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PII: S1466-8564(17)31157-8
DOI: doi:10.1016/j.ifset.2018.03.008
Reference: INNFOO 1945

To appear in: *Innovative Food Science and Emerging Technologies*

Received date: 11 October 2017
Revised date: 19 February 2018
Accepted date: 6 March 2018

Please cite this article as: José Antonio Parrón, Daniel Ripollés, Sergio José Ramos, María Dolores Pérez, Miguel Calvo, Lourdes Sánchez , Effect of thermal and high-pressure treatments on the antirotaviral activity of human milk fractions. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Innfoo(2017), doi:10.1016/j.ifset.2018.03.008

Effect of thermal and high-pressure treatments on the antirotaviral activity of human milk fractions

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ABSTRACT

Rotaviral gastroenteritis is associated with high rate of infant mortality and morbidity. Antirotaviral activity has been associated with some glycoproteins, such as immunoglobulins A (IgA), lactoferrin (LF), mucins and lactadherin of human milk. Although holder pasteurization (HoP, 63 ºC for 30 min) is the treatment currently applied to human milk, it ~~might~~ may lead to a decrease of its bioactive properties. The antirotaviral capacity of human milk ~~appears~~ showed to be mainly associated with the whey fraction, focusing on IgA and LF, with neutralizing values of 100, 100 and 62%, at 1 mg protein/mL, respectively. HoP reduced the antirotaviral activity of human whey, IgA and LF, 30, 98 and 60%, respectively. Interestingly, ~~both pasteurization (HTST) and HHP treatments were less harmful. Thus,~~ ~~H~~high ~~T~~temperature-~~S~~short ~~T~~time (HTST) pasteurization at 75 ºC for 20 s did not affect the antirotaviral activity of samples, while the highest HHP treatment at 600 MPa for 15 min only reduced the activity of human whey, IgA and LF, 9, 40 and 10%, respectively.

Keywords: Human milk / Antirotaviral activity / HoP / HTST / HHP

Abbreviations: α-LA, α-lactalbumin; HoP, Holder pasteurization; HTST, high temperature-short time; HHP, High hydrostatic pressure; ~~Igs, Immunoglobulins;~~ IgA, Immunoglobulins A; LDH, Lactadherin; LF, Lactoferrin; Lyz, Lysozyme; MEM, Minimum essential medium; MFGM, Milk fat globule membrane; MUCs, Mucins; PBS, Phosphate-buffered saline; rhLF, Recombinant human lactoferrin; rhLyz, Recombinant human lysozyme; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

1. Introduction

Rotaviruses are the main cause of severe diarrheal disease in infants and young children, and the second cause of death in children less than 5-years-old (Marcotte & Hammarström, 2016). The morbidity and mortality caused by viral gastroenteritis represent a major public health concern, affecting mainly the poorest countries (Das, Salam, & Bhutta, 2014).

It has been demonstrated that the entry of rotaviruses into intestinal epithelial cells is a multistep process involving the ~~virus~~ outermost ~~layer~~ proteins of the virus, VP7 and VP4, and several cell surface molecules~~,~~ with different requirements depending on the rotavirus strain (Arias, Silva-Ayala, Isa, Díaz-Salinas, & López, 2016). The virus replicates in the enterocytes of the small intestine, causing extensive damage to the microvilli and resulting in malabsorption and loss of fluids and electrolytes. The consequences of the infection include severe dehydration, cerebral edema, hypovolemic shock, renal failure and even death (Bishop, 2009).

The oral introduction of ~~oral~~ live attenuated rotavirus vaccines has significantly reduced the incidence of the disease, especially in developed countries (Payne et al., 2013). ~~Despite this,~~ However, due to financial and logistic problems these vaccines are scarcely used in lower income countries, leaving between a third to a half of children unprotected from severe rotavirus disease ~~in countries with limited resources have left between a third to half of all vaccinated children unprotected~~ (Babji & Kang, 2012). ~~what highlights~~ Thus, the need for alternative approaches to face the rotaviral gastroenteritis disease is of high priority.

It is widely accepted that breastfeeding is the optimal way to provide infants with the essential nutrients for healthy growth and development, also protecting them against a broad spectrum of pathogens (Liu & Newburg, 2013; Peterson, Cheah, Grinyer, & Packer, 2013). When mothers cannot breastfeed their children, the nutrition recommended by the World Health Organization (WHO) can be delivered by ~~is~~ donated breast milk. This is a better

alternative than infant formula, at least for the first six months and especially for low-birth-weight premature infants (Sousa, Delgadillo, & Saraiva, 2014b; WHO, 2003). In particular, the protein fraction of human milk plays an important role on its bioactivity (Lönnerdal, 2016). Caseins and whey proteins represent approximately 40% and 60% of the total protein in human milk, the latter containing the majority of bioactive proteins like α-lactalbumin (α-LA), lactoferrin (LF), immunoglobulins ~~(Igs)~~, human serum albumin, and lysozyme (Lyz). Milk fat globule membrane (MFGM) proteins, including mucins (MUCs), butyrophilin and lactadherin (LDH) represent less than 5% of human milk proteins (Peterson et al., 2013).

One of the ~~protection~~ protective mechanisms attributed to human milk is associated ~~to~~ with glycoconjugates, mainly oligosaccharides and glycoproteins, whose glycan moieties may act as decoy receptors ~~mimetics~~, hampering pathogen binding to the cell surface (Liu & Newburg, 2013; Lönnerdal, 2016). While the protective activity of human milk oligosaccharides has been well established (Kunz, Kuntz, & Rudloff, 2014), human milk glycoproteins have received less attention, even though they seem to play important bioactive roles. ~~At this respect,~~ Although some studies have indicated the role of breastfeeding as a protective factor against rotavirus infection (De Franco et al., 2013; Gianino et al., 2002), there are few studies regarding the antirotaviral activity of the majority of human proteins. Most studies focus on ~~those being mainly focused on~~ IgA (Asensi, Martínez-Costa, & Buesa, 2006; De Franco et al., 2013) or MFGM proteins MUC1 and LDH (Kvistgaard et al., 2004; Newburg et al., 1998; Yolken et al., 1992).

In order to guarantee the hygienic quality of human milk, a pasteurization process at 62.5 ºC for 30 min (holder pasteurization, HoP) is currently applied in human milk banks (Arslanoglu et al., 2010). Nevertheless, there are several studies reporting that thermal treatments usually applied to ~~hygienization~~ pasteurization of human milk ~~might~~ may lead to a

reduction of its beneficial and protective properties, affecting important biological components, such as proteins and in particular, immunoglobulins (Peila et al., 2017).

Consequently, it would be advisable to develop treatments for human milk that can ensure microbial inactivation, while preserving its nutritional, immunological, and bioactive properties. At this respect, different alternative processing methods are currently being studied in order to evaluate their effect on human milk safety and quality, including ~~H~~high ~~T~~temperature-~~S~~short ~~T~~time (72-75 ºC, 15-20 s, HTST) pasteurization and ~~H~~high ~~H~~hydrostatic ~~P~~pressure (HHP) treatments. These treatments seem to maintain most organoleptic and nutritional properties of milk, causing minor degradation of proteins and vitamins than other treatments (Holsinger, Rajkowski, & Stabel, 1997). HHP treatment is a non-thermal processing method that can provide microbiologically safe, nutritionally intact, and products of high sensory quality ~~of high-quality products~~ by applying pressures in the range of 400-600 MPa for 5-10 min (Sousa et al., 2014b). The pressure-induced sterilization and inactivation of non-enveloped and enveloped viruses, including rotaviruses, have been demonstrated in several studies (Pontes et al., 2001; Silva et al., 2014).

