Effect of homogenization and heat treatment on the behavior of protein and fat globules during gastric digestion of milk

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ABSTRACT

The effects of homogenization and heat treatment on the formation and the breakdown of clots during gastric digestion of whole milk were investigated using a human gastric simulator. Homogenization and heat treatment led to formation of coagula with fragmented and crumbled structures compared with the coagulum formed from raw whole milk, but a larger fraction of the protein and more fat globules were incorporated into the coagula induced by action of the milk-clotting enzyme pepsin. The fat globules in the whole milk appeared to be embedded in the clots as they formed. After formation of the clot, the greater numbers of pores in the structures of the clots formed with homogenized milk and heated whole milk led to greater rates of protein hydrolysis by pepsin, which resulted in faster release of fat globules from the clots into the digesta. Coalescence of fat globules occurred both in the digesta and within the protein clots no matter whether they were in homogenized or heated milk samples. The formation of clots with different structures and hence the changes in the rates of protein hydrolysis and the release of milk fat into the digesta in the stomach provide important information for understanding the gastric emptying of milk and the potential to use this knowledge to manipulate the bioavailability of fat and other fat-soluble nutrients in dairy products.

Key words: milk, homogenization, heat treatment, gastric digestion, protein coagulation

INTRODUCTION

Fresh whole milk is usually processed to be safe for human consumption and to extend its shelf life. Commercially available milk is commonly homogenized and pasteurized. Thermal treatments, such as pasteuriza-

tion and UHT processing, lead to denaturation of some milk fat globule membrane (MFGM) proteins and to interactions between the MFGM and milk serum proteins (Ye et al., 2004). Extensive thermal treatment also leads to the denaturation of whey protein and the association of whey protein with casein micelles by interaction with κ -casein (Anema and Li, 2003). Homogenization increases the stability of milk fat by reducing the size of milk fat globules. This process leads to the casein and whey proteins adsorbing onto the surface of fat globules and reducing the amount of MFGM at the fat globule surface (Ye et al., 2008). Homogenization of whole milk followed by heat treatment causes the denatured whey proteins to attach to the adsorbed casein and MFGM proteins via disulfide bonds (Michalski and Januel, 2006).

Recently, claims have been made that raw milk is more nutritious and more easily digested than heat-treated milk because the proteins are not denatured (Smith, 2010). Therefore, studies on the effect of heat and homogenization processing on the digestibility of milk components have attracted some attention (Tunick et al., 2016). Wada and Lönnerdal (2014) showed that protein bands of SDS-PAGE of in-can sterilized milk underwent rapid decrease during in vitro digestion, and this was true to a lesser extent in UHT milk. Proteomic analysis revealed that the level of lactulosyllysine, which reflects a decrease in protein digestibility, in α -LA, β -LG, and caseins was higher in in-can sterilized milk than in UHT milk samples. Thus, industrial heating may improve the digestibility of milk proteins by denaturation, but the improvement is likely to be offset by heat-derived modifications involved in decrease in protein digestibility, for example, the formation of crosslinks such as lysinoalanine and lanthionine (Wada and Lönnerdal, 2014). Miranda and Pélissier (1987) reported that heat treatment results in accelerated gastric emptying in milk digestion and appears to increase the rate of hydrolysis of the caseins in vivo. Barbé et al. (2013) also reported a significant influence of heat treatment on the resistance and sensitivity of both casein and β -LG to hydrolysis in the

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human stomach in an *in vivo* experiment. Tunick et al. (2016) recently investigated the effect of heat and homogenization on *in vitro* digestion of milk protein and fat, showing a faster release in the free fatty acids from homogenized milk in the intestinal fluid. However, these studies used simple *in vitro* models, and limited *in vivo* experiments have been done for understanding why differences are found in the digestibility of milk components after different processing treatments.

