resulted in a final product containing low lactose content, but also containing only trace amounts of COS.

Our group recently developed a novel approach to recover oligosaccharides from bovine whey permeate colostrum in high purity at pilot-scale (companion paper, de Moura Bell et al.). This new approach relies on the integration of optimized processing conditions that favor maximum lactose hydrolysis and monosaccharide fermentation prior to membrane concentration.

Oligosaccharide recovery of 95% with 99% purity (intended as absence of simple digestible sugars such as glucose and galactose) was achieved by using a nanofiltration membrane (500–700 Da) at 35 bar, 50 °C and concentration factor (CF) of 20 (CF = volume of feed/volume of retentate). The integration of these processing techniques solved the major hurdle of removing simple sugars (that lack biological specificity towards commensal bacteria) while concentrating the oligosaccharides by membrane filtration. Our hypothesis was that this new approach could be applied to other milks (besides bovine milk and colostrum) that contain a mixture of bioactive oligosaccharides and other carbohydrates that could be further

converted into fermentable sugars such as lactose. Although the use of microorganisms to ferment digestible sugars, e.g. *S. cerevisiae*, as a strategy to increase the purity of galactooliogosaccharides and fructooligosaccharides has been proposed in the literature (Goulas et al., 2007; Guerrero et al., 2014; Nobre et al., 2016), these studies did not investigate further steps needed either to improve the purification of the target oligosaccharides, in case full removal of undesirable sugars is not achieved (a common case when using complex mixtures), or to recover the target oligosaccharides from the diluted medium prior to final concentration by freeze-drying or spray-drying. Our integrated approach is novel in the sense that it addresses all the steps needed prior to the production of a pool of OS free of simple sugars; it converts lactose into a form than can be fully fermented prior to the recovery of the target OS by nanofiltration. In addition, our approach is novel because is the first to be applied to complex mammalian milk oligosaccharides at pilot-scale.

The aim of the present study was to design and optimize a process to recover purified COS at large-scale for future evaluation of their biological functions. COS were recovered by an innovative approach developed by our research group (companion paper, de Moura Bell et al.) where upstream lactose hydrolysis and fermentation of monosaccharides was conducted prior to concentration of COS by nanofiltration. The specific objectives of this work were to: 1) evaluate the monosaccharide fermentation rate at pilot-scale; 2) investigate the effects of transmembrane pressure (TMP) and feed flow on permeate flux and permeation of oligosaccharides during ultrafiltration; 3) evaluate the effects of TMP on permeate flux and oligosaccharide retention (acidic and neutral) during nanofiltration; and 4) demonstrate the proof-of-concept of the integrated process regarding the recovery of COS at pilot-scale. COS quantification was accomplished by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

2. Material and methods

The recovery of COS at pilot-scale was performed based on an integrated approach relying on optimized processing conditions that favor maximum lactose hydrolysis, monosaccharide fermentation and concentration by nanofiltration to obtain an oligosaccharide-rich fraction free of digestible sugars such as glucose and galactose (Fig. 1).

2.1. Production of defatted goat whey

Two batches of approximately 70 L of raw goat milk (UC Davis Goat Farm, Davis, California, USA) were used to produce goat whey. Whey was obtained from the production of a soft cheese based on a method described by Buriti et al. (2005) with minor modifications. Goat milk was heated to 37 °C in a 95 L stainless steel jacketed tank and a commercial rennet Chy-Max® Extra (100% chymosin, Chr. Hansen, Milwaukee, WI, USA) was added at 8 mL per 10 L of milk. After 40 min, the curd was cut gently into small cubes under constant stirring of 37 rpm for 50 min to encourage syneresis. A sieve was used to separate the curd from the whey, wherein approximately 90% of the initial volume of milk was recovered as whey. The whey was subsequently defatted using a cream separator (GEA

Westfalia Separator, Model CTC 3, Germany) at 420 mL min^{-1}, 0.05–0.1 bar at 50 °C. All steps of the experiment were conducted in duplicate.

2.2. β-Galactosidase treatment of goat whey at pilot-scale

A food grade fungal lactase (Bio-Cat Inc., Troy, Virginia, USA) derived from the fungus *Aspergillus oryzae* was used to hydrolyze lactose into galactose and glucose. The pH of two batches of 70 L of goat whey was adjusted to 4.5 with citric acid before adding 0.2% (w/v) of *β*-galactosidase. The slurry was stirred for 1 h at 60 rpm and kept at 50 °C. Lactose hydrolysis optimized conditions were selected based on our previous work, which also demonstrated that the selected enzyme does not degrade the acidic oligosaccharides 3′-SL, 6′-SL and 6′-SLN (De Moura Bell et al., 2016). After lactose hydrolysis, samples were immediately pasteurized using a continuous UHT/HTST lab pasteurizer system (MicroThermics, Raleigh, NC, USA) at 72 °C for 30 s. Pasteurized samples were refrigerated at 5°C prior to ultrafiltration to produce the goat whey permeate.

