



High-pressure processing of bovine milk: Effects on the coagulation of protein and fat globules during dynamic in vitro gastric digestion

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ABSTRACT

The effect of high-pressure processing (HPP) on the digestion behavior of skim and whole bovine milks was investigated using a human gastric simulator. Both milks formed clots during gastric digestion. HPP treatment led to the formation of a coagulum with a fragmented and crumbled structure, compared with the coagulum formed from untreated milk. At pressures over 400 MPa, more intense pressure resulted in looser and more fragmented gastric clot structures. The weight of the dried clots and the moisture content in the clots of the skim milk treated at 600 MPa were significantly lower and higher than that of untreated skim milk, respectively. The looser and more fragmented gastric clot structures consequently led to faster hydrolysis of the proteins by pepsin during gastric digestion. The denaturation of the whey proteins induced by HPP may have also altered the resistance of α -lactalbumin and β -lactoglobulin in the HPP-treated milk samples to pepsin hydrolysis. This study provides insights into the differences among untreated skim milk, untreated whole milk and HPP-treated milk under in vitro gastric digestion conditions. The structure of the clots formed in the gastric environment affects their breakdown and consequently their emptying rate into the intestine.

1. Introduction

High-hydrostatic-pressure processing (HPP), one of several alternatives and novel processes, has been developed to treat foods that maintain the quality similar to that in traditionally heat-treated products, (Cao et al., 2012; Serna-Hernandez et al., 2021; Yang et al., 2012). HPP is a nonthermal processing method and an alternative to heat treatment; it treats samples at a very high isostatic pressure (from 200 to 800 MPa), depending on the properties of the food product (San Martín-González et al., 2006). The major advantages of high-pressure treatment are elimination or significant reduction of the thermal degradation of food components, and the retention of natural flavors, color, and nutritional value (Huppertz et al., 2002, 2004).

HPP has been applied in the treatment of milk and dairy products. Bovine milk proteins are sensitive to high-pressure treatments. As well as microbial inactivation, the physicochemical and functional characteristics of milk and dairy products are also modified and altered in HPP-treated milk (Serna-Hernandez et al., 2021). However, the type and the

extent of these changes depend mainly on the composition of the milk, the pressure intensity, and the holding time (San Martín-González et al., 2006). A number of studies have shown that treatment of milk or skim milk at pressures >200 MPa causes the disruption of the casein micelles, unfolding and aggregation of the whey proteins, and interactions between the milk fat globule membrane and serum proteins (Desobry-Banon et al., 1994; Gaucheron et al., 1997; Lopez-Fandino et al., 1996; Needs et al., 2000; Scollard et al., 2000; Ye et al., 2004).

High-pressure treatment of milk is being studied increasingly to improve the microbiological quality and technological properties of dairy foods, such as cheese and yogurt (Serna-Hernandez et al., 2021). The changes in the structure and properties of milk components and the induced interactions between milk components have been studied extensively in relation to the quality of milk and dairy products such as the sensory quality, color, and texture (Nuñez et al., 2020; Serna-Hernandez et al., 2021). After HPP, the size, composition, hydration and light scattering properties of casein micelles change dependent on the processing conditions (Goyal et al., 2013; Naik et al., 2013). These

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changes were attributed to the disruption in the hydrophobic bonding of the casein micelles' components and the increase in the solubility of colloidal calcium phosphate (CCP) and caseins; the increase in mineral solubility, ionization and hydration of casein molecules lead to dissociation of casein micelles (Dagleish and Corredig, 2012; Hemar et al., 2020; Huppertz et al., 2006). The dissociation of casein micelles alongside denaturation of whey proteins by high pressure treatments could promote the hydrophobicity, solubility, gelation, hardness, and emulsifying properties (Chawla et al., 2011). However, very little research has been carried out on the impact of HPP on the digestion behavior and nutritional quality of milk and dairy products.

Our previous work has reported that, under dynamic *in vitro* gastric conditions, bovine milk forms a clot with a close-knit network (Ye et al., 2016b), but that heated milk forms a clot with an open and fragmented structure (Ye et al., 2016a, 2017). The structure of the pepsin-induced coagulum is dependent on the interactions between the milk components and the complexes resulting from the pre-processing of the milk (Huppertz and Chia, 2021; Mulet-Cabero et al., 2019; Ye et al., 2019). Interestingly, the coagula with different structures lead to differences in the release rates of nutrients (various proteins and fat), which is reflected in the composition of the digesta (Ye et al., 2016a, 2016b, 2017). For whole milk, milk fat globules are embedded in the clot formed during gastric digestion. We recently reported that the differences in the structures of the clots formed in unheated and heated unhomogenized whole milks led to different rates of protein hydrolysis by pepsin, which also resulted in different rates of release of fat globules from the clots (Ye et al., 2017). The release rate of fat globules from the clots was higher in heated whole milk than in unheated whole milk because of the differences in the structures and the rates of protein hydrolysis of the clots. It has been proposed that these phenomena or digestion behaviors can be attributed to the interactions among the milk components, i.e., whey protein association with the casein micelles during heat treatment (Ye, 2021; Ye et al., 2019). Therefore, we hypothesize that HPP may also lead to changes in milk proteins which in turn would affect the structure of the coagulum induced by pepsin in the stomach environment.

The objectives of this study were to investigate the effect of HPP on the gastric digestion behavior of skim milk and whole milk in a dynamic *in vitro* gastric digestion system. The compositions of the gastric coagulant and the emptied digesta were determined. The dynamics of clot formation and breakdown, the microstructure of the clots, and protein hydrolysis were studied. The impacts of HPP on the coagulation of bovine milk are discussed.

