Recovery of caprine milk oligosaccharides with ceramic membranes

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Abstract

Caprine milk contains a large amount of different sialylated and neutral lactose-derived oligosaccharides compared to cow or sheep milk. In addition, its oligosaccharide profile is very similar to human milk, which suggests it has similar physiological activity. Thus, the caprine milk oligosaccharide fraction is a very promising food ingredient for human nutrition applications, especially for the supplementation of infant formulas. In this research work, a two-step cross-flow filtration process was designed in order to recover the caprine milk oligosaccharides. Tubular ceramic membranes with molecular weight cut-offs of 50 and 1 kDa, respectively, were employed in two separated, consecutive continuous diafiltration steps, in which the cumulated permeate from the first step was the initial feed in the second one. A final retentate containing more than 80% of the original oligosaccharides and less than 4% of the original lactose and protein was obtained.

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1. Introduction

The glucidic fraction of milk contains, along with lactose (the major carbohydrate), nucleotide sugars, glycolipids, glyco-proteins, and oligosaccharides [1]. In particular, human milk is considered to be unique with regard to its high content and singular proportion of complex fucosylated and sialylated lactose-derived oligosaccharides [2]. Although in most cases their structure and/or functions are not yet fully understood, some of these biomolecules are discussed as being involved in the infant’s defence system, the development of a specific intestinal microflora and in inflammatory processes [3]. Besides these properties, some researchers suggest that human milk contains a significantly higher concentration of exogenous sialic acid, than bovine milk or any type of infant formula [4]. In this sense, sialic acid has been demonstrated to participate as an integral part of ganglioside structure in synaptogenesis and neural transmission [5], contributing to the differences in neurodevelopment between breastfed and bottle-fed infants [4].

Caprine milk has recently been reported to contain a large amount of different sialylated and neutral lactose-derived oligosaccharides compared to cow or sheep milk, which is very similar to the oligosaccharide profile in human milk [6]. The caprine milk oligosaccharides seem to inhibit the monocyte adhesion to human umbilical vein endothelial cells, which suggest they may act as anti-inflammatory agent in the newborn infant [7]. Thus, based on these findings, caprine milk oligosaccharides are a very promising functional food ingredient for human nutrition applications, especially for the supplementation of infant formulas.

Within membrane technology, ultrafiltration and nanofiltration processes have proven to be an efficient way in the separation of carbohydrates. Aydogan et al. [8] studied the effect of feed flow rate, operating pressure, and feed concentration on the separation of glucose and sucrose with 500 Da nanofiltration membranes. With respect to oligosaccharides, Goulas et al. [9] demonstrated the potential of cross-flow nanofiltration in the purification of a galacto-oligosaccharide syrup containing contaminants monosaccharides by using cellulose acetate and thin film composite membranes. Goulas et al. [10] developed a process for the production of isomaltooligosaccharides (degree of polymerisation greater than 5) from sucrose in a recycle enzyme reactor coupled with a 10 kDa polyethersulfone/poly sulfone membrane.

On the other hand, membranes have been employed in the extraction of valuable bioactive compounds from milk. For instance, Lal Baruah et al. [11] recovered human IgG fusion protein from transgenic caprine milk employing 0.1 μm tubu-
lar hollow fiber polyethersulfone membranes. Ulber et al. [12] designed a downstream process to recover bovine lactoferrin from sweet whey and evaluated the performance of organic (tubular, spiral, and flat-sheet) and ceramic membranes to this end. Furthermore, hybrid membrane processes like the one described by Sarney et al. [13], based on a combination of enzymatic treatment and nanofiltration, have proven its effectiveness for the isolation of oligosaccharides from human milk.

In this research work, we describe a two-step cross-flow ultrafiltration–nanofiltration process employing tubular ceramic membranes which allows to recover more than 80% of the original caprine milk oligosaccharide fraction. This type of sequential strategy has shown good results in the fractionation of whey proteins [14]. In the first step, starting from skim caprine milk, a 50 kDa membrane preferently retains the proteins, while the oligosaccharides and lactose are collected in the permeate. In the second step, lactose is eluted in the filtrate employing a 1 kDa membrane and the oligosaccharides are finally obtained in the retentate.

2. Experimental

2.1. Materials

The milk used in this study, supplied by Puleva Biotech (Granada, Spain), was pasteurised skim caprine milk from the Murciano-Granadina breed. Before pasteurisation, milk was filtered in order to remove any gross contaminant.

The membranes were multichannel tubular ceramic INSIDE CéRAM™ modules (TAMI Industries, Lyon, France) made of ZrO2–TiO2, 25 cm long, with three channels, and a membrane area of 94 cm².