2.3. Production of goat whey permeate by ultrafiltration

Ultrafiltration experiments were carried out on a pilot-scale tangential membrane filtration system (Model L, GEA Filtration, Hudson, WI, USA) to separate whey proteins and produce the whey permeate. The system was composed of a 2.5″ diameter spiral membrane housing (1–2 m^2 area), a 95 L jacketed stainless steel reactor, a Proline Promass 80E flowmeter for mass flow rate and density (Endress + Hauser, Reinach, Switzerland), a heat exchanger, inlet and outlet manometers, and a 7.0 HP feed pump (Hydra-Cell™, Minneapolis, MN, USA). The whey was concentrated using a 10 kDa spiral wound polyethersulfone ultrafiltration membrane (Hydranautics, Oceanside, CA, USA) with an effective area of 1.86 m^2 to a CF of 6. To evaluate the effects of TMP and feed flow on permeate flux, goat whey was ultrafiltered in recirculation mode with negligible concentration (CF < 1.1). After identification of best TMP and feed flow, ultrafiltration experiments were performed at 50 °C, 2 bar TMP and 6 L min^{-1} feed flow rate. After CF 6 was achieved, the ultrafiltration retentate was diafiltered discontinuously with 50 °C water, at volumes equal to the retentate (8.5 L) to increase the permeation of oligosaccharides from the ultrafiltration retentate into the permeate. Eight discontinuous diafiltrations were performed.

2.4. Monosaccharide fermentation and yeast removal by microfiltration

Monosaccharide fermentation was performed using a 208 L jacketed stainless steel research fermenter system (Cypress, San Jose, CA, USA). Fermentation of hydrolyzed goat whey permeate was performed according to our recently developed method (companion paper, de Moura Bell et al.). Monosaccharide fermentation was performed using 0.4 g L^{-1} of active dry yeast, previously rehydrated before the inoculum, and 0.3 g L^{-1} of yeast extract (Bacto™Becton, Dickinson and Company, Sparks, Maryland), which were mixed into approximately 110 L of whey permeate. Fermentations were conducted in duplicate at 30 °C. The recirculation pump was set up to provide constant stirring (58 min of stirring per hour with 2 min interval). To evaluate the reaction kinetics, samples were withdrawn at time intervals ranging from 2 to 3 h until density measurements values, used as a real time indicator of the fermentation progress, leveled off. The fermentation was cooled down to 4 °C overnight to allow gravity sedimentation of the yeast. Removal of residual yeast in the

supernatant was performed by gross filtration (Millipore® Polysep™ II, Cartridge Filter, pore size 0.5 μm Nominal, Millipore Corporation, Bedford, Massachusetts, USA) followed by subsequent microfiltration.

2.5. Concentration of fermented goat whey permeate by membrane filtration

Microfiltration was performed to ensure the absence of any residual yeast during the nanofiltration of the fermented whey permeate. A tangential filtration system (Model L, GEA Filtration, Hudson, WI, USA) composed of a plate and frame membrane module with 0.036–0.72 m^2 area was used. The fermented whey permeate was microfiltered using a series of flat sheet 150 kDa regenerated cellulose membranes (Mycrodyn-Nadir GmbH, Wiesbaden, Germany) at TMP of 0.8 bar, feed flow of 6.1 L min^{-1}, and temperatures ranging from 10 to 12 °C.

Following yeast removal by microfiltration, the fermented whey permeate was concentrated by nanofiltration using the same equipment used during the ultrafiltration stage. Approximately 100 L of fermented whey permeate were concentrated using a 500–700 Da spiral-wound HYDRACoRe70pHT sulfonated polyethersulfone (SPES) nanofiltration membrane with an effective area of 1.86 m^2 (Hydranautics, Oceanside, CA, USA) to a CF of 10. Nanofiltrations were performed at 50 °C and TMP ranging from 35 to 40 bar and 6.0 L min^{-1} recirculation flow rate.

2.6. Membrane performance parameters

Permeate flux, oligosaccharide retention and purity of the final product were quantified to evaluate the membrane performance (Cohen et al., 2017). Permeate samples were collected and weighed in regular time intervals to determine the permeate flux (J_p) given in units liters per meter squared per hour (L m^{-2} h^{-1}) (Equation (1)):

$$J_p = \frac{V_p}{A * t} \quad (1)$$

where V_p is the permeate volume, A is the membrane area (m2), t is the time (h) for collecting the permeate volume.

The retention of digestible sugars (glucose, galactose and lactose) and acidic (3′-sialyllactose (3′-SL), 6′-sialyllactose (6′-SL), and 6′-sialyl-*N*-acetyllactosamine (6′-SLN)) and neutral oligosaccharides (lacto-*N*-difucohexaose (LNDFH I), lacto-*N*-fucopentaose I (LNFP I), 2′-fucosyllactose (2′-FL), Lacto-*N*-neohexaose (LNnH), lactodifucotetraose (LDFT)) was calculated according to the following equation:

$$R = \left[\left(1 - \frac{C_p}{C_f}\right)\right] * 100 \quad (2)$$

where C_p and C_f are the carbohydrate concentrations in the permeate and feed, respectively.

The average retention of oligosaccharides during the nanofiltration of fermented goat whey permeate was determined according to the following equation (Cheryan, 1998):

$$\log \frac{C_R}{C_f} = R \log (CF) \quad (3)$$

where C_R is the oligosaccharide concentration in the retentate, C_f is the initial concentration of oligosaccharide in the feed, R is the average retention coefficient of oligosaccharides and CF is the concentration factor. Permeate samples were collected at different CF for oligosaccharide determination. Retentate oligosaccharide content was determined by mass balance.

The effects of TMP ranging from 1 to 3 bar and feed flow from 3 to 9 L min^{-1} were evaluated in relation to permeate flux during the ultrafiltration of hydrolyzed whey in a recirculation mode experiment (CF < 1.1). For the nanofiltration experiments, TMP ranging from 5 to 40 bar were investigated in relation to permeate flux and carbohydrate retention in a recirculation mode experiment (CF < 1.1). TMP was chosen per the permeate flux vs. TMP curve and retention of acidic and neutral oligosaccharides obtained in the recirculation mode experiment. Permeate and retentate samples were withdrawn and weighed in regular time intervals for each combination of pressure and feed flow. Experiments were conducted in duplicate.