Although there are several studies on the effect of technological treatments on the protein content of human milk (Peila et al., 2017), to our knowledge there are no studies focusing on its antirotaviral potential. The aim of the present study ~~has been to prove~~ was to evaluate the rotavirus-neutralizing activity of different human milk fractions and proteins, such as IgA, LF, Lyz, α-LA, MUCs, LDH and caseins, and ~~to evaluate~~ compare the effects of several heat and ~~HHP~~ pressure treatments on ~~their~~ antirotaviral activity. This activity ~~has been~~ was determined measuring the ability of milk fractions and proteins to inhibit the infection of MA104 cells by the WC3 bovine rotavirus strain.

2. Materials and methods

2.1. Cell culture and rotavirus propagation

Cell culture and rotavirus propagation have been performed following the procedures described in Parrón et al. (2016). The Rhesus monkey kidney cell line MA104 (ATCC CRL-2378) was used to propagate WC3 bovine rotavirus strain (ATCC VR-2102). Cells were cultured in minimum essential medium (MEM), supplemented with 10% heat-inactivated fetal bovine serum, 1% antibiotics (100 Units/mL penicillin, 100 μg/mL streptomycin), 2 mM L-glutamine, and 0.25 μg/mL amphotericin B. Rotavirus propagation was carried out by inoculating confluent cell monolayers with an aliquot of virus suspension. Serum-free MEM, supplemented with 1% antibiotics, 2 mM L-glutamine, and 0.25 μg/mL amphotericin B was used as diluent in all steps. All cell culture media and supplements were purchased from Gibco (Life Technologies Corporation, Paisley, UK).

2.2. Obtaining human milk fractions: skimmed milk, whey, caseins and MFGM

Transitional (6-15 days after birth) and mature (> 15 days after birth) human milk samples from ten healthy donors were kindly donated and stored at -20 ºC. The Ethical Committee for Clinical Research of the Government of Aragón (CEICA) approved the study and all donors provided informed consent. Frozen human milk was thawed and skimmed by centrifugation at 2500 *g* for 15 min at 4 ºC. Whey was obtained from skimmed milk after precipitation of casein fraction as previously described (Svensson et al., 1999). In brief, human skimmed milk was supplemented with 10% (w/v) potassium oxalate and incubated at 4 ºC overnight. After lowering the pH to 4.3, heating to 32 ºC for 2 h, and incubating at 4 ºC overnight, the casein fraction was precipitated by centrifugation at 5000 *g* for 15 min at 20 ºC. The casein fraction was then washed three times with distilled water and dialyzed against phosphate-buffered saline (PBS) consisting of 0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 8.5. Human whey was filtered through glass wool and dialyzed against PBS pH 7.2. Human MFGM was isolated basically as described by Kvistgaard et al. (2004). Briefly, cream was obtained by centrifugation of human milk for 20 min at 5000 *g*, and washed twice with

10 mM Na_2PO_4, 0.15 M NaCl, pH 7.2. After intense churning and sonication of washed material, human MFGM was obtained by centrifugation at 50000 *g* for 1 h. All fractions were stored at -20 ºC until analysis.

2.3. Purification of human proteins

Human immunoglobulins A (IgA) were isolated by means of conventional fractionation using size-exclusion chromatography, according to Pack (2000). Thus, human ~~Igs~~ immunoglobulins were pelleted from whey by adding ammonium sulfate to 50% (w/v), followed by incubation for 16 h at 4 ºC and centrifugation at 15000 *g* for 1 h at 4 ºC. Afterwards, the pellet was dissolved and dialyzed against 0.02 M Tris-HCl buffer, pH 9.5, and further applied onto a DEAE-Sepharose column equilibrated with the same buffer. Bound IgA were eluted using the Tris buffer with a linear gradient from 0.10 to 0.25 M NaCl.

Human lactoferrin (LF) was purified using cation exchange chromatography on SP-Sepharose following the procedure of Conesa et al. (2008). In brief, milk was skimmed by centrifugation at 2500 *g* for 30 min at 4 ºC, diluted 1:1 with a buffer containing 0.04 M NaH_2PO_4, 0.8 M NaCl, 0.04% (v/v) Tween 20, pH 7.4, and batchwise incubated with SP-Sepharose at 4 ºC overnight. Afterwards, the SP-Sepharose was packed onto a column and washed with a 0.02 M NaH_2PO_4 buffer, with 0.4 M NaCl, 0.02% (v/v) Tween 20, pH 7.4, to wash out the unbound proteins. LF was eluted with a buffer containing 0.02 M NaH_2PO_4, 1 M NaCl, pH 7.4. The iron saturation of LF, estimated from the ratio of absorbances at 280 and 465 nm, was less than 15%.

Three forms of recombinant human LF (rhLF) were also tested. Two forms were obtained from rice and kindly provided by Ventria Bioscience (Sacramento, CA, USA), in the holo form (~80% iron saturation) and as-isolated from rice (~60% iron saturation). Another rhLF from *Aspergillus awamori* (~18% iron saturation) was provided by Agennix (Houston, TX, USA). These proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) showing a main band corresponding to a ~80 kDa protein, so they were used in the experiments without further purification.

Human lysozyme (Lyz) was essentially obtained from human skimmed milk through cation exchange chromatography on SP-Sepharose, as described above for human LF purification, followed by gel-filtration on Sephadex G-50 of fractions excluded from SP-Sepharose column. A recombinant human Lyz (rhLyz) obtained from rice was kindly supplied by Ventria Bioscience, and tested in this study after checking its purity, which was above 95% as determined by SDS-PAGE (~14 kDa).

Human α-lactalbumin (α-LA) was isolated according to Barbana et al. (2008). In short, human whey was obtained after skimming by centrifugation at 2000 *g* for 30 min at 4 ºC and further casein separation by adding $CaCl_2$ and ultracentrifugation at 100000 g for 2 h at 4 ºC. Whey was then subjected to gel filtration chromatography on a Sephadex G-100 column equilibrated with 25 mM sodium acetate buffer, with 50 mM NaCl, pH 6.5, at 4 ºC, and further chromatographed under the same conditions on a Sephadex G-50 column.

A fraction enriched in human mucins (MUCs) was obtained from human MFGM through an adapted method based on Le et al. (2012), including an initial step of enzymatic degradation by incubating first with pepsin (Sigma, St. Louis, MO, USA) at 0.4:100 (w/w) enzyme:protein ratio, for 2 h at 37 ºC with continuous shaking. Afterwards, the mixture was incubated with trypsin (Sigma) at 1:100 (w/w) enzyme:protein ratio, and α-chymotrypsin (Sigma) at 1:100 (w/w) enzyme:protein ratio, for 3.5 h at 37 ºC. Samples were then subjected to ultrafiltration through 30000 MWCO membranes, in order to remove small proteins and peptides.

A fraction enriched in human lactadherin (LDH) was obtained by resuspension of MFGM with PBS containing 0.02 M NaCl and further centrifugation at 25000 *g* for 1 h at 4 ºC. The

resulting supernatant was enriched in LDH. All protein fractions were dialyzed against distilled water, lyophilized and stored at -20 ºC until analysis.

2.4. Rotavirus infectivity assay

The *in vitro* rotavirus infection assay was performed as previously described in the study of Parrón et al. (2016), in which the assay was validated according to international guidelines. Briefly, MA104 cells were seeded into 96-well plates and incubated near confluence. Appropriate dilutions of human milk fractions and proteins were mixed (1:1) with trypsin-activated rotavirus suspension and incubated for 1 h at 37 ºC, to evaluate their neutralizing activity. Afterwards, the samples were transferred into the 96-well cell culture plates and incubated at 37 ºC during 1 h for rotavirus adsorption. Serum-free MEM and rotavirus suspension were included as negative and positive controls, respectively. For cell infection, plates were added with 100 μL per well of MEM containing 2 μg/mL of trypsin and 2% heat inactivated fetal serum, before incubating at 37 ºC in 5% CO_2 for 16 h with gentle rotation. Virus-infected cells were detected afterwards by immunofluorescence. The infectivity percentages were determined by enumerating fluorescent foci (infected cells) in each well using a fluorescence microscope (Eclipse E400) with a FITC-compatible filter, by image analysis using the Zen lite 2012 software.

