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Behaviour of Milk Protein Ingredients and Emulsions Stabilised by Milk Protein Ingredients In the Simulated Gastrointestinal Tract

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Abstract

Milk clotting behaviours in the stomach impact the digestion rates of protein and fat. A variety of milk protein products are applied as functional ingredients in many foods. This research was conducted to investigate the digestion behaviours of various commercial dairy ingredients and lipids in emulsions stabilised by these ingredients using a dynamic *in vitro* digestion model, i.e., a human gastric simulator (HGS), with a focus on the effect of different structures of clots formed in dairy ingredients during gastric digestion on hydrolysis of proteins and/or lipids.

Skim milk powder (SMP), milk protein concentrate (MPC) 4851, MPC 4861, sodium caseinate, whey protein isolate (WPI) and heated (90°C, 20 min) WPI were used in the present study. Results showed that SMP and MPC 4851, which contained casein micelles, formed ball-like clots with a relatively dense network after 10 min of gastric digestion. These clots did not disintegrate after 220 min of digestion. MPC 4861 and sodium caseinate generated clots at around 40 min, and a loose, fragmented structure was observed at the end of the gastric digestion due to a lacking micellar structure of caseins. No clot was observed in WPI or heated WPI after 220 min gastric digestion, although aggregation occurred at around 40 min in heated WPI. These differences in coagulation behaviours apparently affected the rate of gastric emptying and protein hydrolysis by pepsin in the gastric system. In SMP and MPC 4851, the gastric emptying and hydrolysis of caseins was much slower than that observed in MPC 4861 and sodium caseinate. The most rapid gastric emptying of proteins was observed in the WPI samples both with and without heating. This is attributed to the formation of varied structured clots at different times under the gastric conditions.

The effect of protein concentration on the gastric behaviour of these dairy ingredients in solution was then examined, with a particular emphasis on the structure of clots. SMP and MPC 4851 have been selected as model protein ingredients. Their gastric behaviours were investigated over a protein concentration range of 0.5-5.0% (w/w). The results showed that the digestion behaviour of SMP and MPC 4851 followed a similar pattern. The rate of pH changes in the emptied digesta during digestion was protein concentration dependent. With an increase in protein concentration, the decrease in pH slowed. The protein concentration had no apparent impact on the casein clotting time.

Clots were formed in the first 10 min of digestion in all samples. However, in both SMP and MPC 4851, when protein concentration was lower than 2.0% (w/w) the clots consisted of small protein pieces with a loose, porous and open structure after a 220 min digestion. Whereas a cheese ball-like clot with a denser network was observed at the end of gastric digestion when the protein concentration varied from 2.0% to 5.0% (w/w). Such a difference in the structure apparently affected the rate of protein hydrolysis. A more rapid hydrolysis ($P < 0.05$) of the clotted protein was observed when protein concentration was lower than 2.0% (w/w) compared to the samples containing a higher proportion of protein (2.0%-5.0%, w/w).

To study the effect of different coagulation behaviours on the digestion of oil droplets in oil-in-water emulsions, these dairy ingredients (with the exception of SMP) were used to prepare an oil-in-water emulsion (20.0% soy oil and 4.0% protein, w/w). They were digested under the dynamic gastric conditions using the HGS. The gastric digesta was emptied at 20 min intervals. Then all digesta were mixed to investigate the lipid digestion under the small intestinal conditions. Changes in physicochemical properties of emulsions, involving the particle size, the microstructure, the oil content of the emptied gastric digesta and the amount of free fatty acids (FFAs) released during the small intestine stage, were determined using an *in vitro* small intestinal digestion model.

Aggregation of MPC 4851-stabilised emulsion took place after 5 min of digestion in the HGS with the largest size. The aggregates remained in the stomach and did not disappear during the whole gastric digestion. The hydrolysis of the aggregated network by pepsin was largely slowed by the reduced ability of the simulated gastric fluid (SGF, containing pepsin) to diffuse into the larger sized aggregates. MPC 4851-stabilised emulsion thus resulted in the slowest release of oil droplets into the small intestine. In comparison, MPC 4861 and sodium caseinate-stabilised emulsions aggregated in the stomach at approximately 40 min, forming smaller sized aggregates. These aggregates disintegrated at the mid and late-stages of digestion in these two emulsions. Therefore, MPC 4861 and sodium caseinate-stabilised emulsions had a more rapid delivery of oil droplets into the small intestine. In relation to the WPI-stabilised emulsions both with and without heating, the aggregations formed at a similar time to that which was observed in MPC 4861 and sodium caseinate-stabilised-emulsions; i.e., at approximately 40 min. However, they had the smallest sized aggregates amongst all samples and they

disintegrated quickly with further digestion. WPI-stabilised emulsions both with and without heating had the fastest gastric emptying and hydrolysis by pepsin in the early and mid-stages of the gastric digestion process. Thus, the highest level of oil content contained in the emptied gastric digesta was produced from both WPI-stabilised emulsions. In the mixed gastric digesta, which were subjected to the small intestinal digestion, the oil contents contained in the different emulsion samples varied. This difference impacted the extent of lipid digestion by pancreatic lipase. The sample with a higher oil content released a greater amount of FFAs compared to the sample with a lower oil content. The extent of lipid digestion of different emulsion samples adhered to the following pattern: MPC 4851-stabilised emulsion < MPC 4861-stabilised emulsion < sodium caseinate-stabilised emulsion, WPI-stabilised emulsions both with and without heating.

Overall, the gastric behaviours of dairy ingredients either in solutions or emulsions were affected by the formation of structured clots/aggregates. The differences in clotting/aggregation times and their structures were greatly dependent on the component and structure of protein, the processing prior to digestion and the susceptibility to proteases. These differences in protein coagulation/aggregation behaviour impacted the rates of protein hydrolysis and gastric emptying. The oil content and protein composition of the gastric digesta transferred into small intestine and the extent of lipid digestion in small intestine were also affected. These results are important in an application perspective. They provide useful information for the design and development of healthier food products by allowing greater control over the manipulation of protein bioavailability, which subsequently provides greater control over lipid metabolism.

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List of Abbreviations

α-La:	α-lactalbumin
β-Lg:	β-lactoglobulin
BSA:	Bovine serum albumin
CCP:	Colloidal calcium phosphate
HGS:	Human gastric simulator
MPC:	Milk protein concentrate
WPI:	Whey protein isolate
WPNI:	Whey protein nitrogen index
SDS-PAGE:	Sodium dodecyl sulfate-poly acrylamide electrophoresis
SGF:	Simulated gastric fluid
SIF:	Simulated intestinal fluid
$d_{4,3}$:	Average volume-weighted diameter
$d_{3,2}$:	Average surface-weighted diameter
PI:	Isoelectric point
w/w	Weight/weight
w/v	Weight/volume
v/v	Volume/volume

Chapter 1: Introduction

Milk protein is an important source of nutrients for humans through the different stages of life, and digestion behaviours of milk protein have been studied in both *vivo* and *vitro* models (Dangin et al., 2001; Mahé et al., 1995; Ye, Cui, Dalgleish, & Singh, 2016a, 2016b, 2017). Milk protein contains two fractions, caseins and whey proteins. In milk, caseins exist in colloidal particles known as casein micelles, which contain thousands of individual protein molecules (O'Mahony & Fox, 2013). Previous studies have shown that the digestion behaviour of casein in gastrointestinal tract is affected by the state of casein. Because casein micelles are coagulated both by milk-clotting enzyme pepsin (Tam & Whitaker, 1972) and acidic pH (Dalgleish & Corredig, 2012), while the individual casein (e.g. caseinate) is only coagulated by acidic pH not by pepsin.

Recently, a pervious study provided a novel insight into the role of food structure in digestion (Ye et al., 2016b). They proposed that the gastric digestion of milk protein is affected by the structure of the clot induced by the action of pepsin on κ-casein during dynamic *in vitro* gastric digestion. Different structures (e.g. dense or loose) of clots formed in unheated skim milk and skim milk that has been preheated at 90°C for 20 min, leading to a different rate in protein hydrolysis. The further study of digestion behaviour of (raw and heated) whole milk revealed that this difference in the rate of protein hydrolysis induced by a various structured clot under gastric conditions markedly impacted the rate in release of fat globules (Ye et al., 2016a).

Lipids, not only exist in milk but also in most other types of foods, and play a significant role in the human diet, including providing texture, flavour, and mouth-feel. (Singh, Ye, & Horne, 2009). Lipids also perform many important functions in the human body, such as providing a concentrated source of energy (Golding & Wooster, 2010). Meanwhile, overconsumption of lipids has been associated with a variety of diseases and health conditions, e.g. artherosclerosis, hypertension, and in the development of obesity (Shahidi, 2006). Recently, the increasing consumer awareness about the relationships between human health and high-calorie diet has promoted the food industry to design and develop healthier foods with a focus on reducing the adsorption of calorie, and without diminishment of the desirable sensory qualities of food (Chung, Olson, Degner, &

McClements, 2013; Le Révérend, Norton, Cox, & Spyropoulos, 2010; McClements, Decker, Park, & Weiss, 2009; Singh et al., 2009). In many processed foods, lipids exist in the form of emulsified oil droplets, such as mayonnaise, salad dressing, ice cream and soups (McClements, 2005). Milk proteins, as the most common emulsifiers, are applied to prepare food emulsion, due to their excellent emulsification properties.

Digestion behaviour of oil-in-water emulsions in the gastrointestinal tract has received growing interests (Golding & Wooster, 2010; Li, Ye, Lee, & Singh, 2012; Mun, Decker, Park, Weiss, & McClements, 2006; Sarkar, Goh, Singh, & Singh, 2009; Sarkar, Horne, & Singh, 2010b; Sarkar, Ye, & Singh, 2016; Singh et al., 2009; Ye et al., 2016a). Moreover, many attempts have been made to modulate lipid digestion by rational design of the structure of emulsions, such as manipulating interfacial composition (Golding & Wooster, 2010; Maldonado-Valderrama et al., 2008; Singh & Ye, 2013). However, most studies have used a static digestion model to mimic the gastric environment, only very few studies used a dynamic gastric model. Theoretically, most protein-stabilised emulsions will undergo a substantial modification in a more real gastric environment due to the dynamic change in pH value (Singh & Ye, 2009). It can thus be inferred that these alterations in structure of foods during gastric digestion will, to some extent, have an influence on the lipid digestion in the small intestine.

In the present research, an *in vitro* dynamic digestion model was employed to achieve the process of food digestion in the stomach stage. It is preferable to conduct investigations with *in vitro* assays because compared to *in vivo* study, they are faster, simpler and pose no ethical problems. Dairy protein ingredients have been applied in a wide variety of food products. They encompass different proteins (caseins or/and whey proteins), and have been processed through different methods during manufacturing process. Thus, the digestion behaviours of different dairy protein ingredients in the gastrointestinal tract might have some differences. The aim of this study was thus to investigate the behaviour of various of commercial milk protein ingredients in the stomach and lipids digestion in emulsions stabilised by these ingredients in the GI tract, with a focus on the effect of different structures formed in foods during dynamic gastric digestion on protein or/and lipid digestion.

The main objectives of the present study were as follow:

1) To understand the gastric digestion behaviours of different milk protein ingredients with a range of different structures in a dynamic stomach model (human gastric simulator-HGS). The commercial dairy ingredients employed in the present work include skim milk powder (SMP), milk protein concentrate (MPC) 4851, MPC 4861, sodium caseinate, whey protein isolate (WPI) and heated (90°C, 20 min) WPI.

2) To explore the influence of protein concentration on the dynamic gastric digestion behaviours (e.g., protein clotting time and protein hydrolysis rate) of milk protein ingredients in the HGS. SMP and MPC 4851 were selected as model protein ingredients.

3) To investigate the dynamic gastric digestion behaviours of proteins and lipids in emulsions made with different milk protein ingredients in the HGS, and how they affect the subsequent lipid digestion in the small intestine. The oil-in-water emulsions were stabilised by MPC 4851, MPC 4861, sodium caseinate and WPI. WPI-stabilised emulsion was treated by heating at 90°C for 20 min prior to digestion.

These objectives have been completed through three research chapters in this study. The gastric digestion behaviours of different milk protein ingredients have been reported in Chapter 4. The influence of protein concentration on dynamic gastric digestion behaviour of milk protein ingredients was studied in Chapter 5. The digestion behaviour of oil droplets in emulsions stabilised by different milk protein ingredients during gastrointestinal tract was investigated in Chapter 6.

Chapter 2: Literature Review

This chapter covers the knowledge of milk proteins, milk protein ingredients, oil-in-water emulsion and its stability, milk protein emulsifier, and human gastrointestinal tract etc. Moreover, it also reviews *in vitro* and *in vivo* gastric digestion of milk proteins and *in vitro* gastrointestinal digestion of oil-in-water emulsions based on current literature, with a focus on summarizing the behaviour of the milk proteins with different characteristics during digestion process.

2.1 The human gastrointestinal tract

The nutrients that exist in food are involved in different food structures. Digestion is the process of the disintegration of food matrices in the mouth, stomach and intestine, leading to the release of nutrients, which are finally adsorbed into plasma via the intestinal walls (Wickham, Faulks, & Mills, 2009).

2.1.1 Stomach

The human stomach is a "J" shaped muscular bag, which is composed of four principal parts; the cardia, fundus, body, and pylorus. Figure 2.1 shows the anatomy picture of human stomach. After ingestion of a meal, the food enters the stomach through the oesophagus and mixes with the digestive juice, then the chyme is propelled to the small intestine by the pyloric sphincter. The solid or semisolid food is ground and broken down to 1-2 mm sized pieces under gastric peristalsis (Thomas, 2006; van Aken, 2010).

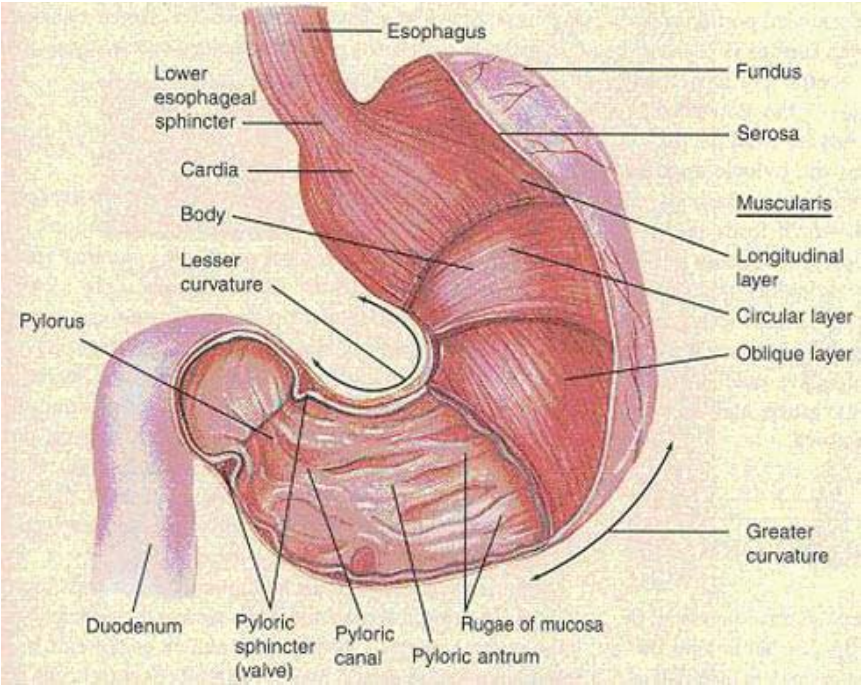


Figure 2.1. The external and internal anatomy of the stomach of human (Tortora & Derrickson, 2008).

2.1.1.1 The pH and ionic strength of gastric fluid

Typically, the pH of human gastric fluid during the fasting state varies from about pH 1 to 3 due to the presence of hydrochloric acid. Such a strongly acidic environment prevents the growth of microorganisms (N'Goma, Amara, Dridi, Jannin, & Carrière, 2012). However, the pH value may alter with the nature and the amount of ingested food, and there may exist significant difference between individuals (McClements, Decker, & Park, 2008). Generally, after taking a meal, the pH value of the gastric content increases to about 5.5-7. After around 60 min of gastric digestion (i.e. the gastric half-emptying time), the pH reduces to about 4-5, and then it drops down to the initial acidic pH with the further gastric emptying, when all digesta leaves the stomach (N'Goma et al., 2012). This acidic condition causes alterations in food structures (e.g. the aggregation of protein or the coalescence of the lipid droplets) and activates the digestive enzymes (Gallier et al., 2013; McClements, Decker, & Park, 2008).

In the fasted state, the typical ionic strength of the gastric fluid is around 100 mM, in which Cl^- is the dominant ion, with a concentration about 100±30 mM (Lindahl, Ungell,

Knutson, & Lennernäs, 1997). However, the ionic strength may further change with the ingestion of food, due to the additional ions from food (Kalantzi et al., 2006).

2.1.1.2 Enzyme

The gastric secretion also contains different enzymes (i.e. pepsin and gastric lipase). Protein may be partially hydrolysed by pepsin in the stomach, and about 10-30% of lipids may be digested to free fatty acids (FFAs) and diacylglycerol by the gastric lipase in the stomach. However, some proteins are not susceptive to catalytic action of pepsin, e.g. β-lactoglobulin (β-Lg) (Mandalari, Mackie, Rigby, Wickham, & Mills, 2009).

For better understanding of human digestion, porcine pepsin has been widely utilised in *in vitro* digestion models (Hollebeeck, Borlon, Schneider, Larondelle, & Rogez, 2013; Li, Ye, Lee, & Singh, 2013; Tan et al., 2017; Ye et al., 2016b). The porcine pepsin is secreted as a catalytically inactive pepsinogen from the hog stomach mucosa. The pepsinogen, with a molecular weight of 40,400, can be converted to pepsin when pH <5.0. This conversion process is catalysed by pepsin (Brown & Ernstrom, 1988). Pepsin is able to induce milk coagulation and hydrolyse the protein under acidic conditions. The optimum pH of activity/stability of porcine pepsin is about pH 2.0. The milk-clotting activity of porcine pepsin was found to be inhibited in cheese making when pH is above 6.3. Normally, pepsin may lose its milk-clotting ability at pH 6.8 (Brown & Ernstrom, 1988).

2.1.2 Small intestine

The small intestine is the major region where the nutrients are digested and converted to an absorbable form. About 70-90% of lipid digestion occurs in the small intestine (Singh & Gallier, 2014). The human intestinal tract is a complex environment consisting of bile salts, pancreatic enzymes, co-enzymes, various salts, phospholipids, yeasts and various bacteria (Singh & Ye, 2013).

2.1.2.1 pH and ionic strength

When partially digested food products pass into small intestine, the pH undergoes a rapid increase from the highly acidic environment (pH 1-3) in the stomach to the neutral environment (pH 6-7.5) in the duodenum, due to the secretion of sodium bicarbonate. This neutral pH provides an optimal environment for the action of pancreatic enzymes

(Golding & Wooster, 2010; Hur, Decker, & McClements, 2009; McClements, Decker, & Park, 2008). The increase in pH may cause some critical changes in physicochemical properties of protein-stabilised emulsions, e.g. a reversal of the protein charge. Most protein-stabilised emulsions exist in anionic form, and will undergo aggregation when the pH is near the isoelectric point region (Singh & Ye, 2013).

Besides, it has been found that the osmolality of the duodenum contents is ~ 180 mOsm/kg during the fasted state, and the ionic strength is ~140 mM (Kalantzi et al., 2006; Lindahl et al., 1997). However, because of the presence of various ions and solutes in ingested food, postprandial osmolality and ionic strength may undergo an appreciable increase. For example, Kalantzi et al. (2006) has reported that, after ingestion of a nutrition beverage, the osmolality of the duodenum had a rapid increase to ~290 mOsm/kg. In addition, the ionic strength in small intestine is known as a particularly significant factor that affects the electrostatic interactions of this system. The multivalent cations (e.g. Ca^{2+}, Mg^{2+}) may reduce the digestibility of long chain saturated fatty acids and bile salts by the formation of precipitations (Karupaiah & Sundram, 2007; Reid, 2004; Vaskonen, 2003).

2.1.2.2 Bile salts

Bile salts play a significant role in both the digestion and adsorption of lipids due to its high surface activity (Sarkar et al., 2016). Bile salts are present in the small intestine and, originates from the liver through the gall bladder (Singh & Ye, 2013). Bile salt is a native biosurfactant (Golding & Wooster, 2010). Unlike other surfactants, it does not contain a hydrophobic head and hydrophilic tail group. Instead, its amphiphilic nature is mainly because of its flat steroidal structure, with methyl groups on the convex side and polar hydroxyl groups on the concave side (Euston, Baird, Campbell, & Kuhns, 2013; Galantini et al., 2015). Bile salts can adsorb readily at the oil-water interface in an emulsion and displace the initial surfactants at the oil droplet surface when is introduced to a simulated intestinal fluid (Hur et al., 2009). Therefore, bile salts facilitate the digestion of lipids by providing the accessibility of the lipase/co-lipase complexes to the bile-coated lipid droplets (Sarkar et al., 2016). Besides this, bile salts can facilitate the deformation of oil droplets under mechanical agitation, which enhances the stability against aggregation and transports the hydrophobic substance by forming micelles

(McClements, Decker, & Park, 2008). It has been reported that bile salts can improve the digestibility of both adsorbed and unadsorbed proteins in an emulsion, e.g. β-Lg-, myoglobin- and bovine serum albumin (BSA)-stabilised emulsions (Gass, Vora, Hofmann, Gray, & Khosla, 2007).

2.1.2.3 Pancreatic lipase

For healthy human adults, the digestion of lipids by the gastric lipases is very limited in the stomach. It mainly (~70-90%) takes place in the small intestine, which is catalysed by pancreatic lipases (Bauer, Jakob, & Mosenthin, 2005; Mun et al., 2006; Singh & Ye, 2009). Pancreatic lipases can work efficiently at about pH 6.5 (Singh & Ye, 2013), although their optimum pH is 8-9 (Patton & Carey, 1981). When the partially digested lipid droplets pass into the small intestine, the pancreatic lipase tends to adsorb at the droplet surface as a complex with co-lipase and/or bile salts (Bauer et al., 2005). Triglycerides and diacylglycerol are then broken down to free fatty acids (FFAs) and 2-monoglycerides. It is reported that pancreatic lipase has no obvious specificity for the chain length of fatty acids, but it is preferential to cleave the positions of sn-1 and sn-3 in the fatty acids (Mu & Høy, 2004). This complexation reaction of pancreatic lipase at lipid droplet interface requires the presence of bile salts, co-lipase and calcium (Hur, Lim, Decker, & McClements, 2011). Its degree of binding appears to depend on the electrical charge of the interface and the competitive adsorption with bile salts, digestion products or other surfactants (McClements, Decker, & Park, 2008). The co-lipase is a kind of coenzyme, which is essential to the action of lipase. It interacts with lipase to form a stoichemetric complex that adsorbs at the oil-water interface, and thereby facilitates the accessibility to the lipid substrate. Co-lipase consists of a hydrophilic group that combines with lipase, and a hydrophobic part that connects with the interfacial layers (Bauer et al., 2005).

Interestingly, the influence of the presence of bile salts on the activity of pancreatic lipase is complex. When bile salts present in a relatively low concentration, they tend to solubilise the products of lipid digestion, such as free fatty acids (FFAs) and 2-monoglycerides, and remove them from the interfacial layers. In that situation, they accelerate the activity of pancreatic lipids. In contrast, a relatively high concentration of bile salts will restrain the digestive ability of pancreatic lipase, which is mainly due to the

competitive adsorption between bile salts and lipases (Gargouri, Julien, Bois, Verger, & Sarda, 1983).

2.2 Milk protein

Milk, serving as an important protein source in the human diet, contains about 3.3% (w/w) protein. Milk protein is divided into two distinct groups: caseins, and whey proteins (serum proteins). Whey proteins are the remainder in the solution after the caseins are precipitated by acid or rennet (Oftedal, 2013). The main components of milk proteins are shown in Figure 2.2.

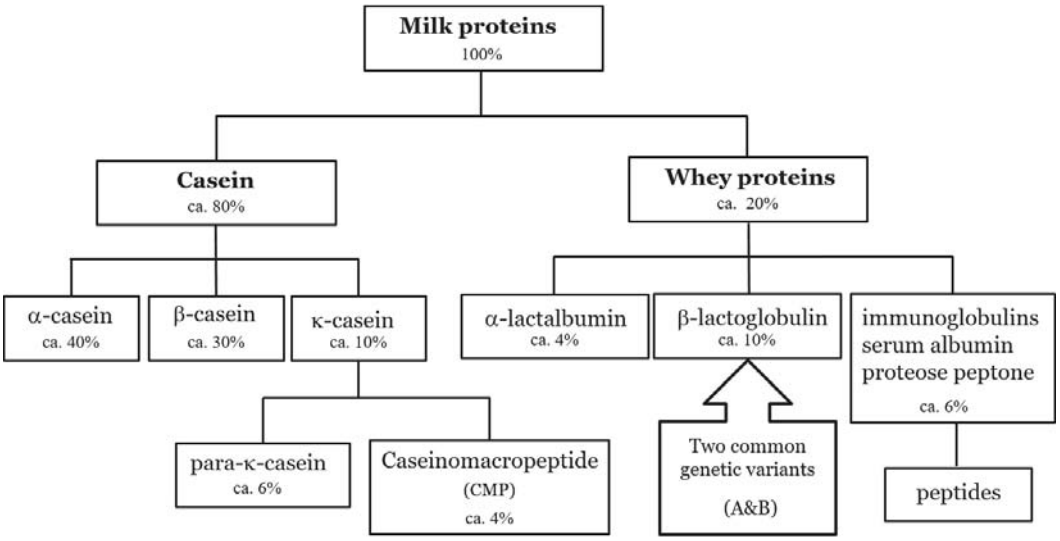


Figure 2.2. Main components of milk proteins showing partitioning into casein and whey fractions (Cheison & Kulozik, 2017).

2.2.1 Casein

Caseins are abundant in milk and constitute about 80% in total milk protein. It can be precipitated from raw milk by adjusting pH to 4.6 at 20°C (Swaisgood, 1992). The caseins of bovine milk can be fractionated into four main classes: α_{s1}-, α_{s2}-, β-, and κ-caseins (Varnam & Sutherland, 2001). Caseins are phosphoproteins and are generally considered to be very hydrophobic proteins, with the exception of β-caseins (O'Mahony & Fox, 2013; Swaisgood, 1992). α_s-Caseins are very sensitive to calcium due to the existence of phosphate groups, and may precipitate at pH 7.0 when calcium ions are

present (Swaisgood, 1992). β-Caseins have high surface hydrophobicity, but they are not exceptionally hydrophobic due to lacking stable secondary and tertiary structures (O'Mahony & Fox, 2013). β-Caseins have been reported to have a strong tendency to bind metal ions, i.e., calcium ions in the milk, due to their high content of phosphate groups (O'Mahony & Fox, 2013). κ-Casein on the other hand, is calcium insensitive (Huppertz, 2013), which only contains one phosphoserine group (Varnam & Sutherland, 2001).

Caseins are susceptible to proteolysis due to their open and flexible structures, which is crucial for their nutritional function and serves as an important source of amino acids (O'Mahony & Fox, 2013). Caseins lack stable tertiary structures; consequently, they are highly thermally stable (Fox, 1981a). Besides, caseins have a strong tendency to associate in aqueous solutions due mainly to their high hydrophobicity (Ye, Srinivasan, & Singh, 2000).

2.2.1.1 Casein micelle structure

In milk, about 95% of caseins exist in colloidal particles known as casein micelles, rather than as individual molecules (O'Mahony & Fox, 2013). The structure and properties of casein micelles have attracted much scientific interest (Holt, 1992; Walstra, 1990). The knowledge of the structure of casein micelles is essential to understand the digestion behaviours of milk in the gastrointestinal tract and nutrient delivery.

The casein micelles are highly hydrated, possessing about 2.0-4.0 g H_2O/g protein. The dry matter of casein micelles is composed of protein (94%) and colloidal calcium phosphate (CCP, 6%) (O'Mahony & Fox, 2013). The casein micelles are generally spherical in shape, with an average diameter of 120 nm. Some of the main properties of casein micelles are summarised in Table 2.1.

Table 2.1. Average characteristics of casein micelles (Fox, 2003).

Characteristic	Value
Diameter	130-160 nm
Surface	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g/cm^3
Mass	$2.2\times10^{-15}g$
Water content	63%
Hydration	3.7 g H_2O/g protein
Voluminosity	4.4 cm^3/g
Molecular weight (hydrated)	1.3×10^9 Da
Molecular weight (dehydrated)	5×10^4 Da
Number of peptide chains (MW: 30,000 Da)	10^4
Number of particles per mL milk	10^{14}-10^{16}
Whole surface of particle	5×10^4 cm^2/mL milk
Mean free distance	240 nm

Many models of the casein micelle structure proposed in the past 50 years were inconsistent and often contradictory, including three main categories: core-coat, internal structure, and submicelles (Dalgleish, 2011; McMahon & Brown, 1984; McMahon & Oommen, 2008; Rollema, 1992; Rose, 1969; Slattery & Evard, 1973). Numerous earlier models proposed that the casein micelle is made up of many smaller sub-micelles, with a size of about 10-15 nm, and a molecular weight about 10^6 Da (Figure 2.3). Morr (1967) first proposed this kind of model in 1967. The CCP is believed to provide the link between sub-micelles, affording an open, porous structure. The CCP may be removed by using some reagents, e.g. EDTA, oxalate, citrate, urea (> 5M) and, ethanol (35% at 70°C), or by increasing the pH to above pH 9. As a result, the casein micelles structure is disrupted (O'Mahony & Fox, 2013).

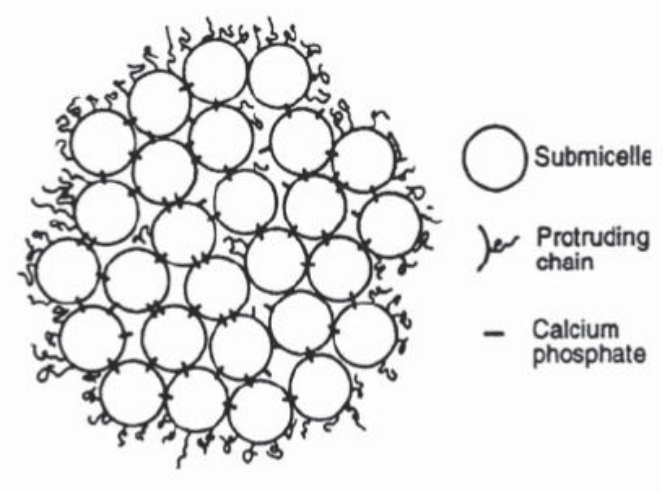


Figure 2.3. The casein micelle schematic diagram (Walstra & Jenness, 1984).

However, the theory of the existence of sub-micelles has been controversial. Hill and Wake (1969) suggested that the amphiphilic structure of κ-caseins plays a major role in stabilising the micellar structure. The information known about the casein micelle structure has continued to be updated and elaborated by scientists with the development of analytical approaches. Walstra and Jenness (1984) and Walstra (1999) assumed that there is a hairy layer with a thickness of 5-10 nm, coated on the surface of micelles, which is composed of a hydrophilic C-terminal area of κ-caseins. The primary responsibility of this hairy layer is to stabilise the casein micelle through steric stabilisation. However, the stabilisation of casein micelles may be disrupted when the hairy layer is collapsed by adding ethanol, or being removed through specific proteases, e.g. rennet or pepsin (Holt & Horne, 1996). Holt (1992) envisaged the casein micelle structure as a crosslinked gel network, which is built by relatively flexible casein molecules. The CCP micro-granules are responsible to keep the stability of the gel-like structure, and the surface of the micelles is covered with a protruding hairy layer, which is comprised of the C-terminal end of the κ-casein.

2.2.1.2 The stability of casein micelles

The micelle is considered to be a very stable system. They are stable to high temperature, compaction, commercial homogenisation and in the presence of high levels of calcium ion concentration (O'Mahony & Fox, 2013). However, casein micelle

properties can be changed under high-pressure processing, low temperature (0-5°C), and freezing. Moreover, in the concentration process of dairy products, casein micelles can be destroyed to some extent by evaporation, ultrafiltration and spray-drying (Fox & Brodkorb, 2008; Havea, 2006).

There is no covalent bonding between CCP and proteins. The acidic pH, specific proteolytic enzyme, and ethanol can result in different degrees of destabilisation of casein micelles. Lowering the pH to casein's isoelectric point (pI=4.6), the polymerisation and precipitation will take place. The CCP can be fully removed when the pH is equal to or lower than 4.9. For acid-induced destabilisation, most of the CCP may be removed without destruction of the micellar structure, because casein micelles are probably rebuilt by increasing the pH to 6.7 under certain conditions. Besides this, some milk-clotting enzymes, e.g. chymosin and pepsin, can specifically catalyse the hydrolysis of κ-casein, which is split into para-κ-casein and macropeptide. As a result, the micellar caseins will coagulate and form a curd-like gel in the presence of calcium or other divalent ions (Lucey, 2011). These properties are significant in the digestion of milk, as both acidic conditions and proteases pepsin exists in the stomach.

2.2.1.3 Enzymatic coagulation of caseins

Milk-clotting is believed to be a complex process, involving a primary enzymatic hydrolysis (first stage) that causes the alteration of κ-casein and a loss of its ability to stabilise the rest of the caseinate complex. Second, the aggregation of the altered caseinate takes place, which is a non-enzymatic step. Then the aggregates of caseins further form a firm cross-linked milk gel, and probably curd syneresis occurs (McMahon & Brown, 1984).

In the first stage (i.e. enzymatic hydrolysis) of the coagulation of milk, the proteases cleave the κ-casein molecules that are on the surface of casein micelles into para-κ-casein and a macropeptide, and hence initiate the milk clotting process:

$$\kappa\text{-casein} \rightarrow \text{para-}\kappa\text{-casein} + \text{macropeptide}$$

The hydrolysis rate is proportional to the enzyme concentration and is dependent on the pH (with an optimal pH at ~5.6) (Carlson, Hill, & Olson, 1987). Chymosin is capable of hydrolysing κ-casein (Holt & Horne, 1996), and uniquely cleaves the Phe105-

Met106 peptide bond, while other milk-clotting enzymes (e.g. pepsin) are less specific. However, all milk-clotting enzymes have the same general functions (Fox, 1981b).

During the secondary step, the aggregation of altered micelles is due mainly to the loss of electrostatic repulsion caused by reducing the pH to its pI (pH~4.6), and the loss of steric repulsion of κ-caseins. The presence of calcium ions facilitates the formation of coagulum by connecting micelles as a bridge and inducing an isoelectric condition (Douglas, 2017).

2.2.2 Whey protein

Whey proteins occupy 20% of the total protein in bovine milk, consisting principally of β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and immunoglobulins (Kinsella & Whitehead, 1989). Whey proteins have a relatively more ordered globular structure in comparison to caseins, which is stabilised by intramolecular disulphide bonds between cysteine residues (Dickinson, 2001). Whey protein is a group of acid-soluble proteins, which can be utilised over a wider pH range than caseins. While caseins are insoluble at their pI (Kinsella & Whitehead, 1989). β-Lg is applied as an emulsifier, thickener or foam stabiliser for nutritional purposes due to its excellent functional properties. While α-La serves as an important source of nitrogen in infant food because of its low allergy potential. (Dickinson, 2001; Foegeding, Davis, Doucet, & McGuffey, 2002; Khalloufi, Alexander, Goff, & Corredig, 2008; Ye, 2008).

2.2.2.1 β-lactoglobulin

β-Lactoglobulin (β-Lg) accounts for approximately 50% of whey protein, and 10% of total protein in milk. It is a major component of whey protein in bovine milk and tends to dominate the characteristics of whey protein products. β-Lg consists of 162 amino acids and its molecular weight is about 18.3 kDa (Hambling, McAlpine, & Sawyer, 1992). It exists as a dimer (MW=36.6 kDa) of two monomeric molecules linked by a non-covalent bond at neutral pH at room temperature (McKenzie & Sawyer, 1967). It contains one free sulfhydryl group at Cys121, and two disulphide (-S-S-) bonds at Cys66-Cys160 and Cys106-Cys119 (Walstra, Wouters, & Geurts, 2005). The secondary structure of β-Lg contains about 6-10% α-helix, 44-52% β-sheet, 8-10% reverse turn and 32-35% of random coil (Casal, Köhler, & Mantsch, 1988; Dong et al., 1996). Changes in the quaternary structure of β-Lg may occur when environmental conditions such as

temperature, pH and ionic strength are altered (McKenzie & Sawyer, 1967). pH can significantly affect the molecular conformation of β-Lg. Although at neutral pH, β-Lg exists as a dimer; when pH is higher than 6.8, or between 2 and 3, it dissociates into monomers (Sawyer, 2003). β-Lg has been reported to be resistant to digestion in the stomach by the action of pepsin due to its stability under acidic pH conditions (Peram, Loveday, Ye, & Singh, 2013).

2.2.2.2 α-lactalbumin

α-Lactalbumin (α-La) is the second major component in bovine whey protein. It is a compactly folded protein molecule of approximately spherical shape (Walstra et al., 2005). Its isoelectric point is about pH 5.1, and it exists as a monomer at its isoelectric region (Nakai & Modler, 1996). α-La is resistant to enzymatic proteolysis, due to its compact globular structure. Numerous attempts have been made to increase its susceptibility to proteolysis, using methods including esterification, binding of zins ions to α-La, or lowering the pH to 2.0 (El-Zahar et al., 2005; Permyakov et al., 1991; Sitohy, Chobert, & Haertle, 2001). α-La has excellent heat stability. Heating has no significant influence on the digestibility of α-La, as the protein will refold during cooling when the heating temperature is lower than 100°C (Schmidt & Poll, 1991; Schmidt & van Markwijk, 1993).

2.2.2.3 Heating induced denaturation of whey protein

Heat treatment of milk proteins is a common industrial processing procedure to ensure food safety. The three-dimensional structure of whey protein can be changed dramatically by extremes of temperature, whereas the highly stable caseins are not markedly affected by thermal treatment (Almaas et al., 2006; Barbé et al., 2013). When being exposed to heating above a certain temperature, whey proteins unfold, denature and aggregate, and form an open conformation (Brodkorb, Croguennec, Bouhallab, & Kehoe, 2016). The thermal denaturation temperature of whey proteins is summarized in Table 2.2.

Table 2.2. Thermal denaturation temperature of whey protein (De Wit, 1984).

Protein	T_D (°C)	T_{tr}(°C)
β-lactoglobulin (β-Lg)	78	93
α-lactalbumin (α-La)	62	68
Bovine serum albumin (BSA)	64	70
Immunoglobulin (Ig)	72	89

T_D represents the initial denaturation temperature.

T_{tr} is the temperature at the DSC peak maximum.

One of the earliest researches on denaturation and aggregation of whey protein was reported by Briggs and Hull (1945), who indicated that the denaturation process is made up of two steps: (1) protein unfolding, and (2) protein aggregation. This two-step process has been accepted for several years. In the first stage, the whey protein denatures through an unfolding step. The hydrophobic interactions between protein molecules increase due to the exposure of hydrophobic groups from the interior of the native molecule onto the molecular surface (Relkin, 1998). Some of the free thiol groups and disulphide bonds in the β-Lg also become exposed during the initial unfolding step; hence, they are available to further form intramolecular disulphide bonds through oxidation of the free sulfhydryl groups or rearrangement of disulphide bonds (Chaudry & Humbert, 1968). The denaturation of whey proteins in milk has been studied. Denatured β-Lg aggregates with κ-casein at the casein micelle surface via disulphide bonds in milk. This process varies greatly with the pH of the serum (Brodkorb et al., 2016). The association extent of β-Lg with casein micelle is predominantly dependent on the heating conditions. Corredig and Dalgleish (1996) reported that heating milk at 75°C to 90°C for 80 min allows all β-Lg to form complexes with casein micelles via κ-casein binding; when heating at 130°C for 100 s, all β-Lg denatures but only half of them associates with κ-caseins (Oldfield, Singh, & Taylor, 2005). Under these heating conditions, all the β-Lg is denatured but it does not all form aggregates with the micellar κ-casein. Sava, Van der Plancken, Claeys, and Hendrickx (2005) indicated that the aggregation reaction of β-Lg is initiated when heating up to 78°C, but β-Lg only unfolds when heating is below 78°C.

In the second stage, the main irreversible aggregation of β -Lg occurs via covalent linkages between thiol groups of cysteine residues. After denaturation, β-Lg aggregates initially into oligomers and subsequently into soluble aggregates, finally generating a

large insoluble colloidal particle under further heating (Baussay, Le Bon, Nicolai, Durand, & Busnel, 2004; Pouzot, Nicolai, Visschers, & Weijers, 2005). The formation of aggregations can be affected by the heating temperature (Sawyer, 1968). Moreover, the free sulfhydryl group also plays an key role in the initial period of aggregation when ionic strength is low and pH is higher than 7.0 (Hoffmann & van Mil, 1997).

By contrast, pure α-La does not aggregate under a mild thermal treatment (e.g. 80°C, pH 6.7) (Calvo, Leaver, & Banks, 1993; Hines & Foegeding, 1993), which is primarily due to the absence of free thiol groups (Singh & Havea, 2003), although its initial denaturation temperature is lower than that of β-Lg.

2.2.3 Milk protein products

A wide range of milk protein products are produced worldwide and utilised as functional ingredients in many foods as shown in Figure 2.4. SMP, MPC and sodium caseinate are dairy ingredients where casein is the major protein component (Hemar, Tamehana, Munro, & Singh, 2001). The micellar structure of casein is retained in SMP and MPC, but it is destroyed during the manufacture of sodium caseinate (Kinsella & Morr, 1984; Mulvihill, 1992). Whey protein isolate (WPI) contains ≥90% protein, which is derived from the cheese whey by ion-exchange chromatographic methods (Mulvihill, 1992).