2. Material and methods

2.1. Materials

Fresh bovine milk was collected from the Massey University Dairy Farm, Palmerston North, New Zealand. The whole bovine milk contained 3.90% fat and 3.55% protein as determined by the Mojonnier method (989.05) and the Kjeldahl method (920.53), respectively (AOAC, 2005). The milk was skimmed by centrifugation with a swing bucket rotor (Thermo Fisher Scientific Multifuge Heraeus 3SR + centrifuge, Thermo Electron LED GmbH) at $3,000 \times g$ and 4°C for 15 min. All the chemicals used were of analytical grade and were obtained from either BDH Chemicals (BDH Ltd., Poole, UK) or Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

2.2. High-pressure treatment of milk samples

Milk samples were transferred to PET bottles (330 mL) and tightly closed. Each bottle was then transferred to a polyethylene bag and vacuum sealed. The samples in the bottles were pressure treated for 15 min in a "Food Lab" high-pressure food processor (Model: S-FL-065-200-9-W; Stansted Fluid Power Ltd., Stansted, Essex, UK). The high-pressure unit was equilibrated to $20 \pm 1^\circ\text{C}$ by recirculating temperature-adjusted

water through the water jacket associated with the unit. The pressure unit and all samples were equilibrated to the desired temperature for at least 1 h before pressurization commenced. The temperature change during pressurization/depressurization cycles was monitored using the thermocouple associated with the unit and standard data logging equipment. After pressure treatment, the unit was automatically depressurized and the samples were processed immediately.

2.3. Determination of particle size

The hydrodynamic size of the casein micelles was analyzed using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK), using a method modified from Bijl et al. (2014). Skim milk samples were diluted 50 times with their own ultrafiltration permeate and were filtered through $0.45\ \mu\text{m}$ polyvinylidene fluoride syringe filters. Samples were measured at 20°C using a scattering angle of 173° . The measurement of each sample was at least in triplicate.

2.4. *In vitro* gastric digestion

Simulated gastric fluid (SGF) was prepared according to an INFOGEST method in a previous study (Minekus et al., 2014) with a slight modification. A solution of a fresh mixture of KCl (6.9 mmol/L), KH_2PO_4 (0.9 mmol/L), NaHCO_3 (25 mmol/L), NaCl (47.2 mmol/L), $\text{MgCl}_2(\text{H}_2\text{O})_6$ (0.1 mmol/L), and $(\text{NH}_4)_2\text{CO}_3$ (0.5 mmol/L) was prepared by dissolving these ingredients in deionized water with stirring for 30 min. The SGF (final volume of 1 L) was made up with water to 800 mL, i.e., a $1.25 \times$ concentrate. The addition of pepsin (4.8 g/L), CaCl_2 (0.15 mmol/L), and water would result in the correct electrolyte concentration. Pepsin and CaCl_2 were added prior to use. The pH of the SGF was adjusted to 1.5 using 1 M HCl/NaOH.

A human gastric simulator (HGS), developed by Kong and Singh (2010), was used for gastric digestion (Ye et al., 2016a). A thin polyester mesh bag (pore size $\sim 1\ \text{mm}$) was placed inside the latex stomach chamber to mimic human gastric sieving, which allows particles only of size $< \sim 1\ \text{mm}$ to pass through to the duodenum (Meyer et al., 1976; Schulze, 2006).

A 200 g milk sample (skim milk or whole milk) was fed into the HGS and was warmed at 37°C for 2 min. The SGF was then added at a rate of 2.5 mL/min (the addition rates of the $1.25 \times$ concentrated SGF and the pepsin were 2.0 and 0.5 mL/min, respectively, and were controlled by two separate pumps). Samples (50 mL) of digesta were removed from the bottom of the stomach chamber at 20-min intervals, equaling an emptied digesta rate of 2.5 mL/min. The gastric contraction frequency was 3 times/min, to mimic the actual contraction of the stomach. The temperature of the HGS was set and maintained at 37°C by a heater and a thermostat during the 220 min of gastric digestion. At each time interval, a sample was removed from the HGS and was then filtered through a mesh with a pore size of 1 mm for further analysis, so that only the solid mass of size $\leq 1\ \text{mm}$ was emptied. In a control experiment, instead of the sample, 200 g of Milli-Q water was fed into the HGS and was digested for 220 min. The emptied digesta samples were collected from the HGS at each time interval, and the pH and the weight of the curd were determined and confocal microscopy observation was carried out immediately before the pepsin inactivation. Then the digesta and curd samples were heated at 90°C for 3 min to inactivate pepsin for further analysis of the samples by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE).

2.5. pH measurement

The initial pH in the HGS was defined as the pH of the freshly prepared milk. With the ingestion of SGF (2.5 mL/min) and gastric emptying (3.0 mL/min), the pH in the HGS at different times was assumed to be that of the emptied digesta, because the set-up (roller contraction) prevented easy access into the HGS.

2.6. Weight of curd

After 220 min of digestion, the curd (if any) was collected and filtered through a sieve with a 1-mm pore size to separate the aqueous phase and the curd. The curd was then rinsed with SGF to remove pepsin from the surface and was weighed immediately. After heating at 90 °C for 3 min to inactivate the pepsin, it was then dried at 105 °C overnight in a vacuum oven to determine its dry weight and for SDS-PAGE analysis.

2.7. Moisture content of the clots

The moisture content of the clots was determined by oven drying the clots at 105 °C for 24 h and calculating the difference between the wet weight and the dry weight. The determination of the moisture content of each sample was duplicated when possible.

2.8. Confocal laser scanning microscopy

The microstructure of the curd obtained from the digestion was studied using a confocal laser scanning microscope (Leica, Heidelberg, Germany). Fast Green (fluorescent dye) was used to stain for protein (He-Ne laser with an excitation line at 633 nm). The images were recorded to observe the change in the microstructure of the samples during digestion. A small piece of curd was stained with 1.0% (wt/vol) Fast Green for 15 min, placed on a concave confocal microscope slide (Sail; Sailing Medical-Lab Industries Co. Ltd., Suzhou, China), covered with a cover slip, and examined with a 63 × magnification lens.