Recently, we reported that the gastric digestion behavior of the protein in skim milk is influenced by the structure of the coagulum that is induced by the action of the milk-clotting enzyme pepsin on κ -casein in gastric conditions (Ye et al., 2016a). The clot formed from the coagulation of unheated milk showed a tightly knit network with numerous small pores interspersed throughout the matrix, whereas a network structure with larger voids was observed in the coagulation of milk that had been heat treated. With increasing digestion time, as the pH decreased further, the structures of the clots from unheated milk tightened and became less permeable to serum and solutes. These changes apparently affected the hydrolysis of casein by pepsin in the gastric system. In unheated milk, casein hydrolysis was much slower than that in the heated milk. Whey proteins, β -LG, and α -LA remained largely intact during the whole digestion period in unheated milk, but whey proteins in heated milk were readily hydrolyzed by pepsin. In heated milk, both casein and whey protein were involved in the formation of the clot.

For whole milk, milk fat globules seem to be embedded in the coagula formed during gastric digestion. We recently reported that the differences in structures of clots formed with untreated and heated unhomogenized whole milk led to different rates of protein hydrolysis by gastric pepsin, which also resulted in different rates of release of fat globules from the clots (Ye et al., 2016b). In heated whole milk, the release rate of fat globules was much higher than that of untreated whole milk, but it was slower than the rate of loss of fat-free matter during digestion. It has been suggested the interaction between the fat globule membrane and serum proteins in heated milk may hinder the release of fat globules from the protein clots.

Several studies have attempted to understand the lipolysis of milk fat globules in the gastrointestinal tract (Michalski, 2009; Ye et al., 2010, 2011; Gallier et al., 2012, 2013). As lipolysis is an interfacial process, the ultrastructure of the MFGM is likely to play a key role in the digestion and absorption of milk fat. Most studies on the digestion of milk fat globules have used *in vitro* digestion models with (Gallier et al., 2012) or without (Berton et al., 2009) the gastric digestion step. It has been reported that milk fat globules undergo

major changes in the stomach because of the possible action of pepsin on the proteins of the MFGM (Ye et al., 2011; Gallier et al., 2012). Apparent flocculation of milk fat globules occurred in the raw milk incubated in simulated gastric fluid (SGF) with pepsin, but no coalescence was observed in both raw milk and cream samples. However, coalescence of fat globules occurred under simulated intestinal conditions, as lipolysis by pancreatic lipase proceeded. The major MFGM proteins were hydrolyzed by pepsin in SGF at different rates, in which the butyrophilin was more resistant to peptic hydrolysis than xanthine oxidase, PAS 6, and PAS 7. This study shows the importance of the MFGM structure in the digestion of the milk fat globules. Therefore, any processing treatment affecting the MFGM structure will influence the way milk fat globules are digested. Changes in the physicochemical properties of fat globules and dispersed hydrolytic products are important in understanding the digestion and absorption of lipids in milk products (Staggers et al., 1990).

This study extends our recent work in which bovine skim milk or whole milk was digested under gastric conditions (Ye et al., 2016a,b). In the present work, the physicochemical behavior and the morphology of milk fat globules and clots formed from homogenized whole milk and heated homogenized whole milk during *in vitro* gastric digestion in a dynamic human gastric simulator (HGS) were investigated. The information obtained from this work will be useful for understanding the digestion and absorption of milk fat and protein from differently processed milks and for the design of products derived from these milks.

MATERIALS AND METHODS

Materials

Bulk whole milk (4.71% fat and 3.51% protein) was collected from the Massey University Dairy Farm, Palmerston North, New Zealand. The homogenized milk was whole milk that had been homogenized by 20/5 MPa at 20°C using a 2-stage valve homogenizer (APV 2000, Copenhagen, Denmark). The average fat globule size of the milk samples was $d_{43} = 3.2 \pm 0.05$ μm and 0.52 ± 0.05 μm for the untreated milk and homogenized milk, respectively. The heated homogenized milk used in the experiments was homogenized milk that had been heated at 90°C for 20 min. Pepsin from porcine gastric mucosa (EC 3.4.23.1; catalog no. P7000, Sigma Chemical Co., St. Louis, MO) had an enzymatic activity of 800 to 2,500 units/mg of protein, as stated by the manufacturer. All other chemicals were obtained from BDH Chemicals (BDH Ltd., Poole, UK) unless otherwise specified. All solutions were prepared from

analytical grade chemicals. Milli-Q water (water purified by treatment with a Milli-Q apparatus; Millipore Corp., Bedford, MA) was used for the preparation of all solutions.