2.5. Treatment of human fractions

The protein concentrations of the samples selected for thermal and HHP treatments were those showing neutralization activity above 80%: pooled human whey at 2 mg protein/mL, IgA at 0.1 mg protein/mL, and LF at 2 mg protein/mL. The heat treatment procedure was based on that previously used (Parrón et al., 2016). The samples were placed in glass vials (~1 mL) and heated in a thermostatic bath (± 0.1 ºC) at: 45 and 55 ºC for 60 min, 63 ºC for 30 min, 72 ºC from 20 s to 60 min, and 75, 80 and 85 ºC for 20 s and 10 min. Temperature was

monitored inside the samples with a digital thermometer. After heating, the samples were cooled rapidly in an iced water bath and stored at -20 ºC until analysis.

High pressure treatment procedure was based on that previously described by Mayayo et al. (2016). Aliquots of pooled human whey (2 mg protein/mL), IgA (0.1 mg protein/mL), and LF (2 mg protein/mL) were introduced into 2-mL polyethylene bags. After eliminating headspace, bags were heat-sealed and pressurised using a Stansted Fluid Power FPG 11500 B (Stansted, Essex, United Kingdom), with propylene glycol/water (70/30, v/v) as the pressure-transmitting fluid. The chamber volume was of 30 mL. Pressure was raised to 400, 500 or 600 MPa and maintained at those values for 5, 10 and 15 min. The pressure was increased at 240 MPa/min, which gave come-up times of 1.6, 2.1 and 2.5 min at 400, 500 and 600 MPa, respectively. The initial temperature of transmitting fluid and samples was equilibrated at 20 ºC, and the approximate temperature increase was 3 ºC per 100 MPa. HHP-treated samples were kept at 4 ºC until analysis.

2.6. SDS-PAGE and Western-blotting

Protein profiles of milk fractions and proteins were analyzed by SDS-PAGE using 4-20% polyacrylamide gels (Mini-Protean TGX, Bio-Rad Laboratories, Hercules, CA, USA), which were Coomassie and Periodic Acid Schiff (PAS) stained, according to standard procedures. Western-blotting procedure was performed to confirm the protein identity, in essence as described by Benfeldt et al. (1995) using PVDF membranes (Immobilon-P, Millipore Corporation, Bedford, MA, USA). Quantification of protein concentration was done by BCA Protein Assay (Pierce Kit, Thermo Scientific). The compositional values of human milk were obtained by infrared spectroscopy using a MilkoScan 4000 (Foss Electric, Hilleroed, Denmark).

2.7. Statistical analysis

Data were analyzed for statistical significance with GraphPad Prism 5 software, by using a Kolmogorov-Smirnov normality test and a One-way ANOVA with Tukey's Multiple Comparison Test.

3. Results and discussion

3.1. Rotaviral neutralizing activity of human milk fractions and proteins

It is widely accepted that the composition of human milk is influenced by many factors, such as genetics, lactation period, and dietary habits. These factors are responsible for the differences in human milk components between individuals, what makes difficult to establish a general composition (Sousa et al., 2014b; Wojcik, Rechtman, Lee, Montoya, & Medo, 2009). The results we have found in the present study when evaluating the rotaviral activity of milks from different donors reflect that variability. In fact, even though there are not clear differences in the electrophoretic profile of human milk fractions from 10 healthy donors, significant differences have been evidenced when analyzing their neutralizing rotaviral activity (Fig. 1), obtaining values ranging from 3 to 100%, at 1 mg of protein/mL. Taking into account these wide differences between donors and in pursuit of achieving representative results, milk from the 10 donors was pooled. This pooled milk was the starting material for subsequent experiments to obtain fractions and proteins, which were subjected to compositional and biological assays.

The electrophoretic profile of the samples tested in this study (Fig. 2) showed that the majority of the human milk proteins are whey proteins, the most abundant being α-LA, LF, IgA and Lyz, what is consistent with results from various studies, which indicate approximate contents of 17, 17, 11 and 5% of total human milk proteins, respectively (Peterson et al., 2013). By contrast, both caseins and MFGM proteins, including MUCs and LDH, represent lower proportion compared to the whey fraction in human milk. Furthermore, the protein, lipid, and lactose percentages of human milk evaluated in this study are in good accordance

with compositional data reported by other authors (Sousa et al., 2014b). Thus, the pooled whole milk presented percentages of protein, lipid, and lactose of 1.1, 2.3 and 5.2% (w/v), respectively.

It is widely accepted that breastfeeding acts as a protective factor against rotavirus infection (De Franco et al., 2013; Gianino et al., 2002). Despite the fact that human milk shows a complex composition, most of the studies performed on its antirotaviral capacity have mainly focused on IgA (Asensi et al., 2006; De Franco et al., 2013), or MFGM proteins MUC1 and LDH (Kvistgaard et al., 2004; Newburg et al., 1998; Yolken et al., 1992). Thus, there is a lack of studies addressing other human milk components, what underlines the need of new evidences to better understand their potential and action mechanisms against rotaviral infections.

In the present study, several human milk fractions have been tested for their ability to reduce rotavirus infectivity (Fig. 3A). Thus, complete neutralization of *in vitro* cell infection with WC3 rotavirus was observed upon incubation either with raw whole milk, skimmed milk or whey, at 1 mg of protein/mL. By contrast, both casein and MFGM fractions showed significantly lower neutralizing values, being of 3 and 2%, respectively, at the same protein concentration, and higher values, of about 36 and 39% when tested at 2 mg of protein/mL. These results suggest that the antirotaviral potential of human milk is mainly associated to the whey fraction components. Moreover, the fat content appears not to be determinant in the overall activity, since no significant differences have been found between the antirotaviral activity of whole and skimmed human milk.

Interestingly, significant interspecies differences in the antirotaviral activity have been found when analyzing the results of the human milk fractions in comparison with those obtained for their bovine counterparts in previous studies we performed under the same conditions (Parrón et al., 2016, 2017). In fact, we observed that bovine raw whole milk,

skimmed milk and whey, showed lower WC3 neutralizing values, being of 74, 73 and 93%, at 1 mg of protein/mL, respectively. On the contrary, both bovine casein and MFGM fractions showed significantly higher antirotaviral activity, of 46 and 29% at the same protein concentration, respectively, than their human counterparts. Despite these divergences, the antirotaviral potential of bovine milk was found to be mainly associated with the whey fraction, as in the case of human milk.

Results obtained with milk fractions are in good agreement with the rotavirus neutralizing activity shown by the isolated proteins assayed in this study (Fig. 3B). At this respect, both IgA and LF, the major human whey proteins, at a concentration of 1 mg/mL, reached neutralization values of 100 and 62%, respectively. However, α-LA and Lyz showed negligible activities, at 1 mg/mL and even at 2 mg/mL. Furthermore, it has been evidenced a certain degree of antirotaviral activity as regards both MUCs-enriched fraction (51%, at 1 mg/mL) and LDH-enriched fraction (78%, at 0.5 mg/mL), which contrasted ~~contrasts~~ with the scarce activity previously attributed to MFGM whole fraction (2%, at 1 mg protein/mL). These MFGM proteins may easily act against rotavirus infection when totally solubilized in whey, better than as MFGM components.

