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Research Article

Comparative study on the biological activity of bovine and ovine PP3 and of their hydrolysates

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ABSTRACT

This study investigated the bioactivity of bovine and ovine proteose-peptone (PPT) fractions, specifically the proteose-peptone component 3 (PP3) and its enzymatic hydrolysate. The antioxidant capacity of the above fractions and their activity against *Cronobacter sakazakii* and rotavirus were evaluated. Results revealed strong interspecies differences on their bioactivity. Thus, antioxidant activity of bovine PPT decreased with the fractionation but increased for ovine PPT, the latter being more active in all cases. Moreover, the antibacterial activity of hydrolyzed bovine PP3 showed high concentration-dependence reaching a maximum of 6.82 logarithmic cycle reduction at 4 mg mL^{-1}, for 24 h of incubation. Conversely, neither intact nor hydrolyzed ovine PP3 were able to reduce the bacterial growth. Finally, both bovine and ovine PP3 neutralized rotavirus in a dose-response way, the former being more effective. Interestingly, the antiviral activity of bovine PP3 increased after hydrolysis, whereas the ovine counterpart lost almost all of its antirotaviral capacity.

1. Introduction

The proteose-peptone (PPT) is a protein that is maintained soluble after heat treatment of milk acidified to pH 4.6 (Pedersen et al., 2012). The first characterization of PPT was made by electrophoresis and revealed the presence of eight components (Girardet & Linden, 1996). Among them, a 28 kDa phosphoglycoprotein was identified and was named proteose-peptone component 3 (PP3) or lactophorin. PP3 consists of 135 amino acids and, unlike other PPT components, is an intact protein that does not derive from enzymatic hydrolysis of milk proteins (Campagna, Cosette, Molle, & Gaillard, 2001; Girardet & Linden, 1996). PP3 migrates under electrophoresis together with a 17 kDa fragment, which is naturally produced in milk by proteolysis of the intact PP3 molecule (Pedersen et al., 2012b). PP3 represents 25 % of the whole PPT fraction, whose concentration in cow's milk is around 300 mg L^{-1} (Sørensen & Petersen, 1993a). Milk fractions with high homology to PP3 have been identified in the milk of other species, such as camel, llama, goat and sheep, though interestingly it is not present in human milk (Campagna et al., 2001).

PP3 is found both in the milk fat globule membrane (MFGM) and whey, as it is weakly anchored to the MFGM and is solubilized easily (Sørensen & Petersen, 1993a). The molecule of PP3 consists of two domains: the N-terminal domain negatively charged (amino acids 1–98) and the C-terminal domain (amino acids 99–135) that has positive charge, amphipathic character and an α-helix structure (Campagna et al., 2001). The primary structure of PP3 contains 5 phosphorylated serines, 1 asparagine-linked N-glycosylation site, 2 threonine-linked and 1 serine-linked O-glycosylation sites (Kjeldsen, Haselmann, Budnik, Sørensen, & Zubarev, 2003). Furthermore, studies of the N-linked carbohydrate moiety have revealed the presence of several biantennary *N*-acetyllactosamine and *N, N′*-diacetyllactosamine type carbohydrates (Valk-Weeber, Nichols, Dijkhuizen, Bijl, & van Leeuwen, 2021).

There are few studies that have addressed the biological role of PP3. However, the proteolysis of the C-terminal domain has been shown to result in a peptide of 3391 Da (amino acids 113–135), termed lactophoricin, with inhibitory activity towards Gram-positive and Gram-negative bacteria (Campagna, Mathot, Fleury, Girardet, & Gaillard, 2004; Pedersen et al., 2012b). PP3 and its hydrolysates have been shown to exert a proliferative effect on microtubule affinity regulating kinase (MARK) 3 hybridoma cells (Mati et al., 1993). The negative charge of the carboxyl groups of its sialic acids might have a function in the stabilization of the active conformation of the glycoprotein and thereby to play

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a role in the simulation of mitosis in MARK 3 hybridoma cells (Mati et al., 1993). A protective activity of PP3 against lipolysis has been demonstrated as it has been found that it can inhibit the lipoprotein-lipase activity (Anderson, 1981; Girardet, Linden, Loye, Courthaudon, & Lorient, 1993). Furthermore, it has been reported that PP3 has a prebiotic activity for *Bifidobacterium animalis*, the digests obtained by enzymatic proteolysis of PP3 still being more bifidogenic (Etienne, Girardet, & Linden, 1994). Some studies have proposed a role for PP3 as an immunostimulant on hybridomas and peripheral lymphocytes (Sugahara et al., 2005) and an inducer of mitogenesis (Pedersen et al., 2012b). The antioxidant activities of different byproducts of the dairy industry (Ripollés et al., 2016) and of dairy proteins (Clausen, Skibsted, & Stagsted, 2009) have been previously evaluated. However, so far, there are no studies regarding the antioxidant activities of PP3 and/or of its hydrolysates. Finally, in addition to the biological functions attributed to PP3, this protein also displays technological functions, such as emulsifying and foaming properties (Innocente, Biasutti, & Blecker, 2011; Innocente, Corradini, Blecker, & Paquot, 1998b; Innocente, Corradini, Blecker, & Paquot, 1998a).

The infant population is particularly susceptible to bacterial and viral infections of the intestinal tract and, in the last decades, two pathogens that have been considered of high relevance are *Cronobacter sakazakii* and rotavirus. The former has low incidence, though it can be lethal (Iversen & Forsythe, 2003); and the latter has very high incidence, though it can be easily treated and cured if adequate measures are rapidly taken with respect to affected infants (Patton, 2012).

C. sakazakii is a Gram-negative bacterium belonging to the Enterobacteriaceae family, and it is characterized by being a facultative anaerobic bacillus, non-sporulated, oxidase negative and catalase positive (Medina, Treviño, & Aguilar, 2014). It is an emergent, opportunistic and ubiquitous pathogen that has been isolated from different environments and transmitted mainly by food consumption. It has been linked with outbreaks of life-threatening necrotizing enterocolitis, meningitis, and sepsis predominately in neonates, infants, but also in other susceptible age groups (Abebe, 2020). The capacity of *C. sakazakii* to form biofilms and its resistance to osmotic stress and to extremely dry environments, allows it to survive for very long periods (Ling et al., 2020). *C. sakazakii* is generally resistant to osmotic stress and dryness; therefore, it can be detected in powdered infant formula even after 2.5 years of storage (Bai, Yu, Guo, Fei, & Shi, 2019). Moreover, *C. sakazakii* has been detected in a wide variety of environmental samples, food and herbs (Baumgartner, Grand, Liniger, & Iversen, 2009; Mayor, 2004).