2.2. Experimental rig

The experimental rig (Fig. 1) consisted of a 2 L feed tank immersed in a thermostatic bath at 30 °C, a precision positive displacement recirculation pump (Procon, TN, USA), a filtration housing, one back-pressure valve and manometers, a flowmeter (Iberfluid, Barcelona, Spain), and two tanks for permeate collection and buffer supply.

2.3. Filtration process

A two-step cross-flow filtration process was selected. The mode of operation consisted of two separated, consecutive continuous diafiltration steps. The molecular weight cut-offs selected were 50 kDa for the first step and 1 kDa for the second one. Skim caprine milk was employed as initial feed in the first step. The cumulated permeate from the first step was collected and employed as initial feed in the second one.

2.3.1. First step

The objective of this step was to retain the protein fraction while obtaining the oligosaccharides and lactose in the permeate (Fig. 2a).

Prior to use, the 50 kDa membrane was conditioned by flushing with demineralised water at 30 °C for 30 min to hydrate the membrane material and remove possible contaminants. Clean membrane resistance was determined by measuring water flux at 30 °C. Transmembrane pressures were set in the 0–250 kPa range and cross-flow velocity was set at 3.3 m/s. Then, skim caprine milk at 30 °C was filtered, recycling both retentate and permeate. Initial permeate flux measurements were taken for 1 min at different transmembrane pressures values up to 250 kPa at a cross-flow velocity of 3.3 m/s.

![Fig. 1. Drawing of the experimental system. (1) Diafiltration water supply, (2) feed tank, (3) recirculation pump, (4) membrane module, (5) permeate tank, (6) back-pressure valve, (7) manometer, (8) manometer, and (9) flowmeter.](image)

![Fig. 2. Scheme of the separation process. (a) First step (50 kDa) and (b) second step (1 kDa).](image)
After cleaning the membranes, in order to start the first filtration step, 2 L of skim caprine milk were added to the retentate tank. Temperature was kept at 30 °C to prevent any possible precipitation of proteins. Velocity of recirculation into channels was fixed at 3.3 m/s in order to reduce fouling effects. Demineralised water was fed to the retentate tank at the same rate of the permeate flow. The system was operated up to 4 diavolumes, i.e. 8 L of permeate were obtained. Samples of retentate and permeate were taken each 0.5 diavolumes for quantification of oligosaccharides, lactose, protein, and calcium.

2.3.2. Second step
The purpose of this step was to elute the lactose in the permeate and to obtain the oligosaccharides in the retentate (Fig. 2b). First, the 1 kDa membrane was flushed with demineralised water at 30 °C for 30 min to hydrate the membrane and remove contaminants. In order to characterise the clean membrane, water flux at 30 °C was measured. Transmembrane pressures were set in the 0–250 kPa range and cross-flow velocity was fixed at 3.3 m/s. Then, cumulated permeate from the first step at 30 °C was filtered in the total recycle mode. Initial permeate flux measurements were taken for 1 min at different transmembrane pressures values up to 400 kPa at a cross-flow velocity of 3.3 m/s.

After cleaning the membranes, in order to start the second filtration step, 2 L of cumulated permeate from the first step were added to the retentate tank. Temperature was kept at 30 °C and velocity of recirculation into channels was fixed at 3.3 m/s. Demineralised water was fed to the retentate tank at the same rate of the permeate flow. The system was operated up to 3 diavolumes, i.e. 6 L of permeate were obtained. Samples of retentate and permeate were taken each 0.5 diavolumes for quantification of oligosaccharides, lactose, calcium, and protein.

2.4. Membrane cleaning
In order to regenerate the membranes after operation, the cleaning procedure consisted of an initial rinse with demineralised water, followed by recirculation of a 20 g/L sodium hydroxide + 0.1 g/L sodium dodecyl sulphate solution at 50 °C for 25 min at neutrality. This cleaning protocol was repeated until the clean membrane resistance was recovered (±10%).

2.5. Analytical methods
2.5.1. Quantification of oligosaccharides
Prior to the quantification, samples (2 mL for the original milk and retentate samples, and 10 mL for permeate samples) were thawed and defatted by centrifugation at 6500 × g for 25 min at 4 °C. Then, the viscous, upper cream layer, consisting primarily of fats and other lipids, was discarded by careful pipetting from the lower aqueous layer, which was completely transferred to sterile tubes. Four millilitres of precooled (4 °C) ethanol was added to the sterile tubes to precipitate the protein fraction, and the sample was kept on ice for 2 h with constant stirring. The ethanol was extracted in a refrigerated vapour trap with vacuum to yield a solution (OS-1 solution) containing the carbohydrate fraction.