With respect to the antirotaviral activity exerted by the recombinant LF (rhLF) tested in this study, significant differences were observed in comparison with LF isolated from human milk. Thus, the three forms of rhLF assayed at 1 mg/mL showed neutralizing values of 95, 95 and 87%, for the rice holo, the rice as isolated, and the *Aspergillus awamori* rhLF, respectively, which are statistically significant higher than that obtained with human milk LF (62% neutralizing activity). These results seem to indicate that there is no correlation between the degree of LF iron saturation and its antirotaviral activity, which is in agreement with results obtained by Superti et al. (2001) who found that complete iron saturation of bovine LF did not modify its antirotaviral activity. By contrast, no significant differences were found

between antirotaviral activity of Lyz from human milk and the recombinant protein obtained from rice.

Our results are in good accordance with those obtained by Asensi et al. (2006) who demonstrated the neutralizing activity of human milk against three rotavirus strains (human Wa and VA70, simian SA11). These authors found no correlation between the antirotaviral activity and the concentrations of IgA in human milk and serum samples, suggesting that antirotavirus IgA were only partly responsible for this activity and pointing out that other bioactive components, such as LF and LDH, could also be involved. Similar results were also reported by De Franco et al. (2013) as they observed that human colostrum and milk samples had the capacity to neutralize the rotavirus independently of their anti-rotaviral IgA levels , indicating a potential protective role of other components besides ~~Igs~~ immunoglobulins. Furthermore, interspecies differences have been reported on the antirotaviral potential of milk IgA. Thus, it has been shown that bovine milk predominant ~~Igs~~ immunoglobulins (IgG) presented a higher activity over 90% at 0.025 mg/mL (Parrón et al., 2016) than those predominant in human milk (IgA), around 55% at 0.025 mg/mL. These differences could be attributed to the vaccination programmes commonly applied in bovine species, resulting in milk with a high titer of specific antirotavirus ~~Igs~~ immunoglobulins.

As regards the antirotaviral capacity of human LF, our results have shown that its neutralizing activity against WC3 strain is close to 40% at a concentration of 0.25 mg/mL. This finding ~~contrasts~~ contrasted with that we obtained previously, which showed that native bovine LF at the same concentration could inhibit about 82% of WC3 rotavirus infection of MA104 cells (Parrón et al., 2016). These results were very similar to those obtained by Superti et al. (2001) who showed that apo and holo forms of bovine LF at 0.2 mg/mL exerted an inhibition of SA-11 strain, from 75 to 85%, in human colon adenocarcinoma cells (HT-29).

With reference to human MUCs, our results showed a WC3 rotavirus-neutralizing activity of 34% at 0.25 mg/mL. In this regard, Yolken et al. (1992) have also attributed antirotaviral activity to a human milk mucin complex fraction containing the MFGM protein MUC1 at 0.1 μg/mL, which showed 50% of inhibition of MA104 cell infection with SA11 rotavirus strain. Furthermore, we have previously demonstrated that infection of MA104 cells by WC3 rotavirus strain was 35% inhibited by bovine MUC1 at 0.25 mg/mL (Parrón et al., 2016). However, no inhibitory effect was observed by Kvistgaard et al. (2004) with a similar fraction, on infection by rotavirus strain Wa, suggesting the great influence of the strain when studying their inhibitory factors.

The present study ~~has proved~~ showed that human LDH at a concentration of 0.1 mg/mL has a WC3 neutralizing activity of 40%. In this respect, Kvistgaard et al. (2004) reported that human LDH inhibited infection of Caco-2 cells by rotavirus strain Wa in a dose-dependent manner (60% inhibition at 0.1 mg/mL). Likewise, Yolken et al. (1992) also suggested that human LDH was able to block the entry of rotavirus into the host cell, activity that was mainly attributed to the presence of sialic acid glycan chains in LDH. Furthermore, Newburg et al. (1998) evaluated the correlation between the concentration of LDH in breast milk and the appearance of symptomatic rotavirus infection. The results obtained showed the existence of significant differences between LDH concentration in milk received by symptomatic (29.2 μg/mL) and asymptomatic (48.4 μg/mL) infected babies. In addition, Parrón et al. (2016) observed that the incubation of WC3 rotavirus with bovine LDH at 0.08 mg/mL resulted in a neutralizing activity of 70%. In contrast, Kvistgaard et al. (2004) observed that bovine LDH could not inhibit Wa rotavirus infection of Caco-2 cells, revealing again the differences that may exist in the sensitivity of strains to inhibitory factors.

Differences in the neutralizing activity exhibited by milk fractions or proteins assayed in several studies could be attributed to either inter or intraspecies divergences in their overall

composition and structure. In particular, differences in the glycosylation degree and microheterogeneity of the attached glycans to proteins should be considered, as several studies pointed out that glycosylation is involved in receptor-ligand and host-pathogen interactions (Liu & Newburg, 2013; Peterson et al., 2013). This could explain the different activities observed in the present study between glycosylated (IgA, LF, MUCs and LDH) and poorly or non-glycosylated proteins (α-LA and Lyz). In addition, it is necessary to take into account the differences between the rotavirus strains used, with regard to their binding preferences to glycans, particularly sialic acid (Arias et al., 2016; Prasad et al., 2014). Furthermore, some discrepancies could derive from differences between the neutralization protocols, protein purification procedures, cell lines, inoculation time and methods to detect infection used.

3.2. Effect of heat treatment on the antirotaviral activity of human milk fractions and proteins

The effect of heat treatments of different intensity applied to human whey on its inhibitory activity against WC3 rotavirus was determined (Fig. 4A). It was observed that HoP (63 ºC for 30 min), caused a significant decrease of the neutralizing activity of human whey, of approximately 30%, whereas HTST pasteurization treatments (72 ºC for 20 s and 75 ºC for 20 s) did not affect its antirotaviral capacity. These results highlight the fact that these last treatments could be used as alternative methods for processing human milk to preserve its bioactivity. Heat treatments of high intensity, between 72 ºC for 10 min and 85 ºC for 10 min, induced higher losses of activity, of 40 and 98%, respectively. The electrophoretic profile of human whey samples subjected to severe heat treatments (Fig. 4B) showed the presence of aggregates that could not enter the wells and a slight decrease in the intensity of some protein bands.

Taking into account that IgA and LF are the proteins that have mainly shown antirotaviral activity in human whey, we also evaluated the effect of different heat treatments on their

activity (Fig. 5A). HoP affected significantly to both proteins, causing a decrease of the neutralizing activity, of 98 and 60%, for IgA and LF, respectively. By contrast, HTST pasteurization, at 75 ºC for 20 s, did not affect the antirotaviral activity of IgA and LF. However, heat treatments of higher intensity (75 ºC for 10 min and 85 ºC for 10 min) caused significant losses of LF and IgA activity, between 82 and 100%. The decrease in the intensity of the electrophoretic bands corresponding to IgA and LF after heat treatments was in accordance with the neutralizing results (Fig. 5B).

Most of the studies performed to determine the effect of technological treatments on human whey proteins have been focused on defensive components, such as IgA, LF and Lyz (Peila et al., 2017), while none of them has focused on their antirotaviral activity. Thus, Mayayo et al. (2016) observed that HoP and HTST pasteurization of human milk caused a decrease of about 57% of IgA immunoreactivity measured by enzyme-linked immunosorbent assay (ELISA), whereas thermal treatments of higher intensity (over 80 ºC for 15 s) produced losses higher than 78%. Although diverse studies have reported similar IgA losses after HoP pasteurization, ranging between 49 and 60% (Contador, Delgado-Adámez, Delgado, Cava, & Ramírez, 2013; Giribaldi et al., 2016; Viazis, Farkas, & Allen, 2007), other studies have evidenced lower losses, ranging from 20 to 28% (Chang et al., 2013; Czank, Prime, Hartmann, Simmer, & Hartmann, 2009; Permanyer et al., 2010; Sousa, Delgadillo, & Saraiva, 2014a), or even no degradation of IgA (Evans, Ryley, Neale, Dodge, & Lewarne, 1978). Furthermore, Giribaldi et al. (2016) showed that HTST pasteurization preserved 79% of IgA immunoreactivity, while HoP pasteurization only preserved 46%; in contrast to other authors that showed similar losses of IgA after applying HoP or HTST treatments (Mayayo et al., 2016; Peila et al., 2017).