2.9. Protein hydrolysis

The time-dependent hydrolysis by pepsin of the proteins in the curd and the emptied digesta was determined by analyzing the protein composition of the samples as a function of the digestion time, using SDS-PAGE. Liquid digesta samples were mixed with sample buffer at a ratio of 1:2 (μL), and 8 μL of the mixture was loaded in each well. For solid curd samples, 4.5 mg of the freeze-dried and ground powder was mixed with 1 mL of sample buffer and 10 μL of this was loaded in each well.

2.10. Statistical analysis

Each experiment was performed at least twice using freshly prepared samples. The results are reported as the calculated means and standard deviations. One-way analysis of variance and the SPSS 19.0 package (IBM, Armonk, NY, USA) were used. Duncan's multiple range tests were used to determine the significant difference of the mean values ($P < 0.05$).

3. Results and discussion

3.1. Effect of HPP on the gastric digestion of skim milk

3.1.1. Size of the casein micelles in skim milk

HPP treatment in the range 200 to 600 MPa resulted in a significant decrease in the casein micelle size of skim milk from ~170 to ~97 nm, especially at pressures >400 MPa (Fig. 1, $P < 0.05$). The increase in the polydispersity index values indicated that the size distribution of the casein micelles became broader after HPP treatment. This result agrees with previous reports (Dagleish et al., 2004; Goyal et al., 2013; Hupertz et al., 2006) which showed that pressures greater than 300 MPa cause an irreversible decrease in the casein micelle size, due to micelle fragmentation.

3.1.2. Change in pH of skim milk during digestion in the HGS

Fig. 2 shows the changes in the pH profiles of the different pressure-

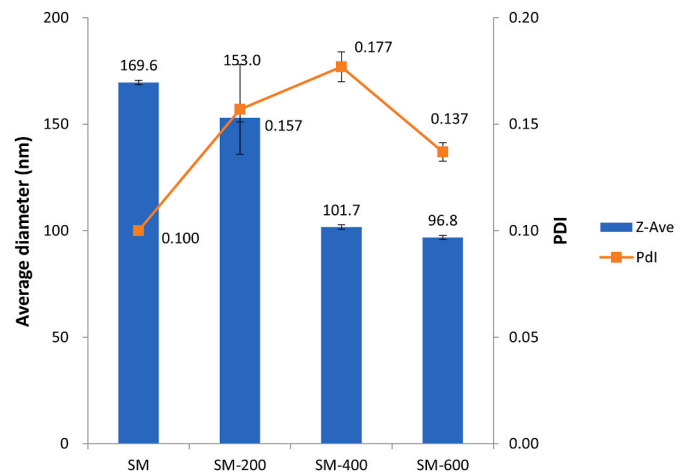


Fig. 1. Average diameter (nm) and polydispersity index (PDI) values of the casein micelles in skim milk after HPP treatment at from 200 to 600 MPa. SM, skim milk.

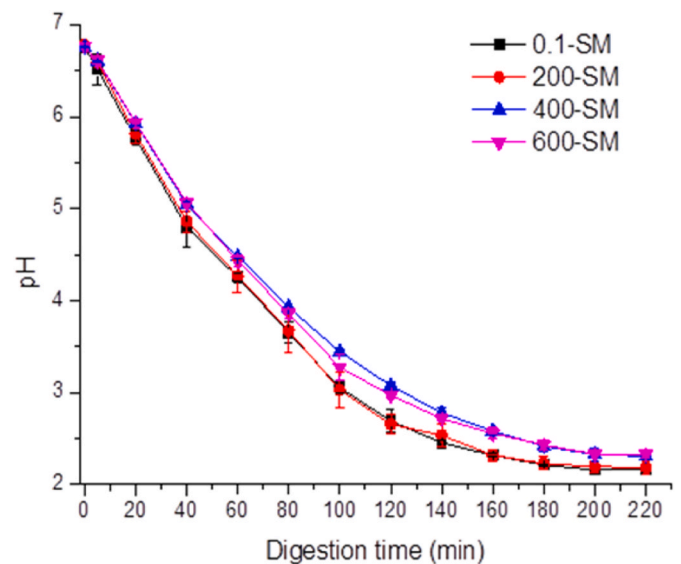


Fig. 2. Changes in pH of skim milk after HPP treatment at from 200 to 600 MPa during gastric digestion in a human gastric simulator. Error bars indicate the standard deviations from triplicate measurements. SM, skim milk.

treated skim milk digesta over 220 min of gastric digestion. The pH of all samples decreased to 2.2 ± 0.10 during 220 min of digestion. The pH of the samples treated at 400 and 600 MPa was 0.2–0.3 units higher than that of the control samples without HPP treatment.

3.1.3. Formation of gastric clots during gastric digestion of skim milk

In the untreated and treated skim milks, protein coagulation, i.e., the formation of clots with various structures and appearances, was visible after 10 min of digestion time (Fig. 3). Almost all the casein micelles had been incorporated into the clots at this stage, as the serum phase became clear. The clot of the untreated skim milk was intact with smooth surfaces, whereas the clots of the HPP-treated skim milks were fragmented and crumbly. The extent of fragmentation of the clots increased with an increase in pressure. More fragmented particles with smaller size were found in the sample treated at 600 MPa [Fig. 3(A)]. The weight of the dried clots and the amount of moisture in the clots from the untreated skim milk and the skim milks treated at 200 and 400 MPa were similar ($P > 0.05$), but the weight of the dried clot and the clot moisture of the skim milk treated at 600 MPa were lower and higher, respectively

