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Effect of ultra-high pressure homogenization on the antirotaviral activity of bovine milk whey

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ABSTRACT

In the present study, ultra-high pressure homogenization (UHPH, 100–250 MPa) was applied on bovine whey. The ability of UHPH-treated whey to neutralize the bovine rotavirus strain WC3 was evaluated *in vitro* using a model of human intestinal epithelium. Results showed that whey homogenized at 100 and 200 MPa inhibited the rotavirus infection in a dose-response way, exhibiting >95% neutralization at 1.5 mg mL^{-1} protein concentration. However, after homogenization of whey at 250 MPa, a clear neutralization pattern could not be observed. This could be attributed to aggregation and/or denaturation of some bioactive proteins, as it has been evidenced in the electrophoretic profile of that sample. Interestingly, the fat fraction obtained from all UHPH-treated wheys, showed almost complete neutralization at 1 mg mL^{-1} protein concentration. Therefore, whey fractions could be used as functional ingredients in novel non-thermal processed products for the control of viral infections.

Industrial relevance: This study provides useful information to increase the commercial value of sweet, skimmed whey as a functional ingredient and its recognition as a natural source of antivirals. The use of emerging processing technologies, such as UHPH, could favor the preservation of certain bioactivity within the dairy by-product.

1. Introduction

Homogenization is widely used in the dairy industry to prevent creaming during storage and also to improve the technological properties of some dairy products. It uses moderate pressures (18–20 MPa) and aims to reduce the milk fat globule (MFG) size. Based on the same principles of conventional homogenization, homogenization using high nominal pressures has been introduced as an emerging technology, which allows continuous processing of pumpable foods. Depending on the pressure level, the technology is usually termed high pressure homogenization (HPH, up to 150–200 MPa) or ultra-high pressure homogenization (UHPH, up to 350–400 MPa) (Trujillo, Roig-Sagués, Zamora, & Ferragut, 2016).

There are many studies supporting that UHPH can be used as a pasteurization method, by investigating microbiological inactivation in several food products, such as liquid egg (Velázquez-Estrada, Hernandez-Herrero, Lopez-Pedemonte, Guamis-Lopez, & Roig-Sagués, 2008), juices and vegetable beverages (Trujillo et al., 2016). In the case of milk and dairy products, these processes proved to be effective in inactivating spoilage bacteria and pathogenic microorganisms, giving a microbial load reduction comparable with pasteurized and sterilized milks by heat treatment, but with improvement in organoleptic quality. Additionally, some studies have dealt with virus inactivation using UHPH. A maximal reduction of 2–4 log cycles was observed for

Abbreviations: ADPH, adipophilin; BCA, bicinchoninic acid assay; BSA, bovine serum albumin; BTN, butyrophilin; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; FF, fat fraction; HHP, high hydrostatic pressure; HPH, high pressure homogenization; IgG, immunoglobulin G; LDH, lactadherin; LF, lactoferrin; MEM, Minimum Essential Medium; MFG, milk fat globule; MFGM, milk fat globule membrane; PBS, phosphate-buffered saline; PP3, proteose peptone component 3; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; T_{in}, inlet temperature; UHPH, ultra-high pressure homogenization; UHT, ultra-high temperature treatment; XOD, xanthine oxidase; α-LA, alpha lactalbumin; β-LG, beta lactoglobulin.

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lactococcal bacteriophages in milk or whey after five homogenization passes at 200 MPa (Moroni, Jean, Autret, & Fliss, 2002); however, these viruses show high resistance to treatments under 100 MPa (Capra et al., 2009). The efficiency of UHPH to inactivate human norovirus surrogates (feline calicivirus strain F9, MS2 coliphage and murine norovirus 1) strongly depended on the type of virus studied (D'Souza, Su, & Harte, 2011; D'Souza, Su, Roach, & Harte, 2009). The authors argued that the mechanism of action exerted by UHPH is the disruption of the viral capsid and the subsequent exposure of the nucleic acid to environmental degradation.

At the same time, there is a growing body of evidence demonstrating that the use of UHPH could be a less aggressive approach compared to heat treatments when considering the effect on the biological activity of milk constituents (Mesa et al., 2020). Thus, it has been shown that UHPH at 300 MPa and T_{in} of 45–85 °C, preserves vitamin C in milk better than thermal pasteurization (90 °C, 15 s) and ultra-high temperature process (UHT; 138 °C, 4 s) (Amador-Espejo, Gallardo-Chacon, Nykänen, Juan, & Trujillo, 2015). Furthermore, Sharabi, Okun, and Shpigelman (2018) explored the effects of UHPH processing on the stability of vitamin C during cold storage (4 °C). They reported that the content of vitamin C in the milk treated at 200 MPa (T_{in} of 25 °C) was >4 times higher after 30 h than that found in the conventionally pasteurized (72 °C, 12 s) and homogenized (40 MPa) milk. In the same study it was also shown that the antioxidant activity of bovine milk after UHPH treatment at 250 MPa (T_{in} of 25 °C) was preserved better than that of thermally pasteurized milk. Amador-Espejo et al. (2015) reported that UHPH treatment at 300 MPa (T_{in} of 45–85 °C) did not induce changes in vitamin B3, folic acid, and cyanocobalamin content until the inlet temperature reached 75 °C and also tocopherol content remained without changes when treated up to 85 °C for the same pressure level, compared to the contents found in raw milk (Amador-Espejo et al., 2015). In another study, bioactive fatty acids, such as conjugated linoleic acid isomers, remained unaffected after a 350 MPa UHPH treatment (T_{in} of 10 °C) (Rodríguez-Alcalá, Harte, & Fontecha, 2009).

Most of the studies on the effect of UHPH treatments on the properties of dairy components have been carried out in milk or the cream fraction since homogenization mainly targets the MFGs (Trujillo et al., 2016). However, there is less knowledge about the impact of this technology on whey, the byproduct of cheese and casein manufacturing. Whey contains 0.4 to 0.5% of residual fat (Rombaut, Dewettinck, & Van Camp, 2007), which consists of small fat globules, lipo-protein particles and milk fat globule membrane (MFGM) fragments (Roesch, Rincon, & Corredig, 2004).

The lipid components of MFGM have recently attracted a lot of attention due to the health benefits they provide to infants, such as the positive effects on their cognitive performance. (Timby et al., 2015; Verardo, Gómez-Caravaca, Arráez-Román, & Hettinga, 2017). Furthermore, components isolated from whey and MFGM have been reported to exert potent activity against rotavirus (Parrón et al., 2017), which is the worldwide leading cause of severe gastroenteritis in infants and children under five years (Nugent & Stewart, 2023). The morbidity and mortality caused by rotaviral infections represent a significant economic and public health burden, occurring regardless of socioeconomic status or environmental conditions, although the outcome and consequences are most severe in developing countries (Sicard, Bryant, Muller, & Quach, 2020).

