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Effect of homogenization and ultra-high-pressure homogenization on the activity of dairy by-products against gastrointestinal pathogens

Laura García[1,3], Laura Carmona[1], Rodrigo Casado[1], María Dolores Pérez[1,3], Antonio J. Trujillo-Mesa[5], M. Manuela Hernández-Herrero[5], Artur X. Roig-Sagués[5], Laura Grasa[2,3,4], Lourdes Sánchez[1,3]

[1]Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria. Universidad de Zaragoza, 50013 Zaragoza, Spain; [2]Departamento de Farmacología, Fisiología y Medicina Legal y Forense, Facultad de Veterinaria, Universidad de Zaragoza, 50013 Zaragoza, Spain; [3]Instituto Agroalimentario de Aragón (IA2) (Universidad de Zaragoza-CITA). Zaragoza, Spain; [4]Instituto de Investigación Sanitaria de Aragón (IIS Aragón), Centro de Investigación Biomédica de Aragón, 50009 Zaragoza, Spain; [5]Centre d'Innovació, Recerca i Transferència en Tecnologia dels Aliments, TECNIO CERTA-UAB, Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, Bellaterra 08193 Barcelona, Spain.

***Corresponding author:** Lourdes Sánchez, PhD, Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, dont Miguel Servet, 177, Zaragoza - 50013, Spain.

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ABSTRACT

Background: The growing emphasis on agri-food sustainability and the circular economy has promoted the valorization of dairy by-products, particularly whey, which has an estimated annual global production of approximately 200 million tons. Whey is a valuable source of proteins with nutritional and bioactive properties, and nearly half of its volume is processed into whey protein concentrate (WPC) and whey protein isolate (WPI). During WPI production, whey protein phospholipid concentrate (WPPC) is generated; however, its functional properties remain poorly characterized. The incorporation of bioactive compounds from bovine milk and its by-products into infant formulas and specialized products may contribute to gut, brain, and immune system health.

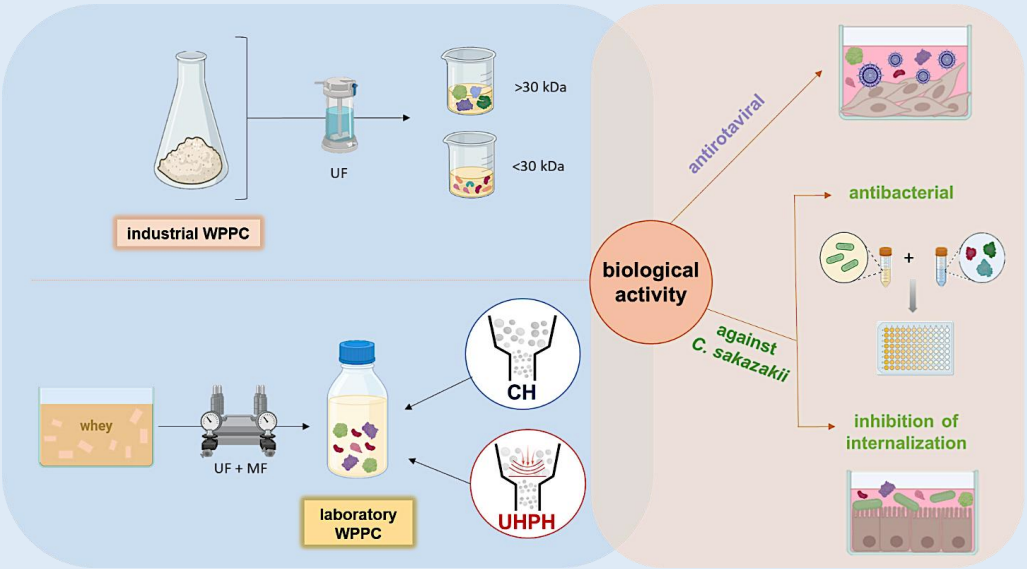
Objective: This study aimed to characterize WPPC obtained from bovine whey and to evaluate its activity against *Cronobacter sakazakii* and rotavirus, as well as to assess the impact of conventional homogenization (CH) and ultra-high-pressure homogenization (UHPH) on this bioactivity.

Methods: WPPC was obtained from industrial cheese whey through ultrafiltration and microfiltration. The resulting fractions were characterized by SDS-PAGE, laser diffraction (Mastersizer), and proteomic analysis, and were further compared with commercial WPPC. Conventional homogenization (25 MPa) and UHPH treatments (100–200 MPa) were applied using pilot plant-scale equipment. Antibacterial activity against *C. sakazakii* was evaluated by measuring bacterial growth and adhesion to Caco-2/TC7 cells, while antiviral activity against bovine rotavirus strain WC3 was assessed in MA104 cells using indirect immunofluorescence.

Results: WPPC at a protein concentration of approximately 5 mg/mL inhibited *C. sakazakii* growth and adhesion to Caco-2/TC7 cells by ~40-50%, with both CH and UHPH enhancing the inhibitory activity of non-commercial WPPC by ~40%. WPPC achieved complete inhibition of rotavirus at concentrations above 0.25 mg/mL. The observed bioactivities appear to be mainly attributable to compounds with molecular weights greater than 30 kDa.

Conclusion: WPPC exhibits significant antibacterial and antiviral activities, with antibacterial effects enhanced by homogenization treatments. UHPH emerges as a promising alternative to thermal pasteurization. These findings further expand current knowledge of WPPC functional properties and support its potential application as a functional ingredient in health-oriented food formulations.

Keywords: cheese whey, WPPC, homogenization, ultra-high-pressure homogenization, rotavirus, *Cronobacter sakazakii*



Graphical abstract: Effect of homogenization and ultra-high-pressure homogenization on the activity of dairy by-products against gastrointestinal pathogens

INTRODUCTION

Sustainability initiatives and circular-economy frameworks have intensified efforts to valorize dairy by-products, particularly whey, the major residue from cheese and yogurt manufacture. Global whey production is estimated at ~200 million tons per year, creating environmental pressure due to its high biological and chemical oxygen demand [1]. Although historically treated as waste, whey retains roughly half of milk's original solids and remains a rich source of nutritionally valuable proteins and bioactive compounds [2].