On the other hand, rotaviruses are the main cause of severe gastroenteritis in infants and present significant healthcare implications worldwide, mainly in developing countries (Caddy, Papa, Borodavka, & Desselberger, 2021; Hallowell, Tate, & Parashar, 2020). The entrance of the rotavirus inside the intestinal epithelial cells is a multistage process in which glycans and several cell surface receptors are involved (Herrmann et al., 2021; Isa, Arias, & López, 2006). Rotavirus replicates inside the enterocytes of the small intestine, provoking great damage to the microvilli and causing malabsorption and loss of fluid and electrolytes (Bishop, 2009). In this view, it has been previously demonstrated an inhibitory activity of milk fractions against rotavirus infection (Kvistgaard et al., 2004). This neutralizing activity has been associated with bioactive glycoproteins present in whey and in the MFGM, such as immunoglobulins, lactoferrin, mucin 1 and lactadherin (Parrón et al., 2016). However, to date, the antirotaviral activity of minor protein fractions, such as PP3 has been scarcely investigated (Inagaki et al., 2010).

The aim of the present study was to extend the knowledge on the biological activity of PPT, and more precisely on PP3 and its hydrolysates, obtained from milk of bovine and ovine origin. The antioxidant activity of those fractions has been studied and also their activity against two pathogens that constitute a high risk for the health of infant population, *C. sakazakii* and rotavirus.

2. Materials and methods

2.1. Proteose peptone and proteose peptone component 3 isolation

For the isolation of bovine and ovine PPT and PP3, the protocol described by Sørensen and Petersen (1993b) was applied with slight modifications. Raw cow's milk was supplied by Villacorona (El Burgo de Ebro, Spain) and raw ewe's milk was obtained from a Lacaune breed flock handled by the Animal Production Unit at the Veterinary Faculty of Zaragoza University (Zaragoza, Spain).

Firstly, milk was warmed to 50 °C in a thermostatic tank and processed in a hermetic centrifuge skimmer (ARR-DES 125, Suministros Químicos Arroyo, Santander, Spain) to remove fat. The skim milk was subjected to heat treatment at 90 °C for 30 min, subsequent acidification to pH 4.6 with 2 M HCl and centrifugation at 1500 *g* for 30 min at 4 °C. The supernatant was recovered and trichloroacetic acid (TCA) was added to reach a final concentration of 13.6 % *(w/v)*. Then, the acidified sample was centrifuged at 1500 *g* for 30 min and the resulting precipitate was resuspended in a 12 % *(w/v)* TCA solution. The mixture was then centrifuged at 3300 *g* for 15 min and the precipitate was washed three times with acetone. Afterwards, to eliminate the remaining acetone, the precipitate was introduced into a drying chamber for 24 h. The dried fraction was crushed and the resulting powder, which constituted the PPT fraction, was stored at −20 °C until use.

PP3 was obtained from bovine and ovine PPT by consecutive chromatographies. Initially, PPT was subjected to molecular exclusion in a Sephadex G-75 column (Ø 2.5 cm × 50 cm) (Sigma Aldrich, St. Louis, MA, USA) by using 0.1 M $(NH_4)HCO_3$ buffer, pH 8.0. The elution flow was maintained at 0.6 mL min^{-1} and the absorbance of the chromatographic fractions (3 mL) was determined at 280 nm using a 6505 UV/Vis Jenway spectrophotometer (Staffordshire, UK). Based on the electrophoretic profiles of the fractions obtained, those containing PP3 were pooled and lyophilized using a Heto Power Dry DW8 (Thermo Fisher Scientific, Rockford, IL, USA).

Afterwards, the lyophilized sample from the previous chromatography fractions (both bovine and ovine) was dissolved in 6 mL of 5 mM $(NH_4)HCO_3$ buffer, pH 8.1 and subjected to ion exchange chromatography in a Q-Sepharose column (Ø 3 cm × 10 cm) previously equilibrated in the same buffer, allowing it to recirculate for 4 h at a flow of 0.15 mL min^{-1}. For the elution of the retained proteins, a linear gradient of $(NH_4)HCO_3$ from 5 mM to 1 M (pH 8.1) was used. The flow was maintained at 0.5 mL min^{-1} and the absorbance of the fractions (3 mL) was measured at 230 nm. The fractions containing PP3 according to the electrophoretic profiles were dialyzed against distilled water, lyophilized and stored at −20 °C until use.

2.2. Enzymatic hydrolysis of PP3

The hydrolysis of PP3 was carried out to release the bioactive peptide contained in the C-terminal domain called lactophoricin, following the procedure of Pedersen, Hansted, et al. (2012). PP3 was prepared at 20 mg mL^{-1} in 0.1 M $(NH_4)HCO_3$ buffer, pH 8.0. Trypsin from porcine pancreas type IX-S (Sigma Aldrich), was dissolved in the same buffer and added at an enzyme:substrate ratio of 1:100 (*w/w*). The mixture was incubated for 3 h at 37 °C with gentle shaking. Afterwards, the mixture was heated at 90 °C for 10 min to stop hydrolysis by enzyme inactivation. The sample was then lyophilized and stored at −20 °C until use.

2.3. Characterization of protein fractions

The protein profiles of the isolated fractions were analyzed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were hand-casted using 30 % acrylamide/bis solution 19:1 (Bio-Rad, Hercules, CA, USA) at a final percentage of 12 %, following the corresponding protocol provided by Bio-Rad. The electrophoresis was carried out in reducing conditions as detailed in the study of Graikini,

Alvarez-Sabatel, Puértolas, Pérez, and Sánchez (2024). Furthermore, in order to verify the nature of the electrophoretic bands, proteomic analysis was performed at the Proteomics Platform of CIBA (IACS-University of Zaragoza, Spain) following the protocol described by Graikini, García, et al. (2024).

Western blotting was performed essentially as previously described by Parrón et al. (2017). In brief, proteins were electroblotted from gels onto polyvinylidene difluoride (PVDF) transfer membranes (Immobilon-P, Millipore Corporation, Bedford, MA, USA) and then, the membranes blocked for 3 min in 50 mM Tris, pH 7.4, 0.5 M NaCl, 2 % Tween 20 (*v/v*). Washing solution was 50 mM Tris, pH 7.4, 0.5 M NaCl, 0.1 % Tween 20. This solution is henceforth termed as TBST. Specific primary antisera obtained in rabbit against bovine or ovine PP3 (diluted 1:100 in 0.1 % TBST) were kindly provided by Jan Trige Rasmussen of University of Aarhus (Aarhus, Denmark). Secondary antiserum was goat anti-rabbit IgG conjugated with peroxidase (diluted 1:1000 in 0.1 % TBST, Sigma Aldrich). The revealing of the bands was done by using a peroxidase substrate composed of 0.06 % (*w/w*) 4-chloro-1-naphthol, 20 % (*v/v*) methanol, 0.1 % (*v/v*) hydrogen peroxide, in phosphate-buffered saline (PBS) composed of 0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and pH 7.4.