The purity of the target oligosaccharides in relation to monosaccharides in the retentate was calculated according to the following equation:

$$\% \, Purity = \left(\frac{C_{CMO}}{C_{CMO} + C_{Mono}} \right) * 100 \quad (4)$$

where C_{CMO} is the summed concentration of all quantified caprine milk oligosaccharides (3′-SL, 6′-SL, 6′-SLN, LNDFH I, LNFP I, 2′-FL, LNnH and LDFT) and C_{Mono} is the summed concentration of quantified monosaccharides (glucose and galactose).

The recovery yield of oligosaccharides in the retentate was calculated based on the following equation:

$$\% \, Yield = \left[\left(\frac{Ci, r * V_R}{Ci, f * Vf} \right) \right] * 100 \quad (5)$$

where $C_{i,r}$ is the concentration of a specific component in the retentate, V_R is the volume of liquid in the retentate) $C_{i,f}$ is the concentration of a specific component in the feed, and V_f is the feed volume.

2.7. Carbohydrate quantification of permeates and retentates

Acidic (containing sialic acid) and neutral CMO as well as simple sugars (glucose, galactose and lactose) were quantified by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD ICS-5000, Thermo Scientific, Sunnyvale, CA, USA) following a previously published protocol (Lee et al., 2015). Samples were diluted between 10 and 1000 times as appropriate and filtered through a 0.2 μm syringe filter (Acrodisc 13 mm PES, Pall Life Sciences, Port Washington, NY, USA) into 2 mL vials with septa. Calibration curves (coefficient of determination ≥ 0.999) were prepared using analytical grade commercial standards for 3′-SL, 6′-SL, 6′-SLN, LNDFH I, LNFP I, 2′-FL (V-Labs, Covington, LA, USA); LNnH and LDFT (ProZyme, Hayward, CA, USA) and glucose, galactose, and lactose (Sigma, St. Louis, MO, USA). 25 μL of diluted, filtered samples were injected into a Carbo-Pac PA200 (Dionex, Sunnyvale, CA, USA) column at 0.5 mL min^{-1} flow rate using 100 mMol NaOH and 10 mMol NaOAc isocratically for acidic oligosaccharides and 100 mMol NaOH and 3mMol NaOAc for neutral oligosaccharides quantification. Glucose, galactose and lactose quantification was carried out on Carbo-Pac PA10 (Dionex, Sunnyvale, CA, USA) column with a flow rate of 1.2 mL min^{-1} and 10 mMol NaOH for the first 12 min and a gradient from 10 to 100 mMol NaOH for 12.5 min.

3. Results and discussion

3.1. Ultrafiltration of goat whey

3.1.1. Effects of TMP and feed flow on permeate flux—Operational parameters such as TMP and feed flow are known to affect permeate flux, with higher pressures and feed flow increasing permeate flux by increasing the removal of solvent and reducing the rate of fouling to a certain point. Although high feed flow typically reduces the rate of fouling due to turbulent flow across the membrane surface favoring higher permeate flux, its effect on permeate flux also depends on the TMP range used (Kuo and Cheryan, 1983). The effects of TMP and feed flow, in recirculation mode, ranging from 1 to 3 bar and 3–9 L min^{-1}, respectively, are shown in Fig. 2. In the pressure range evaluated (1–3 bar), permeate flux was favored by intermediate TMP and higher feed flow, with maximum values (~29 L h^{-1} m^{-2}) observed at 2 bar and 6 or 9 L min^{-1}. At higher TMP (3 bar), a small reduction in permeate flux was seen when increasing the feed flow from 3 to 9 L min^{-1}, likely due to increased fouling. The optimal combination of pressure and feed flow for higher permeate flux was 2 bar and 6 L min^{-1}. Our results are in agreement with the findings of Kuo and Cheryan (1983), who showed that a relative increase in permeate flux was observed at higher flow rates at low and intermediate TMP (2.4 and 3.1 bar). However, an opposite trend was observed at higher TMP (4.85 bar), which could be attributed to increased fouling. In general, the use of higher flow rate has been associated with lower fouling rates at and low to intermediate pressures, while higher fouling rates have been observed at higher flow rate and higher TMP, justifying the decrease in permeate flux.

3.1.2. Concentration and diafiltration—Hydrolyzed goat whey was concentrated by a 10 kDa ultrafiltration membrane to a CF of 6 (Fig. 3) at optimized processing conditions (2 bar and 6 L min^{-1}). Permeate flux decreased from 21.1 to 14.5 L h^{-1} m^{-2} at CF 6, corresponding to an increase in density from 1011.1 to 1021.3 kg m^{-3}. Permeate flux

reduction filtering cheese whey may be attributed to the formation of a polarization concentration layer on the membrane by proteins and salts, which could lead to the formation of salt bridges between the membrane and whey proteins, thus fouling the membrane (Merin and Cheryan, 1979). To increase the recovery of CMO in the ultrafiltration permeate, eight discontinuous diafiltrations were performed (Figure A2). The efficiency of each diafiltration was evaluated by the quantification of acidic and neutral CMO in all permeates. Approximately 98% of the oligosaccharides remaining in the retentate after CF6 were recovered in the permeate after 4 diafiltrations, with complete permeation being achieved with 5 diafiltrations. Although the presented pilot-scale experiment used fresh water for diafiltration, this approach is not the preferred option from a sustainable perspective. In an industrial scenario, the use of a countercurrent diafiltration process, where the permeate from a previous diafiltration is used to diafilter a new retentate and fresh water is used only in the last stage of diafiltrations, could be easily implemented.