The effect of industrial thermal and non-thermal processing on the antirotaviral activity of milk components has been an object of continuous study in our research group (Parrón et al., 2016; Parrón et al., 2018; Parrón et al., 2018). Recently, the influence of thermal and high hydrostatic pressure (HHP) processes on the antirotaviral activity of whey and whey-based preparations using a human intestinal model has been investigated, and it was shown that pasteurization (75 °C, 15 s) or HHP (400–600 MPa, 5 min), had no significant effect on the rotavirus neutralization potential (Graikini et al., 2024).

Hence, in the present study, we aimed to expand the knowledge on the effect of alternative preservation processes, and more specifically that of UHPH, on the rotavirus neutralizing activity of whey. For this, bovine whey was treated by UHPH under three different pressures: 100, 200 and 250 MPa. Subsequently, the residual fat fraction (FF) was isolated from the UHPH-treated whey. After characterization of whey samples, their antirotaviral activity was evaluated *in vitro* using Caco-2/TC7 cells differentiated into enterocytes as a human intestinal epithelial model.

2. Materials and methods

2.1. Whey obtention

Raw bovine milk was provided by the dairy industry Villacorona (El Burgo de Ebro, Zaragoza, Spain). The quality of milk was verified after reception by checking the pH (6.6–6.8), acidity (16–17°Dornic), fat percentage (3.4%), and alkaline phosphatase and lactoperoxidase activities (both positive), and it was processed at the Food Science and Technology Pilot Plant of the University of Zaragoza, located in the Veterinary Faculty. The milk was skimmed using the ARR-DES 125 cream separator (Arroyo Chemical Supplies, Santander, Spain). The fat content of the skimmed milk was about 0.1%. Subsequently, the precipitation of the skim milk caseins was induced by the addition of recombinant chymosin at 1:15,000 (v/v) ratio and $CaCl_2$ 1:8000 (v/v) ratio, and the mixture was left undisturbed for 45 min at 35 °C in a 50 L cheese vat. The whey fraction was recovered by decanting the curd and filtering the whey through cheese cloth and glass wool. Whey was stored at −20 °C until use.

2.2. Ultra-high pressure homogenization (UHPH) treatment

Single stage UHPH treatment of bovine whey was performed in a prototype pilot scale valve-type model (60–100 L/h, depending on the working pressure) fabricated by YPSICON Advanced Technologies (Barcelona, Spain) and installed at AZTI's pilot plant. Before introducing the whey into the equipment, it was filtered using a metal mesh sieve (224 μm), to remove aggregates and ensure optimal processing. For each experimental condition a total of 12 L of bovine whey was used. The processing conditions are shown in Table 1. It is important to note that it is not possible to register the temperature in the homogenization valve due to the design of the equipment. The presence of any temperature probe inside the homogenization valve would cause changes in the homogenization forces and therefore in the effectiveness of the treatments. It is estimated that in this point there will be higher temperature than in the output, though for <1 s, according to manufacturer. For this reason, the temperature indicated in Table 1 called T homogenizer is that of the output right after the homogenizer. After this, an integrated cooling system restores the temperature of the sample back to its initial level, as indicated by the T outlet.

2.3. Obtention of the whey fat fraction (FF)

To obtain the residual FF, non-treated (control) and UHPH-treated whey samples were subjected to the process illustrated in Fig. 1. Whey samples were centrifuged at 4000 rpm for 20 min at 4 °C and the fat layer was carefully collected, and it was called "first fat fraction" (FF1). The whey remaining between the cream layer and the protein

Table 1
Temperature (T) registers during the different UHPH treatments.

Treatment-Pressure (MPa)	T_{in} (°C)	T homogenizer (°C)	T outlet (°C)
100	18	43	13
200	19	70	15
250	20	89	22

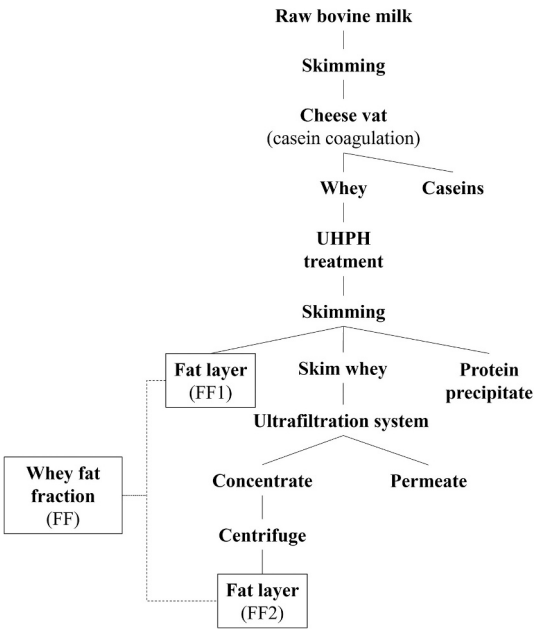

Fig. 1. Flow chart of the fractionation process of whey after UHPH treatment.

precipitate was then subjected to ultrafiltration using the model DC2/CD2A hollow fiber dialyzer/ concentrator Millipore Amicon (Danvers, MA, USA) system, where it was concentrated 15-fold. The hollow fiber membrane was of 100 kDa. The resultant concentrate was then centrifuged at 13,000 rpm for 10 min at 4 °C and the fat layer was carefully separated and called "second fat fraction" (FF2). Finally, FF1 and FF2 derived from each treated sample were combined resulting in FF.

2.4. Determination of protein content and SDS electrophoresis

The bicinchoninic acid assay (BCA) method was used to determine the protein content of samples. The assay was carried out using the commercial Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA) following the manufacturer instructions.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the procedure described by Laemmli (1970). Briefly, samples were diluted 1:1 with a 0.12 M Tris-HCl buffer, pH 6.8, containing 20% (v/v) glycerol, 4% (w/v) SDS and 0.02% (w/v) bromophenol blue. For electrophoresis performed in reducing conditions, β-mercaptoethanol (10%, v/v) was used. Samples were heated at 100 °C for 5 min and the process was carried out using 4–20% polyacrylamide gels (Mini-Protean TGX, Bio-Rad Laboratories, Hercules, CA, USA), following the recommendations established by the manufacturer. The molecular weight marker used was PageRuler™ Prestained Protein Ladder, from 10 to 180 kDa, (Thermo Fisher Scientific, Vilnius, Lithuania). The protein bands were stained with Coomassie Brilliant Blue R-250 (Serva Blue R, Serva Feinbiochemica GmbH & Co, Heidelberg, Germany).