Industrial processing has transformed whey into high-value ingredients such as whey protein concentrate (WPC, 60–85% protein) and whey protein isolate (WPI, ≥90% protein), both of which are widely used. Used for their nutritional and functional properties. WPCC is generated during WPI manufacture and is enriched in proteins (≥50%), lipids (≥12%), and milk fat globule membrane (MFGM)-derived proteins and phospholipids [3]. (MFGM)-associated components have been linked to antimicrobial and anti-inflammatory activities and may support intestinal and neurodevelopmental outcomes, increasing interest in their use as functional ingredients [4,5]. Consequently, WPCC represents a potentially valuable co-product for sustainable ingredient development, alongside other whey valorization routes [6].

Whey-derived ingredients are commonly subjected to thermal treatments, fractionation, and enzymatic hydrolysis. However, non-thermal processing technologies–such as pulsed electric fields, pulsed light, high hydrostatic pressure, high-pressure homogenization (HPH), and ultrasounds–are increasingly used to preserve nutritional quality while enabling new functionality and improved stability [7]. Among these, HPH and ultra-high-pressure homogenization (UHPH) are fluid-mechanical techniques that disrupt liquid suspensions by forcing them through micrometric valves at pressures ranging from 20–60 MPa (HPH) to 350–400 MPa (UHPH) [8]. This process reduces milk fat globule size from an average of 3.5 µm to <1 µm, effectively preventing fat separation and improving product stability. Beyond particle disruption, high- and ultra-high-pressure homogenization (HPH/UHPH), induce structural modifications in proteins, altering hydrophobic, electrostatic, and intra- and intermolecular interactions [9]. Apart from processing benefits, the application of these technologies to food industry byproducts may also address pressing public health challenges. Infectious gastroenteritis remains the most prevalent gastrointestinal disorder in children, with rotavirus being the leading global cause of severe diarrhea and diarrheal mortality in infants and young children [10]. It is also a significant concern in older adults [11]. Although vaccination and oral rehydration therapy are the primary preventive and therapeutic strategies, their limited accessibility and variable efficacy highlight the need for alternative anti-rotaviral agents. In this context, bioactive compounds from whey and its by-products represent promising candidates for novel therapeutic approaches [11]. Concurrently, *Cronobacter* spp. has gained attention as an emerging foodborne pathogen of critical concern, particularly for high-risk groups such as infants, preterm neonates, and elderly individuals. *Cronobacter sakazakii* accounts for approximately 0.5–2.4% of total foodborne diseases and is associated with severe clinical outcomes, including meningitis, septicemia, and necrotizing enterocolitis, with neonatal mortality rates reaching approximately 25% [12]. This pathogen has been detected in powdered infant formula, cereals, reconstituted formula, a wide range of food products, water, and hospital or household environments [12,13].

Given the growing challenges of antimicrobial resistance and safety concerns associated with synthetic preservatives, research has increasingly focused on natural antimicrobials for food safety applications,

particularly in infant nutrition. Compounds of animal origin (e.g., lactoferrin.), plant origin (e.g., essential oils), and microbial origin (e.g., bacteriocins) have demonstrated inhibitory activity against foodborne pathogens, either individually or in combination with complementary preservation strategies [14].

The objective of this study was to characterize WPPC derived from cheese whey and to evaluate its antibacterial activity against the emerging pathogen *C. sakazakii*, as well as its antiviral activity against rotavirus, in comparison with commercial WPPC. In addition, the study aimed to assess the influence of conventional homogenization and ultra-high-pressure homogenization on these biological activities.

MATERIALS AND METHODS

Preparation and characterization of WPPC: Whey was provided by the cheese industry VillaCorona S.A. (El Burgo de Ebro, Spain) and was derived from the manufacture of low-salt fresh cheese produced by rennet coagulation of pasteurized cow's milk. Whey was fractionated to obtain whey protein phospholipid concentrate (WPPC) using ultrafiltration and microfiltration with 10 kDa and 1000 kDa membranes (Millipore, Burlington, MA, USA). In the ultrafiltration step, two fractions were obtained: a whey protein permeate and a retentate corresponding to whey protein concentrate (WPC). The retentate was subsequently processed by microfiltration to generate two additional fractions: a permeate corresponding to whey protein isolate (WPI) and a retentate corresponding to WPPC hereafter referred to as non-commercial WPPC. Vivinal® MFGM (FrieslandCampina, Amersfoort, The Netherlands), which is analogous to WPPC and obtained from pasteurized cheese whey, was donated by the company and used as commercial WPPC. Commercial WPPC was rehydrated according to the manufacturer's recommendations and fractionated using an ultrafiltration cell (Amicon Inc., Beverly, MA, USA) fitted with a 30 kDa membrane (Millipore). This yielded two fractions: a >30 kDa fraction and a <30 kDa fraction. Subsequently, three diafiltration steps were performed by adding three volumes of PBS relative to the initial sample volume, after which both fractions were concentrated.

Non-commercial WPPC was tempered to 50 °C and subjected to conventional homogenization (CH) at 25 MPa using a Panda homogenizer (Niro Soavi, Brazil) operating at a production flow rate of 10 L/h at the Pilot Plant for Food Science and Technology (Faculty of Veterinary, Universidad de Zaragoza, Spain). Non-commercial WPPC was also processed by ultra-high-pressure homogenization (UHPH) at 100, 150, and 200 MPa using a prototype valve-based system (60–100 L/h, depending on operating pressure) manufactured by YPSICON Advanced Technologies (Barcelona, Spain) and located at the Pilot Plant for Food Technology (Universitat Autònoma de Barcelona, Spain). Prior to UHPH treatment, WPPC was filtered through a metallic mesh sieve (117 µm) to remove aggregates and ensure optimal processing. The different dairy fractions obtained were characterized by SDS-PAGE according to the procedure described by Dimitra et al. (2024) [15], using commercial Mini-Protean TGX 4–20% polyacrylamide gels and a Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, Hercules, CA, USA). Samples were diluted at a 1:1 (v/v) ratio in a buffer containing 125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.02% bromophenol blue, and 5% 2-mercaptoethanol. The PageRuler Prestained Protein Ladder (GE Healthcare, Buckinghamshire, UK) was used as the molecular weight standard. After electrophoresis, proteins were stained with a Coomassie Blue solution containing 0.65 g/L Coomassie Blue R (Serva Feinbiochemica, Heidelberg, Germany) and destained according to standard procedures.

The particle size distribution of non-commercial WPPC was determined by laser diffraction following the procedure described by Graikini et al. (2024) [15], using a

Mastersizer 3000E system (Malvern Instruments, Malvern, UK) equipped with a wet dispersion unit.