2.4. Antioxidant activity

Bovine and ovine PPT were dissolved at a concentration of 80 mg mL^{-1} in 0.1 M $(NH_4)HCO_3$ buffer, pH 8.0. On the other hand, the derived PP3 and its hydrolysates were dissolved at 10 mg mL^{-1} in 5 mM $(NH_4)HCO_3$ buffer, pH 8.1 These solutions were mixed, in a 96-well plate, with a solution of 0.11 mM 2,2 diphenyl 1 picrylhydrazyl (DPPH, Sigma Aldrich) in 50 % (*v/v*) ethanol, which was prepared in the absence of light. A 50 % (*v/v*) ethanol solution was used as control. The plate was incubated at 37 °C for 60 min in the absence of light, and afterwards the absorbance was read at 535 nm using a Tecan GENios Microplate Reader (MTX, Bradenton, USA). The color shift, from the DPPH purple to a yellowish color, measured by spectrophotometry, is a function of the antioxidant capacity of the samples.

2.5. Antibacterial activity

C. sakazakii culture was obtained from frozen criovials containing the lyophilized CECT 858 strain supplied by the Colección Española de Cultivos Tipo (CECT, Valencia, Spain). A microsphere was transferred from the cryovial to a tube containing 10 mL of Tryptone Soya Broth (TSB) (Merck, Darmstadt, Germany) supplemented with 0.6 % yeast extract (YE) (Oxoid, Basingstoke, UK) and was incubated at 37 °C for 24 h. Afterwards, the suspension was seeded onto Tryptone Soy Agar (TSA) (Merck) with 0.6 % YE and the plates incubated at 37 °C for 20 h to obtain isolated colonies of *C. sakazakii*. Finally, a colony was transferred to 50 mL of TSB and diluted 1:100 in 1 % peptone water.

For the antibacterial assay, 50 μL of the bacterial suspension was mixed with 50 μL of the protein samples, previously filtered through 0.22 μm (Pall Life Sciences, New York, USA), in a 96-well assay plate (TPP, Trasadingen, Switzerland). The final concentrations of the samples tested were: 1, 2, 4, and 8 mg mL^{-1} for the bovine and ovine PP3; 1, 2, 3 and 4 mg mL^{-1} for the bovine hydrolyzed PP3; and 1 and 4 mg mL^{-1} for the ovine hydrolyzed PP3. Next, the plate was incubated for 24 h at 37 °C. Aliquots of 20 μL of the incubated samples were extracted after 4, 8 and 24 h and after preparing the appropriate dilutions, the samples were seeded on TSA plates and the colonies counted after 37 °C for 24 h. Additionally, for the control of bacterial growth, the plates were read at 620 nm, after 4, 8 and 24 h in an ELISA reader (Labsystems Multiskan MS, Thermo Scientific).

2.6. Antirotaviral activity

The Rhesus monkey kidney cell line MA104 (CRL-2378) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). This cell line was employed for this assay as it is particularly susceptible to rotavirus infection. Cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10 % (*v/v*) fetal calf serum (FCS), 1 % (*v/v*) 2 mM L-glutamine, 1 % (*v/v*) antibiotic solution (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin), and 0.25 μg mL^{-1} amphotericin B (all purchased from Thermo Fisher). Cells were maintained in 25 cm^2 culture vessels at 37 °C in a Heraeus B5060 EK/CO_2 thermostatic incubator with 5 % CO_2. The bovine rotavirus used was the WC3 strain (VR-2102) purchased from the ATCC and it was propagated in MA104 cells as previously described by Parrón et al. (2016).

For the antirotaviral assay, a 96-well plate was seeded with 3.2×10^4 cells mL^{-1} and incubated at 37 °C for 3 d until 95 % confluence. The night before infection, the cell monolayers were washed twice with 200 μL of EMEM *per* well and were incubated in this media (200 μL *per* well) until the following day. The rotavirus neutralization procedure was carried out according to Graikini, Soro, Sivagnanam, Tiwari, and Sánchez (2023). Briefly, a volume of 25 μL of the samples to be assayed was incubated with 25 μL of a trypsin-activated bovine rotavirus suspension (at a multiplicity of infection or MOI, of 0.02) in a 96-well plate of conical bottom and incubated for 1 h at 37 °C. Afterwards, the content of the wells was transferred to the plate containing the MA104 cell monolayers previously prepared and incubated for 1 h at 37 °C under gentle agitation. Next, 100 μL of EMEM supplemented with 4 % FCS and 1 μg mL^{-1} trypsin was added to each well. The plate was incubated for 1 h at 37 °C under gentle agitation. Finally, to facilitate the infection after adsorption of the virus to cells, 100 μL of EMEM supplemented with 4 % (*v/v*) FCS and 2 μg mL^{-1} trypsin was added *per* well and the plate was incubated for 16 h at 37 °C, under gentle agitation.

The infection was revealed by an indirect immunofluorescence assay. Briefly, the medium was removed after the 16 h of incubation, and the cells were washed once using PBS. The cell monolayer was then fixed using a mixture of acetone:methanol:formaldeyde (1:1:1, *v/v/v*). After two washes, the monolayers were incubated with lamb anti-bovine rotavirus polyclonal antisera diluted 1:300 in PBS containing 2 % gelatin (Sigma Aldrich) for 2 h at 37 °C with gentle agitation. After two washings with PBS, the cells were incubated with donkey anti-lamb IgG labelled with fluorescein isothiocianate (FITC) (Sigma Aldrich) diluted at a ratio of 1:300 in PBS containing 2 % of gelatin for 1 h at 37 °C. Finally, after washing three times with PBS, the fluorescent cells were counted in the Eclipse E400 fluorescence microscope with a Nikon FITC filter (Nikon, Tokyo, Japan), and the Zen lite 2012 image processing software (ZEISS, Jena, Germany). The infectivity percentages were determined by enumerating fluorescent foci (infected cells) in each well and were expressed with respect to the infectivity obtained with the controls, which consisted of cells infected by the virus suspension without neutralizing agent, considered as 100 %.

The laboratories located at the Department of Animal Production and Food Science, in the Faculty of Veterinary Medicine, have the approval to work with the pathogens investigated in the present study by the Biosafety Committee of the University of Zaragoza.