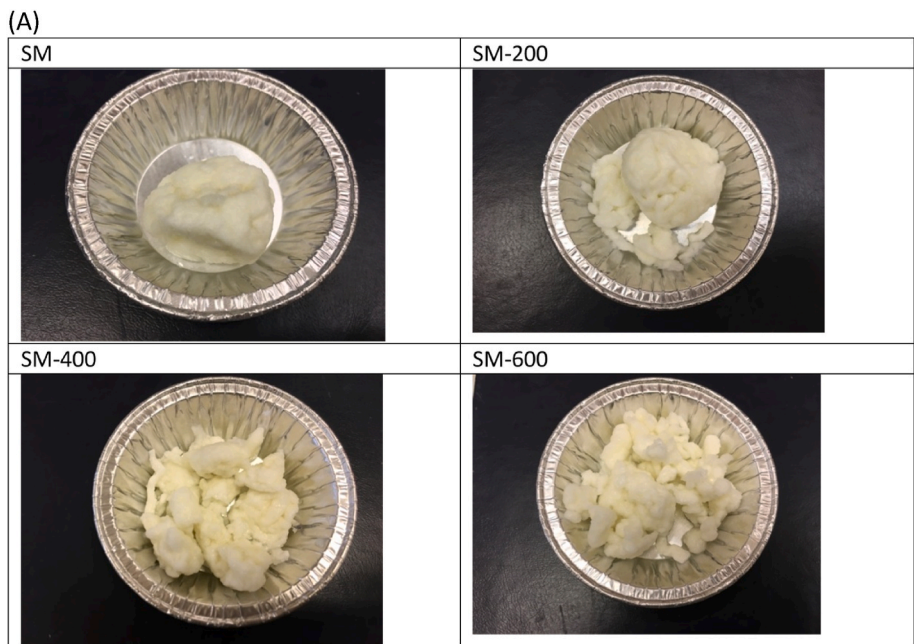


Fig. 3. (A) Images of gastric clots formed by differently HPP-treated milks at 220 min of digestion, and (B) confocal micrographs of gastric clots formed by differently HPP-treated milks at 5 and 220 min. Green represent proteins; scale bars indicate 20 μm . SM, skim milk; SM 200, skim milk treated at 200 MPa; SM 400, skim milk treated at 400 MPa; SM 600, skim milk treated at 600 MPa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

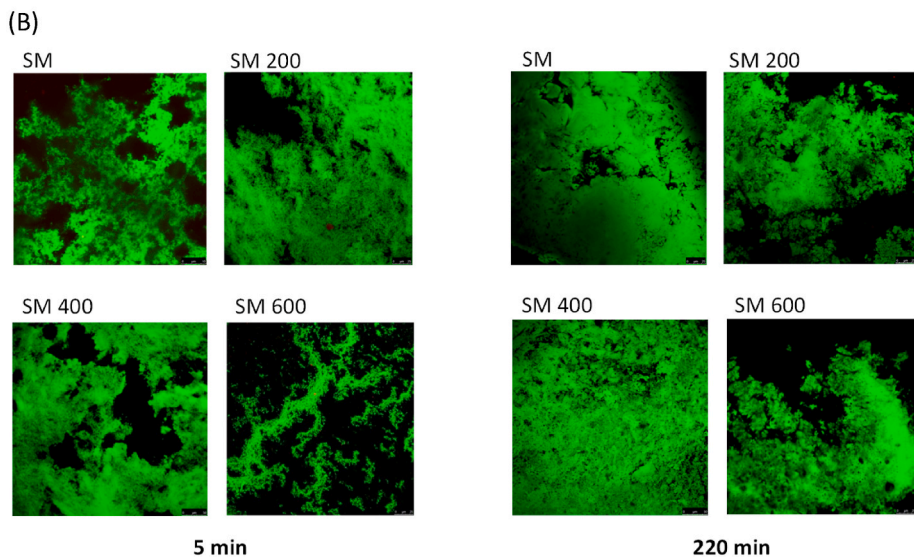


Table 1
Weight and moisture of clots formed from skim milk and HPP-treated skim milk.

	Weight of wet clot after 220 min of digestion (g)	Weight of dried clot after 220 of min digestion (g)	Moisture of clot after 220 min of digestion (%)
SM	14.15	5.17	63.46
SM-200	14.74	5.52	62.55
SM-400	14.92	5.53	62.94
SM-600	15.42	4.92	68.09

(Table 1). This indicated the significant difference in the structure of the clot obtained from the skim milk treated at 600 MPa.

The microstructure of the clots was also greatly affected by the HPP pressure [Fig. 3(B)]. A similar structure with water pores within the protein aggregate network was observed for all samples at the beginning of the digestion (5 min). At 220 min, the protein aggregate matrices had become more compact for all samples, but the HPP-treated skim milk clots had more open and porous structures.

3.1.4. Protein profiles of clots and emptied digesta from skim milk

Fig. 4 shows the SDS-PAGE patterns under reducing conditions of the clots at 220 min of digestion. For the untreated skim milk, the clots were composed mainly of caseins and proteins with bands at ~ 20 and ~ 14 kDa, but there was no κ -casein band. For the HPP-treated samples during digestion, there was a very pronounced decrease in the intensity of the casein bands with increasing pressure. The major whey protein, β -lactoglobulin (β -LG), was also observed in the samples that were treated with high pressure (400 and 600 MPa). In addition, more peptide bands were observed in the sample treated at 600 MPa. Therefore,