3.2. Pilot-scale fermentation of hydrolyzed goat whey permeate and yeast removal by microfiltration

The complete fermentation of monosaccharides released from the hydrolysis of bovine colostrum whey permeate lactose has been successfully demonstrated at pilot-scale as an essential step to improve the purity of oligosaccharides concentrated by membrane filtration (companion paper, de Moura Bell et al.). The rate of fermentation of hydrolyzed goat whey permeate is shown in Fig. 4. HPAEC-PAD results demonstrated that nearly complete fermentation of glucose (12.9660–0.0150 g L^{-1}) and galactose (13.0755–0.0120 g L^{-1}) in goat whey permeate was achieved at 9 and 16 h, respectively when using 0.4 g L^{-1} of active dry yeast and 3.0 g L^{-1} of yeast extract. The initial density of the hydrolyzed whey permeate decreased from 1.0092 ± 0.0002 in the starting material to 0.9988 ± 0.0 Kg m^{-3} at 16 h of fermentation, leveling off from 16 to 22 h. Constant density measurements have shown to be a reliable method for monitoring the fermentation progress (companion paper, de Moura Bell et al.). These results corroborate our recent work (companion paper, de Moura Bell et al.) in that nearly complete fermentation of glucose and galactose was observed at 10 and 15 h of fermentation, respectively. The starting materials of both studies, bovine colostrum and goat whey permeates, were very similar in terms of density (1.0123 vs. 1.0092 Kg m^{-3}) which facilitates the comparison of both results. In addition, similar optimized fermentation conditions (amount of yeast, yeast extract, stirring conditions, temperature, and fermentation reactor) were used in both studies. As expected, galactose fermentation started after glucose was almost completely depleted, being in agreement with reports in the literature demonstrating *Saccharomyces cerevisiae* preference for glucose upon galactose.

In addition to the utilization of the monosaccharides in the whey permeate, a reduction in the initial COS concentration from 0.0740 to 0.0361 g L^{-1} was measured by HPAEC-PAD after the fermentation. No changes in the concentration of the acidic COS was observed, which is in agreement with our recent work (companion paper, de Moura Bell et al.) in that no reduction in the concentration of the major acidic oligosaccharides in bovine whey permeate from colostrum was observed after fermentation. Reduction on the total COS after fermentation has been associated with the consumption of the neutral COS by *Saccharomyces cerevisiae*. Total COS and monosaccharide concentrations of 0.0361 g L^{-1}

and 0.0278 g L^{-1}, respectively, were observed after fermentation. The fermentation of nearly all monosaccharides increased the whey permeate purity from 0.28 to 56.64%, in relation to fermentable sugars. Approximately 7.5% of the initial volume of whey permeate was lost during the separation of the yeast by sedimentation. Residual yeast removal was accomplished by microfiltration with minimum loss of whey permeate (~1% of the initial volume) and a permeate flux of 90 L h^{-1} m^{-2}.

3.3. Nanofiltration of whey permeate

3.3.1. Effects of TMP on permeate flux and retention of oligosaccharides—

TMP has a direct impact on permeate flux and solute retention. Increased TMP can lead to membrane compaction and reduced membrane thickness, which might result in increased permeate flux. However, membrane compaction can also lead to an effective pore size reduction, thus affecting the retention of target components (Goulas et al., 2002). The effect of TMP on the permeate flux of goat whey permeate and oligosaccharide retention using a 500–700 Da NF membrane is shown in Fig. 5. Permeate flux increased from 1.92 to 11.33 L h^{-1} m^{-2} when TMP increased from 5 to 35 bar and leveled off from 35 to 40 bar. A similar trend was observed for oligosaccharide retention, which increased from 50.5 to 89.2% when TMP increased from 5 to 40 bar, with marginal increases observed from 30 to 40 bar. Increased carbohydrate retention with increasing TMP is likely due to the combination of polarization concentration and membrane compaction. Higher TMP would result in a denser gel layer and reduced membrane pore size which would in turn reduce the convective transport of oligosaccharides through the membrane pores. The consistent oligosaccharide retention observed from 30 to 40 bar may indicate the transition from a convective to a diffusive transport of solutes (Pontalier et al., 1997). TMP of 35 bar and feed flow of 6 L min^{-1} was used to concentrate the fermented goat whey permeate.

Goat milk contains both acidic and neutral oligosaccharides, so electrostatic interactions between acidic oligosaccharides and the membrane charge may play a role in the retention of charged components. Electrostatic interactions might affect the retention of charged solutes when using charged membranes. This effect arises from the interaction between the charged solutes (i.e. acidic oligosaccharides) and the membrane charge, in our case a negatively charged membrane (Verliefde et al., 2008). The retention of acidic and neutral oligosaccharides at different TMP is shown in Fig. 6. In general, acidic oligosaccharides showed a higher retention than neutral oligosaccharides (40–77% vs. 20–44%) when concentrated by the negatively charged sulfonated polyethersulfone nanofiltration membrane. This trend was observed across all TMP but was more pronounced at lower TMP values. Increased membrane compaction at higher TMP increased the retention of all oligosaccharides potentially masking the effect of the solute charge on the retention of acidic oligosaccharides. In addition to the role of electrostatic interactions between the acidic COS and the negatively charged membrane, differences in the individual COS composition of the fermented whey permeate (0.0167 g L^{-1} for 3′-SL, 0.0027 g L^{-1} 6′-SL, 0.0019 g L^{-1} 6′-SLN, 0.0060 g L^{-1} LNDFH I, 0.0014 g L^{-1} LNFP I, 0.0024 g L^{-1} 2′-FL, and 0.0050 g L^{-1} LDFT) may also have contributed to differences in the retention of acidic and neutral COS. Higher COS concentration in the feed, as the ones observed for LNDFH I and LDFT might have contributed to reduced retention of neutral COS compared with the acidic ones.