Culture of *Cronobacter sakazakii* and antibacterial activity assay: Antibacterial activity assays of the dairy fractions were performed using *C. sakazakii* CECT 853, obtained from the Spanish Type Culture Collection (Valencia, Spain) and preserved in cryobeads at −80 °C. For culture, a cryobead was transferred to 10 mL of tryptic soy broth (TSB; Oxoid, Basingstoke, UK) supplemented with 0.6% (w/v) yeast extract (YE; Oxoid) and incubated at 37 °C for 24 h. Subsequently, streak plating was performed on tryptic soy agar (TSA; Oxoid) supplemented with 0.6% (w/v) YE to obtain isolated colonies, followed by incubation at 37 °C for 24 h. All experiments involving *C. sakazakii* were conducted under sterile conditions in a Telstar PV-30/70 laminar flow cabinet (Thermo Fisher Scientific). Assays were carried out using *C. sakazakii* in the stationary phase. An isolated colony was inoculated in 10 mL TSB supplemented with 0.6% (w/v) YE and incubated at 37 °C for 18–20 h. The resulting suspension was diluted to approximately 10^4 CFU/mL. The antibacterial activity of non-commercial WPPC fractions treated by CH and UHPH was evaluated at protein concentrations of 1.15, 2.30, and 4.60 mg/mL. The antibacterial activity of commercial WPPC and its fractions was assessed at concentrations of 1.25, 2.50, 5.00, and 10.00 mg/mL. Each treatment was performed in sterile 96-well plates by mixing 100 µL of bacterial suspension with 100 µL of sample, in duplicate, followed by incubation at 37 °C for 4 or 24 h. After incubation, serial dilutions were plated on TSA supplemented with 0.6% (w/v) YE and incubated at 37 °C for 24 h. Colony counts were expressed as CFU/mL, and antibacterial activity was calculated as the percentage reduction relative to the untreated control.

Inhibition of *C. sakazakii* internalization assay: For the inhibition assays of *C. sakazakii* adhesion to a model of human intestinal epithelium, the Caco-2/TC7 cell line, derived from human colon adenocarcinoma and differentiated into enterocyte-like cells, was employed. This cell line was kindly provided by Dr. Rousset's group at INSERM U178 (Villejuif, France). Cells were cultured in 25 cm^2 flasks (TPP) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Life Technologies, Paisley, UK) supplemented with 20% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), 1% (v/v) non-essential amino acids (NEAA), 2 mM L-glutamine, amphotericin and antibiotic solution (100 U/mL penicillin and 100 µg/mL streptomycin (Gibco Life Technologies). This medium is hereafter referred to as supplemented DMEM. Culture flasks were maintained in a Heraeus thermostatic incubator (Hanau, Germany) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. In parallel, a culture medium referred to as basic DMEM was prepared, consisting of DMEM supplemented with 1% (v/v) NEAA, 2 mM L-glutamine, and amphotericin. Cell viability of differentiated Caco-2/TC7 cells exposed to dairy fractions was assessed using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) kit (Promega, Madison, WI, USA) in 96-well plates, following the manufacturer's instructions. Formazan production was quantified by measuring absorbance at 492 nm in a Multiskan MS ELISA plate reader, with color intensity proportional to the number of viable cells.

C. sakazakii internalization assays were performed in differentiated Caco-2/TC7 cells cultured for 15–17 days in 24-well plates with supplemented DMEM. Cells were pre-incubated for 2 h at 37 °C in 2 mL antibiotic-free basic DMEM, washed with sterile PBS, and treated in duplicate with 150 µL of each test sample for 1 h at 37 °C.

Stationary-phase *C. sakazakii* was obtained from an overnight culture (TSB supplemented with 0.6% YE, 37 °C, 18–20 h). Bacteria were harvested by centrifugation (5000 × g, 10 min), washed twice with PBS, and resuspended in 1% (w/v) peptone water. Serial dilutions were prepared to obtain a final concentration of approximately 10^5 CFU/mL. After treatment of the cells with the samples, wells were washed with PBS and infected with 50 µL of bacterial suspension in 150 µL antibiotic-free basic DMEM supplemented with 1%

mannose (Thermo Fisher, Kandel, Germany), followed by incubation for 4 h at 37 °C. Non-internalized and surface-adherent bacteria were removed by washing with PBS and treating the cells with gentamicin (150 µg/mL; Sigma-Aldrich) for 2 h at 37 °C. After antibiotic removal and washing, cells were lysed with 0.2% Triton X-100 (Sigma-Aldrich) in PBS (500 µL/well) for 15 min at room temperature. Serial dilutions of the lysates were plated in duplicate on TSA supplemented with 0.6% (w/v) YE and incubated at 37 °C for 24 h to determine CFU counts.

Antirotaviral activity assay: The bovine rotavirus (BRV) used in the assays was the WC3 strain (ATCC VR-2102) provided by the American Type Culture Collection (ATCC). Antirotaviral activity was assessed in the rhesus monkey fetal kidney epithelial cell line MA104 (CLR-2378), also obtained by ATCC. MA104 cells were cultured in Minimal Essential Medium (MEM; Gibco Life Technologies, Paisley, UK). Supplemented MEM consisted of MEM with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin), 1% (v/v) 2 mM L-glutamine, and 0.25 µg/mL amphotericin B. Basic MEM was prepared identically but without FBS. Prior to evaluating the activity of the dairy fractions, their effect on cell viability was assessed using the same method as described for the Caco-2/TC7 cell line. First, the supplemented MEM was removed from 96-well plates containing MA104 cells at approximately 95% confluence, and 200 µL/well of basic MEM was added to facilitate the removal of FBS from the culture. Plates were incubated with this medium at 37 °C for 2 h. During this period, BRV was activated by adding porcine pancreatic trypsin (Sigma-Aldrich) at a final concentration of 20µg/mL for 1 h at 37 °C, with manual agitation every 10 min. This activation step allows trypsin-mediated cleavage of the rotavirus VP4 protein, enabling the virus to acquire its infectious conformation. The activated BRV suspension was then diluted 1:60 in basic MEM for infection of MA104 cells.