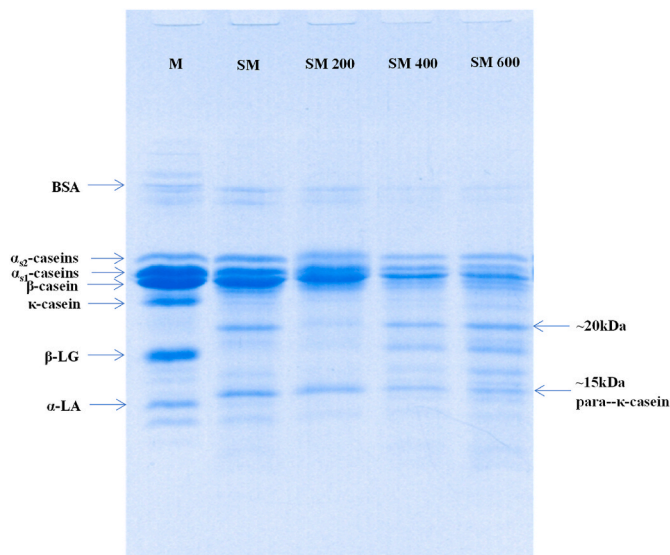


Fig. 4. SDS-PAGE patterns under reducing conditions of the clots obtained from differently HPP-treated milks at 220 min. SM, skim milk; SM 200, skim milk treated at 200 MPa; SM 400, skim milk treated at 400 MPa; SM 600, skim milk treated at 600 MPa.

compared with the untreated skim milk, HPP resulted in more rapid hydrolysis of the caseins from the clots.

Fig. 5 shows the SDS-PAGE profiles of the emptied digesta. The digesta of the untreated skim milk and the skim milk treated at 200 MPa had almost identical protein profiles (Fig. 5, SM and SM 200). Caseins

were not observed in the digesta after 20 min of digestion, presumably because of the formation of clots involving almost all the caseins at an early digestion time. β-LG was abundant in the digesta initially and its band intensity decreased gradually over the 220 min of digestion. This was consistent with previous studies on skim milk; that is, native β-LG resists hydrolysis by pepsin and the dilution effect in a dynamic digestion system causes the reduction in its concentration (Ye et al., 2016a). α-Lactalbumin (α-LA) was abundant in the digesta at 20 and 40 min but had disappeared completely after 80 min of digestion. For the samples treated at 400 and 600 MPa, caseins were observed in the digesta at 20 min of digestion and were more obvious in the sample treated at 600 MPa (Fig. 5). However, these caseins were not observed after 40 min.

The proportions of β-LG in the emptied digesta of the samples treated at 400 and 600 MPa appeared to be lower than those in the untreated skim milk digesta and decreased more rapidly over time (Fig. 5). Interestingly, an α-LA band was visible at 80 min of digestion in the emptied digesta of the samples treated at 400 and 600 MPa, in contrast to the untreated milk digesta in which this band had totally disappeared after 40 min. These results indicate that β-LG remained intact during the whole digestion period for the untreated skim milk and the sample treated at 200 MPa. The decrease in β-LG with time was probably because of dilution of the stomach contents by the gastric juices. For the samples treated at 400 and 600 MPa, lesser amounts of β-LG were observed in the emptied digesta, and β-LG band had almost disappeared at 80 min; this was due to hydrolysis by pepsin. In contrast, α-LA was hydrolyzed by pepsin earlier in the untreated skim milk and the sample treated at 200 MPa than in the milks treated at 400 and 600 MPa.

