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Intragastric restructuring dictates the digestive kinetics of heat-set milk protein gels of contrasting textures

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ABSTRACT

The gelation of milk proteins can be achieved by various means, enabling the development of diverse products. In this study, heat-set milk protein gels (15 % protein) of diverse textures were made by pH modulation and two gels were selected for dynamic *in vitro* gastric digestion: a spoonable soft gel (SG, pH 6.55′ G' of ~100 Pa) and a sliceable firm gel (FG, pH 5.65; G' of ~7000 Pa). The two gels displayed markedly different structural changes and digestion kinetics during gastric digestion. The SG underwent substantial structural compaction during the first 120 min of gastric digestion into a denser and firmer gastric chyme (26.3 % crude protein, G* of ~8500 Pa) than the chyme of the FG (15.7 % crude protein, G* of ~3000 Pa). These contrasting intragastric structural changes of the gels reversed their original textural differences, which led to slower digestion and gastric emptying of proteins from the SG compared with the FG. The different intragastric pH profiles during the digestion of the two gels likely played a key role by modulating the proteolytic activity and specificity (to κ-casein) of pepsin. Preferential early cleavage of κ-casein in SG stimulated coagulation and compaction of solid chyme, whereas rapid hydrolysis of α_S- and β-caseins in the FG weakened coagulation. This study provided new insights into controlling the structural development of dairy-based foods during gastric digestion and modulating digestion kinetics.

1. Introduction

The gelation function of milk proteins allows the development of milk-based foods with diverse structural and textural properties, such as cheese and yoghurt. Gelation of the milk proteins is achieved by means that destabilize their colloidal stability (Lucey, 2020). Rennet-induced gelation of the casein micelles in milk is caused by the specific enzymatic cleavage of κ-casein, which provides steric and electrostatic stabilization at the casein micelle surface. Acidification of milk towards the isoelectric point of the caseins at pH 4.6 reduces the negative charge and collapses the κ-casein surface layer of the casein micelles, causing their aggregation and gelation. Heat-induced gelation of the whey proteins, primarily β-lactoglobulin (β-LG), arises from the unfolding of their native globular structure and their subsequent aggregation via disulphide bonding and hydrophobic interactions.

Research interest in the heat-induced gelation of the micellar casein has increased recently (Nicolai & Chassenieux, 2021). Concentrated dispersions of the casein micelles form a gel under heat treatment. pH, and salts, and the presence of whey proteins plays important roles in modulating the rheological properties of the resulting gels (Balakrishnan et al., 2018; Ji et al., 2016; Nicolai & Chassenieux, 2021). For example, firmer heat-set milk protein gels form at low pH, which has been attributed to the effect of pH in altering the structure of whey proteins and their interactions with the caseins during heat treatment (Ji et al., 2016). This provides the opportunity to develop heat-set milk protein gels with high protein content and diverse textural and sensory profiles. Sufficient protein intake is crucial for the healthy ageing of senior citizens, alleviating sarcopenia and its associated risks (Wolfe et al., 2008).

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However, decreased food intake and dysphagia are prevalent in the elderly population, which makes meeting nutritional requirements difficult. The heat-set milk protein gel has great potential to deliver high protein in a small-portion snack format for senior citizens.

The stomach serves an important role during food digestion, including the secretion of gastric acid and the digestive enzyme pepsin, the size reduction of food particles by gastric motility and control of the gastric emptying into the duodenum (Gallier et al., 2013; Guo et al., 2020). The texture of foods influences their gastric breakdown and emptying and consequently affects the overall digestive dynamics and nutrient absorption (Loveday, 2022; Mulet-Cabero, Mackie, et al., 2020). Previous studies have shown that dairy foods with a hard texture are digested more slowly compared with their softer counterparts, e.g. cheese compared with yoghurt (Horstman et al., 2021; Lamothe et al., 2017) and a hard whey protein gel compared with a soft whey protein gel (Guo et al., 2015). These observations have been attributed to the greater resistance to physical breakdown and the limited access to digestive enzymes of the harder gel matrices. However, structural changes of the food bolus in the stomach are not limited to breakdown and emptying. Previously broken-down food particles, molecules or hydrolysates may undergo "restructuring" behaviours, such as bolus formation, coagulation and phase separation, when subjected to the gastric physiological environment, i.e., ionic composition, acid, enzyme and gastric motility (Guo et al., 2020; Acevedo-Fani & Singh, 2021).

A classic example of intragastric restructuring is ruminant milk, which coagulates in the stomach before further digestion and emptying (Huppertz & Chia, 2020; Li, Pan, et al., 2022; Ye, 2021). This is mainly driven by the hydrolysis of κ-caseins at the casein micelle surface by pepsin in the gastric fluid and it occurs early during gastric digestion in 10–20 min. Furthermore, casein-based gels were reported to digest differently in the stomach depending on the means of inducing gelation. Rennet- and acid-induced milk gels with similar firmness and viscoelastic properties displayed different gastric digestion behaviours in a dynamic *in vitro* model (Qazi et al., 2021) and a pig model (Barbé et al., 2014); the rennet gels were digested more slowly because of their structural compaction in the stomach. The lower pH of acid gels (<pH 4.6) compared with rennet gels has been suggested to promote their digestion, because the proteolytic activity of pepsin increases markedly at pH 4.0 and the internal bonding formed by colloidal calcium phosphate (CCP) weakens at a lower pH (Barbé et al., 2014; Qazi et al., 2021). These studies indicate that an understanding of gastric digestion behaviours is essential in order to predict the digestive dynamics and the nutritional outcomes of casein-based gels.

This study aimed to investigate the gastric digestion behaviours of two heat-set milk protein gels with contrasting textures modulated by pH, a soft gel (SG; pH 6.55) and a firm gel (FG; pH 5.65), using a dynamic gastric digestion system, the human gastric simulator (HGS). We hypothesize that the counterbalancing effects of gel texture and pH will determine whether the SG or the FG digests faster. On the one hand, the firmer texture of the FG would delay its gastric emptying and digestion. On the other, the lower pH of the FG would promote pepsin proteolytic activity and accelerate digestion. This study will provide insights into the influence of gel structure, texture and pH on the digestive dynamics of heat-set milk protein gels. These insights can be applied generally to milk-protein-based foods, and used to modulate digestive dynamics through food structure design.

2. Materials and methods

2.1. Materials

Milk protein concentrate (80 %) and whey protein concentrate (80 %) were supplied by Fonterra Co-operative Group (Auckland, New Zealand). The pepsin (P7000, Sigma-Aldrich, St. Louis, MO, USA) used for the digestion experiment had an activity of 504 U/mg, determined as described previously (Brodkorb et al., 2019). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of heat-set milk protein gels

Reconstitution of the ingredients and preparation of the protein gels were conducted as modified from Ji et al. (2016). The formulation, which is presented in Table 1, had a protein content of 15 % (w/w) with a casein:whey protein ratio of 70:30. The ingredient powders were added to reverse osmosis water warmed to 50 °C and mixed well to disperse. Sodium azide (0.02 %, w/v) was added to the milk protein solutions as a preservative. The reconstituted protein solutions were kept at ambient temperature for an hour to rehydrate before further use. Glucono-δ-lactone (GDL) was used to acidify the reconstituted protein solutions. Samples were held at 60 °C for 40 min following GDL addition to allow the complete hydrolysis of the GDL and stabilization of the pH. Trials were conducted to determine the amounts of GDL required to attain final pH values ranging from pH 5.5 to pH 6.7.

Milk protein gels were made by heat treatment following acidification. During the development stage, the gels were made in an Anton Paar Physica MCR 302 rheometer paired with a conical concentric cylinder geometry (DIN 53019, Anton Paar, Graz, Austria). The rheometer cup was pre-heated to 60 °C before the sample was added. The sample was then heated to 90 °C at a rate of 6 °C/min and held at 90 °C for 20 min. Then, the sample was cooled to 20 °C (4 °C/min) and held for 20 min. The strain was set at 0.025 % and the frequency was 0.1 Hz. Following gel formation, the viscoelastic properties of the final gel were determined using a frequency sweep test from 0.01 to 10 Hz (at 20 °C, 0.1 % strain).