Human LF has also been reported to be altered after thermal treatments. Several studies agreed that HoP pasteurization of human milk causes a decrease of LF immunoreactivity,

with losses ranging from 57 to 80% (Chang et al., 2013; Mayayo et al., 2014; Peila et al., 2016). Furthermore, several authors have pointed out that even HTST pasteurization may affect LF immunoreactivity, while to a lesser extent than HoP, causing losses from 35-60% (Mayayo et al., 2014; Peila et al., 2016). Heat treatments at lower temperatures, such as 40 ºC or 60 ºC for 30 min, did not cause significant changes in LF and IgA immunoreactivity (Chang et al., 2013).

At this respect, the present study points out that HTST pasteurization treatment preserves the antirotaviral activity of human whey, IgA and LF, better than HoP, what should be taken into account in the design of human milk processing conditions in order to maintain its bioactivity.

3.3. Effect of HHP treatment on the antirotaviral activity of human milk fractions and proteins

HHP treatments of different intensity were applied to human whey in order to evaluate the effect on its rotaviral neutralizing activity (Fig. 6A). HHP treatments were slightly harmful to the antirotaviral activity of samples. In fact, HHP treatments at 400 and 500 MPa, for 5, 10 and 15 min, did not affect significantly the activity of human whey, as previously observed for HTST pasteurization. Likewise, the more severe HHP treatments of whey at 600 MPa for 5, 10 and 15 min, led to slight decreases of neutralizing activity, of 6, 6 and 9%, respectively, which is much lower than that produced by HoP (30%). The electrophoretic profile of HHP treated human whey (Fig. 6B) was in good accordance with the neutralizing results, since no apparent changes were observed in the intensity of the electrophoretic bands.

The effect of different HHP treatments on the rotavirus-neutralizing activity of native IgA and LF was different (Fig. 7A). It has been shown ~~evidenced~~ that HHP treatment seems to be more harmful to IgA than to LF. Thus, HHP treatments at 400, 500 and 600 MPa for 15 min caused significantly losses of the antirotaviral activity of IgA, of 21, 30 and 40%, respectively.

These losses were much lower than those produced after HoP (98%), and significantly higher than those obtained after HTST pasteurization (0% of loss). However, none of the HHP treatments tested on LF, 400, 500 and 600 MPa for 15 min, affected significantly its activity, similarly to results obtained after HTST pasteurization, while in contrast with the 60% loss produced after HoP. Furthermore, no decrease in the intensity of the electrophoretic bands corresponding to human IgA and LF after HHP treatments was observed (Fig. 7B), what may correlate with a minor damage of samples, in accordance with the neutralizing results.

Several studies have been carried out on the effect of applying HHP treatment to human milk, specifically determining IgA and LF content and immunoreactivity. Even though HHP has been reported to be less harmful to proteins than HoP, as we have observed in our study, some divergences exist among results from different studies. Thus, Sousa et al. (2014a) found that immunoreactivity of IgA was fully preserved after HHP treatment of human colostrum at 200 and 400 MPa up to 30 min, and even after pressures up to 600 MPa for 2.5 min. By contrast, in the same study it was shown that HHP treatment at 600 MPa for 15 min led to similar IgA losses than HoP pasteurization (20%). Furthermore, Viazis et al. (2007) showed that HHP processing of human milk at 400 MPa for 30, 60, 90, and 120 min resulted in losses of 14, 13, 19 and 25% IgA immunoreactivity, respectively, being lower than the decrease produced after HoP pasteurization (49%). Other studies have also indicated a high maintenance of IgA after HHP treatment of human milk. Thus, no loss of IgA was observed after treatment at 400 MPa for 5 min, while losses of 12 and 31% were produced after 500 and 600 MPa for 5 min, respectively (Permanyer et al., 2010). Contador et al. (2013) showed that human milk treated at 400 MPa for 6 min maintained IgA levels, determined by ELISA, higher than those resulting after HoP; whereas at 600 MPa for 6 min the decrease was similar to that obtained after HoP (50-60%). Similar results were observed by Mayayo et al. (2016) who showed that HHP treatments at 300, 400, 500 and 600 MPa for 30 min led to a decrease

of IgA concentration of 20, 42, 53 and 64%, respectively, whereas HoP pasteurization caused a decrease of approximately 57%.

The effect of HHP on LF denaturation has been also investigated. HHP treatment of human milk at pressures of 300, 400, 500 and 600 MPa for 15 min resulted in 9, 23, 34 and 48% of LF denaturation, respectively, whereas HoP pasteurization denatured 80% (Mayayo et al., 2014). The same study also pointed out that LF aggregation did not take place after HHP, whereas aggregates involving disulphide bonds occurred during thermal treatments.

Divergences in results obtained between studies could be mainly attributed to different treatment conditions and equipment, with particular focus on holding times and initial temperature of the samples, and transmitting fluid in the pressure chamber. In fact, it is known that temperature reached during pressure treatment due to adiabatic compression is an important parameter that influences protein denaturation, as it has been observed for IgA (Delgado et al., 2013).

Results derived from the present study clearly suggest that HHP treatment, at pressures <600 MPa for 15 min, is a potential alternative to HoP pasteurization of human milk, in terms of antirotaviral activity retention. However, more studies are needed to elucidate whether these conditions for treatment also respect the overall nutritional and hygienic quality criteria demanded by human milk banks.

4. Conclusions

The results obtained in this study demonstrated that human milk is a valuable source of antirotaviral compounds, with whey fraction and the proteins IgA and LF showing the higher capacity. HoP pasteurization, which is currently recommended in all international human milk bank guidelines, has been proved to significantly decrease the antirotaviral activity of human whey, IgA and LF. Both HTST pasteurization and HHP treatment, at pressures <600 MPa, have shown promising results as potential alternatives to HoP pasteurization of human milk,

in terms of retention of antirotaviral activity. Despite that, more research is necessary in order to know which technological treatments and conditions should be applied to human milk to ensure microbiological safety, as well as maintenance of biological activity, making those treatments a reliable alternative to the conventional HoP pasteurization.

Funding

The work described herein was supported by Spanish government under a FPU predoctoral grant, a CICYT project (AGL2010-20835) and the European Social Fund.

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Figure captions

Fig. 1. (A) Effect of human milk fractions from ten donors (numbered 1-10) on neutralizing WC3 rotaviral infection of MA104 cells. (▬) Whole milk at 1 mg protein/mL; (▭) skimmed milk at 1 mg protein/mL; (▭) whey at 0.5 mg protein/mL; (▭) casein fraction at 4 mg protein/mL. Results are shown as mean ± standard deviation of triplicates from two independent experiments (n=6). (B) Electrophoretic profile of skimmed human milk from ten donors (numbered 1-10). SDS-PAGE was performed under reducing conditions in 4-20% polyacrylamide gradient gel. PAS + Coomassie staining. LF, lactoferrin; IgA, immunoglobulins A; α-LA, α-lactalbumin; Lyz, lysozyme; M, molecular weight marker.

Fig. 2. Electrophoretic profile of human fractions and proteins. Lane 1, whole milk; lane 2, skimmed milk; lane 3, whey; lane 4, casein fraction; lane 5, MFGM fraction; lane 6, immunoglobulins A (IgA); lane 7, lactoferrin (LF); lane 8, lysozyme (Lyz); lane 9, α-lactalbumin (α-LA); lane 10, mucins enriched fraction (MUCs); lane 11, lactadherin enriched fraction (LDH); M, molecular weight marker. SDS-PAGE was performed under non-reducing conditions in 4-20% polyacrylamide gradient gel. PAS + Coomassie staining.