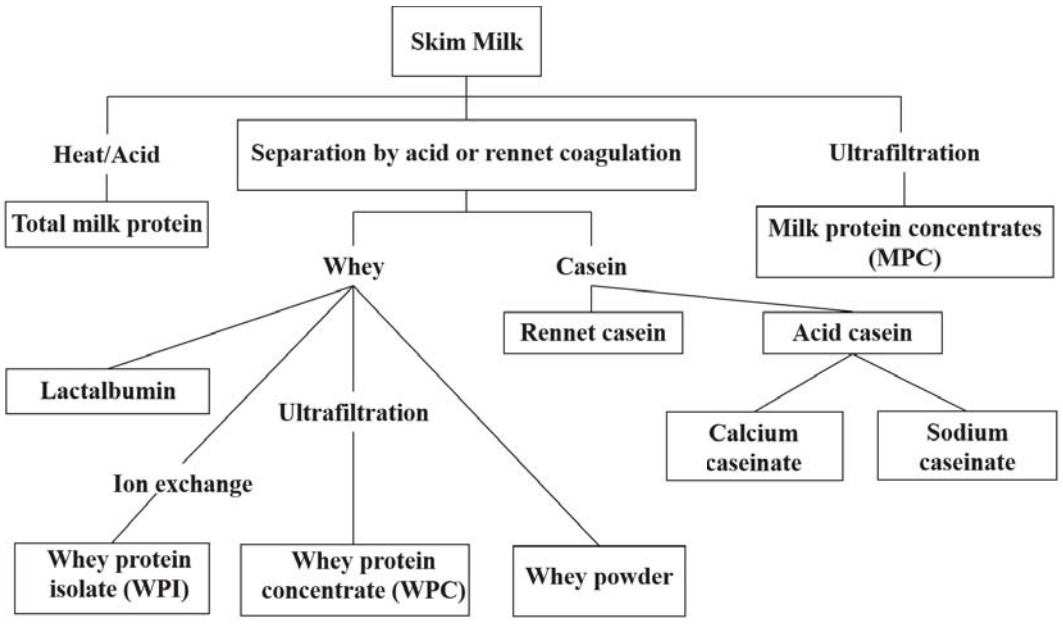


Figure 2.4. Functional protein ingredients manufactured from skim milk (derived from Singh (2005))

2.2.3.1 Skim milk powder

Milk powders are defined as "milk products that can be obtained by the partly removal of water from milk" by the Codex Standard 207-1999 (Codex Alimentarius Commission, 1999). SMP and whole milk powder (WMP) are two primary types of commercial milk powder. SMP is the most widely applied as a functional dairy ingredient (Singh & Creamer, 1991). The manufacture of SMP involves heat treatment (normally known as preheating), evaporation, and spray drying. SMPs are normally classified as low-, medium- and high-heat powder according to the whey protein nitrogen index (WPNI). SMP with different preheat treatment conditions designed to meet the specific needs of the food industry has gained wide acceptability in different types of food applications (in Table 2.3). The heating conditions play an essential role in determining the functional properties of milk powder. The most important effect is the induction of denaturation of whey protein, achieving a partially denatured whey protein, which could simply self-aggregate and/or associate with casein micelles via micellar κ-casein (Singh, 2007).

Table 2.3. Food applications of skim milk powder of different heat classes (Kelly & Fox, 2016).

Heat classification	Heat treatments typically applied	WPNI	Functional properties	Food applications
Low heat	70°C for 15 s	>6.0 mg/ml	Solubility, lack of cooked flavour	Recombined milk, milk standardisation, cheese making
Medium heat	85°C for 1 min 90°C for 30 s 105°C for 30 s	1.5-6.0 mg/ml	Foaming, emulsification, flavour, colour, viscosity	Chocolate, ice cream, confectionery
High heat	90°C for 5 min 120°C for 1 min 135°C for 30 s	<1.5 mg/ml	Gelation, heat stability, water adsorption	Recombined evaporated milk
High-high heat		<1.5 mg/ml	Colour, flavour, water binding	Recombined evaporated milk, bakery

WPNI, whey protein nitrogen index.

In milk powder, the micellar structures of caseins largely retain their integrity during processing, conferring a number of unique functional properties, such as heat stability, curd formation and emulsification. Whey proteins may already be somewhat denatured by the process of preheating, depending on the heat treatment conditions (Singh, 2007). The interaction of denatured whey proteins with κ-casein at the surface of the casein micelles may modify the properties of casein micelles. This interaction may impair the clot formation and increase the rennet coagulation times, thereby milk powder is not suitable for the preparation of recombined cheese milk (Singh, 2007). The denatured whey protein-casein micelle complexes have a high water-binding capacity (Schkoda, 1999), which increases the gel strength and viscosity of yoghurt and other fermented milk products (Singh, 2007). Milk powder is inferior to caseinate in the emulsifying property due to the micellar structure of caseins in milk powder (Mulvihill & Murphy, 1991). However, Euston and Hirst (1999) suggested that the stability of milk powder-stabilised emulsions against creaming is superior than caseinate-stabilised emulsion systems, which is probably because of the formation of a weak gel network or the lower susceptibility to depletion flocculation in milk powder-stabilised system. Singh and Newstead (1992) reported that milk powder exhibits better emulsifying properties than sodium caseinate when pH is close to 5.2, which may be due to the dissociation of casein micelles around this pH value.

2.2.3.2 Milk protein concentrate

Milk protein concentrate (MPC) was the earliest casein-based product obtained by membrane technique on the market (Carr & Golding, 2016). MPCs are considered an essential source of calcium, and are widely applied as an ingredient for milk extension in cheese manufacture, nutritional beverages, frozen desserts and yoghurt production (Huffman & Harper, 1999; Ye, 2011). MPC has protein content in the range of approximately 50-85%. It is processed directly through the partial removal of lactose and soluble salts from skim milk using a combined ultrafiltration/diafiltration process (Mulvihill, 1992; O'Donnell & Butler, 1996) prior to evaporation and spray drying. In MPCs, casein is in a micellar form as that found in milk, casein and whey protein ratio remains as in milk (about 80:20) (Carr & Golding, 2016), and is similar to that in SMP (Singh, 2007). However, a fraction of CCP in casein micelle may be dissolved during the ultrafiltration and diafiltration process, which leads to the loose structure of casein micelles, resulting in a smaller fragmented micellar structure. Whey proteins in MPC remains largely in their native state, as the manufacturing process does not involve a preheat treatment (Singh, 2007).

Recently, in order to improve the functional properties (e.g. solubility) the casein micelles in some new MPC products have been dissociated to a certain extent by removing the calcium content (Ye, 2011). This type of product refers to "MPC 4861" in the present study. MPC with a micellar structure can be produced from a native micelle structure and altered to a structure closer to sodium caseinate, depending on the level of calcium depletion (Carr & Golding, 2016). However, a significant difference between MPCs and caseinate is that MPCs contain phosphate, whereas caseinates contain a reduced level of phosphate because of the acidic precipitation and subsequent washing. Micellar phosphate levels can increase buffer capacity because of the formation of dihydrogenphosphate (Ferreira, Oliveira, & Rocha, 2003) on acidic solubilisation of CCP (Carr & Golding, 2016).

2.2.3.3 Sodium caseinate

Traditionally, caseins are separated using precipitation techniques such as rennet or acid precipitation (Mulvihill, 1992), which destroy the native micellar structure, and the products produced in this manner contain individual casein molecules. Sodium

caseinate is one of the most commonly used ingredients in foods. It is the water-soluble form of casein, which is prepared by solubilising acid casein with NaOH (Mulvihill & Ennis, 2003). The casein curd separated from milk by acidification to pH 4.6 is further separated through a process of dewheying, and is then washed to remove whey protein, minerals, lactose and residual acid. Next, the casein curd from a de-watering device is minced and mixed with NaOH, followed by a process of spray drying to form caseinate (Carr & Golding, 2016; Mulvihill & Ennis, 2003). The excellent heat stability is one of the most important functional benefits of the caseinates, which limits modifications to product properties in consequence of thermal processing (Carr & Golding, 2016).

2.2.3.4 Whey protein isolate

Whey is the liquid remaining after removing caseins from milk. Whey protein isolate (WPI) is a concentrated form of whey protein component, which is highly soluble, with a high level of protein concentration that is above 90% (Bansal & Bhandari, 2016). WPI is processed from ultrafiltration (UF) and sometimes diafiltration (DF), or microfiltration, or can be obtained from whey by ion-exchange (IX) chromatography (Fox & McSweeney, 1998). Whey protein in WPI is in its native state; thus it retains, to a great extent, its functional properties. WPI is widely applied in the food industries, because of its high protein content, excellent water-binding capacity, emulsification, gelling and foaming properties (Singh, 2005).

2.2.4 The digestion behaviours of milk protein during gastric digestion

The digestion behaviour of milk proteins has recently been the subject of many investigations in *in vitro* and *in vivo* (Dangin et al., 2001; Mahé et al., 1995; Ye et al., 2016a, b, 2017). The different milk protein has a different hydrolysis rate induced by pepsin and can cause different gastric emptying rates. It has been reported that caseins caused a delayed delivery of amino acid to the small intestine in an *in vivo* digestion (Mahé et al., 1996). Thus, casein is considered as a “slow” digested protein. This “slow” digestion of casein can be explained by that casein micelles coagulate under gastric conditions, and remain for several hours in the stomach. Casein micelles have been reported as able to be coagulated by the milk-clotting enzyme pepsin (Tam & Whitaker, 1972) and acidic pH (Dalgleish & Corredig, 2012). Both them exist in the stomach. However, caseinate is only coagulated by acid and not by pepsin, because of its existence

in the form of individual casein molecules. This digestion behaviour of casein has been observed in an *in vivo* study (Miranda & Pelissier, 1981). The gastric emptying rate and hydrolysis rate of a mixture of individual caseins in a rat stomach was faster than that of casein in the skim milk samples (Miranda & Pelissier, 1981). On the contrary, whey protein is considered as a "fast" digested protein. It remains soluble in the presence of pepsin at acidic conditions and can pass rapidly from stomach into small intestine without being hydrolysed by digestive enzymes. Thus, this enables a fast delivery of proteins to the small intestine, where it is further digested and absorbed (Boirie et al., 1997).

Previous studies have shown that the digestion behaviour of protein in the gastrointestinal tract can be affected by its structure (Kitabatake & Kinekawa, 1998; Schmidt & van Markwijk, 1993; Tunick et al., 2016; Zeece, Huppertz, & Kelly, 2008). The structure of protein in food is dependent on the processing treatment and its source. Different proteins have different conformational properties; therefore, the nature of the protein may markedly affect its susceptibility to proteolysis by pepsin. Casein has a highly flexible, disordered conformation (Modler, 1985). It is more exposed to gastric hydrolysis by pepsin in the stomach (Mahé et al., 1996). In contrast, β-Lg is highly resistance to pepsin action due to its compact, folded tertiary structure. However, the molecular flexibility and the susceptibility to proteases can be modified by physical processing (e.g. emulsification, exposure under high-pressure, or heating) (Peram et al., 2013; Zeece et al., 2008). For example, heat treatment can improve the availability of enzymatic cleavage sites of β-Lg, and lead to an increase in the susceptibility to proteolysis by pepsin (Barbé et al., 2013; Li et al., 2013; Peram et al., 2013; Schmidt & van Markwijk, 1993). Miranda and Pelissier (1987) reported that heat treatment can accelerate gastric emptying of milk and increase the hydrolysis rate of caseins in an *in vivo* digestion study.

Recently, studies in our lab showed the gastric digestion behaviour of unheated and heated skim milk (90°C for 20 min) using a dynamic digestive model-Human Gastric Simulator (HGS) (Ye et al., 2016b). The results provide a novel insight into the influence of the structured clot on the protein hydrolysis rate. Ye et al. (2016b) found that unheated skim milk formed a firm, dense, cheese-like clot with a porous network structure. Such a structure prevented the accessibility of the pepsin to the interior of the clot. In contrast, the heated skim milk, in which whey protein had been denatured, formed a looser, fragmented network-structured clot with numerous larger voids. With further digestion,

the curd became more tightened and less permeable to serum and solute with increasing digestion time, in particular, in unheated skim milk. The hydrolysis rate of caseins and whey proteins by pepsin varied from the formation of different structured clots under the gastric conditions. The hydrolysis of caseins in heated milk became much faster than that in unheated milk, which is mainly because of the formation of the clot with an open, fragmented structure. Such a structure increased the effective contact area with simulated gastric fluid (SGF, containing pepsin). Pepsin was thus prone to diffuse and act on the clot. Additionally, the denatured whey protein in heated milk was hydrolysed rapidly while β-Lg and α-La were maintained intact during the whole digestion period in unheated milk. Besides, in the heated milk, casein and serum proteins can be observed in the coagula, but the emptied digesta did not contain intact casein and whey proteins. This further confirmed that the digestibility of casein and β-Lg can be considerably improved by thermal treatment (Barbé et al., 2013).

The gastric digestion of whole milk has also recently been investigated in our lab with respect to the effect of pre-treatment on the behaviour of protein and fat globules during dynamic digestion (Ye et al., 2016a, 2017). It was found that the rates of protein hydrolysis and the release of milk fat globules from the curd into the small intestine can be modified by different pretreatment (e.g. homogenisation and heat treatment) prior to the digestion of milk. This is because homogenisation and heating treatments affect the formation of structured clot in the stomach. The curds produced in the homogenised milk and heated homogenised milk had a more crumbled and fragmented structure than that produced in the raw whole milk. The fat globules were embedded in the curds as they generated. After the formation of the curd, a further quantity of voids was found in the structured clots formed from the homogenised milk and heated milk, which gave rise to a greater rate of protein hydrolysis by pepsin. This led to a more rapid release of incorporated fat globules from the curds into the digesta. The formation of clots with different structures cause the changes in the rate of protein hydrolysis and release of milk fat into the digesta in the stomach (Ye et al., 2017).

2.3 Emulsion

An emulsion refers to an intimate dispersion of at least one immiscible liquid in another in the form of discrete droplets (McClements, 2005). In most foods, the droplet

size of an emulsion is usually ranged between 0.1 and 100 µm in diameter (Dickinson, 1992; Dickinson & Stainsby, 1982; Friberg & Larsson, 1997). An emulsion consists of a discontinuous (dispersed or internal) phase and a continuous (external) phase. The former is made up of droplets, while the latter refers to the surrounding liquid. According to different spatial distribution of water and oil phases, emulsions can be divided classically into two types. An emulsion composed of oil droplets dispersed in an aqueous phase is referred to as an oil-in-water emulsion (e.g. milk, mayonnaise, beverages, sauce and cream). An emulsion made up of water droplets dispersed in an oil phase is referred to as a water-in-oil emulsion (e.g. butter and margarine) (McClements, 2005).

2.3.1 Emulsion formation

Normally, when pure water and pure oil are poured into a container, a layer of oil (lower density) will be rapidly separated onto the top on the layer of water (higher density), due to their tendency to arrangement in the most thermodynamically stable state. Make an emulsion requires an intense shear and the presence of emulsifiers. The intense shear may increase the interfacial area between oil and water phases, leading the dispersed oil phase into tiny oil droplets. The shear may be provided through mechanical agitation, such as high pressure valve homogenisers, high speed blenders and colloid mills (Singh & Ye, 2013). Homogenisation is known as the process of transformation of two separate liquid immiscible phases (usually oil and water) into an emulsion, or of reducing the droplet size of a premixed emulsion. An emulsion can be kinetically stable for a period of time when the stabilisers are present. An emulsifier or a texture modifier (e.g. thickening agents) can be a stabiliser to improve the stability of the emulsion. An emulsifier is a surfactant that can adsorb onto newly formed emulsified droplets and form a protective membrane against aggregation and coalescence of droplets (McClements, 2005). Moreover, it contributes to lowering the interfacial tension and Laplace pressure and, hence facilitates to break the droplets into smaller ones (Walstra, 2003).

Emulsion formations may include a single step or several consecutive steps, depending on the methods employed to make it, the nature of the ingredients and the desired droplet size. Homogenisation is conveniently classified into two categories; primary, and secondary homogenisation. The primary homogenisation is the process of converting two separated liquids into a coarse emulsion that involves some fairly large

droplets by using a homogeniser (e.g. high-speed blender). The secondary homogenisation is employed to reduce the droplet size of an already existing emulsion by using another type of homogeniser; for example, a two-stage homogeniser (Walstra, 2003).

2.3.2 Emulsion stability

The term emulsion stability is known as the ability to an emulsion to resist modifications in its properties over time (Dickinson, 2003; McClements, 1999). For food emulsion systems, because they are thermodynamically labile systems, the emulsion breakdown will eventually take place with the passage of enough time (McClements, 2005). Even though some emulsion systems appear fairly stable, so that the product has a long shelf life. In fact, the total number of droplets, the size distribution and the spatial arrangement are always changing.

The destabilisation of an emulsion normally involves two aspects: physical instability, and chemical instability. The most important physical instability mechanisms include creaming, flocculation, coalescence, phase inversion, and Ostwald ripening. It may change the spatial distribution of oil and water phases, or lead to the structural re-organisation of molecules (Dickinson, 1992; Dickinson & Stainsby, 1982; Walstra, 1996, 2003). Chemical instability normally involves oxidation, or hydrolysis, and results in the generation of new molecules in the system (Fennema, 1996). The extent of destabilisation of an emulsion during its lifetime is dependent on its composition, microstructure and external environment (e.g. storage conditions and temperature) (McClements & Decker, 2000).

The stabilisation and destabilisation of protein-stabilised emulsions is driven by a number of factors, such as the strength and type of interactions occurring between droplets (e.g. van der Waals attractive forces, electrostatic interaction, steric repulsion, hydration forces), and these factors are dependent on the composition, structure, and concentration of adsorbed layer (Leman, Kinsella, & Kilara, 1989).

2.3.2.1 Gravitational separation

Generally, in an emulsion, the droplets in a discontinuous phase do not have the same density as the liquid surrounding them. Therefore, a net gravitational force has an essential influence on the stability of the system (Dickinson, 1992; Dickinson & Stainsby,

1982; Hunter, 1989; Walstra, 1996). Gravitational separation involves creaming and sedimentation. Creaming refers to the upward movement of emulsified droplets without an alteration in droplet size, because the density of droplets is lower than the density of liquid that surrounds them (McClements, 2005; Walstra, 1987). On the contrary, when the surrounding liquid has a lower density, the droplets have a tendency to move down, which is known as sedimentation. Creaming is more common than sedimentation in a food emulsion system. The density of water is higher than that of most liquid oils. In the case of oil-in-water emulsion, oil droplets tend to cream and suspend on the top in the system when the water goes to the bottom (McClements, 2005).

The creaming rate of an isolated rigid spherical droplet in an ideal liquid is descried in Stockes' law equation (Equation 2.1), which is determined by the balance of frictional force and gravitational force. This equation is applied in food emulsion to estimate the stability against creaming:

$$v_{\text{Stokes}} = -\frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1}$$

Where v_{Stokes} is the velocity of creaming, g is the acceleration due to gravity, r is the radius of the emulsion droplet, ρ_1 is the density of the continuous phase, ρ_2 is the density of discontinuous phase and η_1 is the shear viscosity of continuous phase (McClements, 2005).

The creaming rate can be affected by a number of factors, such as droplet flocculation, droplet fluidity, electrical charge of droplets, fat crystallisation and Brownian motion (McClements, 2005).

The gravitational separation is an instability problem, which accelerates the process of flocculation or coalescence, and eventual oiling off. A number of methods can be used to control gravitational separation, such as reducing the particle size, minimising the density difference between the suspended droplets and the liquid phase, increasing the droplet concentration and modifying the rheology of the continuous phase using a thickening agent (McClements, 2005).

2.3.2.2 Flocculation

Flocculation is the process in which destabilised suspended droplets associated with each, but maintain their individual integrity (McClements, 2005; Tadros & Vincent, 1983). In diluted emulsion, flocculation accelerates the rate of gravitational separation, and therefore, its shelf life is drastically reduced (Luyten, Jonkman, Kloek, & Van Vliet, 1993; Tan, 2004). It can also lead to a great increase of the viscosity of food emulsion, and may even accelerate the development of a gel network (Demetriades, Coupland, & McClements, 1997). Although flocculation modifies the psychical properties of an emulsion, the droplet size may maintain unaltered and the flocs may be dispersed because the interaction force is weak (Walstra, 1987).

The following mathematical model may describe the droplets flocculation rate in a colloidal system that includes monodisperse globose particles (Evans & Wennerström, 1999):

$$\frac{dn_T}{dt} = -\frac{1}{2}FE$$

Where dn_T/dt is the droplet flocculation rate, t is time, n_T is the total amount of droplets per unit volume, F represents collision frequency and E represents collision efficiency. The factor ½ refers to the collision between two droplets leading to one droplet decreasing in the total amount of droplets.

According to the above equation, the flocculation rate is dependent on the collision frequency between particles and the collision extent that causes aggregation. The collision frequency refers to the total amount of particles in contact with their neighbours per unit time per unit volume of emulsion. Molecular movement can induce the collision between particles; hence, collision is caused by Brownian motion, gravitational separation, or mechanical agitation (McClements, 2005). If each encounter between particles may result in flocculation, then the emulsion will rapidly become unstable. Thus, a high enough repulsive energy barrier is necessary against the droplets coming too close together. The likelihood of flocculation induced by droplet collision is known as collision efficiency. The collision efficiency is highly dependent on the height of the energy barrier.

In protein-stabilised emulsions, the net charge of protein is zero at its pI, is positive at low pH values, and is negative at high pH values. Such a change in droplet charge has a substantial influence on the emulsion stability against flocculation. When the pH is near the pI of protein, the electrostatic repulsive force is no longer strong enough to prevent flocculation because the net charge on the droplets is not high enough (Demetriades et al., 1997; Kulmyrzaev, Chanamai, & McClements, 2000).

However, flocculation may be retarded by controlling the collision frequency. In addition, the droplet-droplet interactions (i.e., electrostatic interactions, steric interactions, hydrophobic interactions, depletion interactions, hydrodynamic interactions, biopolymer bridging interactions, and covalent interactions) play a crucial role on the flocculation rate of the emulsion system (McClements, 2005).

2.3.2.3 Coalescence

Coalescence is another type of droplet aggregation where two or more emulsified droplets irreversibly merged into a single larger droplet. Coalescence may result in the increase of droplet size over time, thus accelerating the creaming and sedimentation process. In the oil-in-water emulsion, it gradually leads to the separation of oil phase and the aqueous phase. Besides this, the contact area between the dispersed phase and the continuous phase decreases in the coalescence process; the emulsion is moving towards the most thermodynamically stable state. Coalescence requires that the droplets are close enough and the thickness of separation membrane between droplets is thin enough to be ruptured (McClements, 2005; Walstra, 1993). Protein-stabilised emulsions normally have desirable stability against coalescence over long-term storage (Singh & Ye, 2013).

2.3.3 Protein emulsifier

Proteins are employed widely as an emulsifying agent in food industries. Milk proteins are widely valued as functional ingredients to facilitate the formation and stabilisation of food emulsions. The ability of milk protein products to adsorb at the oil-water interface and to stabilise emulsions is affected by the structures, aggregation state and flexibility of the constituent proteins (Singh & Ye, 2009). During emulsification, surface-active proteins, either in molecular form or in protein aggregates, can readily spread to cover on the surface of droplets. Subsequently, they form a relatively thin and electrically charged interfacial membrane (Claesson, Blomberg, & Poptoshev, 2004;

Singh, 2005). The adsorbed protein lowers the surface tension of the oil-water interface and, consequently, facilitates the further disruption of oil droplets during the emulsification process (Dalgleish, 2006). The formation of the protective membrane can effectively prevent the coalescence of emulsion droplets (Singh & Ye, 2013). Normally, milk protein-stabilised emulsions have a good stability against coalescence over long-term storage in the presence of sufficient protein. However, they are susceptible to different types of flocculation. Bridging flocculation may take place in the emulsion containing insufficient protein to fully cover the oil-water interface. In emulsion prepared with aggregated milk protein ingredients (e.g. MPC or calcium caseinate), bridging flocculation is the most probable process to occur. However, depletion flocculation may occur in the emulsion containing excess unadsorbed protein (Singh & Ye, 2009). Protein-stabilised emulsion is also fairly susceptive to pH and ionic strength, and has a high tendency to flocculate when pH is close to the pI of adsorbed protein (McClements, 2005). In addition, the presence of certain ions, in particular of calcium, may considerably affect the stability of protein-stabilised emulsions, which can be explained by the ion-binding capacity of protein (Singh & Ye, 2013).

Proteins are amphipathic molecules. During emulsification, the hydrophilic sides of protein are in contact with the water phase and the hydrophobic sides are in contact with the oil phase (Dickinson, 1992). Once adsorbed on the interface of an emulsion droplet, the adsorbed protein undergoes unfolding and rearrangement to adapt to the new environment (Dalgleish, 1996; Dickinson, 1992). The degree of unfolding is dependent on the molecule flexibility of adsorbed protein (Singh & Ye, 2009). The molecular flexibility refers to the ability of the protein to undergo an alteration in conformation in a changed environment, i.e. the strength of the forces maintaining the secondary and tertiary structure (Dalgleish, 1996; Damodaran, 1997). For example, caseins undergo rapidly an conformational alteration upon adsorption to interface due to their more disordered, open structure, and may form an extended layer with thickness up to 10 nm (Dalgleish, 1990). In contrast, whey protein (e.g. β-Lg) only can change its conformation and unfold to some degree, forming a relatively thin adsorbed layer with a thickness about 2 nm, because of its relatively rigid structure (Dalgleish, 1995, 1996; Dalgleish & Leaver, 1993; Dickinson & McClements, 1995; Mackie, Mingins, Dann, & North, 1993). After it is adsorbed at the interface, whey protein lies somewhere in the intermediate between its native and

denatured states, and may have both the native secondary structure and the denatured tertiary structure (Dickinson, 1998). The unfolding of protein molecules would cause the exposure of the sulfhydryl group, and the adsorbed protein slowly polymerises at the aged interface through sulfydryl-disulfide interaction (Dickinson & Matsumura, 1991; McClements, Monahan, & Kinsella, 1993). The knowledge of interfacial behaviour of proteins is essential to understand the digestion behaviour of protein-stabilised emulsion.

2.3.3.1 MPC

In MPC, proteins (mainly micellar caseins) exist as cross-linked aggregates caused by calcium-induced interactions. Such an aggregated protein can also coat the surface of emulsified droplets. Its emulsifying stability is much lower than that of sodium caseinate and WPI. Therefore, a higher protein concentration is required to form a stable emulsion using MPC. Meanwhile, the average droplet size of MPC-stabilised emulsion is much greater (Euston & Hirst, 1999). In MPC-stabilised emulsions, two or more adjacent droplets normally tend to share an adsorbed protein aggregate if the emulsion made at a low protein/oil ratio, this refers to bridging flocculation. Bridging flocculation cause a considerable increase in particle size (Singh & Ye, 2013). These aggregated proteins are laid together at the oil-water interface via calcium bonds and/or colloidal calcium phosphate. These aggregated proteins exist in a highly stable conformation since these bonds are stable during emulsification. Therefore, the spreading of protein is very limited, and thus leads to a decreased emulsifying ability (Euston & Hirst, 1999; Magesh Srinivasan, Singh, & Munro, 1999). However, no depletion flocculation has been observed in MPC-based emulsion, probably because the size of micelles is too large to induce the depletion flocculation (Singh, 2005). The creaming stability of the emulsion made with a high concentration of MPC is higher than that made with whey proteins, because of the formation of a thick, dense protective layer on the droplet surface with an increased droplet density (Singh & Ye, 2009). The formation of this membrane also contributes to the extension in the storage life of emulsions by increasing the stability against coalescence, via electrostatic and steric interactions (Singh & Ye, 2013). It has also been reported that no obvious evidence of competitive adsorption between proteins is observed in aggregate-like caseins-stabilised emulsions (e.g. MPC) (Euston & Hirst, 1999; Magesh Srinivasan et al., 1999).

Calcium-depleted MPC is a newly developed commercial product, which exhibits an improved emulsifying properties (Ye, 2011). In such MPCs, caseins exist in smaller particles, because the integrity of casein micelle has been destroyed by the depletion of colloidal calcium phosphate during processing. The emulsions stabilised by this type of MPC exhibit remarkably enhanced stability with a smaller droplet size. In the case of depleted 80% calcium MPC-stabilised emulsion, there is a similar surface protein concentration to that in the sodium caseinate-stabilised emulsion. It is worth noting that in calcium-depleted MPC, depletion flocculation may be induced by small casein aggregates (Ye, 2011).

2.3.3.2 Sodium caseinate

Sodium caseinate has excellent emulsifying ability. It may stable an emulsion at a relatively low protein/oil ratio (1:60) (Singh, 2005). Caseinates-based emulsions show a superior stability against heating than that of whey proteins-based emulsions (McClements, 2005). It has been reported that emulsions made with 2.0% (w/w) sodium caseinate and 20.0% (w/w) oil have excellent stability when being subjected to a thermal treatment at 90°C for 30 min or 121°C for 15 min (Hunt & Dalgleish, 1995; Magesh Srinivasan, Singh, & Munro, 2002). The fact that there are no heating induced conformational alterations in caseinate molecules may be the consequence for highly flexibility of caseins (McClements, 2005).

The surface protein composition and structure of adsorbed protein membrane are crucial factors that impact the stability of sodium caseinate-stabilised emulsions (Dickinson, 1999). The surface protein composition is influenced by protein concentration and the association state of protein in the aqueous phase (Singh & Ye, 2009). The competitive adsorption of caseins in a sodium caseinate-stabilised emulsion has been reported as having a strong correlation with the protein/oil ratio (Euston, Singh, Munro, & Dalgleish, 1995; Magesh Srinivasan et al., 1999). During homogenisation, β-caseins are firstly adsorbed at the oil-water interface of an emulsion prepared with a low protein/oil ratio (1:60). Whereas α_{s1}-caseins are preferentially adsorbed when protein content is more than the minimum amount of that required for saturation coverage of the interface (Magesh Srinivasan et al., 1999). The behaviour of preferential adsorption of β-casein is mainly attributed to its high surface activity. This evidence is only observed at

low protein content, because caseins may exist as monomers at low concentration (Lucey, Srinivasan, Singh, & Munro, 2000). However, caseins have a strong tendency to associate to form complexes with the increasing protein content (Lucey et al., 2000). For example, β-caseins self-associate or associate with other caseins to form micelles, and thus further loss of their competitive ability (Singh & Ye, 2009). As a result, the surface activity of casein complexes and aggregates are more decisive factors in the surface composition of high levels of sodium caseinate-stabilised emulsions.

Depletion flocculation has been reported to occur in the sodium caseinate-stabilised emulsions, rather than in the emulsion stabilised by MPC or whey proteins (Singh, 2005). The stability of sodium caseinate-based emulsion against flocculation has a strong correlation in protein concentration. In emulsion containing 2.0% (w/w) sodium caseinate, caseins can form a thick layer to protect droplets from flocculation, coalescence and creaming over a period of several weeks via the steric stabilisation mechanism. However, when protein concentration is above 3.0% (w/w), depletion flocculation may be caused by casein aggregates that are formed from unadsorbed sodium caseinates in the aqueous phase. This gives rise to an increase in droplet size, and further induces creaming with a further increase in protein from 3.0 to 5.0% (w/w). When caseinate concentration is above 6.0% (w/w), a high level of depletion flocculation may be observed, and with the further formation of a strong particle network (Dickinson & Golding, 1997; Singh & Ye, 2009). However, depletion flocculation in sodium caseinate-stabilised emulsion may be controlled under certain conditions, such as in the presence of certain levels of sodium chloride, or a small amount of calcium chloride, or lowering the pH. The emulsion thus has an enhanced resistance to creaming (Singh, 2005; Srinivasan, Singh, & Munro, 2000; Ye & Singh, 2001).

2.3.3.3 Whey protein isolate

WPI is amongst the most employed emulsifiers in food industries. It is capable of stabilising an emulsion at a relatively low protein concentration (Singh, 2005). Whey protein-stabilised emulsion possesses high heat stability at neutral pH, in a low ionic strength and/or a low protein content. However, it may loss its stability, and forms a gel in the presence of 10 mM of KCl (Hunt & Dalgleish, 1995). In the emulsion prepared with whey protein, both adsorbed and unadsorbed proteins may form aggregation under

heating condition. The level of aggregation increases with the increasing concentration of whey protein. However, the extent of droplet aggregation decreases when the unadsorbed protein in the aqueous phase being removed. Interestingly, the formation of aggregations during heating is mainly through interactions between emulsified droplets and unadsorbed whey proteins, rather than between two protein-adsorbed particles. This phenomenon is because of the different surface hydrophobicities between unadsorbed denatured protein and protein-adsorbed droplets (Euston, Finnigan, & Hirst, 2000).

The influence of heating temperature on stability of WPI-based emulsions has been widely studied (Kyros Demetriades & McClements, 1998; Monahan, McClements, & German, 1996; Sliwinski, Roubos, Zoet, Van Boekel, & Wouters, 2003). When heating at temperatures ranging from 75 to 80°C, not all adsorbed whey proteins are unfolded, and hydrophobic amino acid residues only partially contact with the oil phase. Emulsified droplets are thus more prone to aggregate, which results in an increase in viscosity and induces creaming instability. However, when heating temperature is above 80°C, the rate of aggregation decreases with a corresponding increase in creaming stability. This can be explained by the full unfolding of proteins at a relatively high temperature, with full exposure of hydrophobic residues in the oil phase, which increases the aggregation stability (Kyros Demetriades & McClements, 1998).

Heating treatment can cause modifications in the surface protein composition of whey protein-coated droplets. During heating process, there is an increase in the number of adsorbed β-Lg. Simultaneously, a considerable decrease is observed in the number of adsorbed α-La, probably due to α-La being replaced by β-Lg at the oil-water interface when heating up to 90°C (Ye, 2010; Ye & Singh, 2006).

2.3.4 The digestion behaviours of milk protein-stabilised emulsions

The formation, stability, microstructure, and applications of food emulsions have been widely studied (Dickinson, 1998, 1999; McClements, 1999; Singh & Ye, 2009). However, the knowledge about how the structural properties of emulsions change while passing through the human gastrointestinal tract is still not fully understood. In this section, we review the existing literature and provide an overview of possible changes of lipids in emulsion systems during gastrointestinal digestion.

2.3.4.1 Milk protein-stabilised emulsions in the gastric environment

When an emulsion enters the stomach, its structure and stability can be affected profoundly by the gastric environment. As mentioned earlier, the stomach environment is highly acid (pH between 1 and 3), containing various ions, proteolytic enzyme (i.e., pepsin) and lipolytic enzyme, and providing some mechanical agitation (Ekmekcioglu, 2002; Kalantzi et al., 2006; Pal, Brasseur, & Abrahamsson, 2007). The mechanical agitation offers a shear force, providing a better mixing with other constituents in the stomach. Proteases pepsin is the most significant factor that affects the stability and digestibility of emulsions. Its action induces alterations in properties of emulsified droplets and adsorbed protein layers. The environmental pH is another important factor that affects the digestion behaviour of an emulsion. At a neutral pH, most protein-stabilised emulsions are negatively charged. This charge will undergo a substantial modification when pH falls to below 2.0, and the droplets may aggregate when pH is near the isoelectric points of the protein-stabilised emulsions (Singh & Ye, 2009).

The major digestion of emulsions stabilised by milk proteins start in the stomach by the action of pepsin (Nik, Wright, & Corredig, 2010a). Milk protein stabilised emulsions (e.g. WPI, sodium caseinate) have a strong tendency to lose their stability against flocculation and coalescence during gastric digestion. Interfacial hydrolysis of the proteins at the interface by pepsin was proposed by the authors as a major reason for the emulsion destabilisation (Li et al., 2012; Nik, Wright, & Corredig, 2010b; Sarkar et al., 2009). Proteins have different susceptibility to digestive enzymes and the liberation of peptides, and amino acids from proteolysis is affected by a number of factors. For instance, β-Lg in solution is resistant to gastric digestion and *in vitro* peptic hydrolysis, due to its highly folded structure (Reddy, Kella, & Kinsella, 1988a; Schmidt & Poll, 1991). In contrast, β-Lg appears more prone to hydrolysis by pepsin when it exists as an emulsifier lying somewhere at the oil-water interface (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Sarkar et al., 2009). This might be explained by that the conformation of adsorbed β-Lg at interface undergoes some alterations during the emulsification process (Macierzanka et al., 2009). However, α-La is less prone to hydrolysis by pepsin when it is adsorbed at the interface in an emulsion than in its native state in the solution (Nik et al., 2010b). Besides, the adsorbed BSA and caseins at the oil-in-water interface were

found to undergo a rapid hydrolysis by pepsin (Kenmogne-Domguia, Meynier, Viau, Llamas, & Genot, 2012; Li et al., 2012).

2.3.4.2 Milk protein-stabilised emulsions in the intestinal environment

The partially hydrolysed emulsified lipids then enter the small intestine, in which they are mixed with duodenal and bile fluids. These emulsions may experience a higher pH as the sodium bicarbonate neutralizers the pH from a highly acidic pH (1-3) in the stomach to neutral pH (6-7) in the small intestine. Pancreatic proteolytic and lipolytic enzymes can work most efficiently in such a neutral pH region, and thus induces a series of enzymes catalysed reactions (Bauer et al., 2005; Krondahl, Orzechowski, Ekström, & Lennernäs, 1997). The hydrolysis reactions induced by enzymes are complex. Both adsorbed and unadsorbed protein and peptides in an emulsion are hydrolysed by pancreatic proteinases (i.e., trypsin and chymotrypsin) in the small intestine. The pancreatic lipase tends to combine with co-lipase and /or bile salts via complexation reaction, thus adsorbing at the oil-in-water interface to hydrolyse triglycerides into a mixture of 2-monoglycerides and free fatty acids. Simultaneously, the remaining proteins and peptides at the emulsified lipids surface are displaced by bile salts. Whey protein was reported being more prone to be displaced by bile salts than caseinates at the interface in an emulsions during *in vitro* digestion (Mun, Decker, & McClements, 2007).

The stability of milk protein-stabilised emulsions will be markedly affected when the environmental pH and ionic strength are altered. As reported, in emulsions prepared by β-Lg, caseinate, and lecithin, the rate of lipid hydrolysis showed an increase with increasing concentration of $CaCl_2$ (0-20 *mM*) (Hu, Li, Decker, & McClements, 2010).

A number of studies have suggested that the stability of emulsions against flocculation and coalescence has a strong correlation with the emulsifier type (Macierzanka et al., 2009; Mun et al., 2007; Sarkar et al., 2009). For example, WPI-stabilised emulsion was found to be the least stable compared to the emulsion stabilised by sodium caseinate, lecithin and Tween 20. The WPI-stabilised emulsions appeared to be highly prone to droplet coalescence; while the caseinate-based emulsions are more prone to flocculation than to coalescence. However, the extent of lipid digestion was similar in both WPI- and caseinate-stabilised emulsions, even that the WPI-stabilised emulsion was more less stable to coalescence (Mun et al., 2007). The particle size of

emulsions was found to have little change in $d_{3,2,}$ but a considerable increase in $d_{4,3}$ under pancreatic hydrolysis conditions. The extent of alteration in droplet size is highly dependent on the type of emulsifier. Moreover, the microstructure may have a substantial change during the pancreatic lipolysis, and the extent of alteration is, as well, dependent on the type of emulsifier (Mun et al., 2007). However, Hur et al. (2009) suggested that the initial emulsifier used to stabilised an oil-in-water emulsion only to have a limited effect on the alterations in its microstructure during *in vitro* digestion. The difficulty in simulating the real gastrointestinal environment was explained by the authors as a possible reason for this inconsistence.

The interfacial composition of the oil droplets plays a key role in the ability of pancreatic enzymes to digest oil droplets at the interface. When emulsion pass through the gastrointestinal tract, the interfacial layer composition of the droplets can have an appreciate change. The rate and extent of lipid hydrolysis can be determined by the nature and structure of initial emulsifier to stable an emulsion. It was reported the rate of lipid digestion of emulsion stabilised by protein is higher than that stabilised by Tween 20 (McClements, Decker, Park, & Weiss, 2008; Mun et al., 2007).

Bile salts have a crucial effect on the interfacial dynamic during lipid hydrolysis. At the initial period of the digestion, bile salts can accelerate the action of pancreatic lipase by replacing other surface active materials from the oil-water interface. However, it is highly dependent on the interfacial layer. Bile salt is reported being able to displace milk protein from the interface (Sarkar et al., 2010b). It can permeate and disrupt the interfacial layer of an emulsion stabilised by β-Lg, and thereby replacing the adsorbed protein at the oil droplet surface (Sarkar, Horne, & Singh, 2010a). In addition, in the *in vitro* intestinal digestion system, although protein-stabilised emulsion initially to some extent underwent a droplet coalescence, with further digestion, however, all flocculated or aggregated particles were finally broken down to form a uniform dispersion of small lipid droplets. The adsorbed bile salts can prevent these small droplets from further aggregations via electrostatic repulsion (Golding & Wooster, 2010; Sarkar et al., 2010b).

The type of oil is also a factor in determination of the rate and extent of lipolysis during the lipid digestion. However, the effect of oil type on lipid digestion in gastrointestinal tract beyond the scope of the present study.