3.3.2. Concentration of goat whey permeate by nanofiltration—Fig. 7(a and b) shows the nanofiltration of goat whey permeate using a 500–700 Da nanofiltration membrane to a CF of 10, feed flow of 6 L min^{-1}, 50 °C and 35 bar, with total time of filtration of 5 h and 45 min. Polarization concentration due to increased solids concentration in the retentate at CF 10 had a pronounced effect on permeate flux. Permeate flux decreased from 10.22 to 5.33 L h^{-1} m^{-2} when the retentate density increased from 988.3 to 1010.2 kg m^{-3} at CF 10 (Fig. 7a). Our results are in agreement with those in the companion paper (de Moura Bell et al.) in which the permeate flux of fermented bovine whey from colostrum decreased from 13.8 to 7.05 L h^{-1} m^{-2} at similar processing conditions. At CF 10, total oligosaccharide yield in the retentate (acidic + neutral) was approximately 75% compared with the starting material. Fig. 7b shows the effect of CF on the recovery yield of each individual oligosaccharide. Final yields of 61.7, 80.2 and 82.0% were observed for the acidic oligosaccharides 6′-SLN, 6′-SL, and 3′-SL, respectively. Final yields for neutral oligosaccharides were 58.7, 62.5, 78.7, and 73.9% for LNDFH I, LDFT, 2′-FL, and LNFP I, respectively. Average oligosaccharide retention, as shown by the slope of the equation in Fig. A1, was 89%. The recovery yield of acidic oligosaccharides (~75% average) observed in this work was lower than observed with bovine colostrum whey permeate, in which ~ 95% of acidic oligosaccharides were recovered from the starting material. Although the same processing conditions were used in both studies (TMP, membrane type, feed flow), compositional differences in the starting materials may be responsible for the differences in oligosaccharide retention.

The total concentration and individual oligosaccharide composition of the final retentate at CF 10 is shown in Fig. 8(a and b). Total oligosaccharide concentration increased from 0.036 to 0.27 g L^{-1} at CF 10, evidencing 75% oligosaccharide recovery when using TMP of 35 bar (Fig. 8a). 67.6 and 34.4% of the oligosaccharides quantified in the final retentate were acidic and neutral, respectively (Fig. 8b). Negligible concentration of monosaccharides were detected in the final retentate (0.07 g L^{-1}), evidencing an oligosaccharide-rich fraction nearly free of digestible sugars. The concentration of COS by nanofiltration increased the purity of the fermented whey permeate from 56.64 to 78.38% in relation to digestible sugars. The residual amount of monosaccharides in the final retentate represented 0.3% of the initial amount of lactose in the whey permeate. The recovery yield observed using our approach (75%) was similar to those obtained by Martinez-Ferez et al. (2006) in which 82% of oligosaccharides were recovered from goat milk by a two-stage membrane filtration followed by diafiltration. However, in their final retentate, 5% of the initial amount of lactose remained therefore reducing the final product purity. Although the recovery yield of oligosaccharides was not reported, an oligosaccharide fraction containing 10% of the initial amount of lactose was obtained by Oliveira et al. (2012) using two-stage membrane filtration.

4. Conclusions

A recently developed approach to purify oligosaccharides from bovine colostrum has been successfully applied to recover caprine milk oligosaccharides at pilot scale. This method relies on the use of enzymatic hydrolysis, monosaccharide fermentation, and membrane concentration. The concentration of whey permeate by NF enabled the recovery of 75% of

the oligosaccharides initially present in the whey permeate with minimum contamination of glucose and galactose (equivalent to 0.3% of the initial amount of lactose). A final retentate containing 0.27 g L^{-1} of oligosaccharides, of which approximately 67.6% and 34.4% were acidic and neutral respectively, was obtained. This process will enable the production of gram quantities of oligosaccharides with minimum contamination by digestible sugars for future functional tests and commercialization.

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Appendix A

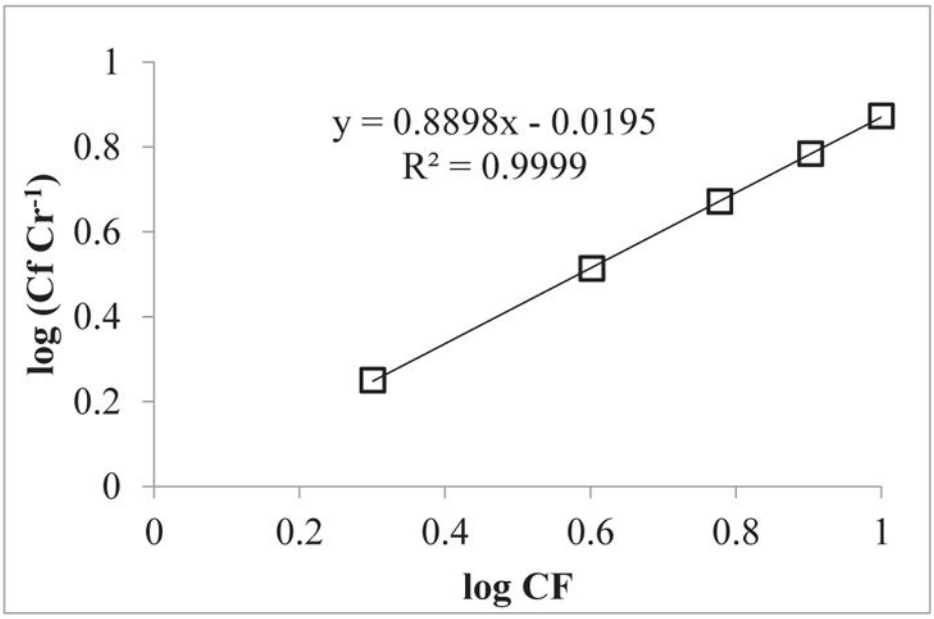

Fig. A.1.