Fig. 3. Dose-response of human samples on neutralizing WC3 rotaviral strain infectivity of MA104 cells. (A) Human milk pooled fractions: (⊖) whole milk; (●) skimmed milk; (⊟) whey; (■) casein fraction; (▲) MFGM defatted fraction. (B) Human milk proteins: (⊖) immunoglobulins A; (●) lactoferrin; (⊟) lysozyme; (■) α-lactalbumin; (▲) mucins enriched fraction; (▲) lactadherin enriched fraction. Results are shown as mean ± standard deviation of triplicates from two independent experiments (n=6).

Fig. 4. Effect of heat treatment on the neutralizing activity of human whey against WC3 rotaviral strain infection of MA104 cells. (A) Results are expressed in relation to control (non-treated whey) as mean ± standard deviation of triplicates from two independent experiments (n=6). Asterisks indicate statistically significant differences (***$p < 0.001$) in relation to control. Whey was treated at 2 mg/mL protein concentration. (B) Electrophoretic profile of heat treated human whey. SDS-PAGE was performed under non-reducing conditions in 4-20% polyacrylamide gradient gel. PAS + Coomassie staining. LF, lactoferrin; IgA, immunoglobulins A; M, molecular weight marker. The code number corresponds to the numbers in brackets as regards thermal treatments.

Fig. 5. Effect of heat treatment on the neutralizing activity of human immunoglobulins A (▬) and lactoferrin (▭) against WC3 rotaviral strain infection of MA104 cells. (A) Dose-response results expressed in relation to control (non-treated proteins) as mean ± standard deviation of triplicates from two independent experiments (n=6). Asterisks indicate statistically significant differences (***$p < 0.001$; **$p < 0.01$) in relation to control. Immunoglobulins A (IgA) and lactoferrin (LF) were treated at 0.1 and 2 mg/mL, respectively. (B) Electrophoretic profile of heat treated human proteins. SDS-PAGE was performed under non-reducing conditions in 4-20% polyacrylamide gradient gel. Coomassie staining. The code number corresponds to the numbers in brackets as regards thermal treatments.

Fig. 6. Effect of HHP treatment on the neutralizing activity of human whey against WC3 rotaviral strain infection of MA104 cells. (A) Results expressed in relation to control (non-treated whey) as mean ± standard deviation of triplicates from two experiments (n=6). Asterisks indicate statistically significant differences (***$p < 0.001$) in relation to controls. Whey was treated at 2 mg/mL of protein concentration. (B) Electrophoretic profile of HHP

treated human whey. SDS-PAGE was performed under non-reducing conditions in 4-20% polyacrylamide gradient gel. PAS + Coomassie staining. LF, lactoferrin; IgA, immunoglobulins A; M, molecular weight marker. The code number corresponds to the numbers in brackets as regards HHP treatments.

Fig. 7. Effect of HHP treatment on the neutralizing activity of human immunoglobulins A (▬) and lactoferrin (▬) against WC3 rotaviral strain infection of MA104 cells. (A) Results expressed in relation to control (non-treated proteins) as mean ± standard deviation of triplicates from two experiments (n=6). Asterisks indicate statistically significant differences (***$p < 0.001$) in relation to controls. Immunoglobulins A (IgA) and lactoferrin (LF) were treated at 0.1 and 2 mg/mL, respectively. (B) Electrophoretic profile of HHP treated human proteins. SDS-PAGE was performed under non-reducing conditions in 4-20% polyacrylamide gradient gel. Coomassie staining. The code number corresponds to the numbers in brackets as regards HHP treatments.

Highlights

- The antirotaviral activity of several human milk fractions and proteins was proved.
- Human whey fraction, at 1 mg protein/mL, showed 100% rotavirus neutralization.
- HoP significantly decreased the antirotaviral activity of human whey, IgA and LF.
- The percentage of decrease upon HoP ranged 30-98% for human whey, IgA and LF.
- HTST and HHP are promising alternatives to HoP for human milk treatment.

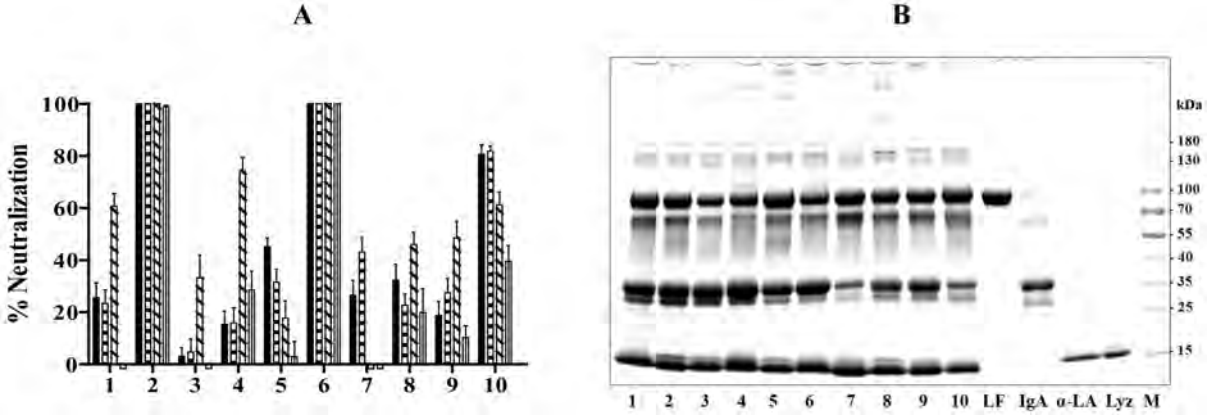
A
% Neutralization
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
B
kDa
180
130
100
70
55
40
35
25
15
1 2 3 4 5 6 7 8 9 10 LF IgA α-LA Lyz M