The viscoelastic properties of the milk protein gels were modulated by different levels of acidification. After the development of a range of acid heat-set milk protein gels with diverse rheological profiles, two gels with contrasting viscoelastic properties were selected for *in vitro* gastric digestion study: a spoonable soft gel (SG) made at pH 6.55 and a sliceable firm gel (FG) made at pH 5.65. Their rheological properties are presented in Section 3.1.

A method to scale up the gel preparation in a water bath was developed for the *in vitro* digestion study. After reconstitution and GDL addition, as described above, the protein solutions were transferred into aluminium disposable rheometer cups EMB-Z4 (Anton Paar, Graz, Austria). They were held for 40 min in a water bath at 60 °C for GDL hydrolysis, transferred to a water bath at 90 °C, heated for 20 min without stirring and then removed from the water bath and left to cool at ambient temperature overnight before further analysis. To validate the viscoelastic properties of the gels prepared using this method, a vane geometry (ST22-4V-40/113, Anton Paar) was used to conduct frequency sweep tests as described above on the pre-formed gels.

2.3. Dynamic in vitro gastric digestion

A 200 g portion of milk protein gel was used for each digestion experiment, starting with simulated oral processing. Simulated salivary

Table 1
Formulation and composition of milk protein gels.

Ingredient formulation (g/1000 g)	
Milk protein concentrate 80 %	160.4
Whey protein concentrate 80 %	23.4
Water	816.2
Composition (%)	
Protein	15.0
Fat	0.4
Lactose	0.8
Ash	1.2
Moisture/water	82.6

fluid (SSF) was prepared as described by Brodkorb et al. (2019) without amylase. The SSF was warmed to 37 °C and mixed with each gel bolus equal to its solids content (35 g of SSF in 200 g of gel bolus), as recommended by Mulet-Cabero, Egger, et al. (2020). Gel texture affects chewing time, chewing motion, and the particle size distribution of the bolus delivered to the stomach (Luo, Ye, Wolber, and Singh, 2019), which subsequently impacts gastric digestion kinetics (Luo et al., 2021; Guo et al., 2014). Considering this, we simulated the mastication process differently for the SG and the FG to represent real-life scenarios. For the SG, the oral processing was simulated by gently mixing with a spatula (approximately 30 revolutions per min) for 15 s, based on previously reported oral processing times of spoonable yoghurt (Aguayo-Mendoza et al., 2020; Saint-Eve et al., 2006). For the FG, mastication was simulated as modified from Luo et al. (2021). FG samples were taken from the disposable rheometer cups, cut into cylinders of 20 mm height, and then four cylindrical gel samples were added at a time into a food processer (Mini Wizz BFP100, Breville Group Ltd, Sydney, Australia) that had different mixing functions of chop, whisk and grind. The oral processing of the FG was simulated in the sequence chop (1 s)–chop (1 s)–grind (1 s)–grind (1 s), and the resulting gel bolus was used for gastric digestion. This mastication simulation condition was selected based on preliminary trials to obtain a particle size distribution that was similar to that of the in vivo masticated bolus of protein gels of a comparable G' level reported previously (Guo et al., 2014).

Following the simulated oral phase of digestion, the gel bolus was transferred to the HGS (Kong & Singh, 2010) for gastric digestion, using a method modified from previous studies (Li, Ye, et al., 2022; Mulet-Cabero, Egger, et al., 2020). The gastric digestion was performed for 240 min at 37 °C. Gastric motility was simulated with moving rollers on four sides of the gastric chamber at a frequency of 3 cycles/min. A mesh bag (pore size ≈ 1 mm) was placed inside the gastric chamber to mimic gastric sieving. Simulated gastric fluid (SGF) was prepared as a 1.25 × electrolyte stock (Brodkorb et al., 2019), which was mixed with water, $CaCl_2$ and pepsin, as controlled by two pumps to reach the intended concentrations during digestion. The final SGF contained 2000 U pepsin/mL and was added gradually at a rate of 2.5 mL/min. Gastric emptying was simulated by collecting the digesta through a 1-mm sieve at 20-min intervals at a rate of 3.0 mL/min. Preliminary digestion trials of the gels were conducted in the HGS for 240 min using SGF with different amounts of HCl to determine the amount of the HCl required to attain a final pH of approximately 2.0, as recommended by Mulet-Cabero, Egger, et al. (2020).

Samples of the solid chyme and the emptied digesta were collected at different time points for further analysis. Solid chyme refers to the food chyme that was retained in the stomach during the dynamic gastric digestion and did not pass through the 1-mm gastric sieve. Emptied digesta refers to the sample that was emptied through the 1-mm sieve at the quantity defined above at 20-min intervals, including solid particles below 1 mm. Liquid chyme refers to the fraction of gastric chyme that was retained in the stomach after each gastric emptying but was able to pass through the 1-mm gastric sieve, including solid particles below 1 mm.

2.4. Analyses of the solid chyme

At 20, 120 and 240 min, the solid chyme was collected by draining the whole gastric contents through a 1-mm sieve for further analyses, while not applying excess force to avoid pressing out the moisture held in the solid chyme structure. The wet weight of the fresh solid chyme was determined immediately after sampling. The moisture content (w/w %) was determined by oven drying two sub-samples of each solid chyme at 105 °C for 24 h. The dry weight of the solid chyme was then calculated from the wet weight and moisture content. Photographs and confocal laser scanning microscopy (CLSM) images of the fresh solid chyme were taken, as described previously for milk curds formed during gastric digestion (Pan et al., 2021). The total nitrogen content of the solid chyme was analysed using the Kjeldahl method (AOAC International, 2006). A conversion factor of 6.38 was used to calculate the amounts of crude protein (including intact proteins and peptides) in the samples.

The rheological properties of the gels after simulated oral processing (immediately before gastric digestion) and the solid chymes sampled at 20, 120 and 240 min of digestion were determined using an HR20 rheometer (TA Instruments, New Castle, DE, USA) paired with a 40-mm parallel plate geometry using a method modified from previous studies (Li, Pan, et al., 2022; Mulet-Cabero et al., 2019). The sample was gently mixed and a portion of 3–4 g was placed at the centre of the Peltier plate. The geometry was lowered to a gap of 1.5 mm when possible and the excess sample was carefully removed using a spatula. The temperature was set at 37 °C and the samples were equilibrated for 120 s. The oscillation test was initiated only when the normal force was below 5 N. The complex modulus (G*) after oscillation for 10 min at 0.5 strain and 1.0 Hz frequency was reported. A few samples were not measurable at a gap of 1.5 mm because of their high firmness and difficulty in reaching the axial force target; they were measured at a gap of 2.0 mm instead. Trials on six other samples showed that the G* values measured using a 2.0-mm gap were on average 9.8 % lower than those of the same samples measured using a 1.5-mm gap, which was negligible relative to the magnitude of the difference between samples.

The protein composition and the extent of protein hydrolysis were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (Ye et al., 2017) with one modification. The samples were diluted differently with the sample buffer to the same crude protein concentration. Precision Plus Protein™ Dual Xtra Prestained Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were loaded with the samples to indicate the molecular weights of the proteins and peptides. The SDS-PAGE gels were scanned using a Gel Doc XR + system and the images were analysed for staining intensity of bands with Image Lab software (Bio-Rad Laboratories).

2.5. Analyses of the emptied digesta

The pHs of all digesta samples emptied at 20-min intervals were measured immediately. The emptied digesta collected at 20, 40, 60, 120, 180 and 240 min were used for more detailed analyses.

The particle size distribution of the emptied digesta was analysed immediately using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). Other analyses of the emptied digesta were conducted similarly to those of the solid chyme, as described above. Photographs and CLSM images of the emptied digesta were taken. The concentrations of total solids and crude protein in the emptied digesta were determined by oven drying and the Kjeldahl method respectively. The digesta were also analysed for protein hydrolysis using SDS-PAGE, as described above.