2.7. Statistical analysis

Statistical analysis was conducted through the GraphPad Prism v8.0.2 software (GraphPad Software, San Diego, CA, USA) and results are presented as the mean ± standard deviation. The normality of the data was tested through the Shapiro-Wilk test. For the comparison of the antioxidant activity of the several proteins and fractions, a Two-Way Analysis of variance (ANOVA) followed by Tukey's multiple comparisons test were applied. For the comparison of the antibacterial activity of the several proteins and fractions, the One-Way ANOVA followed by Tukey's multiple comparisons test were applied. Differences with a p value ≤ 0.05 were considered statistically significant.

3. Results and discussion

3.1. Isolation of proteose peptone and proteose peptone component 3

Isolation and purification of PP3 has been attempted in many different ways and mostly using classic liquid chromatography. Heating and acidifying skim milk, followed by steps of ammonium sulphate precipitation and preparative scale polyacrylamide gel-electrophoresis has been described (Ng, Brunner, & Rhee, 1970). Additionally, the hydrophobic properties of PP3 allow for its isolation by hydrophobic interaction chromatography and subsequent gel filtration (Paquet, Alais, & Aubert, 1982). Affinity chromatography using Concanavalin A was used by Kester and Brunner (1982) to isolate a glycoprotein from PPT and from the soluble fraction of the MFGM, obtaining a glycosylated fraction identical to PP3. Furthermore, hydrophobic interaction fast protein liquid chromatography followed by affinity chromatography has been previously used to isolate PP3 (Girardet, Mati, Sanogo, Etienne, & Linden, 1991; Girardet et al., 1994). More recently, PP3 was purified from cheese whey by hydrophobic interaction chromatography on an epoxy-Sepharose column using a step dual-salt gradient (Sousa et al., 2008).

The purification method in the present study was based on gel filtration of crude PPT protein (Sørensen & Petersen, 1993b). Bovine PP3 was isolated from the PPT fraction with a yield of approximately 1 g L^{-1} of raw cow's milk, in agreement with that obtained by Sørensen and Petersen (1993b), who reported a yield of 1.1 g L^{-1}. The yield obtained in the isolation of ovine PPT was higher than that of bovine origin, being of approximately 2 g L^{-1} of raw sheep milk. The yield obtained in the present study is consistent with the protein concentration of ovine whey, which can be up to double than the protein concentration of bovine whey (Hernández-Ledesma, Ramos, & Gómez-Ruiz, 2011). Furthermore, MFGM proteins have also been reported to be in higher concentration in ovine milk compared to bovine milk (Pisanu et al., 2011). The isolation of bovine PP3 was carried out by several consecutive chromatographies. Initially, the PPT fraction was subjected to molecular exclusion chromatography using a Sephadex G-75 column (Fig. 1A) and the fractions containing PP3 (bands of 28 and 17 kDa, framed bands in Fig. 1C) were pooled and subjected to Q-Sepharose. Accordingly, after observing that fractions 39–65 of the Q-Sepharose elution were those containing PP3 (framed bands in Fig. 1D), they were pooled and henceforth considered as the PP3 sample.

The results after the various stages of isolation and purification are summarized in the electrophoresis of Fig. 2A and the Western blotting of Fig. 2B. It can be observed that bovine PP3 was obtained with its two fragments, with apparent molecular weights at 28 and 17 kDa, and high degree of purity (Fig. 2A, lane 4). In order to confirm the identity of the isolated proteins, a Western blotting was performed using polyclonal specific antibodies against bovine PP3 (Fig. 2B). Both the chromatographic and electrophoretic profiles obtained during the isolation of bovine PP3 are consistent with those previously reported by Sørensen

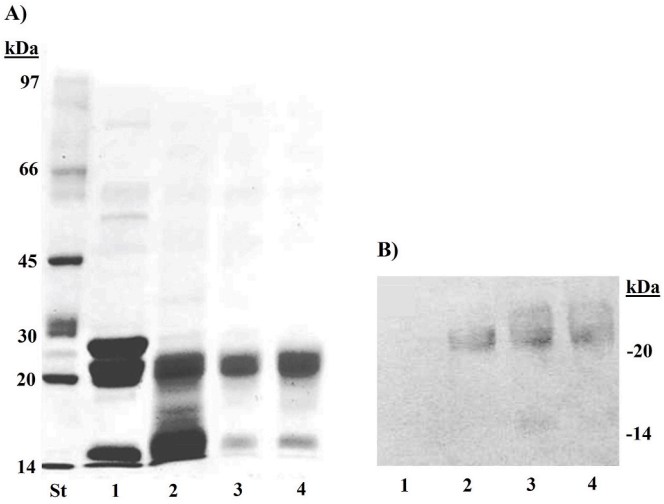

Fig. 2. A) SDS-PAGE in 12 % polyacrylamide gel of the different fractions obtained during the isolation of bovine proteose-peptone component 3 (PP3) (Coomassie blue staining). B) Western-blotting with anti-bovine PP3 antibody. Fractions: St: low molecular weight standard (kDa); 1. Raw bovine milk; 2. PPT; 3. Sephadex G-75 fraction (number 35), 4. PP3: Q-Sepharose fraction (number 45).

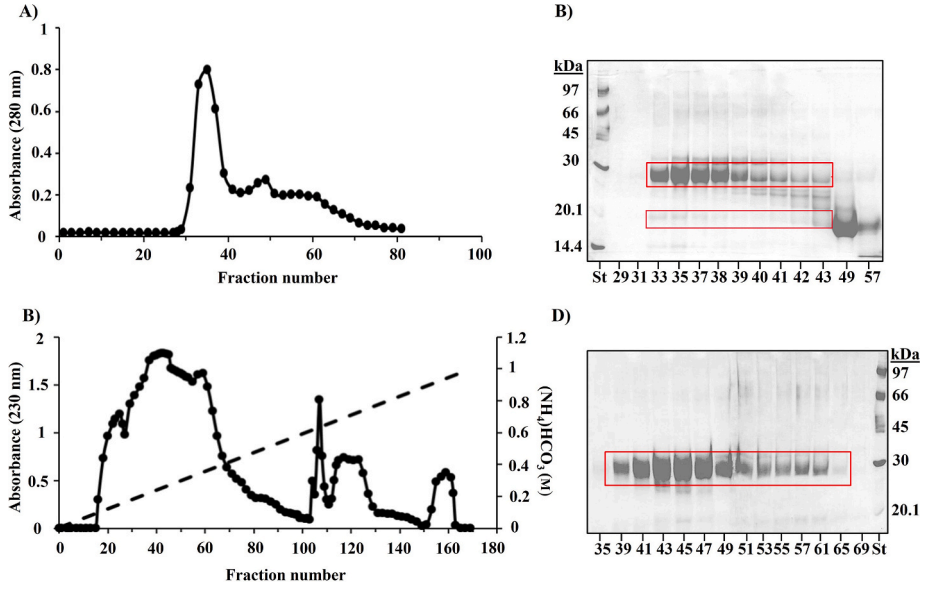

Fig. 1. Isolation of bovine proteose-peptone component 3 (PP3). A) Elution profile obtained from the Sephadex G-75 column. B) Elution profile obtained from the Q-Sepharose chromatography with a linear gradient of $(NH_4)HCO_3$ (dashed line). The sample loaded in the chromatography was the fraction enriched in PP3 from the filtration in Sephadex G-75. C, D) SDS-PAGE in 12 % polyacrylamide gels of the fractions obtained after Sephadex G-75 and Q-Sepharose chromatographies, respectively; the bands corresponding to PP3 are framed with a red box (Coomassie blue staining). The numbers below the lanes of the gel correspond to the chromatography fraction numbers. St: low molecular weight standard (kDa).