Figure 1

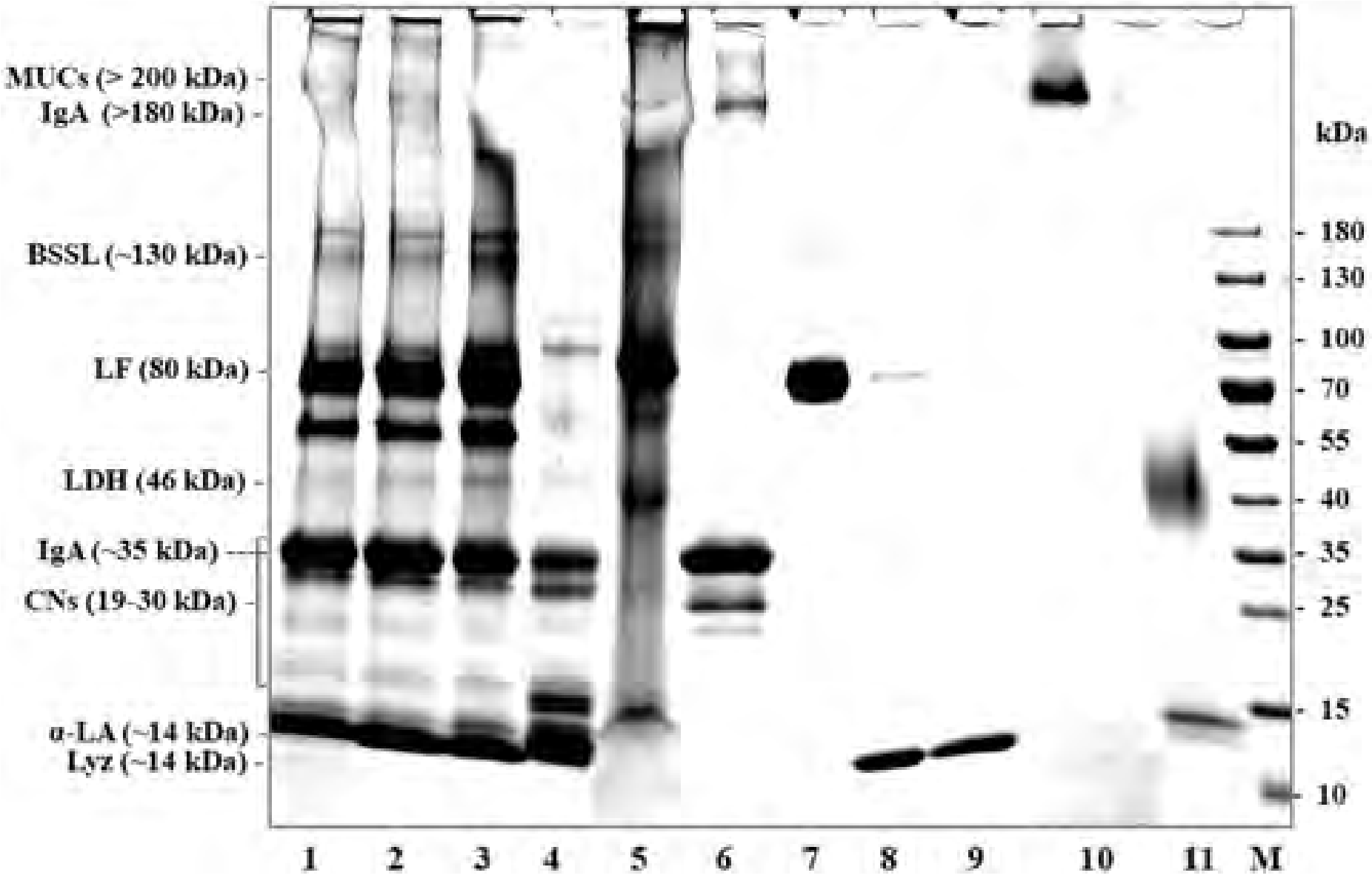
MUCs (> 200 kDa)
IgA (>180 kDa)
BSSL (~130 kDa)
LF (80 kDa)
LDH (46 kDa)
IgA (~35 kDa)
CNs (19-30 kDa)
α-LA (~14 kDa)
Lyz (~14 kDa)
kDa
180
130
100
70
55
40
35
25
15
10
1
2
3
4
5
6
7
8
9
10
11
M

Figure 2

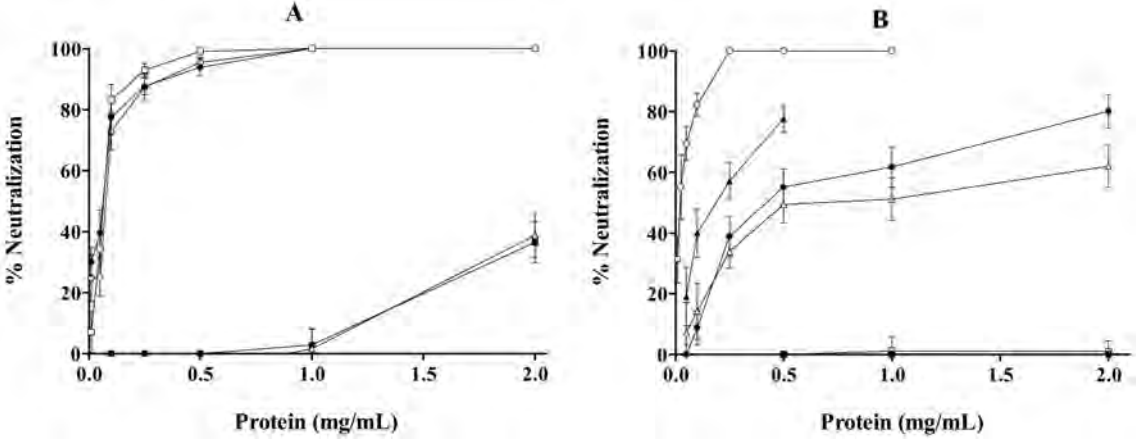
A
% Neutralization
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Protein (mg/mL)
B
% Neutralization
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Protein (mg/mL)

Figure 3

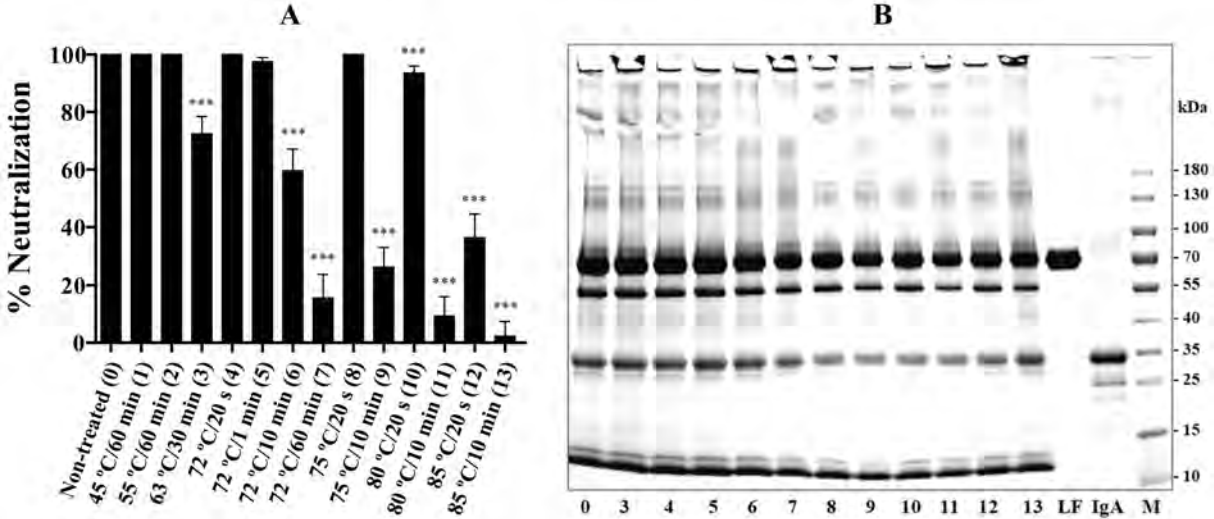
A
% Neutralization
100
80
60
40
20
0
***
Non-treated (0)
45 °C/60 min (1)
55 °C/60 min (2)
63 °C/30 min (3)
72 °C/20 s (4)
72 °C/1 min (5)
72 °C/10 min (6)
72 °C/60 min (7)
75 °C/20 s (8)
75 °C/10 min (9)
80 °C/20 s (10)
80 °C/10 min (11)
85 °C/20 s (12)
85 °C/10 min (13)
B
kDa
180
130
100
70
55
40
35
25
15
10
0 3 4 5 6 7 8 9 10 11 12 13 LF IgA M