As reviewed above, the mechanisms of emulsified lipid digestion have been attempted to understand. However, most of these studies on the digestion behaviour of emulsions employed a static gastric digestion model. The information about behaviour of lipids in a milk protein-stabilised emulsion under dynamic gastric conditions, and how gastric digestion behaviour of the adsorbed protein layers influence subsequent lipid digestion in small intestine is still not fully understood.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Dairy ingredients

In this study, commercial dairy ingredients including skim milk powder (SMP), milk protein concentrate (MPC) 4851, MPC 4861, sodium caseinate (180) and whey protein isolate (WPI) 895 were used, which were purchased from Fonterra Co-operative Group Ltd. (Auckland, New Zealand). The compositions of these dairy ingredients, as stated by the manufacturer, are given in Table 3.1. MPC 4851 contains 2,160 mg calcium/100 g, and MPC 4861 contains 1,260 mg calcium/100 g. In this study, MPC 4861 is referred as a calcium-reduced MPC, which was manufactured using cation exchange to replace the divalent ions with monovalent ions and then ultrafiltration/diafiltration (Dybing, Bhaskar, Dunlop, Fayerman, & Whitton, 2002).

Table 3. 1. The composition of dairy ingredients

	Protein (g 100 g^{-1})	Moisture (g 100 g^{-1})	Fat (g 100 g^{-1})	Total carbohydrate (g 100 g^{-1})	Ash (g 100 g^{-1})
SMP	33.4	3.8	0.8	54.1	7.9
MPC 4851	82.9	5.6	1.4	3.3	6.8
MPC 4861	81.8	5.8	1.4	4.0	7.0
Sodium caseinate (SureProtein ™ 180)	Min 95.0	Max 5.5	Max 1.5	-	Max 4.0
WPI (Instantised) 895	93.0	4.8	1.0	0.3	1.7

SMP, skim milk powder; MPC, milk protein concentrate; WPI, whey protein isolate.

3.1.2 Soybean oil

Soybean oil was purchased from Davis Trading Company, Palmerston North, New Zealand.

3.1.3 Chemicals

Water was purified by treatment with a Milli-Q apparatus (Millipore Corp., Bedford, MA) and was used for all experiments. All chemicals used were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO) or BDH Chemicals (BDH Ltd., Poole, England) unless otherwise specified.

3.1.4 Enzymes

Pepsin from porcine gastric mucosa (EC 3.4.23.1; catalogue no.1.07185.0100) was purchased from Merck (Darmstadt, Germany); it had an activity of 0.7 FIP-U/mg, as stated by the manufacturer. Pancreatin (EC 232.468.9, P1750) from porcine pancreas (4×USP specifications) is a combination of enzymes including trypsin, amylase and lipase, ribonuclease, and protease, and was supplied by Sigma Aldrich (St. Louis, MO). Bile extract porcine (EC 232.369.0, B8631) contains glycine and taurine conjugates of hyodeoxycholic acid and other bile salts, which was purchased from Sigma Aldrich (St. Louis, MO) and was used without further purification.

3.1.5 Simulated gastric fluid (SGF)

SGF was prepared according to a method in a previous study (Minekus et al., 2014) with a slight modification. A solution of a fresh mixture of KCl (6.9 mmol/L), KH_2PO_4 (0.9 mmol/L), $NaHCO_3$ (25 mmol/L), NaCl (47.2 mmol/L), $MgCl_2$ $(H_2O)_6$ (0.1 mmol/L), and $(NH_4)_2CO_3$ (0.5 mmol/L) was prepared by dissolving these ingredients in deionized water with stirring for 30 min. The SGF (a final volume of 1L) was made up with water to 800 mL, i.e., a 1.25×concentrate. The addition of pepsin (4.8 g/L) and $CaCl_2$ (0.15 mmol/L) and water would result in the correct electrolyte concentration. Pepsin and $CaCl_2$ were added prior to use. The pH of the SGF was adjusted to 1.5 using 1 *M* HCl/NaOH.

3.1.6 Simulated intestinal fluid (SIF)

Simulated small intestinal fluid (SIF) was prepared according to USP Convention (1995) with some slight modifications. 1L of SIF consisted of 39 mM K_2HPO_4 and150 mM NaCl, and was made up with water to 800 mL, i.e., a 1.25 × concentrate. The addition of bile extract, $CaCl_2$, pancreatin and water will obtain a correct electrolyte concentration of the SIF. The pH of SIF was then adjusted to 7.5 with 1.0 *M* NaOH. The bile extract was added in a concentration of 5 mg/mL in SIF. The pancreatin (1.6 mg/mL in SIF) was prepared immediately before use to avoid denaturation and was added in a powder form. $CaCl_2$ of was added in 5 mM/L in SIF.

3.2 Methods

3.2.1 Preparation of protein solution (Chapter 4 and 5)

A 200 g protein solution was prepared by dissolving the milk protein ingredients at a given protein concentration [3.0% (w/w) protein in Chapter 4; 0.5-5.0% (w/w) protein in Chapter 5] in Milli-Q water. It was stirred and placed in a water bath at constant temperature (50°C) for 2 h to ensure complete dissolution.

3.2.2 Preparation of emulsions

3.2.2.1 Protein solution preparation (Chapter 6)

MPC 4851, MPC 4861, sodium caseinate and WPI were used as an emulsifier to make an oil-in-water emulsion. A 500 g aqueous solutions of emulsifiers (5.0% protein, w/w) was prepared by dissolving the milk protein ingredients in Milli-Q water at 50°C and stirring 2 h to achieve complete dissolution.

3.2.2.2 Emulsion preparation

The coarse emulsion containing 4.0% (w/w) protein and 20.0% (w/w) soybean oil was produced by using a high speed mixer (D500 series, Labserv Germany) to pre-homogenise for 3 min at 12,000 rpm at room temperature. The pre-emulsion was then homogenised with 2 passes through a two-stage value homogenizer (Homolab 2, FEF ITALIA SRL, Sala Baganza, Parma-Italy) at pressures: the first-stage/second stage pressures were 200/40 bar. The emulsion made with different dairy ingredients had an oil droplet size $d_{4,3}$ ranging from 0.78 ± 0.14 μm to 1.36 ± 0.16 μm. The stock emulsions contained 0.02% sodium azide and were stored at 4°C for further use.

3.2.3 *In vitro* gastric digestion

A schematic diagram of digestion process is shown in Figure 3.1

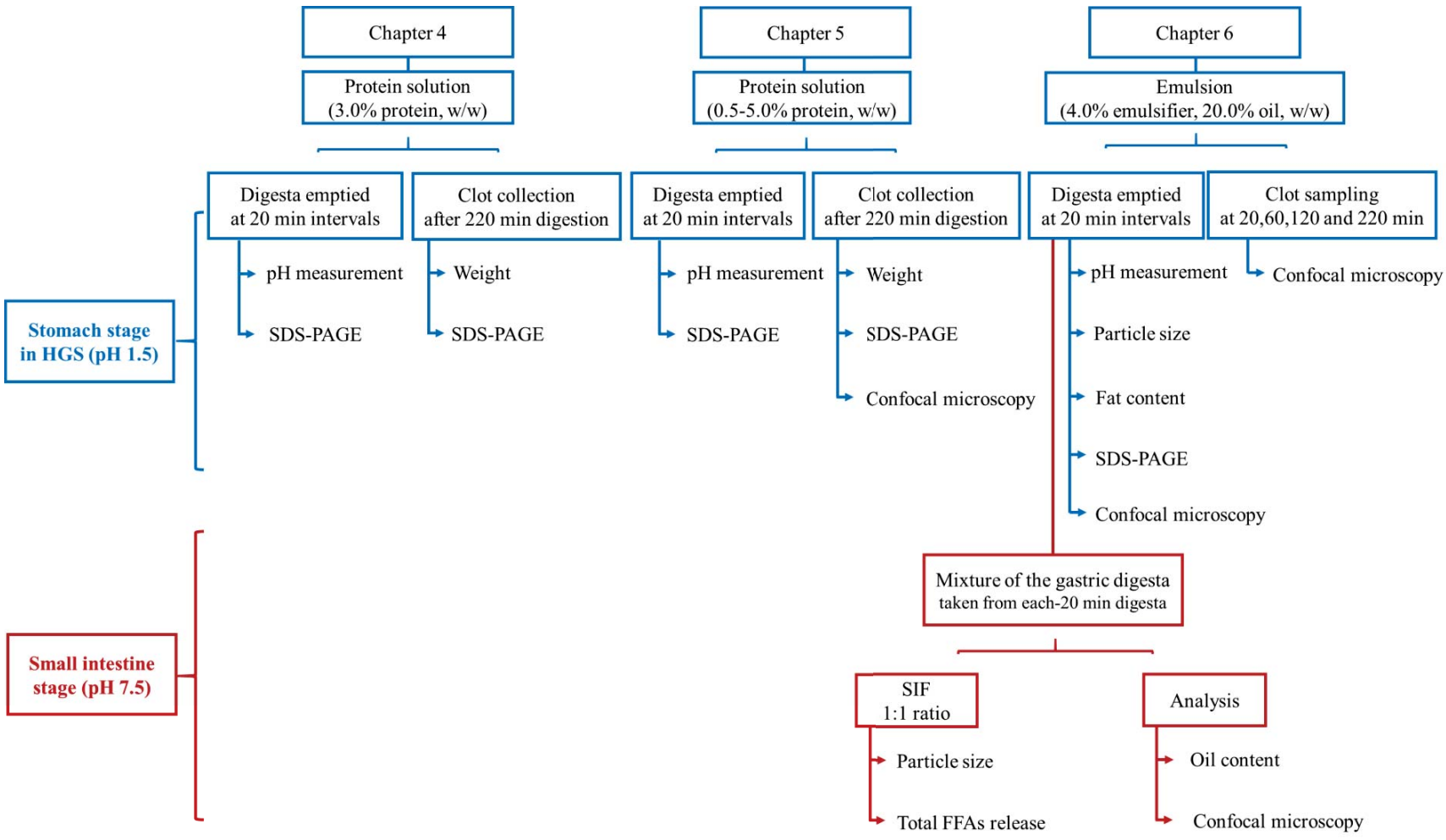


Figure 3. 1. The schematic diagram of digestion process.

3.2.3.1 Human gastric simulator (HGS)

A HGS, designed by Kong and Singh (2010), was employed for *in vitro* gastric digestion (Figure 3.2). The driving system of the HGS consists of 12 rollers, 4 belts, driving shafts, and a pulley system, mimicking a relatively real environment of gastric peristaltic contraction. The driving system was installed on four sides of the latex stomach chamber. The rollers were screwed on the belts distributed along the four equally spaced sides of the stomach chamber. Each roller contains two side wheels (length 9 mm) that were 12 mm apart from each other. Each belt was 81 cm in length, being screwed three equally distanced rollers. A pair of opposite rollers was placed 30 mm higher than the pair at right angles to it. When the simulator contracted, the gap between two opposite rollers was 10 mm. A latex stomach chamber was placed inside of the simulator, a plastic tube was connected the bottom of the chamber to the outside, for controlling the gastric emptying. To mimic human gastric sieving, a thin polyester mesh bag with a pore size of 1 mm was placed inside of the stomach chamber. So that only particles of size less than 1 mm can pass through to the duodenum (Meyer, MacGregor, Gueller, Martin, & Cavalieri, 1976; Schulze, 2006).

A 200-g sample (a protein solution in Chapter 4 and 5, an emulsion in Chapter 6) was fed into the HGS and was warmed at 37°C for 2 min. The SGF was then added at a rate of 2.5 mL/min (the addition rates of the 1.25 × concentrated SGF and the pepsin were 2.0 and 0.5 mL/min, respectively, and were controlled by 2 separate pumps). Samples (50 mL) of digesta were removed from the bottom of the stomach chamber at 20 min intervals, equalling an emptied digesta rate of 2.5 mL/min. The gastric contraction frequency was 3 times/min, to mimic the actual contraction of the stomach. The temperature of the HGS was set and maintained at 37°C by a heater and a thermostat during the 220 min of gastric digestion. At each time interval, the sample was removed from the HGS and was then filtered through a mesh with a pore size of 1 mm for further analysis, so that only the solid mass of size $\leqslant$ 1 mm was emptied. In a control experiment, instead of the sample, 200-g of Milli-Q water was fed into the HGS and was digested for 220 min.

Figure 3.2. Image of a HGS (A) and schematic illustration of a latex stomach chamber (B). (1) SGF; (2) plastic tubes for secretion; (3) pump; (4) latex stomach chamber; (5) mesh bag; (6) roller; (7) belt; (8) pulley; (9) shaft; (10) angle gear; (11) Lovejoy joint; (12) fan heater for temperature control. Picture derived from Ye et al. (2016b).

3.2.3.2 pH measurement

The initial pH refers to the pH of the freshly prepared samples. With the introduction of SGF into the HGS (2.5 mL/min) and the gastric empting (2.5 mL/ min), the pH in the HGS at different digestion time points was defined as the pH of the emptied digesta. Because the access of the HGS was prevented by the simulated gastric contraction (movement of the rollers).

3.2.3.3 Weight of clot

After 220 min of digestion, the clot (if any) was collected and filtered through a sieve with a 1-mm pore size to separate the aqueous phase and the clot. The curd was then rinsed with SGF to remove pepsin from the surface and was weighed immediately. It was then dried at 105°C overnight in a vacuum oven to determine its dry weight. Samples of curds were also collected by a blade for confocal microscopic examination and SDS-PAGE analysis.

3.2.3.4 Measurement of oil content (Chapter 6)

In emulsion (Chapter 6), emptied digesta samples were collected at selected time points (20, 60, 120 and 220 min) during gastric digestion to determine their oil contents. The oil content was measured according to Mojonnier ether extraction method (AACC 30-10).

(1) Preparation of samples

10 mL of emptied digesta was placed into a dry Mojonnier tube and weight. Each sample was prepared in duplicate. 2 mL of ammonium hydroxide was added and mixed well in the lower bulb, and then the tube was placed in a 60°C water bath for ~5 min and was swilled occasionally. Few drops of phenolphthalein indicator were added when the tube was cool. Then, 10 mL of ethanol was added and mixed to allow the liquid to flow backwards and forwards between the two bulbs. 25 mL of diethyl ether was added, and then stopper the tube and rock gently for ~1 min.

(2) Oils extraction

In the oils extraction procedure, 25 mL of petroleum ether was placed into the Mojonnier tube, and then rock the tube gently for 30 sec. The tubes were then centrifuged at 600 rpm for 2 min. After centrifuging, the tubes were checked to make sure the interface of aqueous phase and solvent phase was in line with the junction of the tubes. If it was below this, a little water was added to allow the interface to grow. Then, the stopper of the tube was removed and the organic solvent layer was transferred carefully to a pre-weighted conical flask by gradually bringing the cylindrical bulb of the cube into a horizontal position.

According to the AOAC method, 5 mL ethanol was then added to the Mojonnier tube and mixed to prevent the formation of emulsion. The sample was extracted repeatedly with 15 mL of diethyl ether and 15 mL of petroleum ether. The second extracted product was added into the same flask as used before. All solvents were removed from flask by evaporation using a steam bath. The flask was then dried in the oven at 100°C for 90 min. Before placing the flasks in the oven, it is necessary to ensure that all traces of solvent vapour has been removed. When the flask was cooled to room temperature, it was weighted and the fat content was recorded. A blank determination was conducted at the same time, in which 10ml distilled water was used to replace the digesta sample.

(3) Calculation of the oil content

$$\%\ \text{oil in sample} = \frac{w_2 - w_1}{w_3} \times 100$$

Where w_1 is the weight of emptied flask (g); w_2 is the weight of flask and oil (g); w_3 is the weight of sample taken (g).

3.2.4 *In vitro* intestinal digestion (Chapter 6)

20 ml of gastric digesta taken from the emptied digesta was collected at each 20 min during the gastric digestion stage. They were mixed well for the subsequent small intestinal digestion. The pH of gastric digesta (from each 20 min) was adjusted to pH 7.5 immediately using NaOH (1 *M* and 0.1 *M*) to inactive the pepsin after emptying from the HGS.

3.2.4.1 Measurement of free fatty acid release

20 mL of the mixture of gastric digesta (pH 7.5) was mixed with 16 mL of the 1.25 ×concentrated SIF. It was then transferred to a temperature-controlled chamber for testing fatty acid release. 4 mL of bile extract (25 mg/mL) solution and 11.1 mg of $CaCl_2$ was then added into the cell. Then, 32 mg of pancreatin was added into the final digestion mixture. The final concentration of bile extract, $CaCl_2$ and pancreatin in the final digestion mixture was 2.5 mg/mL, 0.28 mg/mL and 0.8 mg/mL, respectively. The small intestinal digestion was conducted at 37°C for 2 h. The pH of the digestion mixture was monitored and maintained constant at pH 7.5. The volume of 0.25 *M* NaOH to neutralize the free fatty acids (FFAs) released during the lipids digestion was recorded using a pH-stat automatic titration unit (TitraLab 856; Radiometer Analytical, Villeurbanne, France). The quantity of FFAs released per ml digestion mixture was calculated according to the following equation:

$$\frac{\mu mol_{fatty\ acid}}{ml_{digestion\ mixture}} = \frac{V_{NaOH}\,(t) \times C_{NaOH} \times 1000}{V_{digestion\ mixture}}$$

Where: $V_{NaOH}\,(t)$ is the volume of NaOH added into the reaction mixture at the digestion time t for the sample in the SIF; C_{NaOH} is the molar concentration of the NaOH solution (0.25 *M*) used; $V_{digestion\ mixture}$ is the volume of the digestion mixture.

The amount of the fatty acid released per gram oil in the digestion mixture was calculated according to the following equation:

$$\text{total FFA released (\%)} = \frac{V_{NaOH}\,(t) \times C_{NaOH} \times m_{w\ lipid}}{2m_{liquid}} \times 100$$

Where: $V_{NaOH}\,(t)$ is the volume of NaOH added into the reaction mixture at the digestion time t for the sample in the SIF; C_{NaOH} is the molar concentration of the NaOH solution (0.25 *M*) used; $M_{w\ lipid}$ is the average molecular weight of the lipid (881 g/mol); and m_{lipid} is the total mass of oil in the reaction mixture.

3.2.5 Particle size measurements

The mean particle size and size distribution of emulsions and digesta were measured immediately after the preparation of the emulsion or during *in vitro* gastrointestinal digestion by a static light scattering technique using a Mastersizer (2000S, Malvern Instruments, Worcestershire, UK). The particle size of emulsified droplets was characterised by the volume-weighted average diameter $d_{4,3}$ (μm) and the volume-surface average diameter $d_{3,2}$ (μm), respectively, which were calculated according to the following equations:

$$d_{4,3} = \sum \frac{n_i {d_i}^4}{n_i {d_i}^3}$$

$$d_{3,2} = \sum \frac{n_i {d_i}^3}{n_i {d_i}^2}$$

Where n_i is the number of particles with the diameter of d_i.

Particle size distribution of gastric digesta emptied from the HGS at 5, 20, 40, 80, 120, 160 and 220 min were through a sieve (with a pore size ~1 mm), and were then measured immediately without pH adjustment. During the small intestinal stage, the particle size distribution of the digestion mixture at 1, 10, 30, 60 and 120 min were measured immediately after sampling. The refractive index of the gastric and small intestinal digesta was set as 1.47, equalling to that of protein-stabilised emulsion (Ye & Taylor, 2009). $d_{4,3}$ was reported for the particle size distributions.

3.2.6 Confocal laser scanning microscopy

The microstructure of the clots and emptied digesta during gastrointestinal digestion were examined using a confocal laser scanning microscope (Leica SP5 DM6000B, Leica microsystems, Heidelberg, Germany).

Method (Chapter 5)

For the clot collected after 220 min of the gastric digestion of 200-g of different protein solutions, a fluorescent dye Fast Green (1.0%, w/v) was used to stain protein (He-Ne laser with an excitation at 633 nm)(Ye et al., 2016b). A small piece of clot was stained with Fast Green for 15 min, and was then placed on a concave confocal microscope slide (Sail; Sailing Medical-Lab Industries Co. Ltd, Suzhou, China), and covered with a cover slip. The confocal images acquired using a digital image processing software (Leica LAS AF) consisted of 1024×1024 pixels. Image analysis was performed using the Image J software. At least three images were taken from each sample. The samples were observed using the ×10 lens and ×40 oil immersion lens.

Method (Chapter 6)

The microstructure of emulsion, emptied digesta removed from the HGS and the chyme in the stomach at 20, 60, 120 and 220 min, and the mixture of gastric digesta were examined. Samples for confocal microscopic examination were not pretreated (i.e. heating or pH adjustment), they were placed into an ice bath to arrest the enzymatic action of pepsin temporarily before proceeding with analyses.

A fluorescent dye Nile red, dissolved in acetone (0.1%, w/v), was added the sample to stain the oil phase (Argon laser with an excitation line of 488 nm). Fast green (1.0%, w/v) was used to stain the protein (He-Ne laser with an excitation at 633 nm). For liquid samples. 200 μL sample was transferred into an Eppendorf tube, and adding 5 μL of 1.0% (w/v) Fast Green and 10 μL of 0.1% (w/v) Neil Red. The samples were stained for at least 5 min. For solid chyme, i.e. aggregated emulsion, an aliquot sample, taken using a blade, was stained with Fast Green and Neil Red for at least 10 min. A drop or a piece of stained sample were placed on a concave confocal microscope slide (Sail; Sailing Medical-Lab Industries Co. Ltd., Suzhou, China), and covered with a cover slides. The samples were observed using the ×40 and ×100 oil immersion lens.

3.2.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The time-dependent hydrolysis by pepsin of proteins in the emptied digesta was determined by observing the protein composition of samples as a function of the digestion time, using SDS-PAGE. After 220 min of *in vitro* gastric digestion, the protein composition of the clot was also analysed (Ye et al., 2016a, 2016b).

3.2.7.1 Preparation of stock solutions

(1) 10 % Sodium Dodecyl Sulfate (SDS)

10 g of SDS was dissolved in Milli-Q water under gentle stirring, and made up to 100 mL. Store at room temperature.

(2) 1.5 M Tris-HCL Buffer (resolving gel buffer), pH 8.8

18.15 g of Tris was dissolved in 60 mL of milli-Q water. The pH was adjusted to 8.8 using 6 *M* HCl, and was made up the volume to 100 mL. The buffer was stored at 4°C.

(3) 0.5 M Tris-HCL Buffer (stacking gel buffer), pH 6.8

6.05 g of Tris was dissolved in 60 ml of Milli-Q water and mixed well. The pH was then adjusted to 6.8 using 6 *M* HCl, and was made up the volume to 100 mL. The buffer was stored at 4°C.

(4) 10% Ammonium Persulphate (APS)

100 mg of APS was dissolved in 1 mL of Milli-Q water. The APS solution was freshly prepared before use.

(5) 0.1% Bromophenol blue solution

0.08 g of Bromophenol blue was dissolved in 0.35 mL 0.1 *M* NaOH, and was made up the volume to 80 mL using Milli-Q water.

(6) Stain solution-Coomassie Brilliant Blue Solution (0.3%)

3.0 g of Coomassie Brilliant Blue R was dissolved in a mixture of 700 mL of Milli-Q water, 200 mL of Isoproponol and 100 mL of glacial acetic acid. The solution was then filtered through Whatman Filter Paper (Grade 4) and was stored in a dark bottle.

(7) Destaining solution

100 mL of Isoproponol and 100 mL of glacial acetic acid was added in 800 mL Milli-Q water. The solution was stored in a well closed container.

(8) 5X Electrode buffer pH 8.3 ±0.2

7.5 g Tris, 36 g Glycine and 2.5 g SDS was dissolved in Milli-Q water and then made up the volume to 500 mL. Dilute 70 mL of concentrated electrode buffer to 350 mL with Milli-Q water before use.

(9) Acrylamide/bisacrylamide (37.5:1) solution

29.2 g of Acrylamide and 0.8 g of bisacrylamide was added into 100 mL of Milli-Q water and then mixed well. The solution was stored at 4°C in a dark bottle.

(10) Sample buffer

6 mL of glycerol, 7.5 mL of 0.5 *M* Tris-HCl buffer (pH 6.8), 12 mL of 10% SDS, 1.5 mL of Bromophenol Blue solution, and 1.5 ml of β-mercaptoethanol was mixed, and then made up the volume to 60 mL with Milli-Q water. The solution was stored at 4°C. The β-mercaptoethanol was added in the buffer with a ratio of 1:19 (v: v) prior to use for reducing conditions.

3.2.7.2 Gel preparation

(1) Resolving gel (16%)

Resolving gel was prepared by mixing 4.04 ml milli-Q water, 5 mL of 1.5*M* Tris-HCl buffer, 200 µL of 10% SDS and 10.6 mL of Acrylamide/bisacrylamide solution. The mixture was stirred and then degassed for 15 min. 10 µL of TEMED and 100 µL of freshly prepared 10% APS was added in the mixture before the gel casting.

(2) Stacking gel (4%)

1.3mL of acrylamide/bisacrylamide solution, 2.5 mL of 0.5 *M* Tris-HCl buffer, 100 µL of 10% SDS and 6.1 mL of Milli-Q water were mixed well and degassed for 15 min. Then, 10 µL of TEMED and 50 µL of freshly prepared 10% APS was added in the mixed gel solution prior to gel casting.

3.2.7.3 Sample preparation

Liquid sample for SDS-PAGE analysis included freshly prepared protein solutions (Chapter 4 and 5), emulsions (Chapter 6), and the emptied gastric digesta samples removed from the HGS at 20, 40, 80, 120, 160, and 220 min during gastric digestion.

During the gastric digestion of protein solution (Chapter 4 and 5), once the gastric digesta was removed from the HGS at different time points, 5 mL of digesta was transferred into a glass tube. It was heated at 90°C for 3 min to inactive the pepsin. During the gastric digestion of emulsions (Chapter 6), after the digesta was emptied from the HGS, the pH value of the sample was immediately adjusted to 7.5 using 1 *M* NaOH to inactive the pepsin. The freshly prepared protein solutions and emulsions were not pretreated (i.e. without pH adjustment or heating). The samples were then mixed with the sample buffer (Tris-HCl buffer at pH 6.8, consisting of glycerol, SDS, bromophenol blue and β-mercaptoethanol (19:1, v: v)) at an appropriate ratio. Then, they were heated at 90°C for 5 min (Ye et al., 2016b) and were cooled to room temperature for further analysis.

The solid samples, i.e. clot, after 220 min of digestion, the clot was freeze-dried using a freeze drier (Telstar Cryodos-80, Terrassa, Spain) for 72 h, and was then ground to powder in a mortar (Ye et al., 2016b). 4.5 mg of powder was mixed with 1mL sample buffer, and heated at 90°C for 5 min.

3.2.7.4 Running of electrophoresis, staining and destaining

10 μL of sample was loaded onto a resolving gel previously prepared on a Mini PROTEIN II system (Bio-Rad Laboratories, Richmond, CA, USA). The electrophoresis analysis was conducted at 125 V for approximately 60 min. After running, the gel was stained using a Coomassie Brilliant Blue R staining solution (0.003% (w/v) in 10% acetic acid and 20% isopropanol) for 60 min under gentle shaking. Then the gel was destained with a destaining solution (consisting of 10% acetic acid and 10% isopropanol) for 1 h, and then soaking the gel in a fresh destaining solution for overnight. The gel was then scanned using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories).

3.2.8 Statistical analysis

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

Chapter 4: Behaviours of Different Milk Protein Ingredients during in vitro Gastric Digestion

4.1 Abstract

The coagulation behaviours and the kinetics of protein hydrolysis of skim milk powder (SMP), milk protein concentrate (MPC) 4851, MPC 4861, sodium caseinate, whey protein isolate (WPI), and heated (90°C, 20 min) WPI under the gastric conditions were examined using an advanced dynamic digestion model, i.e., a human gastric simulator (HGS). During gastric digestion, these protein ingredients exhibited various pH profiles as a function of the digestion time. SMP and MPC 4851, which contained casein micelles, formed ball-like curds with a dense structure after 10 min of digestion; these curds did not disintegrate over 220 min of digestion. MPC 4861 and sodium caseinate, which lacked an intact casein micellar structure, formed curds at approximately 40 min and a loose, fragmented structure in the curds after 220 min of digestion. In contrast, no curds were formed in either WPI or heated WPI after 220 min of digestion. In addition, the hydrolysis rates and the compositions of the digesta released from the HGS were different for the various protein ingredients, as detected by sodium dodecyl sulfate PAGE. SMP and MPC 4851 exhibited slower hydrolysis rates than MPC 4861 and sodium caseinate. The most rapid hydrolysis occurred in the WPI (with and without heating). This was attributed to the formation of different structured curds under gastric conditions.

4.2 Introduction

Milk protein is an important source of nutrients for humans through the different stages of life. Milk protein ingredients are not just useful for improving the functional properties of, but also provide excellent nutritional value for, food and nutritional products.

SMP is the most widely applied functional dairy ingredient (Singh & Creamer, 1991). Its manufacture involves heat treatment, normally known as preheating, evaporation, and spray drying. The most important effect of the preheating is the induction of the denaturation of the whey proteins, to give partially denatured whey proteins, which can simply self-aggregate or associate with the casein micelles via micellar κ-casein or both (Singh, 2007).

MPC was the first membrane-produced, casein-based product on the market (Carr & Golding, 2016). The caseins and whey proteins are in the same proportions as in milk. The casein is in a micellar form and the whey protein is in its native state as the manufacturing process does not involve preheating. However, a fraction of the colloidal calcium phosphate in MPC may be dissolved during the manufacturing process, leading to loose casein micelle structures and, resulting in a smaller fragmented micellar structure (Singh, 2007).

To improve the functional properties of MPC, the casein micelles in some new products have been dissociated to a certain extent by removing the calcium (Ye, 2011). This calcium reduced product refers to "MPC 4861" in the present study. The micellar structure of MPC can be converted from the native structure to a structure that is closer to that of sodium caseinate, depending on the level of calcium depletion (Carr & Golding, 2016). However, a significant difference between caseinates and MPCs is that MPCs contain phosphate, whereas caseinates have reduced phosphate levels because of acidic precipitation and subsequent washing. Micellar phosphate increases the buffer capacity because of the formation of dihydrogen phosphate (Ferreira et al., 2003) on the acid-mediated solubilisation of colloidal calcium phosphate (Carr & Golding, 2016).

Sodium caseinate, the water-soluble form of casein that is most commonly used in foods, is usually prepared by solubilising acid casein with NaOH (Mulvihill & Ennis, 2003). The products produced in this manner contain individual casein molecules because

the native micellar structure is disrupted during the manufacturing process. The excellent heat stability of caseinates is one of their most important functional benefits, it limits alterations to their properties as a consequence of thermal processing (Carr & Golding, 2016).

Whey is obtained after removing casein from milk. Whey protein ingredients that contain ≥90% protein are known as WPI. To produce WPI, whey is skimmed by centrifugation or microfiltration and then demineralized by ion exchange, electrodialysis or nanofiltration. The proteins are concentrated by membrane filtration or ion exchange chromatographic methods (Bansal & Bhandari, 2016). As the whey protein in WPI is in its native state, its functional properties are largely retained.

Whey protein and casein are usually used as model proteins in studies investigating the digestion and absorption rate of protein (He & Giuseppin, 2014). Boirie et al. (1997) proposed the concepts of "slow" casein and "fast" whey protein according to the different digestion rates of these proteins. As whey protein is reported to induce a dramatic but short increase in plasma amino acids after ingestion, is classified as a "fast" protein. It has a fast gastric emptying rate, because it stays soluble in the stomach and passes into the small intestine rapidly without being hydrolysed by pepsin (Boirie et al., 1997; He & Giuseppin, 2014). β-Lactoglobulin (β-Lg) is a globular protein and it comprises almost 50% of the total whey protein in bovine milk (Sawyer, 2003). Native β-Lg is resistant to some proteases, particularly pepsin, because of its unique structural stability at low pH levels (Miranda & Pelissier, 1983; Mohan Reddy, Kella, & Kinsella, 1988b). Most of the hydrophobic amino acids, which are potential cleavage sites for pepsin, are buried inside the hydrophobic core and are not readily accessible. Heating, has been reported to increase the susceptibility of β-Lg to hydrolysis by pepsin (Guo, Fox, Flynn, & Kindstedt, 1995; Ye et al., 2016b). Since heat treatments induce conformational changes in β-Lg, resulting in increased exposure of peptic cleavage sites, this increases susceptibility to pepsin action. In addition, the hydrolysis of α-lactalbumin (α-La) by proteases (e.g. pepsin) is reported in correlation with protein conformation (Miranda, Hazé, Scanff, & Pelissier, 1989).

In contrast, casein is classified as a "slow" protein for digestion. Casein clots in the stomach, which greatly reduces the gastric emptying rate, probably resulting in a

slower release of amino acids (He & Giuseppin, 2014). In caseinate, caseins exist in the form of the individual protein molecules, their coagulation behaviour is induced by acid (Ye et al., 2016b). However, in milk, caseins together with colloidal calcium phosphate form the particles known as casein micelles, which contain thousands of individual protein molecules (Dalgleish & Corredig, 2012). Similarly, caseins exist in large colloidal micelles, with the calcium phosphate in SMP and MPC, as with those found in milk (Singh, 2007).

Casein micelles have been reported to have different digestion behaviours from the individual caseins (Miranda & Pelissier, 1981). In an *in vivo* gastric digestion study, in a rat stomach, the coagulation of skim milk was much greater than that of a sodium caseinate solution. In addition, the gastric emptying rate and the hydrolysis rate of a mixture of individual caseins were much faster than those of skim milk (Miranda & Pelissier, 1981). This difference in digestion behaviour between caseinates and casein micelles occurs primarily because casein micelles can be coagulated by the milk-clotting enzyme pepsin (Tam & Whitaker, 1972) and an acidic pH (Dalgleish & Corredig, 2012), whereas caseinate is coagulated only by low pH and not by an enzyme.

In recent work in our laboratory, digestion behaviour of unheated and heated (90 ºC, 20 min) skim milk was investigated using a dynamic *in vitro* gastric digestion model. The results revealed that the different structure of the curds that are induced by the action of the milk-clotting enzyme pepsin can significantly affect the rate of protein hydrolysis. It was found that unheated skim milk formed a firm, tightened, cheese-like round clot with a porous network structure. Such a structure means that the accessibility of pepsin to the inside of the curd was very limited. In contrast, the heated (90 ºC, 20 min) milk formed a looser, fragmented network-structured clot with numerous larger voids. The coagula became tighter and less permeable to serum and solute with an increasing digestion time, particularly in the case of unheated skim milk. The formation of different structured clots had apparently affected the hydrolysis rate of caseins and whey proteins, induced by the action of pepsin on κ-casein under gastric environment. The hydrolysis of caseins in heated milk became much faster than that in unheated milk. Furthermore, the whey proteins have different digestion behaviour in heated milk due to the association with casein micelles during heat treatment (Ye et al., 2016b).

This present study extends recent work in our lab on gastric digestion of skim milk and whole milk (Ye et al., 2016a, b). In the present work, the physicochemical behaviour and the curd formation of different commercial dairy protein ingredients during *in vitro* gastric digestion were investigated using a dynamic digestion model, i.e., a human gastric simulator (HGS). The dynamic model allows a more "real" environment in which to simulate the human gastric digestion process, mimicking gastric contraction, the continuous addition of fresh simulated gastric fluid (SGF) that contains pepsin, and simulated gastric emptying. The information obtained from this study will be useful in understanding the digestion of different commercial dairy ingredients, and for the design and development of different products derived from these dairy ingredients.

4.3 Results

4.3.1 pH profiles

Figure 4.1 shows the changes in the pH of the emptied digesta from the different dairy ingredients as a function of the digestion time. In general, with the gradual addition of SGF at a rate of 2.5 mL/min, the pH of all ingredients (i.e., the pH of the emptied digesta) decreased with an increase in the digestion time. The initial pH in the stomach represented the pH of the different dairy ingredient solutions containing 3.0% (w/w) protein and ranged from pH 6.79 ± 0.18 to pH 7.05 ± 0.03. The pH profiles of the different dairy ingredients were different during the early digestion period. MPC 4861 had a slower decrease in pH with increasing digestion time than the other dairy ingredients, reaching pH ~5.6 within 40 min and pH ~2.0 after 140 min. In contrast, heated WPI had the fastest decrease in pH, dropping to pH ~ 3.4 within the first 40 min and to close to pH ~ 2.0 at 120 min. SMP, MPC 4851, sodium caseinate, and WPI had only slight differences their pH profiles. The decrease in pH was slower in SMP and sodium caseinate than in MPC 4851 and WPI. However, with further digestion, the pH of all ingredients did not change significantly from 120 to 220 min.

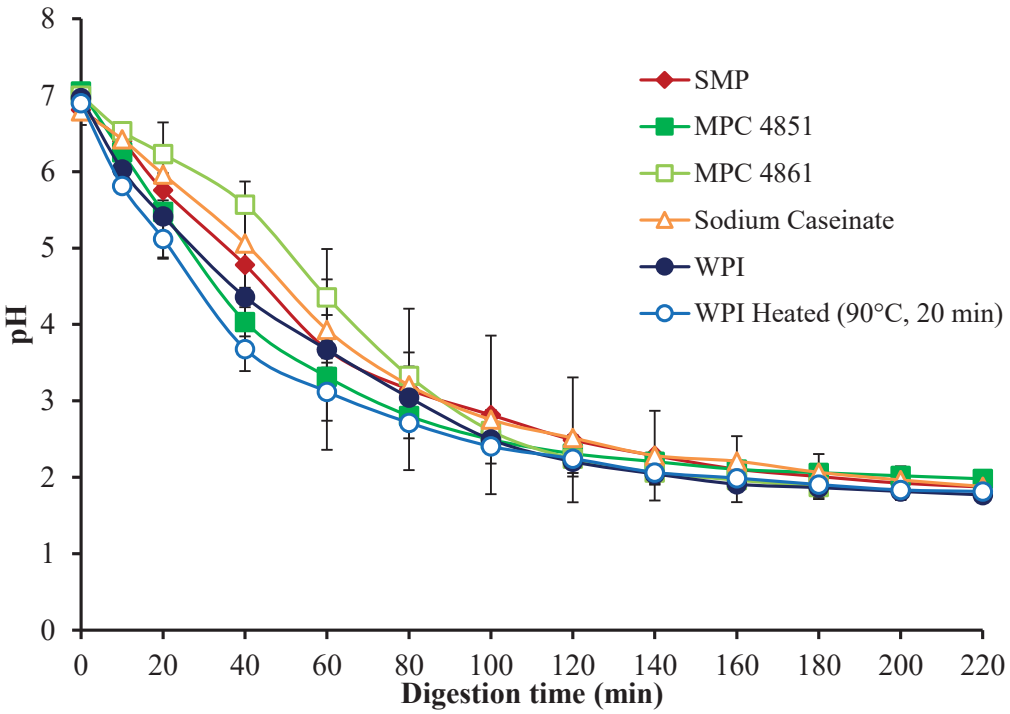


Figure 4.1. Changes in pH during the gastric digestion of different dairy ingredient solutions with 3.0% (w/w) protein.

4.3.2 Coagulation behaviour of different milk protein ingredients

Figure 4.2 shows the clots obtained from 200 g of the dairy ingredient solutions after 220 min of gastric digestion. In general, the appearances of the clots from all ingredients were different. For SMP and MPC 4851, protein coagulation was visible in the first 10 min of digestion, and the serum phase gradually became clear during this period. The pHs at 10 min of digestion were 6.40 and 6.22 for SMP and MPC 4851, respectively. Over the 220 min of digestion, a cheese-like curd with a closely knit network and a ball-like shape was observed in both SMP and MPC 4851. However, there was a slight difference in appearance between these 2 curds. The curd from SMP contained numerous loose, crumbled fragments, many more than were observed in MPC 4851. The curd obtained from MPC 4851 was a more integrated ball-like pellet (Figure 4.2).

For MPC 4861, protein coagulation was visible after 40 min of digestion. In sodium caseinate, with the introduction of SGF into the HGS, the caseins formed some

visible white floccules and started to form a small, spongy and elastic pellet at about 15 min of digestion. With further digestion, sodium caseinate in the serum phase produced a large amount of white precipitations after 40 min, and the serum phase became clear again at nearly 60 min of digestion. However, over 220 min of digestion, a loose, fragmented protein matrix with a number of large voids was consistently observed in the curds formed from MPC 4861 and sodium caseinate (Figure 4.2).

No curd was observed in unheated WPI and heated WPI after 220 min of digestion. However, it is worth noting that aggregation occurred in heated WPI after 40 min of digestion, but no aggregation was observed after 120 min, whereas there was no aggregation in native WPI during the whole digestion period.

