ABSTRACT

Gastric digestion of 2 commercial ultrafiltered milks and milk enriched with skim milk powder (to simulate concentration by reverse osmosis) was investigated and compared with the digestion of nonconcentrated milk. Curd formation and proteolysis of high-protein milks in simulated gastric conditions were studied using oscillatory rheology, extrusion testing, and gel electrophoresis. The presence of pepsin in the gastric fluid triggered coagulation at pH >6 and the elastic modulus of gels from high-protein milks was ~5 times larger than the gel from reference milk. Despite similar protein concentrations, the coagulum from milk enriched with skim milk powder showed higher resistance to shear deformation than the coagulum from ultrafiltered milks. The gel structure was also more heterogeneous. During digestion, the degradation of coagula from high-protein milks was slowed down compared with the coagulum from reference milk, and intact milk proteins were still detected after 120 min. Differences in the digestion patterns of coagula from high-protein milks were observed and were associated with the proportion of minerals bound to caseins and the denaturation rate of whey proteins.

Key words: gastric digestion, coagulation, milk protein, rheological properties

INTRODUCTION

Consumer demand for high-protein dairy foods has increased significantly over the past few years. Indeed, Greek-style yogurt is very popular worldwide, but recently, high-protein milks have been marketed to consumers with twice as much protein as regular milk. Such a product offers an alternative to consumers for meeting their daily protein needs with high-quality protein, providing them with essential amino acids, potentially bioactive peptides, and easily absorbed calcium (Ye, 2021). Milk proteins have also been recognized with satiating properties that can regulate appetite and food intake to maintain BW or for specific nutrition needs (Kondrashina et al., 2020).

Protein and lipid digestion kinetics are influenced by the original food structure, but the effect of the structure formed during digestion has recently been the focus of recent studies (Ye, 2021). Under gastric conditions, the combined action of pepsin and acidic pH, induces the coagulation of milk which affects the kinetics of digestion (Ye et al., 2016b). Major milk proteins, such as αS1- and β-caseins, together with calcium phosphate, form micelles with κ-casein present at their surface to provide steric and electrostatic stabilization (Lucey, 2014). At the beginning of digestion, pepsin hydrolyzes κ-casein and destabilizes casein micelles, resulting in aggregation in the presence of calcium ions (Ye et al., 2020). Gastric coagulation is initially driven by pepsin action on κ-casein in the early stages of gastric digestion when the pH is still high (~6.3; Ye et al., 2016b). Lowering the pH during gastric digestion increases the rate of coagulum formation due to higher pepsin activity and pH that is closer to the isoelectric point of casein (pH = 4.6). In contrast, native whey proteins do not participate in the coagulum formation under gastric conditions and remain soluble, but they become integrated into the gel structure following denaturation when milk is sufficiently heated (Ye et al., 2016b; Mulet-Cabero et al., 2019). Different heat treatments are used in the dairy industry such as pasteurization (72°C–80°C, 15–30 s) for most fluid milk products and UHT processing (135°C–150°C, 1–10 s) for commercially sterile products (IDF, 2022). Heating milk at temperatures >70°C causes the denaturation of whey proteins that form complexes with κ-casein through disulfide bond formation (Anema, 2020). Such interactions can affect the structure of the coagulum formed under gastric conditions. Previous studies have shown that UHT milk formed a coagulum with a looser, fragmented structure and larger pores than raw or pasteurized milks (Ye et al., 2016b; Mulet-Cabero et al., 2019; Ye et al., 2019). A more open and fragmented structure was less resistant to disintegration and promoted the diffusion of gastric fluid which increased the rate of pepsin proteolysis.

Recently, Huppertz and Lambers (2020) observed that in vitro gastric coagulation was strongly influ-
enced by the mineralization of casein micelles. They found that reducing the content of micellar calcium phosphate in milk samples resulted in the formation of weaker coagula leading to faster casein digestion under gastric conditions. They attributed these results to higher levels of nonmicellar casein, which impaired pepsin coagulation of casein micelles. Similarly, Wang et al. (2018a) reported different kinetics of protein digestion between milk ingredients having different levels of colloidal calcium. Milk protein concentrate ingredients (high level of colloidal calcium) was more rapidly coagulated under gastric conditions and formed a stronger coagulum than calcium-reduced milk protein concentrate or sodium caseinate (no colloidal calcium). These studies suggest that pepsin induces the coagulation of micellar casein, but not individual caseins for which coagulation occurs later when the pH gets closer to the isoelectric point (pH <5). High-pressure treatment, spray drying, and ultrafiltration are processes widely used by the dairy industry, and they can modify casein micelle structure and affect milk coagulation by acid and rennet (Singh, 2007; Wang et al., 2018a; Li and Zhao, 2019). Such treatments are therefore susceptible to change milk coagulation behavior under gastric conditions and may have consequences for the coagulum structure and the rate of protein digestion.