2.6. Statistical analysis

Three batches of milk protein gels were independently prepared and digested. Statistical analysis was performed using Minitab® software (Version 19.1.1). Significant differences between samples at different digestion time points were determined using one-way analysis of variance and the Tukey post-hoc test. The impact of gel type (SG and FG) was analysed by t-tests. Correlation analysis was performed to determine significant correlations and the Pearson correlation coefficients (r) between parameters. Significance was defined at the $P < 0.05$ level unless otherwise specified. Standard deviations are plotted in the figures as error bars.

3. Results

3.1. Rheological properties of heat-set milk protein gels and selection for in vitro digestion

The gelation profiles of the SG and the FG made in situ in the rheometer are presented in Fig. 1. Gelation occurred when the heating process reached 90 °C and the firmness of the gel, presented as G', increased continuously during the cooling phase.The final FG had a G' of 7200 Pa whereas the final SG had a G' of 138 Pa. The phase angle of the FG (16.2 ± 0.9°) was higher than that of the SG (12.3 ± 1.2°), indicating a greater increase in the viscous properties proportionally to the elastic properties as the pH decreased. The gels prepared using the water bath method had similar G' values to those prepared in the rheometer (results not shown) and were used for the *in vitro* digestion studies.

3.2. Structures of the solid gel chymes during gastric digestion

Photographs of the milk protein gels following simulated oral processing and of the solid chymes retained at different stages of gastric digestion were taken to illustrate the macrostructural development of the gels (Fig. 2). Overall, the macrostructure of the SG bolus changed markedly during the gastric digestion whereas the changes in the FG bolus structure were less noticeable. Following simulated oral processing, the bolus of the FG (0 min) was composed of small pieces of broken gel, which largely resembled the FG solid chyme at 20 min of gastric digestion. At 120 and 240 min, the quantity of FG solid chyme had decreased markedly, and the chyme appeared to be composed of large oval particles held together by other finer particles. In contrast, the SG transformed from a yoghurt-like texture after oral processing to a soft and hydrated chyme after 20 min of digestion, and further developed into large pieces of dry chyme from 120 min of digestion. More solid chyme was retained following the gastric digestion of the SG than that of the FG. These observations of chyme quantity and dryness are supported by quantitative data described below (Fig. 3).

The microstructures of the gel boluses and the solid chyme, imaged using CLSM, reflected the macrostructural observations (Fig. 2). Before gastric digestion, masticated FG bolus had a porous network formed by aggregated proteins. The SG bolus had a more continuous protein network with smoother edges around fewer gaps. It also had a greenish background, which indicated the presence of free proteins in the aqueous phase. During the gastric digestion, the microstructure of the FG solid chyme did not change markedly, apart from a tightening of the network in some parts at 120 and 240 min. For the SG, the solid chyme at 20 min of digestion had smaller gaps and no longer had a greenish background, which suggested that the free proteins had been either incorporated into or drained from the solid chyme phase. At 120 min of digestion, the SG solid chyme had the most compact structure among all samples determined. At 240 min, the SG chyme was looser than after 120 min but still more compact than the FG chyme.

3.3. Weight and composition of solid chyme

The SG and the FG displayed different dynamics of gastric digestion and emptying, as indicated by the composition of the solid chyme (Fig. 3). The wet weight of the FG solid chyme was slightly higher than that of the SG solid chyme at 20 min but decreased at a faster rate during digestion (Fig. 3A). At 240 min, the quantity of solid chyme that remained was significantly greater for the SG (29.1 g) than for the FG (7.6 g). The difference between the SG and FG solid chymes was more apparent in the crude protein content (Fig. 3B). More crude protein was retained in the FG solid chyme at 20 min (21.6 g), and crude protein retention rapidly decreased to 6.2 g at 120 min and to 1.0 g at 240 min of digestion. In contrast, the crude protein content of SG solid chyme did not decrease significantly from 20 to 120 min (14.3 and 13.8 g respectively). After 240 min of digestion, 6.2 g of crude protein remained in the SG solid chyme, similar to that of the FG solid chyme at 120 min.

The moisture contents of the gel chymes were different for the SG and the FG during digestion (Fig. 3C). At 20 min of digestion, the FG solid chyme contained less moisture (78.4 %) than the SG solid chyme (84.0 %). During further gastric digestion, the moisture content of the FG solid chyme had a slightly increasing trend but did not change significantly ($P > 0.05$). In contrast, the moisture content of the SG solid chyme decreased significantly from 20 to 120 min of digestion, reaching

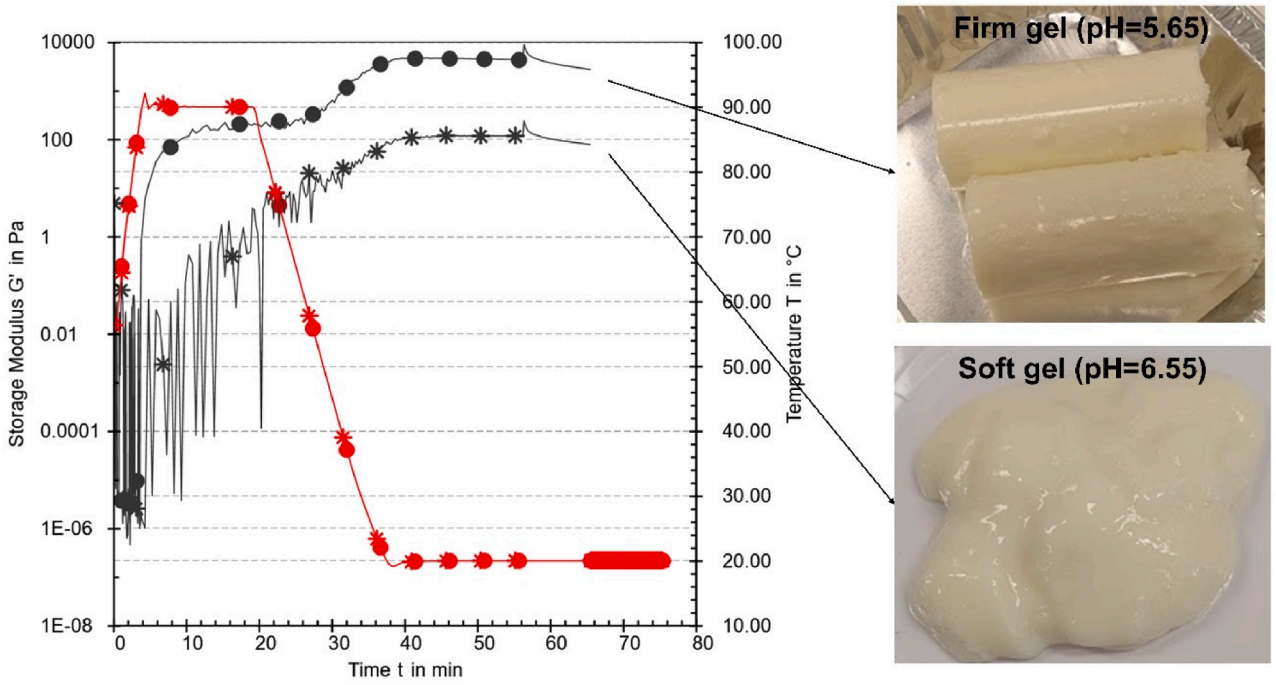

Fig. 1. In situ gelation profiles of milk protein gels made in the rheometer. Black lines indicate the storage modulus values of the firm gel made at pH 5.65 (●) and the soft gel made at pH 6.55 (✳). The red line indicates temperature. Images show the textural differences between the firm gel and the soft gel.