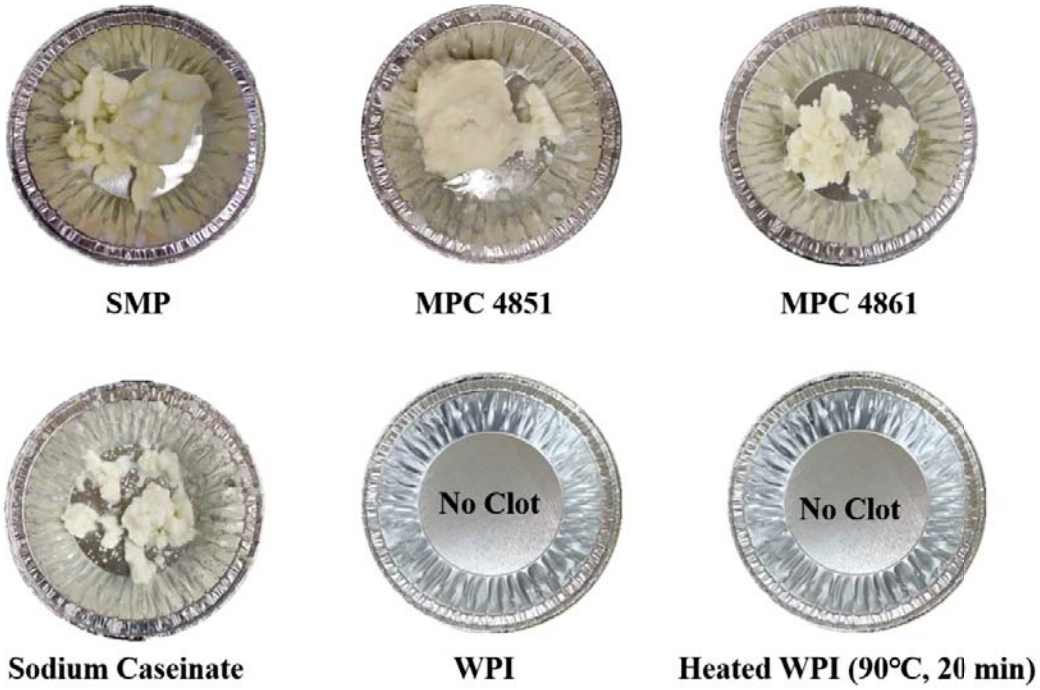


Figure 4.2. Images of clots obtained from 200 g of dairy ingredient solutions containing 3.0% (w/w) protein after 220 min of *in vitro* gastric digestion.

The wet weights of the clots obtained from the different dairy ingredients after 220 min of digestion showed a significant difference ($P < 0.01$) (Figure 4.3). However, no difference ($P > 0.05$) was observed in wet weights between SMP and MPC 4851. Both

of them were significantly (P < 0.05) higher than those of MPC 4861 and sodium caseinate.

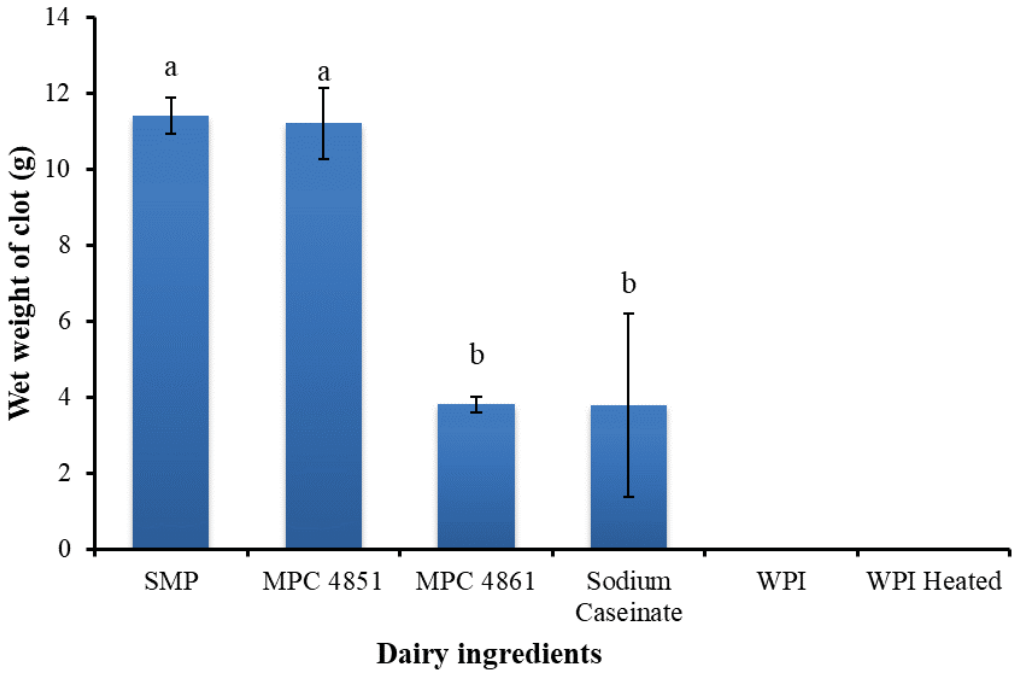


Figure 4.3. Wet weights of clots obtained from 200 g of dairy ingredient solutions containing 3.0% (w/w) protein after 220 min of *in vitro* gastric digestion. Different lowercase letters indicate significant difference (P < 0.05) among different dairy ingredients.

The dried weights, using a vacuum oven to dry the curds at 105°C overnight, showed a similar pattern to those observed with the wet weights (Figure 4.4). The dried weights of the SMP and MPC 4851 curds were about 3.24 and 2.85 g, respectively. Both were significantly greater (P < 0.05) than those of the MPC 4861 and sodium caseinate curds.

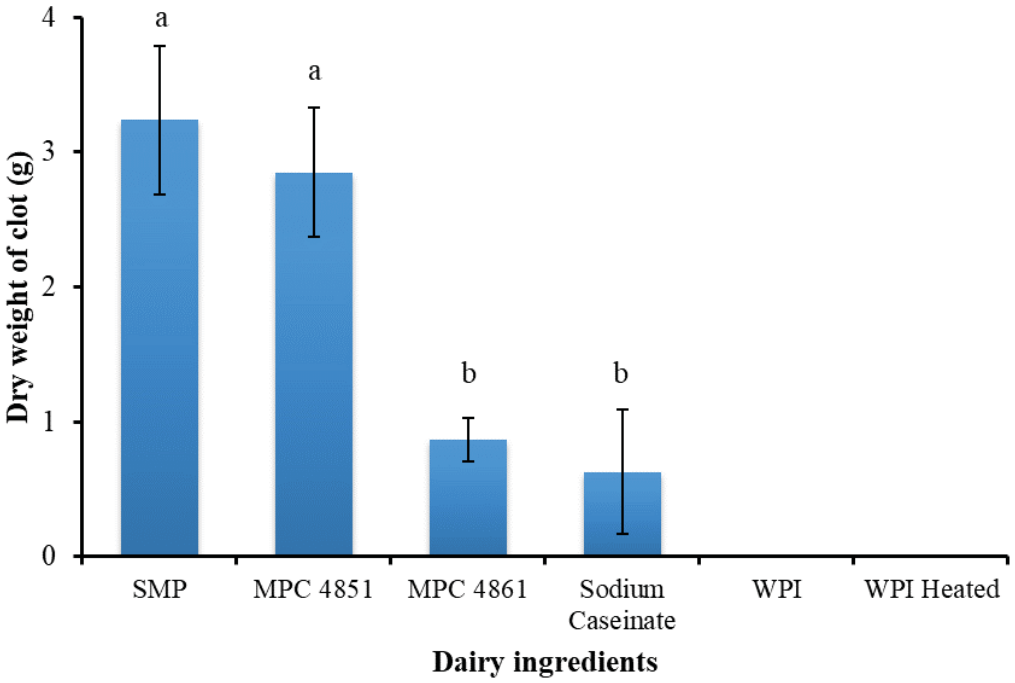


Figure 4.4. Dry weight of clots obtained from 200 g of dairy ingredient solutions containing 3.0% (w/w) protein after 220 min of *in vitro* gastric digestion. Different lowercase letters indicate significant difference ($P < 0.05$) among the different dairy ingredients.

4.3.3 Protein hydrolysis

4.3.3.1 SDS-PAGE pattern of clots

SDS-PAGE under reducing conditions was used to determine the protein composition and the hydrolysis of the curds obtained after 220 min of gastric digestion in the HGS (Figure 4.5). For all ingredients, the κ-casein band was faint and a new band appeared at around 15 kDa; it was probably para-κ-casein (Miranda & Pelissier, 1983). α_s-Caseins (α_{s1}-casein+α_{s2}-casein) and β-Lg were observed for SMP and MPC 4851, but were much less apparent for MPC 4861 and sodium caseinate. Furthermore, multiple peptide bands with different molecular weights (ranging from 10 to 20 kDa) were clearly seen for all ingredients. However, the relative band intensities of the peptides were much greater for MPC 4861 and sodium caseinate than for SMP and MPC 4851.

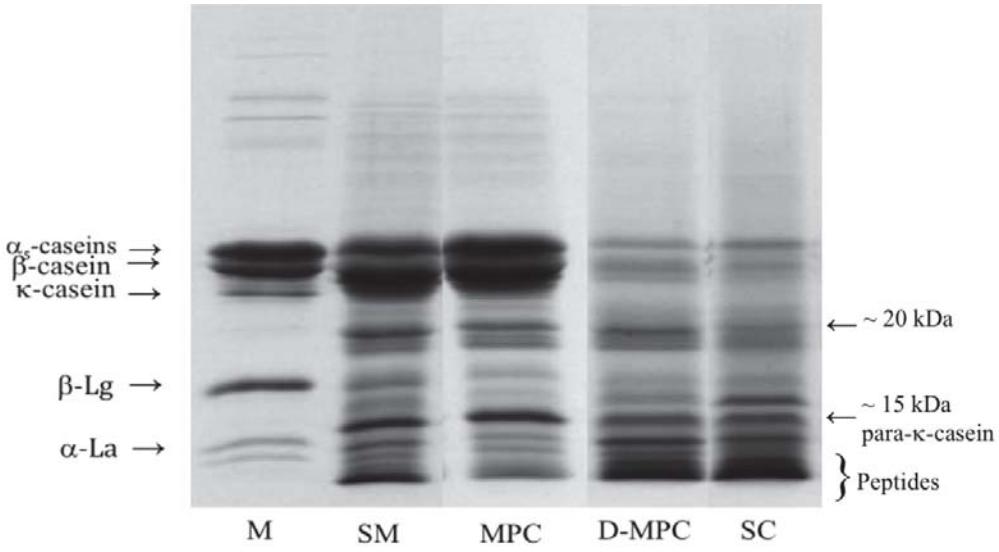


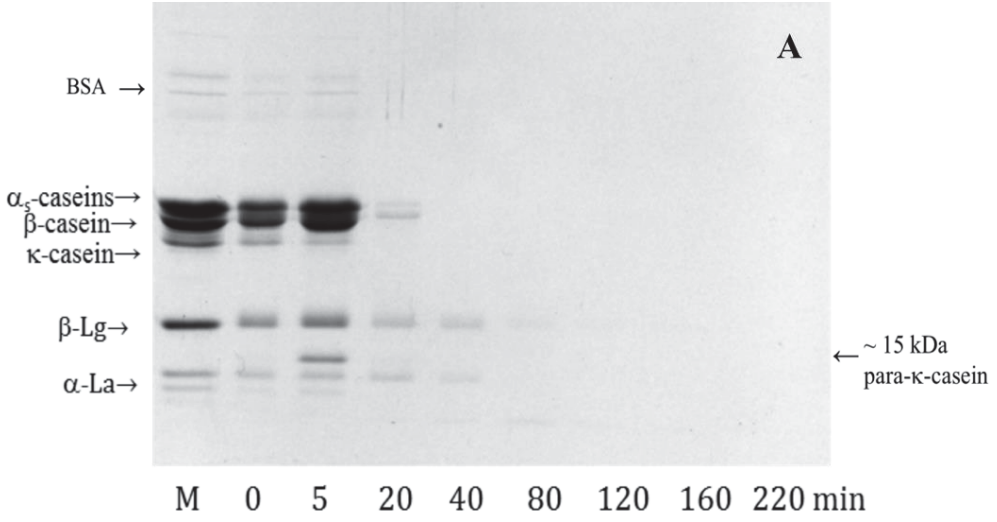
Figure 4.5. SDS-PAGE patterns of clots collected from different dairy ingredients after 220 min of gastric digestion in the HGS. M, trim milk; SM, skim milk powder; MPC, milk protein concentrate 4851; D-MPC, milk protein concentrate 4861; SC, sodium caseinate.

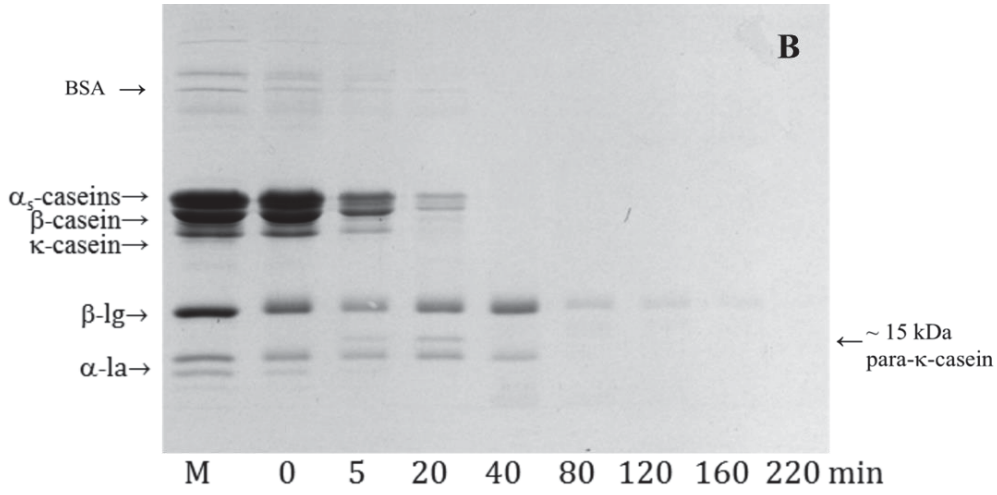
4.3.3.2 SDS-PAGE patterns of emptied digesta

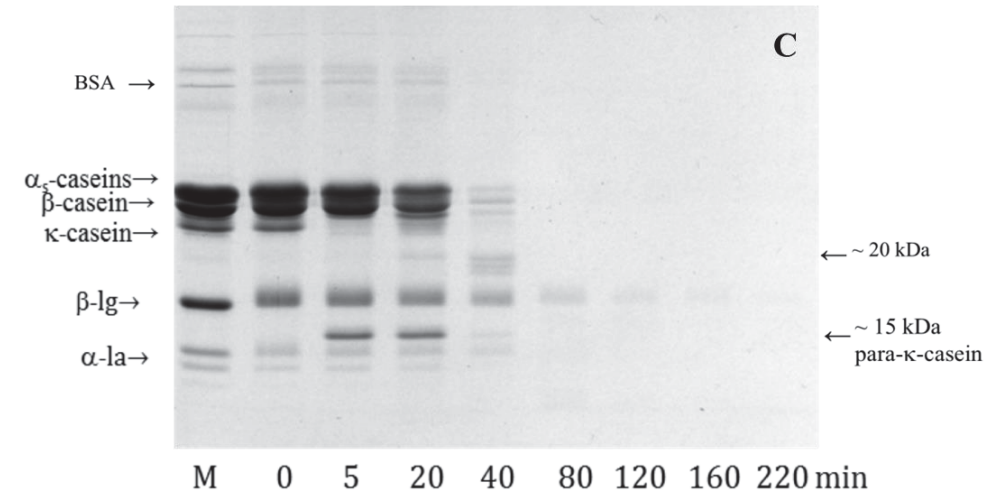
SDS-PAGE patterns under reducing conditions of the emptied digesta of different dairy ingredients as a function of digestion time are shown in Figure 4.6. After 5 min of digestion, the digesta from SMP, MPC 4851, MPC 4861, and sodium caseinate contained intact α_s-casein and β-casein, and relatively intact κ-casein. The intensity of the κ-casein band was much less than that observed before digestion and a newly formed band appeared at around ~15 kDa, probably consisting of para-κ-casein. With increasing digestion time, the intensity of the para-κ-casein band decreased very rapidly and was not observed in the digesta from these 4 ingredients after 80 min of digestion.

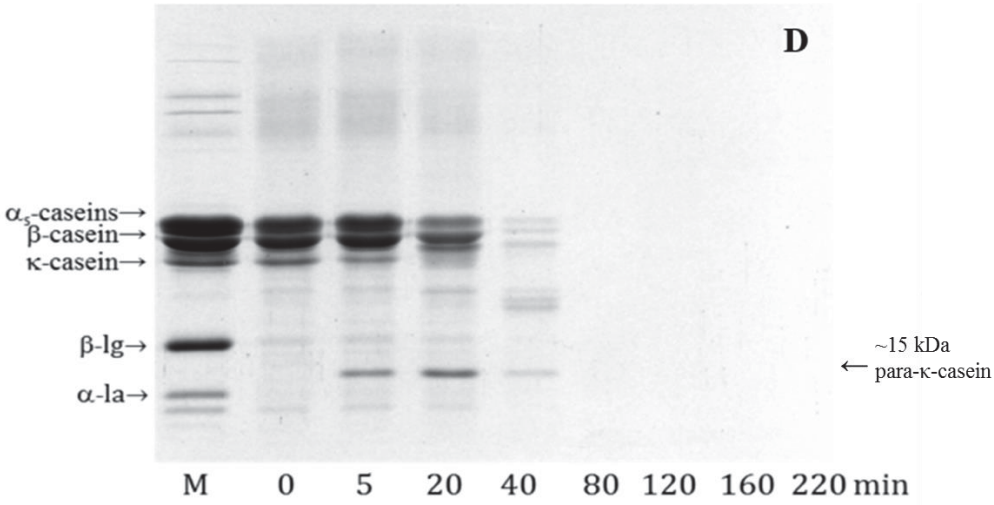
For SMP and MPC 4851, after 20 min of digestion, the α_s-casein and β-casein bands had become very faint, whereas the κ-casein and para-κ-casein bands could still be observed for MPC 4851 but had disappeared for SMP [Figures 4.6 (A) and (B)]. All casein bands in the emptied digesta had disappeared after 40 min of digestion for both SMP and MPC 4851. Intact β-Lg, α-La and BSA were present in the emptied digesta for

both SMP and MPC 4851 at 5 min of digestion. With further digestion, the band intensities of α-La and BSA decreased rapidly, with the α-La band disappearing after 80 min for both SMP and MPC 4851. However, the BSA band was not observed after 20 and 40 min of digestion for SMP and MPC 4851, respectively. β-Lg was observed in the emptied digesta for MPC 4851 until 220 min of digestion, whereas it was absent at 120 min for SMP.






C
BSA →
α_s-caseins→
β-casein→
κ-casein→
β-lg→
α-la→
← ~ 20 kDa
← ~ 15 kDa
para-κ-casein
M 0 5 20 40 80 120 160 220 min


D
α_s-caseins→
β-casein→
κ-casein→
β-lg→
α-la→
~15 kDa
← para-κ-casein
M 0 5 20 40 80 120 160 220 min

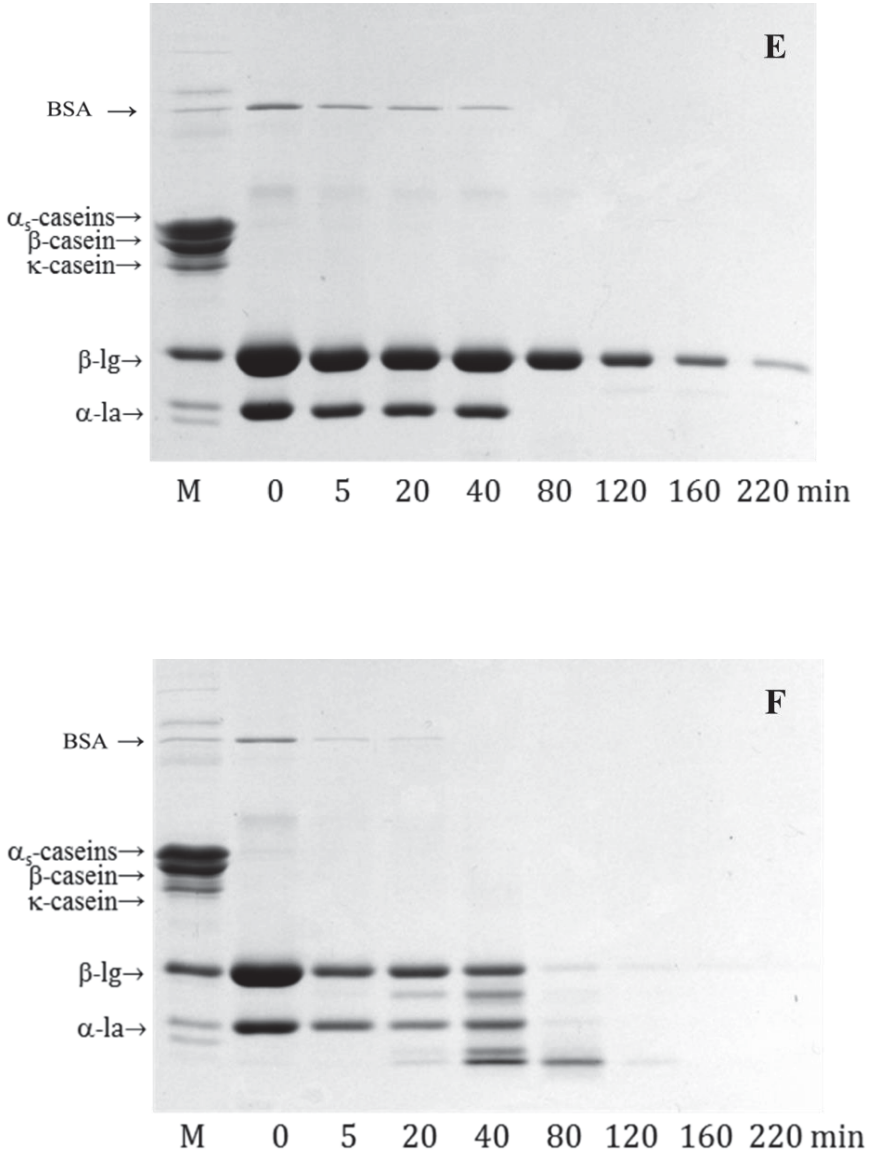


Figure 4.6. SDS-PAGE patterns of the emptied digesta collected from different dairy ingredients during 220 min of gastric digestion in the HGS. M, trim milk. BSA, bovine serum albumin. A, skim milk powder; B, MPC 4851; C, MPC 4861; D, sodium caseinate; E, WPI; F, WPI heated (90°C, 20 min).

Compared to what was observed in SMP and MPC 4851, the electrophoresis of the emptied digesta from MPC 4861 [Figure 4.6 (C)] showed a similar pattern to that of

the emptied digesta from sodium caseinate [Figure 4.6 (D)]. The intensities of the casein bands decreased gradually with an increase in digestion time, and becoming very faint at 40 min and disappearing at 80 min for both MPC 4861 and sodium caseinate. Furthermore, numerous peptide bands with molecular weights ranging from ~10 to 15 kDa were observed at 40 min of digestion for both ingredients. For MPC 4861, BSA and α-La bands were not observed at 40 and 80 min, respectively; the β-Lg band was retained for longer, disappearing at 220 min of digestion.

Figures 4.6 E and F present the SDS-PAGE patterns of the emptied digesta obtained from unheated WPI and heated WPI, respectively. The hydrolysis of the native WPI showed a remarkable difference from that of the heated WPI. After 5 min of digestion, intact β-Lg, α-La and BSA were clearly visible in the digesta for both native and heated WPI. For unheated WPI, β-Lg remained intact during the whole digestion period and appeared to decrease gradually in intensity with an increase in the digestion time. In contrast, from 5-40 min, the intensity of the β-Lg band for heated WPI was much less than that for unheated WPI, it had nearly disappeared at 80 min of digestion. For both unheated WPI and heated WPI, clear and intact α-La bands were present during the first 40 min of digestion but had disappeared at 80 min. BSA remained intact during the first 20 min of digestion for both unheated WPI and heated WPI, but had disappeared from the emptied digesta at 80 and 40 min of digestion for unheated WPI and heated WPI, respectively. Furthermore, a large amount of peptides was clearly visible in the emptied digesta from heated WPI at 20 min of digestion, whereas no peptides were observed in the digesta from unheated WPI during the whole digestion period.

4.4 Discussion

The different dairy ingredients showed significant differences in digestion behaviour during the dynamic gastric digestion. In general, for all dairy protein ingredients, there was a reduction in pH with an increase in the digestion time; however, the rate of decrease was different for the different ingredients (Figure 4.1). This difference can be attributed to the formation of curds with different structures in the different dairy ingredients. Therefore, the diffusions of ions and molecules into and out of the coagulum were different (Ye et al., 2016b). This difference is probably also related to the different buffer capacities of the various milk proteins in the ingredients. The changes in pH apparently influence the activity of pepsin. Pepsin activity was relatively low at the early period of the gastric digestion due to the high pH (pH > 6), and the activity of pepsin increased with the decrease of pH in the HGS at a longer digestion time.

SMP and MPC 4851 showed similar digestion behaviours during the 220 min of gastric digestion. In general, considerable protein coagulation was visible within the first 10 min of digestion. The emptied digesta contained very little protein after 20 min of digestion. The curd remained in the stomach as a ball-like shape with a closely knit network over the 220 min of digestion. Protein coagulation started at pHs of ~6.40 and 6.22 for SMP and MPC 4851, respectively, which are far above the pH region of acid coagulation (isoelectric point of caseins, pI 4.6). Furthermore, as observed from the SDS-PAGE under reducing conditions, newly formed bands occurred at about ~15 kDa and the κ-casein bands were faint in the emptied digesta from both SMP and MPC 4851 after 5 min of digestion [Figures 4.6A and 4.6B]. These bands at 15kDa were probably κ-casein's degradation product, para-κ-casein (Miranda & Pelissier, 1983). The initial hydrolysis rate of κ-casein by pepsin is known to be faster than that of other caseins at pH 6.0 during *in vitro* digestion (Tam & Whitaker, 1972). These observations indicate that the initial protein coagulation behaviour was driven by the action of the milk-clotting enzyme pepsin on the micellar κ-casein, causing destabilisation of the casein micelles (Tam & Whitaker, 1972). These changes in the milk proteins in the early stages of digestion are consistent with the digestion of skim milk and whole milk reported in previous studies in our laboratory (Ye et al., 2016a, b, 2017).

The coagulation behaviour of SMP and MPC 4851 catalysed by pepsin in the early stage of digestion (pH > 6) can be attribute to the existence of casein micelles. Casein micelles are known to be coagulated both by the milk-clotting enzyme, pepsin (Tam & Whitaker, 1972) and an acidic pH (Dalgleish & Corredig, 2012). Therefore, with the increasing addition of pepsin within SGF into the HGS, a large amount of protein was coagulated very quickly and formed a pellet-like clot under the mechanical contraction of the stomach. As a result, the digesta (aqueous phase) only contained a very limited amount of proteins; thus, only faint protein bands can be detected by SDS-PAGE after 20 min of digestion in both SMP and MPC 4851 [Figure 4.6 (A) and (B)].

For both SMP and MPC 4851, over the 220 min of digestion, the clots did not break down into small pieces or disappear. After 220 min, both the wet weight and the dry weight of the curds obtained from SMP and MPC 4851 were significantly greater ($P < 0.05$) than those obtained from sodium caseinate and MPC 4861 (Figures 4.3 and 4.4). The curds obtained from SMP and MPC 4851 remained intact during the whole digestion period, which can be attributed to their characteristic structure. The curds formed from the SMP and MPC 4851 showed an integrated, closely knit structure, which was like a cheese-ball. Previous work in our laboratory demonstrated that the clot formed from skim milk under gastric conditions was degraded only on its surface, because of its relatively limited surface area, leading to a relatively slow proteolysis of caseins by pepsin during *in vitro* digestion (Ye et al., 2016b). Such a dense pellet-like structure allows only a limited amount of pepsin from SGF to diffuse and permeate to the inside of the curd and hydrolyse the caseins. This was confirmed by the electrophoretic analysis results for the clots (Figure 4.5), which showed that caseins were still present in the curds in considerable amounts after 220 min of digestion.

However, there were some slight differences in the structures of the curds derived from SMP and MPC 4851. The SMP curd contained a much larger number of fragmented, aggregated protein blocks than the MPC 4851 curd (Figure 4.2). This difference can be attributed primarily to the different processing technologies applied to the different ingredients. It is worth noting that all ingredients used in the present study were commercial dairy protein ingredients; that is, they had been processed by thermal treatment under specific temperature conditions or by a membrane technique. The SMP used in the present study has a whey protein nitrogen index of between 1.51 and 5.99

mg/g, which indicates that the whey proteins had been partly denatured by preheating during the manufacturing process. Thus, denatured β-Lg was involved in the formation of the curd of the SMP curd, which was detected by SDS-PAGE (Figure 4.5), because a conformational unfolding is induced by preheating and causes the association of β-Lg with casein micelles via micellar κ-casein (Singh, 2007). These small, fragmented, aggregated protein blocks remained separated in the loose, crumbled structure during the whole digestion period, as the clot formed in the renneting of heated milk is less cohesive (Anema, Lee, & Klostermeyer, 2007).

MPC is manufactured using a combined ultrafiltration/diafiltration process. During these processes, colloidal calcium phosphate may be partly dissolved, resulting in loose casein micelle structures and a smaller fragmented micellar structure (Singh, 2007). However, it is likely that MPC 4851 still contains a relatively intact casein micelle structure. It may be coagulated by pepsin and may form an integrated ball-like clot under gastric conditions, as observed in skim milk (Ye et al., 2016b).

Compared with MPC 4851, most of the casein micelles are dissociated when the calcium is removed in MPC 4861 (Ye, 2011). Therefore, the digestion behaviour of MPC 4861 appears to be significantly different from that of MPC 4851 but similar to that of sodium caseinate. For MPC 4861 and sodium caseinate, although a few precipitations can be observed in the sodium caseinate in earlier time periods, precipitation and coagulation of the caseins occurred mainly at longer digestion times (about 40 min of digestion). This observation was further confirmed by SDS-PAGE of evacuated digesta [Figure 4.6 (C) and (D)]. At this digestion time, the pH values (pH < 5) in the stomach were close to the pI of caseins. This difference in digestion behaviour between MPC 4861, sodium caseinate and MPC 4851 may be attributed to there being no intact casein micelles but only individual caseins in MPC 4861 and sodium caseinate. The coagulation behaviour of casein molecules is different from that of casein micelles. That is, casein is coagulated only by acidic pH and not by pepsin, while casein micelles can be coagulated not only by low pH (Dalgleish & Corredig, 2012), but also by pepsin (Tam & Whitaker, 1972). Therefore, coagulation occurred in MPC 4861 and sodium caseinate at longer digestion times, as the pH decreased gradually to pH < 5.0 with the addition of SGF.

Over the 220 min of digestion, the clots observed in MPC 4861 and sodium caseinate were a few separated, aggregated protein blocks of small size, with a loose, open network. Such a structure was more accessible to pepsin hydrolysis, resulting in a large amount of hydrolysis product in the curds derived from both MPC 4861 and sodium caseinate (Figure 4.5).

There was no clot obtained in unheated WPI and heated WPI after 220 min of gastric digestion. Both WPI contained more protein in the digesta compared to the other four ingredients, which emptied rapidly from the stomach into the small intestine. This is consistent with the previous research (Ye et al., 2016b). For unheated WPI, β-Lg remained intact without being degraded during the whole digestion period, as native β-Lg is resistant to hydrolysis by pepsin because of its compact globular structure (Reddy et al., 1988b). However, for heated WPI, a lesser amount of β-Lg was observed in the emptied digesta, which can be attributed to the hydrolysis by pepsin. Because the conformation of the protein may unfold after heating a β-LG solution above its denaturation temperature for 10-15 min, some potential cleavage sites for pepsin (i.e., buried hydrophobic amino acid residues) may be exposed. This makes β-Lg more susceptible to pepsin hydrolysis (Anema & Li, 2003b; Guo et al., 1995). Therefore, for heated WPI, a considerable amount of enzymatic degradation products (i.e., peptides) was detected by electrophoretic analysis in the emptied digesta after 20 min of digestion [Figure 4.6 (F)].

The digestion behaviour of α-La did not show any significant difference between unheated WPI and heated WPI. α-La is considered to be the most heat stable whey protein (Cheison & Kulozik, 2017). When heating temperature is below its irreversible denaturation temperature (i.e. 100°C) (Boye, Alli, & Ismail, 1997), α-La has a strong tendency to refold during the cooling stage. Therefore, heat treatment at such a temperature (90°C in this case) does not improve its susceptibility to hydrolysis significantly (Schmidt & Poll, 1991; Schmidt & van Markwijk, 1993). The disappearance or a faintness of the α-La band was observed at about 80 min of digestion in the emptied digesta for both unheated WPI and heated WPI. At this time point, the pHs of the digesta for unheated WPI and heated WPI were about pH 3.05 and pH 2.51, respectively. This probably occurred because, when the pH of digestion is lower than 4.0, α-La and β-Lg

are more accessible to hydrolysis by pepsin, which is similar to as suggested by Miranda et al. (1989).

It is worth noting that digesta was evacuated from the HGS each 20 min during the whole dynamic digestion. The amount of soluble proteins gradually decreased in the stomach because of the dilution of stomach contents with increasing digestion time. With the gastric emptying, many peptides were also evacuated from the HGS. In particular, at longer digestion time, pepsin had higher activity at low pH. One of the limitations is that these hydrolysis products cannot be shown in SDS-PAGE patterns due to having too small a molecular weight.

In addition, in heated WPI, there was some aggregates formed during the digestion from 40 to 120 min, when the pH approached the pI of whey proteins (~5.1). The formation of aggregation is probably because heating at above 80°C (90°C in this case) may lead to the full exposure of the hydrophobic groups caused by the conformational change, and may increase the hydrophobic interactions between molecules (André, Thomas, Said, & Joseph, 2016). Therefore, when the pH approached the pI of whey proteins, the aggregation formed in heated WPI, and subsequently disappeared when the pH was further decreased with digestion. This pH-dependent aggregation in heated WPI delays the delivery rate of whey protein to the duodenum compared to that of native WPI. However, the hydrolysis rate in heated WPI by pepsin was considerably more than that in unheated WPI.

4.5 Conclusions

The difference in the coagulation of protein and structure of clots during dynamic gastric digestion of various protein ingredients has been explored, and found to have some influences on protein digestibility. During dynamic gastric digestion, SMP and MPC 4851, which contained casein micelles, started to form a coagulum in the early digestion period (in the first 10 min) and at high pH region (pH > 6). However, MPC 4861 and sodium caseinate, which contained no intact casein micellar structure, formed a coagulum at pH < 5 after 40 min of digestion. The clots observed in SMP and MPC 4851 had a closely knit structure, resembling a firm cheese ball, and had a much greater weight than the clots observed in MPC 4861 and sodium caseinate. After 220 min of digestion, the clots for MPC 4861 and sodium caseinate consisted of numerous small, loose, fragmented protein blocks. Such different structures apparently influence the rate of protein hydrolysis by pepsin of the different dairy protein ingredients under gastric conditions. The hydrolysis of the clots derived from SMP and MPC 4851 was much slower than that of those derived from MPC 4861 and sodium caseinate.

Native WPI remained soluble in the stomach and passed through the stomach; the emptied digesta indicated that β-Lg was not hydrolysed by pepsin during the whole period of gastric digestion. In contrast, heated WPI was hydrolysed rapidly by pepsin and formed aggregates when the pH was close to the pI of the whey proteins in the stomach. However, the aggregations were further hydrolysed by pepsin with further digestion.

These results suggest that curd formation occurs at different initial times and pHs in different milk protein ingredients, and that the structure of the clot is dependent on the protein composition, the integrity of the casein micelle, and the processing technology (preheating or membrane technique). These differences lead to different rates of protein hydrolysis of different milk protein ingredients in the stomach, resulting in different compositions of the digesta delivered to the next digestion step.

Chapter 5. The Dynamic Gastric Digestion Behaviours of Skim Milk Powder and Milk Protein Concentrate: the Influence of Protein Concentration

5.1 Abstract

The aim of this study was to investigate the influence of protein concentration (0.5-5.0%, w/w) on the gastric digestion behaviours of skim milk powder (SMP) and milk protein concentrate (MPC) 4851, using a dynamic *in vitro* digestion model, i.e., a human gastric simulator (HGS). The evolution of pH as a function of digestion time, and the protein coagulation and hydrolysis behaviour during gastric digestion were examined. The results showed that the digestion behaviours of SMP and MPC 4851 followed a similar pattern. The rate of pH changes in emptied digesta during digestion was protein concentration dependent. With an increase in protein concentration, the decrease in pH slowed. The protein concentration had no apparent impact on the protein clotting time. The clots formed in the first 10 min of digestion in all of the samples. However, when protein concentration was lower than 2.0% (w/w), the clot consisted of small protein pieces, with a loose, porous and open structure. In contrast, a cheese ball-like clot with a denser network was observed when the protein concentration varied from 2.0% to 5.0% (w/w). Such a difference in the structure apparently affected the rate of protein hydrolysis. A more rapid hydrolysis ($P < 0.05$) of protein was observed when the protein concentration was lower than 2.0% (w/w) compared to the samples containing a higher protein (2.0%-5.0%, w/w). These results suggest that protein concentration does not appreciably affect the clotting time in the SMP and MPC 4851, respectively. However, when protein concentration is very low (i.e., < 2.0% w/w), the structure of the clot would become more loose and fragmented. Such a loose structure causes a significantly increase ($P < 0.05$) in the rate of protein hydrolysis.

5.2 Introduction

Milk protein is an important source of nutrients for human diet. Skim milk powders (SMPs) and milk protein concentrates (MPCs) are two kinds of primary dry dairy ingredients, which are used in a wide range of food applications, e.g. cheese and yogurt manufacture (Oldfield & Singh, 2005).

The digestion behaviours of SMP and MPC with 3.0% (w/w) protein under the simulated gastric conditions have been studied in Chapter 4. It has known that SMP and MPC 4851 can form a clot during dynamic gastric digestion, which is induced by the action of milk-clotting enzyme, pepsin. SMP formed a porous ball-like clot consisting of numerous smaller protein pieces, while a cohesive, cheese-like pellet with dense structure was observed in the MPC 4851.

The objective of the study in this Chapter was to determine digestion behaviour of SMP and MPC 4851 at different protein concentrations.

5.3 Results and Discussion

5.3.1 Skim milk powder

5.3.1.1 Results

(1) pH changes during digestion

Initially, the evolution of pH value of emptied digesta from SMP containing different protein concentrations (0.5-5.0%, w/w) was monitored at different digestion time points of the simulated gastric digestion (Figure 5.1). The initial pH in the stomach represented the pH of the freshly prepared SMP samples, which ranged from pH 6.57 ± 0.18 to pH 7.15 ± 0.08. In general, the pH of all samples (i.e., pH of the emptied digesta) decreased with an increase in digestion time. However, there was a difference in the pH profiles among the samples with a different level of protein. With an increase in protein concentration, the decrease of pH became slower, in particular, at the early stage of digestion. For example, the pH of SMP containing 0.5% (w/w) protein decreased to ~pH 2 at about 80 min and then changed only slightly during further digestion up to 220 min. SMP containing 5.0% (w/w) protein reached to ~ pH 2 at 220 min.

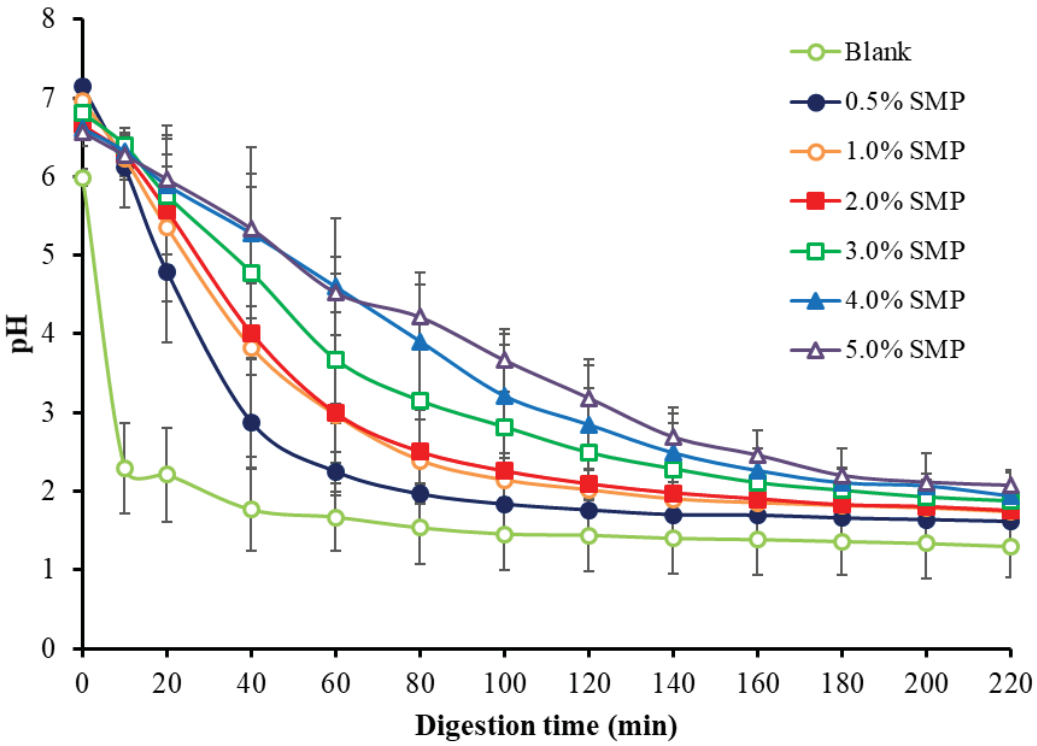


Figure 5.1. Changes in pH of emptied digesta obtained from SMP with different protein concentrations (0.5-5.0%, w/w) as a function of digestion time. Blank refers to a control experiment carried out without any added skim milk powder. SMP, skim milk powder.