2.5. Particle size distribution

The particle size distribution of the FF was measured using a laser light diffraction method in a Mastersizer 3000E equipment (Malvern Instruments, Malvern, UK) using wet dispersion system. The refractive index for material and dispersant was set to 1.460 (particles) and 1.330 (deionized water) respectively, while the absorption index of the disperse phase was set at 0.001 (Hayes & Kelly, 2003a). Obscuration was maintained at 7.5% with a residual value below 0.32%. The size distribution was measured in five replicates for each sample.

2.6. Proteomic analysis

The identification of some protein bands after SDS-PAGE of the UHPH treated samples was done by proteomic analysis at the Proteomics Platform at CIBA (IACS-Universidad de Zaragoza). Protein identification was performed by analyzing the peptide fingerprint and the fragmentation spectra of the peptides generated after trypsin digestion of the polyacrylamide gel bands in an automatic digester (Intavis, Bioanalytical Instruments, Cologne, Germany). The protocol that was followed is described in the study by Graikini, García, et al. (2024).

2.7. Cell culture and virus replication

The human adenocarcinoma cell line Caco-2 clone TC7 (Caco-2/TC7) (Chantret et al., 1994) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine 2 mM, 1% (v/v) non-essential amino acid solution, 1% (v/v) antibiotic solution (100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) and 1 μg mL^{-1} amphotericin B. The rhesus monkey epithelial cell line MA104 (ATCC CRL-2378) was cultured in Minimum Essential Medium (MEM) supplemented with 10% (v/v) FBS, 1% (v/v) 2 mM L-glutamine, 1% (v/v) antibiotic solution (100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) and 0.25 μg mL^{-1} amphotericin B. Both cell lines were maintained in 25 cm^2 culture vessels at 37 °C in a Heraeus B5060 EK/CO_2 thermostatic incubator with 5% CO_2. All cell culture media and supplements were purchased from Gibco (Thermo Fisher Scientific, Paisley, UK).

The replication of bovine rotavirus WC3 strain (ATCC VR-2102) was carried out by inoculating confluent MA104 monolayers with an aliquot of virus suspension, according to previously described procedures (Graikini, Soro, Sivagnanam, Tiwari, & Sánchez, 2023). FBS-free MEM, supplemented with 1% antibiotics, 2 mM L-glutamine, and 0.25 μg mL^{-1} amphotericin B, was used as diluent in all steps of the replication process.

2.8. Rotavirus neutralization assay

The antirotaviral activity of the whey fractions was tested using the Caco-2/TC7 cells. The *in vitro* neutralization assay was performed as previously described (Graikini, Conesa, Abad, Pérez, & Sánchez, 2024). Briefly, cells were seeded in 96-well plates at a density of 1.4×10^4 cells/cm^2 and cultured for 15 days until reaching the differentiation stage and morphology similar to functional enterocytes (Mesonero et al., 1994). Before the assay, cells were serum-starved for 2 h using FBS-free culture medium. Meanwhile, appropriate dilutions of samples were mixed (1:1) with a trypsin-activated rotavirus suspension (diluted in DMEM to achieve a final multiplicity of infection of 0.02) and incubated for 1 h at 37 °C. FBS-free DMEM was used as negative control and activated rotavirus suspension diluted 1:1 with FBS-free DMEM as positive control. After the incubation period, the samples were transferred to the plate containing the previously serum-starved cells and incubated at 37 °C for 1 h. Next, plates were added with 100 μL of DMEM containing 2 μg mL^{-1} trypsin and 6% FBS per well and incubated at 37 °C in 5% CO_2 for 12 h with gentle agitation. Subsequently, virus-infected cells were detected by indirect immunofluorescence.

2.9. Detection of rotavirus infection by indirect immunofluorescence

After the 12 h of the infection period, cells were washed with 200 μL per well of sterile phosphate-buffered saline (PBS) composed of 0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, and KH_2PO_4, pH 8.5. Cell fixation was carried out by adding 300 μL per well of a solution of acetone: methanol:formaldehyde (1:1:1) and incubating for 3 min at 4 °C. The plate was then washed twice with sterile PBS and incubated with 100 μL per well of bovine anti-rotavirus antiserum obtained in lamb, kindly donated by Dr. Snodgrass (Moredun Research Institute, Penicuik, UK) at

37 °C for 2 h under gentle agitation. Next, the wells were washed three times with sterile PBS and incubated with 100 μL per well of FITC-conjugated donkey anti-sheep IgG antibody (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C under gentle agitation. Finally, fluorescent cells were counted in the Eclipse E400 fluorescence microscope (Nikon Corp., Tokio, Japan) with a Nikon FITC filter, and the Zen lite 2012 image processing software (Carl Zeiss AG, Oberkochen, Germany). The infectivity percentages were determined by enumerating fluorescent foci (infected cells) in each well in relation to the 100% infectivity obtained with the positive control, which consisted of the virus suspension without neutralizing agent.

2.10. Statistical analysis

For the comparison of the means of neutralization values obtained from the *in vitro* assays, statistical analysis was conducted through the GraphPad Prism v8.0.2 software (GraphPad Software, San Diego, CA, USA). Results are presented as the mean ± standard deviation. The normality of the data was tested through the Shapiro-Wilk test. For the variables characterized as normal, the Student's *t*-test was performed. Variables that did not follow a normal distribution were analyzed using the Mann Witnney *U* test. Differences with p value ≤ 0.05 were considered statistically significant.

3. Results & discussion

3.1. Fat recovery from UHPH-treated whey

From the total 12 L of whey that was subjected to UHPH treatment, approximately 6 L were recovered and considered the final sample, as 3 L of the initial and 3 L of the final outlet liquids were discarded to avoid dilution with the washing water. The samples were immediately aliquoted and placed at 4 °C once obtained from the UHPH equipment. Therefore, all the further analyses of this study were performed using the refrigerated samples along a week. Specifically, the bioactivity assays were performed within the two following days of the treatment.