The various high-protein milks available on the market propose nutritional benefits that appear to be similar. However, variations in the processing steps of these milks may have an effect on digestion and physiological outcomes. Recent studies have shown the significance of the coagulation behavior of milk under gastric conditions in the kinetics of protein hydrolysis during in vitro digestion. Coagulation properties of milk induced by the rennet enzyme and acidification have been widely studied (Lucey, 2014). However, little information is available on the rheological properties of gels formed with acid and pepsin and their behavior during gastric digestion. The objective of this study was to compare the coagulum formation and rate of protein digestion of commercially available high-protein milks in a simulated gastric environment. The relationship between the characteristics of the milks (composition, coagulation properties) and coagulum degradation during gastric digestion was also studied.

MATERIALS AND METHODS

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

Materials

Standard solutions for mineral analysis were purchased from SCP Science (Baie-d’Urfé). Pepsin from porcine gastric mucosa (3,872 U/mg) and all other chemicals were obtained from the Sigma-Aldrich Chemical Company.

Milk Samples

Two high-protein milks produced by ultrafiltration (ultrafiltered milk A and B; UFA and UFB), and a standard pasteurized milk (reference milk) were purchased from a local store. These commercial products were partly skimmed and contained 2% milk fat. To simulate high-protein milk produced by reverse osmosis (RO), low heat skim milk powder (Agropur) was added to reference milk (R milk) to reach 7% (wt/wt) total protein (sRO milk). The mixture was stirred for 2 h at room temperature and stored at 4°C overnight.

Milk Composition

Milk samples were analyzed for total protein, acid (pH 4.6) soluble protein, and nonprotein nitrogen [soluble in 12% TCA (wt/wt)] by the Kjeldahl method (AOAC International, 2000), using a nitrogen-to-protein conversion factor of 6.38. True protein was calculated from the difference between total and nonprotein nitrogen. Native whey protein was calculated from the difference between acid soluble and nonprotein nitrogen. Lactose, galactose, and glucose contents were measured using an HPLC (Agilent, model 1260) equipped with a refractometer detector (G1362A, Agilent) fitted with an ICE-Ion 300 column (ICSep) at 40°C, according to the method of Doyon et al. (1991). A mobile phase consisting of 0.02 N sulfuric acid was used at a flow rate of 0.4 mL/min. Total calcium and phosphorus contents were analyzed using an inductively coupled plasma–optical emission spectrometer (Prism High Dispersion ICP; Teledyne Leeman Labs, Hudson). Minerals were determined from the ash obtained by incineration of milk samples at 550°C for 18 h in a muffle furnace. The ash was solubilized in 0.23 N nitric acid (1/50 dilution) before analysis. The concentrations of soluble calcium and phosphorus were also determined. Milk samples were centrifuged at 100,000 × g for 1 h at 34°C using an ultracentrifuge Beckman Coulter Optima LE-80K (Beckman Coulter). The supernatants (soluble phase) were recovered and filtered through a 0.45-µm and a 0.22-µm membrane filter (Krishnankutty Nair and Corredig, 2015). The supernatants were diluted (1/10 dilution) in 0.23 N nitric acid and centrifuged 3,000 × g
for 15 min at room temperature to precipitate the protein before analysis.

**Milk Coagulation**

Milk coagulation induced by pepsin and glucono-δ-lactone (GDL), or by GDL alone, was monitored using a rheometer (Physica MCR 301, Anton Paar). The simulated gastric fluid (SGF) for adult static in vitro digestion was prepared according to Brodkorb et al. (2019) with slight modifications. Gastric lipase was not used in this study because the focus was on curd formation and proteolysis. The electrolyte stock solution for gastric fluid (pH 3.0) was prepared to a 1.25× concentration, warmed to 37°C, and mixed with the pepsin solution (kept on ice until use) to reach pepsin activity of 4,000 U/mL. Pepsin-free SGF was also prepared to study coagulation with GDL alone. Forty mL of milk were warmed to 37°C, and 1 mL of SGF or pepsin-free SGF was added with GDL, mixed by inversions. The ratio of milk to SGF was chosen to simulate early stages of gastric digestion. The milk sample was then transferred into a cup and vane measuring geometry (ST22–4V-40, Anton Paar) and maintained at 37°C. The amount of GDL required to reach a pH of ~5.2 after 30 min at 37°C was determined in a preliminary experiment and ranged from 2 to 3% (wt/wt) depending on the milk sample. Similar acidification profiles (pH vs. time) were observed for the milk samples being studied. The elastic modulus ($G'$) and loss tangent ($\tan \delta$) of the coagulum were recorded every 30 s for 30 min at a strain of 1% and a frequency of 1 Hz. The coagulation was measured for 30 min to study the effect of the compositional and structural differences among high-protein milks on the gelation properties.