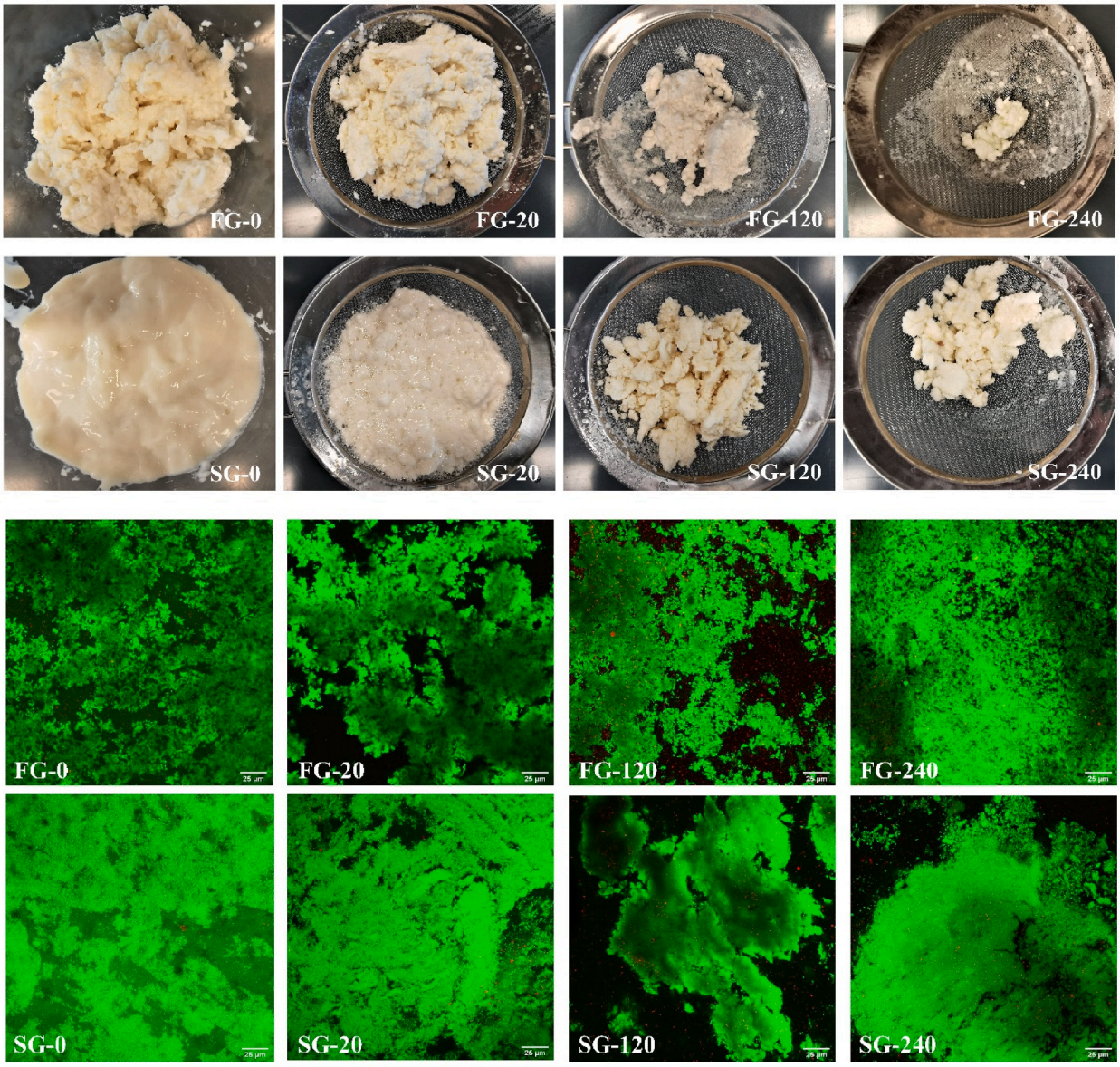

Fig. 2. Photographs and confocal laser scanning microscopy images of the firm gel (FG) and the soft gel (SG) at different stages of dynamic gastric digestion (numbers indicate the digestion time). Samples at 0 min were the gel boluses following simulated oral processing; samples at 20, 120 and 240 min were the solid gel chymes retained in the stomach chamber by a 1-mm sieve.

the lowest level of all samples (70.0 %), reflecting its dry appearance as presented in Fig. 2. The moisture content of the SG solid chyme increased from 120 to 240 min of digestion to 75.3 %. At both 120 and 240 min, the SG solid chyme contained significantly less moisture than the FG solid chyme ($P < 0.05$). The crude protein percentages in the solid chymes displayed the opposite trend to their moisture contents (Fig. 3D). For the FG, crude proteins made up 17.9 % of the chyme weight at 20 min and demonstrated an insignificant ($P > 0.05$) decreasing trend during digestion to 15.7 % at 120 min and 14.0 % at 240 min. In contrast, the SG solid chyme contained 13.5 % crude proteins at 20 min, which almost doubled at 120 min (26.3 %) and then decreased to 21.3 % at 240 min.

3.4. Rheological properties of digested milk protein gels

The G* values of the milk protein gel boluses following simulated oral processing and of the solid chymes at 20, 120 and 240 min of gastric digestion were analysed to indicate their consistency (Fig. 4). As expected, the G* of the masticated FG bolus (727 Pa) was markedly higher than that of the masticated SG bolus (31 Pa) before gastric digestion.

The G* of the FG solid chyme was 980 Pa at 20 min of digestion, slightly higher than at 0 min, increased to about 3000 Pa at 120 min and decreased to 1160 Pa at 240 min of digestion. In contrast, the G* of the SG solid chyme at 20 min of digestion (166 Pa) was five times greater than that of the gel bolus at 0 min (Fig. 4); it increased dramatically to over 8500 Pa at 120 min of digestion, markedly higher than that of its FG counterpart at 120 min. At 240 min, the G* of the SG solid chyme (4785 Pa) was still higher than that of the FG solid chyme.

3.5. Emptied digesta – appearance, composition and pH profile

The milk components were emptied in different forms and quantities during the gastric digestion of the SG and the FG, as illustrated in the photographs of the emptied digesta (Fig. 5A); this reflected the crude protein percentages of the digesta (Fig. 5B). The SG digesta at 20 min

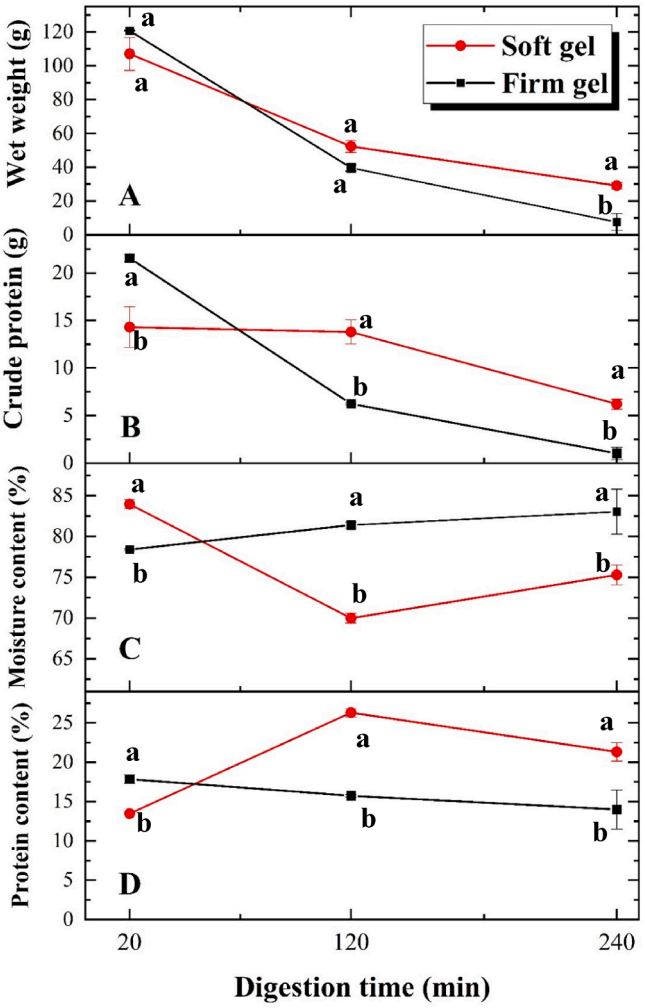

Fig. 3. Weight and composition of the solid chyme retained at different stages during the dynamic gastric digestion of the soft gel and the firm gel: wet weight (A), crude protein (B), moisture content percentage (C) and crude protein percentage (D) in the solid chyme weight. Different letters (a, b) indicate significant differences between the gels at the same time point.