In a first step, the whey fat was isolated by centrifugation of the UHPH-treated whey obtaining a very thin layer (FF1) difficult to separate, unlike the non-treated whey used as control that gave a top layer thicker and easier to separate. Afterwards, the defatted whey was concentrated by ultrafiltration and centrifugated to obtain the FF2. In all cases, the final FF used in the following assays, consisted of mixing FF1 and FF2, with the exception of the FF from whey homogenized at 200 MPa, for which no FF2 could be recovered from treated whey. Similar difficulties were also reported in a previous study of Zamora, Ferragut, Guamis, and Trujillo (2012) where the authors intended to isolate the fat from UHPH treated milks to obtain later the MFGM, though they encountered a very thin cream layer after skimming. The authors attributed this to the increase of the density of the MFG near to that of the serum phase, which was provoked by their reduction after homogenization and suggested the use of density gradients during the isolation process in order to optimize the technique.

3.2. Protein profile and total protein content

The total protein content and the pH of the UHPH-treated samples, measured immediately after treatment, are shown in Table 2. A slight increase in the total protein content was observed for all treated wheys respect to the control sample. This effect was not treatment-dependent, with the 200 MPa-treated whey presenting the largest increase, followed by 100 MPa and 250 MPa-treated wheys.

With regards to the FFs, a decrease in the protein content was observed for whey treated at 100 MPa (7%) and 200 MPa (54%) compared to that of control. In turn, UHPH treatment caused a high increase in the protein content in the FF from whey homogenized at 250 MPa (104%). A similar effect was observed in the study of Zamora et al. (2012), where the total protein content of washed cream from bovine milk was slightly decreased after homogenization at 100 MPa, but significantly increased when pressure level was at 300 MPa (T_{in} of 20 °C). This effect of protein increase in the FF would have been attributed to the incorporation of whey proteins and caseins into the MFGM, which is dependent on the applied pressure among other factors (Ye, Anema, & Singh, 2004). However, the present results regarding the protein content of the FF from the 200 MPa-treated whey differed from what it was observed in the above studies. As mentioned earlier, the obtention of the FF from whey treated at 200 MPa differed from the rest of the samples; therefore, such lower protein content could be primarily attributed to the isolation process rather than to the effect of UHPH.

Table 2
Values of pH and total protein content of the UHPH-treated samples and the fractions derived from them.

UHPH treatment	pH	Protein content (mg mL^{-1})			
		Whole fraction	Fat fraction (FF)	Ultrafiltration concentrate	Ultrafiltration permeate
Control	6.42	30.89	90.58	110.08	22.51
100 MPa	6.38	32.15	88.43	126.79	17.49
200 MPa	6.30	34.80	32.71	118.83	18.23
250 MPa	6.46	31.14	184.63	114.56	23.89

The protein profile of UHPH-treated wheys (whole fraction) and their corresponding protein precipitates obtained after centrifugation at 4000 rpm for 20 min was analyzed by SDS-PAGE (Fig. 2). No major differences, considering the type and intensity of the protein bands, were observed between the non-treated whey (lane 1) and whey homogenized at 100 and 200 MPa (lanes 2 and 3, respectively). However, the bands corresponding to the whey homogenized at 250 MPa (lane 4) appeared in weaker intensity, especially those of xanthine oxidase, lactoferrin and immunoglobulin G. Furthermore, greater variability was observed between the profiles of the protein precipitates obtained by centrifugation of the treated wheys (lanes 6–9). The precipitate from whey treated at 100 MPa (lane 7) presented a clear band of

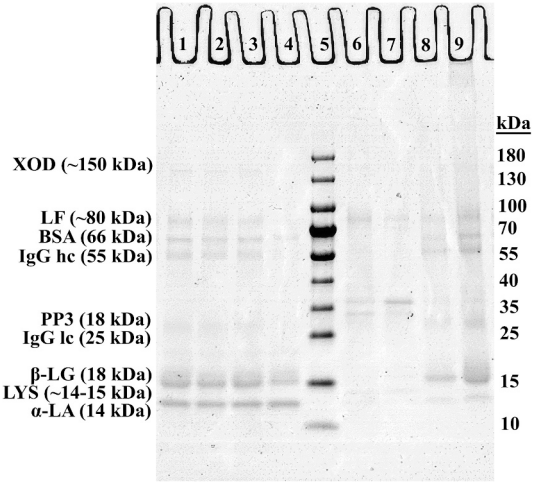

Fig. 2. SDS-PAGE of UHPH-treated bovine wheys (whole fraction) and of the corresponding protein precipitates obtained by centrifugation. 1: control (non-treated whey); 2: whey homogenized at 100 MPa; 3: whey homogenized at 200 MPa; 4: whey homogenized at 250 MPa; 5: molecular weight standard; 6: precipitate of control (non-treated whey); 7: precipitate of whey homogenized at 100 MPa; 8: precipitate of whey homogenized at 200 MPa; 9: precipitate of whey homogenized at 250 MPa. Electrophoresis was performed using 4–20% polyacrylamide gel and carried out under reducing conditions. Abbreviations: α-lactalbumin (α-LA); β-lactoglobulin (β-LG); immunoglobulin G (IgG)-light chain (lc) and heavy chain (hc); proteose-peptone component 3 (PP3); bovine serum albumin (BSA); lactoferrin (LF); xanthine oxidase (XOD).

approximately 36 kDa, probably corresponding to caseins, and a less intense band of 80 kDa corresponding to lactoferrin. Notably, protein precipitates obtained from the homogenization of whey at 200 and 250 MPa (lanes 8 and 9, respectively) contained all proteins present in the whole whey fraction. However, it seems that the sample run in lane 9 also contained proteins trapped in the stacking gel indicating increased aggregation of proteins. Therefore, the low amount of protein observed in the whey homogenized at 250 MPa (lane 4) could be explained by the loss of solubility of some proteins that appear in the precipitate.

The FF obtained from the UHPH-treated wheys was also subjected to SDS-PAGE. As it can be seen in Fig. 3A, no major differences were observed when the electrophoresis was performed under non-reducing conditions, except for the existence of an additional band of ~35 kDa in lane 5, corresponding to the FF from whey homogenized at 250 kDa. It is likely that the band corresponds to caseins, which are solubilized after the more intense UHPH treatment. Furthermore, in this electrophoresis it was observed that in some lanes there was some protein that did not enter the running gel. The FFs were also analyzed under reducing conditions (Fig. 3B). As it can be observed, the profile of the FF from the whey homogenized at 100 MPa (lane 2) was very similar to that of the non-treated whey (lane 1), although some bands appeared less intense, especially those corresponding to β-lactoglobulin. The profile of the FF originating from whey homogenized at 200 MPa (lane 3) lacked one band of ~23 kDa, which was present in the profiles of the other samples. Finally, in the profile of the FF from whey homogenized at 250 MPa (lane 4), an additional band with molecular weight ~ 33 kDa was observed. The latter band was subsequently excised from the gel (dashed box in Fig. 3B) and subjected to proteomic analysis. It was revealed that the band did not correspond exclusively to one protein, but rather to a mixture of various proteins. Among these, β-lactoglobulin, bovine serum albumin and lactoferrin were the main proteins, followed by butyrophilin, lactadherin, αs1 and αs2 caseins, and xanthine oxidase.