**In Vitro Gastric Digestion**

The gastric digestion model was a 2-step process. First, two 20-g milk samples were placed in two 60-mL plastic syringes at 37°C. The internal diameters of syringe barrel and tip were respectively 26.7 and 7.0 mm. Simulated gastric fluid and GDL were added to milk samples in the same concentrations as indicated in the previous section and mixed by inversion. The mixtures were incubated at 37°C for 10 min to allow gel formation. Then, the samples were extruded by pressing the piston of the syringe at a constant speed of 0.75 mm/s, using a TA-XT2 texture analyzer (Stable Micro Systems Ltd.). At the time of the extrusion, the coagulum pH was 5.53 ± 0.02. The linear velocity of the gel in the syringe tip was 1.1 cm/s, corresponding to a shear rate of 12.5 s⁻¹. This extrusion procedure was performed to simulate retropulsion movements in the stomach. The pressure required to extrude the sample was recorded during the process and an example of an extrusion profile is presented in Figure 1. The plateau portion of the profile (between 15 and 45 s) corresponds to the coagulum extrusion pressure, which is an indication of its resistance to high shear deformation. An index of coagulum heterogeneity was also defined as the average pressure variation measured at 1-s intervals over the same portion of the profile. The equation used to calculate the average pressure variation is inserted in Figure 1. For the second step of the digestion process, the extruded coagula were recovered in plastic dishes and transferred into 50-mL conical centrifuge tubes containing 19.5-mL SGF at 37°C to reach a 1:1 (vol/vol) ratio of SGF to milk. The pH was rapidly adjusted to 3 by adding a volume of 5 N HCl specific to each milk sample (determined in preliminary tests), and the mixtures were rotated head-over-heels (55 rpm) at 37°C. After a fixed digestion time (10, 20, 50, or 110 min), the 2 tubes were removed from agitation. The first tube was used to determine coagulum degradation and the second to analyze the protein composition of both the coagulum and the liquid chyme.

**Coagulum Degradation**

Coagulum matrix degradation was determined from the proportion of nonsugar solids released in the gastric fluid, according to the method of Lamothe et al. (2012). Briefly, the total content of the digestion tube was filtered using a sieve (1.5 × 1.5 mm mesh) to separate out larger coagulum particles. Pepsin was removed from the surface of coagulum particles by rinsing with
pepsin-free SGF. Blotting paper was placed around the sieve for 5 min to allow residual gastric fluid to drain. The coagulum particles were transferred to an aluminum dish, weighed, put in a forced air oven at 100°C for 5 h, and weighed again to determine the mass of solids retained by the sieve. The coagulum degradation, corresponding to the proportion of coagulum solids passing through the sieve, was calculated after subtracting the amount of sugar present in the aqueous phase of coagulum and liquid chyme. This correction was applied to eliminate the bias from the differences in sugar content between milk samples.

**Protein Composition**

As for coagulum degradation, the total content of a digestion tube was filtered with a sieve to separate out larger coagulum particles. The pH of the filtrate was rapidly raised to 7.5 with 5 N NaOH to stop pepsin activity. The coagulum was mixed with 5 or 10 mL of NaOH 0.1 N (at 80°C) and incubated in a boiling bath for 3 min to inactivate pepsin (Ye et al., 2016a). The dispersion was cooled to room temperature, and 2% (wt/vol) trisodium citrate was added to promote the dissociation of the caseins. The mixture was stored at 4°C overnight and homogenized using an Ultra-Turrax T25 homogenizer (IKA) at 20,000 rpm for 1 min. The liquid chyme filtrate and coagulum were stored at −20°C until analysis.

The protein and peptide composition in the initial (nondigested) milks and gastric digesta (coagulum and liquid chyme) was analyzed by SDS-PAGE under reducing conditions. Liquid chyme from the R milk sample was diluted at a ratio of 1:4 with Laemmli buffer (Bio-Rad Laboratories) and 5% β-mercaptoethanol. Liquid chymes from the other milk samples were diluted at a ratio of 1:8 with the buffer. Coagulum samples were diluted with the buffer to reach 3.5 µg of solids/µL. The mixtures were heated in a boiling bath for 5 min, loaded (10 µL) onto 4–15% polyacrylamide precast gel (Bio-Rad), and run for 45 min at 150 mV. Then, the gel was immersed in a fixing solution (10% acetic acid and 40% methanol in water) for 15 min, followed by staining in Coomassie Brilliant Blue R-250 (Bio-Rad) for 60 min. The gel was de-stained for 90 min with a solution of 10% acetic acid and 40% methanol in water.