had the most opaque appearance, indicating the presence of a large quantity of free proteins. The SG digesta rapidly became transparent at 40 min of digestion with a small amount of sediment particles (Fig. 5A), corresponding to the sharp drop in protein content in the digesta (Fig. 5B); this was largely maintained throughout the digestion. At 240 min, the SG digesta appeared to be slightly cloudier and the crude protein percentage in the digesta increased (Fig. 5A and 5B). In comparison, the FG digesta at 20 min was more transparent (Fig. 5A), corresponding to its lower protein content (Fig. 5B); it became cloudier and contained more sediment particles than the SG digesta from 40 to 120 min of digestion (Fig. 5A). This suggested greater gastric emptying of the milk components as small particles during the digestion of the FG, which was supported by its higher crude protein percentage than that of the SG during this period ($P < 0.01$, Fig. 5B). At 180 min, the FG digesta was cloudier than the SG digesta but they contained similar amounts of sediment. At 240 min of digestion, slightly fewer particles were found in the FG digesta than in the SG digesta, again in line with their crude protein percentages.

The gradual decreases in pH during the digestion of the SG and the FG were measured in their emptied digesta (Fig. 5C). The shape of the pH–time curve during dynamic gastric digestion in the HGS is indicative of the structural changes (e.g. coagulation or breakdown) of ingested foods (Li, Ye, et al., 2022; Ye et al., 2016). As expected, the SG had both a

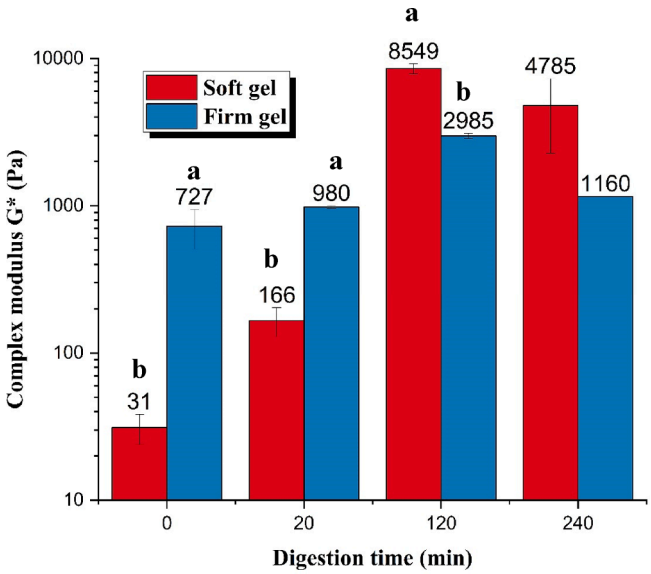

Fig. 4. Complex modulus (G*) of milk protein gels following oral processing (0 min) and the solid chymes at 20, 120 and 240 min of gastric digestion. Error bars represent standard deviations. Different letters (a, b) indicate significant differences between the gels at the same time point. No statistical analysis was conducted for Firm gel–240 min because there was sufficient sample in only one run.

higher initial pH (6.5 ± 0.1) and a higher final pH (2.3 ± 0.1) than the FG (5.6 ± 0.1 at 0 min; 1.7 ± 0.2 at 240 min). However, from 80 to 120 min of digestion, the pH did not differ significantly between the SG and the FG ($P \geq 0.1$). This resulted from the different rates of pH decrease during the early stages of digestion (Fig. 5C). The pH of the SG digesta decreased more rapidly than that of the FG digesta from 20 to 60 min, and then decreased linearly. In contrast, FG digesta has a slow rate of pH decrease during early digestion, which then increased over time. This difference was probably because of the depletion of proteins (the main buffering component) from the SG digesta at 40 min of digestion; in contrast, FG broke down faster from 40 to 120 min of digestion that increased the buffering capacity of the liquid phase (Fig. 5B). During the later stages of digestion (from 160 min), the more rapid decrease in pH of the FG digesta than the SG digesta (Fig. 5C) was probably the result of the lower retention of the FG chyme in the gastric chamber (Figs. 2 and 3) that reduced buffering capacity.

3.6. *Emptied digesta – microstructure and particle size distribution*

There were microstructural differences in the CLSM images of the emptied digesta (Fig. 6A). The FG digesta was composed of loose protein aggregates from 20 to 120 min of digestion, which were smaller and tighter at 60 and 120 min. At 180 and 240 min of digestion, the FG digesta contained mainly small particles, although some large but loose structures were also found at 240 min (Fig. 6A). In contrast, SG digesta had a greenish background during early digestion, similar to that of the SG bolus before gastric digestion (Fig. 2), indicating the presence of free proteins or small protein aggregates. At 60 and 120 min of digestion, the SG digesta contained large and highly compact particles; they became smaller towards the end of digestion (180 and 240 min), but were larger and more compact than those in their FG counterparts.

The particle size distributions of the FG digesta and the SG digesta developed differently during digestion (Fig. 6B and 6C). The particle size of the FG digesta decreased consistently throughout the digestion. The main population shifted gradually from 200–300 μm at 20 min to 10–20 μm at 240 min of digestion. In contrast, the changes in the particle size distribution of the SG digesta over time displayed a coagulation phase followed by a breakdown phase. The SG digesta at 20 min was composed