Average oligosaccharide retention during nanofiltration (500–700 Da sulfonated polyethersulfone) of fermented goat whey permeate.

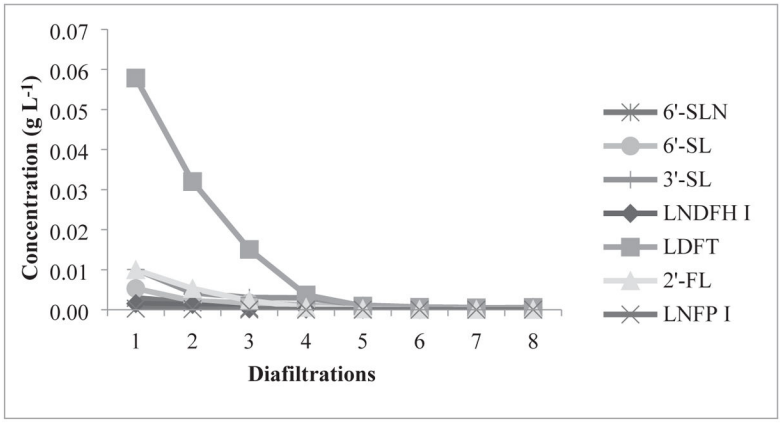

Fig. A.2.

Effects of diafiltrations on the permeation of oligosaccharides from the ultrafiltration retentate.

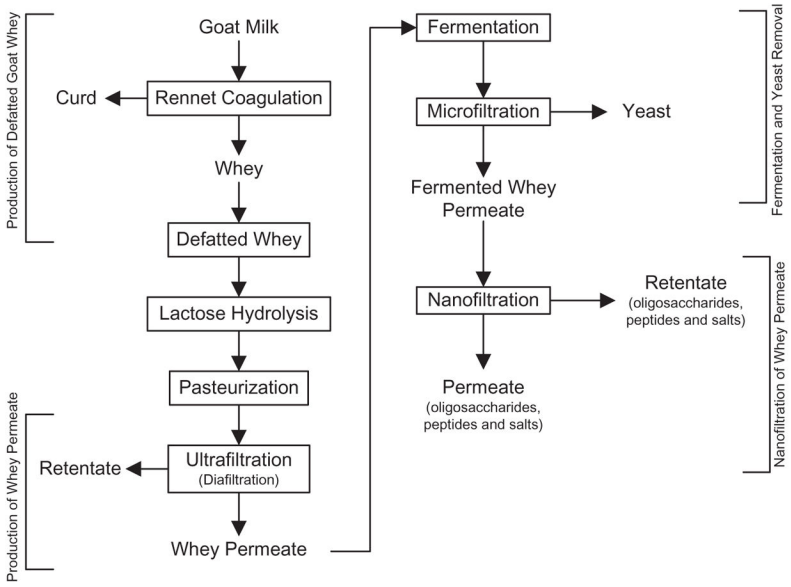

Fig. 1.

Pilot-scale recovery of COS by an integrated approach based on enzymatic hydrolysis, monosaccharide fermentation and nanofiltration.

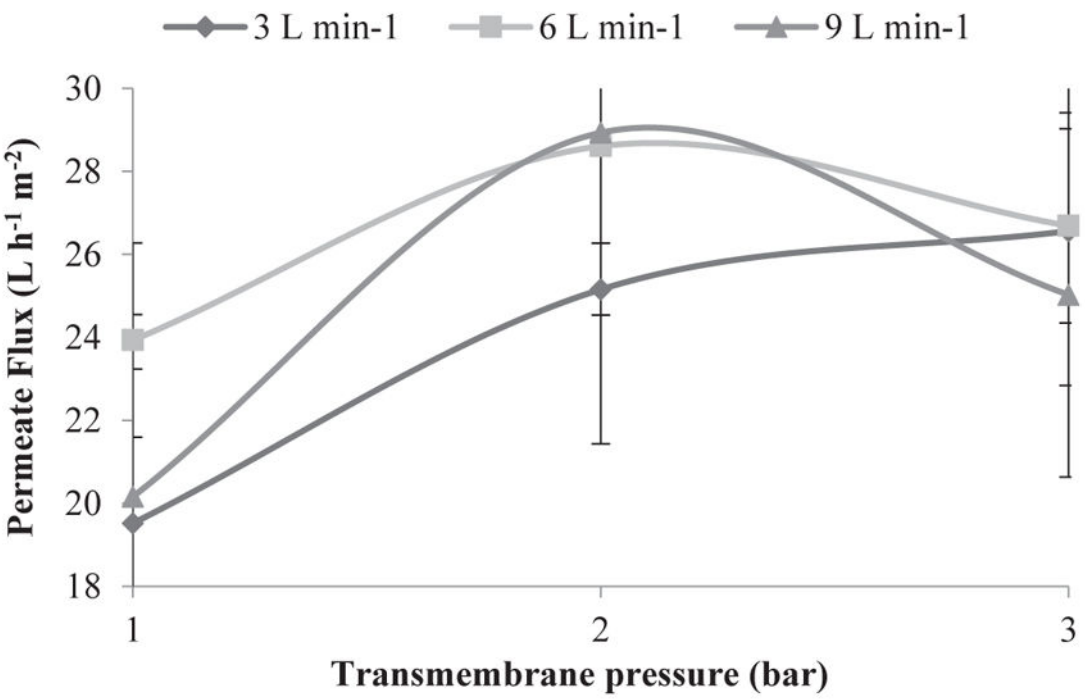

Fig. 2.