Subsequently, the OS-1 solution was diluted to 1 mL with Milli-Q water for purification from lactose and salts. The diluted OS-1 solution was applied onto a Sephadex G-25 column (900 mm × 25 mm i.d.) connected to a Fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) and eluted with Milli-Q water at a flow rate of 1.0 mL/min [15], where lactose and salts were removed. The resulting fractions were then pooled and further concentrated by freeze-drying. The final powder (OS powder) containing caprine milk oligosaccharides was weighed and dissolved in 0.5 mL of Milli-Q water (OS-2 solution) and stored at −20 °C for quantification.

Quantification of oligosaccharides was performed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac PA-1 column (250 mm × 4.6 mm i.d.) connected to a Dionex System (Sunnyvale, CA, USA) equipped with pulsed electrochemical detection (PED2). A 20 μL sample from the OS-2 solution indicated above was injected in the system and a flow rate of 1.0 mL/min was always used. Neutral and acidic oligosaccharides structures were separated in the column at room temperature using the following conditions: eluent A, 100 mM NaOH; eluent B, 100 mM NaOH, 250 mM sodium acetate. The elution programme began with 3 mL eluent A, followed by a 30 min linear gradient (0–100%) of eluent B, and finally 4 mL of eluent B. A re-equilibration volume of 3 mL of eluent A was chosen [16].

For the original milk, the total area under the chromatogram was calculated and referred to the concentration of oligosaccharides (mg in the OS powder/sample volume). For retentate and permeate samples, the total area under the chromatogram was also calculated and compared with the original milk area, which allows the calculation of the concentration of each sample.

2.5.2. Quantification of other components
Lactose was determined using a commercial kit from Roche Boehringer Mannheim (Basel, Switzerland). Total protein content was measured by the Kjeldahl method. Calcium was analysed by atomic absorption spectroscopy using an AAnalyst® 800 Spectrophotometer (Perkin-Elmer, MA, USA).

3. Results and discussion
3.1. Composition of the original caprine milk
The composition of the initial pasteurised skim caprine milk that was fed to the two-step filtration process is shown in Table 1. As it can be seen, the contents of protein, lactose, and calcium are lower compared to the bovine milk.
cium were similar to the values reported for bovine milk [17]. However, the concentration of oligosaccharides was almost five times the value for bovine milk [6]. The molecular weight of the caprine milk oligosaccharide fraction ranged from 504 (corresponding to galactosyl-lactose) to approximately 1200 (corresponding to di-sialyl-hexosyl-lactose) [6].

3.2. First step (50 kDa membrane)

3.2.1. Membrane characterization

Fig. 3a shows the correlation between water permeate flux \( (J_{H2O}) \) and transmembrane pressure \( (P_T) \) for the 50 kDa membrane. The clean membrane resistance \( (R_M) \) was calculated according to [18]:

\[
J_{H2O} = \frac{P_T}{\mu R_M}
\]

where \( \mu = 0.000798 \text{ kg/(m s)} \) is the viscosity of water at 30\(^\circ\)C. The value \( R_M = 5.65 \times 10^9 \text{ m}^{-1} \) was obtained \( (r^2 = 0.995) \).

The observed initial permeate flux with skim caprine milk \( (J_0) \) against transmembrane pressure is shown in Fig. 3b. The decreasing slope of the curve suggested that the resistances in series model [18] could be applied:

\[
J_0 = \frac{P_T}{\alpha + \beta P_T} \]  

where the parameter \( \alpha \) takes into account the membrane and fouling resistances and \( \beta \) is involved in the computation of the polarization layer resistance (which is considered to be proportional to the transmembrane pressure). The values obtained for these parameters \( (\alpha = 1.05 \text{ kPa/(L/m}^2 \text{ h)}, \beta = 0.00366 \text{ m}^2 \text{ h/L}) \) were employed to calculate the solid line represented in Fig. 3b. In order to work in the pressure controlled region, the transmembrane pressure selected to perform the diafiltration process was 100 kPa since higher pressure values could lead to severe fouling due to the compaction of the fouling layer. It is worth to note that \( \alpha \) is much greater than the inverse of the slope of the curve \( J_{H2O} \) versus \( P_T \) (0.0752).