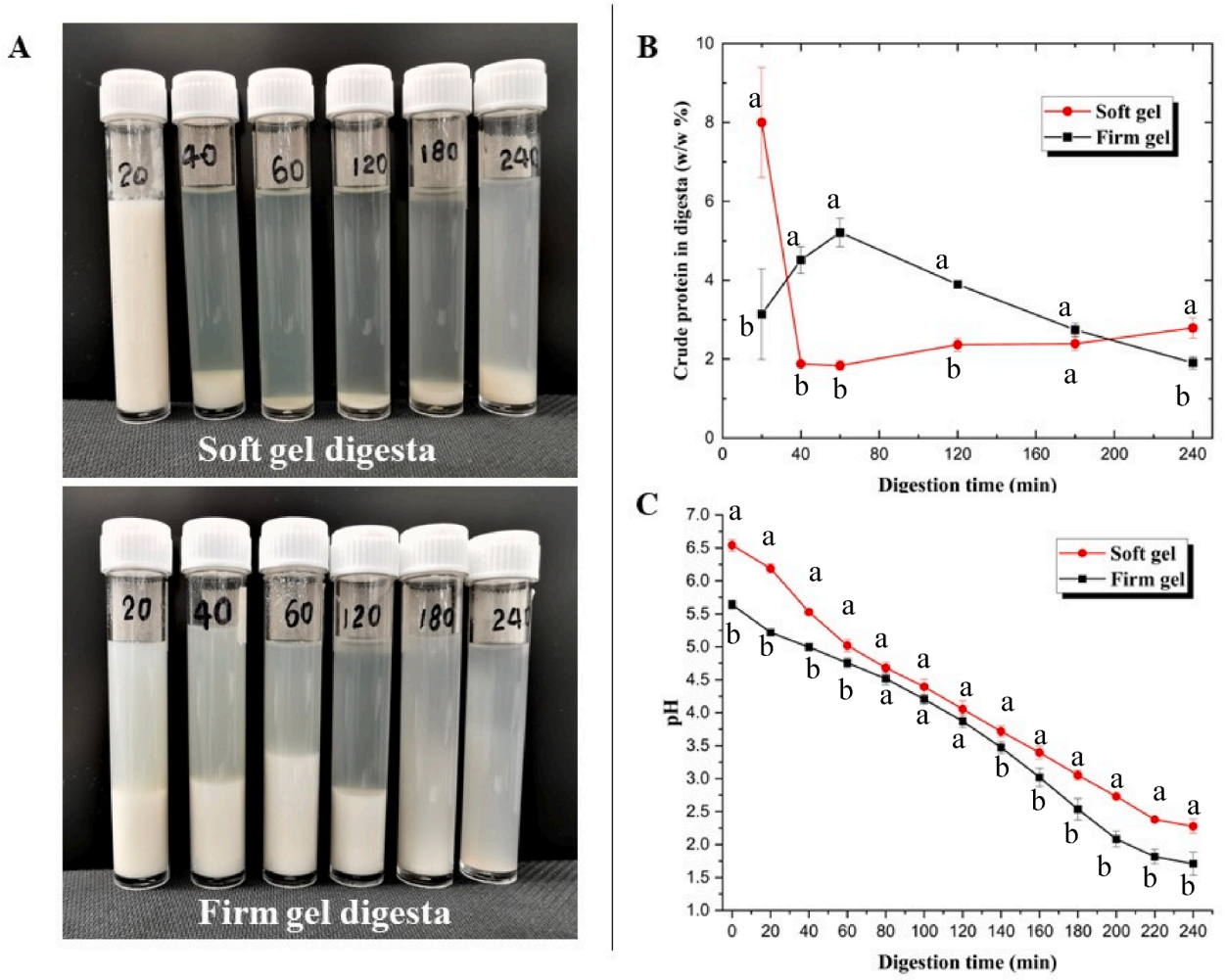

Fig. 5. Photographs (A), crude protein contents (B) and pH profiles (C) of the emptied digesta sampled during the gastric digestion of the soft gel and the firm gel. The pH at 0 min was measured in the gel bolus following simulated oral processing. Different letters (a, b) indicate significant differences between the gels at the same time point.

mainly of particles of ~20 μm. It was also the only sample that had a population at ~0.3 μm, which probably indicated native or small aggregates of casein micelles, in line with the micrograph (Fig. 6A). The main population in the SG digesta gradually increased to around 200 μm from 40 to 120 min of digestion. At 180 and 240 min of digestion, the population of large particles of >100 μm remained, but the population of small particles at 10–20 μm again increased.

3.7. SDS-PAGE profiles

Fig. 7 presents the protein profiles of the solid chyme and the emptied digesta as analysed using SDS-PAGE. Major differences already occurred in 20 min of digestion. New peptide bands resulting from casein hydrolysis (15–25 kDa) appeared in the solid chymes of both gels (enlarged lanes, Fig. 7). The FG solid chyme contained less intact α_S-casein and β-casein and more peptides than the SG solid chyme. The emptied digesta showed an even greater difference. The FG digesta at 20 min contained much more peptides (including small peptides of ~10 kDa) and fewer intact proteins than the SG digesta. The results indicated greater proteolysis of the FG than the SG at 20 min of digestion. Comparing the SG digesta and the SG solid chyme at 20 min of digestion (enlarged lanes, Fig. 7), the κ-casein band was more pronounced in the SG digesta than in the solid chyme. Staining intensity analysis estimated that the proportion of intact κ-casein in the digesta was 28 % higher than that in the solid chyme. To lesser extents, other intact proteins were also proportionally more abundant in the SG digesta than in the solid chyme at 20 min.

From 40 min of digestion, the SDS-PAGE profiles of the two gels became more similar, both composed of more peptides while intact proteins were gradually hydrolysed. Smaller peptides (≤12 kDa) made up a much higher proportion in the SG solid chyme and digesta than in the FG counterparts (Fig. 7). This indicated that proteins and larger peptides were better retained in the SG solid chyme and only the highly digested peptides were emptied. In contrast, the FG was more easily emptied without extensive proteolysis.

Regarding whey proteins, β-LG appeared to be more resistant to pepsin digestion in the FG than in the SG. β-LG was present in higher proportions in the FG digesta than in the SG digesta at each time point from 40 to 120 min. From 120 min of digestion, β-LG band was visible in both the solid chyme (dashed rectangles, Fig. 7) and the digesta of FG but had disappeared from the SG. In contrast, α-lactalbumin (α-LA) behaved similarly in both gels, largely disappeared from 120 min from both solid chyme and emptied digesta, when the pH had decreased to about 4.0. This is in agreement with previous studies that α-LA becomes susceptible to pepsin hydrolysis at pH < 4.0 (Li et al., 2021; Roy et al., 2021).

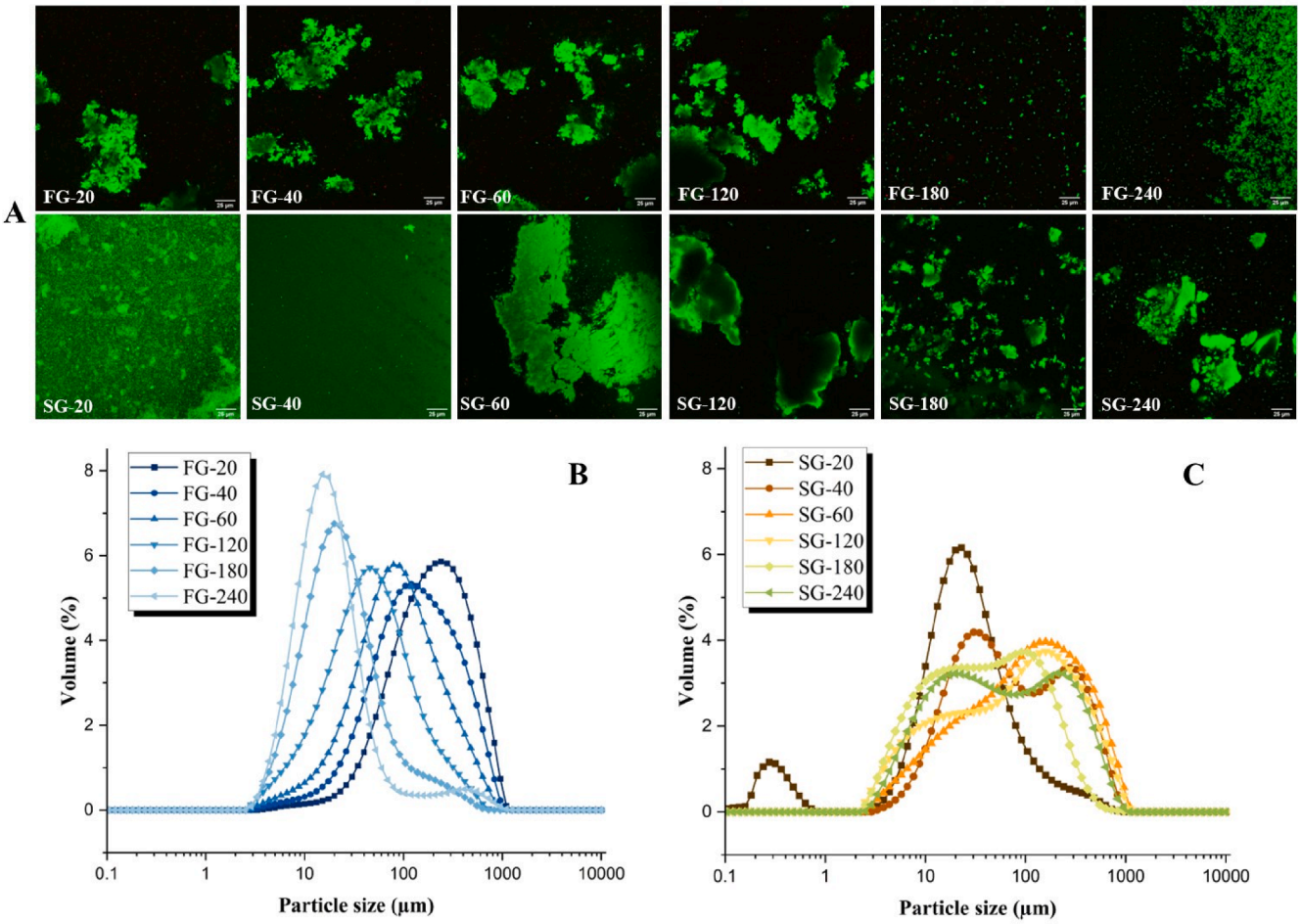

Fig. 6. Confocal laser scanning microscopy images (A) and particle size distributions (B and C) of the digesta emptied at different time points (from 20 to 240 min) during gastric digestion of the firm gel (FG) and the soft gel (SG). Proteins are indicated in green in the confocal images.