Simulated gastric fluid (SGF) was prepared by adding 3.8 mL of 37% HCl and 8.775 g of NaCl to 500 mL of water and then making up to 1 L with water; 3 g of pepsin was added to this solution before use, with stirring for 30 min, and then the pH of the SGF was adjusted to 1.5 using 1 N HCl/NaOH (Ye et al., 2011).

Methods

Human Gastric Simulator. An HGS, developed by Kong and Singh (2010), was used for gastric digestion. The driving system of the HGS, consisting of 12 rollers, 4 belts, driving shafts, and a pulley system, was installed to create peristaltic contractions on 4 sides of the latex stomach chamber. The rollers were screwed on belts distributed along the 4 equally spaced sides of the stomach. Each roller consisted of 2 side wheels that were 9 mm in length and 12 mm apart from each other. Each belt (length 81 cm) had 3 equally spaced rollers. A pair of opposite rollers was placed 30 mm higher than the pair at right angles to it. The gap between 2 opposite rollers was 10 mm when they contracted. A thin polyester mesh bag (pore size ~ 1 mm) was placed inside the latex stomach chamber to mimic human gastric sieving, which allows particles only of size < 1 mm to pass through to the duodenum (Meyer et al., 1976; Schulze, 2006).

A 200-g sample of 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. Because of the biphasic nature of gastric emptying (Collins et al., 1983; Siegel et al., 1988), the emptying of the digesta was started after 30 min. For accurate control of the gastric emptying, the digesta were removed from the bottom of the stomach at 45 mL/15 min, equaling the natural gastric emptying rate of 3.0 mL/min. The contraction frequency of the HGS was 3 times/min, simulating the actual contraction of the stomach. The temperature of the HGS was maintained at 37°C by a heater and a thermostat. The maximum digestion time was 220 min, but most experiments were terminated at shorter times so that the clot of coagulated milk could be collected for further analysis. For each time interval, the total sample was removed from the HGS and was then filtered through a mesh with a pore size of 0.5 mm in diameter to obtain both solid mass and an aqueous phase for further analysis. In addition, to observe the effect of mechanical processing alone on the digestion of milk, experiments were carried out without the addition of pepsin

and served as control experiments. The samples were prepared in duplicate. All tests were replicated twice.

pH Measurement. The initial pH in the HGS was defined as the pH of the 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. These pH values only refer to the fluid part of the HGS contents because it is not possible to measure the pH within the coagula.

Weight of Clots. The clots obtained after the digestion of untreated whole milk for different times were very firm and were lifted out, rinsed with pepsin-free SGF to remove pepsin from the surface, and weighed immediately. For the homogenized and heated milk, at the end of each run, the contents of the HGS were collected and passed through a filter with a 1-mm pore size to separate the clot and the aqueous phase. The clot was then weighed immediately and samples were taken for microscopy. All coagulum samples were then heated at 90°C for 3 min to inactivate pepsin. Each clot obtained from milk sample, at different time intervals, was dried at 105°C for 12 h in a vacuum oven so that the dry weight could be determined.

Fat Content of Clots. The fat content of the clots was determined using the Mojonnier method (IDF Standard, 1987).

Confocal Laser Scanning Microscopy. The microstructure of the clot with digesta obtained from the digestion of milk was studied using a confocal laser scanning microscope (Leica, Heidelberg, Germany). Clots with digesta samples for observation of microstructure by confocal microscopy were collected immediately by a blade without inactivating pepsin to mix with staining solutions. Nile Red was used to stain for oil (argon laser with an excitation line at 488 nm) and Fast Green 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 milks during digestion. Mixtures of clot and digesta was stained with 1.0% (wt/vol) Fast Green and Nile Red for 15 min, placed on a concave confocal microscope slide (Sail; Sailing Medical-Lab Industries Co. Ltd., Suzhou, China), covered with a coverslip, and examined with a 63 $\times$ magnification lens.