3.2.2. Continuous diafiltration

During the continuous diafiltration process performed in the first step, a progressive decrease of the permeate flow with time was observed (Fig. 4). The cake filtration model [18] is expressed in mathematical terms as:

\[
J = \frac{J_0}{\sqrt{1 + \frac{A J_0^2 b t}{R_M}}} = \frac{J_0}{\sqrt{1 + kt}}
\]

where \( J_0 \) is the initial flux, \( A \) the membrane area, \( b \) the fouling index (which can be correlated with operating variables), and \( k \) is a lumped parameter. This model fitted the data well \( (k = 0.173 \text{ h}^{-1}, r^2 = 0.983) \). The model assumes that the membrane surface is covered by a layer of foulant, which grows although if back-transport of solute takes place. Besides proteins, soluble calcium (not linked to casein micelles) is an important foulant in dairy processing due to precipitation as tricalcium phosphate and the formation of salt bridges between the membrane and proteins [18].

The evolution of the normalised retentate concentration \( (C_R/C_{R0}) \) with the number of dia volumes \( (N) \) is represented in Fig. 5. More than 98% of the lactose and oligosaccharides were eluted after 4 dia volumes. On the other hand, 94% of the original protein content was retained in this step. In a similar way, 76% of the calcium did not cross the membrane since its major fraction is linked to the large casein micelles. The calcium fraction eluted (24%) corresponded to its soluble form.
Fig. 5. Evolution of the normalised retentate concentration for protein (■), calcium (♦), lactose (□), and oligosaccharides (●) during the first step.

The transmission coefficients \( T = \frac{C_P}{C_R} \) of the different compounds during the continuous diafiltration process can be assessed considering the expression:

\[
T = \frac{d}{dN} \left( \ln \frac{C_R}{C_R^0} \right)
\]

which is derived from a differential material balance in the retentate tank. Thus, \( T \) results to be the slope of \( \ln(C_R/C_R^0) \) versus \( N \) plots. Effectively, in Fig. 6a it can be seen that a slope 1 straight line fitted both oligosaccharides and lactose series, which involved full transmission during the entire operation. In the case of the retained calcium and protein (Fig. 6b), only partial transmission was observed. Calcium transmission dropped from an average value of 0.20 in the first 0.5 diavolumes to 0.049 at the end of the process. With respect to protein, transmission dropped from 0.0079 to 0.0013. The reduction observed in both cases, which reaches practically null values, could be related to membrane fouling. However, it seems that this fouling did not have any influence in the transmission of oligosaccharides and lactose, probably due to the small size of these molecules.

3.3. Second step (1 kDa membrane)

3.3.1. Membrane characterization

The calibration of water permeate flux versus transmembrane pressure for the 1 kDa membrane is represented in Fig. 7a. As mentioned above, the clean membrane resistance was calculated and resulted to be \( R_M = 7.32 \times 10^{10} \text{ m}^{-1} \) \( (r^2 = 0.997) \). Obviously, this resistance is higher than the obtained for the 50 kDa membrane due to the smaller pore size.

Fig. 7b shows the initial permeate flux of cumulated permeate from the first step as a function of transmembrane pressure. Taking into account the cut-off of the membrane (1 kDa), the osmotic pressure model\[18\] was considered:

\[
J_0 = \frac{P_T - \Delta \pi}{\delta}
\]

The straight trendline suggested that the osmotic pressure term \( \Delta \pi \) could be neglected. The inverse of the slope gave the value of the parameter \( \delta = 5.19 \text{ kPa/(L/(m}^2 \text{ h})} \) \( (r^2 = 0.998) \).

In order to prevent an excessive compaction of the fouling layer, the transmembrane pressure selected to perform the diafiltration process in this step was 150 kPa.

3.3.2. Continuous diafiltration

The time evolution of permeate flux in the second step is plotted in Fig. 8. It can be seen that a steep drop occurred in the 0–10h interval, followed by a steady state (18.8 L/(m\(^2\) h)) until the end of operation. This behaviour could be correlated to an empirical model as follows:

\[
J = (J_0 - J_{SS}) \exp(-\gamma t) + J_{SS}
\]

where \( \gamma = 0.371 \text{ h}^{-1} \) \( (r^2 = 0.995) \).

Effectively, Eq. (6) fits an initial adsorption of foulants which involves an exponential flux decay. Then, the existence of the steady state suggests that an equilibrium between membrane fouling and back-transport of foulants was achieved.