(2) Coagulation behaviours

Images of the clots taken from 200 g of SMP containing different concentrations of protein after 220 min of gastric digestion are shown in Figure 5.2. In all samples, protein coagulation occurred in the first 10 min of digestion. Initially, the proteins formed floccules, and then produced a mass of small protein pieces with smooth surface, and finally these protein pieces were adhered to a cheese-like clot under the mechanical gastric peristalsis and contraction. Once the formation of the clot, the serum phase in the stomach gradually became clear, which means the casein micelles have been removed from the solution and incorporated into the coagulum. The serum phase in the all samples was completely clear after digestion in the HGS for about 40 min, no matter what concentration of protein was used. After 220 min of digestion, these structured clots were still observed and, collected from the simulated stomach. The size of the coagulum

appeared to increase along with an increase in protein concentration (Figure 5.2). In the clot collected from the sample containing 0.5% (w/w) and 1.0% (w/w) protein, only a few small protein pieces were observed. While the protein concentration in the samples varied from 2.0% to 5.0% (w/w), a ball-like clot consisting of numerous protein fragments were observed, along with some separated protein fragments.

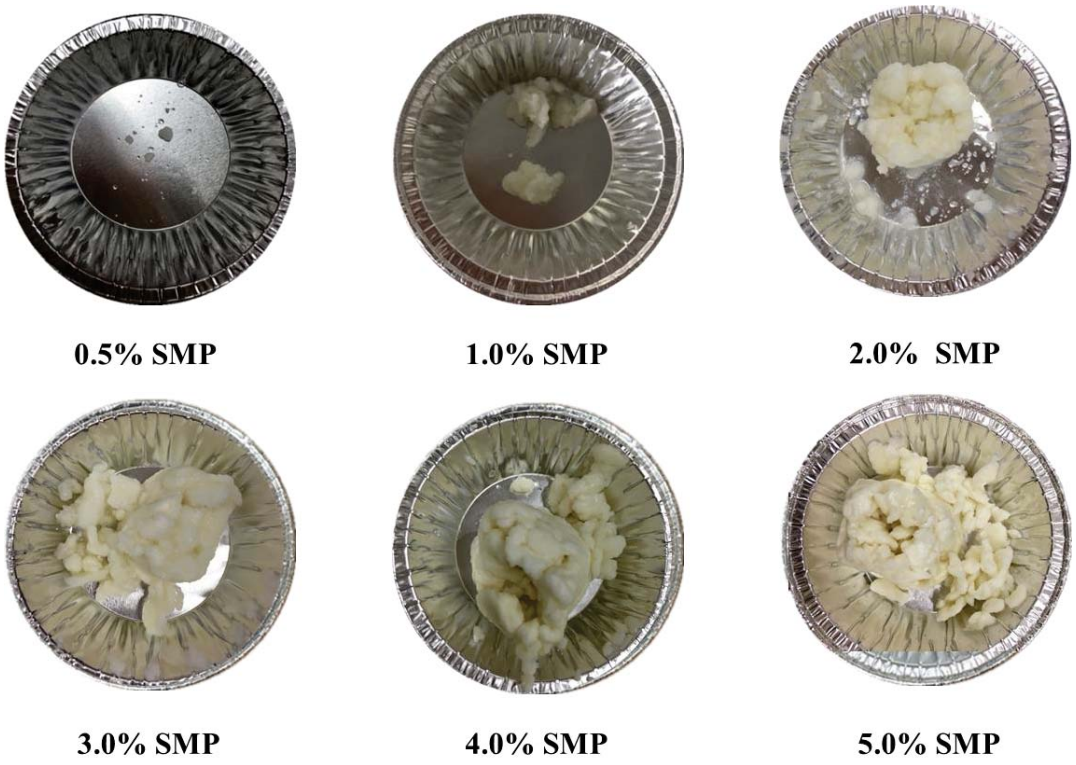


Figure 5.2 Images of the clots obtained from 200 g of SMP containing a different level of protein (0.5-5.0%, w/w) after 220 min of *in vitro* gastric digestion

The wet weights and dry weights of the clots are present in Figure 5.3 and 5.4. Both of these weights showed an increase with increasing protein concentration from 0.5% to 5.0% (w/w). However, no statistically significant difference ($P > 0.05$) was found in the wet weight and dry weight of the samples containing protein between 0.5% and 1.0% (w/w), and between 4.0% and 5.0% (w/w). After 220 min of digestion, the curd weight ratio is calculated and shown in Figure 5.5, which refers to the ratio of dry weight of the curd gained after 220 min of digestion to the protein weight in the initial sample. The protein weight in initial sample can be calculated. For example, a 200 g of SMP sample

with 1.0% (w/w) protein concentration contains 2 g of protein. This ratio represents the extent of undigested protein (i.e. mainly clot) in the stomach after the whole digestion process. With an increase in protein concentration from 0.5% to 2.0% (w/w), the curd weight ratio significantly increased ($P < 0.05$). However, when the protein concentration varied from 2.0% to 5.0% (w/w), no remarkable difference ($P > 0.05$) was observed in the curd weight ratio.

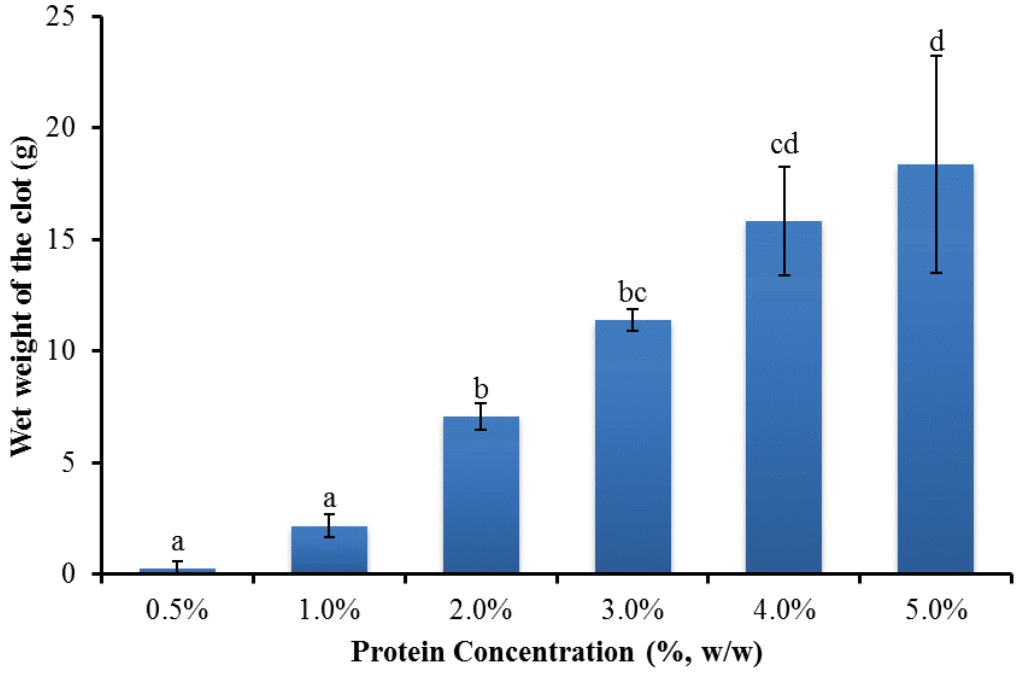


Figure 5.3. Wet weights of the clots obtained after 220 min of gastric digestion of SMP containing different protein concentrations (0.5-5.0%, w/w). Different lowercase letters indicate significant difference ($P < 0.05$) in the wet weight of clots.

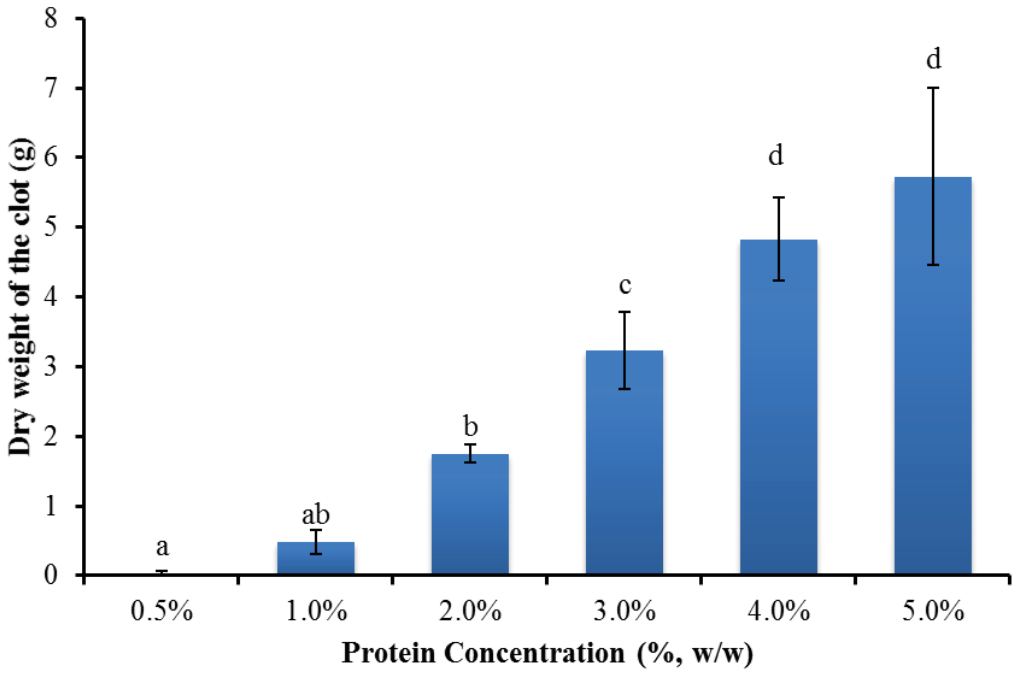


Figure 5.4. Dry weights of the clots obtained after 220 min of *in vitro* gastric digestion of SMP containing different protein concentrations (0.5-5.0%, w/w). Different lowercase letters indicate significant difference ($P < 0.05$) in the dry weight of clots.

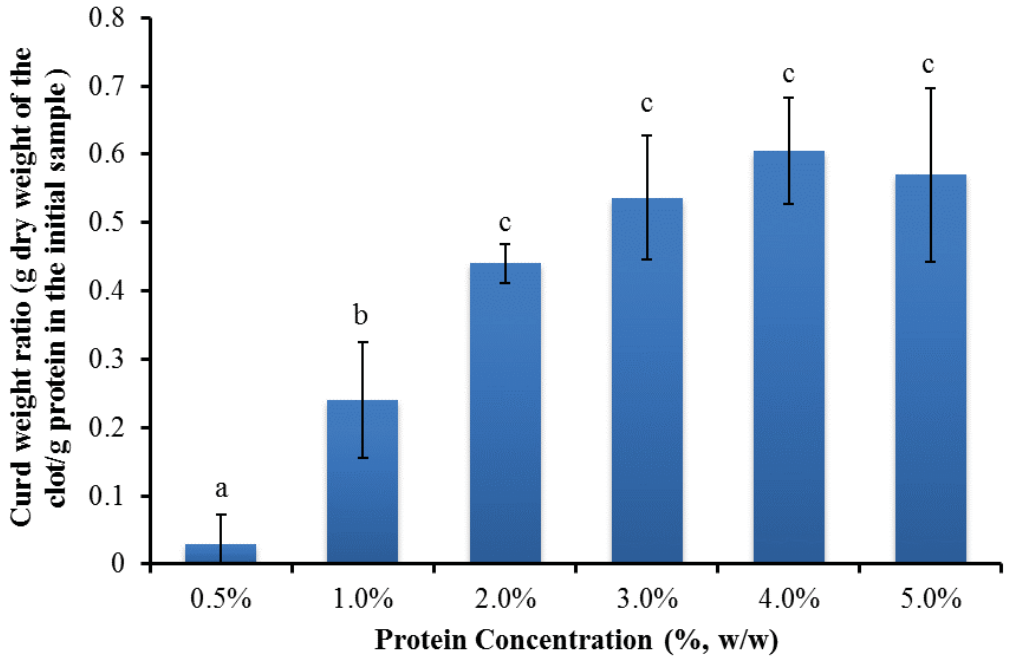


Figure 5.5. The curd weight ratio after 220 min of *in vitro* gastric digestion of SMP containing different protein concentrations (0.5-5.0%, w/w). Different lowercase letters indicate significant difference ($P < 0.05$) in the curd weight ratio (g dry matter in the clot/g protein in the initial sample).

(3) The microstructure of the clots

The microstructure of the clot obtained after 220 min of gastric digestion from the selected protein concentration (0.5%, 2.0% and 5.0%, w/w) of the SMP samples were observed using confocal laser scanning microscopy (Figure 5.6). The curd produced in the sample containing 0.5% (w/w) protein showed an open, porous and ragged matrix network, with a mass of small pores, along with some large voids. There was no considerable difference in the microstructure of the clots generated in SMP containing 2.0% and 5.0% (w/w) protein. Both of them showed a higher density and a larger size than that was observed in the SMP sample containing 0.5% (w/w) protein. Moreover, the curds derived from the SMP containing 2.0% and 5.0% (w/w) protein consisted of some closely knitted protein blocks, with more smooth surface and less pores throughout the matrix.

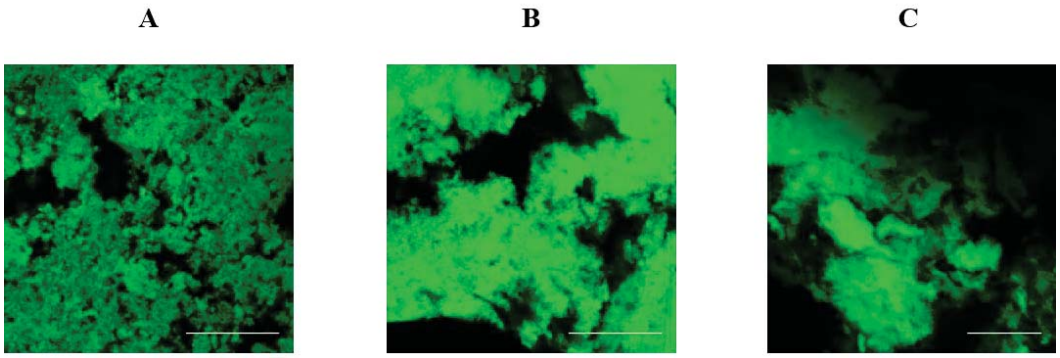


Figure 5.6. The microstructure of the clots obtained from 200 g of SMP solution containing (A) 0.5%, (B) 2.0%, and (C) 5.0% (w/w) protein after 220 min of *in vitro* gastric digestion. The scale bar in all images is 50 μm.

(4) Protein hydrolysis

SDS-PAGE pattern of clots

The protein composition of the clot produced from the SMP samples containing a different level of protein was determined using electrophoretic analysis under reducing conditions (Figure 5.7). In all samples collected from SMP, α_s-casein (α_{s1}-casein+α_{s2}-casein) and β-casein bands were observed. However, in the SMP samples containing protein from 0.5% to 2.0% (w/w), the intensities of α_s-casein and β-casein bands were less than that of the samples containing protein from 3.0% to 5.0% (w/w). As a response, the SMP samples with a protein concentration from 0.5% to 2.0% (w/w) generated more abundant peptides compared to the samples containing a protein concentration from 3.0% to 5.0% (w/w). The κ-casein bands were not observed in the samples with a lower protein concertation (0.5%-2.0%, w/w). However, the faint κ-casein bands were seen in the samples with a higher protein concentration (3.0%-5.0%, w/w). A newly formed band was observed at around 15 kDa in the clot from all SMP samples, correspondingly. This was probably the degradation product of κ-casein, para-κ-casein (Miranda & Pelissier, 1983). Moreover, there was another newly generated band appeared at around 20 kDa in all samples, probably consisting of the hydrolysis product from caseins (Ye et al., 2016b). In addition, the clear whey proteins (β-Lg and α-La) bands were observed in the curds of all samples.

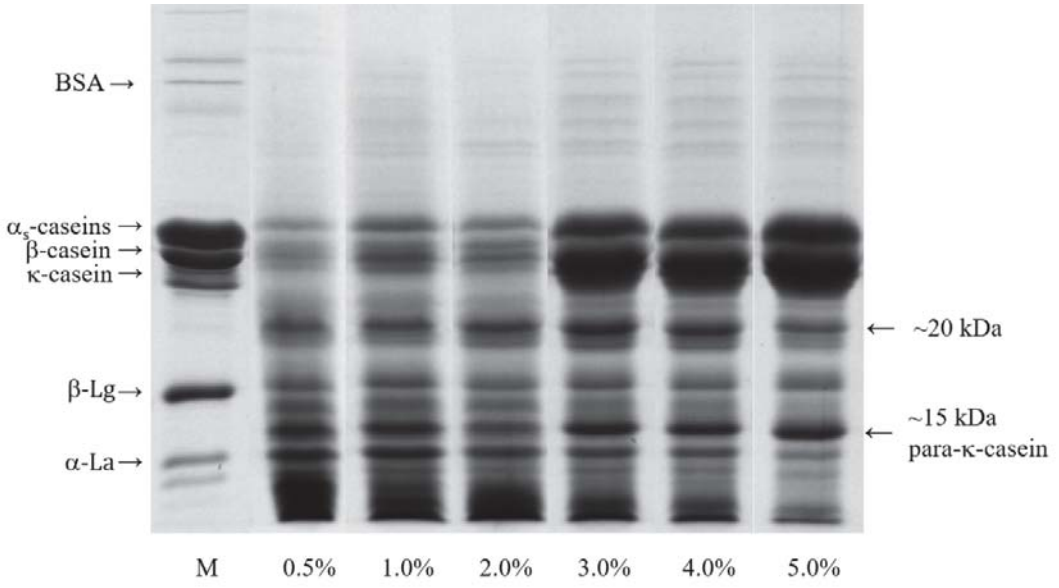
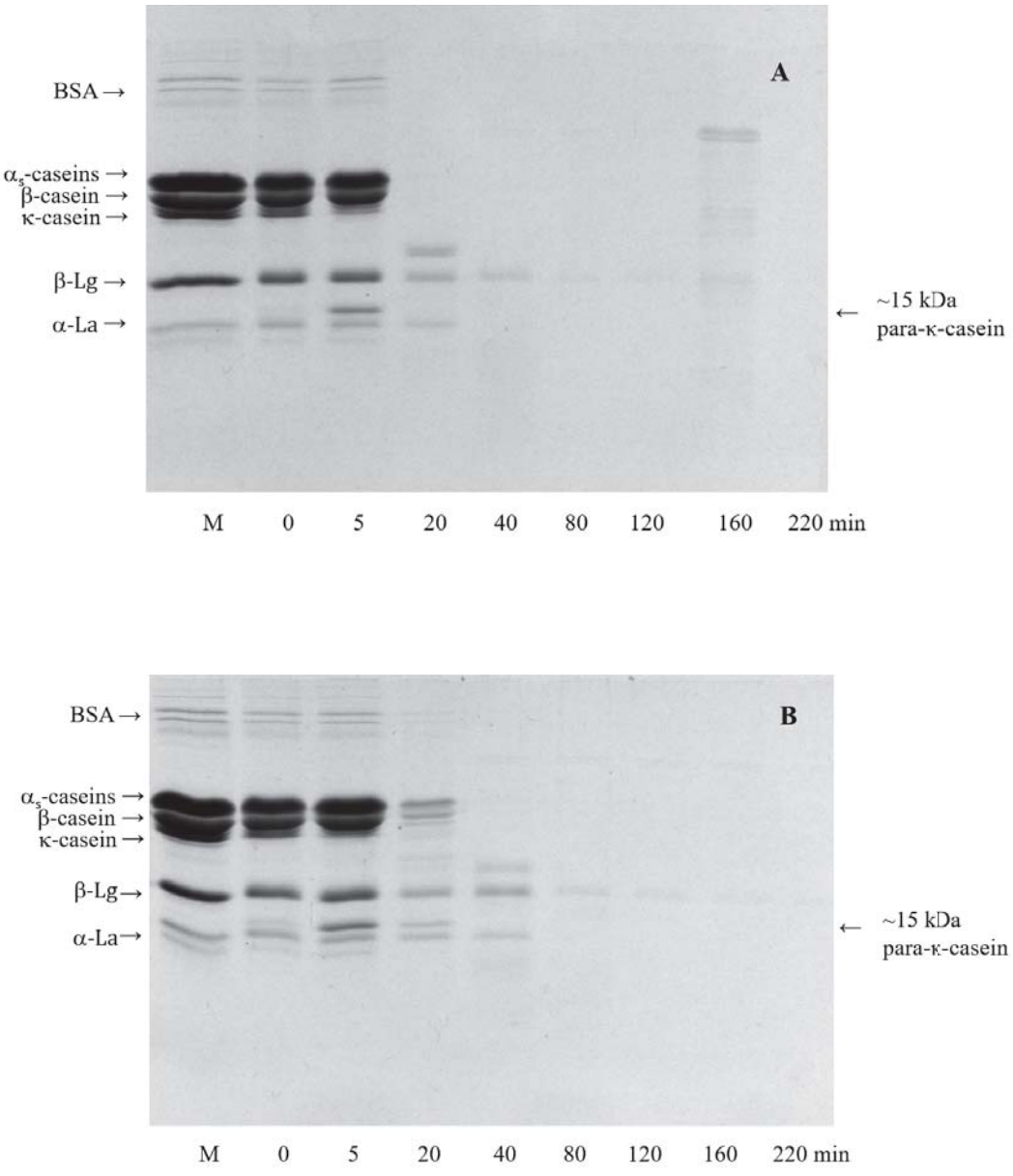
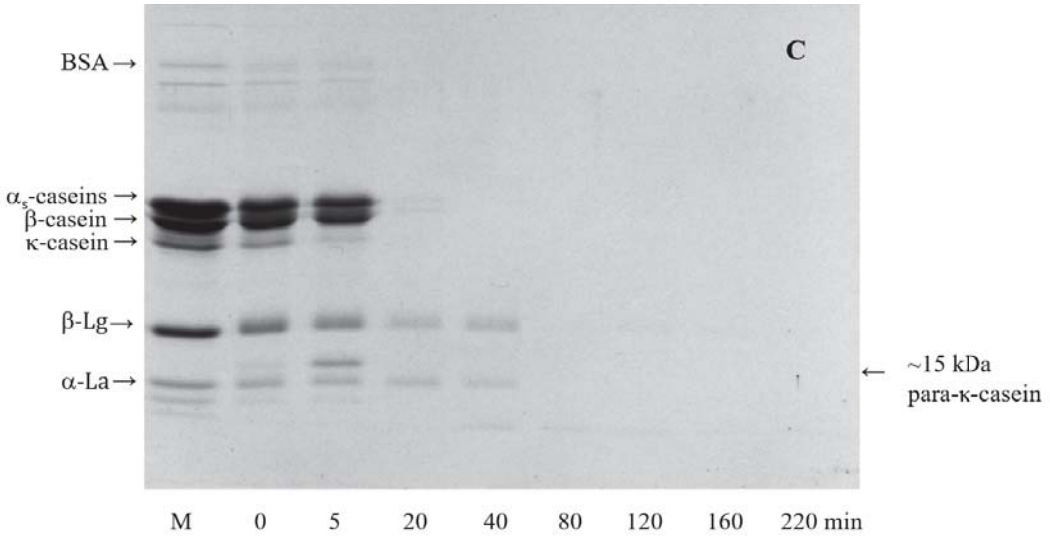


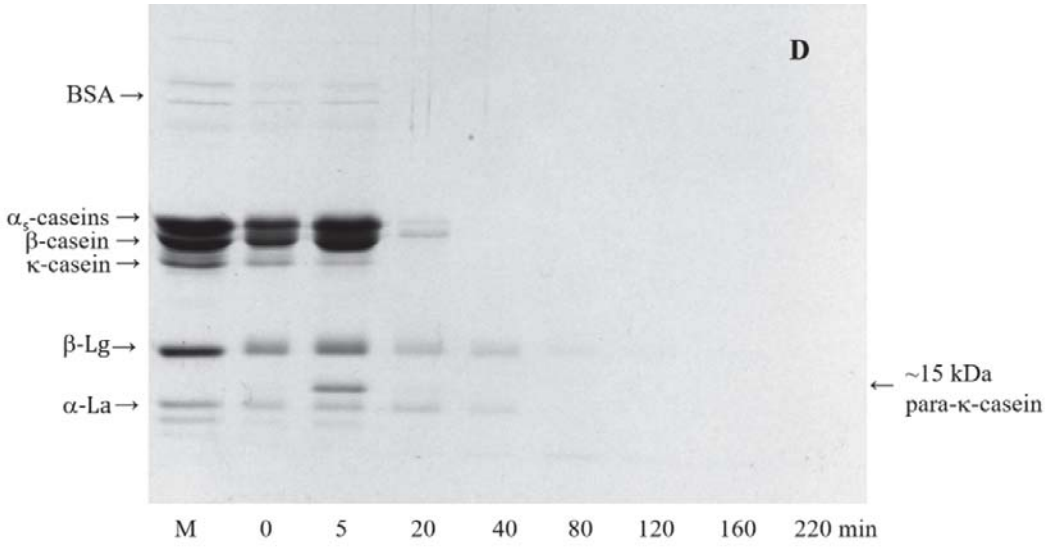
Figure 5. 7. The SDS-PAGE pattern under reducing conditions of the clots collected from the SMP samples with different protein concentrations (0.5-5.0%, w/w) after 220 min of gastric digestion in the HGS. M, trim milk; BSA, bovine serum albumin.

SDS-PAGE pattern of emptied digesta

The emptied digesta from SMP with different protein concentrations (0.5%-5.0%, w/w) showed no considerable differences in protein composition and hydrolysis profile during digestion (Figure 5.8). At 5 min of digestion, the κ-casein band became less intense, with the appearance of a new band at around 15 kDa, probably consisting of para-κ-casein (Miranda & Pelissier, 1983). With further digestion, the α_s-caseins and β-casein bands almost disappear at 20 min. All of the casein bands and para-κ-casein bands were not observed at 40 min in the samples containing 0.5% to 3.0% (w/w) protein, and very faint in the samples containing 4.0% to 5.0% (w/w) protein. For whey proteins, the intact β-Lg, α-La and bovine serum albumin (BSA) bands were involved in the digesta emptied from all samples at 5 min of digestion. With further digestion, the BSA band was almost invisible at 20 min, the α-La band disappeared at 40 min in the emptied digesta from the sample containing 0.5% (w/w) protein, and disappeared after 40 min in other samples. The band intensity of β-Lg gradually decreased with increasing digestion time, and almost cannot be detected at 120 min.


A
BSA →
α_s-caseins →
β-casein →
κ-casein →
β-Lg →
α-La →
← ~15 kDa
para-κ-casein
M 0 5 20 40 80 120 160 220 min
B
BSA →
α_s-caseins →
β-casein →
κ-casein →
β-Lg →
α-La →
← ~15 kDa
para-κ-casein
M 0 5 20 40 80 120 160 220 min


C
BSA →
α_s-caseins →
β-casein →
κ-casein →
β-Lg →
α-La →
← ~15 kDa
para-κ-casein
M
0
5
20
40
80
120
160
220 min


D
BSA →
α_s-caseins →
β-casein →
κ-casein →
β-Lg →
α-La →
← ~15 kDa
para-κ-casein
M
0
5
20
40
80
120
160
220 min

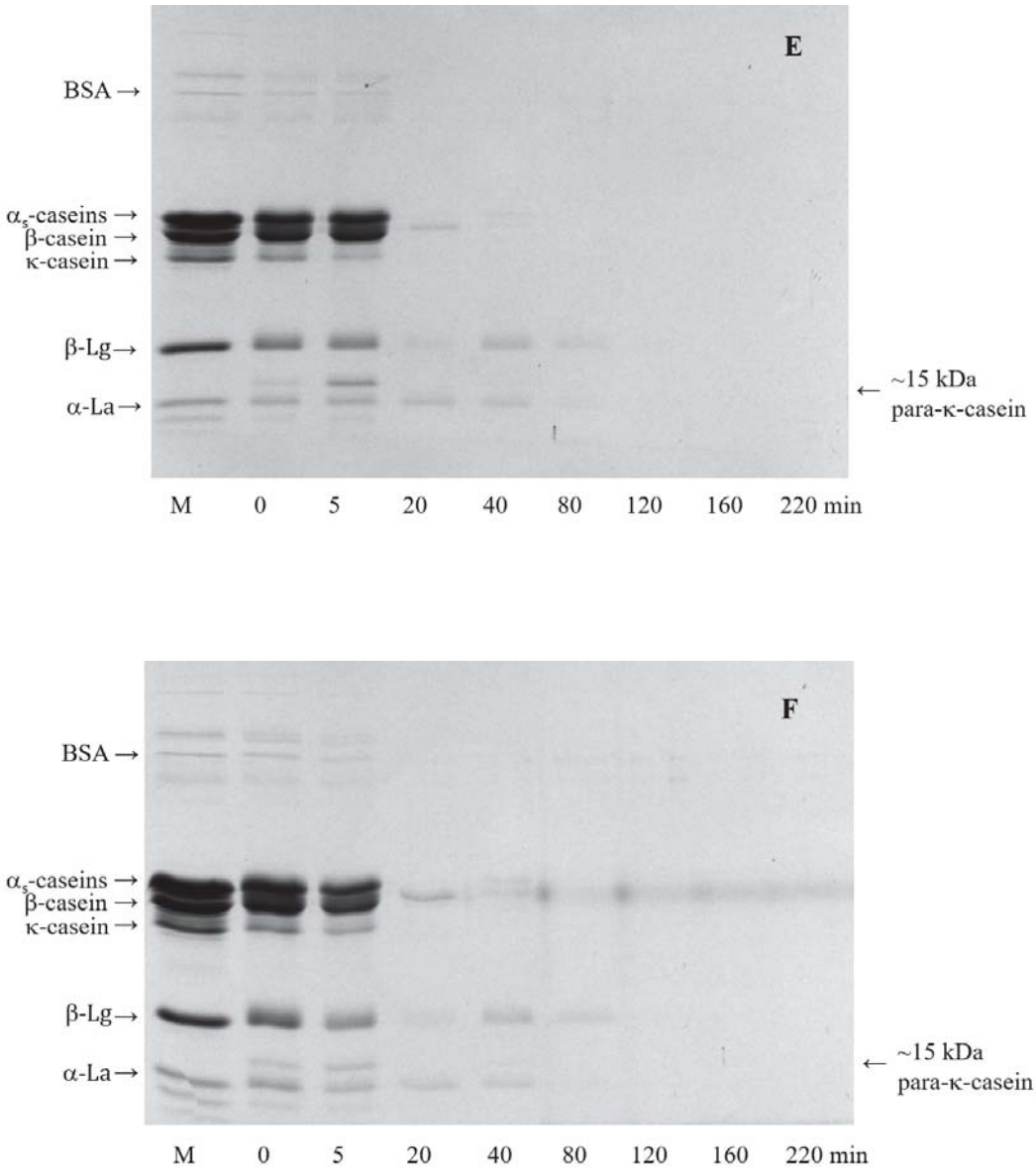


Figure 5.8. SDS-PAGE patterns of emptied digesta collected from the SMP samples containing (A) 0.5%, (B) 1.0%, (C) 2.0%, (D) 3.0%, (E) 4.0% and (F) 5.0% (w/w) protein during 220 min of gastric digestion in the HGS. M. trim milk; BSA, bovine serum albumin.

5.3.1.2 Discussion

(1) Influence of protein concentration on pH profiles during gastric digestion

Protein concentration plays an important role in the rate of pH decrease during dynamic gastric digestion. The decrease in pH slowed with an increase in protein concentration from 0.5% to 5.0% (w/w), in particular at the early stage of digestion. The difference may be attributed to the buffer capacity of milk proteins. With increasing protein concentration, the amount of the constituents in the milk protein which are responsible for buffer capacity would increase, such as some acidic amino acids of whey protein and phosphoserine residues of caseins (Clark, Thompson, & Rokahr, 1983). This difference may also be contributed to the structure of the clot. The clot taken from SMP with a lower level of protein showed a smaller size, which means that the contact area to the surrounding liquid was limited. So that the proteins in the clot may only exert a limited buffering action. In contrast, the clot obtained from the samples containing a higher protein concentration had a larger size, leading to a greater buffering action and a slowdown of the decrease in the pH (Ye et al., 2016b).

(2) Influence of protein concentration on protein coagulation during digestion

The impact of protein concentration on the protein coagulation behaviour in the HGS is discussed from the perspectives of coagulation time and the structure of the clots. Firstly, in SMP containing different concentrations of protein (0.5%-5.0%, w/w), the onset time of protein coagulation had no considerable difference, occurring within the first 10 min of gastric digestion. Furthermore, along with the formation of the clot, almost all casein micelles and some whey protein were incorporated in the first 40 min and remained in the stomach, no matter what concentration of protein was used. It is consistent to electrophoretic results (Figure 5.9). The possible reasons of whey protein being incorporated in the curd have been discussed in the Chapter 4. These observations indicated that the protein concentration (0.5%-5.0%, w/w) of SMP has no essential effect on protein clotting time, in particular in the onset time of coagulation.

When the protein concentration was 0.5% and 1.0% (w/w), many small protein fragments were observed in the clots. The structure of the clots was more loose and open. However, when the protein concentration varied from 2.0% to 5.0% (w/w), a ball-like clot consisting of numerous protein fragments was observed, with a more compact

structure. It is probable that under same conditions of the mechanical gastric contraction, with an increase in the protein concentration, the size of the clot increased, so that the structure of the clot was considered to become more compact.

(3) Influence of protein concentration on protein hydrolysis

As observed in SMP with a lower level of protein (0.5% and 1.0%, w/w) (Figure 5.3), at the end of digestion (220 min), the clots were no longer ball-like structure, they instead consisted of some smaller protein pieces. The breakdown of the clots, presumably, might follow such a process: the clot with a cheese-ball structure was broken into some smaller pieces and finally disappeared if the digestion time was long enough.

Our results (i.e., curd weight ratio) indicated that the protein concentration had a great impact on the extent of proteolysis of the clot (Figure 5.5). The hydrolysis of the clot from SMP sample with a lower protein concentration (0.5% and 1.0%, w/w) was significantly more efficient ($P < 0.05$) than that of the samples with a higher protein concentration (2.0%-5.0%, w/w). After 220 min of digestion, the weight of undigested clotted protein (i.e., mainly casein) increased progressively ($P < 0.05$) with the protein concentration increased from 0.5% to 2.0% (w/w). However, no further increase ($P > 0.05$) was observed when protein concentration varied from 2.0% and 5.0% (w/w) (Figure 5.5). This difference will be discussed from two aspects: (i) the structure of the clot and (ii) enzymatic reaction activity affected by pH.

Firstly, the different extent of proteolysis was probably because the clot formed from a sample containing a relatively low protein concentration had a more fragmented, open and loose structure. Over 220 min of digestion process, the size of the curd increased with increasing concentration of protein. The clots were comprised of more or less protein crumbles (Figure 5.2), which is probably caused by the heat treatment during manufacturing process. It was reported that the rennet clot from heated milk is not as cohesive as that formed from unheated milk (Anema et al., 2007). However, it can still be bonded to a ball-like structure. The structure of the clot played an important role on the hydrolysis degree of the curd. The clot appeared to have a more compact structure at a higher protein concentration (2.0%-5.0% w/w), with a larger size. The hydrolysis of the milk coagulum by pepsin has been shown to proceed from surface to interior of the clot

(Ye et al., 2016b). Such a clot with a larger size and a denser structure appears to prevent the diffusion of pepsin into the protein matrix. In contrast, when the protein concentration varied from 1.0% to 0.5% (w/w), the structure of the clot became more open, porous and loose. The clots were made up of numerous small protein pieces. Such a looser structure provides more abundant channel for the surrounding liquid to diffuse on the surface of the curd. As a result, the contact area of protein pieces with pepsin increased, leading to an increase in the hydrolysis extent.

Secondly, the pepsin activity affected by the reduction rate of pH would have an effect on hydrolysis of the clotted protein. As discussed above, with an increase in the protein concentration, the reduction rate of pH during the digestion process decreased (Figure 5.1). The samples with a higher level of protein was probably in a higher pH environment, leading to a slower digestion by pepsin. Because the proteolytic activity of pepsin is pH dependent. It has an optimum of proteolytic activity at pH 2, a considerable activity at pH 3.5 (Axelsson, Axelsen, Szecsi, & Foltmann, 1983). It is able to digesta proteins at pH 5.0 or less but inhibited above pH 5.0 (Northrop, 1931).

5.3.2 MPC 4851

5.3.2.1 Results

(1) pH change during digestion

The initial pH in the stomach represented the pH value of MPC 4851 samples containing various protein concentrations (0.5-5.0%, w/w), which ranged from pH 6.96 ± 0.06 to pH 7.33 ± 0.13 (in Figure 5.9). The pH of all samples (i.e., pH of the emptied digesta) decreased generally with an increase in digestion time. However, there was a noticeable difference in pH profiles among the samples with a different protein concentration. In all samples, the rate of pH decreases showed an apparent decrease with increasing protein concentration, in particular at the early stage of digestion. For example, the pH of MPC 4851 sample containing 0.5% (w/w) protein decreased to ~ pH 2.5 at about 40 min and then varied slightly with further digestion. Whereas the MPC 4851 containing 5.0% (w/w) protein showed a markedly lesser reduction in pH, reaching ~pH 2.5 at about 180 min.

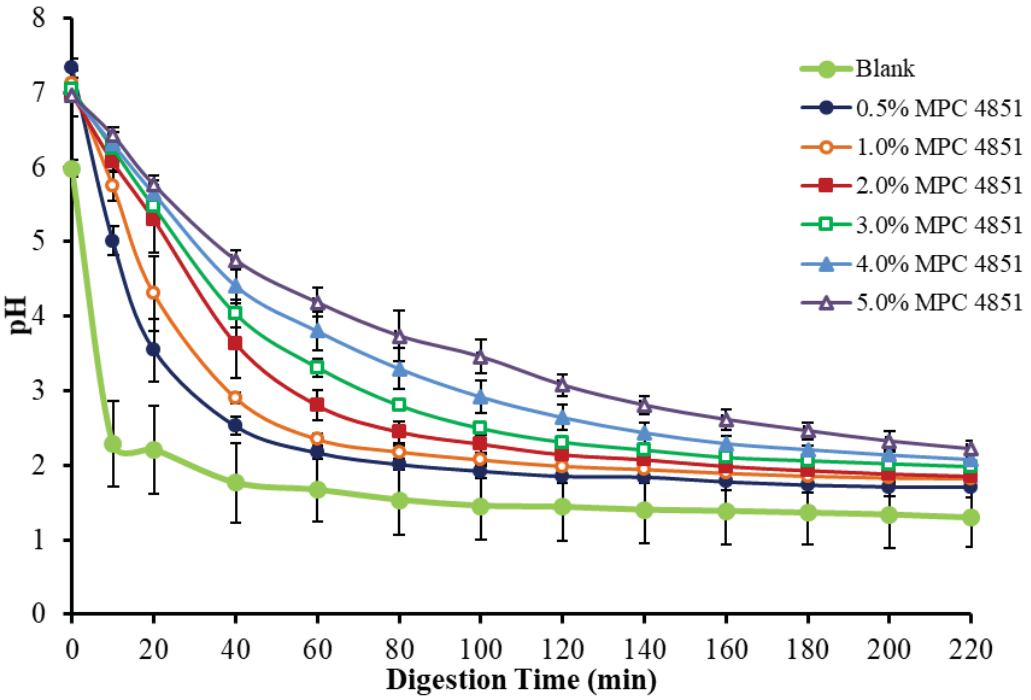


Figure 5.9. Changes in pH of emptied digesta obtained from MPC 4851 samples with different protein concentrations (0.5-5.0%, w/w) as a function of digestion time. Blank refers to a control experiment carried out without any added MPC 4851.

(2) Coagulation behaviours

Images of the clots collected from MPC 4851 containing different protein concentrations (0.5-5.0%, w/w) after 220 min of gastric digestion are shown in Figure 5.10. In all samples, the initial protein coagulation occurred within the first 10 min of digestion in the HGS. Over the 220 min of digestion, these curds did not disappear. The curd generated from MPC 4851 with 0.5% (w/w) protein consisted of some smaller protein fragments. For MPC 4851 sample containing 1.0% (w/w) protein, a larger clot fragment was observed. However, when MPC 4851 samples contained a higher concentration of protein (2.0-5.0%, w/w), a highly dense structured curd was observed, resembling a cheese-like pellet, with a cohesive and elastic texture, along with some individual protein fragments. The size of the clot showed an increase with increasing protein concentration.