As mentioned above, UHPH induces changes in the protein structure of whey proteins and MFGM and can lead to their denaturation/aggregation depending on the treatment conditions applied (Trujillo et al., 2016). If only the heat effect is considered, whey proteins start to denature at ~65 °C and interact with casein micelles (Singh, 1993). However, simultaneous heating and homogenization processes take place in UHPH, inducing denaturation and interaction between proteins. Hayes, Fox, and Kelly (2005) treated milk up to 250 MPa (T_{in} of 45 °C) that reached a temperature of 83.6 °C and suggested that the physical forces experienced by whole milk during UHPH produced the denaturation of β-lactoglobulin (Hayes et al., 2005). Furthermore, Zamora, Ferragut, Jaramillo, Guamis, and Trujillo (2007) reported that denaturation of β-lactoglobulin was much greater (17%) in whey homogenized at 200 MPa, which reached approximately 75 °C for a very short time (~0.7 s), compared to pasteurized milk treated at 72 °C for 15 s. The denaturation of β-lactoglobulin was ~35% for UHPH treatment at pressures of 230 and 330 MPa (Zamora et al., 2007). Accordingly, in the present study, β-lactoglobulin, was identified by proteomic analysis in a higher molecular weight band in the FF from 250 MPa-treated whey (T_{in} of 20 °C, T homogenizer at 89 °C) presumably due to phenomena of aggregation with other proteins during the UHPH process.

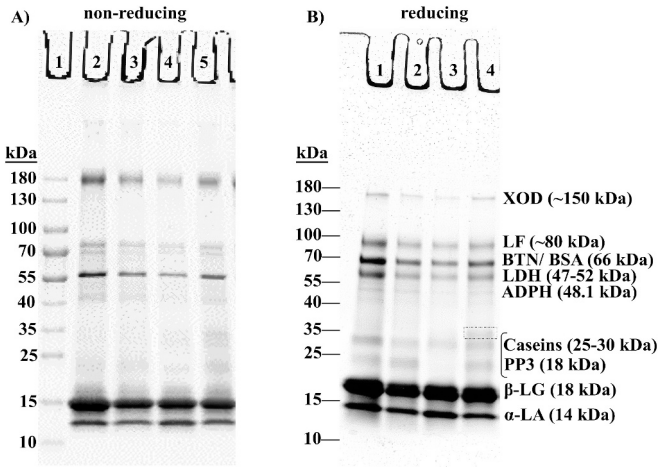

Fig. 3. SDS-PAGE of the fat fraction (FF) from UHPH-treated bovine wheys. A) 1: molecular weight standard; 2: FF from control; 3: FF from whey homogenized at 100 MPa; 4: FF from whey homogenized at 200 MPa; 5: FF from whey homogenized at 250 MPa. Electrophoresis was performed using 4–20% polyacrylamide gel and carried out under non-reducing conditions. B) 1: FF from control; 2: FF from whey homogenized at 100 MPa; 3: FF from whey homogenized at 200 MPa; 4: FF from whey homogenized at 250 MPa. Electrophoresis was performed using 4–20% polyacrylamide gel and carried out under reducing conditions. Framed band in lane 4 was subjected to proteomic analysis. Abbreviations: α-lactalbumin (α-LA); β-lactoglobulin (β-LG); proteose-peptone component 3 (PP3); adipophilin (ADPH); lactadherin (LDH); butyrophilin (BTN); bovine serum albumin (BSA); lactoferrin (LF); xanthine oxidase (XOD).

Finally, in a study of Grácia-Juliá et al. (2008), it was found that UHPH treatments up to 300 MPa, did not cause lactoferrin or immunoglobulin aggregation/denaturation in a whey protein isolate. These findings are not in full agreement with the present results, as in the electrophoretic profile of whey homogenized at 250 MPa, it was observed that the band corresponding to lactoferrin and immunoglobulin G appeared with much less intensity compared to the rest of the samples (Fig. 2). Notably, those authors have reported that for processing pressures at 250–300 MPa, the temperature of their samples measured right after the homogenization valve ranged at 66–76.5 °C, which is considerably lower than that expected for the whey homogenized at 250 MPa in the present study (T homogenizer 89 °C).

3.3. Particle size distribution of fat fraction samples

The particle size distribution (vol%), the mean volume-weighted diameter [D (4,3)] and the mean surface-weighted diameter [D (3,2)] of the FF isolated from the different UHPH-treated wheys are summarized in Fig. 4 and Table 3. This analysis was performed in multiple modes since the Malvern 3000E software allows selecting specific populations of the particle distribution; therefore, the several peaks appear enumerated. The control FF, from non-treated whey, and the FF obtained from whey homogenized at 100 MPa displayed a bimodal distribution with two main peaks (Fig. 4A and B). In this distribution, the first smaller peak represents the casein micelles, and the second larger peak corresponds to the MFGs. Interestingly, homogenization at 100 MPa did not reduce the mean diameter of the second peak as expected, but lead to an increase of the D (3,2) value, from 3.7 to 4.91 μm. A similar increase was also observed in the case of the FF from whey homogenized at 250 MPa, which showed a D (3,2) of 4.88 μm; however, this sample differed in the morphology of the distribution as it presented a single narrow peak (Fig. 4D). Finally, the FF from whey homogenized at 200 MPa had the least uniform distribution, displaying two partially overlapping peaks with a D (3,2) of 0.44 and 2.42 μm, which corresponded to the size in which appeared the first two peaks in the control,