Effects of TMP and feed flow on permeate flux during ultrafiltration of hydrolyzed goat whey (CF < 1.1). Error bars represent the standard deviation of two analytic replicates.

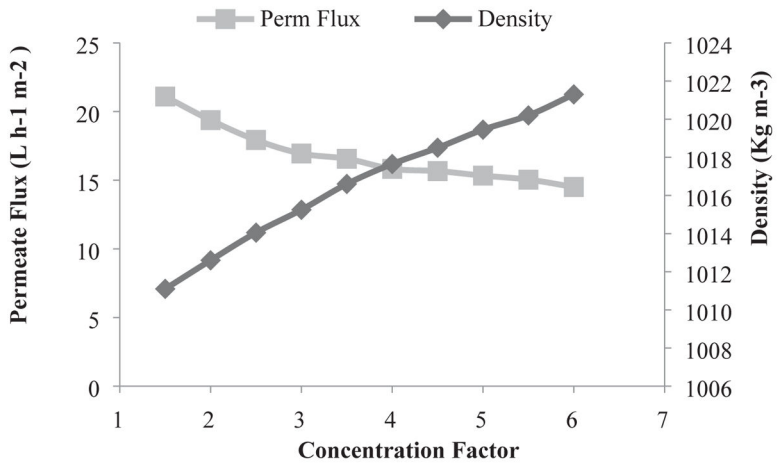

Fig. 3.

Effects of concentration factor on permeate flux and density of hydrolyzed goat whey using a 10 kDa membrane.

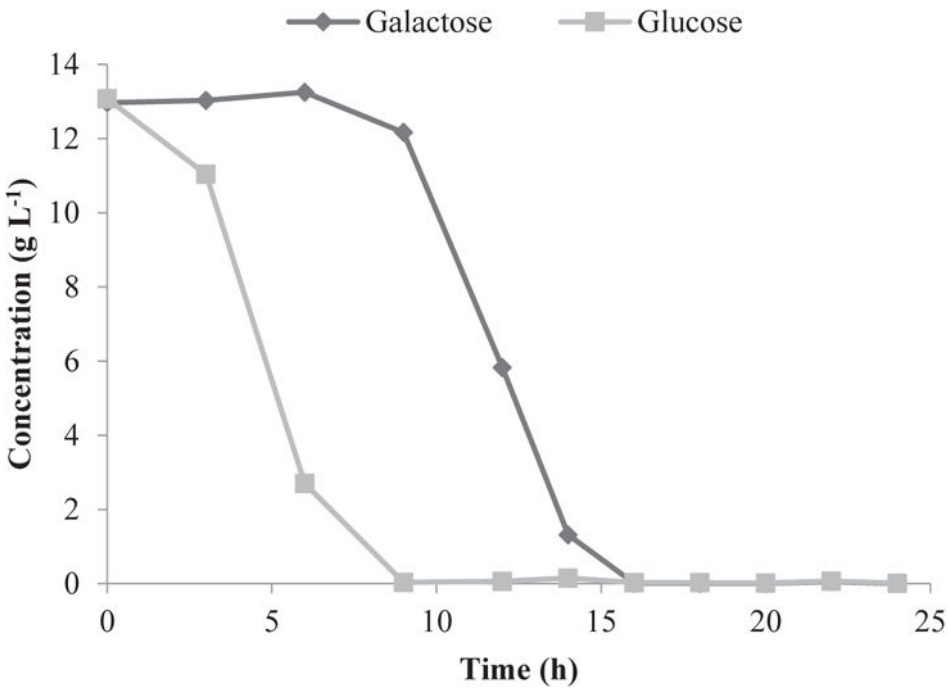

Fig. 4.
Pilot-scale fermentation of hydrolyzed goat whey permeate.

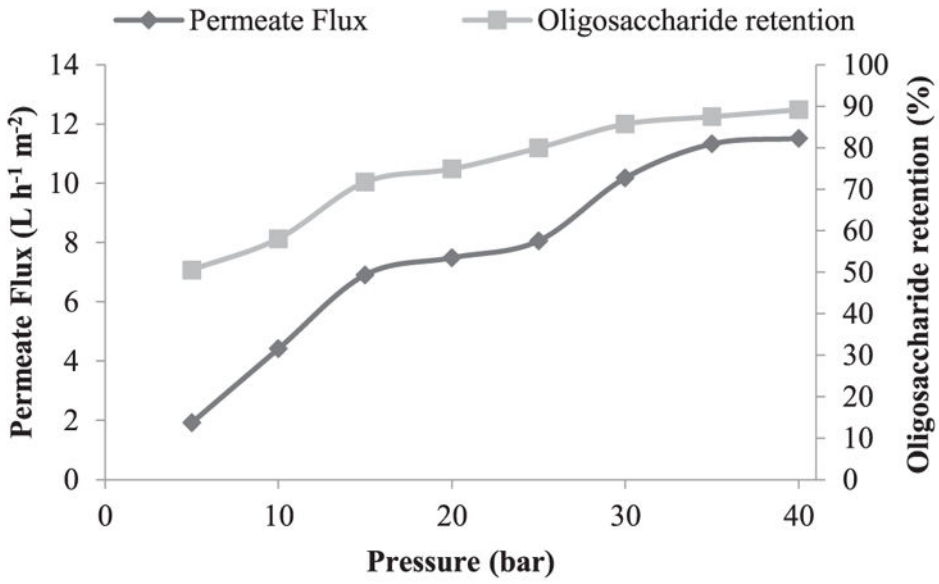

Fig. 5.