and Petersen (1993b). Furthermore, the band of 28 kD was obtained from the gel and identified as ruminant glycosylation-dependent cell adhesion molecule 1 or milk glycoprotein PP3 by proteomic analysis.

The ovine PP3 was isolated by the same procedure as the bovine counterpart, with similar elution profiles. The electrophoretic pattern of the ovine milk derived fractions obtained at each stage of isolation can be seen in Fig. 3, where the ovine PP3 is shown with its two fragments, with apparent molecular weights at 28 and 17 kDa (Fig. 3, lane 4). It is worth noticing the greater intensity of the electrophoretic band of the 17 kDa fragment in the SDS-PAGE of the ovine PP3 compared to that of the bovine counterpart. This difference in the intensity of the 17 kDa band between bovine and ovine PP3 isolates has also been observed previously (Sørensen, Rasmussen, Møllerm, & Petersen, 1997). Markedly, the Western blot analysis of the ovine PP3 revealed the presence of an additional band at aproximatelly 26 kDa (Fig. 3B, lane 4) which was not visible in the electrophoretic profile of the same sample (Fig. 3A, lane 4). It is possible that the ~26 kDa band could represent a contaminant protein in the sample, although the isolation process followed was the same for bovine and ovine samples.

3.2. Enzymatic hydrolysis of bovine and ovine proteose peptone component 3

The enzymatic hydrolysis of bovine and ovine PPT and PP3 was carried out to evaluate the biological activity of hydrolysates compared to intact proteins. The efficiency of hydrolysis was evaluated by electrophoresis. As can be seen in Fig. 4, the profile of the hydrolyzed samples shows very faint bands (almost negligible) in the area corresponding to a molecular weight of less than 10 kDa. In comparison with their respective untreated samples, it can be concluded that hydrolysis was effective on all the fractions.

The protocol for the hydrolysis of PP3 followed the methodology previously described in the studies of Pedersen et al. (2012a, 2012b), where the peptide named Lpcin P was purified from trypsinated PP3 and contained the residues 110–135 of PP3. In those studies, analysis using circular dichroism showed that PP3 and Lpcin P adopted a conformation calculated to contain 27.4 and 22 % α-helical structure in aqueous solution, respectively. Notably, these percentages increased in the presense of anionic and organic solvents and, in the case of Lpcin P, also in the presence of lipid vesicles. Furthermore, the same group showed that the α-helical structure of PP3 either refolded to or remained in its native state after being exposed to extreme temperatures and pH conditions.

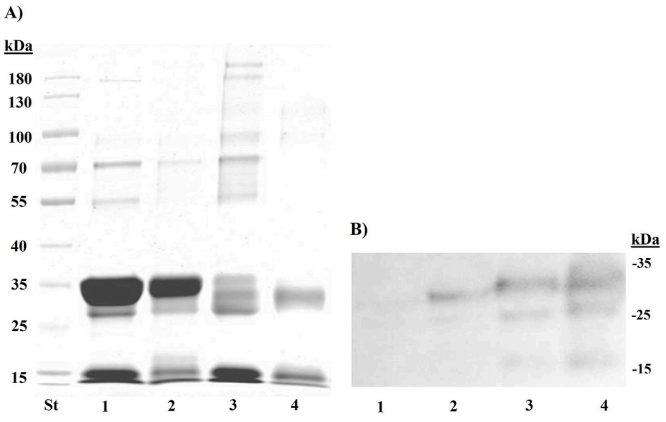

Fig. 3. A) SDS-PAGE in 12 % polyacrylamide gel of the fractions obtained in the isolation of ovine proteose-peptone component 3 (PP3) (Coomassie blue staining). B) Western-blotting with anti-ovine PP3 antibody. Fractions: St: low molecular weight standard (kDa); 1. Raw ovine milk; 2. PPT; 3. Sephadex G-75 fraction (number 25); 4. PP3: Q-Sepharose fraction (number 34).

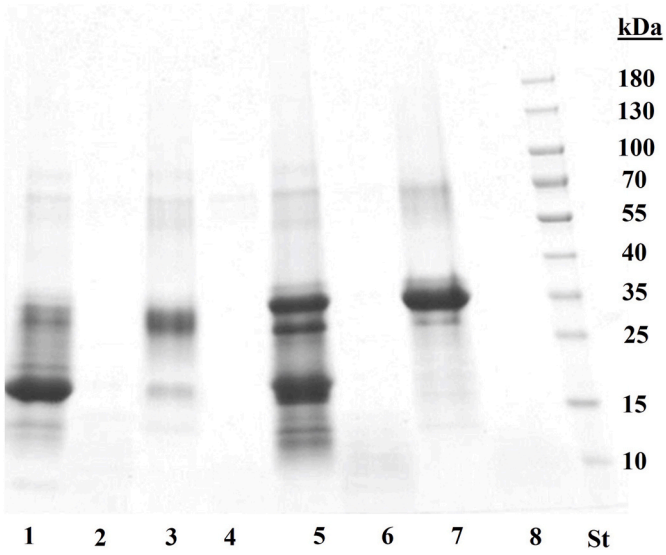

Fig. 4. SDS-PAGE in 12 % polyacrylamide gel of proteose-peptone (PPT), proteose-peptone component 3 (PP3) and their respective hydrolyzed samples from bovine and ovine origin. Fractions: 1. Bovine PPT; 2. Hydrolyzed bovine PPT; 3. Bovine PP3; 4. Hydrolyzed bovine PP3; 5. Ovine PPT; 6. Hydrolyzed ovine PPT; 7. Ovine PP3; 8. Hydrolyzed ovine PP3.