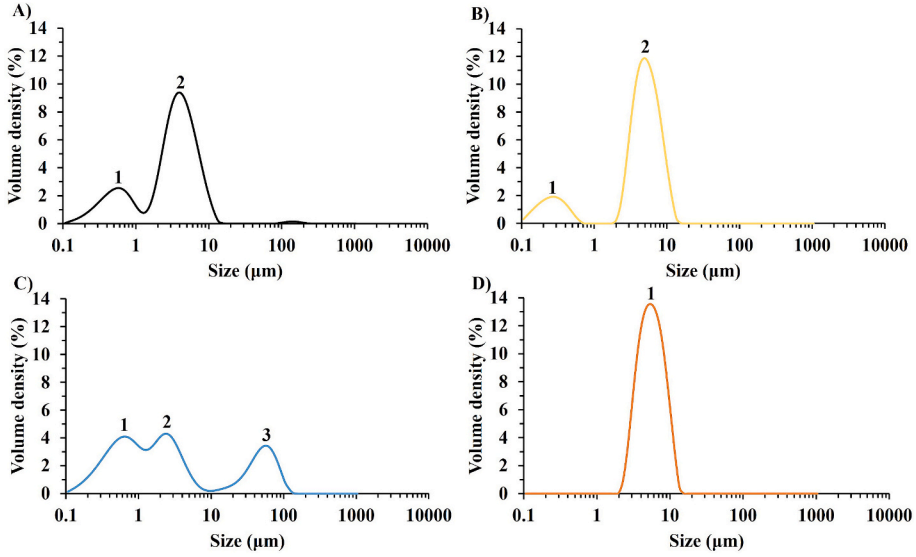

Fig. 4. Particle size distribution of the fat fraction (FF) from UHPH-treated bovine whey. A) Control (non-treated whey); B) FF of UHPH-treated whey at 100 MPa; c) FF of UHPH-treated whey at 200 MPa; D) FF of UHPH-treated whey at 250 MPa.

Table 3
Surface-weighted mean diameter [D (3,2)] and volume-weighted mean diameter [D (4,3)] of the fat fraction (FF) obtained from UHPH-whey treated at different pressure. Since analysis was performed with multimodal distribution in the Malvern 3000E software, the percentage of each mode (peak) is also shown. The number of each peak corresponds to that shown in Fig. 4.

UHPH treatment	Peak N°	Percent of each peak (%)	D (3,2) (μm)	D (4,3) (μm)
FF-control	1	21.06	0.43	0.55
	2	78.94	3.70	4.59
FF-100 MPa	1	13.78	0.23	0.27
	2	86.22	4.91	5.70
FF-200 MPa	1	39.33	0.44	0.60
	2	38.01	2.42	2.93
	3	22.66	42.28	48.73
FF-250 MPa	1	100.00	4.88	5.62

and a third separated peak with a mean diameter of 42.28 μm (Fig. 4C).

Overall, the results regarding the particle size of the FF from UHPH-treated samples were not as expected, with the mean diameter of the particles being increased in all cases in comparison to the non-homogenized control. It is possible that this phenomenon is due to the development of large particles of fat aggregates that were formed after the disruption of the MFGs by the homogenization process, either by coalescence or flocculation (Keenan, Moon, & Dylewski, 1983; Walstra, 1995). These aggregates are usually formed due to the lack of protein, which results insufficient to cover the new fat globules formed right after homogenization.

In agreement with the present results, Thiebaud, Dumay, Picart, Guiraud, and Cheftel (2003) reported that the MFG size distribution in milk samples homogenized at 300 MPa (T_{in} of 24 °C) displayed large particles, with a mean diameter of ~5 μm. The authors stated that the large particles observed in their study might correspond to fat clusters formed through interaction between proteins adsorbed to their surface, and that the addition of SDS solution (5 g L^{-1}) or a solution containing EDTA (50 mM) and urea (8 M) could disrupt these particles. Furthermore, it has been reported that the T_{in} during UHPH treatment plays a significant role on the reduction of the MFG size (Datta, Hayes, Deeth, & Kelly, 2005; Hayes & Kelly, 2003b). The milk fat is a mixture of crystallized and liquid fat depending on the temperature; therefore, its state prior to homogenization could significantly impact the extent of globule size reduction, despite the rapid increase in temperature of the milk at the primary valve. Thus, in the study of Datta et al. (2005), no reduction in size was observed for homogenization at 200 MPa with increasing T_{in} above 35 °C (resulting in outlet temperatures of >70 °C), which is beyond the melting point of milk fat.

Finally, it is important to note that, in the present study, the UHPH treatment was not applied directly to the FF samples, but to the whey from which fat was isolated. The subsequent steps for the obtention of the FF, such as ultrafiltration and centrifugations, may have further contributed to the aggregation phenomena and the overall increased particle size. Furthermore, the storage of the FF samples at 4 °C for up to six days before the size distribution measurement might have also produced the formation of large particles due to cold agglutination (Meena & Upadhyay, 2022). Collectively, the above factors could justify the particle size observed for the different FFs. However, since the primary aim of this study was to investigate the effect of UHPH on the biological activity of the FF from whey, we did not go deeper into aspects related to the size of the particles contained in the different FFs from UHPH-treated whey.

3.4. Antirotaviral activity of fractions from UHPH-treated whey

In the present study, whey after UHPH treatment was tested for its ability to reduce rotavirus infectivity using a model of human intestinal epithelium. The intestinal barrier was simulated using a culture of Caco-2/TC7 cells, which have the capacity to differentiate into a monolayer of polarized cells with morphological and functional characteristics of enterocytes (Ferruzza, Rossi, Scarino, & Sambuy, 2012). The concentrations in which whey was applied to the cells were considered safe for their viability as they did not show cytotoxic effect according to a previous study (Graikini, García, et al., 2024).

As seen in Fig. 5A, the non-treated whey and the whey homogenized at 100 and 200 MPa presented a dose-response antirotaviral activity with neutralization values of 98.4, 96.2 and 94.9%, respectively, at a protein concentration of 1.5 mg mL^{-1}. At all tested concentrations, the neutralization activity of the UHPH-treated samples was slightly lower than that of the control. Nevertheless, these differences were significant only in the case of the 0.5 mg mL^{-1} concentration, at which whey treated at 100 and 200 MPa had an activity loss of 26.4 and 19.2%, respectively. This decrease might be due to the partial denaturation of the most active whey proteins against rotavirus, such as lactoferrin (Graikini, Conesa, et al., 2024; Parrón, Ripollés, Ramos, et al., 2018) and immunoglobulin G (Bojsen et al., 2007; Parrón et al., 2018). The latter