4. Discussion

4.1. Different structural development during gastric digestion of the milk protein gels

The structural and compositional analyses consistently demonstrated two contrasting processes of restructuring and destructuring during the gastric digestion of the FG and the SG. The FG gradually broke down and was emptied as particles from the beginning of the gastric digestion whereas the SG underwent coagulation and firming of the structures during the first 120 min of gastric digestion before apparent breakdown took place in the last 120 min.

The main difference in the structural development of the two gels occurred in the first 120 min of digestion, resulting in the SG forming a much larger and firmer solid chyme than the FG (Figs. 2 and 4), which reversed their initial textural differences. The intragastric coagulation and compaction of the SG resulted in a pronounced increase in the percentage of crude protein in the solid chyme from 20 to 120 min (Fig. 3D), which fused into a compact structure (Fig. 2) that expelled moisture (Fig. 3C) and had the highest consistency (Fig. 4). For the FG, its structure, composition and consistency did not change markedly during digestion, except for a smaller increase in consistency at 120 min (Fig. 4).

Analyses of the emptied digesta indicated that the compaction of the SG limited gastric emptying of proteins in the first 120 min of digestion, when major destructuring and emptying occurred for the FG (Fig. 5). From the FG solid chyme, 64.2 % of the total ingested protein was released from the stomach from 20 to 120 min. Meanwhile, after gastric emptying at 20 min, 33 % of the total protein was present in the SG liquid chyme, much higher than in the FG liquid chyme (4 %). Most of these liquid-phase proteins had presumably been incorporated into the SG solid chyme later, resulting in the sudden decrease in emptied proteins at 40 min (Fig. 5), and the increased percentage of protein in the SG solid chyme from 20 to 120 min (Fig. 3D). The compact structures of the SG solid chyme and the SG particles (Figs. 2 and 6) probably limited the access to pepsin and resisted physical breakdown, contributing to their delayed gastric digestion and emptying. The results highlighted that the digestive dynamics of casein-based milk protein gels can be dictated by their structural changes during gastric digestion rather than their initial structures.

4.2. Possible mechanisms for the contrasting gastric digestion behaviours

4.2.1. Role of pH profile during gastric digestion

We propose that the pH contrast between the SG (pH 6.55) and the FG (pH 5.65) played a key role in modulating their structural changes during the first 120 min of digestion, via its impacts on the proteolytic activity and the specificity (to κ-casein) of pepsin and the calcium equilibrium.

The proteolytic activity of pepsin is highly pH-dependent. Pepsin is barely active at pH $>$ 6.0, starts to increase in activity at pH $<$ 5.5, reaches a major activity peak at pH 4.0–4.5 (70 % of the maximum) and exhibits maximal activity at pH 2.0 (Piper & Fenton, 1965; Salelles et al., 2021). As a result, different pH profiles during dynamic gastric digestion can play a crucial role in determining the rate of digestion. Previous studies have reported that the more rapid gastric digestion of acid-

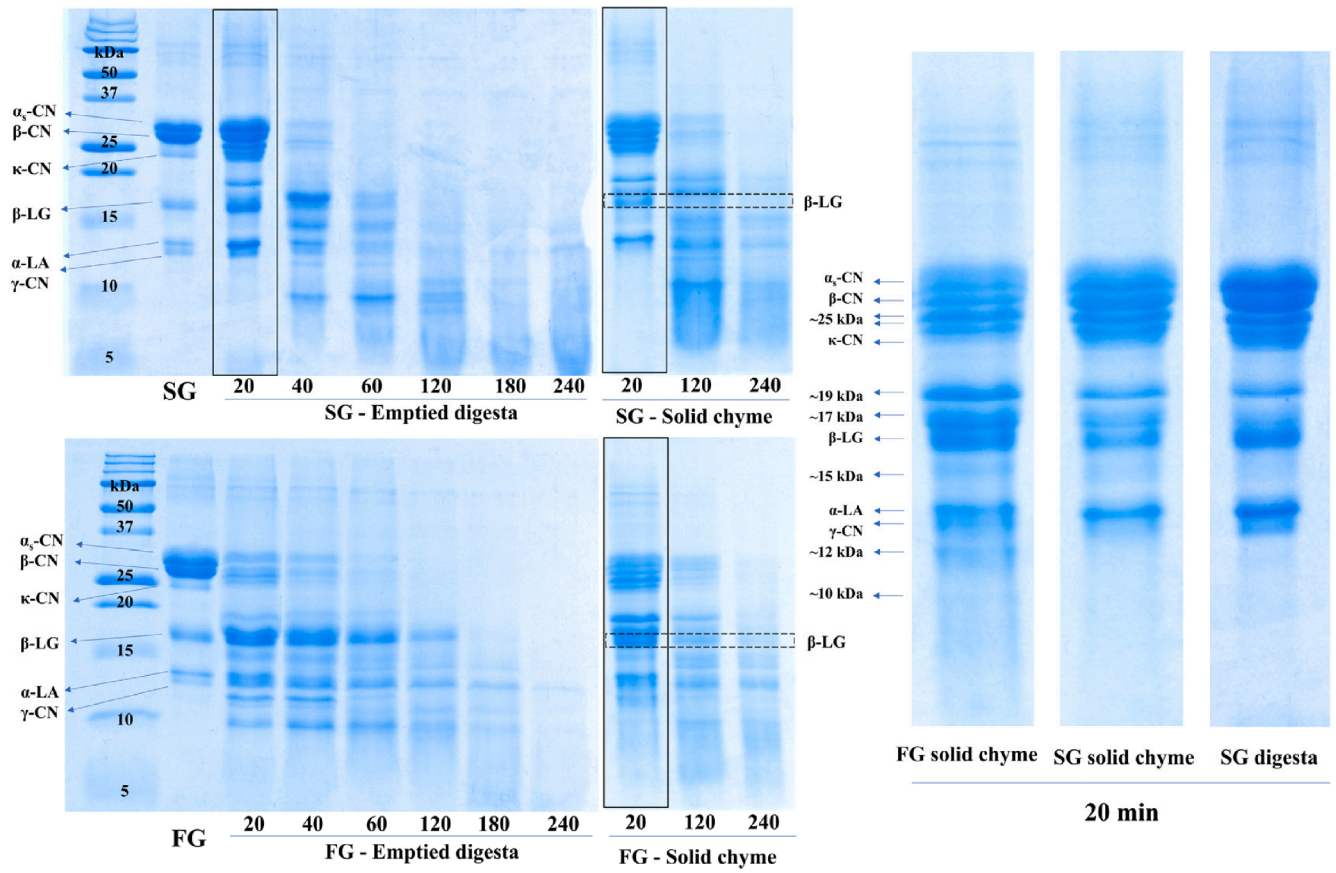

Fig. 7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis profiles of the gastric solid chyme and the emptied digesta at different time points (min) during the dynamic gastric digestion of the soft gel (SG) (top) and the firm gel (FG) (bottom). Lanes of three samples at 20 min of digestion are enlarged on the right-hand side for better comparison. Peptide bands referred to in the text are labelled in the enlarged images.

induced milk gels (pH 4.0–4.6) than of rennet milk gels (pH > 6) arises partially from the greater pepsin activity at pH 4 or below, which is reached much earlier during the digestion of acid gels (Barbé et al., 2014; Qazi et al., 2021). However, in the current study, the FG had a pH of 5.65, considerably higher than that of typical acid milk gels. The pHs of the SG digesta and the FG digesta did not differ significantly during 80–120 min of digestion when the pH of both gels decreased from around 4.6 to around 4.0 (Fig. 5C). This indicated that the time to reach the peak of pepsin activity at pH 4.0 was not a key contributor to the contrasting digestion behaviours during early digestion.