**Statistical Analysis**

All analyses were performed on duplicate samples, and the experiment was repeated 3 times. The SAS software (SAS Institute Inc., version 3.8) was used to perform ANOVA. Data from digestion experiments were analyzed according to a factorial design to determine the differences between milk samples as a function of digestion time. Differences were considered significant at \( P \leq 0.05 \). Standard deviations were obtained from the statistical model and are indicated in the tables or shown as error bars in the figures.

**RESULTS AND DISCUSSION**

**Milk Samples Composition**

The composition of the various milk samples is shown in Table 1. The concentration of true protein in R milk was 3.14%, and it increased by a factor of ~2.16 in UF milks, while the total sugar content was reduced from 4.7 to ~3.2% \( (P < 0.05) \). To reduce the sugar content in high-protein milk produced by ultrafiltration, it is common practice to exceed the target protein concentration during filtration and then dilute the retentate with water. The ratio of true protein to total sugar in UF milks suggests that milk was first concentrated up to ~10% protein before dilution to the final protein concentration (~6.8%). As evidenced by the sugar composition, UFB milk was treated with lactase to offer an alternative to consumers with lactose intolerance. As expected, sRO milk showed the highest concentration of lactose, because only water is removed during the concentration process.

Heat treatments are applied to drinking milk to ensure product safety and increase shelf-life. Heating milk at temperatures above 70°C promotes the denaturation of whey proteins that form soluble aggregates or bind with casein micelles (Anema 2020). Compared with R milk, UFA and UFB milks showed a significantly lower ratio of native whey protein to true protein \( (P < 0.05) \), indicating that a more severe heat treatment was applied. Such degree of whey protein denaturation corresponds to heating conditions in between pasteurization and UHT processing, which are commonly used for the production of extended shelf-life milk (IDF, 2022).

In regular milk, about two-thirds of the calcium and half of the phosphorus is bound to caseins in the form of micellar calcium phosphate (Gaucheron, 2005). Consequently, the concentrations of calcium and phosphorus were higher in high-protein milks than in R milk and increased in the following order: UFA milk < UFB milk < sRO milk \( (P < 0.05) \). The ratios of colloidal calcium to protein in R milk and UFA milk were similar \( (P > 0.05) \), whereas they were 16% higher in UFB and sRO milk \( (P < 0.05) \). A similar trend was observed for the ratio of colloidal phosphorus to protein. Different factors can affect the mineral equilibrium of milk, such as temperature (Liu et al., 2014). Decreasing milk temperature is known to promote the dissociation of micellar calcium phosphate. It is then likely that UFA
milk was ultrafiltered at a lower temperature than UFB milk. The highest ratios of colloidal calcium and phosphorus to protein were observed in sRO milk. Because milk is already saturated with calcium phosphates, the enrichment with skim milk powder promotes the precipitation of these minerals onto casein micelles. The precipitation of calcium phosphate also causes the release of protons (dissociation of phosphoric acids), which is responsible for the lower pH of the RO milk ($P < 0.05$).

**Milk Coagulation**

Milks were coagulated by GDL alone or by a GDL-pepsin combination. The evolution of $G'$ and tan $\delta$ for milks acidified by GDL alone are shown in Figure 2. Acidification causes the reduction of the electrostatic repulsion between the casein micelles, which results in aggregation and gel formation when pH is close to the isoelectric point (Lucey, 2014). The R milk showed no sign of coagulation over the 30-min period allowed for coagulation (Figure 2A). The low concentration of protein in the R milk, which is further reduced by dilution in pepsin-free SGF, could explain the absence of coagulation. However, the absence of coagulation was also noted for sRO milk, which contains twice the protein content. In contrast, UFA milk and UF milk started to coagulate after 15 min, as the pH reached 5.3. Such differences in gelation properties are attributed to the denaturation of whey proteins. UFA milk and UF milk were processed at higher temperatures to extend shelf life, as evidenced by lower levels of native whey protein when compared with R milk and sRO milk (Table 1).