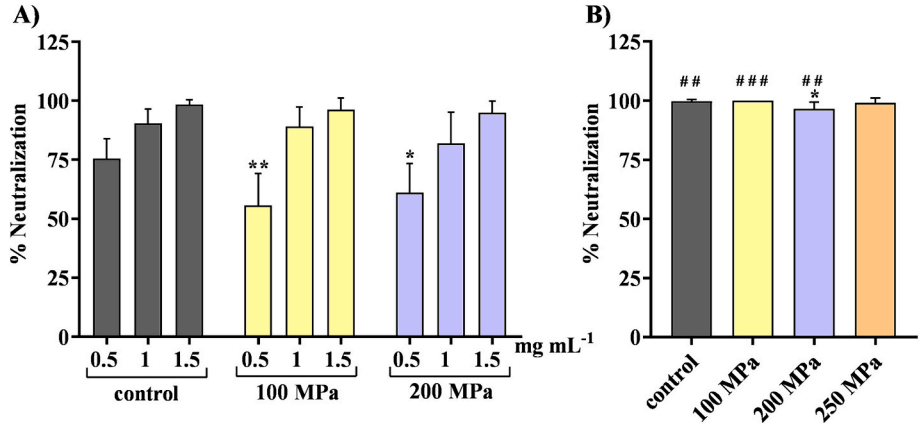

Fig. 5. Effect of UHPH on the neutralization activity of whey fractions against the infection of Caco-2/TC7 cells by the rotavirus strain WC3. A) UHPH-treated whey (whole fraction) tested at three protein concentrations (0.5, 1 and 1.5 mg mL-1). Results are shown as the mean ± standard deviation of duplicates of at least three independent experiments ($n \geq 6$). B) Fat fraction (FF) obtained from the UHPH-treated whey. The samples were tested at 1 mg mL-1 protein concentration. Results are shown as the mean ± standard deviation of duplicates of four independent experiments ($n = 8$). *Indicate significant differences (*$p < 0.05$; **$p < 0.01$) between the neutralization values of UHPH-treated samples and those of the non-treated control. # Indicate significant differences (# #p < 0.01; # # #p < 0.01) between the neutralization values of the FF (B) in comparison to those of the whole whey (A) at the corresponding protein concentration (1 mg mL-1).

was found in the electrophoretic profile of the protein precipitate of the skim whey treated at 200 MPa, while the former was found in both electrophoretic profiles of the protein precipitate from 100 and 200 MPa-treated wheys. Furthermore, as it has been reported, UHPH allows for a partial dissociation of the micelles and a release of free caseins in the serum phase, the degree of which depends on the UHPH pressure applied (Touhami, Doyen, Pouliot, & Brisson, 2023). Therefore, an additional reason for the lower neutralizing activity of the treated samples might be due to the stereochemical obstruction of the main antiviral components caused by the solubilized caseins. Notably, no significant differences were found between the neutralization activities of the two UHPH treated whole whey samples (100 and 200 MPa) at the same concentration.

Contrary to the whey subjected to lower pressure homogenization treatments, there was no clear pattern of neutralization in the case of whey homogenized at 250 MPa. Instead, a great variability was observed in the antiviral activity of this sample, with neutralization percentages ranging from 0 to 100% at all tested concentrations. Although the *in vitro* tests were repeated several times in this case, the variability in the neutralization values remained high. Results from the SDS-PAGE and proteomic analysis indicated that this sample underwent changes in the protein structure either due to fragmentation of some proteins and/or interaction between them. It is well known that the structure of proteins directly affects their bioactivity (Zanabria, Griffiths, & Corredig, 2020), consequently the different exposure of the protein fractions contained in the 250 MPa-treated whey may have been responsible for the variation in the neutralization activity of the sample between different assays.

The results obtained in the *in vitro* tests using the FF as antirotaviral agent showed almost complete virus inhibition (Fig. 5B). Specifically, the neutralization activity of the control FF, and of FF from whey treated at 100, 200 and 250 MPa were 100, 100, 97 and 99%, respectively. Significant differences between the neutralization activity of the UHPH-treated samples and that of the control was observed only in the case of the FF from whey homogenized at 200 MPa. Furthermore, the neutralization activity of the FF samples (control, 100 and 200 MPa) was significantly higher than that observed for the whole whey fraction at the protein concentration of 1 mg mL^{-1}.

Previous studies have reported that the glycoproteins associated with bovine MFGM, such as mucin 1, lactadherin, PP3 and xanthine oxidase have potent antirotaviral activity (Inagaki et al., 2010; Kvistgaard et al., 2004; Parrón et al., 2017). Many types of rotaviruses rely on binding to either terminal or subterminal sialic acid residues in glycoconjugates to initiate the infection of the host cell, although glycans have also been reported to be recognized by some rotavirus strains (Isa, Arias, & López, 2006; Yu & Blanchard, 2014). In this view, glycosylation together with sialylation on these molecules have been described as important factors responsible for the antirotaviral activity of the milk proteins (Graikini, Conesa, et al., 2024; Parrón, Ripollés, Sánchez, et al., 2018; Sun, Li, & Duan, 2021). It is important to note that the antirotaviral activity of the MFGM proteins may also be strain dependent. For example, in the study of Kvistgaard et al. (2004), bovine lactadherin did not inhibit the RRV rotavirus strain. However, as shown in another study, bovine lactadherin neutralized the infectivity of UK, WC3, Wa, B223, and NCDV strains by 83–97% at a concentration as low as 0.04 mg mL^{-1}, though it was less potent against the RRV strain (Parrón, Ripollés, Sánchez, et al., 2018). Similarly, mucin 1 exerted a mild neutralizing activity on WC3 (Parrón et al., 2016), while it was highly effective inhibiting the infectivity of the EMcN murine rotavirus (Bojsen et al., 2007).

At the same time, MFGM as a whole fraction has also exhibited antirotaviral activity against several rotavirus strains, although it was necessary to apply higher concentrations of this fraction in order to reach the same neutralization values as the isolated MFGM proteins (Parrón, Ripollés, Sánchez, et al., 2018). Based on this observation, it could be hypothesized that the distribution of the proteins on native MFGM, partially or totally embedded in the triple phospholipid membrane, could prevent their full potential as rotavirus neutralizing agents. Technological treatments, such as UHPH, could expose the active regions of MFGM proteins, as proposed in Fig. 6. It was previously shown that low intensity thermal and HHP treatments applied to an enriched buttermilk preparation resulted in enhanced antirotaviral activity (Graikini, García, et al., 2024). It has been also recommended that, the development of mildly processed milk and infant nutrition products could become part of preventive strategies to reduce the incidence of allergic disease (van Neerven, Knol, Heck, & Savelkoul, 2012). In the present study, partial destabilization of the triple phospholipid layer of the MFGM caused by UHPH followed by ultrafiltration could expose bioactive regions of MFGM proteins present in the FF.