3.3. Antioxidant activity of bovine and ovine proteose peptone and proteose peptone component 3

The antioxidant activity of bovine and ovine PPT was determined at various concentrations from 0.1 to 20.0 mg mL^{-1} and the results are depicted in Fig. 5A. This allowed for adjusting the concentrations of PP3 and its hydrolyzed samples for subsequent tests. The results showed that both bovine and ovine PPT exerted antioxidant activity, measured as reduction of the DPPH radical, in a dose-response manner. Overall, bovine and ovine PPT showed similar antioxidant activity. However, there were some differences in activity at the concentrations of 10 and 20 mg mL^{-1}, at which ovine PPT had significantly higher values compared to the bovine protein, while the opposite was observed for the concentration of 1 mg mL^{-1}.

Afterwards, the antioxidant activity of bovine and ovine PP3 was also evaluated. As can be seen in Fig. 5B, the two proteins presented a dose-response profile. At the lowest concentration tested (0.1 mg mL^{-1}) there was no significant difference between the activities of bovine and ovine PP3; however, the latter presented significantly higher activity at 1 and 2.5 mg mL^{-1}.

Finally, the antioxidant activity of the different samples (PPT, PP3 and hydrolysates) at a fixed concentration of 2.5 mg mL^{-1} was compared, as shown in Fig. 6. It is interesting to note the inter-species variability. While bovine samples lost the antioxidant activity with the isolation and purification process (PPT > PP3 > hydrolyzed PP3), the ovine sample did not show the same pattern (PP3 > PPT > hydrolyzed PP3). In general, the ovine PP3 and its hydrolyzed fractions presented significantly higher antioxidant activity than the bovine counterparts, while the activity of PPT was similar regardless of the origin of the milk.

3.4. Antibacterial activity of bovine and ovine proteose peptone component 3 and their hydrolyzed fractions

In the present study, the antibacterial activities of bovine and ovine PP3 and of their respective hydrolysates have been evaluated against *C. sakazakii*. Bovine PP3 only presented antibacterial activity when added at 8 mg mL^{-1} after 4 h of incubation at 37 °C, significantly reducing the bacterial growth by 0.43 logarithmic cycles (Fig. 7A), compared to the control.

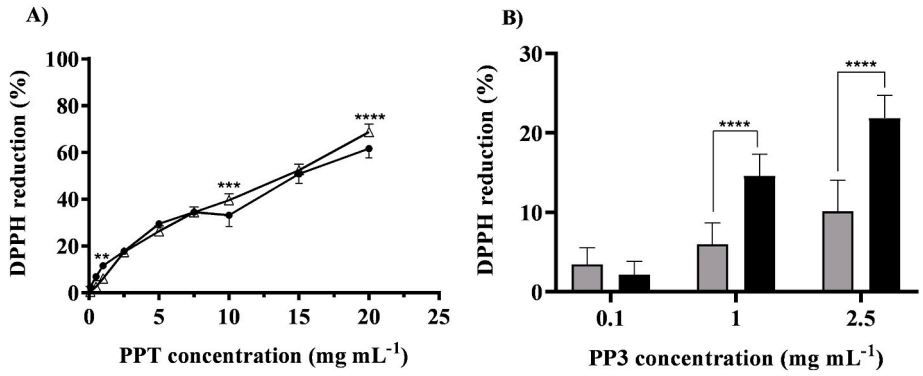

Fig. 5. Antioxidant activity of A) proteose-peptone (PPT) of bovine (white triangles) and ovine (black circles) origin and B) proteose-peptone component 3 (PP3) of bovine (grey bars) and ovine (black bars) origin. Results are presented as mean ± standard deviation of six replicates in three independent experiments. Significant differences between the means of same concentrations are indicated with: $^{**}p < 0.01$; $^{***}p < 0.001$ and $^{****}p < 0.0001$.

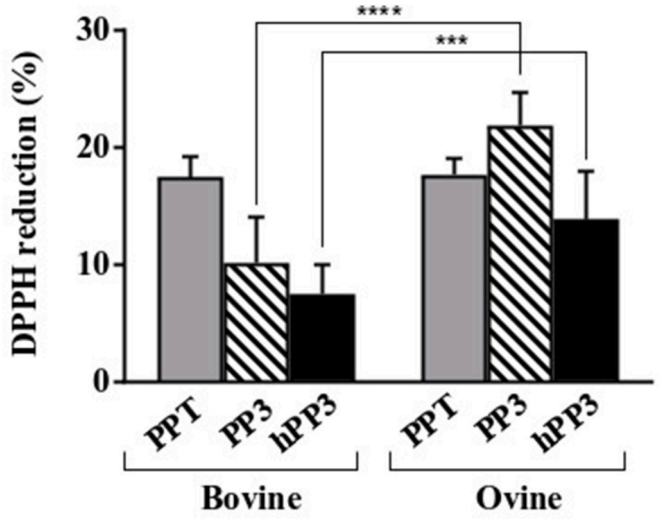

Fig. 6. Comparison of antioxidant activity of proteose-peptone (PPT, grey bars), proteose-peptone component 3 (PP3, dashed bars) and the hydrolyzed fraction of PP3 (hPP3, black bars) from bovine and ovine origin. The samples were analyzed at a concentration of 2.5 mg mL^{-1}. Results are presented as mean ± standard deviation of six replicates in three independent experiments. Significant differences between the means of same protein samples are indicated with: $^{***}p < 0.001$ and $^{****}p < 0.0001$.

However, when bovine PP3 was hydrolyzed with trypsin, a considerable increase in the antibacterial activity was observed compared to intact PP3 (Fig. 7B). After 2, 3 and 4 h incubation intervals, the inhibition of *C. sakazakii* presented by all samples was significantly higher compared to the control. The greatest inhibitory effect corresponded to the concentration of 4 mg mL^{-1} after 24 h of incubation at 37 °C, with a growth reduction of 6.82 logarithmic cycles. Overall, a dose-response effect was observed for each time interval (4, 8 and 24 h).

Surprisingly, the activity of the ovine samples (PP3 and hydrolyzed PP3) was very different from those of bovine origin. Thus, no inhibition of *C. sakazakii* growth was observed at any ovine protein concentration or incubation interval evaluated (Fig. 7C and D). In contrast, a small but significant increase in the logarithmic cycles was observed in the incubation for 4 h with ovine PP3 at all concentrations, and in all cases with the hydrolyzed PP3.