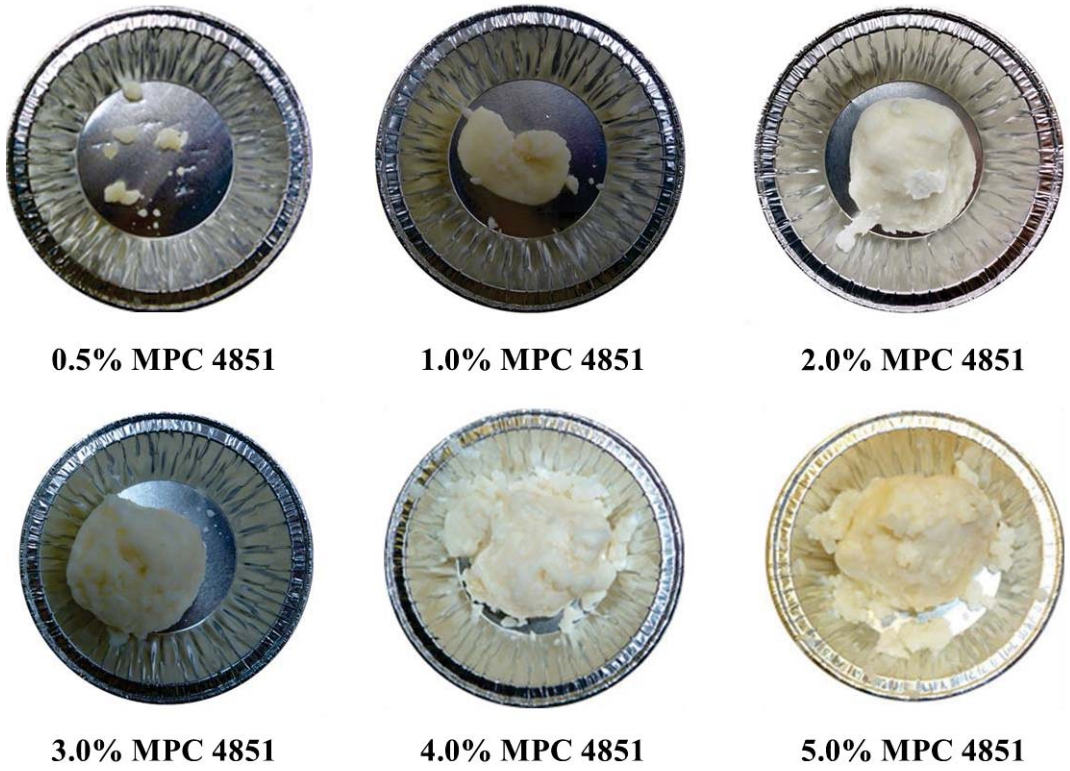


Figure 5 10. Images of clots obtained from 200 g of MPC 4851 solutions containing a different level of protein (0.5-5.0%, w/w) after 220 min of gastric digestion in the HGS.

Both wet weight and dry weight of the clots taken from MPC 4851 showed significant increases ($P < 0.05$) with an increase in protein concentration from 1.0% to 5.0% (w/w) (Figures 5.11 and 5.12). However, no considerable difference ($P > 0.05$) was observed in the both wet weight and dry weight of the clots when the protein concentration was equal or less than 1.0% (w/w). The curd weight ratio (i.e., the ratio of dry weight of the curd obtained after 220 min of digestion to the protein weight in the initial sample) is calculated and shown in Figure 5.13. There was no appreciable difference ($P > 0.05$) in the curd weight ratio among MPC 4851 samples containing a higher concentration of protein (2.0%-5.0%, w/w). However, the curd weight ratio showed a considerable decrease ($P < 0.05$) with decreasing protein concentration from 2.0% to 0.5% (w/w).

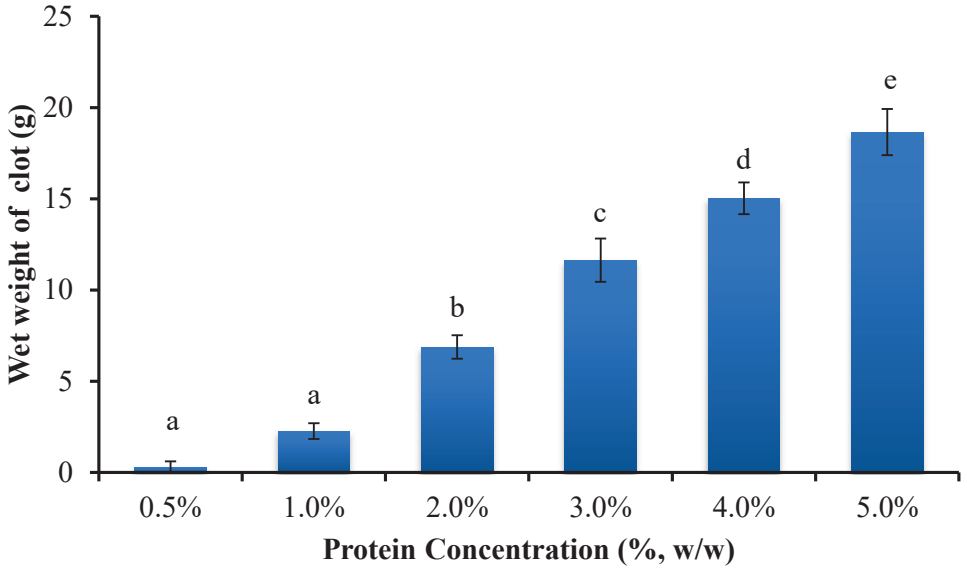


Figure 5 11. Wet weights of the clots obtained after 220 min of in vitro gastric digestion of MPC 4851 samples containing different protein concentrations (0.5-5.0%, w/w). Different lowercase letters indicate significant difference ($P < 0.05$) in the wet weight of the clot.

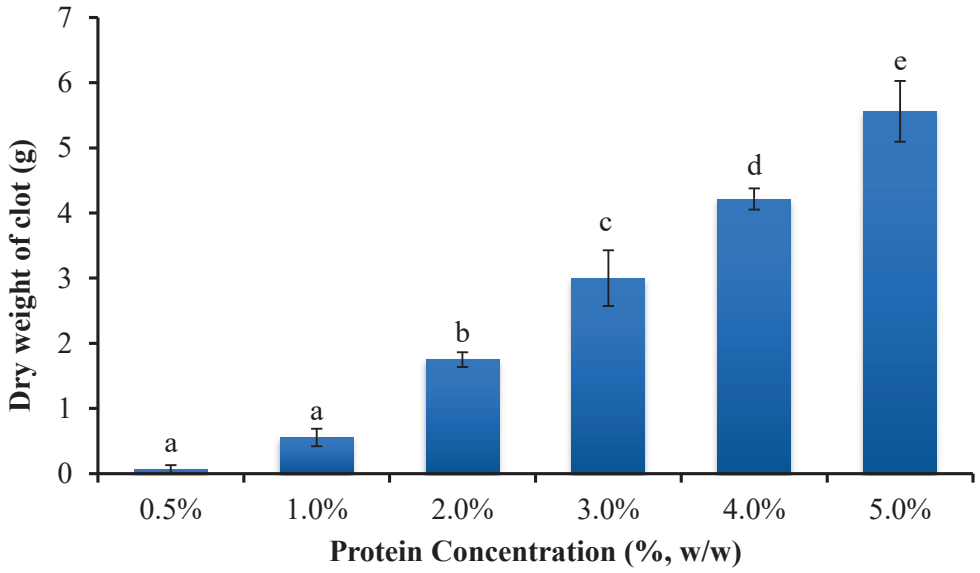


Figure 5.12. Dry weights of the clots obtained after 220 min of *in vitro* gastric digestion of MPC 4851 samples containing different protein concentrations (0.5-5.0%, w/w).

Different lowercase letters indicate significant difference ($P < 0.05$) in the dry weight of the clot.

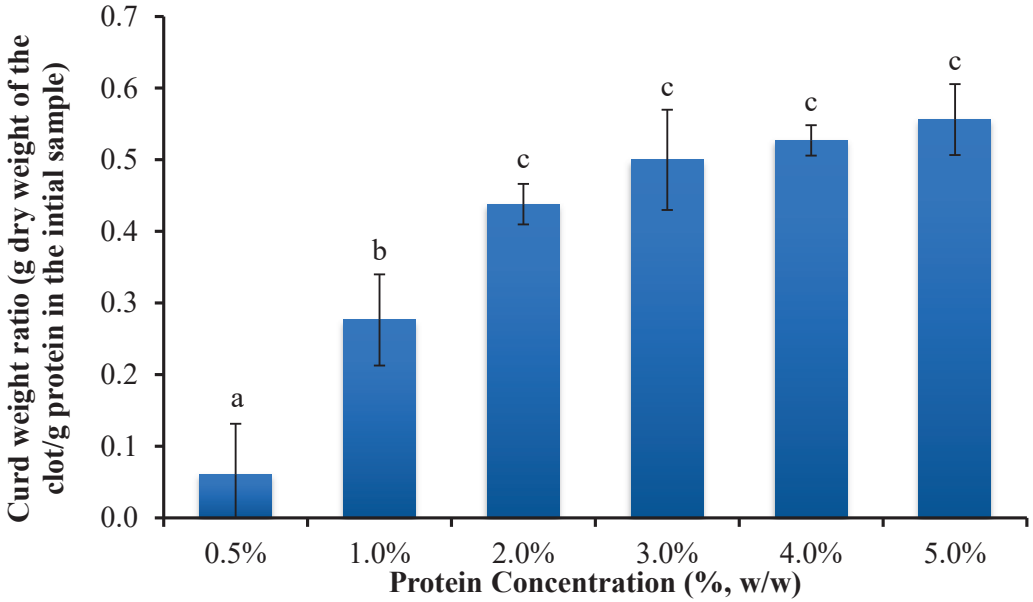


Figure 5.13. The curd weight ratio of MPC 4851 samples containing different protein concentrations (0.5-5.0%, w/w). Different lowercase letters indicate significant difference ($P < 0.05$) in the curd weight ratio (g dry weight of the clot/g protein in the initial sample).

(3) The microstructure of the clots

The microstructure of the clots obtained after 220 min of gastric digestion from the selected protein concentration (0.5%, 2.0% and 5.0%, w/w) of MPC 4851 samples were observed using confocal laser scanning microscopy (Figure 5.14). In the clot sample produced from MPC 4851 containing 0.5% (w/w) protein, the structured protein matrix was uneven and relatively ragged, consisting of some protein blocks and crumbled pieces, with some pores on the surface. However, no remarkable difference was observed in the microstructure of the clot formed from MPC 4851 with 2.0% and 5.0% (w/w) protein. Some greater protein blocks, separated from the aqueous phase, with a highly dense structure and a larger size were observed in both clots, in which almost no voids were observed.

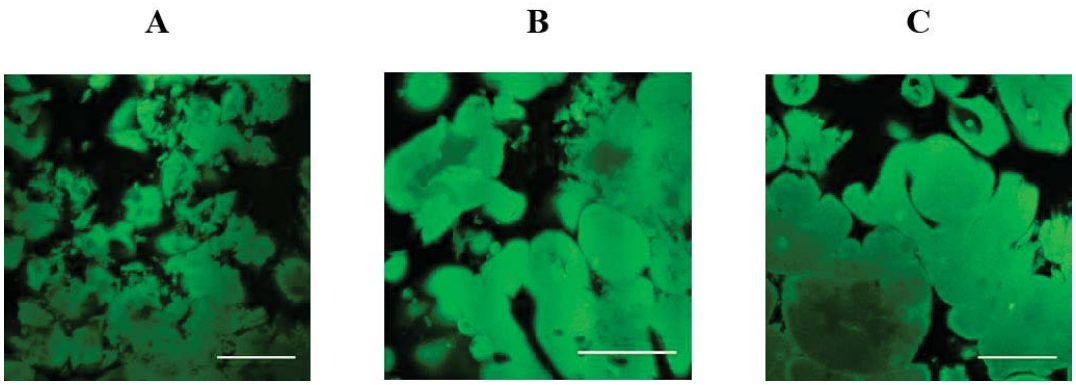


Figure 5.14. The microstructure of the clots obtained from 200 g of MPC 4851 samples containing (A) 0.5%, (B) 2.0%, and (C) 5.0% (w/w) protein after 220 min of gastric digestion. The scale bar in all images is 50 μm.

(4) Protein hydrolysis

SDS-PAGE pattern of clots

In all samples, clear α_s-casein (α_{s1}-casein+α_{s2}-casein) and β-casein bands were observed (Figure 5.15). The κ-casein band was very faint, as a response, a newly formed band with a strong intensity appeared at around 15 kDa, probably consisting of para-κ-casein (Miranda & Pelissier, 1983). Another newly produced band at around 20 kDa was also observed, which probably consisted mainly of hydrolysed products from caseins (Ye et al., 2016b). The faint β-Lg and α-La bands were also observed from all MPC 4851 samples. Compared to the 0.5% (w/w) MPC 4851, no significant differences were observed in the samples with a protein concentration from 1.0% to 5.0% (w/w). However, the intensities of the casein bands in the MPC 4851 sample containing 0.5% (w/w) protein were less than that of the other samples, correspondingly, its peptide bands were more intense than the other samples.

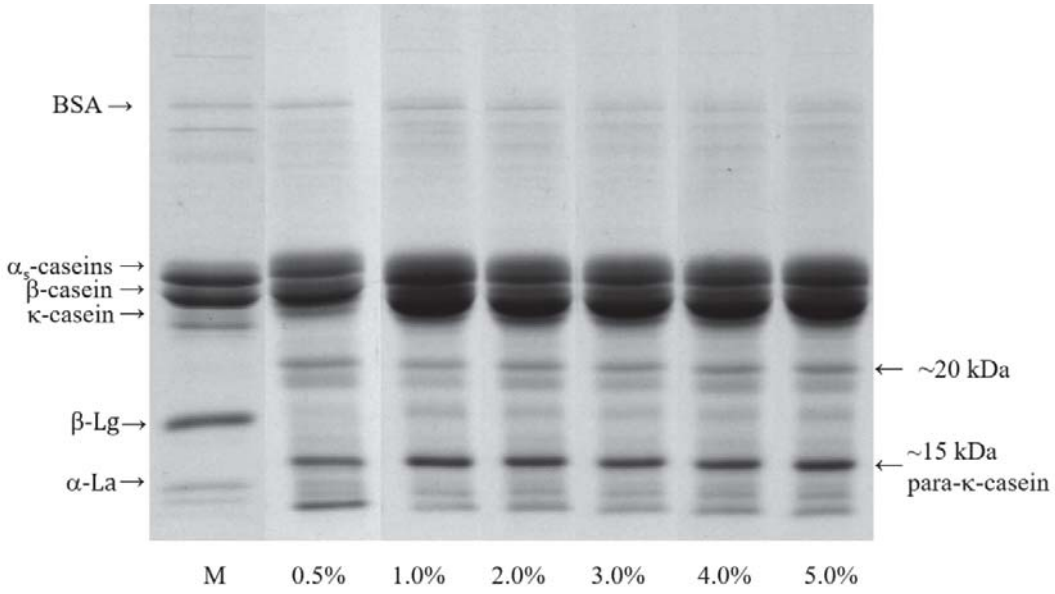
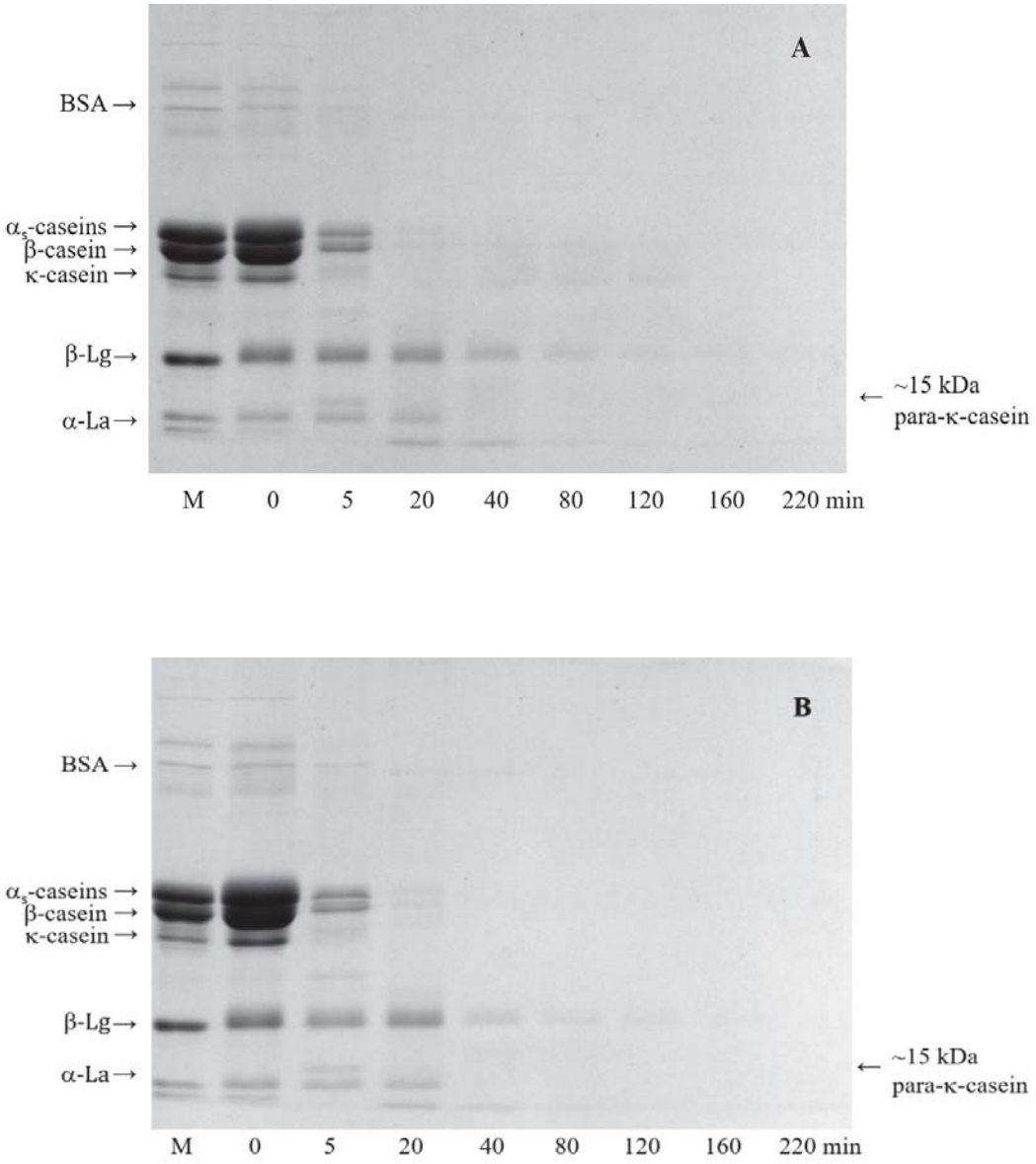
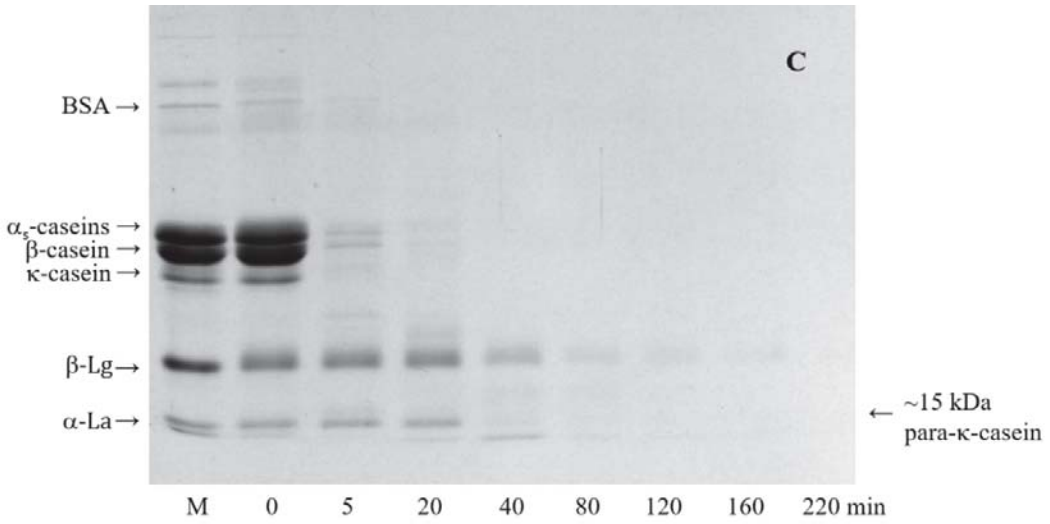


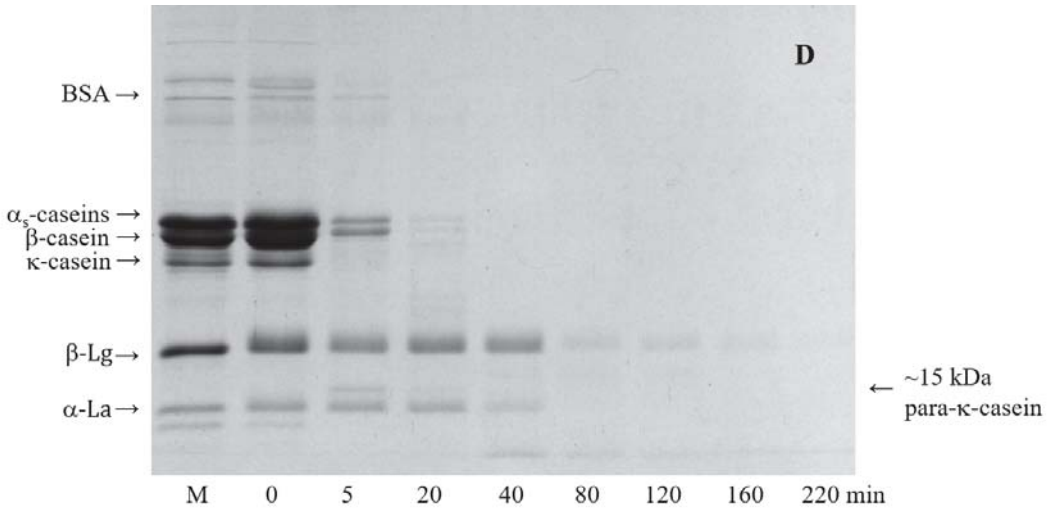
Figure 5.15. The SDS-PAGE pattern of the clots collected from the MPC 4851 samples containing different protein concentrations (0.5-5.0%, w/w) after 220 min of gastric digestion in the HGS. M, trim milk. BSA, bovine serum albumin.

SDS-PAGE pattern of emptied digesta

There was no significant difference observed in the SDS-PAGE patterns of the emptied digesta in all samples during 220 min digestion (Figure 5.16). At 5 min of digestion, the emptied gastric digesta samples contained intact caseins (α_s-, β- and κ-casein), and whey proteins (β-Lg, α-La and BSA) bands. The α_s-caseins and β-casein bands were less intense than that was observed at 0 min. The κ-casein band was almost not observed in all samples at 5 min, along with the appearance of the newly formed para-κ-casein band at about 15 kDa. With an increase in digestion time, all of the casein bands and para-κ-casein bands were almost invisible at 20 min and disappeared completely at 40 min. The band intensity of β-Lg gradually decreased until disappearing at 220 min. While BSA band was not observed at 40 min and α-La band was absent at 80 min in all MPC 4851 samples.


A
BSA →
α_s-caseins →
β-casein →
κ-casein →
β-Lg →
α-La →
← ~15 kDa
para-κ-casein
M 0 5 20 40 80 120 160 220 min
B
BSA →
α_s-caseins →
β-casein →
κ-casein →
β-Lg →
α-La →
← ~15 kDa
para-κ-casein
M 0 5 20 40 80 120 160 220 min


C
BSA →
α_s-caseins →
β-casein →
κ-casein →
β-Lg →
α-La →
← ~15 kDa
para-κ-casein
M 0 5 20 40 80 120 160 220 min


D
BSA →
α_s-caseins →
β-casein →
κ-casein →
β-Lg →
α-La →
← ~15 kDa
para-κ-casein
M 0 5 20 40 80 120 160 220 min

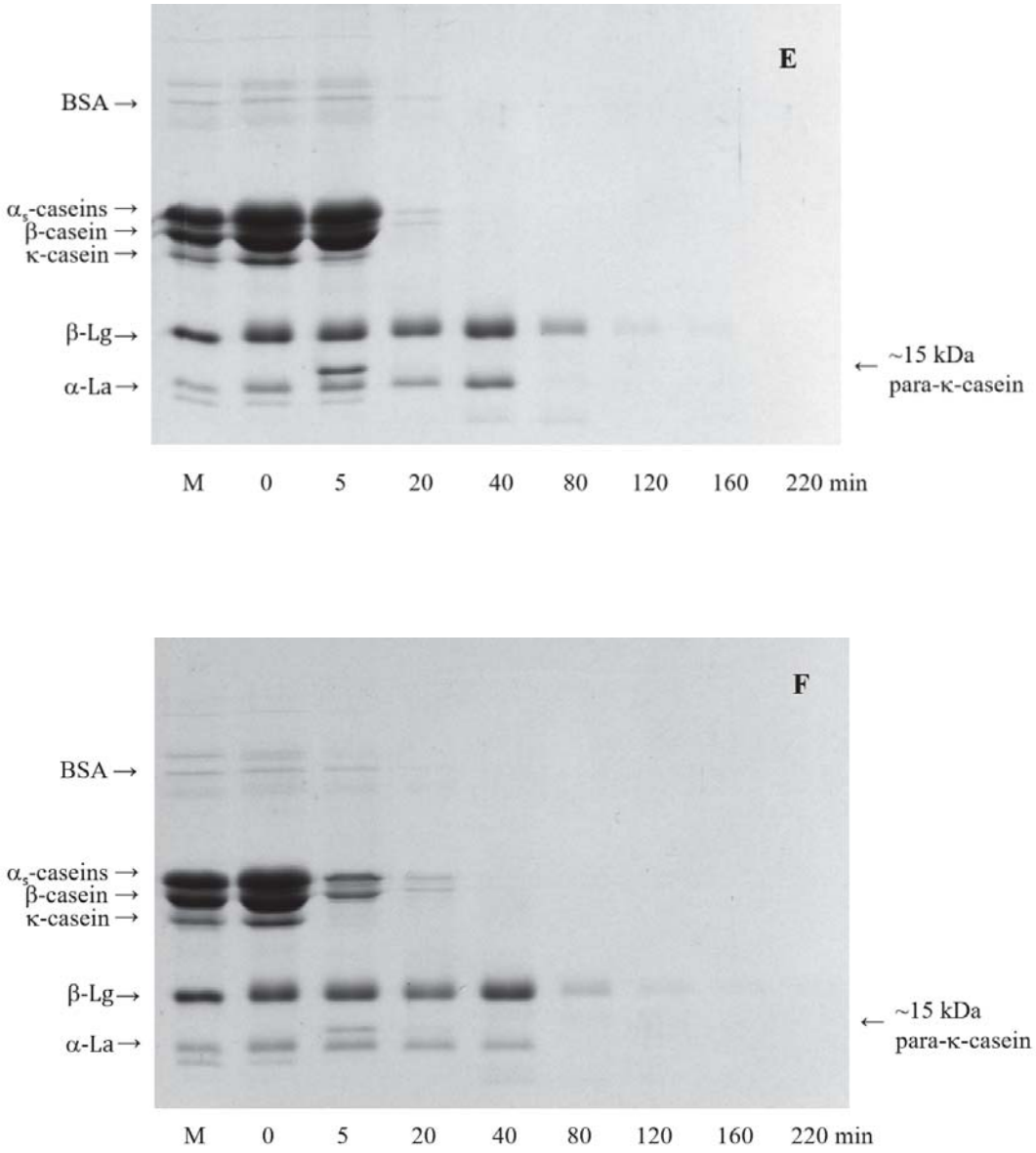


Figure 5.16. SDS-PAGE patterns of the emptied digesta collected from the MPC 4851 samples containing (A) 0.5%, (B) 1.0%, (C) 2.0%, (D) 3.0%, (E) 4.0% and (F) 5.0% (w/w) during 220 min of gastric digestion in the HGS. M. trim milk. BSA, bovine serum albumin.

5.3.2.2 Discussion

(1) Influence of protein concentration on pH profiles during digestion

The relationship between the pH and protein concentration in MPC 4851 (Figure 5.9) is similar to that observed in SMP. The decrease rate of pH in the emptied digesta was affected by the protein concentration of the samples. The decrease in pH became more pronounced with decreasing protein concentration from 5.0% to 0.5% (w/w). This difference is best explained by two factors: the buffer capacity of milk proteins and the structure of the clot formed in MPC 4851. First, the buffer action of milk protein is considered as the most important factor. As mentioned in section 5.3.1.2(1), the amount of the constituents in milk protein being responsible for the buffer capacity would increase with increasing protein concentration. MPC 4851 containing a higher protein concentration presents a more prominent buffering action than the sample with a lower protein concentration. Second, the structure of the curd formed in MPC 4851 may be another contributor to the different reduction rate in pH. The clot collected from MPC 4851 containing a lower level of protein showed a smaller size, thereby the accessibility of the curd to the surrounding liquid was very limited. As a result, the proteins in the clot only can exert a limited buffering action. In contrast, the clot obtained from the samples containing a higher protein concentration had a larger size, resulting in a greater buffering action and a slowdown of the reduction of pH (Ye et al., 2016b).

(2) Influence of protein concentration on protein coagulation behaviour during digestion

In all samples, protein coagulated in the first 10 min after being digested in the HGS. The majority of the casein micelles have been incorporated in the clot network in the first 20 min. The full development of the clot was in the first 40 min of digestion, regardless of the concentration of protein in the samples. These results suggest that the protein concentration does not have a great impact on protein coagulation time in MPC 4851.

However, the protein concentration had some impacts on the structure of the clot produced in MPC 4851 when protein concentration was very low (1.0% and 0.5%, w/w) (Figure 5.10). The curds obtained from MPC 4851 containing 0.5% and 1.0% (w/w) protein showed a looser and crumbled structure. However, when protein concentration

was equal or higher than 2.0% (w/w), it had a limit effect on the structure of the curd. These clots were spherical in shape to resemble a smooth cheese-ball.

(3) Influence of protein concentration on protein hydrolysis during digestion

The results of the curd weight ratio (Figure 5.13) indicated that the protein concentration had no significant influences ($P > 0.05$) on the hydrolysis rate of the clot when protein concentration varied from 2.0% (w/w) to 5.0% (w/w). In contrast, a lower protein concentration (1.0% and 0.5%, w/w) may cause a more rapid hydrolysis ($P < 0.05$) during gastric digestion.

This pattern can be explained from the structure of the curd, and the enzyme activity. First, the structure of the curd is considered to be a major factor in determining the digestion rate of clotted caseins. The sizes of the clots formed in MPC 4851 increased with an increase in protein concentration from 0.5% to 5.0% (w/w). When the protein concentration was very low (1.0% and 0.5% w/w), the structure of the clot was loose. Such a fragmented and loose structure means that more open water channels were provided for the diffusion of the pepsin during gastric digestion, accelerating the protein hydrolysis. Previous study demonstrated that the milk clot was degraded from its surface under gastric conditions (Ye et al., 2016b). When the protein concentration was higher (2.0%-5.0%, w/w), the clots resembled a tight, inclosed cheese-ball. The dense protein network had almost no voids, so that water could not be filled in the interior of the curd easily. It is likely that this more compact structure of clot prevented the permeation and diffusion of pepsin into its interior, resulting in a slowdown of the hydrolysis of clotted protein in the stomach.

Second, the pepsin activity, which is affected by pH, is a possible factor affecting hydrolysis of the clot. The rate of pH changes is governed by the protein concentration in MPC 4851, i.e., the more the protein content, the slower the decrease in pH of the emptied digesta. The MPC 4851 sample with a lower protein concentration had a more suitable pH (a lower pH in this case) to pepsin (Axelsson et al., 1983). This probably accelerated the proteolytic action of pepsin, promoting the digestion degree driven by pepsin.

It is noting that, as mentioned above, the results of the curd weight ratio (i.e., undigested clotted protein) showed that both 0.5% and 1.0% (w/w) samples had a smaller ($P < 0.05$) curd weight ratio than other samples. However, no difference in hydrolysis

profile or protein composition of the clots was detected by the SDS-PAGE among all samples, with an exception of 0.5% (w/w) MPC 4851 sample (Figure 5.15). This is probably because, although there is an increase in the rate of protein hydrolysis in the MPC 4851 sample containing 1.0% (w/w) protein; however, this increase is not enough to result in a greater extent of degradation similar to the sample containing 0.5% (w/w) protein.

Interestingly, although in both SMP and MPC 4851, casein exists in a micellar form, casein and whey protein ratio remains as in milk (about 80:20) (Carr & Golding, 2016), and both of them had a more rapid hydrolysis of the clots when the protein was lower than 2.0% (w/w). However, the protein composition and hydrolysis pathway of the clots taken from SMP and MPC 4851 followed a different pattern with increasing protein concentration (Figure 5.7 and 5.15). A large amount of peptides bands observed in the clots of SMP containing a protein concentration from 0.5% to 2.0% (w/w) indicates more considerable degradation occurred in these samples. By contrast, in MPC 4851, only the sample with the lowest protein concentration of 0.5% (w/w) had slightly more degradation. This difference may be explained from the different structure of the clot induced by various processing techniques. The curd in SMP was made up of many crumbled protein pieces, rather than an inclosed cheese-like pellet. This is probably because the commercial SMP has been preheated during the manufacturing process. The possible effects include: (1) unlike unheated milk, the clot formed from the heated milk showed a more open, fragmented structure (Ye et al., 2016b), such a structure provides more channels for diffusion of SGF; (2) the denatured whey protein/κ-casein complexes incorporated in the clot in the SMP samples are able to imbibe a remarkable quantity of water (Singh, 2007), allowing the pepsin to gain access to the interior of the protein matrix. So that, the action of pepsin to diffuse and permeate the curd is easier for SMP in comparison to MPC 4851. In comparison, MPC is a membrane-produced product (Carr & Golding, 2016). Its manufacturing process does not involve preheating. Therefore, the structure of the curd was very dense, and less porous, preventing the action of pepsin, in particular in the interior of the clots. Only when the protein concentration was very low, the structure of the clot was crumbled, resulting in a more efficient hydrolysis by pepsin.

Besides, it should be mentioned that base on the results shown in Figures 5.4, 5.7, 5.13 and 5.15, the quantity of degradation products from the clotted protein, presumably,

was different. However, with the simulated gastric emptying at 20 min intervals, many peptides and soluble proteins were gradually evacuated from the HGS. These hydrolysis products cannot have detected by SDS-PAGE due to having too small a molecular weight, which is one of the limitation of electrophoretic analysis.

5.4 Conclusions

This study presented an overview of the influence of protein concentration on the digestion behaviour of SMP and MPC 4851 in a HGS. Protein concentration plays an important role in the structure of the clot formed in the gastric environment. The gastric hydrolysis of protein was greatly influenced by the clot structure. In both SMP and MPC 4851, the clot had a fragmented, loose and porous structure when protein concentration was lower than 2.0% (w/w). An intact ball-like clot had a more compact, dense structure observed when protein concentration varied from 2.0% to 5.0% (w/w). The curd with a loose, fragmented structure can cause a more rapid ($P < 0.05$) protein hydrolysis compared to the clot with a dense ball-like structure. These results suggest that a low protein content in protein solution can induce a more rapid proteolysis in the stomach.

Chapter 6: Behaviours of Oil-in-Water Emulsions Stabilised by Different Milk Protein Ingredients during in Vitro Gastrointestinal Digestion

6.1 Abstract

The aim of this study was to investigate the lipid digestion in emulsions stabilised by different dairy ingredients under simulated intestinal conditions after simulated gastric digestion by using an advanced dynamic digestion model, i.e., a human gastric simulator (HGS). Emulsions were prepared by using milk protein concentrate (MPC) 4851, MPC 4861, sodium caseinate and whey protein isolate (WPI). The various dairy ingredients-stabilised emulsions aggregated at different digestion times and with a range of different sizes induced by the milk-clotting enzyme pepsin and/or acidic pH in the stomach. The oil droplets in the emulsions appeared to be embedded in the aggregates once they formed. The formation of aggregates with a different structure and time in various emulsions cause a different rate of protein hydrolysis by pepsin in the stomach. The oil droplets were thus released from the aggregates and entered the small intestine at a different rate. This caused a various extent of lipid digestion within the small intestinal environment.

The aggregation in MPC 4851-stabilised emulsion took place after 5 min digestion in the HGS, with the largest size, and remained in the stomach during whole gastric digestion. MPC 4851-stabilised emulsion thus had a slowest release of oil droplets into the small intestine. In comparison, MPC 4861 and sodium caseinate-stabilised emulsions aggregated in the stomach at approximately 40 min, with a relative small size. These aggregates disintegrated at the mid- or late-stages of gastric digestion process in these two emulsions. MPC 4861 and sodium caseinate-stabilised emulsions had a more rapid oil droplets delivery into the small intestine. In relation to WPI- and heated WPI-stabilised emulsions, the aggregation formed at a similar time to that which was observed in MPC 4861 and sodium caseinate-stabilised-emulsions, i.e., approximately 40 min. There had the smallest size among all of the samples, and disintegrated quickly with the further digestion. Both WPI-stabilised emulsions with and without heating had the fastest hydrolysis by pepsin in the early and mid-stages of the gastric digestion process, thus contained the highest level of oil content in the emptied gastric digesta. In the mixed

gastric digesta, which were subjected to small intestinal digestion, the oil contents contained in different emulsion samples varied. These differences affected the extent of lipid digestion in the small intestine. The sample with a higher oil content released a greater amount of free fatty acids (FFAs) than the sample with a lower oil content. The extent of lipid digestion of different emulsion samples followed the following pattern: MPC 4851-stabilised emulsion < MPC 4861-stabilised emulsion < sodium caseinate-stabilised emulsion and WPI-stabilised emulsions both with and without heating.

6.2 Introduction

Lipids exist in almost all types of foods and play a key role in human diet. They provide flavour, texture and mouth-feel. Lipids also perform many important functions in the human body, such as providing a concentrated source of energy (Singh et al., 2009) and act as a carrier of oil-soluble vitamins, such as vitamins A, D, E and K (Richards, 2006). However, an excess consumption of dietary fat may result in increased food-related diseases, e.g. obesity and type 2 diabetes (Révérend et al., 2010; McClements et al., 2009). Recently, the increasing consumer awareness about the relationships between human health and a high-calorie diet has promoted the food industry to design and develop healthier foods with a focus on reducing the adsorption of calories, without diminishment of the desirable sensory qualities of food (Chung et al., 2013; Révérend et al., 2010; McClements et al., 2009; Singh et al., 2009).

In modern processed foods, fat may be normally incorporated within the food matrix in the form of an emulsion, such as spreads, salad dressings or soups (McClements et al., 2009). In addition, milk as a native food, is also known as a classical oil-in-water emulsion, which consists of milk fat globules, stabilised by a complex protein/phospholipid membrane, milk fat globule membrane (Mather, 2000; Ye, Singh, Taylor, & Anema, 2002). It is therefore important to understand the digestion and adsorption behaviour of lipids in emulsion during their transition through the gastrointestinal tract.

Recently, there have been some attempts to understand the food emulsion behaviors during gastrointestinal digestion (Golding & Wooster, 2010; Li et al., 2012; Mun et al., 2006; Sarkar et al., 2009; Sarkar et al., 2010b; Sarkar et al., 2016; Singh et al., 2009; Ye et al., 2016a). When the emulsion is mixed with the gastric fluid, which contains proteolytic enzymes (i.e., pepsin), gastric lipase (Mandalari et al., 2009), and various ions (e.g. Na^+, Ca^{2+}, Cl^-), the pH of the emulsion undergoes a dramatic change from about pH 6-7 to pH 1-3. Meanwhile, the emulsion is exposed to mechanical agitation in the stomach because of the gastric contractions (Ekmekcioglu, 2002; Kalantzi et al., 2006; Pal et al., 2007). The protease pepsin is a factor that significantly affects the stability of emulsions, as its action alters the properties of the emulsified droplets and adsorbed protein layers. At neutral pH, most protein-stabilised emulsions are negatively charged. Their charge

will undergo substantial modifications when the pH falls to a lower level (pH < 2.0), and particles aggregate when the pH is near their isoelectric points (pI) (Singh & Ye, 2009).

Although 10-30% of lipids may be hydrolysed to free fatty acids (FFA)s and diacylglycerol by the gastric lipase in the stomach (Mandalari et al., 2009); lipid digestion takes place mainly in small intestine, catalysed by pancreatic lipases (Bauer et al., 2005; Mun et al., 2006; Singh & Ye, 2009). Pancreatic lipase is an interface-active enzyme, which can work efficiently at about pH 6.5 (Singh & Ye, 2013). It preferentially cleaves the sn-1 and sn-3 positions of triglycerides and diacylglycerol and generates free fatty acids (FFAs) and 2-monoglycerides (Mu & Høy, 2004), which would precipitate at oil-water interface and result in a limited accessibility of pancreatic lipase to oil droplets (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Bile salt is a kind of biosurfactant, which plays a significant role in digestion and adsorption of lipids (Sarkar et al., 2016). It can adsorb readily at the oil-water interface when it is introduced to a simulated intestinal fluid (SIF) (Hur et al., 2009). It is able to solubilise the lipolytic products, such as FFAs and 2-monoglycerides, and remove them from interfacial layers (Gargouri et al., 1983), accelerating the lipid digestion.

Milk protein is an important source of nutrients for human through the different stages of life. Dairy ingredients do not just add excellent nutritional value to food and nutritional products but are also useful for important functional properties, e.g. as emulsifiers to stabilise emulsion.

In MPC, caseins and whey proteins are in the same proportions as those found in milk. Caseins retain much of their micellar structure, and whey protein is in its native state as the manufacturing process does not involve preheating (Euston & Hirst, 1999; Ye, 2011). During emulsification, these micellar caseins can coat the surface of emulsified droplets, but the spreading of casein micelles is very limited (Dalgleish, 1996).

The casein micelles in some new MPC products have been dissociated to a certain extent by removing the calcium content for improving the functional properties (Ye, 2011). This calcium reduced product refers to MPC 4861 in the present study. The emulsion made with calcium reduced MPCs exhibits remarkably enhanced stability with smaller droplet size in comparison to the MPC-stabilised emulsion. In the case of reduced 80% calcium MPC-stabilised emulsion, it has a similar surface protein concentration to

that in the sodium caseinate-stabilised emulsion. However, in calcium reduced MPC, depletion flocculation may be induced by small casein aggregates (Ye, 2011).