As well as affecting the overall pepsin proteolytic activity, pH is also crucial for the specificity of pepsin to κ-casein (Tam & Whitaker, 1972; Yang et al., 2022). Pepsin can completely hydrolyse the κ-casein in milk during prolonged incubation at pH 6.0 while not hydrolysing other milk proteins (Yang et al., 2022). The specific rate of pepsin hydrolysis of κ-casein is slightly lower at pH 6.3 and is significantly lower at pH 5.3 (Yang et al., 2022). The preferential hydrolysis of κ-casein by pepsin at a fairly high pH (>6.0) is responsible for the gastric coagulation of casein micelles (Huppertz & Chia, 2020; Ye, 2021). For milk clotting enzymes, a high ratio of milk clotting activity (for specific κ-casein hydrolysis) to their overall proteolytic activity is crucial for their coagulating properties (Guinee & Wilkinson, 1992). The porcine pepsin used in *in vitro* digestion studies has greater proteolytic activity than rennet (Guinee & Wilkinson, 1992) and thus is prone to result in excessive hydrolysis of α_S- and β-caseins, which impairs coagulation. Therefore, the time for which the pH remains in the range for preferential κ-casein hydrolysis of pepsin is crucial for the structural changes during gastric digestion. In the present study, the pH of the SG digesta was 6.2 at 20 min and remained above pH 5.5 at 40 min, whereas the pH of the FG digesta was already reduced to 5.2 at 20 min (Fig. 5C). The higher pH during the early stages of digestion of the SG probably favoured preferential hydrolysis of κ-casein while not markedly hydrolysing the other caseins as demonstrated with SDS-PAGE (Fig. 7). The high concentration of intact caseins in the SG digesta at 20 min (Fig. 7) provided the potential for further coagulation, which occurred, as evidenced by the sharp decrease in the protein content in the digesta at 40 min (Fig. 5B) and the greater percentage of proteins in the SG solid chyme at 120 min (Fig. 3D). In contrast, the pH during the digestion of the FG quickly fell out of the optimal range that favours coagulation and much greater proteolysis of other caseins occurred during the first 20 min of digestion (Fig. 7). Consequently, the FG lacked the driving force for strong compaction during the early stages of digestion, as indicated by the smaller change in structure and texture of the solid chyme (Figs. 2 and 4) and the increase in gastric emptying from 20 to 60 min (Fig. 5).

The pH difference between the SG and the FG would also have affected the calcium equilibrium, i.e. the dissolution of CCP. Around 70 % of milk calcium is insoluble at pH 6.7, which decreases to 22 % at pH 5.6 (Choi et al., 2007). The SG with its higher pH would have contained a greater quantity of CCP, which probably contributed to its greater intragastric compaction during early digestion. This is supported by the study of Huppertz and Lambers (2020), who reported that a model infant formula made from unadjusted skim milk (pH 6.7) underwent coagulation during gastric digestion, but a model infant formula made from skim milk equilibrated at pH 5.7 to partially remove the CCP did not.

From 120 to 240 min of digestion, all the results consistently showed that both milk protein gels underwent breakdown and protein digestion. This can be explained primarily by the markedly higher proteolytic activity of pepsin during this period when the pH was below 4.0 (Fig. 5C). Furthermore, there may have been swelling of the solid chyme structure

as the pH decreased further away from the isoelectric point of the proteins (pH 4.6–5.3) (van der Sman et al., 2020). It could have contributed to the higher moisture content and lower consistency of the solid chyme (Figs. 3C and 4) towards the end of digestion.

4.2.2. Role of whey proteins

Whey proteins can contribute to the structure of heat-set gels formed by casein micelles even without gelling themselves (Ji et al., 2016; Nicolai & Chassenieux, 2021). It was interesting to observe a markedly higher proportion and longer survival of β-LG in the FG solid chyme and digesta than in the SG counterparts, whereas α-LA behaved similarly during the digestion of the two gels (Fig. 7). The greater emptying of β-LG from the SG at 20 min of digestion (Fig. 7) probably contributed to the lower retention of β-LG in the SG solid chyme. However, this cannot explain the greater resistance of β-LG to pepsin hydrolysis in the FG than in the SG. It is well known that heat-induced denaturation renders β-LG susceptible to gastric digestion by exposing the cleavage sites of pepsin (Deng et al., 2020; Kitabatake & Kinekawa, 1998; Li et al., 2021). The pH of heat treatment is known to affect the structures of heat-induced whey protein gels (Langton & Hermansson, 1992), which can affect their susceptibility to gastrointestinal digestion (Macierzanka et al., 2012). When heated in the presence of casein micelles, denatured whey proteins partially associate with them, and the extent of association is greater at a lower pH (Corredig & Dalgleish, 1996; Vasbinder & de Kruif, 2003). The different pHs during the gelation of the SG and the FG may have affected the state of aggregation and the micelle association of denatured β-LG, which resulted in the different susceptibilities of β-LG to pepsin hydrolysis.

The greater incorporation of denatured whey proteins in gastric milk clots has been well demonstrated to loosen the clot structure by hindering the fusion of the casein matrix (Li et al., 2021; Ye et al., 2017). Hence, the higher proportion of β-LG in the FG solid chyme may have contributed to its looser structure and lower consistency than the SG from 120 min (Figs. 2 and 4). However, rennet milk gels have been reported to undergo stronger compaction than acid milk gels during gastric digestion, regardless of whether they are made from unheated (Qazi et al., 2021) or intensely heated (90 °C for 10 min) (Barbé et al., 2014) milk, indicating that the involvement of whey proteins did not play the main role.

4.2.3. Role of the initial gel texture

The different textures of the SG and the FG and their masticated boluses before gastric digestion may also have affected their gastric digestion behaviours. The mastication of the two gels was simulated differently to reflect their textural differences (Fig. 1), which would influence the particle size of the bolus in the gastric phase and their subsequent digestion. The weaker consistency of the masticated SG bolus and the presence of free proteins and small protein aggregates (Figs. 2 and 4) may have allowed pepsin to access and hydrolyse κ-casein more readily, and the relatively high mobility of intact proteins within the weak gel facilitated optimal coagulation and compaction during early digestion. The impact of the initial gel structures can be further investigated by studying liquid milk systems adjusted to different pH levels similar to those of the SG and the FG.

5. Conclusions

The present study demonstrated that the kinetics of protein digestion and gastric emptying of two heat-set milk protein gels with different textures were dominated by their different structural changes under gastric conditions. The FG (pH 5.65) was continuously broken down during gastric digestion. In contrast, the SG (pH 6.55) underwent coagulation and structural compaction during the first 120 min of gastric digestion, forming firmer gastric chyme that retained greater quantities of protein than the FG. This contrast in textural developments resulted in a slower digestion of the SG than the FG. The effect of pH on pepsin activity and specificity probably played a key role, as supported by the more rapid digestion of intact caseins in the FG during early digestion. the findings of this study provided a deeper understanding of the physicochemical leavers to modulate the structural changes of milk protein gels during digestion, which could enable novel food designs of unique texture and digestive outcomes.

CRediT authorship contribution statement

Siqi Li: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Tanyaradzwa Mungure:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Aiqian Ye:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Simon M. Loveday:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Ashling Ellis:** Writing – review & editing, Methodology. **Mike Weeks:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Harjinder Singh:** Writing – review & editing, Resources.

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.

Data availability

Data will be made available on request.

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