Protein Hydrolysis. The time-dependent hydrolysis by pepsin of the proteins in the clot and the emptied digesta was determined by analyzing the protein composition of the milks as a function of the digestion time, using SDS-PAGE. The sample buffer used was 0.2 M Tris-HCl buffer, pH 6.8, 40% glycerol, 2% SDS, 0.04% bromophenol blue, and β -mercaptoethanol (19:1,

vol:vol). Samples of digesta were treated with this buffer and were heated at 90°C for 5 min. Aliquots of liquid digesta were mixed with sample buffer at a ratio of 1:2 and 8 μ L of the mixture was loaded in each well of the electrophoresis gel. For samples of solid clot, 4.5 mg of the freeze-dried and ground powder was mixed with 1 mL of sample buffer and 10 μ L of this was loaded in each well after the heat treatment. The electrophoresis gels were previously prepared on a Mini-PROTEAN II system (Bio-Rad Laboratories, Richmond, CA). The resolving gel contained 16.0% acrylamide, made up in Tris/HCl buffer, and the stacking gel was made up of 4.0% acrylamide in Tris/HCl buffer. After running, the gels were stained for 60 min with a solution of Coomassie Brilliant Blue R-250 [0.003% (wt/vol) in 10% acetic acid and 20% isopropanol (Merck)]. The gel was destained with a solution of 10% acetic acid and 10% isopropanol and was scanned using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories). Bands were assigned on the basis of previous studies of milk and milk proteins (Ye et al., 2016a).

Statistical Analysis. The *t*-tests and ANOVA tests were carried out using Minitab software (Minitab version 16, Minitab Inc., State College, PA) to determine the significance of the differences. Significance was established at $P < 0.05$. The samples were prepared in duplicate. All tests were replicated twice.

Figure 1. Change in pH of whole milk during gastric digestion in the human gastric simulator: untreated whole milk (●), homogenized (Homo) milk (○), and heated Homo milk (90°C for 20 min; ■). Each data point is the average $\pm$ SE of 2 determinations on 2 separate samples.

RESULTS AND DISCUSSION

pH Profiles

Changes in the pH of milk samples (fluids) in the HGS as a function of digestion time are shown in Figure 1. In general, the pH of all samples decreased with increasing digestion time, but a difference was found in the pH profiles of milks after homogenization and heat treatment. The pH of the untreated whole milk mixed with SGF containing pepsin decreased to about pH 2 at about 150 min, then changed slightly during further digestion up to 220 min. The change in the pH of homogenized milk was slightly different compared with the untreated milk. However, the heated homogenized milk showed significantly less decrease in pH with digestion time compared with untreated whole milk. The pH only reached about 3 at the end of digestion (220 min). This difference could be ascribed to the formation of coagula with different structures in the samples with different treatments, so that the diffusion of molecules and ions in and out of the clots was different (Ye et al., 2016a).

Coagulation Behavior of Milk

In untreated whole milk, protein coagulation was visible after 8 min of digestion time and a firm clot with a smooth surface was formed after 10 min; at this stage the serum phase became clear, so that it appeared that all of the casein micelles were incorporated into the clot. The pH after 10 min of digestion was 6.2. The size of the clot reduced gradually with increasing digestion time, but the clot was not broken up into smaller pieces as the digestion progressed up to 220 min (Figure 2). For the homogenized whole milk sample, protein coagulation was also observed after about 10 min, when the pH was around 6.2. The coagulation also resulted in an integrated clot, but it appeared to be more porous than the clot from untreated whole milk. The clots became less integrated and separated into several small pieces at longer digestion times (Figure 2). The coagulum from the heated homogenized milk was much looser and fragmented and had a crumbled structure. The amount of coagulated material decreased markedly and the size of the crumbs became smaller as the digestion time increased.

The clots formed in the milk samples during digestion were dried at 105°C for 12 h in a vacuum oven. The changes in the weight of the dried matter obtained from 100 g of milk sample as a function of digestion time are shown in Figure 3. After 10 min of digestion, the weights of the dried matter obtained from milk samples were ~8, ~9, and ~10.2 g for untreated, homogenized,

Figure 2. Images of clots formed during the gastric digestion (simulated gastric fluid with pepsin) of 200 g of untreated whole milk, homogenized milk, and heated homogenized milk (90°C for 20 min) at different digestion times. Color version available online.