Subsequently, in conical-bottom 96-well plates, the test samples were mixed with the activated BRV suspension at a 1:1 ratio (25 µL each) and incubated for 1 h at 37 °C under gentle agitation. After incubation, 50 µL of the sample–virus mixture was transferred to the cell plates, from which the previously added basic medium had been removed. The plates were then incubated at 37 °C for 1 h with mild agitation. Next, 100 µL/well of facilitation medium was added, consisting of MEM supplemented with 4% FBS and trypsin (2 µg/mL), and plates were incubated at 37 °C with gentle agitation for 16 h. The antiviral activity of the samples against BRV was evaluated by detecting viral infection in cells using an indirect immunofluorescence assay after 16 h of incubation. Briefly, the contents of the wells were removed and washed with 200 µL/well of sterile PBS. Cells were then fixed by adding 300 µL/well of an acetone:methanol:formalin solution (1:1:1) and incubated at 4 °C for 4 min. The fixative was removed, and the plates were washed three times with 300 µL of PBS.

Infected cells were detected using a mouse monoclonal anti-BRV antibody [A2] (Abcam, Cambridge, UK) as the primary antibody at a 1:500 dilution in PBS containing 3% (w/v) commercial gelatin (Sigma-Aldrich), adding 50 µL/well and incubating at 37 °C for 2 h under gentle agitation. Wells were then washed three times with PBS, and 50 µL/well of goat anti-mouse IgG conjugated with FITC (Abcam), diluted in PBS with 3% (w/v) gelatin, was added as the secondary antibody. Plates were incubated at 37 °C for 1 h under gentle agitation and protected from light. Subsequently, the plates were washed three times with PBS, and fluorescent cells were counted using a phase-contrast and fluorescence microscope (Nikon Eclipse E400, Nikon Corporation, Japan) equipped with a high-resolution AxioCam MRc camera (Zeiss, Germany) and ZEN 2012 software (Zeiss, Germany) for image processing. Images were acquired from three fields per well, ensuring the integrity of the cell monolayer prior to selection. Infectivity percentages were determined by enumerating infected cells per well relative to the positive control,

consisting of BRV suspension without any antiviral treatment, which was considered 100% infectivity.

Statistical analysis: Data were analyzed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Data normality was assessed using the Shapiro–Wilk test. For normally distributed variables, Student's t-test or one-way analysis of variance (ANOVA), followed by Tukey's or Dunnett's multiple-comparison test, was applied. For non-normally distributed variables, the Mann–Whitney U test or Kruskal–Wallis test, followed by Dunn's multiple-comparison test, was used. Differences with $p \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

WPPC characterization: The electrophoretic profile of commercial WPPC (Figure 1A) showed a protein composition consistent with the typical profile of this by-product [5,16,17], including β-LG (~18 kDa), α-LA (~14 kDa), and several higher molecular weight proteins derived from whey and MFGM, such as xanthine oxidase (XO; 140 kDa). Ultrafiltration resulted in partial size-based separation, with proteins of varying molecular weights detected in both fractions, indicating incomplete resolution or protein interactions. In the <30 kDa fractions, a greater reduction of higher molecular weight bands was observed compared with the >30 kDa fractions (Figure 1B).

The electrophoretic analysis of non-commercial WPPC, both untreated and treated by homogenization, was conducted to evaluate potential modifications in the protein profile after treatment, with results shown in Figure 1C. The electrophoretic patterns of the homogenized samples exhibited a high degree of similarity among treatments and with untreated WPPC, suggesting that, within the applied pressure range, no significant changes in protein composition were detectable by this method. These findings are consistent with those of Sato et al. (2021) [18], who reported no major alterations in the electrophoretic profile of WPI samples treated at pressures up to 240 MPa, indicating that whey proteins in WPI and WPPC are not denatured under these conditions. However, in samples 5 and 6, corresponding to UHPH treatments at 100 and 150 MPa, respectively, an increased intensity of the XO band was observed, which may indicate the release of this protein from the MFGM.

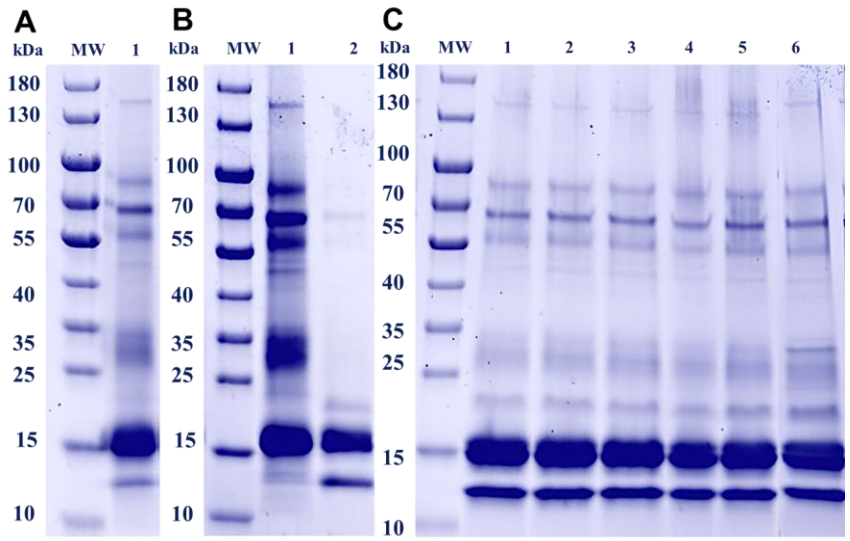


Figure 1. SDS-PAGE of A: 1) Commercial WPPC. B: 1) Fraction >30 kDa; 2) fraction<30 kDa. C: Non-commercial WPPC. 1) WPPC; 2) WPPC CH 25 MPa; 3) WPPC UHPH 100 MPa; 4) WPPC UHPH 150 MPa; 5) WPPC UHPH 200 MPa; 6) Whey. MW: molecular weight marker.

The results of proteomic analysis of non-commercial WPPC revealed a similar protein composition between the untreated and treated fractions. We focused mainly on the composition of MFGM proteins, finding that FABP was the most abundant, followed by adipophilin, butyrophilin, and lactadherin.

Assessment of particle size distribution in WPPC, including UHPH-treated samples, is particularly relevant because UHPH reduces fat globule size to <1 µm. This reduction can improve uniformity and stability and may induce protein structural changes that increase exposure of hydrophobic groups [19].