3.2. Effect of HPP on the gastric digestion of whole milk

After HPP at 600 MPa, whole milk became more transparent in

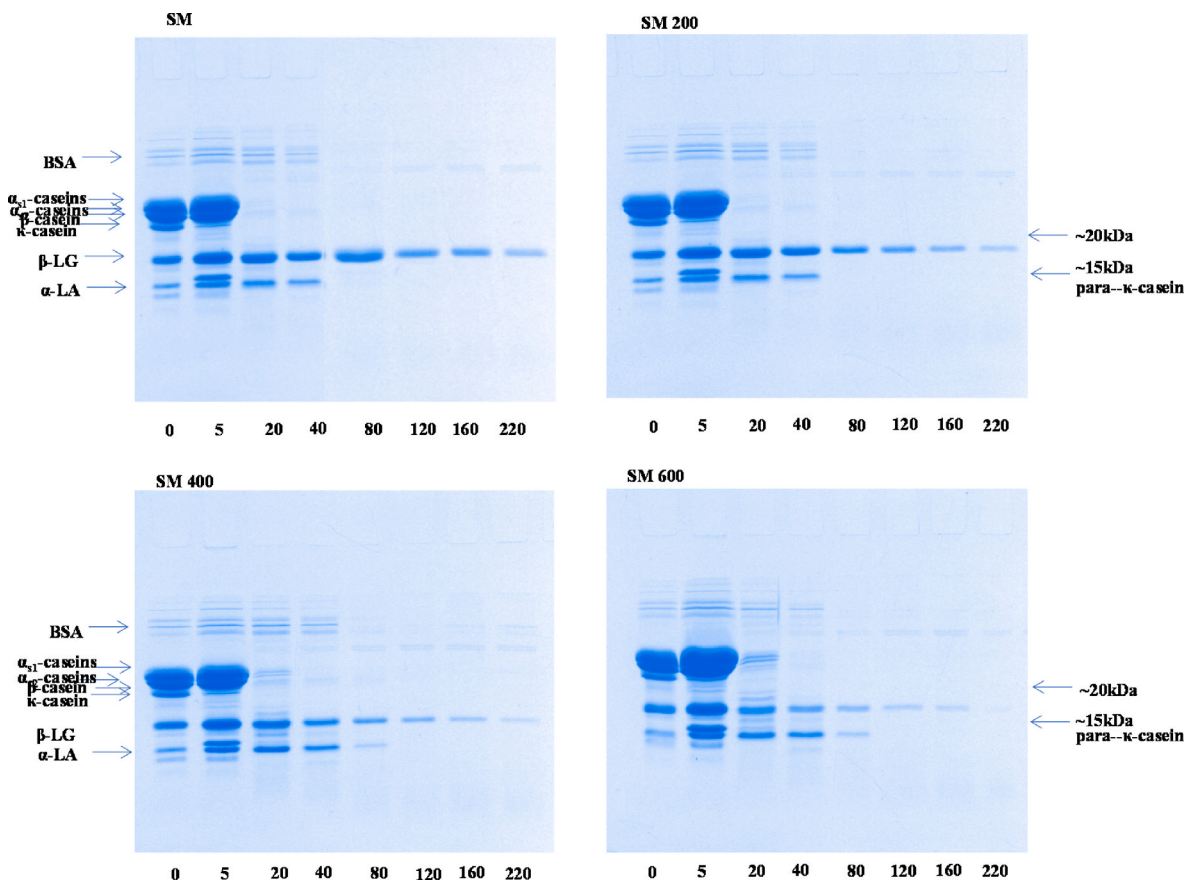


Fig. 5. SDS-PAGE patterns under reducing conditions of the digesta obtained during the gastric digestion (0, 5, 20, 40, 80, 120, 160, 220 min) of differently HPP-treated skim milks. SM, skim milk; SM 200, skim milk treated at 200 MPa; SM 400, skim milk treated at 400 MPa; SM 600, skim milk treated at 600 MPa.

appearance than untreated whole milk [Fig. 6(A)], which is in agreement with previous reports (Iturmendi et al., 2020; Ye et al., 2004) and was due to the reduction in size of the casein micelles. The similar extents of creaming observed in the untreated whole milk and the HPP-treated whole milk suggested that the fat globules had similar size in both milk samples, as HPP does not change the size of milk fat globules (Ye et al., 2004).

During gastric digestion, the decrease in the pH of the whole milk treated at 600 MPa was slower than that of the untreated whole milk [Fig. 6(B)], which was consistent with the pH profiles of the skim milk samples under gastric conditions (Fig. 2). Similar to the clot formation for the skim milks, whole milk treated at 600 MPa formed a fragmented and crumbly clot, whereas the clot formed by the untreated whole milk was intact with smooth surfaces [Fig. 6(C)].

The microstructure observed using confocal scanning laser microscopy showed that the fat globules in the whole milk were involved in the formation of the clot in both the HPP-treated milk and the untreated milk, although there was some coalescence of the fat globules. A more open structure was clearly observed in the clot of the HPP-treated whole milk than in the clot of the untreated whole milk (Fig. 7). For the digesta, the fat globules were evenly distributed in the digesta samples released from the stomach and the size of the fat globules did not change during the 220 min of gastric digestion for both the untreated whole milk and the HPP-treated whole milk. Little protein was observed in the digesta after 220 min of digestion (no green color), probably because of dilution by the gastric fluid and hydrolysis by pepsin. Compared with the untreated whole milk, HPP (600 MPa) treatment resulted in more rapid hydrolysis of the caseins. In addition, more peptides were found in the clots of the HPP-treated whole milk (Fig. 8).

4. Discussion

HPP treatment led to gastric clots with fragmented structures and open microstructures for both the skim milk and the whole milk (Figs. 3 and 6); this resulted in fast hydrolysis of the casein during gastric digestion. This phenomenon has also been observed in the digestion of heat-treated milk (Li et al., 2021; Ye et al., 2019). Mild heat treatment such as pasteurization has a small effect on the structure of the clot, but more intense heating, such as UHT treatment and heating at 90 °C for 5 min, has a large impact on the formation and the structure of the clot. As heat treatment results in a fragmented curd under gastric conditions, protein hydrolysis is promoted during gastric digestion (Li et al., 2021; Ye et al., 2019). The present results also demonstrate that the effect of HPP on the gastric milk clot depends on the pressure, but that the effect of HPP appears to be less pronounced compared to that of heat treatment. We proposed that the loosening of the clot structure occur because of the incorporation of the heat-denatured whey proteins with casein micelles during heat treatment, which may lead to the steric hindrance between casein–casein and casein–fat globule interactions during coagulation of casein micelles and induced by pepsin (Ye et al., 2019). The association of whey protein with the casein micelles is also probably the reason for the loosening of the clot structure in HPP-treated milk. If this is the case, the impact should be less than for heat treatment as the level of whey protein denaturation and association with the casein micelles is generally lower for HPP treatment than for heat treatment (Huppertz et al., 2004). As the intensity of the HPP treatment increases, the rennet coagulation time of skim milk increases, similar to that for heat-treated milk, because more extensive whey protein denaturation causes interaction between β -LG and κ -casein, which, in turn, results