and heated homogenized milks, respectively. A gradual decrease in the dried weight with digestion time was observed in all milk samples, but the decrease in the unheated milk was much slower than that in the heated milk; for example, after 220 min, the weights of the clots from untreated, homogenized, and heated homogenized milks were about 5.2, 4.3, and 2.9 g, respectively. Because of the difference in the initial weight of clots formed in milk samples, the loss in the coagulated materials during digestion is best displayed as percentages of the original clot weight as a function of digestion time (Figure 3B). In the heated homogenized milk, only about 20% material remained after 220 min, but in untreated milk about 70% of the clot material and in homogenized milk about 50% of the clot materials remained after 220 min digestion. These different rates of breakdown reflect the structures of the clots. The untreated milk forms a very tight clot that contains very little water and consequently offers little chance for pepsin to diffuse into it, so that breakdown is slow (Ye et al., 2016a,b). The somewhat more open structure of the clot from homogenized milk allows greater penetration by pepsin and faster breakdown, and this is accentuated in the even looser structure formed by the heated homogenized milk.

In the gastric environment, milk proteins aggregate and precipitate to form the clot due to the low pH and the presence of the proteolytic enzyme, pepsin. In previ-

ous reports using simple in vitro gastric models, it was proposed that the precipitation of milk protein during digestion is induced by low pH (Tunick et al., 2016). However, the coagulation of milk started at a pH of 6.2 in the present work using the dynamic in vitro model, which is well above the pH at which acidic coagulation of either heated or unheated milk can occur. The coagulation of milk proteins therefore must be the result of the proteolytic activity of the pepsin in the milk (Ye et al., 2016a). It has been found the pepsin can rapidly hydrolyze the κ -casein to para- κ -casein in vivo gastric digestion of milk (Delfour et al., 1966; Miranda and Pélissier, 1983). The hydrolysis of κ -casein by pepsin at pH 6 was also confirmed in an in vitro work (Tam and Whitaker, 1972), which showed the hydrolysis of κ -casein by pepsin is faster than the hydrolysis of other caseins at pH 6. This activity of pepsin on κ -casein causes the coagulation of casein micelles (Mulvihill and Fox, 1979). Our previous work (Ye et al., 2016a), by comparing SGF with and without pepsin, showed that the structures of clots induced by pepsin and low pH are different under dynamic gastric conditions.

The present results showed that the coagulation behavior of casein micelles under gastric conditions was not dependent on the presence of fat globules. However, the weights of the clots in untreated whole milk were significantly higher than that formed from skim milk (Ye et al., 2016a), indicating the involvement of fat

globules in the formation of clots in the whole milk samples. Further, the higher weights of clots obtained from homogenized milk samples were presumably due to the incorporation of more fat. This may be because of the smaller size of fat globules in the homogenized milk and also because the surfaces of the fat globules of homogenized milk were mainly coated by casein and whey proteins, rather than the original MFGM. In rennet gels, natural milk fat globules with native MFGM from untreated whole milk have been considered to act as inactive fillers in the gel matrix (Michalski et al., 2002). In the present study, the fact that native fat globules of untreated whole milk did not affect the

formation of the pepsin-induced clots agreed with this theory. In contrast, the fat globules coated by casein and whey proteins in homogenized milk cannot be considered as inactive fillers because they will interact with one another and with destabilized casein micelles to form the gel matrix. It is therefore not surprising that the properties of the gels formed by homogenized milk differ with those of the gel formed by untreated whole milk.

The heated homogenized milk has a larger clot weight than either of the other samples, and this presumably arises from the incorporation of whey proteins within the gel. From the pH at which this milk coagulated, it appears that the coagulation of heated milk was caused by pepsin, but as is found with the renneting of heated milk (Anema et al., 2007), the clot formed was not as cohesive as in the whole milk and the unheated homogenized milk; we found a similar result in the clot formed from heated skim milk (Ye et al., 2016a). The amount of coagulated material decreased more rapidly than in the other samples, and the size of the crumbs became smaller with an increase in the digestion time (Figure 3B), as a result of the faster diffusion of pepsin and consequently greater hydrolysis rate of protein in the more open structure of the coagulum.