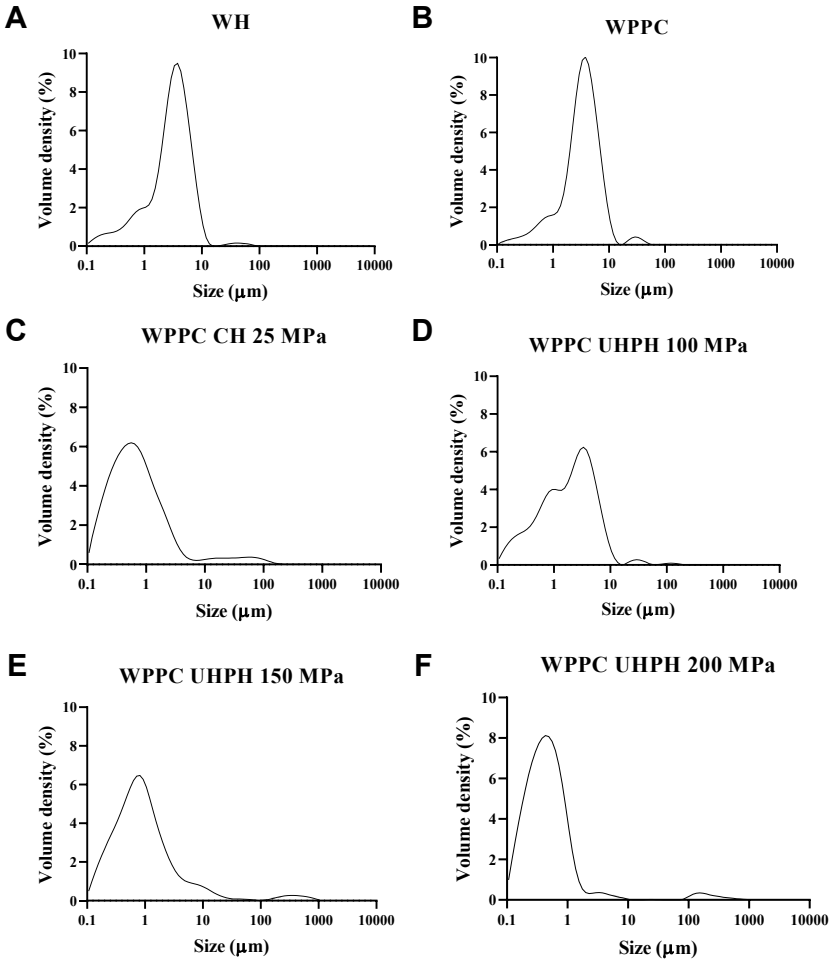


Figure 2. Analysis of volume density and particle size distribution of whey, non-commercial WPPC, and non-commercial WPPC treated by conventional homogenization (CH) and ultra-high-pressure homogenization (UHPH), represented on a semi-logarithmic scale. (A) Whey (WH), (B) WPPC, (C) WPPC CH 25 MPa, (D) WPPC UHPH 100 MPa, (E) WPPC UHPH 150 MPa, (F) WPPC UHPH 200 MPa.

As shown in Figure 2, whey and untreated WPPC exhibited very similar particle size distribution profiles, ranging from 1 to 10 µm. However, after the application of CH and UHPH, a clear reduction in particle size was observed. Specifically, after homogenization, particle size was reduced to the 0.1–1 µm range. It is noteworthy that, following UHPH treatment at 100 MPa, although a decrease in fat globule size was evident, a considerable proportion of particles between 1 and 10 µm remained. In contrast, CH treatment at 25 MPa produced a homogenization effect comparable to that observed with UHPH at 150 MPa, with a marked reduction in particle size. Finally, UHPH at 200 MPa resulted in the greatestdecrease in fat globule size, yielding a particle size distribution centered below 1 µm.

Antibacterial activity of WPPC against *C. sakazakii*: The results of the antibacterial activity of commercial WPPC against *C. sakazakii* are presented in Figure 3. When comparing activity at 4 h and 24 h for each sample and concentration, significant differences ($p < 0.05$) were observed at concentrations of 1.25 and 5 mg/mL. This behavior suggests that the activity observed at 4 h may be associated with a bacteriostatic effect. These findings are consistent with those reported by Abad et al. (2023) [20], who evaluated the effect of bovine lactoferrin on *C. sakazakii* and observed a similar trend. Nevertheless, the levels of antibacterial activity were considerably higher for pure lactoferrin compared to WPPC. Although WPPC contains lactoferrin, its concentration is low, and it is present in combination with other proteins, which may interact with and interfere with lactoferrin activity.

A concentration-dependent reduction in CFU counts was observed, as higher WPPC concentrations generally resulted in greater antibacterial activity. An exception was noted as at 24 h, where WPPC exhibited an inverse trend, with reduced activity at 2.5 and 5 mg/mL, whereas activity increased at 1.25 mg/mL. This outcome could be explained by the induction of bacterial resistance mechanisms under prolonged incubation, which may be more strongly activated at higher concentrations of bioactive compounds.

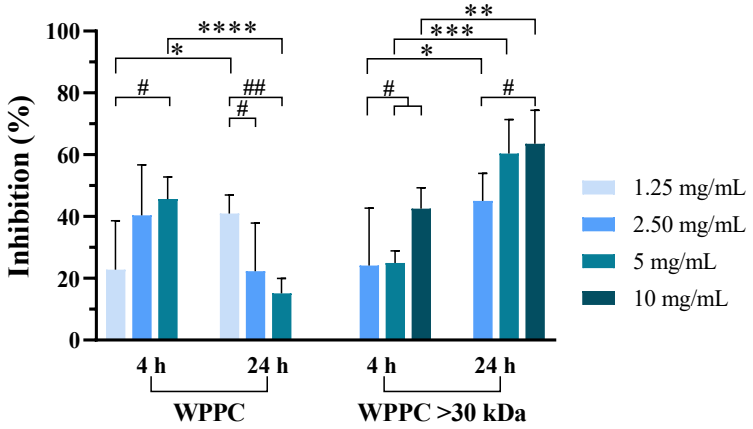


Figure 3. Activity of commercial WPPC and its >30 kDa fraction against *C. sakazakii* at 4 and 24 h of incubation. The concentration of the samples is expressed as mg protein/mL. Results are expressed as the percentage of inhibition relative to the control and reported as the mean ± standard deviation of three independent experiments (n=6). *Indicates significant differences in antibacterial activity between incubation times for each sample and concentration (*p<0.05; **p<0.01; ***p<0.001; **p<0.0001). #Indicates significant differences in antibacterial activity among the evaluated concentrations for each sample and incubation time (#p<0.05; ##p<0.01).