Figure 4

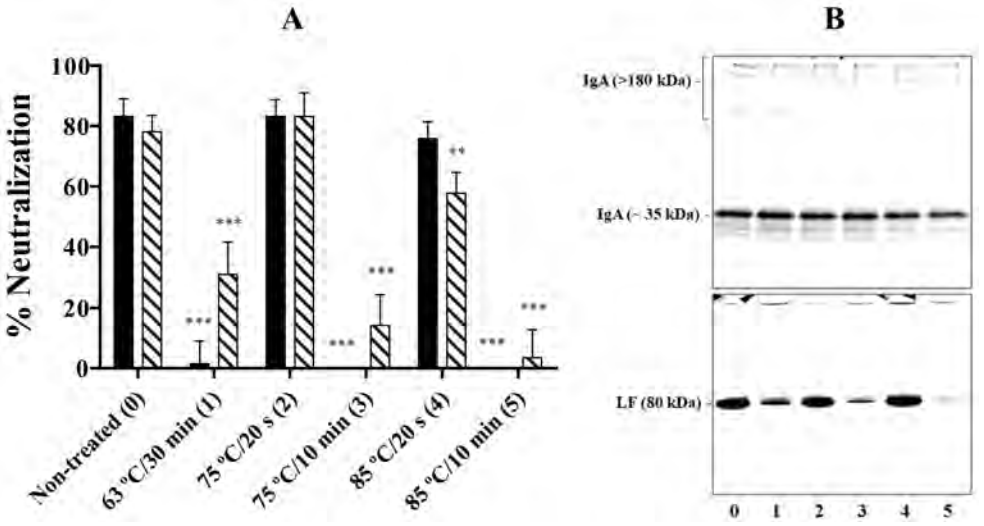
A
B
% Neutralization
100
80
60
40
20
0
***
***
***
***
**
***
***
Non-treated (0)
63 °C/30 min (1)
75 °C/20 s (2)
75 °C/10 min (3)
85 °C/20 s (4)
85 °C/10 min (5)
IgA (>180 kDa)
IgA (~ 35 kDa)
LF (80 kDa)
0
1
2
3
4
5

Figure 5

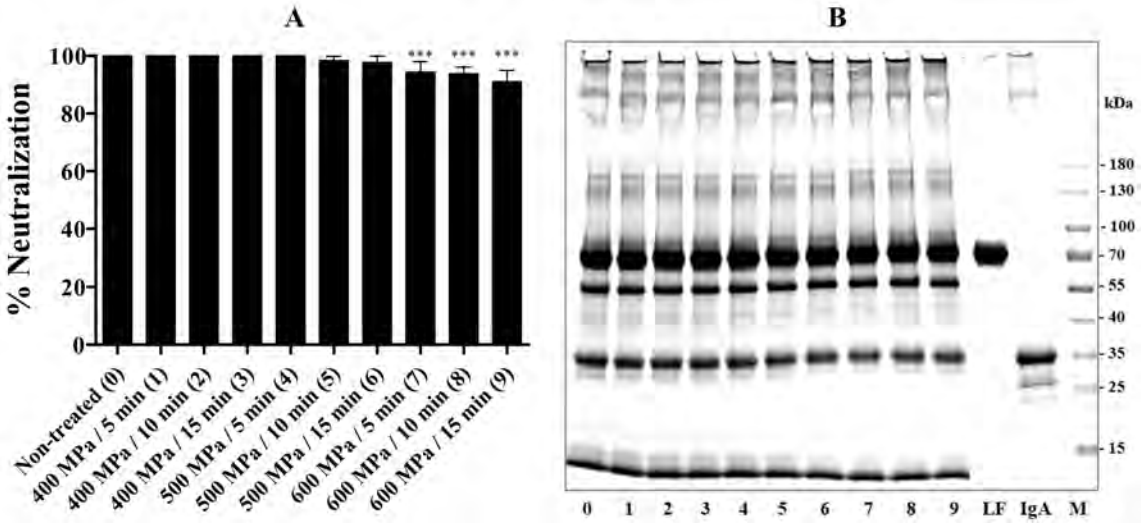
A
% Neutralization
100
80
60
40
20
0
***
***
***
Non-treated (0)
400 MPa / 5 min (1)
400 MPa / 10 min (2)
400 MPa / 15 min (3)
500 MPa / 5 min (4)
500 MPa / 10 min (5)
500 MPa / 15 min (6)
600 MPa / 5 min (7)
600 MPa / 10 min (8)
600 MPa / 15 min (9)
B
kDa
180
130
100
70
55
40
35
25
15
0
1
2
3
4
5
6
7
8
9
LF
IgA
M

Figure 6

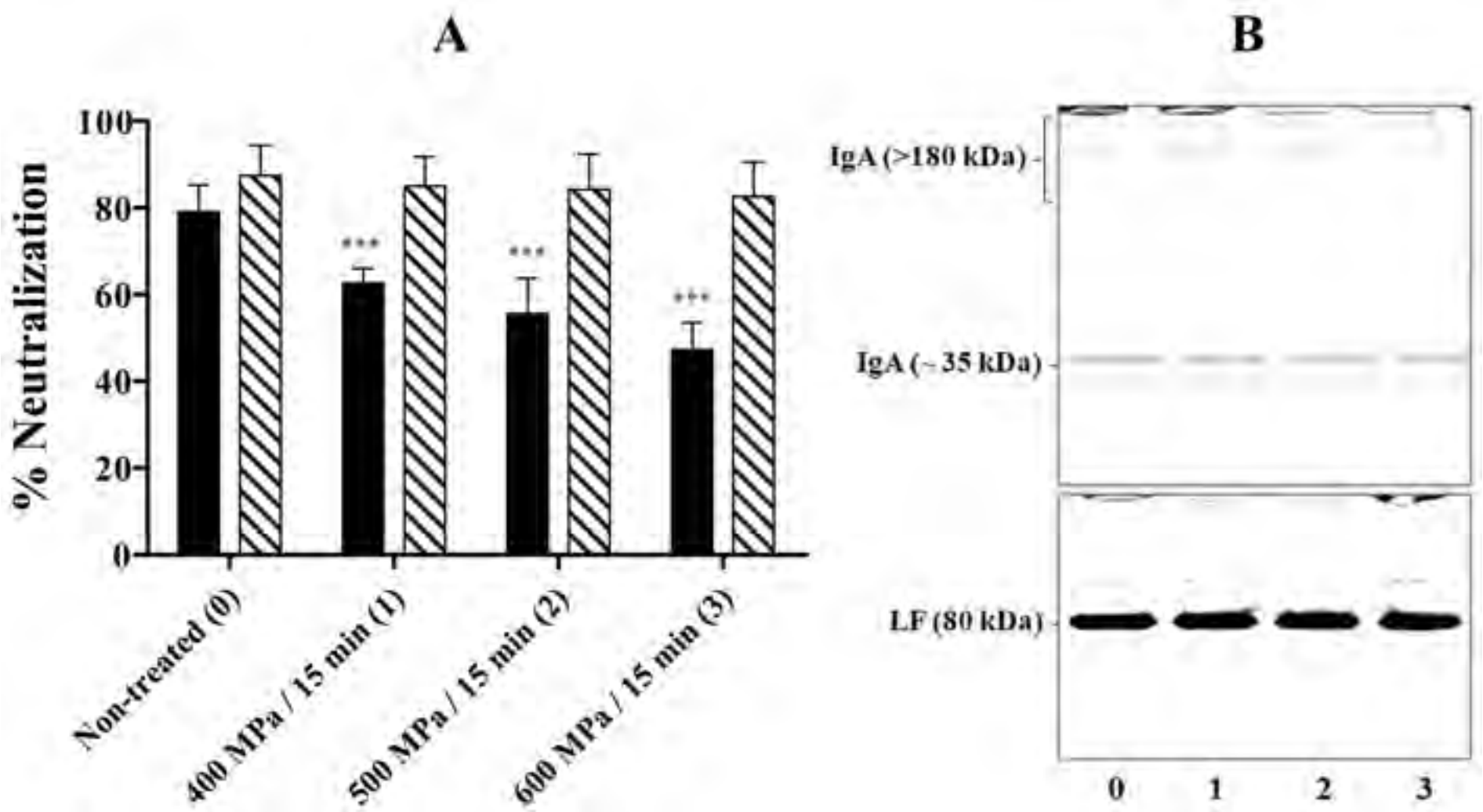
A
% Neutralization
100
80
60
40
20
0
***
***
***
Non-treated (0)
400 MPa / 15 min (1)
500 MPa / 15 min (2)
600 MPa / 15 min (3)
B
IgA (>180 kDa)
IgA (~ 35 kDa)
LF (80 kDa)
0
1
2
3

Figure 7