In heated milk, whey protein denaturation and adsorption onto casein micelles allow coagulation to occur at a higher pH than in nonheated milk, due to the isoelectric pH of the main whey protein, $\beta$-LG (~5.3; Lucey, 2014). The loss tangent is the ratio of the loss modulus and the viscous modulus ($G''/G'$) and is a useful indicator of the relaxation behavior of the protein-protein bonds in the gel (Lucey et al., 2000). Slight and similar increase of tan $\delta$ was observed for both UF milks as the pH decreased (Figure 2B). In a previous study, this behavior was attributed to the dissociation of micellar calcium phosphate, which increased the capacity of casein bonds to relax (Lucey, 2014).

High-protein milks were also coagulated by the combination of pepsin and GDL, which is more representative of the gastric environment. The rheological profiles are provided in Figure 3. Compared with GDL alone, the combination with pepsin induced faster coagulation and increased coagulum firmness (Figure 3A), which is in agreement with Roy et al. (2020). The initial $G'$ value (measured at 1 min; pH 6.1) ranged between 68

### Table 1. Composition of reference milk and high-protein milks ($n = 3$)

<table>
<thead>
<tr>
<th>Item</th>
<th>Reference milk</th>
<th>High-protein UFA</th>
<th>High-protein UFB</th>
<th>High-protein sRO</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.72 ± 0.03$^c$</td>
<td>6.83 ± 0.05$^a$</td>
<td>6.77 ± 0.04$^b$</td>
<td>6.55 ± 0.04$^d$</td>
</tr>
<tr>
<td>TS (%)</td>
<td>10.76 ± 0.12$^b$</td>
<td>12.87 ± 0.11$^b$</td>
<td>12.86 ± 0.05$^b$</td>
<td>20.32 ± 0.14$^b$</td>
</tr>
<tr>
<td>Lactose (%)</td>
<td>4.68 ± 0.00$^b$</td>
<td>3.22 ± 0.00$^a$</td>
<td>0.04 ± 0.00$^d$</td>
<td>8.81 ± 0.01$^a$</td>
</tr>
<tr>
<td>Glucose (%)</td>
<td>0.01 ± 0.00$^d$</td>
<td>0.01 ± 0.00$^d$</td>
<td>1.56 ± 0.00$^d$</td>
<td>0.17 ± 0.00$^d$</td>
</tr>
<tr>
<td>Galactose (%)</td>
<td>0.01 ± 0.00$^d$</td>
<td>0.01 ± 0.00$^d$</td>
<td>1.51 ± 0.00$^d$</td>
<td>0.21 ± 0.00$^d$</td>
</tr>
<tr>
<td>Total N (%)</td>
<td>3.31 ± 0.03$^b$</td>
<td>6.89 ± 0.05$^b$</td>
<td>6.93 ± 0.04$^b$</td>
<td>6.86 ± 0.03$^b$</td>
</tr>
<tr>
<td>NPN (%)</td>
<td>0.16 ± 0.00$^b$</td>
<td>0.12 ± 0.00$^b$</td>
<td>0.12 ± 0.01$^b$</td>
<td>0.34 ± 0.01$^a$</td>
</tr>
<tr>
<td>True protein (%)</td>
<td>3.14 ± 0.04$^a$</td>
<td>6.76 ± 0.05$^a$</td>
<td>6.81 ± 0.04$^a$</td>
<td>6.51 ± 0.03$^a$</td>
</tr>
<tr>
<td>pH 4.6 insoluble (%)</td>
<td>2.67 ± 0.03$^b$</td>
<td>6.33 ± 0.05$^b$</td>
<td>6.42 ± 0.01$^b$</td>
<td>5.45 ± 0.03$^b$</td>
</tr>
<tr>
<td>pH 4.6 soluble (native whey protein; %)</td>
<td>0.48 ± 0.00$^b$</td>
<td>0.43 ± 0.00$^c$</td>
<td>0.39 ± 0.03$^d$</td>
<td>1.06 ± 0.00$^a$</td>
</tr>
<tr>
<td>Native whey/true protein (%)</td>
<td>15.1$^b$</td>
<td>6.4$^a$</td>
<td>5.7$^a$</td>
<td>16.3$^a$</td>
</tr>
<tr>
<td>Total ash (%)</td>
<td>0.71 ± 0.03$^d$</td>
<td>1.06 ± 0.03$^b$</td>
<td>0.94 ± 0.01$^c$</td>
<td>1.52 ± 0.02$^a$</td>
</tr>
<tr>
<td>Calcium (mg/kg)</td>
<td>1,000 ± 46$^b$</td>
<td>1,770 ± 28$^a$</td>
<td>1,990 ± 53$^b$</td>
<td>2,180 ± 56$^a$</td>
</tr>
<tr>
<td>Soluble</td>
<td>322 ± 8$^b$</td>
<td>309 ± 12$^b$</td>
<td>291 ± 11$^b$</td>
<td>539 ± 15$^b$</td>
</tr>
<tr>
<td>Colloidal</td>
<td>680 ± 40$^b$</td>
<td>1,462 ± 17$^b$</td>
<td>1,697 ± 52$^b$</td>
<td>1,645 ± 55$^b$</td>
</tr>
<tr>
<td>$C_{\text{cal}}$/true protein (mg/g)</td>
<td>21.6$^b$</td>
<td>21.6$^b$</td>
<td>24.9$^b$</td>
<td>25.3$^b$</td>
</tr>
<tr>
<td>Phosphorus (mg/kg)</td>
<td>835 ± 34$^d$</td>
<td>1,281 ± 16$^c$</td>
<td>1,432 ± 28$^b$</td>
<td>1,840 ± 37$^a$</td>
</tr>
<tr>
<td>Soluble</td>
<td>399 ± 9$^b$</td>
<td>729 ± 7$^a$</td>
<td>774 ± 13$^a$</td>
<td>759 ± 21$^a$</td>
</tr>
<tr>
<td>Colloidal</td>
<td>436 ± 27$^c$</td>
<td>951 ± 9$^b$</td>
<td>1,098 ± 29$^a$</td>
<td>1,081 ± 40$^a$</td>
</tr>
<tr>
<td>$P_{\text{cal}}$/true protein (mg/g)</td>
<td>13.9$^b$</td>
<td>14.1$^b$</td>
<td>16.1$^a$</td>
<td>16.6$^a$</td>
</tr>
</tbody>
</table>