In addition, there are more components of the MFGM that could be responsible for its bioactivity. For example, the gangliosides, a minor component of the MFGM, are present in neonatal piglet intestine and serve as specific receptors for the attachment of the sialic acid-dependent group A porcine rotavirus to intestinal epithelial cells (Rolsma, Gelberg, & Kuhlenschmidt, 1994; Rolsma, Kuhlenschmidt, Gelberg, & Kuhlenschmidt, 1998). In the study of Fuller, Kuhlenschmidt, Kuhlenschmidt, Jiménez-Flores, and Donovan (2013), the whole bovine

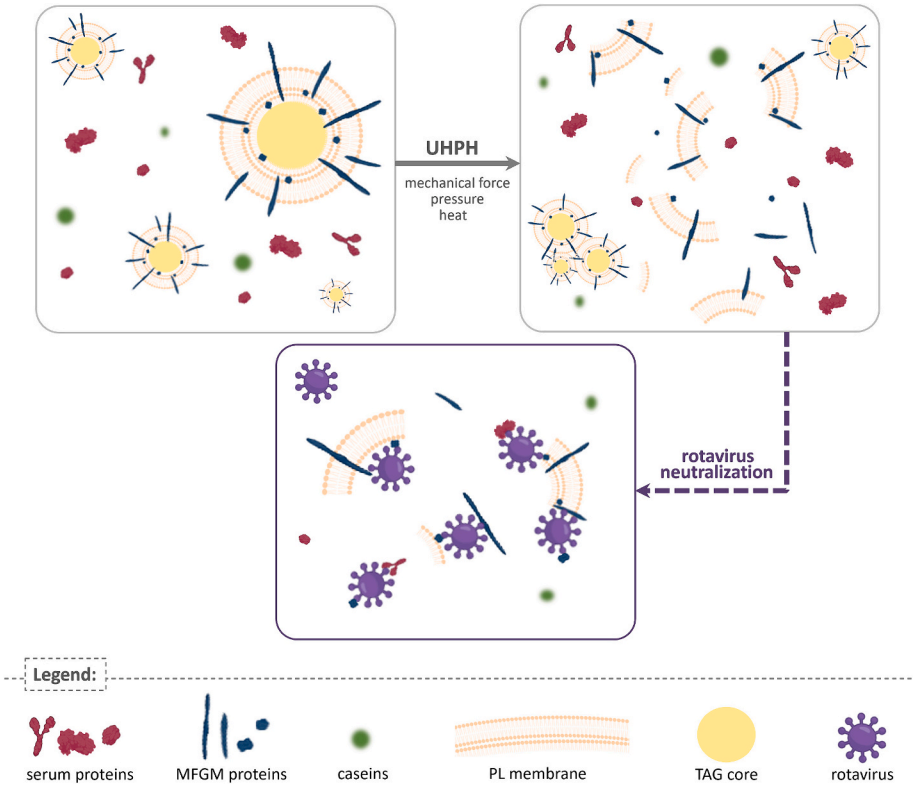

Fig. 6. Graphical representation of the hypothesis on the effect of ultra-high pressure treatment (UHPH) on the antirotaviral activity of the whey fat fraction. MFGM: milk fat globule membrane; PL: phospholipid; TAG: triacylglycerols. Created with BioRender.com.

MFGM isolated from either cheese whey or buttermilk, effectively inhibited the OSU porcine rotavirus infection of MA104 cells. When the lipid and aqueous phases of the MFGM complex were compared, all antirotaviral activity was recovered in the lipid phase, whereas the aqueous phase did not exert any significant inhibitory effect. Therefore, the authors argued that the lipid components of the MFGM are mainly responsible for the antirotaviral activity (Fuller et al., 2013). In the present study, the FF of the whey demonstrated higher neutralization activity compared to the whole whey fractions, both for the non-treated and the UHPH-treated samples. Accordingly, Touhami et al. (2023) found that the total phospholipid content recovered from buttermilk cream increased significantly after UHPH (100, 200 and 300 MPa) treatment and ultracentrifugation (Touhami et al., 2023). Collectively, these observations could explain the higher antirotaviral activity observed in the present study in the FF obtained from whey (both control and UHPH-treated) compared to the whey as a whole fraction.

To the best of the author's knowledge, this is the first study investigating the effect of UHPH on the antiviral activity of dairy fractions, particularly of whey. In the study of Iucci, Patrignani, Vallicelli, Guerzoni, and Lanciotti (2007), it was reported that the antimicrobial activities of lactoferrin and lysozyme were enhanced by low intensity UHPH treatment at 100 MPa (Iucci et al., 2007). Recently, it has been reported that the antirotaviral activity of buttermilk-based formulas increases with low intensity heat and pressure treatments (Graikini, García, et al., 2024). Together, these observations indicate the potential of dairy fractions in the fight against infectious diseases along with the possibility to preserve their bioactivity with industrial processing. Microbial safety and retention of bioactive compounds are essential requirements for the development of functional food products. Therefore, future studies should focus on evaluating the antimicrobial activity of milk components recovered from byproducts from dairy industry with the objective to promote circular economy.

4. Conclusions

In conclusion, it was revealed that when UHPH treatments of lower intensities (100 and 200 MPa) are applied to bovine whey from cheese manufacture, its strong antirotaviral activity is maintained. However, homogenization at a pressure of 250 MPa results in the loss of such bioactivity, which may have been the result of fragmentation and/or aggregation of several bioactive whey proteins. At the same time, the residual FF present in the whey appears to be unaffected by those UHPH treatments, regarding the rotavirus neutralizing activity, but experiments with a greater concentration-dependence range should be performed in order to clarify the exact effect of the UHPH treatment on this specific whey fraction. Overall, this study provides useful information to increase the commercial value of sweet, skimmed whey as a functional ingredient and its recognition as a natural source of antivirals. Furthermore, the use of emerging processing technologies, such as UHPH, could support the preservation of the antiviral activity of this dairy byproduct. It would be interesting to continue investigating the effect of non-thermal technologies on the bioactivity of more types of whey generated by the dairy industry. Moreover, detailed characterization of the molecular changes that take place within the dairy fractions will be crucial in establishing its future potential.

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CRediT authorship contribution statement

Dimitra Graikini: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Saioa Alvarez-Sabatel:** Writing – review & editing, Methodology. **Eduardo Puértolas:** Writing – review & editing, Methodology. **María Dolores Pérez:** Writing – review & editing. **Lourdes Sánchez:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

There are no conflicts to declare.

Data availability

Data will be made available on request.

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