Release of Milk Fat Globules from the Clots

The amounts of fat in the clots were determined as a function of gastric digestion time (Figure 4). After 10 min, about 3.8 g fat was included in the clot obtained from 100 g of untreated milk, and slightly larger amounts of fat (~4.2 g/100 g of milk) were contained in the coagulated material of homogenized milk, but no difference was found between the homogenized milk samples without and with heat treatment. During further digestion, the fat content of the clots decreased at different rates. For untreated milk, the fat content in the clot reduced to ~2.5 g/100 g of milk at 220 min. Lower fat content was observed in homogenized milks, which were ~2.0 g/100 g of milk and ~1.0 g/100 g of milk for unheated and heated samples, respectively.

Similar amount of fat in the clots of untreated milk and homogenized milk samples indicated that the amounts of fat-free matter involved the formation of clots were different. Figure 4B shows that the weight of fat-free matter in the clots of homogenized milks was higher than that of untreated milk at 10 min when the clots just formed. About 6.3 g/100 g of milk was observed in heated homogenized milk, compared with only about 4 g/100 g of milk in untreated milk at 10 min digestion. Compared with the decrease in the fat content of the clots during digestion, different rates of loss in weight of fat-free matter were also observed in

Figure 3. Change in dried pellet weights (g/100 g of milk; A) and decrease in the dried matter of clots (%) B) of untreated whole milk (●), homogenized (homo) milk (○), and heated homo milk (90°C for 20 min; ■) during digestion in simulated gastric fluid containing pepsin. Each data point is the average $\pm$ SE of 2 determinations on 2 separate samples.

Figure 4. Changes in fat content (g/100 g of milk; A) and fat-free matter content (g/100 g of milk; B) in clots obtained from untreated whole milk (●), homogenized (homo) milk (○), and heated homo milk (90°C for 20 min; ■) during digestion in simulated gastric fluid containing pepsin. Each data point is the average $\pm$ SE of 2 determinations on 2 separate samples.

the milk samples with different treatments (Figure 4). Untreated milk had much less decrease in the amount of fat-free matter compared with that of heated homogenized milk; the result for homogenized milk was between the untreated milk and heated homogenized milk.

The relationships between the amounts of fat remaining in the clot and the total weights of the clots as a function of digestion time are shown in Figure 5. All milk samples exhibited a linear correlation between fat and fat-free matter in the clots. No statistically significant difference was present between the different

Figure 5. The relationship between the loss of fat and the loss of total weight of the clots of untreated whole milk (●) and homogenized milk (○) and heated homogenized milk (90°C for 20 min; ■) during digestion in simulated gastric fluid containing pepsin. Each data point is the average $\pm$ SE of 2 determinations on 2 separate samples.

milk samples, which shows that the fat in all of the samples was lost proportionally to the rate of digestion of the whole clot, and that the clot structure was not of importance in this respect. However, it is also clear from Figure 3B that the clot structure is important in determining the overall rate of breakdown, so that the greatest release of fat was from the clot that breaks down fastest (the clot from heated homogenized milk). When the results from all of the samples were combined, a least squares linear fit to Figure 5 gives a gradient of 1.07 ($R^2 = 0.965$). Because the slope was close to 1, this suggests that as the protein from the clot was digested, fat was liberated in proportion.

These results suggest that, perhaps surprisingly, the manner in which the fat globules are incorporated within the clot matrix does not control their release. In the untreated milk, the fat globules are trapped within the clot matrix formed of caseins, but there are no interactions between protein and fat. In comparison, in the homogenized milk, the surfaces of the fat globules are covered with casein, so that the globules themselves help to form the matrix as the clot is formed. In the heated homogenized milk, the fat globule surfaces contain both denatured whey proteins and caseins, and again participate in formation of the clot matrix.

Hydrolysis of Protein in Clots

Our previous study showed the reduction in the weight of fat-free matter in the clot materials was mainly attributable to the peptic hydrolysis of protein, and that

Figure 6. The SDS-PAGE patterns under reducing conditions of the clots obtained during the gastric digestion (simulated gastric fluid with pepsin) of untreated whole milk (A), homogenized milk (B), and heated homogenized milk (90°C for 20 min; C) at different times. M, untreated milk.

the rates of protein breakdown were significantly different in the clots formed from heated and unheated skim milk (Ye et al., 2016b). In the present study, consisting of fat globules with different processing treatments, the hydrolysis of protein in the clots, determined using SDS-PAGE under reducing conditions, during digestion is shown in Figure 6.