In Fig. 9, the normalised retentate concentration \( (C_R/C_R^0) \) during the second step is represented. After 3 diavolumes, 94% of the lactose and 96% of the calcium were collected in the permeate and, more importantly, 84% of the oligosaccharides...
Fig. 7. Calibration with (a) water and (b) cumulated permeate from the first step of the 1 kDa membrane.

remained in the retentate. The percentage of oligosaccharides that crossed the membrane (16%) most likely corresponded to small structures as those described by Martinez-Ferez et al. [6], i.e. galactosyl-lactose and sialyl-lactose. These structures contain three monomers and have a molecular weight around 500 and 650, respectively.

It has to be noted that the optimal number of diafiltration volumes to be performed in this second step depends on the trade-off between two opposite facts: the greater the number of diafiltrations, (a) the greater the elution of lactose, (b) the lower the retention of oligosaccharides.

The evolution of the transmission coefficients was also evaluated for this second step. It can be seen that lactose showed again full transmission during the process (Fig. 10a). Similarly, calcium freely permeated through the 1 kDa membrane since no casein linked calcium was present. This result suggested that fouling did not affect the sieving properties of the membrane for lactose and calcium. On the other hand, the low oligosaccharides transmission (Fig. 10b) varied, as a consequence of fouling, from 0.18 in the starting half diafiltration to only 0.0064 in the last one. It is worth to mention that the selectivity of this membrane (defined as the ratio between the transmissions of lactose and oligosaccharides) is rather high compared to the values reported in the literature for similar separations [9,10]. With respect to protein transmission (Fig. 10b), it decreased from 0.37 to 0.026.

As a result of the two diafiltration steps, the global yield for the oligosaccharide fraction was 82%. The composition of the resulting final retentate is shown in Table 2. By comparing with Table 1, it can be seen that the oligosaccharides/lactose ratio was increased 14 times from 0.0054 in the original caprine milk to 0.076. Regarding the oligosaccharides/protein ratio, it raised from 0.0073 to 0.1608, which represents a factor of 22. In terms of dry matter, the two-step process involved an increase of the oligosaccharide content of 16 times from 0.3% to 4.9%.

Table 2

<table>
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<th>Component</th>
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<tr>
<td>Total protein</td>
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</tr>
<tr>
<td>Calcium</td>
<td>0.0033</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.66</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>0.050</td>
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</table>

Fig. 8. Time evolution of permeate flux in the second step: experimental (♦) and calculated (—) values.

Fig. 9. Evolution of the normalised retentate concentration for oligosaccharides (●), proteins (■), lactose (□), and calcium (♦) during the second step.
4. Conclusion

It has been shown that a two-step cross-flow filtration process could be employed to recover more than 80% of the oligosaccharide fraction present in skim caprine milk. In the first step, operating in continuous diafiltration for 4 diavolumes with a 50 kDa ceramic tubular membrane, 94% of the protein was retained while the oligosaccharides and lactose permeated freely. In the second step, using a 1 kDa membrane, 94% of the lactose was eluted after 3 diavolumes and the final oligosaccharide product was recovered in the retentate.

The cake filtration model and a model considering an initial exponential drop followed by a steady-state regime, fitted the flux data with time for the first and second steps, respectively.

With respect to the variation of the sieving properties of the membrane, protein and calcium transmission decreased during the first step, while oligosaccharide and protein transmission decreased through the second one.

Acknowledgments

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Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
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<tr>
<td>A</td>
<td>membrane area (m²)</td>
</tr>
<tr>
<td>b</td>
<td>fouling index (kPa h m²)</td>
</tr>
<tr>
<td>C_p</td>
<td>permeate concentration (g/L)</td>
</tr>
<tr>
<td>C_R</td>
<td>retentate concentration (g/L)</td>
</tr>
<tr>
<td>C_R0</td>
<td>initial feed concentration (g/L)</td>
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<tr>
<td>J</td>
<td>permeate flux (L/m² h)</td>
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<tr>
<td>J_0</td>
<td>initial permeate flux (L/m² h)</td>
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<td>J_H2O</td>
<td>steady state permeate flux (L/m² h)</td>
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<td>k</td>
<td>fouling parameter in Eq. (3) (h⁻¹)</td>
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<td>number of diavolumes</td>
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<td>T</td>
<td>transmission coefficient</td>
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Greek letters

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<th>Symbol</th>
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<td>α</td>
<td>parameter in Eq. (2) (kPa/L/m² h))</td>
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<tr>
<td>β</td>
<td>parameter in Eq. (2) (m² h/L)</td>
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<tr>
<td>δ</td>
<td>parameter in Eq. (5) (kPa/L/m² h))</td>
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<td>γ</td>
<td>fouling parameter in Eq. (6) (h⁻¹)</td>
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References