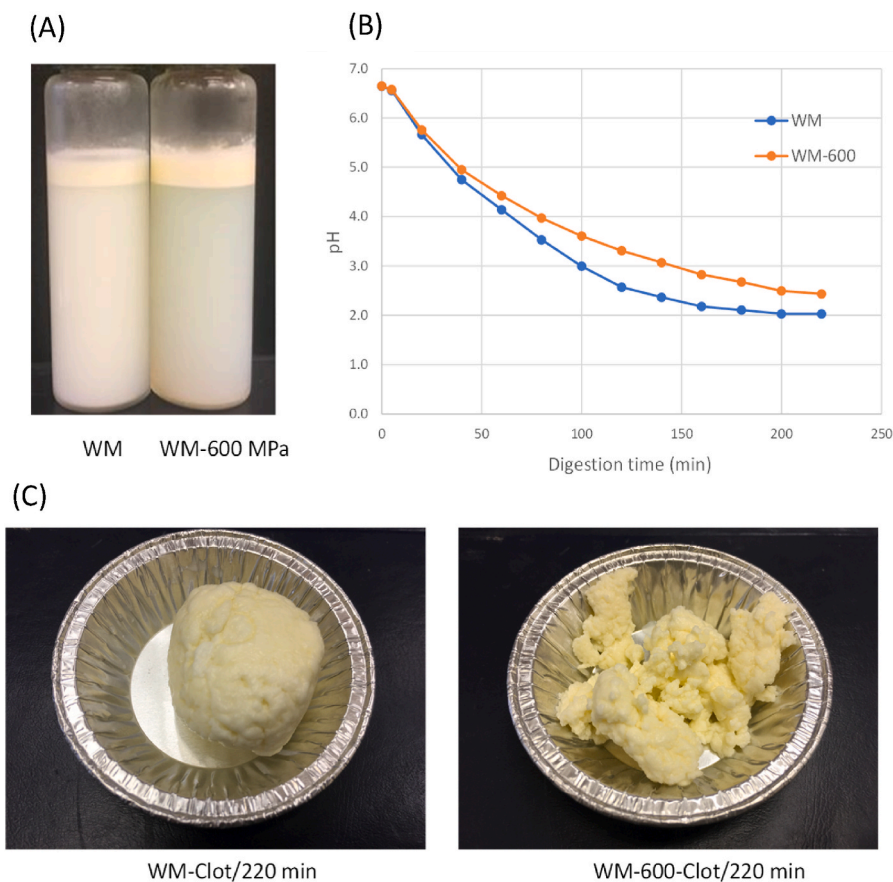


Fig. 6. (A) Appearance of untreated whole milk and whole milk treated at 600 MPa, (B) changes in pH of untreated whole milk and whole milk treated at 600 MPa during gastric digestion in a human gastric simulator, and (C) images of gastric clots formed from untreated whole milk and whole milk treated at 600 MPa (220 min of digestion).

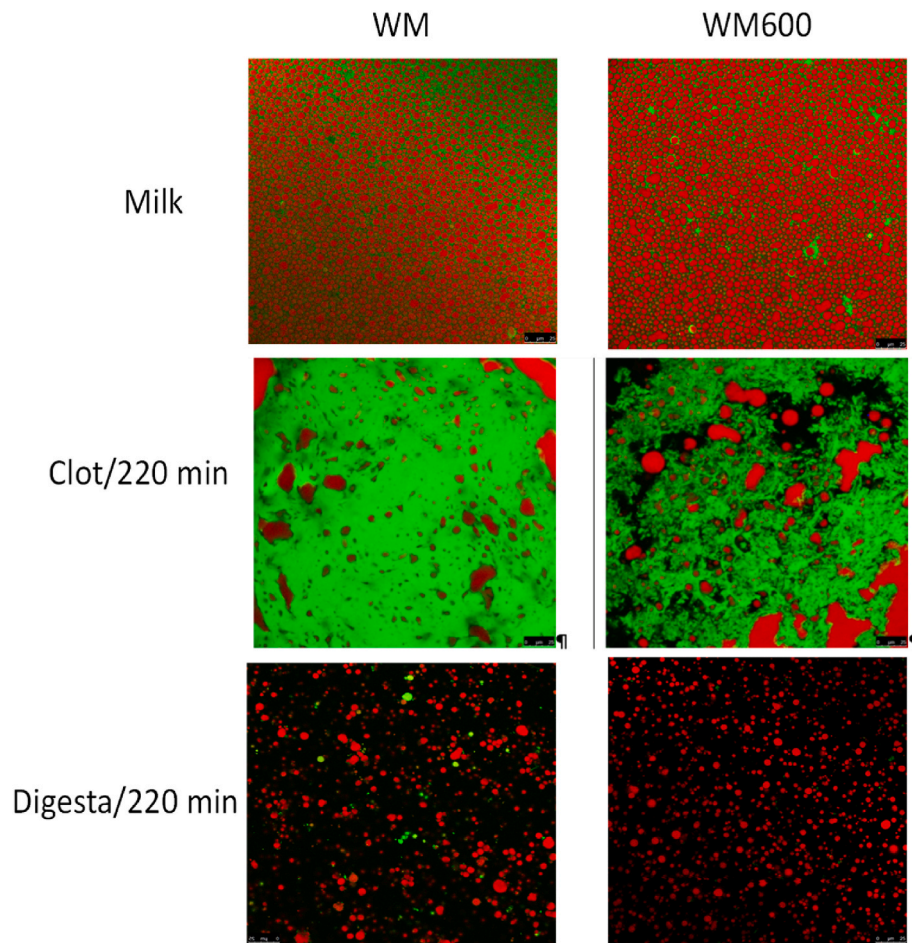


Fig. 7. Confocal micrographs of whole milk, whole milk treated at 600 MPa, and their clot and digesta after 220 min of digestion. Scale bars indicate 20 μm ; green and red represent proteins and lipids, respectively. WM, whole milk; WM-600, whole milk treated at 600 MPa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

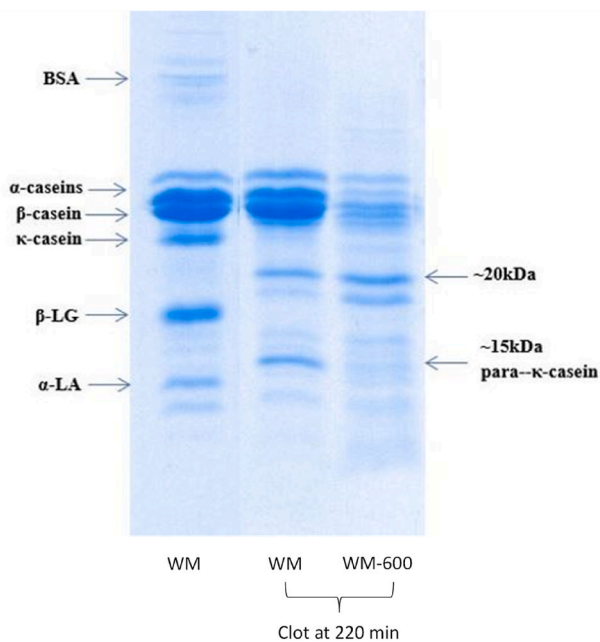


Fig. 8. SDS-PAGE patterns under reducing conditions of the clots obtained during the gastric digestion of whole milk and whole milk treated at 600 MPa. WM, whole milk; WM-600, whole milk treated at 600 MPa.

slows the release of the casein macropeptide (Lopez-Fandino et al., 1996; Naik et al., 2013; San Martín-González et al., 2006).