Similar protein composition and bands were observed for untreated whole milk samples and homogenized milk samples, except that some decreases occurred in band intensities of caseins and para- κ -CN (15 kDa) in homogenized milk after 80 min of digestion. These changes in protein bands during digestion have been observed consistently in the digestion of skim milk and whole milk reported in previous work (Ye et al., 2016a,b). The analysis shows that the early action of pepsin is on κ -CN, whose band intensity decreases

in the first 20 min, along with the appearance of a para- κ -CN band at about 15 kDa. The reduction in the protein bands in homogenized milk at long digestion time suggested that greater hydrolysis of these proteins occurred in the homogenized milk than in the untreated milk, in agreement with the more rapid breakdown of the clot (Figure 3). Very faint β -LG and α -LA bands were observed in the clots from both untreated milk and homogenized milk, indicating that whey proteins were not significantly involved in the formation of the clots and remained in the serum phase (digesta).

In contrast to unheated homogenized milk, large amounts of β -LG and α -LA were initially present in the clot of heated homogenized milk, but these proteins were degraded gradually along with the caseins during digestion. This result was also in agreement with the behavior of heated whole milk (Ye et al., 2016b), in which the whey proteins were involved in the formation of the clot, partly because of the association of whey proteins with casein micelles during heat treatment (Anema and Li, 2003; Donato et al., 2007), and partly because the non-micelle-bound whey protein/ κ -CN complexes formed during heating associate with the micelles during renneting (Kethireddipalli et al., 2011) and at pH values below ~ 5.3 (Vasbinder et al., 2001; Donato et al., 2007).

Figure 7 showed the protein compositions of the material (digesta) removed from the HGS during the digestion process. Little difference was found between untreated milk and homogenized milk at early stages of digestion, in which intact β -LG and α -LA were seen to be present, and then evacuated from the stomach. For both milks, the intensity of the serum albumin bands decreased rapidly and these bands disappeared from the digesta after 20 min; the intensity of the α -LA and β -LG bands decreased gradually with increasing digestion time. However, there were smaller amounts of intact serum proteins in the digesta from homogenized milk. In contrast, the digesta evacuated from the HGS before 40 min of digestion of the heated homogenized milk (Figure 7B) contained only small amounts of intact caseins and whey proteins, and very few bands were observed in the samples at longer digestion times. It should be noted that many peptides were evacuated from the stomach, in particular at long digestion times, which were too small to be detected by SDS-PAGE.

In the early stages of digestion of untreated milk and homogenized milk, the activity of pepsin was low because of the high pH (>6 ; Figure 1). After 20 min of digestion, the disappearance of BSA from the digesta could be attributed to its hydrolysis by pepsin, because the activity of pepsin was increased with the decrease in pH. Only β -LG remained in the digesta at long digestion times, probably because of its resistance

Figure 7. The SDS-PAGE patterns under reducing conditions of the digesta obtained during the gastric digestion (simulated gastric fluid with pepsin) of untreated whole milk (A), homogenized milk (B), and heated homogenized milk (90°C for 20 min; C) at different times. M, unheated milk.

to pepsin hydrolysis. However, it should be noted that the amount of β -LG and the other soluble proteins in the digesta decreased because of the dilution of the digesta with increasing digestion time, as digesta are withdrawn and fresh SGF is added to the contents of the stomach. The difference in the protein composition of digesta between unheated milks and heated milk is caused by the interaction of casein and whey proteins during the heating process. In unheated milk, β -LG

and α -LA were not involved in the formation of the clot, remained in the serum phase under gastric conditions, and were drained from the HGS. In contrast, the whey proteins were involved in the formation of the clot in heated milk, and the small amounts of intact whey proteins in the digesta of heated milk reflected the marked increase in their susceptibility to hydrolysis by pepsin caused by their heat denaturation. Barbé et al. (2013) have also reported a significant influence of heat treatment on the resistance and sensitivity of both casein and β -LG to hydrolysis in the human stomach in an in vivo experiment. Miranda and Pélissier (1983) reported that β -LG and α -LA were still present in considerable amounts in the stomach contents of rats after 30 min of digestion of skim milk, suggesting that the whey proteins are probably preferentially evacuated from the stomach. Mahé et al. (1996) tested the exogenous protein fraction recovered from the upper jejunum of humans after the ingestion of casein and β -LG purified by membrane microfiltration. They also found that casein empties from the stomach mainly as peptides, whereas β -LG empties mainly as intact protein.