$^a$–$^d$Values within a row with different superscripts are significantly different ($P < 0.05$).

$^1$Values are reported as mean ± SD.

$^2$UFA = ultrafiltered milk A.

$^3$UF = ultrafiltered milk B.

$^4$sRO = simulated reverse osmosis milk.

$^5$C$_{\text{cal}}$ = colloidal calcium.

$^6$P$_{\text{cal}}$ = colloidal phosphorus.
and 308 Pa. The initial G' value was proportional to the concentration of calcium bound to protein (mg of colloidal Ca/g of protein; Table 1). High colloidal calcium phosphate (CCP) content reduces the negative charge of casein micelles and promotes aggregation (Lucey et al., 2000). Appreciable coagulation at high pH (~6.0) was previously reported in in vitro gastric digestion studies (Wang et al., 2018b; Li et al., 2022). These results clearly support the fact that milk coagulation under gastric conditions is mostly driven by pepsin. Indeed, acidification contributes to milk coagulation because lower pH increases pepsin activity and the rate of κ-casein hydrolysis, which promotes micelles aggregation (Roy et al., 2020). Acidification also solubilizes some CCP, which increases the Ca^{2+} content and promotes calcium bridging, which is essential for casein aggregation (Lucey, 2014). In addition, the gradual CCP solubilization facilitates the fusion of casein particles due to the greater capacity of the gel structure to rearrange itself (Lucey et al., 2000). As expected, high-protein milks produced stiffer gels (higher G') than R milk. After 5 min coagulation, the G' values of gels from high-protein milks were on average 5 times higher than the gel from R milk (Figure 3A). Higher protein concentration increases casein volume fraction and the number of bonds between micelles (Sandra et al., 2011). In the last 15 min of the coagulation profile, all milks showed a gradual decrease of G', with a simultaneous increase of tan δ (Figure 3B). This behavior is characteristic of milk systems coagulating at pH ≥5.3 (Lucey et al., 2000) and is attributed to the loosening of the casein network induced by the dissociation of micellar calcium phosphate. After complete dissociation of micellar calcium phosphate, G' is expected to rise, and tan δ to decrease. However, because the concentration of GDL was chosen to reach pH ~5.2 after 30 min, the dissociation of CCP was not completed at that point and a maximum could not be observed in the tan δ profile. Interestingly, another transition was observed in the first 10 min of the rheological profile. A maximum in tan δ (Figure 4B) occurred at 4 min for R milk, corresponding to pH 5.8. In this study, the gelation conditions (GDL and pepsin concentrations) induced gelation at pH >6.0 followed by a relatively slow acidification between pH 6.0 and 5.5, which allowed the observation of this transition. This early transition may be attributed to the softening of the gel network due to the solubilization of precipitated calcium phosphate. Precipitated calcium phosphate is different from native micellar calcium phosphate and is solubilized at higher pH, close to pH 6 (Hassan et al., 2004). The precipitation of calcium phosphate onto the casein micelles is promoted by heat treatments, such as pasteurization (Gautcherson, 2005). Although the reaction is reversible, heat precipitated calcium phosphate is less soluble than the native micellar calcium phosphate (Van Hooydonk et al., 1987) and, as a result, a portion of the native micellar calcium phosphate is replaced by precipitated calcium phosphate after cooling pasteurized milk. Compared with R milk, a larger transition peak was observed in the tan δ profile during the gelation of sRO milk (Figure 3B). Higher concentrations of calcium and phosphorus in sRO milk (Table 1) increased the proportion of precipitated calcium phosphate, which in turn increased the gel network softening effect during acidification. UFB milk showed a very small transition, whereas no transition was observed during the acidification of UFA milk. These results were unexpected be-
cause UF milks received intense heat treatment, which should have increased calcium phosphate precipitation. However, during ultrafiltration, the mineral equilibrium is altered and the dissociation of insoluble calcium phosphate is promoted (Liu et al., 2014). Furthermore, ultrafiltration drastically reduces the ratio between soluble calcium and casein. The amount of precipitated calcium phosphate (per gram casein) in UF milks after heat treatment is then expected to be lower than in nonconcentrated or RO concentrated milks. Consequently, the early transition in the tan δ profile during the gelation of UF milks was hardly detected (UFB milk) or totally absent (UFA milk).