In addition, the fragmentation of the casein micelles that is induced by HPP may also influence the gastric coagulation of the milk under gastric conditions. HPP treatment at 600 MPa resulted in a significant decrease in the casein micelle size to ~ 97 nm (Fig. 1), which has been attributed to dissociation of casein micelles due to the increase in the solubility of CCP and caseins (Chawla et al., 2011). The smaller particles dissociated from casein micelles and the high solubility and hydration of casein molecules probably impaired the pepsin induced coagulation. This could be similar to that observed in the coagulation of calcium-depleted milk protein concentrate (MPC) and sodium caseinate under gastric conditions (Wang et al., 2018). It was reported that the casein micelles in calcium-depleted MPC were partially fragmented and their size was reduced to 50–100 nm from native casein micelles (Ye, 2011). Under gastric conditions, the coagulation behavior and the structure of the clot formed from calcium-depleted MPC or sodium caseinate were different from those normal MPC, which are not induced by pepsin hydrolysis at $\text{pH} > 6$ and the structure of formed curd induced by low pH is more fragmented and looser (Wang et al., 2018).

β -LG remained intact during the whole digestion period in untreated skim milk and the milk treated at 200 MPa; this suggests that native β -LG is resistant to hydrolysis by pepsin because of its compact globular structure, in agreement with previous reports (Reddy et al., 1988; Zeece et al., 2008). The β -LG in the skim milks treated at 400 and 600 MPa was hydrolyzed by pepsin after 40 min of digestion ($\text{pH} < 5$). This occurred because the conformation of the protein may unfold after the HPP

treatment of milk for 10–15 min, exposing some potential cleavage sites for pepsin (i.e., buried hydrophobic amino acid residues. This makes β -LG more susceptible to pepsin hydrolysis (Anema and Li, 2003; Guo et al., 1995). It has been reported that, on the HPP treatment of milk (pH 6.7) in the range 100–800 MPa and 20 °C, β -LG was considerably less stable to denaturation than α -LA; denaturation of β -LG occurred at pressures >100 MPa and reached almost 100% after treatment at 600 MPa (Huppertz et al., 2004).

In contrast to the behavior of β -LG, α -LA was hydrolyzed by pepsin earlier in the untreated skim milk and the milk treated at 200 MPa than in the milks treated at 400 and 600 MPa (Fig. 5). This suggested that the native state of α -LA in the untreated milk was more sensitive to pepsin hydrolysis than β -LG. However, it appeared that the α -LA in the skim milks treated at 400 and 600 MPa was more resistant to pepsin hydrolysis. It is known that α -LA is better digested in the stomach than β -LG; the rate is influenced by the heat treatment to some extent but is dominated by the pH during the gastric phase. Under adult in vitro digestion conditions (pH < 3), native α -LA has commonly been reported to be rapidly digested under gastric conditions (Kim et al., 2007; Kopf-Bolan et al., 2014; Tunick et al., 2016; Wada and Lönnerdal, 2014). In our present study, the slight difference in pH between the untreated skim milk and the milks treated at 400 and 600 MPa for 80 min (Fig. 2) might be the critical factor for the difference in the pepsin hydrolysis of α -LA in these milk samples. Kitabatake and Kinekawa et al. (1998) also reported the peptic hydrolysis of α -LA at different pH values. SDS-PAGE indicated that α -LA was hydrolyzed by pepsin to a certain extent at pH 4–4.5 whereas it was completely digested at pH 3.5. Miranda et al. (1989) demonstrated a rapid increase in the rate of α -LA hydrolysis by pepsin when the pH was reduced from 4.0 to 3.5 and attributed it to the pH-induced conformational change of α -LA. Furthermore, the partial conformational change in the structure of α -LA (partial unfolding) induced by high-pressure treatment may also lead to the increase in its resistance to pepsin hydrolysis; this was found in studies on the digestion of α -LA in emulsions stabilized by whey protein (Nik et al., 2010; Ye et al., 2020). In these studies, α -LA appeared to be more resistant to gastric digestion by pepsin when it was adsorbed and located at the oil–water interface than when it was in solution. Under high-pressure treatment, α -LA is considerably more stable to denaturation than β -LG; denaturation of α -LA starts to occur only at pressures >400 MPa and reaches 72% after 30 min at 800 MPa (Huppertz et al., 2004). Partial unfolding of the α -LA structure may occur only under our present treatment conditions.

5. Conclusions

The present study demonstrated the impacts of HPP treatment on the structural properties of the clots formed by both skim milk and whole milk during dynamic in vitro gastric digestion. HPP resulted in clot structures that were looser and more fragmented as the pressure increased. The fragmented clot structure led to the fast hydrolysis of proteins by pepsin during gastric digestion. The structural differences in the clots appeared to be associated with the interactions of casein micelles and whey protein during HPP and the disruption of the casein micelles by HPP. The whey protein denaturation that is induced by HPP may also alter the resistance of α -LA and β -LG to pepsin hydrolysis in HPP-treated milk samples.

CRedit authorship contribution statement

Xiaoye He: Methodology, Validation, Formal analysis, Investigation, Data curation, Visualisation, Writing – review & editing. **Mengxiao Yang:** Data curation, Writing – review & editing. **Fang Yuan:** Supervision, Writing – review & editing. **Harjinder Singh:** Supervision, Funding acquisition, Writing – review & editing. **Aiqian Ye:** Conceptualization, Supervision, Resources, Funding acquisition, Project administration, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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