While the antibacterial potential of bovine lactophoricin has been previously demonstrated, there are no reports considering that of the ovine counterpart. Campagna et al. (2004) found that a commercial lactophoricin peptide corresponding to the PPT fragment 113–135, exerted potent concentration-dependent antibacterial activity against several gram-positive (*Streptococcus thermophilus, Listeria innocua* and *Staphylococcus aureus*) and gram-negative (*Pseudomonas aeruginosa* and *Salmonella* St Paul) bacteria. In their study, a concentration range of 0.14–678 μM (approximately 0.38–1840 μg mL^{-1}) and a 24 h incubation time at 37 °C was applied. The authors attributed the relatively high peptide concentrations required in some cases to limit bacterial growth, which was also observed in the present study, to the alteration of the peptide structure by bacterial proteases excreted in the medium or present at the cell surface, finding previously reported in the study of Andreu and Rivas (1998). Furthermore, in a comparative study, Pedersen et al. (2012a) investigated the membrane-perturbing activities of trypsin-hydrolyzed PP3 (named Lpcin P) and full-length PP3 by their ability to permeabilize large unilamellar vesicles using the calcein-release assay. It was found that, while both molecules were able to permeabilize the vesicles, the release caused by PP3 was much slower (on the scale of minutes) and reached a plateau of only approximately 50 %, illustrating that PP3 needs to be activated by proteolytic cleavage to efficiently permeabilize lipid bilayers. These authors also showed that Lpcin P produced a marked reduction in the growth of *Streptococcus thermophilus,* but did not inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*, while the PP3 full-length protein had no antibacterial effect against any of those bacteria.

3.5. Antirotaviral activity of bovine and ovine PP3 and their hydrolyzed fractions

In the present study, the antirotaviral activity of bovine and ovine PP3 and of their respective hydrolyzed fractions has been evaluated. The neutralization assay has been carried out with the bovine rotavirus strain WC3 on the MA104 cell line, and the detection of the infection extent was visualized by an indirect immunofluorescence assay, based on a technique previously validated in our laboratory (Parrón et al., 2016). The results of the effect of bovine and ovine PP3 and their respective hydrolysates at several concentrations against rotavirus are shown in Fig. 8. The inhibitory activity was found greater for bovine samples, compared to the ovine ones, at all concentrations tested. Furthermore, it is interesting to highlight the increase in activity of the bovine hydrolyzed PP3, in comparison to the intact protein, whereas for the ovine samples, the hydrolyzed PP3 showed less activity than its respective intact PP3.

With regards to the activity of bovine PP3 against the WC3 rotavirus, it was observed that at a concentration of 1.5 mg mL^{-1} it exerted 58 % neutralization, which reached approximately 75 % at the highest concentration (3 mg mL^{-1}). However, for the ovine PP3, higher concentrations were needed (9 mg mL^{-1}) for a 67 % neutralization to be achieved. The bovine and ovine PP3 had an IC_{50} of 0.94 and 3.05 mg mL^{-1}, respectively. When the proteolytically treated bovine PP3 was tested, it demonstrated higher antirotaviral activity compared to its

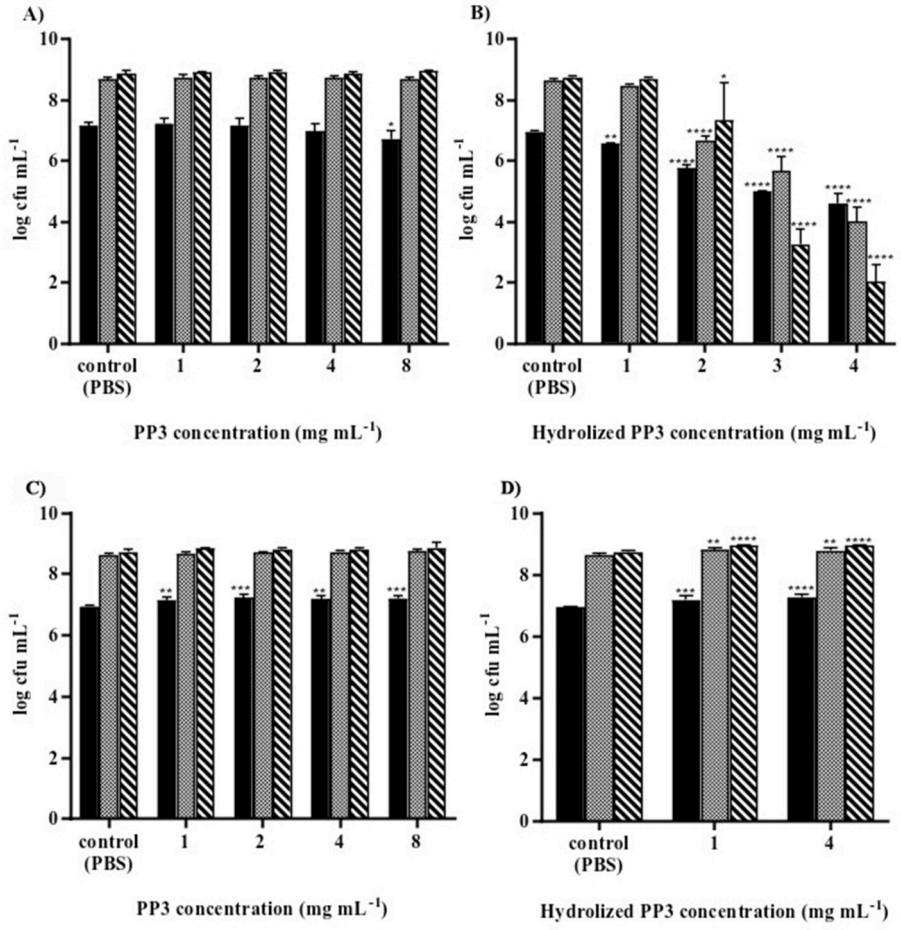

Fig. 7. Antibacterial activity of A) bovine proteose-peptone component 3 (PP3); B) bovine hydrolyzed PP3; C) ovine PP3 and D) ovine hydrolyzed PP3 against *Cronobacter sakazakii* at different incubation intervals: 4 h (black bars); 8 h (checkerboard bars); 24 h (dashed bars). Results are presented as mean ± standard deviation of three replicates in two independent experiments (n = 6). Significant differences between the means of each sample in comparison to the control constituted of phosphate buffer saline (PBS) are indicated with: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ and $****p < 0.0001$.