Traditionally, caseins are separated using techniques such as acid or rennet precipitation (Mulvihill, 1992), which destroy the native micellar structure. The products produced in this way contain individual casein molecules. Sodium caseinate is the water-soluble form of caseins, and is the most commonly applied ingredient in food industry (Mulvihill & Ennis, 2003). Sodium caseinate is known as an excellent emulsifier, and is able to make a stable emulsion at a relatively low protein to oil ratio (1:60) (Singh, 2005).

Whey is obtained after casein is removed from milk. WPI is also an excellent emulsifier. During emulsification, globular β-lactoglobulin (β-Lg) in WPI tends to unfold partially, and adsorbs at the oil-water interface in an intermediate state between the native state and fully denatured conformation. This thus results in compact adsorbed layers (Dickinson, 1998; Dickinson & McClements, 1995a; Fang & Dalgleish, 1998).

Whey proteins and caseins are usually employed as model proteins in studies investigating and understanding human digestion in the gastrointestinal tract (He & Giuseppin, 2014). Whey protein and caseins possess different digestion behaviour. It is known that native β-Lg in solution is resistant to acid and proteolytic enzymes (e.g. pepsin) under stomach conditions (Nik et al., 2010b; Reddy et al., 1988b). However, β-Lg appears to be more prone to hydrolysis by pepsin when it exists as an emulsifier to lay somewhere at the oil-water interface (Macierzanka et al., 2009; Sarkar et al., 2009). This might be explained by the fact that the conformation of adsorbed β-Lg at interface undergoes some alterations and thus leads to the exposure of peptic cleavage sites during the emulsification process (Macierzanka et al., 2009; Sarkar et al., 2010b). Nevertheless, α-lactalbumin (α-La) is less prone to hydrolysis by pepsin when it adsorbs at the oil-water interface than its native state in solution (Nik et al., 2010b).

During gastric digestion, whey protein solutions remain soluble in the stomach and passes into small intestine rapidly without hydrolysis by pepsin (Boirie et al., 1997; He & Giuseppin, 2014). However, during emulsification, a portion of the hydrophobic groups at the core of the native whey protein molecule become exposed on the surface induced by partial unfolding (Dickinson, 1992). Heating at above 80 °C is also known to lead to the full exposure the hydrophobic groups. Thus the hydrophobic interactions

between the molecules are accelerated (André et al., 2016). In the dynamic *in vitro* digestion, the WPI-stabilised emulsions undergo a dramatic change in pH, on mixing with the gastric fluid (Lindahl et al., 1997). Thus, the digestion behaviours of the WPI-stabilised emulsions with and without heating (90°C, 20 min) are expected to be different, especially when the pH is near to the pI of whey proteins (~5.1).

Caseins, compared to whey protein, have been previously reported to exhibit different hydrolysis behaviour under the gastric conditions. After ingestion, caseins clot in the stomach, which greatly reduce the gastric emptying rate and probably result in a slower release of amino acids (He & Giuseppin, 2014). The delayed delivery rate of caseins into the small intestine induced by coagulation of casein micelles in SMP and MPC solution was observed during simulated *in vitro* gastric digestion, which has been presented in Chapter 4.

Recent work in our laboratory has demonstrated that during the dynamic *in vitro* gastric digestion, when casein micelles in the fresh/heated (90°C, 20 min) whole milk were coagulated on the protein fraction by pepsin, the fat globules appeared to be embedded in the clots (Ye et al., 2016a). Further, Ye et al. (2016a) found that the structure of the clots formed varies in unheated and heated whole milk, leading to a different rate of protein hydrolysis by protease pepsin. This results in various release rates of fat globules from the protein matrix into the emptied digesta. The capture and subsequent liberation of the fat globules from the protein matrix could affect their availability for lipolysis by gastric and pancreatic lipases (Ye et al., 2016a).

It has been reported that the behaviours of emulsions in the static *in vitro* gastric digestion does not influence the rate and extent of lipid digestion in the small intestine (Li et al., 2012, 2013). However, the pepsin hydrolysis in the simulated gastric digestion accelerated coalescence of emulsified droplets during the intestinal digestion (Li et al., 2013). In addition, Kenmogne-Domguia et al. (2012) suggested the kinetic of lipolysis of bovine serum albumin (BSA)-stabilised oil-in-water emulsions within the small intestinal environments may be modified by previous gastric digestion with different conditions. However, the various gastric conditions (pH 2.5 or 4.0) only have a limited effect on the final extent of the lipid digestion. To date there is limited knowledge about the influence of the dynamic gastric digestion of protein-stabilised emulsions on the subsequent lipid

digestion within small intestinal environments, as most studies use the static digestive model to simulate human digestion of emulsions.

In the present study, we aimed to understand behaviour of the lipid digestion in emulsion made with different milk protein ingredients, with a focus on the influence of milk protein aggregation formed during the dynamic gastric digestion on physicochemical behaviour, morphology of oil droplets and subsequent lipolysis in the small intestine. A dynamic human gastric simulator (HGS) model was employed to mimic the gastric digestion before exposing the emulsions to the small intestinal environment. The information obtained from this study will be useful for understanding lipid digestion in emulsions made with different commercial dairy ingredients, and design and development of healthy products derived from these dairy ingredients.

6.3 Results

6.3.1 *In vitro* gastric digestion of emulsions

6.3.1.1 pH profiles

Overall, the pH of all samples decreased gradually with increasing digestion time (Figure 6.1). The initial pH in the stomach represented the pH value of different dairy ingredients-stabilised emulsions with 4.0% (w/w) protein and ranged from pH 6.64 ± 0.04 to pH 6.95 ± 0.01. The pH profile of the gastric digesta emptied from MPC 4851-stabilised emulsion showed a slower decrease during the 220 min of digestion than the other dairy ingredients. The pH decreased to about ~pH 5.1 at 80 min of digestion and only dropped to ~pH 2.5 at the end of digestion.

The digesta emptied from MPC 4861-, sodium caseinate-, WPI- and heated (90°C, 20 min) WPI-stabilised emulsions exhibited a more rapid decrease in pH. Among these four samples, the decrease in pH with each time point was different from 40 min to 160 min. The pH values did not differ markedly between 160 min and the end of digestion. There was a more rapid decrease in the pH value of the emptied digesta from WPI-stabilised emulsion compared to that of other emulsions. It dropped to ~ pH 5 within 40 min and reached to ~pH 2 after 120 min, whereas the pH in the digesta of MPC 4861-stabilised emulsion had the slowest decrease among these four samples, which reduced to ~pH 5 within 60 min and dropped to ~pH 2 after 180 min. Furthermore, there was no difference in the changes of pH in the emptied digesta from sodium caseinate-stabilised emulsion and heated WPI-stabilised emulsion, both showed a more rapid decrease in pH than MPC 4861-stabilised emulsion.

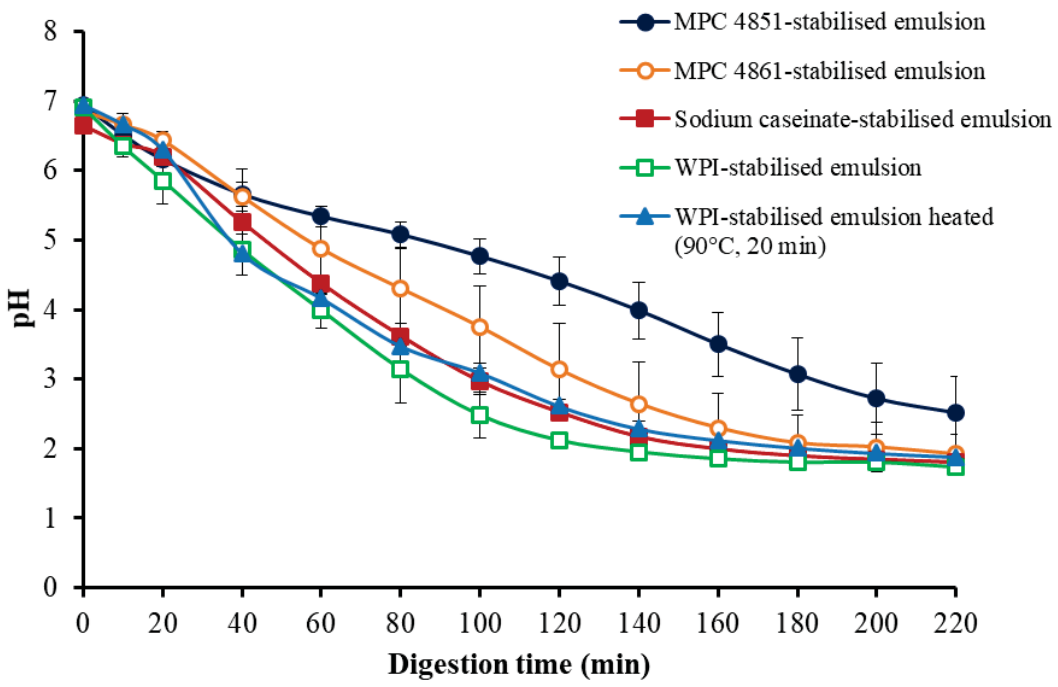


Figure 6.1. Changes in pH of digesta emptied from different milk protein ingredients-stabilised emulsions during 220 min of gastric digestion

6.3.1.2 Changes in emulsions during gastric digestion

There were appreciable differences in aggregation behaviours among emulsions made with different protein ingredients over 220 min of gastric digestion. The most important distinction is that a large amount of aggregated emulsion could only be collected from MPC 4851-stabilised emulsion after 220 min of digestion, but not from the other emulsions.

During the digestion of MPC 4851-stabilised emulsion, aggregation was observed during the first 5 min, and most of the emulsion droplets were incorporated into aggregates within the first 20 min, when the pH was about 6.2. The aggregated emulsion consisted of many tiny granules of irregular shape, with a range of different sizes (Figure 6.2). These aggregates did not disintegrate completely during the entire digestion period. After 220 min, the aggregated emulsion with a wet weight of ~ 7.1 g and a dry weight of ~3.0 g was obtained.

For MPC 4861 and sodium caseinate-stabilised emulsions, a similar aggregation behaviour was observed at the early stage of digestion. The emulsion aggregation was first visible at around 20 min of digestion, with a larger extent of aggregation observed in the stomach between 40 and 60 min, when the pH approached 5. With an increase in digestion time, the aggregates had disappeared after 100 min in sodium caseinate-stabilised emulsion, while aggregates disintegrated after 200 min in MPC 4861-stabilised emulsion.

In unheated and heated emulsions made with WPI, emulsion aggregation took place at approximately 40 min of digestion, when the pH was ~4.8. There was a greater amount of emulsion aggregates observed in the heated emulsion. The aggregates had disappeared after 80 min in unheated WPI-stabilised emulsion sample but disappeared after 100 min in heated WPI-stabilised emulsion.

Figure 6. 2. The aggregates taken from MPC 4851-stabilised emulsion after 220 min of gastric digestion in the HGS.

6.3.1.3 Average droplet size of emulsions in SGF

The average volume mean diameter ($d_{4,3}$) of different protein ingredient-stabilised emulsions ranged from 0.78 ± 0.14 to 1.36 ± 0.16 μm, and the average surface mean diameter ($d_{3,2}$) ranged from 0.43 ± 0.07 to 0.74 ± 0.08 μm (Table 6.1).

Table 6.1. The volume ($d_{4,3}$) and surface ($d_{3,2}$) mean diameters of original emulsion stabilised by different dairy ingredients (20.0% soybean oil and 4.0% protein, w/w).

	MPC 4851-stabilised emulsion	MPC 4861-stabilised emulsion	Sodium caseinate-stabilised emulsion	WPI-stabilised emulsion	Heated WPI-stabilised emulsion
$d_{4,3}$ (μm)	1.36 ± 0.16	1.04 ± 0.04	0.78 ± 0.14	0.81 ± 0.19	0.88 ± 0.09
$d_{3,2}$ (μm)	0.74 ± 0.08	0.59 ± 0.04	0.48 ± 0.09	0.43 ± 0.07	0.45 ± 0.05

Values are means and standard deviations (SD). MPC, milk protein concentrate; WPI, whey protein isolate.

The changes in particle size of emptied digesta from emulsions made with various protein ingredients as a function of time during gastric digestion are shown in Figure 6.3. In general, the average particle size changed significantly ($P < 0.05$) in all samples during the whole gastric digestion process. In MPC4851-stabilised emulsion, the particle size ($d_{4,3}$) of digesta showed a severe increase from ~1.36 to ~197.09 μm ($P < 0.05$) in the first 20 min of digestion, and followed by a decrease to ~122.05 μm ($P < 0.05$) at 40 min [Figure 6.3 (A)]. With further digestion, the particle size decreased to 56.00 μm at the end of digestion.

In the digesta emptied from emulsions stabilised by MPC 4861 [Figure 6.3 (B)] and sodium caseinate [Figure 6.3 (C)], the particle size ($d_{4,3}$) was not changed ($P > 0.05$) in the first 20 min of digestion. With further digestion, the average particle size ($d_{4,3}$) of emptied digesta increased to a maximum value of ~ 88.63 μm and ~ 85.99 μm ($P < 0.05$) at 80 min, followed by a decrease to ~46.40 μm and ~20.92 μm ($P < 0.05$) at 160 min for MPC 4861 and sodium caseinate-stabilised emulsions, respectively.

For WPI-stabilised emulsions both with and without heating [Figures 6.3 (D) and (E)], there was an increase in the particle size ($d_{4,3}$) ($P < 0.05$) of the emptied digesta at 40 min. The average droplet size of unheated WPI-stabilised emulsion (~35.13 μm) at 40 min was smaller than that of heated WPI-stabilised emulsion (~91.49 μm). With further digestion, the value of $d_{4,3}$ of the emptied digesta rapidly decreased to ~2.08 and ~27.41μm at 80 min ($P < 0.05$) for both unheated and heated WPI-stabilised emulsions, respectively. However, no difference ($P > 0.05$) in the particle size ($d_{4,3}$) of the emptied

digesta from WPI-stabilised emulsion was observed with further digestion, while a slight decrease in the particle size ($d_{4,3}$) of the emptied digesta was observed in heated WPI-stabilised emulsion at 120 min.

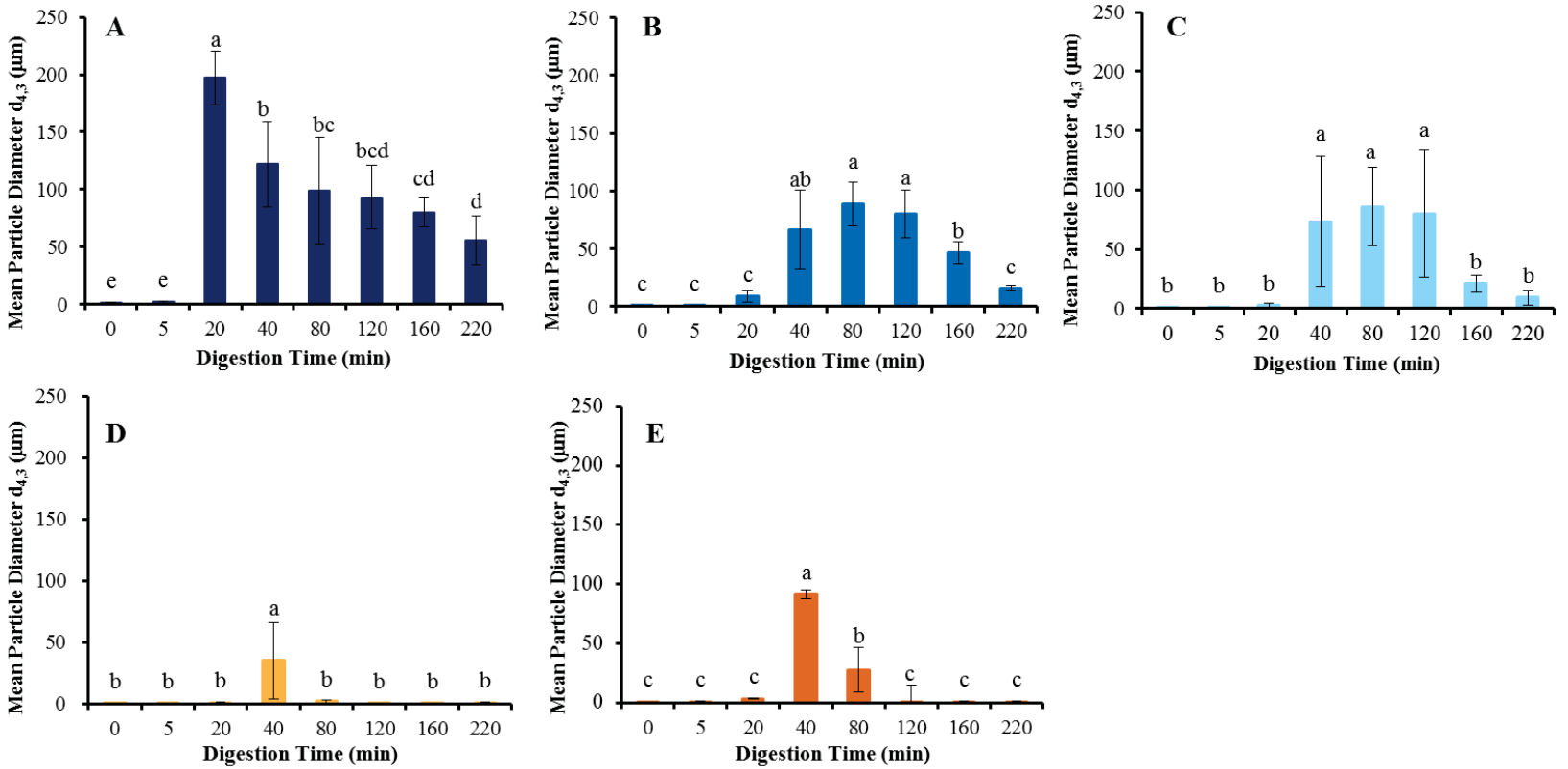


Figure 6. 3. Changes in mean diameters volume ($d_{4,3}$) of emptied digesta obtained from different milk protein ingredients-stabilised emulsions (20.0% soybean oil and 4.0% protein, w/w) during 220 min of gastric digestion in the HGS. (A) MPC 4851-stabilised emulsion; (B) MPC 4861-stabilised emulsion; (C) sodium caseinate-stabilised emulsion; (D) WPI-stabilised emulsion; (E) WPI-stabilised emulsion heated (90°C, 20 min). Different lowercase letters indicate significant difference ($P < 0.05$) on the mean diameters volume ($d_{4,3}$) of digesta emptied from emulsions between different digestion time points within the same emulsifier.

6.3.1.4 Particle size distribution

For MPC 4851-stabilised emulsion, the initial emulsion had a monomodal distribution of particle sizes and did not change significantly within the first 5 min of digestion [Figure 6.4 (A)]. However, the size distribution shifted to a larger size region after 20 min of digestion, and the peak in this distribution showed the size has increased. This distribution peak became slightly broader at 40 min. When the emulsion was digested for 80 min to 160 min, the particle size distribution of emptied digesta became multimodal, with two peaks at 1-15 μm region and at around the 1000 μm region. With further digestion to 220 min, the droplet size distribution still exhibited a multimodal peak, with a peak in the 0.04-1 μm region and a peak in a broad range.

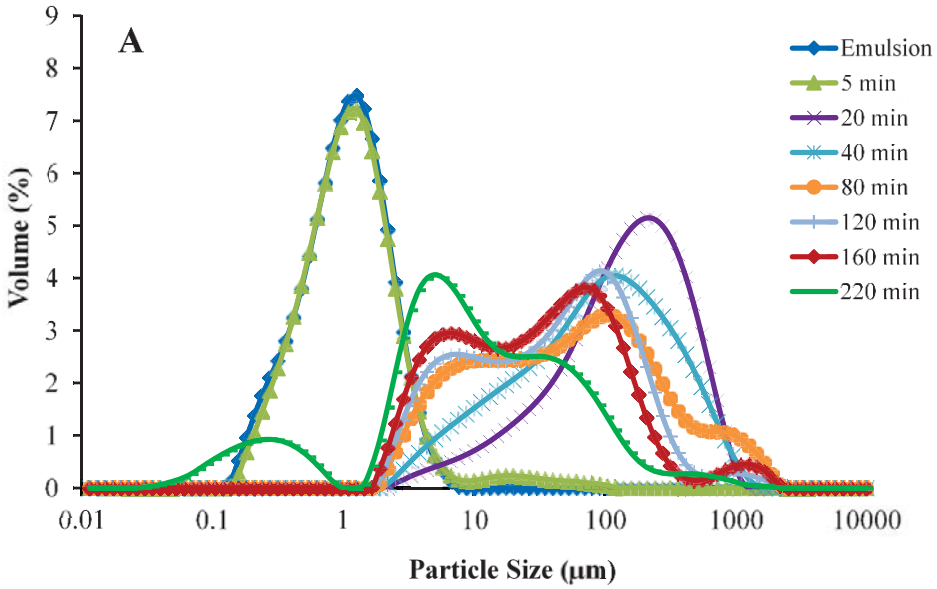
For MPC 4861-stabilised emulsion sample, the droplet size distribution of initial emulsion had a narrow single peak around 0.1-10 μm, and the size distribution did not change compared to the original emulsion within the first 20 min of digestion [Figure 6.4 (B)]. However, at 40 min of digestion, the size distribution of emptied digesta became bimodal, with the second peak at around 10-500 μm region and a corresponding reduction in the area of the first peak. The area of the second peak increased at 80 min, along with the disappearance of the first peak which was located in 0.1-5 μm region. In addition, the droplet size distribution at 80 min had a broad unimodal peak in a large size region. However, at 120 min of digestion, we observed that the droplet size distribution became bimodal with a newly formed peak centred approximately 10 μm along with a corresponding decrease in the area of the previous peak at 80 min. With further digestion, the area of this newly formed peak increased in response to a relative reduction in the area of the previous peak until the previous peak disappeared at the end of digestion.

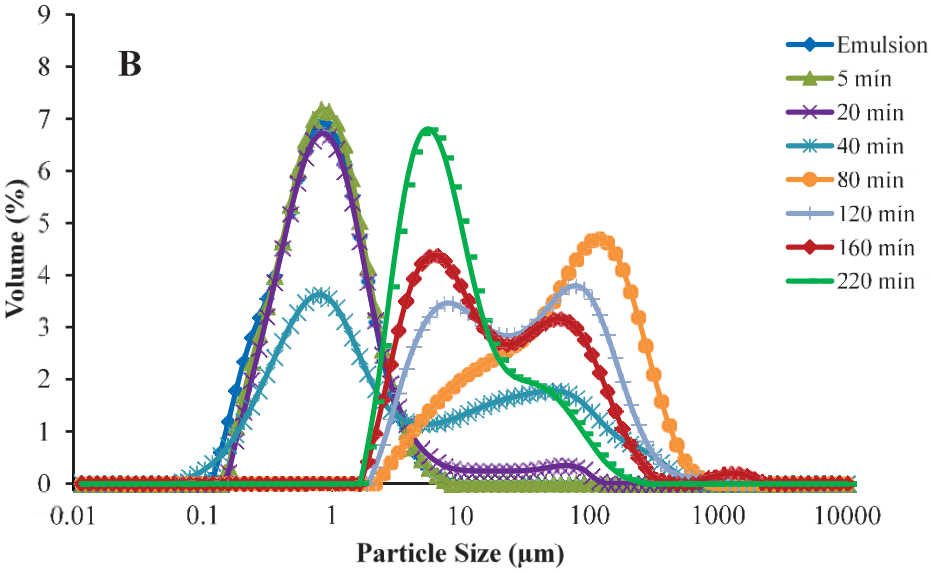
The initial emulsion made with sodium caseinate had a narrow unimodal droplet size distribution [Figure 6.4 (C)]. In the first 20 min of digestion, the size distribution of emptied digesta did not significantly change compared with the original emulsion. With increasing digestion time, this size distribution peak shifted to a larger size region which centred at about 30 μm at 40 min. And the size distribution became multimodal with the further digestion to 80 min, with a small wide peak in 0.1-1 μm region and two other peaks in 1-400 μm region. The size distribution became bimodal again after 120 min along with the disappearance of the smallest peak. The digesta droplet size distribution

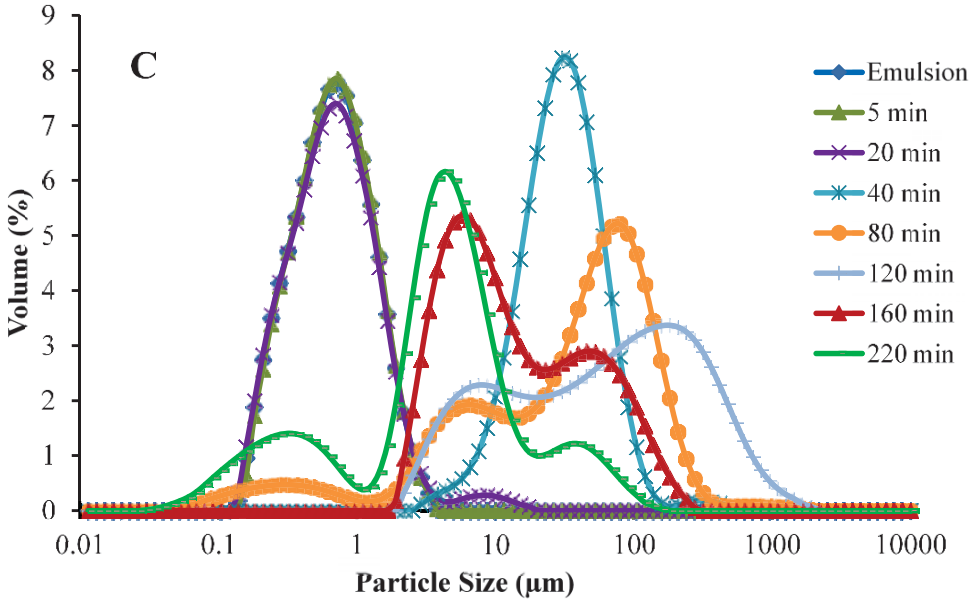
peak appeared to be composed of two overlapping peaks, which had the first peak between 2-20 μm, and the second wider peak in 20-1900 μm region. There was an increase in the area of the first peak with a corresponding decrease in the area of the second peak at 160 min of digestion, and the second peak located in the range of 25-300 μm became narrower. This trend in change of size distribution was observed until 220 min. At 220 min, a consistent increase in the area of the first peak in response to the decline in the area of the second peak can be found, and an additional small peak appeared in the region of 0.05-1μm.

The size distribution of emulsion made with WPI did not change during whole digestion period except at 40 min [Figure 6.4 (D)]. The droplet size distribution of initial emulsion had a single narrow peak at 0.1-10 μm region. At 40 min, the peak shifted to a larger size region, located in the range of about 1-120 μm. The peak moved to its original size region after 80 min and remained unchanged until the end of digestion.

The initial heated WPI-stabilised emulsion had a monomodal droplet size distribution, with a peak during 0.06-3.8 μm [Figure 6.4 (E)]. With an increase in digestion time, the size distribution became bimodal at 40 min, and the peak appeared to be composed of two overlapping peaks, which located in the region of 2-830 μm. At 80 min of digestion, the size distribution peak consisted of an additional peak in the 0.06-3.8 μm region with a decrease in the area of the peak observed in the 40 min-sample. However, at 120 min of digestion, the digesta droplet size distribution peak shifted to its original size region and was not observed to change.


A
Volume (%)
Particle Size (µm)
0.01
0.1
1
10
100
1000
10000
Emulsion
5 min
20 min
40 min
80 min
120 min
160 min
220 min


B
Volume (%)
Particle Size (µm)
0.01
0.1
1
10
100
1000
10000
Emulsion
5 min
20 min
40 min
80 min
120 min
160 min
220 min


C
Volume (%)
Particle Size (μm)
0
1
2
3
4
5
6
7
8
9
0.01
0.1
1
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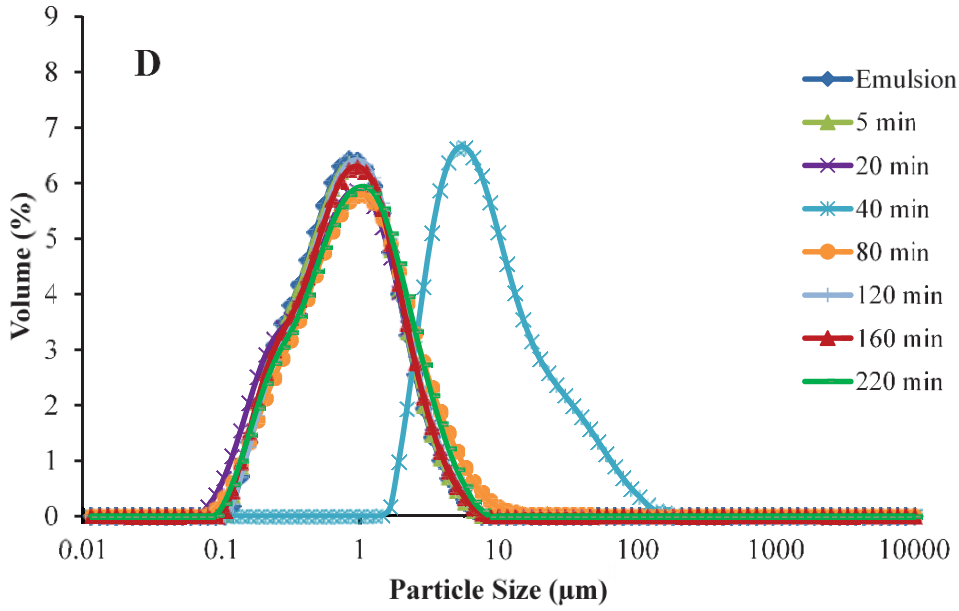
100
1000
10000
Emulsion
5 min
20 min
40 min
80 min
120 min
160 min
220 min


D
Volume (%)
Particle Size (μm)
0
1
2
3
4
5
6
7
8
9
0.01
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Emulsion
5 min
20 min
40 min
80 min
120 min
160 min
220 min

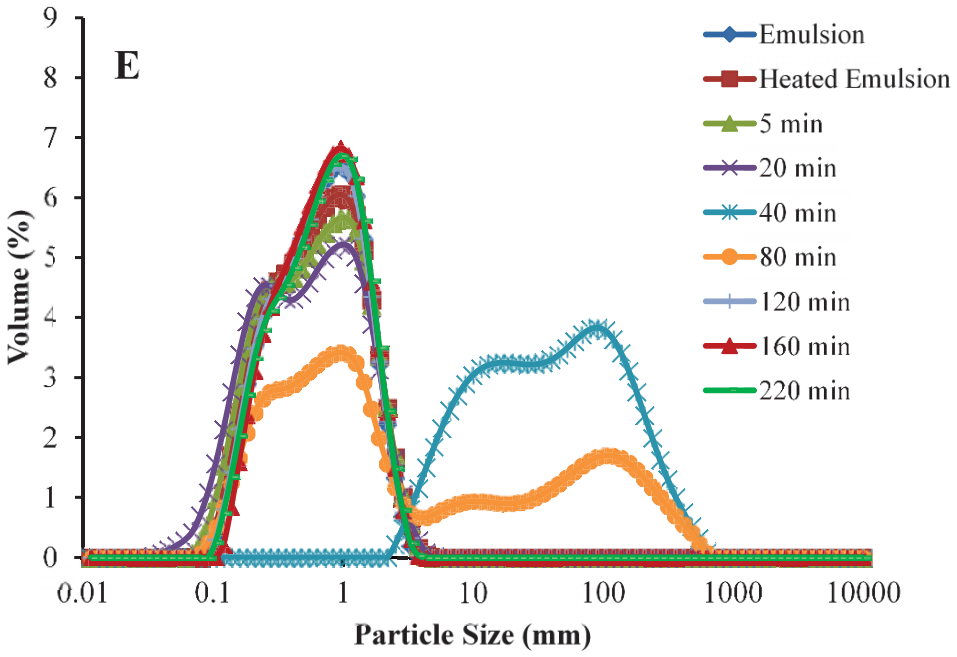


Figure 6. 4. Particle size distribution of emptied digesta obtained from 220 min gastric digestion of different protein stabilised emulsions (20.0% soybean oil and 4.0 % protein, w/w) in the HGS: (A), MPC 4851-stabilised emulsion; (B), MPC 4861-stabilised emulsion; (C), sodium caseinate-stabilised emulsion; (D), WPI-stabilised emulsion; (E), WPI-stabilised emulsion with heating (90°C, 20 min).

6.3.1.5 Oil content of emptied digesta

The oil contents of emptied digesta derived from emulsions stabilised by different dairy ingredients as a function of digestion time were present in Figure 6.5, which represented the amount of oil delivered to the small intestine at different time points.

In MPC 4851-stabilised emulsion, the oil content of emptied digesta at 20 min was significantly ($P < 0.05$) lower than other emulsions. The oil content decreased from~ 20.0 to ~1.9 g oil per 100 g digesta within the first 20 min, while the oil content of the other dairy ingredient-stabilised emulsions did not decrease below ~14.6 g oil per 100 g digesta within this same period. The oil content of the emptied digesta from MPC 4851-stabilised emulsion, then increased to ~3.7 g oil per 100 g digesta at 120 min, and was not observed to change until the end of the gastric digestion.

MPC 4861 and sodium caseinate-stabilised emulsions did not differ in change in oil content of the emptied digesta during the whole period of gastric digestion. Both them had a slightly decrease ($P < 0.05$) in oil content at 20 min, to 15.3 g and 14.6 g oil per 100 g digesta, followed a more rapid decrease ($P < 0.05$) at 60 min, to about 1.7 g and 4.3 g oil per 100 g digesta for MPC 4861 and sodium caseinate-stabilised emulsions, respectively. The oil content of emptied digesta from MPC 4861-stabilised emulsion was not observed to change ($P > 0.05$) with further digestion, whereas the oil content of the emptied digesta from sodium caseinate-stabilised emulsion decreased to 1.5 g oil per 100 g digesta at 220 min.

Compared to the other dairy ingredients, WPI-stabilised emulsions both with and without heating showed the slowest decrease in the oil content of the emptied digesta over 220 min of digestion. In the first 20 min, a more rapid decrease ($P < 0.05$) in the oil content was observed in heated WPI-stabilised emulsion, compared to unheated WPI-stabilised emulsion. At 20 min, unheated WPI-stabilised emulsion had a highest value ($P < 0.05$) of oil content among all dairy ingredient-stabilised emulsions. With further digestion, both heated and unheated WPI-stabilised emulsions showed a decrease in the oil content of emptied digesta; however, no significant difference ($P < 0.05$) was observed between these two WPI samples at each time point.

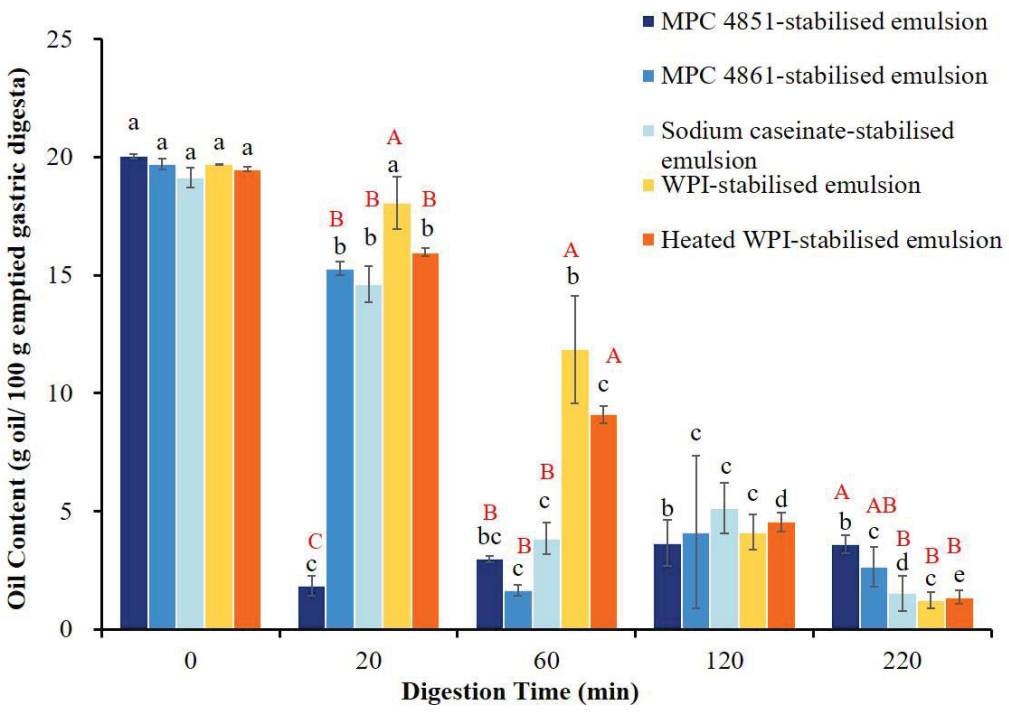


Figure 6.5. Oil content (g oil/100 g emptied gastric digesta) of the emptied gastric digesta at different digestion time points. Different capital letters indicate significant difference ($P < 0.05$) on the oil content between the gastric digesta emptied from the different dairy ingredients-stabilised emulsions within the same digestion time point. Different lowercase letters indicate significant difference ($P < 0.05$) on the oil content of the emptied digesta between the different digestion time points within the same emulsifier.

6.3.1.6 Microstructure of digestive residue in the stomach

Figure 6.6 shows confocal micrographs of the digestive residue of the emulsion made with different protein ingredients remained in the stomach at different digestion time point.

(a) MPC 4851-stabilised emulsion

The initial MPC 4851-stabilised emulsion had fine uniformly dispersed oil droplets [Figure 6.6 (MPC 4851-0 min)]. At 20 min of digestion, aggregation was observed, with a closely-knit network, in which a large amount of oil droplets were entrapped. A portion of oil droplets within the aggregated network appeared to coalesce,

while some free oil droplets in a larger size appeared to be suspended in the aqueous phase [Figure 6.6 (MPC 4851-20 min)]. These aggregated blocks remained relative intact without disappearance over 220 min of gastric digestion. The amount of coalesced oil droplets within aggregated matrix increased with further digestion [Figure 6.6 (MPC 4851-60 min) and (MPC 4851-120 min)]. However, at a longer digestion time (220 min), the structure of aggregated network became more open and porous, with more voids.

(b) MPC 4861-stabilised emulsion

A very small amount of aggregation was observed in the initial MPC 4861-stabilised emulsion [Figure 6.6 (MPC 4861-0 min)]. At 20 min of digestion, a few small aggregates were observed in the emulsion. With further digestion to 60 min, the emulsion samples formed numerous large block of aggregates, with a relatively open, porous structure, in which most of the oil droplets were trapped while only a small amount of larger oil droplets were suspended in the aqueous phase. With increasing digestion time, the cluster of aggregates remained in the stomach without breaking down or disappearing until 120 min and with some relatively large oil droplets observed in the aqueous phase. These large aggregates seemed to be broken down to tiny aggregates and dispersed in the stomach at 220 min.

(c) Sodium caseinate-stabilised emulsion

For sodium caseinate-stabilised emulsions [Figure 6.6 (sodium caseinate-0 min)], noticeable flocculation of emulsion droplets was observed in the original emulsion. The flocculation disappeared at 20 min of digestion with the digestion in the HGS. Fine and evenly distributed oil droplets ($d_{4,3}$ ~2.5 μm) were present in the stomach. With further digestion, the emulsion formed a large amount of aggregates with an open, porous network at 60 min. At this time point, most of the oil droplets were entrapped within the domain of aggregates while only some larger oil droplets were suspended in the aqueous phase. At longer digestive period (120 min), the cluster of aggregates disappeared. At the end of gastric digestion, the digestive residue in the stomach consisted of numerous tiny, evenly dispersed aggregates, which contained some oil droplets by entrapment.

(d) WPI-stabilised emulsion

In WPI-stabilised emulsion [Figure 6.6 (WPI)], fine emulsified oil droplets were observed in the stomach within the first 20 min. At 60 min, the emulsion generated large

blocks of aggregates in the stomach and separated from the aqueous phase. The blocks of aggregate had a closely-knitted network with a smooth and dense structure, in which many oil droplets were contained by entrapment. Meanwhile, the suspension of large pools of oil between the gaps of separated domains of aggregation can be observed. With an increase in digestion time, the cluster of aggregates disappeared at 120 min. Instead, many tiny aggregates containing small oil droplets were present in the stomach.

(e) Heated WPI-stabilised emulsion

For heated WPI-stabilised emulsion [Figure 6.6 (WPI Heated)], the cluster of cross-linked aggregation blocks with an open, porous structure was produced at 60 min in the stomach, forming a highly ragged and uneven matrix. Almost all of the oil droplets were entrapped within the blocks. With further digestion, the aggregates disappeared at 120 min. Tiny oil droplets were evenly dispersed in the stomach at this time. At the end of gastric digestion, a few minor protein aggregates were observed in the stomach.