In Vitro Gastric Digestion

Extrusion of Milk Coagulum. Milk samples were incubated with GDL and pepsin for 10 min, and then extruded in controlled conditions through a syringe to simulate the retropulsion movements in the stomach. Extrusion pressure was recorded during the process and the extrusion parameters are included in Table 2. The extrusion pressure is an important parameter related to the coagulum structure and indicates resistance to high shear deformation, while the pressure variation can be used as an index of gel heterogeneity. As expected, coagulum from R milk showed the lowest extrusion pressure \( (P < 0.05) \) and heterogeneity index \( (P < 0.05) \), suggesting a weak and homogeneous structure due to its low protein content. The extrusion pressure was significantly higher for gels from high-protein milks and varied in the following order: UFA milk < UFB milk < sRO milk \( (P < 0.05) \). The UFA milk coagulum showed lower extrusion pressure and lower pressure variation than UFB milk \( (P < 0.05) \). In comparison to UFB milk coagulum, smaller aggregates could be visually observed in UFA milk coagulum after extrusion, which is consistent with a weaker and more homogeneous structure. In the first 10 min of coagulation, UFA milk showed lower tan δ values than UFB milk (Figure 3B), suggesting lower susceptibility to structural rearrangements and resulting in a more homogeneous matrix with smaller pores. Despite a \( G' \) value at 10 min lower than those from UF milks (Figure 3A), gel from sRO milk showed greater resistance to deformation.

Figure 3. Changes in storage modulus (\( G' \)) and loss tangent (tan \( \delta \)) during the coagulation of reference milk (○), high-protein UFA milk (■), high-protein UFB milk (□), and high-protein simulated reverse osmosis milk (●) gels made at 37°C induced by a combination of glucono-δ-lactone and simulated gastric fluid with pepsin. The dotted line represents the change in pH. Values depicted are mean ± SD \( (n = 3) \). UFA = ultrafiltered milk A; UFB = ultrafiltered milk B.
The sRO milk contains more than twice the amount of sugar compared with the other milk samples (Table 1), which is likely to increase the viscosity of the aqueous phase and the gel resistance to high shear deformation (Siamand et al., 2014). The sRO milk also showed the largest pressure variation during extrusion, suggesting a more heterogeneous texture ($P < 0.05$). The high tan $\delta$ value observed in the first 10 min of coagulation (Figure 3B) suggests a rapid rearrangement of the casein network, increasing the tendency toward micro- and macrosyneresis (Gastaldi et al., 2003) and the formation of dense protein clusters. Indeed, visual observation of extruded coagulum showed areas with large and tightly packed aggregates surrounded by serum.

**Coagulum Degradation and Proteolysis.** The coagulum degradation was monitored at different times during digestion (Figure 4), and the protein composition of both the coagulum and the liquid chyme were analyzed using SDS-PAGE under reducing conditions (Figure 5). Immediately after the extrusion of the coagulum from R milk (10 min), the proportion of nonsugar solids in liquid chyme was ~20%. The release of native whey proteins is responsible for this high value, as evidenced by the strong intensity of $\beta$-LG and $\alpha$-LA bands on the SDS-PAGE pattern of the liquid chyme (Figures 5B). A strong $\beta$-LG band was still detected after 120-min digestion, which is consistent with previous studies reporting that the compact globular structure of native $\beta$-LG increases its resistance to pepsin hydrolysis (Ye et al., 2016b; Mulet-Cabero et al., 2020). The appearance of a clear band at about 15 kDa in the coagulum protein profile (Figure 5A) corresponds to para-$\kappa$-casein and confirms that pepsin was involved in milk coagulation through the hydrolysis of the $\kappa$-casein, as previously reported (Ye et al., 2016b; Roy et al., 2021). This band was also observed on the SDS-PAGE patterns of coagula from UF- (Figure 5C, E) and RO- (Figure 5G) milks. The degradation of the coagulum from R milk was fast and, after 30 min, it reached 85% with almost no intact protein remaining (Figure 5A). At this point, residual coagulum probably consisted of flocculated fat, which resisted digestion due to absence of gastric lipase. At the end of digestion (120 min), the degradation of R milk coagulum was close to 100%, indicating a complete dispersion in the gastric fluid. Agitation during digestion provided the energy to disrupt fat aggregates and allowed them to pass through the sieve used to separate the coagulum from liquid chyme.