Effects of TMP on permeate flux and total oligosacccharide retention using a 500–700 Da membrane.

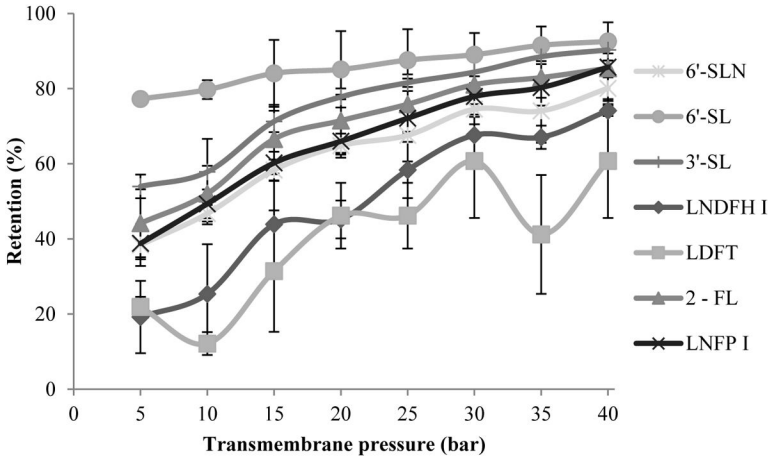

Fig. 6.

Effects of TMP on the retention of COS using a 500–700 Da sulfonated polyethersulfone membrane (CF < 1.1). Error bars represent the standard deviation of two analytic replicates.

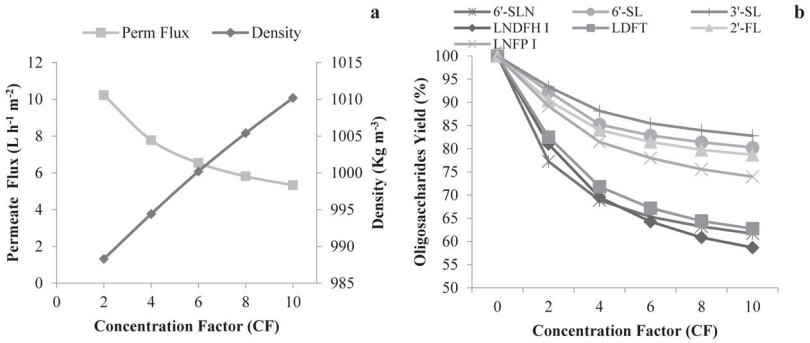

Fig. 7.

Effects of concentration factor on permeate flux and density (a) and on the retention of acidic and neutral oligosaccharides (b) in the retentate using a 500–700 Da sulfonated polyethersulfone membrane.

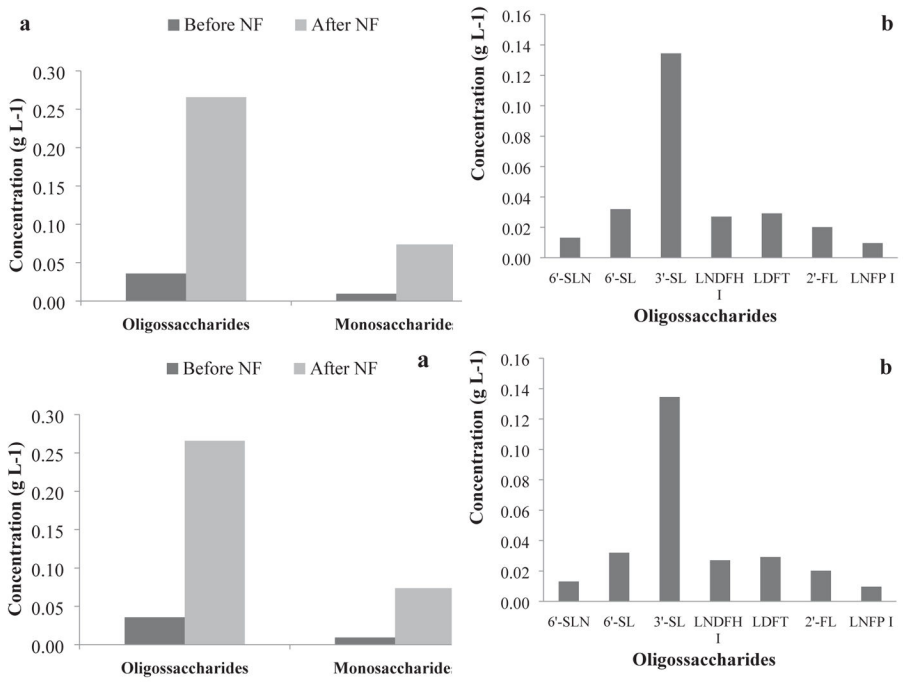

Fig. 8.

Total oligosaccharide and monosaccharide concentration before and after nanofiltration (a) and individual oligosaccharide concentration (6′-SLN, 6′-SL, 3′-SL, LNDFH I, LDFT, 2′-FL and LNFP I (b) in the final retentate (CF 10).