In addition, the >30 kDa fraction of commercial WPPC showed a statistically significant ($p < 0.05$) increase in activity at 24 h compared with 4 h, for all tested concentrations, in contrast to the whole sample. This result suggests that, upon removal of proteins with molecular weights <30 kDa, the remaining proteins retain and may even enhance their antibacterial effect over time. Moreover, no statistically significant differences were observed between WPPC and the >30 kDa WPPC fraction at comparable concentrations (2.5 and 5 mg/mL) at either time point. It should be noted that results for the <30 kDa fraction were not included, as preliminary assays showed no antibacterial activity for this fraction. Overall, these findings indicate that the proteins responsible for the observed bioactivity are predominantly associated with molecules with a molecular weight greater than 30 kDa.

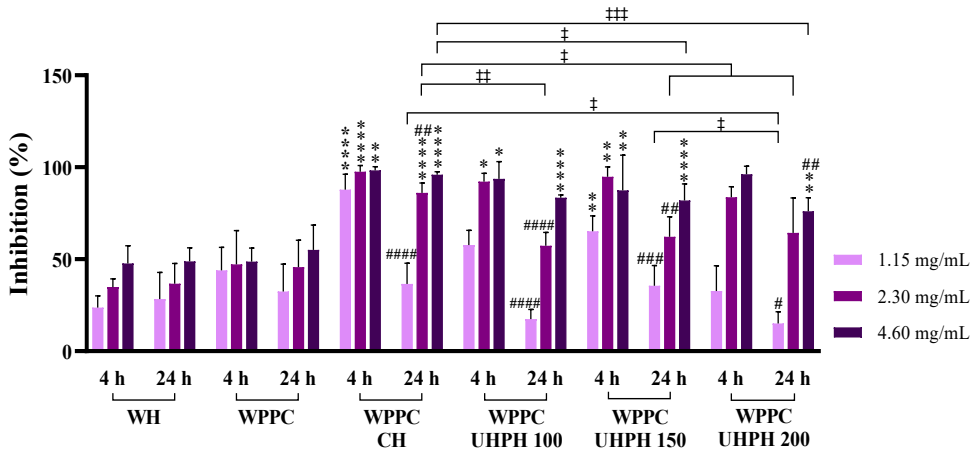


Figure 4. Antibacterial activity of whey (WH), non-commercial WPPC, and non-commercial WPPC treated by conventional homogenization (CH) and by ultra-high-pressure homogenization (UHPH) against *C. sakazakii* after 4 and 24 h of incubation. Sample concentrations are expressed as mg protein/mL. Results are expressed as the percentage of inhibition relative to the control and reported as the mean ± standard deviation of three independent experiments (n=6). *Indicates significant differences in antibacterial activity of treated WPPCs compared with untreated WPPC for each incubation time and concentration (*p<0.05; **p<0.01; ****p<0.0001). #Indicates significant differences in antibacterial activity between the two incubation times for each sample and concentration (#p<0.05; ##p<0.01; ###p<0.001; ####p<0.0001). ‡Indicates significant differences in antibacterial activity among the treated samples for each incubation time and concentration (‡p<0.05; ‡‡p<0.01; ‡‡‡p<0.001).

Figure 4 shows the results obtained after exposing *C. sakazakii* to whey, non-commercial WPPC, and WPPC samples subjected to homogenization treatments. A reduction in bacterial counts was observed under all tested conditions, indicating that these samples exhibited antibacterial activity. First, the activity of WPPC was compared with that of the starting whey; both displayed very similar inhibition levels, with no statistically significant differences between them. Second, the effect of CH and UHPH treatments on the antibacterial activity of WPPC was evaluated. The results indicated that these treatments enhanced the inhibitory capacity of WPPC against *C. sakazakii*. CH increased the activity by over 40% at all concentrations after 4 hours. In the case of UHPH-treated samples, activity was elevated by 35–40% at 4 hours at concentrations of 2.3 and 4.6 mg/mL. After 24 hours, all treatments resulted in an enhancement of inhibitory activity at the two highest concentrations tested, with CH showing the most pronounced effect, exceeding a 40% increase in activity.

These findings are consistent with Iucci et al. (2007) [21], who reported increased antibacterial activity of lactoferrin after ultra-high-pressure homogenization at 100 MPa, attributed to pressure-induced disruption of the supramolecular structure, which increases molecular mobility and exposes hydrophobic regions. Although UHPH has been extensively investigated for its effects on technological properties of dairy systems, its influence on antimicrobial functionality remains comparatively underexplored. Dimitra et al. (2024) [15] evaluated the effect of UHPH on whey antirotaviral activity and concluded that the treatment preserved the antiviral activity naturally present in whey.

UHPH treatments may induce reversible or irreversible modifications in the tertiary and quaternary structures of proteins, which could facilitate their functionalization and potentially enhance bioactivity. Such structural changes may increase the exposure of antimicrobial domains in whey proteins and contribute to the observed improvements in antimicrobial activity. In addition, UHPH-induced alterations in the MFGM, as well as in protein–protein and protein–membrane interactions, may influence the availability and functionality of antimicrobial components. While UHPH has been generally explored as an alternative to conventional thermal treatments, the findings of the present study suggest that this technology may not only

preserve, but also enhance, the bioactivity of food components [15,22].

When the antibacterial activity of treated WPPCs was compared with that of untreated WPPC at 4 h, statistically significant differences ($p < 0.05$) were observed at all tested concentrations, except for WPPCs treated by UHPH at 100 MPa and 200 MPa, at 1.15 mg/mL. After 24 h of incubation, significant differences in antibacterial activity were detected for all treated samples at 4.6 mg/mL, and only for CH-treated WPPC at 2.3 mg/mL, whereas no significant differences were observed at 1.15 mg/mL compared with untreated WPPC.

Furthermore, when comparing the 24 h results with those at 4 h for each sample and concentration, a statistically significant reduction in antibacterial activity was observed for all samples at concentrations of 1.15 and 2.3 mg/mL. In contrast, at 4.6 mg/mL, no significant differences were found, as inhibition values were close to 100% at both time points. However, for WPPC treated by UHPH at 200 MPa, significant differences were observed at 1.15 and 4.6 mg/mL but not at 2.3 mg/mL, where the reduction in antibacterial activity was not statistically significant.