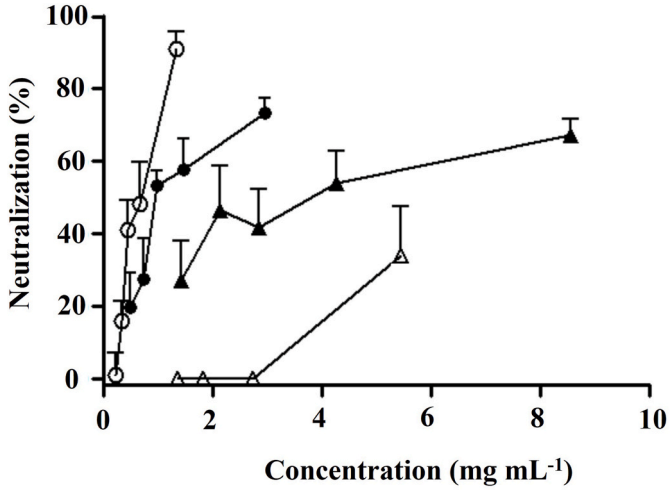

Fig. 8. Dose-response of the activity of bovine and ovine proteose-peptone component 3 (PP3) and of their respective hydrolyzed fractions on neutralizing the bovine rotavirus WC3 strain MA104 cells infection. Bovine PP3 (black circles); hydrolyzed bovine PP3 (white circles); ovine PP3 (black triangles); hydrolyzed ovine PP3 (white triangles). Results are shown as mean ± standard deviation of four replicates in three independent experiments (n = 12).

intact form in all tested concentrations, with a maximum of approximately 90 % neutralization at 1.5 mg mL^{-1} and an IC_{50} of 0.72 mg mL^{-1}. Finally, the ovine hydrolyzed PP3 had a minor effect in rotavirus infection with a mild inhibition observed only at 5.5 mg mL^{-1} (35 %).

There are many studies showing that various dairy fractions and proteins present antirotavirus activity (Graikini, García, et al., 2024; Kvistgaard et al., 2004; Parrón et al., 2018). To the best of the authors' knowledge, there are no publications regarding the antirotaviral activity of PP3 from ovine origin; however, some studies have been conducted considering the bovine protein. Therefore, the results obtained in the present study are in good accordance with those reported previously by Parrón et al. (2017), who used similar infection conditions. In addition, Inagaki et al. (2010) identified PP3, and more specifically a 16 kDa N-terminal fragment, as one of the components responsible for the inhibitory activity of a high molecular weight glycoprotein derived from bovine whey against replication of the human rotavirus strain MO, but the exact mechanisms of action of this fraction was unclear. However, their study showed inhibitory values higher than those reported by Parrón et al. (2017) and those of our study. Thus, in the study of Inagaki et al. (2010), a ~50 % inhibition at 1 μg mL^{-1} and >70 % at 10 μg mL^{-1} was achieved; however, the potential of an enzymatic derivative of that molecule has not been evaluated.

PP3 is a heavily glycosylated molecule and contains variable levels of sialic acid (Girardet et al., 1995). It exhibits a great microheterogeneity of the glycan structures. Specifically, it has been recently reported that PP3 contributes approximately 78% of the total number of glycans in the

overall whey protein N-linked pool obtained from cows (Valk-Weeber et al., 2021). It contains a common octasaccharide core comprised by *N*-acetylglucosamine (GlcNAc), fucose (Fuc) and mannose (Man) units linked to *N*-acetylgalactosamine (GalNAc), galactose (Gal) or *N*-acetylneuraminic acid (NeuAc) as terminal units, as it has been determined by NMR (Girardet et al., 1995). A comparative analysis on the carbohydrate composition of PPT (or the "acid stable, heat-stable polypeptides" as referred in the study) obtained from bovine and ovine milk, showed that the carbohydrate content of bovine PPT, 4.00 mg 100 mg^{-1} of protein, was lower than that of the ovine counterpart, 7.30 mg 100 mg^{-1} of protein. However, the percentage of sialic acids in the total carbohydrate content was higher in bovine milk than in ovine milk (Ramos, Sánchez, Olano, Sanz, & Martinez-Castro, 1988).

Sialic acids are of vital importance for the growth and development of the newborn (Wang & Brand-Miller, 2003) and have been correlated with a variety of biological functions (Nwosu et al., 2012; Varki et al., 1999); however, the exact mechanism of their action is still not fully elucidated. The *N*-glycans are known to interact with pattern-recognition receptors of the innate immune system, serving as immune-modulating agents (de Kvit, Kraneveld, Garssen, & Willemsen, 2001; Figueroa-Lozano, Valk-Weeber, van Leeuwen, Dijkhuizen, & de Vos, 2018; Figueroa-Lozano et al., 2020) and, interestingly, their modification with sialic acids is known to affect this interaction with receptors (Varki & Gagneux, 2012; Falconer, Subedi, Marcella, & Barb, 2018). In this view, a recent study conducted by our research group, has shown that in the case of lactoferrin, another heavily glycosylated and sialylated whey protein, there is a direct relationship between rotavirus neutralization activity and its content of sialic acid, regarding lactoferrins from different mammalian origin (Graikini, Conesa, Abad, Pérez, & Sánchez, 2024). Notably, the first step of rotavirus infection in the host cell involves interactions of the viral surface protein VP4 (VP8 domain) with a sialylated cellular receptor, thus mediating the initial binding of the infectious particle to the cell surface. In the post-binding phase, both VP4 (VP5 domain) and VP7 interact with several cell surface molecules, including integrins and gangliosides, which seem to act as cellular co-receptors that allow the virus to gain access into the cell (Isa et al., 2006). Therefore, it can be hypothesized that one of the mechanisms of rotavirus inhibition might be through the interaction of the glycans and/or the terminal sialic acids on the PP3 or hydrolyzed PP3 molecule with the viral surface proteins at an early stage of the infection. Furthermore, the differences in the overall antimicrobial activity of the bovine and ovine PP3 and their different response on the hydrolysis can be attributed to the different glycosylation and sialylation profiles of molecules of the two species.

4. Conclusions

Bioactive peptides are a rapidly developing field of research which should contribute significantly to the availability of functional foods on the market in the near future. Accordingly, the present study, expanded the current knowledge about the bioactivity of bovine and ovine PPT generated peptides and fractions. Thus, enzymatic hydrolysis of bovine PP3 generated antimicrobial peptides against *C. sakazakii* and rotavirus. Hydrolyzed ovine PP3, on the other hand, presented low bioactivity against these microbes, but possessed high antioxidant activity. The latter is a rather important finding since there is limited information with respect to the bioactivity of ovine milk peptides. However, further research should be done in order to identify which peptides are formed during the digestion that could exert bioactivity and also to obtain a better understanding of their mechanism of action in more complex biological systems.

CRediT authorship contribution statement

D. Graikini: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **M. Hadidian:** Methodology, Investigation. **I. Abad:** Methodology, Investigation. **J.A. Parrón:** Methodology, Investigation, Data curation. **D. Ripollés:** Software, Methodology, Investigation, Data curation. **M.D. Pérez:** Writing – review & editing, Conceptualization. **M. Calvo:** Funding acquisition, Conceptualization. **L. Sánchez:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the paper entitled " Comparative study on the biological activity of bovine and ovine PP3 and of their hydrolysates " by Graikini et al.

Data availability

Data will be made available on request.

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