Compared with the coagulum from R milk, the proportion of nonsugar solids in liquid chyme immediately after the extrusion (10 min) of coagula from UF milks was much lower ($P < 0.05$; Figure 4), and only faint bands of intact whey proteins could be observed on the SDS-PAGE patterns (Figure 5D, F). UF milks were processed at higher temperatures than R milk, which induced the denaturation of whey proteins (Table 1). Denatured whey proteins were integrated into the coagulum and were not released at the beginning of digestion. The degradation of coagula from UF milks increased with digestion time but remained lower than the degradation of the coagulum from R milk ($P < 0.05$). Intact protein bands could still be observed on the SDS-PAGE patterns of coagula from UF milks after 120-min digestion (Figure 5C, E). Slower degradation and proteolysis of coagulum from UF milks are explained by the high-protein content of these milks, which reduces the enzyme-to-substrate ratio. Also, the dense and strong network of high-protein coagula slows down the penetration and diffusion of pepsin. The degradation of the coagulum from UFB milk was slower than the degradation of the coagulum from UFA milk ($P < 0.05$; Figure 4). Proteolysis was also slower in coagulum from UFB milk, as evidenced by the higher intensity of intact protein bands after 120 min digestion (Figure 5E), compared with the coagulum from UFA milk (Figure 5C). The coagulum from UFB milk showed higher resistance to extrusion and higher pressure variation (Table 2), suggesting a stronger matrix, leading to larger particles after extrusion, which is expected to reduce the surface area available for pepsin activity.

Immediately after the extrusion of the coagulum from sRO milk (10 min), the proportion of solids in the liquid chyme was high and similar to that observed for R milk coagulum (~20%; Figure 4). As for R milk,
Figure 5. The SDS-PAGE patterns under reducing conditions of the coagula (A, C, E, G) and liquid chymes (B, D, F, H) obtained after 10, 20, 30, 60, and 120 min simulated gastric digestion of reference milk (A, B), high-protein UFA milk (C, D), high-protein UFB milk (E, F), and high-protein simulated reverse osmosis milk (G, H). M = reference milk before digestion. UFA = ultrafiltered milk A; UFB = ultrafiltered milk B.
the proportion of native whey protein in sRO milk is high (Table 1), and native whey proteins were rapidly released in liquid chyme (Figure 5H). After this point, the rate of degradation was reduced compared with R milk coagulum, due to the high-protein content of sRO milk. At the end of gastric digestion (120 min), the coagulum degradation was significantly lower than for UF milks (P < 0.05; Figure 4) and strong intact protein bands could still be observed on the SDS-PAGE pattern of sRO milk coagulum (Figure 5G). The coagulum from sRO milk showed the highest pressure and pressure variation during extrusion (Table 2), supporting the premise that the resistance to high shear deformation is an important factor controlling the gastric digestion of milk coagulum.

CONCLUSIONS

High-protein milks offer an alternative to consumers for meeting their daily protein needs. The present study demonstrates that the gastric digestion of these products is different from the gastric digestion of nonconcentrated milk. A much stronger coagulum is produced and the resistance to mechanical degradation is increased. Pepsin-induced proteolysis is also slower and intact milk proteins are still observed after 120 min. These differences can have significant physiological effects because it may influence the kinetics of gastric emptying and nutrient release. The processing conditions used for the production of high-protein milk also affects gastric digestion. Despite similar protein concentration, high-protein milks under study showed different behavior during gastric digestion. The type of filtration, the temperature during filtration and the severity of heat treatment used to extend shelf life affect the concentration of minerals bound to caseins and the denaturation rate of whey proteins. These factors are likely responsible for the observed differences. According to our results, it would be possible to optimize the processing conditions of high-protein milk to meet specific requirements regarding the kinetics of coagulation and degradation in the gastric environment.

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