Inhibition of *C. sakazakii* internalization into Caco-2/TC7 cells: Cell viability was evaluated by MTS assay prior to bacterial internalization experiments in differentiated Caco-2/TC7 cells. As shown in Figure 5A, exposure to commercial WPPC and its >30 kDa fraction resulted in viability values consistently above 90%, with only minor decreases to approximately 90% in a few cases. These results are within the acceptable range (>85%) reported previously [23,24], confirming the absence of relevant cytotoxic effects under the experimental conditions.

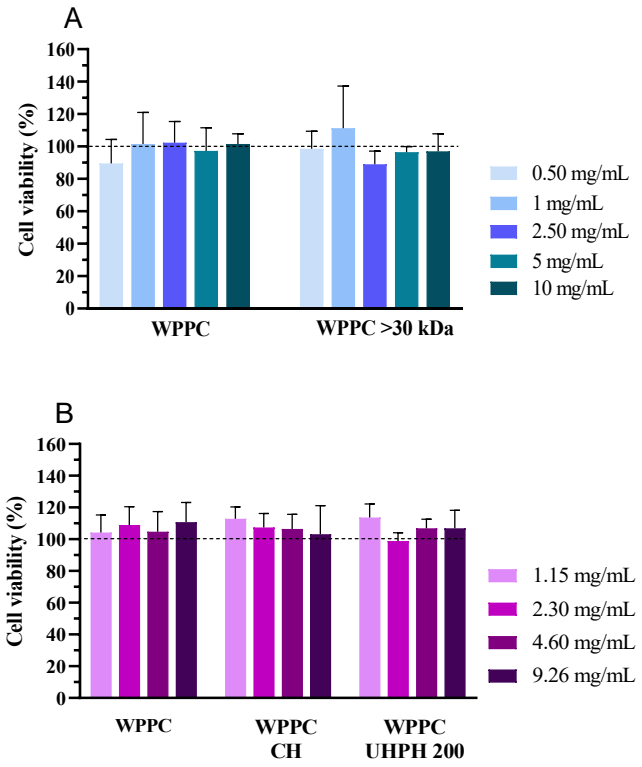


Figure 5. A) Viability of differentiated Caco-2/TC7 cells after incubation with commercial WPPC and its >30 kDa fraction. The concentration of the dairy samples is expressed as mg protein/mL for the whole WPPC and as mg protein/mL for the >30 kDa fraction. Results are expressed as the percentage of cell viability relative to the control and reported as the mean ± standard deviation of two independent experiments (n=6). B) Viability of differentiated Caco-2/TC7 cells after incubation with non-commercial WPPC treated by CH at 25 MPa and UHPH at 200 MPa. Sample concentrations are expressed as mg protein/mL. Results are expressed as the percentage of cell viability relative to the control and reported as the mean ± standard deviation of two independent experiments (n=6).

Cell viability remained above 100% for untreated non-commercial WPPC and for WPPC treated by CH (25 MPa) or UHPH (200 MPa) (Figure 5B). The only exception was UHPH-treated WPPC (200 MPa) at 2.3 mg/mL, which yielded a mean viability of 98.9%. These findings indicate that the treatments did not produce cytotoxic components under the tested conditions.

Adhesion to host cell surfaces constitutes a critical step in the pathogenesis of gastrointestinal infections [23]. Once attached, pathogens can readily access nutrients, release toxins, and eventually cross the intestinal barrier to enter the bloodstream, leading to disease development [25]. In the case of *C. sakazakii*, the outer membrane proteins have been described as a key factor in the invasion of intestinal cells [26]. Notably, adhesion inhibitors of pathogens have been identified in certain foods, including human milk [27].

The capacity of commercial and non-commercial WPPC, including CH- and UHPH-treated samples, to inhibit *C. sakazakii* internalization into differentiated Caco-2/TC7 cells was evaluated. As shown in Figure 6, commercial WPPC and its >30 kDa fraction did not display a concentration-dependent response, since inhibition levels did not differ significantly across concentrations within each sample.

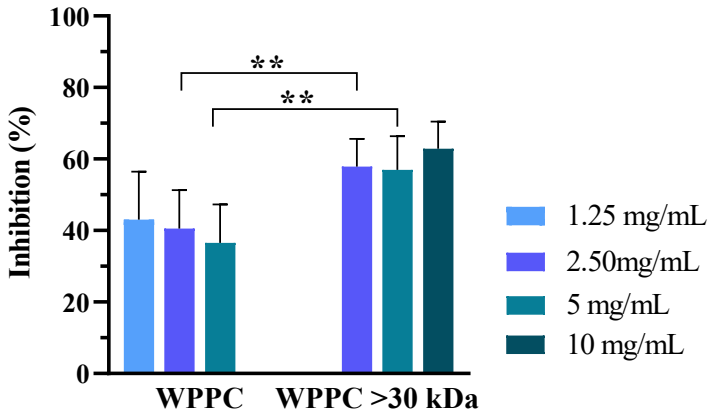


Figure 6. Inhibition assay of *C. sakazakii* internalization in differentiated Caco-2/TC7 cells by commercial WPPC. The concentration of the samples is expressed as mg protein/mL. Results are expressed as the percentage of inhibition relative to the control and reported as the mean ± standard deviation of three independent experiments (n=6). *Indicates significant differences in the inhibition of internalization between the WPPC and its >30 kDa fraction for each WPPC and concentration (**p<0.01).

To our knowledge, the anti-internalization activity of WPPC in intestinal cell models has not been previously reported. McEvoy et al. (2016) [23] showed that WPC reduced *C. sakazakii* adhesion to Caco-2 cells, in line with our results. WPC has also been reported to inhibit internalization of other pathogens—including *Cronobacter malonaticus* (ATCC 18702) [28] and *Salmonella enterica* serovar Typhimurium [29] in Caco-2 cells, with concurrent reductions in adhesion.

It is also important to note that several WPPC components have been shown to exert inhibitory activity when studied individually. For instance, α-lactalbumin (α-LA) and glycomacropeptide (GMP) have demonstrated anti-adhesive properties against various pathogens [30]. Another key component of WPPC is lactoferrin, whose inhibitory activity against *C. sakazakii* has been confirmed in Caco-2/TC7 cells [24]. Taken together, these findings suggest that the observed inhibitory activity observed may result from a synergistic action of the bioactive compounds present in WPPC.