Microstructure of Fat Globules in Clots with Digesta

Figure 8 shows confocal microscopy images of the clots, containing fat globules, formed from untreated whole milk, homogenized milk, and heated homogenized milk at different times of digestion. The fat globules were distributed homogeneously in the protein aqueous phases of all of the milks before digestion. At an early stage in digestion (20 min), a closely knit network of protein was observed for untreated whole milk and unheated homogenized milk. Some fat globules appeared to have coalesced within the matrix of the clot, as evidenced by the large area of fat seen in the samples; in contrast, other fat globules appear within the aqueous phase (Figure 8, 20 min); this possibly indicates that some micro-phase separation occurred during the formation of the clot. In heated homogenized milk after 20 min of digestion, the structure of the clots was much more open, with blocks of aggregated protein. The fat globules appeared to be clumped in separate areas and did not seem to be contained within the protein matrix, although they seemed to remain associated with it. The sizes of these fat globules in the aqueous phase were apparently larger than the fat globules in the homogenized milk before digestion, suggesting that coalescence was taking place as the membrane surrounding the fat globules was attacked by pepsin.

With an increase in the digestion time, the knitted network in all of the samples became loose and the

fat globules within the matrix tended to aggregate and coalesce, so that large pools of fat could be observed especially after 220 min of digestion. The protein matrix appeared to decrease, whereas increasing amounts of fat globules were released from the protein matrix into the aqueous phase in all samples. These fat globules present in the aqueous phase appeared to increase in size with increasing digestion time. In comparison, a degree of coalescence and phase separation of oil droplets was also observed in whey protein emulsion gels during gastric digestion as the oil droplets were released from the gel matrix (Guo et al., 2014a,b). A similar behavior appears to occur in whole milks, since coalescence occurred both within the clot matrix and in

the more open pores containing the fat globules (Guo et al., 2015). After the oil droplets had been released from the gel matrix, they behaved in a similar manner to a protein-stabilized oil-in-water emulsion or to the fat globules derived from cream and milk under gastric conditions (i.e., destabilization of the oil droplets occurred as the adsorbed protein was broken down; Ye et al., 2011; Gallier et al., 2012; Li et al., 2012, 2013). It has been reported that the MFGM proteins of fat globules are totally hydrolyzed by pepsin during the *in vitro* gastric digestion of milk and cream. Only peptides remain at the surface of the fat globules, which may lead to their destabilization and coalescence as observed here (Ye et al., 2011; Gallier et al., 2012).

Figure 8. Confocal microscopy images of untreated whole milk, homogenized milk, and heated homogenized milk (90°C for 20 min) at different times during gastric digestion from 0 to 220 min. Dark gray (red) shows the fat, and light gray (green) shows the protein. The scale bar in all images is 20 μ m. Color version available online.

CONCLUSIONS

The changes in fat globules and protein of whole milk due to homogenization and heat treatment influence the coagulation of protein and the formation of clots combined with protein and fat globules during the digestion in stomach. The fat globules in whole milk were incorporated into the firm clots when they were formed, but more fragmented and crumbled structures were formed from homogenized milk and heated homogenized milk. In untreated milk, the fat globules are simply trapped within the gel structure formed by the casein micelles, whereas in the homogenized milks the fat globules are coated with caseins and actively are incorporated into the gel matrix. The differences in the structures of the clots formed with untreated whole milk, homogenized milk, and heated homogenized milk led to different rates of protein hydrolysis by pepsin and hence in different rates of release of fat from the clots. However, the relative rates of release of fat were independent of the treatment given to the milk. Coalescence of fat globules occurred both in the digesta and within the protein clots in all samples. The formation of clots with different structures and hence the changes in the rates of protein hydrolysis and the release of milk fat from the clots into the digesta in the stomach are relevant to gastric emptying and the digestion of fat in the intestine. The present study may provide some understanding of the effect of processing not only on the properties of milk but also on its digestibility.

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