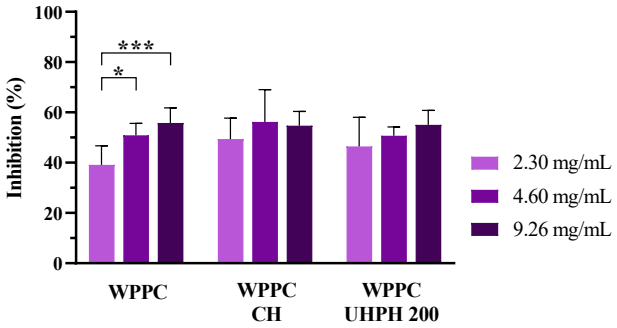


Figure 7. Inhibition assay of *C. sakazakii* internalization in differentiated Caco-2/TC7 cells by non-commercial WPPC and non-commercial WPPC treated by conventional homogenization (CH) and ultra-high-pressure homogenization (UHPH). Sample concentrations are expressed as mg protein/mL. Results are expressed as the percentage of inhibition relative to the control and reported as the mean ± standard deviation of three independent experiments (n=6). *Indicates significant differences in internalization inhibition among the evaluated concentrations for each sample (*p<0.05; ***p<0.001).

The inhibitory capacity of non-commercial WPPC against the internalization of *C. sakazakii* into Caco-2/TC7 cells was also evaluated (Figure 7). Similar levels of inhibition were observed for untreated WPPC, WPPC treated by CH at 25 MPa, and WPPC treated by UHPH at 200 MPa, with no statistically significant differences among them. This suggests that, although homogenization treatments may release components with antibacterial activity against *C. sakazakii*, such components do not appear to be involved in the inhibition of bacterial internalization into Caco-2/TC7 cells. Moreover, no clear dose–response relationship was detected for treated non-commercial WPPCs, as no significant differences were found among the concentrations tested for each sample. However, the activity of the untreated non-commercial WPPC showed significant differences ($p < 0.05$) between 2.3 and 4.6 mg/mL, and between 2.3 and 9.26 mg/mL.

Antirotaviral activity of WPPC: MA104 cell viability was assessed after exposure to the tested dairy fractions (0.25–5 mg/mL). Viability exceeded 90% for non-commercial WPPC and for the >30 and <30 kDa fractions of commercial WPPC. Viability was slightly lower for commercial WPPC, but remained >85% under all conditions. Antirotaviral activity against the WC3 BRV strain is presented in Figure 8.

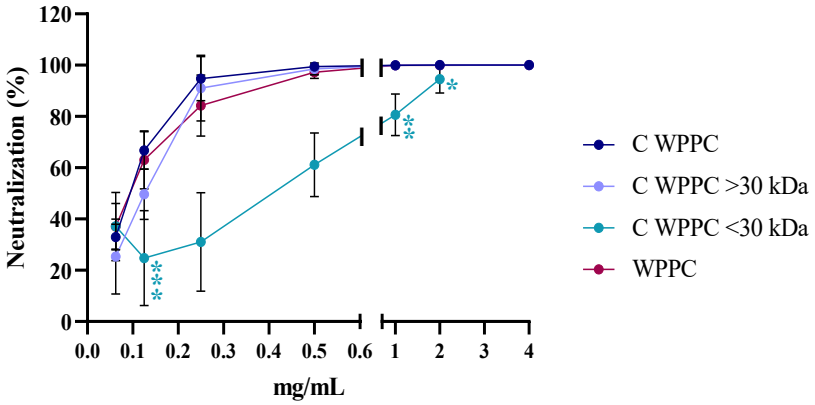


Figure 8. Dose-response effect of dairy fractions on the neutralization of the WC3 bovine rotavirus strain infectivity in MA104 cells. Commercial WPPC (C WPPC), >30 kDa fraction of commercial WPPC (C WPPC >30 kDa), <30 kDa fraction of commercial WPPC (C WPPC <30 kDa), non-commercial WPPC (WPPC).

Both commercial and non-commercial WPPC exhibited comparable activity, achieving 100% inhibition at protein concentrations above 0.5 mg/mL. Most of this activity was associated with the >30 kDa fraction, although some activity was also detected in the <30 kDa fraction, in contrast to the results obtained for the antibacterial activity of this fraction against *C. sakazakii*. These findings are consistent with those reported by Kramer et al. (2024) [31], who demonstrated a dose-dependent effect of Vivinal® MFGM on the neutralization of rotavirus infection in MA104 cells. The percentage of neutralization they reported at concentrations comparable to those used in our study for both commercial and non-commercial WPPC was somewhat higher, which can be attributed to differences in the sensitivities of the rotavirus strains employed, as reported in previous studies [32].

The FFC 17-Step Model proposed by Martirosyan and Alvarado (2023) [33] offers a practical framework for functional food development. In this context, our results correspond to Step 3, which links protein concentrations across WPPC samples with the measured antibacterial effect.

CONCLUSIONS

The findings of this study demonstrate that WPPC, whether produced at the laboratory or at industrial scale, exhibits strong activity against *C. sakazakii in vitro*. Furthermore, the application of conventional and ultra-high-pressure homogenization enhances this activity. The main advantage of UHPH lies in its potential as an alternative to pasteurization. The observed antirotaviral activity of both WPPC preparations *in vitro* is highly promising; however, further research is required to elucidate the specific effects of homogenization. Overall, these results expand current knowledge on WPPC properties and support its potential use as a valuable ingredient in functional products. Nevertheless, it is necessary to perform complementary sensory evaluations to ensure that the addition of UHPH-treated WPPC to foods results in products that are acceptable to consumers.

List of Abbreviations: WPC, whey protein concentrate; WPI, whey protein isolate; WPPC, whey protein phospholipid concentrate; CH, conventional homogenization; UHPH, ultra-high-pressure homogenization; MFGM, milk fat globule membrane; BRV, bovine rotavirus.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: LG, conceptualization, methodology, investigation, software, and writing – original draft; LC, methodology, investigation, software, and writing – original draft; RC, methodology, investigation; MDP, methodology, investigation; AJT, methodology, investigation, MMH, methodology, investigation; AXR, methodology, investigation; LG supervision, writing –review and editing, funding acquisition, and project administration; LS, conceptualization, methodology, validation, supervision, writing –review and editing, funding acquisition, and project administration.

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