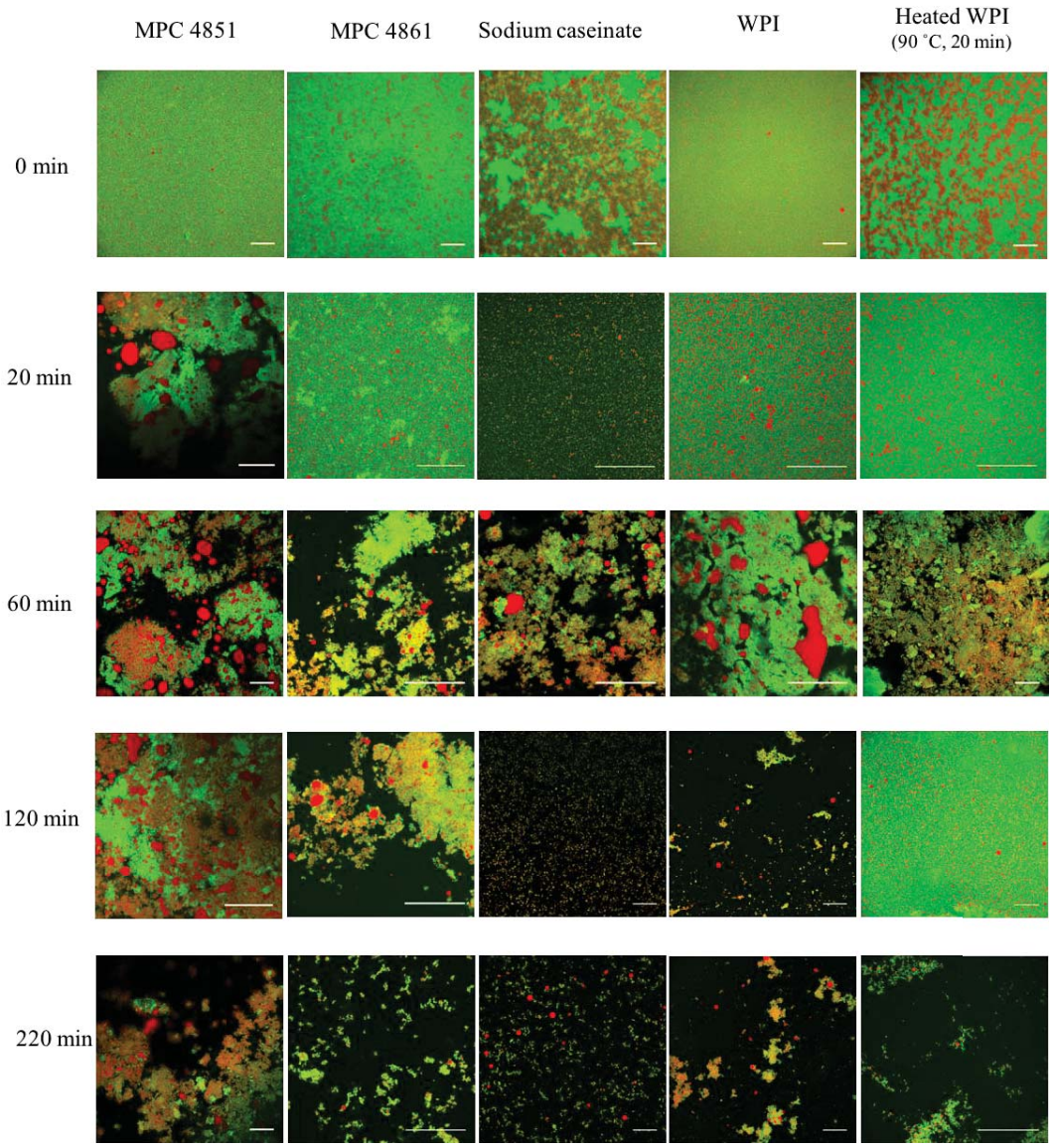


Figure 6. 6. Confocal microscopy images of digestion residues of different milk protein ingredients-stabilised emulsions in the stomach at different times during gastric digestion from 0 to 220 min. All samples were stained with Nile Red (for oil) and Fast Green (for protein). The scale bar in all images is 50 μm.

6.3.1.7 Microstructure of emptied digesta

Figure 6.7 shows confocal micrographs of emptied digesta of different emulsions samples released from the stomach at different digestion time points. This represents the emptied gastric content that enters into the small intestine.

(a) MPC 4851-stabilised emulsion

The initial MPC 4851-stabilised emulsion had fine and uniformly dispersed oil droplets (Figure 6.7 (MPC 4851-0 min)). At 20 min of digestion [Figure 6.7 (MPC 4851-20 min)], a few aggregates were present among the black background, in which the oil droplets were trapped. With an increase in digestion time, the distribution density of aggregates in emptied digesta increased, and some aggregates with larger size were visible at 60 min. At this time point, most oil droplets in the emptied digesta appeared to be evenly incorporated in the aggregates by embedment, whereas only a few "free" oil droplets were dispersed in the aqueous phase. With further digestion, the size of aggregates decreased with the gradual breakdown of aggregates to a smaller size until the end of digestion. During this period, tiny oil droplets were observed in the emulsion aggregates, only a limited amount of free oil droplets dispersed at the aqueous phase.

(b) MPC 4861-stabilised emulsion

In the initial emulsion, fine oil droplets stabilised by MPC 4861 were evenly distributed throughout the system [Figure 6.7 (MPC 4861)]. At 20 min of digestion, the digesta contained emptied emulsion with a few small aggregate particles. With further digestion to 60 min, numerous blocks of aggregate particles with a range of sizes and shapes were observed in the emptied digesta, in which most of the small oil droplets were embedded. At a longer digestion time (120 min), the density and size of blocks of aggregated particles in emptied digesta had decreased [Figure 6.7 (MPC 4861-120 min)], and the aqueous phase surrounding the aggregated particles became green. Meanwhile, some free oil droplets appeared in the continuous phase. At 220 min, tiny evenly dispersed oil-filled particles and some separated free oil droplets were visible in the emptied digesta.

(c) Sodium caseinate-stabilised emulsion

For sodium caseinate-stabilised emulsion, a remarkable flocculation took place in the original emulsion. At 20 min of digestion, fine and uniformly distributed oil droplets

with an average size ($d_{4,3}$) approximately 2.5 μm were observed in the emptied digesta, along with the disappearance of the flocculation. With further digestion to 60 min, only a very small amount of tiny aggregated particles among the black background can be observed from the emptied digesta, with some small free oil droplets dispersed in the aqueous phase. With an increase in digestion time, these small aggregated particles underwent a considerable increase in the distribution density, and most of the oil droplets were trapped within evenly dispersed emulsion aggregates at 120 min. At the end of gastric digestion, the density of particles gradually decreased with further digestion, and some larger free oil droplets were present in the aqueous phase.

(d) WPI-stabilised emulsion

In WPI-stabilised emulsion [Figure 6.7 (WPI)], fine, uniformly distributed oil droplets were observed in the 20-min emptied digesta sample. At 60 min of digestion, the emptied digesta contained some separated blocks of aggregates, and the oil droplets were embedded within the aggregated matrix. With further digestion, the oil-filled aggregates appeared to be broken down to a smaller size, along with the release of small oil droplets in the aqueous phase [Figure 6.7 (WPI-120 min)]. At 220 min, some particles aggregated, along with some larger sized oil droplets present in the emptied digesta.

(e) Heated WPI-stabilised emulsion

For WPI-stabilised emulsion with heating [Figure 6.7 (Heated WPI)], many separated, ragged aggregates of a relatively small size were found in the emptied digesta at 60 min of digestion. At this time point, there existed some large cross-linked emulsion aggregates. These protein aggregates (either individual or cross-linked) trapped most oil droplets, although a few released “free” oil droplets that were suspended in the aqueous phase. With further digestion to 120 min, the aqueous phase became green, the fine and evenly distributed oil droplets can be seen in the emptied digesta, along with the majority of disassociated protein aggregates. There were a few emulsion aggregates in the digesta at this time. At a longer time of digestion (220 min), some emulsified particles were observed to aggregate with further hydrolysis.

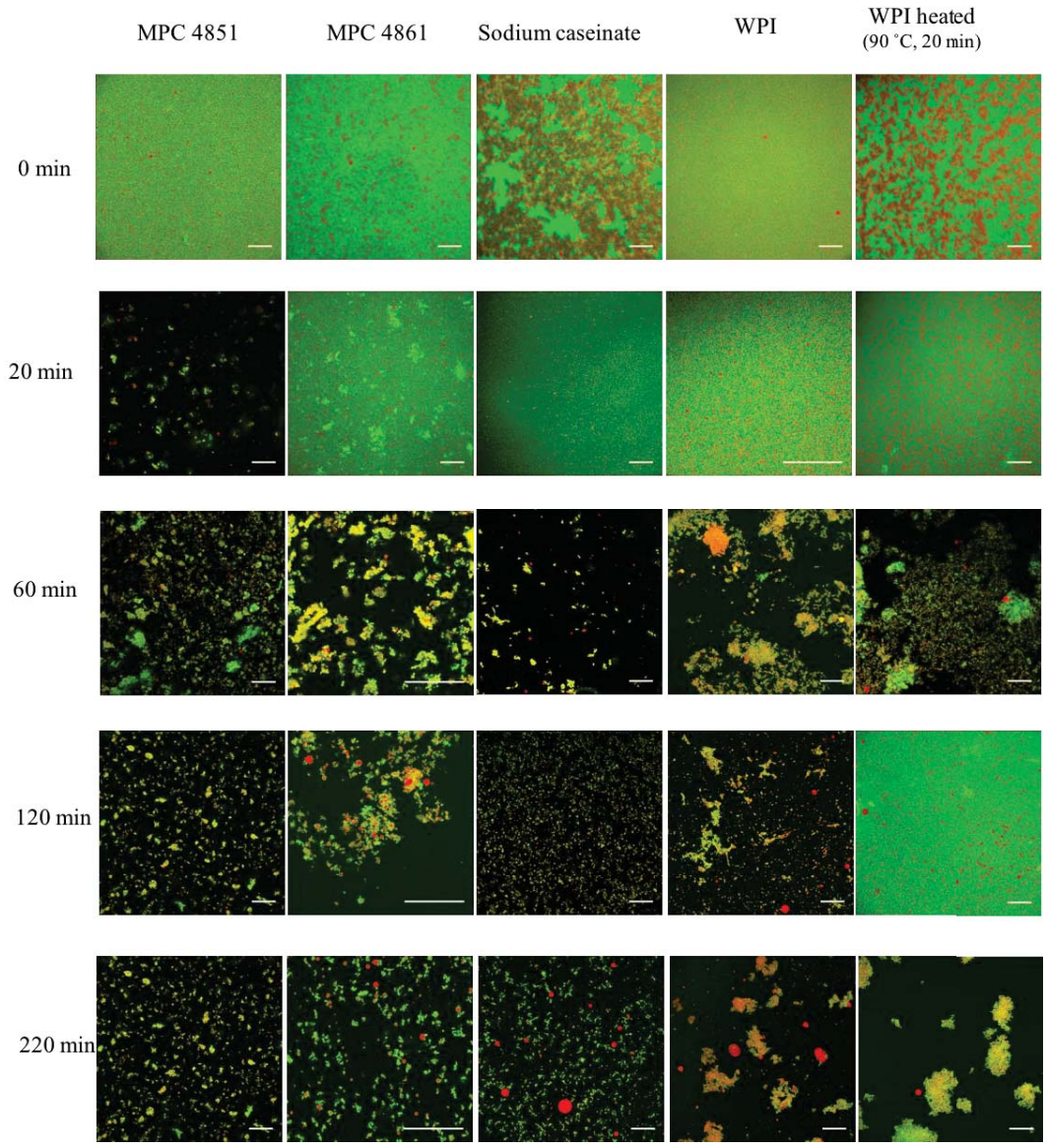


Figure 6.7. Confocal microscopy images of the emptied digesta of different milk protein ingredients-stabilised emulsions at different time points during the gastric digestion from 0 to 220 min. Fat is stained red, protein is stained green. The scale bar in all images is 50 μm.

6.3.1.8 SDS-PAGE patterns of emptied digesta

The protein composition and hydrolysis pathway of emptied digesta derived from emulsions stabilised by different protein ingredients as a function of digestion time, investigated by electrophoretic analysis under reducing conditions, were shown in Figure 6.8.

(a) MPC 4851-stabilised emulsion

In the SDS-PAGE pattern of MPC 4851-stabilised emulsion [Figure 6.8 (A)], the initial emulsion sample contains clear α_s-caseins (α_{s1}- + α_{s2}-casein), β-casein and κ-casein bands. The κ-casein band was less intense at 5 min. These components almost cannot be detected at 20 min of digestion and disappeared completely after 40 min. The intact whey protein (β-Lg, α-La and BSA) bands were observed within the first 5 min. The intensity of BSA band had become weaker with further digestion at 20 min, and cannot be detected at 40 min. The intensities of β-Lg and α-La bands gradually decreased with an increase in digestion time, α-La band cannot be visually observed at 80 min of digestion while β-Lg band was not observed at 220 min.

(b) MPC 4861-stabilised emulsion

For MPC 4861-stabilised emulsion, intact α_s-caseins, β-casein and κ-casein bands were observed at the first 5 min [Figure 6.8 (B)]. At 20 min, the κ-casein band was less intense than those were observed at 0 min and 5 min. A newly formed band occurred at around ~15 kDa, which probably consisted of para-κ-casein. Also, at this period, there was a remarkable decrease in band intensity of α_s-caseins and β-casein. With further digestion, casein bands became gradually less intense and was not observed at 120 min. In contrast, intact β-Lg and α-La bands were observed during whole digestion period, although the intensities of them decreased steadily with increasing digestion time, while BSA band was not observed from 40 min to the end of the gastric digestion.

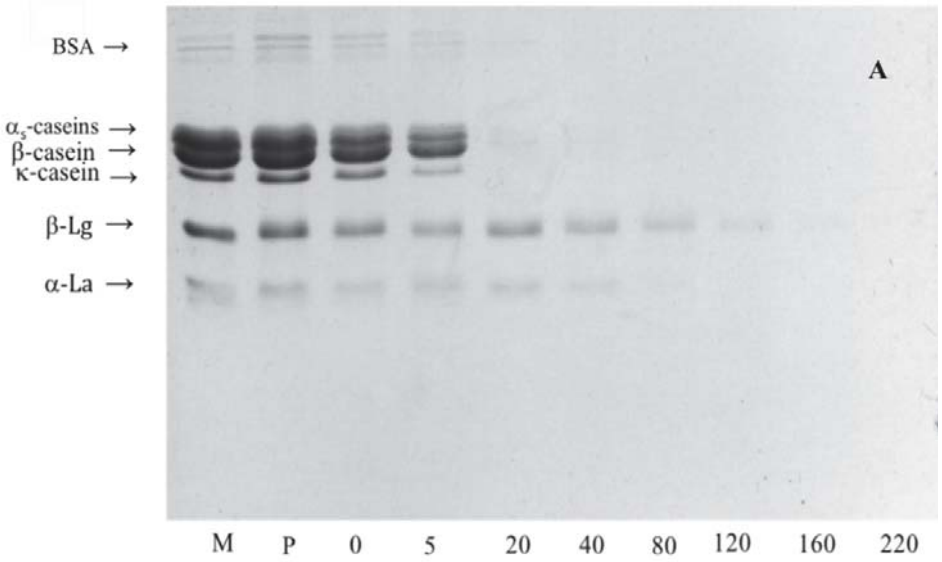
(c) Sodium caseinate-stabilised emulsion

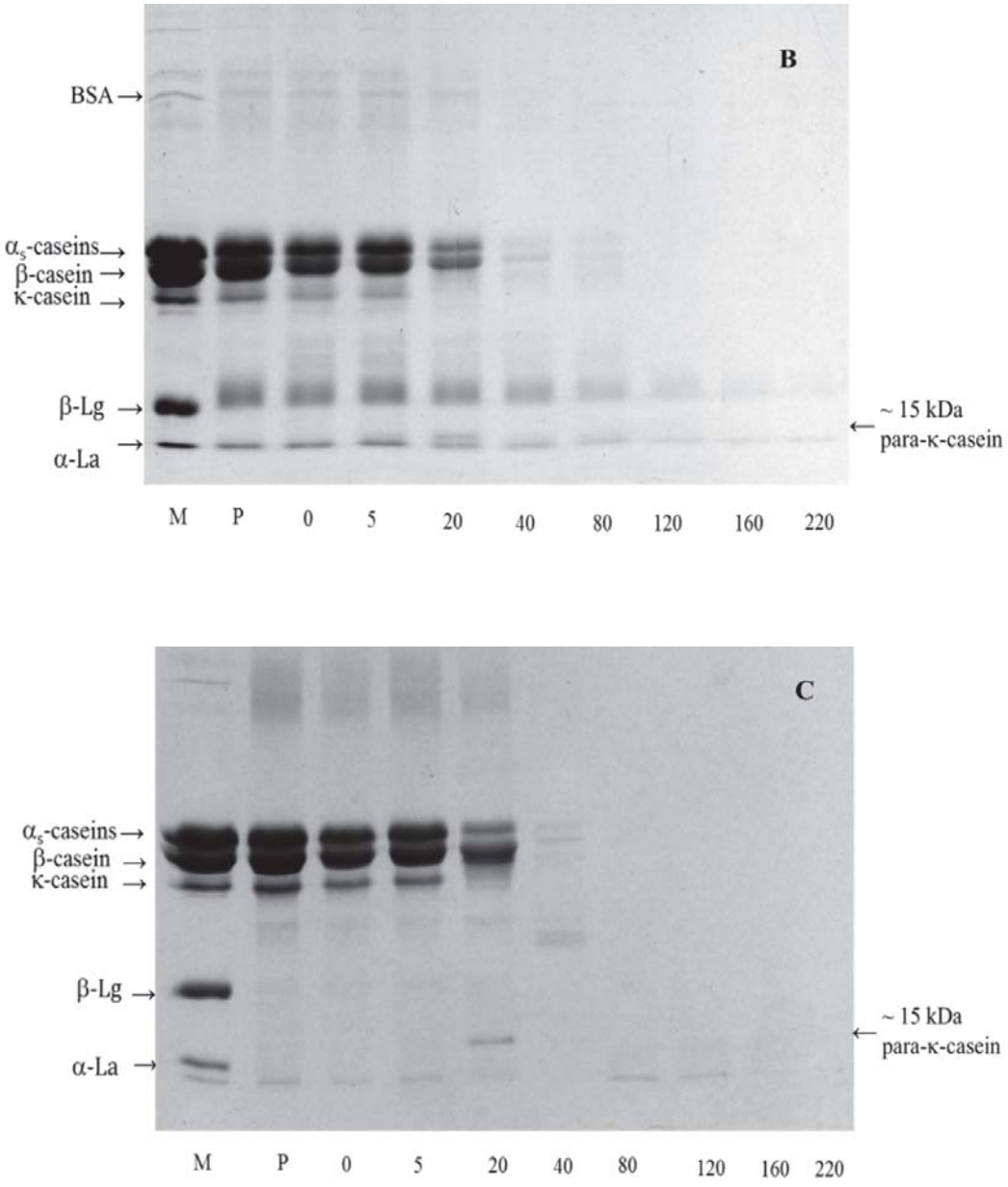
There were clear α_s-caseins, β-casein and κ-casein bands [Figure 6.8 (C)] observed in the emptied digesta from sodium caseinate-stabilised emulsion at 0 min and 5 min. At 20 min of digestion, a newly formed band occurred at approximately 15 kDa and the κ-casein bands were faint. A considerable reduction in intensities of α_s-caseins

and β-casein bands were observed at this time point. With further digestion, all casein bands were almost invisible at 40 min and were not observed at 80 min of digestion.

(d) WPI-stabilised emulsion with and without heating

At 5 min of digestion, intact β-Lg and α-La bands can be observed in the emptied digesta from both unheated and heated WPI-stabilised emulsions. In the emptied digesta of unheated emulsion, β-Lg band was visible at each time point of digestion and a steady reduction in the band intensities with an increase in digestion time was observed. In the emptied digesta of heated emulsion made with WPI [Figure 6.8 (E)], β-Lg band had become less intense with increasing digestion time to 120 min. Faint peptide bands can be observed at 20 min in the emptied digesta of heated emulsion, and the number of peptide bands increased from 40 min to 220 min, whereas almost no clear peptide bands were observed in the digesta of unheated WPI-stabilised emulsion. There was a decrease in intensity of α-La band with the increase in digestion time in the emptied digesta of both unheated and heated emulsions, although the band intensities of the latter were slightly less than those of the former. In the emptied digesta of unheated WPI-stabilised emulsion, α-La band was observed up to 40 min, but was no longer observed at 80 min, whereas in the emptied digesta of heated emulsion, α-La band was observed up to 80 min and no longer observed at 120 min. In both unheated and heated WPI-stabilised emulsions, BSA bands were observed up to 40 min but cannot observed at 80 min.




B
BSA
α_s-caseins
β-casein
κ-casein
β-Lg
α-La
~ 15 kDa
para-κ-casein
M P 0 5 20 40 80 120 160 220
C
α_s-caseins
β-casein
κ-casein
β-Lg
α-La
~ 15 kDa
para-κ-casein
M P 0 5 20 40 80 120 160 220

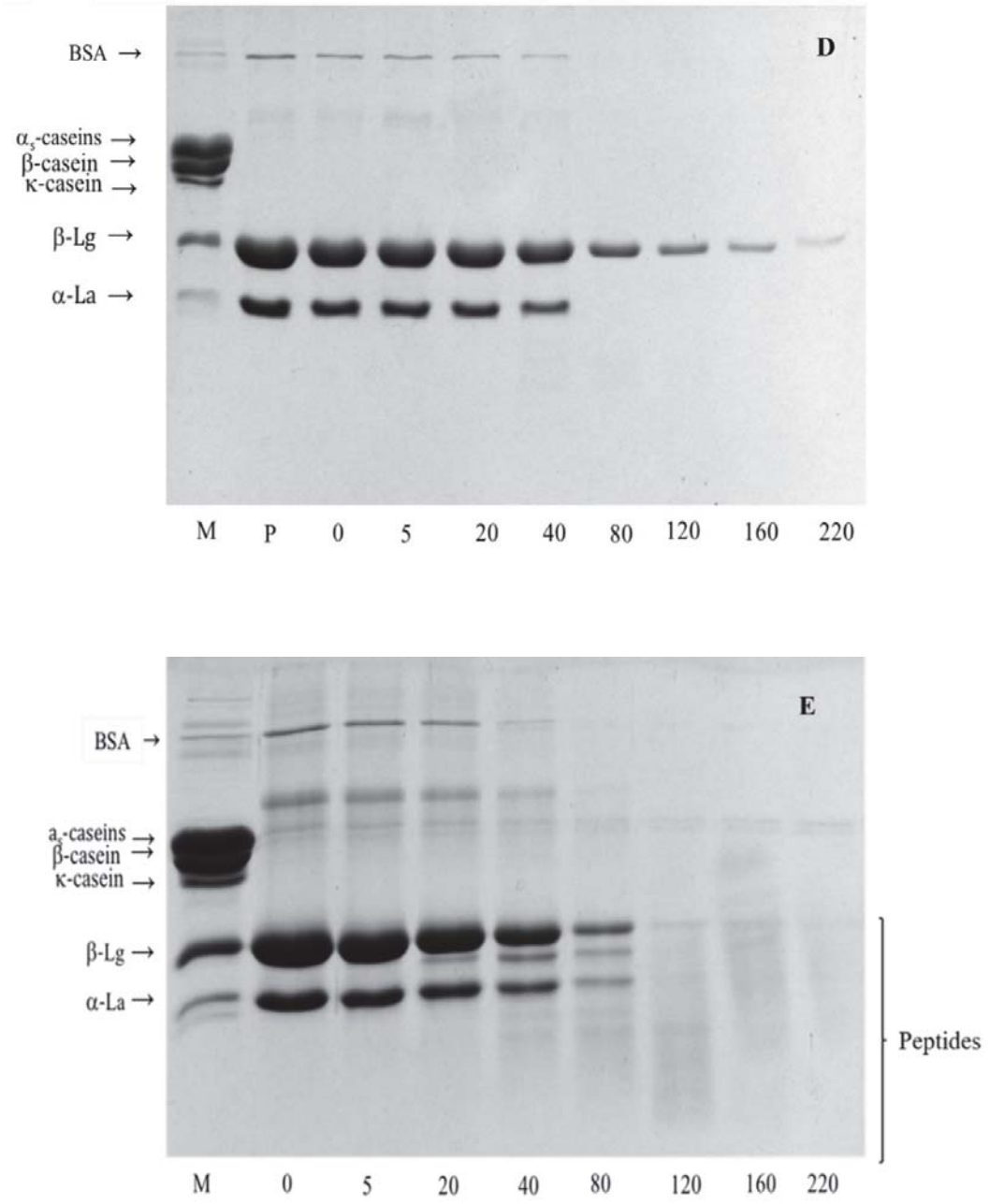


Figure 6.8. The SDS-PAGE patterns under reducing conditions of the emptied digesta obtained at the different time points during 220 min of gastric digestion from: (A) MPC 4851-stabilised emulsion; (B) MPC 4861-stabilised emulsion; (C) sodium caseinate-stabilised emulsion; (D) WPI-stabilised emulsion; (E) heated (90°C, 20 min) WPI-stabilised emulsion. BSA, bovine serum albumin; M, trim milk.

6.3.2 *In vitro* small intestinal digestion of emulsions

The emptied gastric digesta from emulsion made with different dairy ingredients were collected at each 20 min interval up to 220 min of gastric digestion and mixed well, then were digested in the simulated intestinal fluid (SIF) containing pancreatic lipase for 120 min.

6.3.2.1 The characteristic of mixed-emptied gastric digesta

The particle size ($d_{4,3}$) of mixed gastric digesta taken from different emulsions followed a pattern described below: MPC 4851-stabilised emulsion > MPC 4861-stabilised emulsion > sodium caseinate-stabilised emulsion, WPI-stabilised emulsions both with and without heating (Table 6.2).

The oil contents of the mixed gastric digesta taken from different emulsions adhered to the following pattern: MPC 4851-stabilised emulsion < MPC 4861-stabilised emulsion < sodium caseinate-stabilised emulsion, WPI-stabilised emulsions both with and without heating (Table 6.2).

Table 6.2. The volume ($d_{4,3}$) mean diameters and oil content of the mixed gastric digesta emptied from emulsions made with different dairy ingredients.

	MPC 4851-stabilised emulsion	MPC 4861-stabilised emulsion	Sodium caseinate-stabilised emulsion	WPI-stabilised emulsion	Heated WPI-stabilised emulsion
Droplet size ($d_{4,3}$) (μm)	54.21 ± 31.07	18.35 ± 10.06	1.33 ± 1.20	0.93 ± 0.10	1.17 ± 0.37
Oil content (g oil/100 mL digesta)	3.41 ± 0.22	4.38 ± 0.88	5.28 ± 2.00	6.05 ± 0.60	5.91 ± 0.11

Values are means and standard deviations (SD).

6.3.2.2 Microstructure of initial mixed-digesta in SIF

Many aggregate particles were observed from the mixture of emptied digesta from MPC 4851-, MPC 4861- and sodium caseinate-stabilised emulsions, in which oil droplets were incorporated by entrapment (Figure 6.9). In the mixture of emptied digesta from WPI-stabilised emulsion, some flocs were observed along with some aggregated matrixes. The mixed emptied gastric digesta of heated WPI-stabilised emulsion consisted of tiny and evenly dispersed oil droplets.

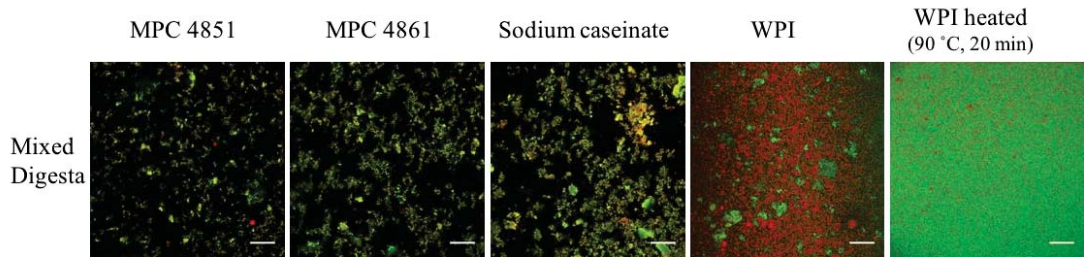


Figure 6.9. Confocal microscopy images of initial mixed-digesta (0 min) in SIF of different milk protein ingredients-stabilised emulsions. All samples were stained with Nile Red (for oil) and Fast Green (for protein). The scale bar in all images is 50 μm.

6.3.2.3 The change in average droplet size of emulsion during intestinal digestion

The average droplet size ($d_{4,3}$) of the mixture of emptied digesta from emulsion made with different dairy ingredients increased at mid and late-stages of intestinal digestion. For MPC 4851-stabilised emulsion [Figure 6.11 (A1)], the droplet size decreased ($P < 0.05$) from ~54.2 to ~4.4 μm at 1 min of digestion. The droplet size distribution of the initial sample was multimodal, with peaks at ~ 0.5 to 1000 μm. At 1 min, the size distribution was bimodal, with peaks at about 0.5 to 100 μm. With further digestion, the particle size distribution became unimodal at 10 min. At 120 min, the droplet size was ~19.4 μm at 120 min, and the droplet size distribution became bimodal again, with a newly formed peak at about 0.05 to 1 μm region.

The droplet size of the mixture of the emptied digesta from MPC 4861-stabilised emulsion [Figures 6.10 and 6.11 (B)] underwent a rapid decrease ($P < 0.05$) from ~18.4 to ~2.7 μm at 1 min. The particle size distribution of initial sample was bimodal, with peaks at about 1 to 1000 μm. At 1 min, the distribution became a unimodal peak located in a smaller size region (about 1 to 15 μm). With the increase in digestion time, the particle size ($d_{4,3}$) was not observed to change. At 120 min, the particle size distribution was bimodal, with peaks at about 0.1 to 100 μm regions.

For sodium caseinate-stabilised emulsion [Figures 6.10 and 6.11 (C)], the droplet size ($d_{4,3}$) did not show significant change ($P > 0.05$) over 120 min of the small intestinal digestion. However, the droplet size distribution became unimodal with a peak at about 0.1 to 10 μm region at 1 min from an initial bimodal distribution at about 0.1 to 100 μm

region. With an increase in digestion time to 120 min, the droplet size distribution was bimodal with peaks at about 0.01 to 100 μm.

For WPI-stabilised emulsion [Figures 6.10 and 6.11 (D)], the droplet size ($d_{4,3}$) was ~ 0.9 μm at 0 min. With increasing digestion time, the droplet size increased to ~13.2 μm ($P < 0.05$) at the end of digestion. The particle size distribution had no significant change within the first 60 min but was located at a larger size region at 120 min.

For heated WPI-stabilised emulsion [Figures 6.10 and 6.11 (E)], the droplet size ($d_{4,3}$) was ~0.9 μm at 1 min, followed by an increase to ~6.6 μm ($P < 0.05$) at 120 min. The initial droplet size distribution was unimodal, with a peak at around 0.1 to 10 μm. With further digestion to 60 min, the distribution became bimodal, with a newly formed peak at around 0.05 to 1μm regions. At 120 min, the droplet size distribution was multimodal, with peaks at around 0.01 to 1000 μm.

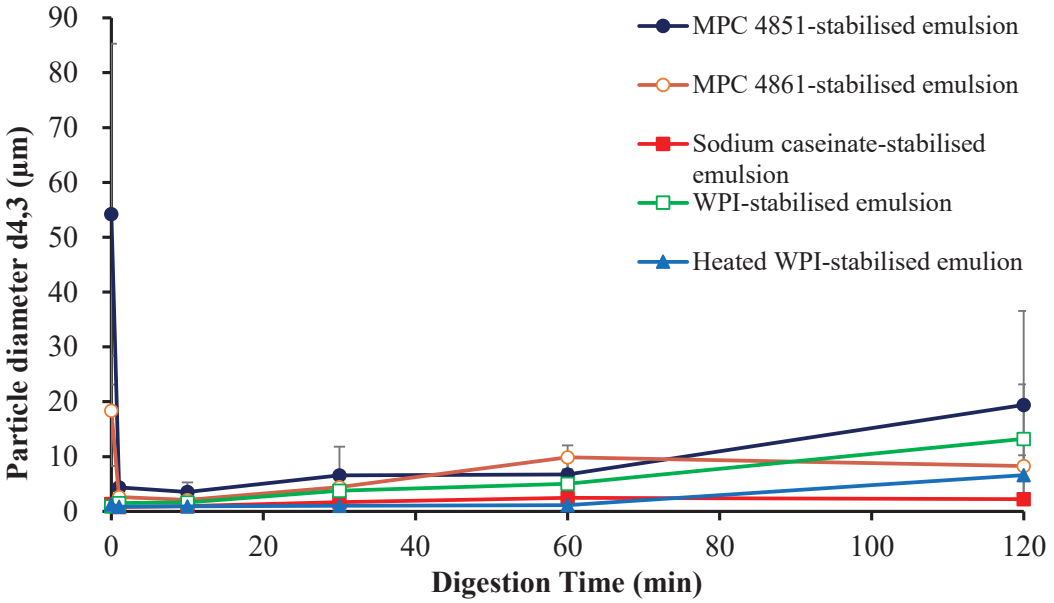
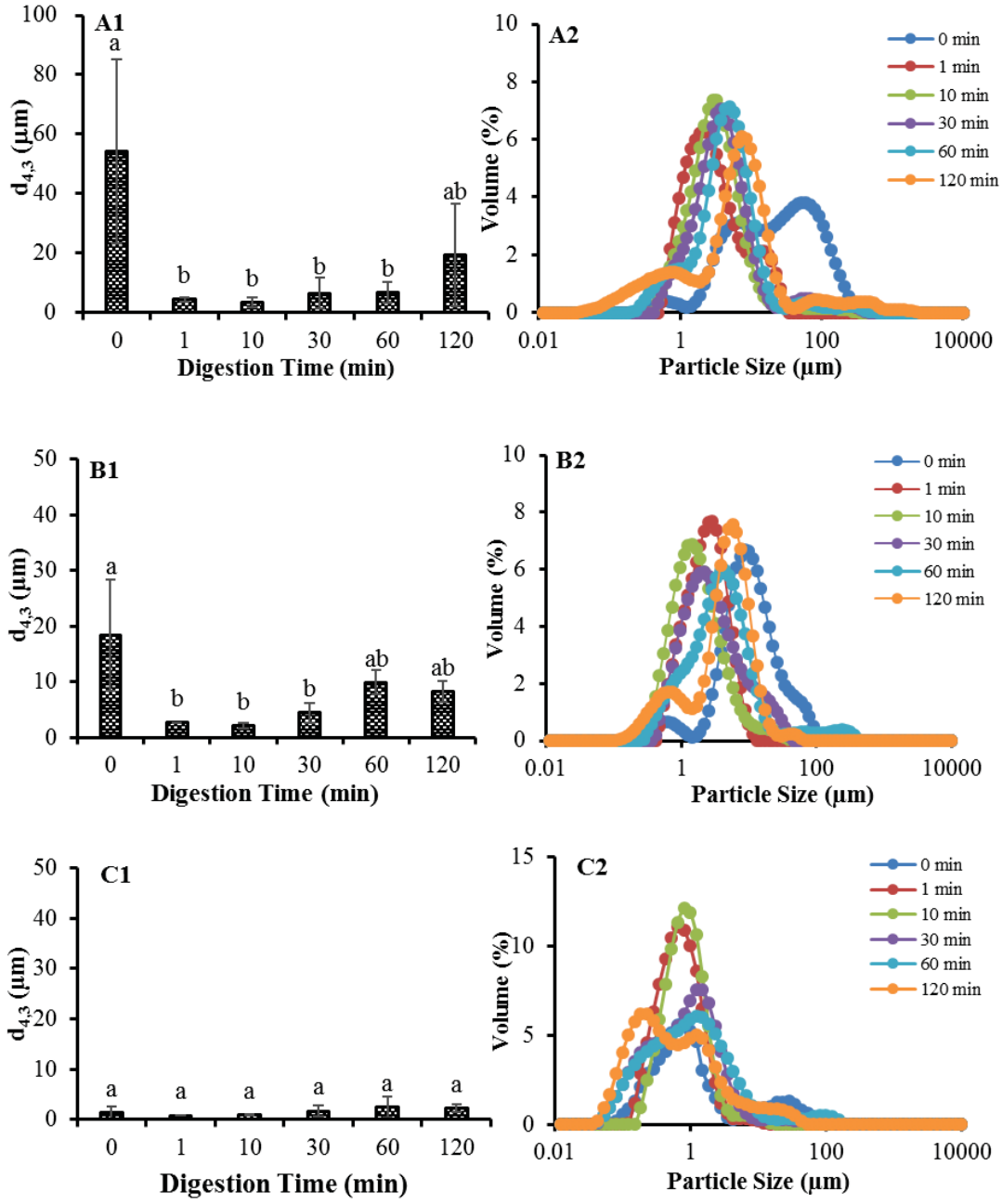


Figure 6.10. Changes in the average droplet diameter ($d_{4,3}$) of the mixed gastric digesta emptied from emulsions stabilised by different dairy ingredients during the digestion in SIF.


A1
a
b
b
b
b
ab
$d_{4,3}$ (μm)
Digestion Time (min)
A2
Volume (%)
Particle Size (μm)
0 min
1 min
10 min
30 min
60 min
120 min
B1
a
b
b
b
ab
ab
$d_{4,3}$ (μm)
Digestion Time (min)
B2
Volume (%)
Particle Size (μm)
C1
a
a
a
a
a
a
$d_{4,3}$ (μm)
Digestion Time (min)
C2
Volume (%)
Particle Size (μm)

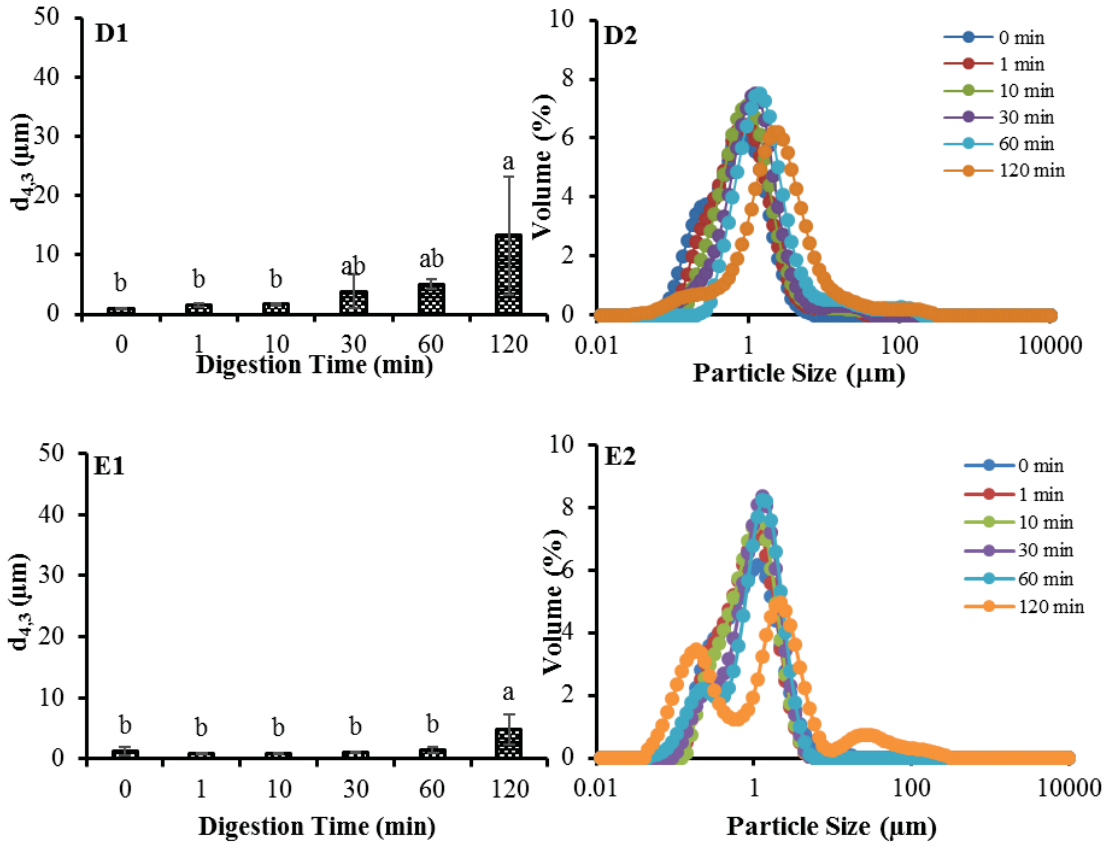


Figure 6. 11. Changes in volume ($d_{4,3}$) mean diameters (-1) and the size distributions (-2) of oil droplets in the emptied digesta from different milk protein ingredients-stabilised emulsions (20.0% soybean oil and 4.0% protein, w/w) during 120 min of intestinal digestion: (A) MPC 4851-stabilised emulsion; (B) MPC 4861-stabilised emulsion; (C) sodium caseinate-stabilised emulsion; (D) WPI-stabilised emulsion; (E) WPI-stabilised emulsion with heating (90°C, 20 min). Different lowercase letters indicate significant difference ($P < 0.05$) on the volume ($d_{4,3}$) mean diameters of oil droplets in emptied digesta between different digestion time points within the same emulsifier type.

6.3.2.4 Release of free fatty acid during small intestinal digestion

The MPC 4851-stabilised emulsion showed the slowest increase in the amount of released free fatty acids (FFAs), and had the lowest value of ~65 µmol FFA/ mL digesta among all emulsions at the end of digestion (120 min) (Figure 6.12). Compared to MPC 4851, MPC 4861-stabilised emulsion showed a slightly rapid release of FFAs during all

stages of the digestion and achieved a higher value of ~70 μmol FFA/ mL digesta at the end of the digestion (120 min). In comparison, the total amounts of FFA released from sodium caseinate-stabilised emulsion, unheated and heated WPI-stabilised emulsion were the highest among all samples. At the mid-digestion stage, FFA was released from sodium caseinate-stabilised emulsion at the most rapid rate than other four emulsions, in particular between 50 and 80 min. However, the rate of FFA release decreased with the further digestion, and arrived ~ 81 μmol FFA/mL digesta at 120 min. The lipid digestion profiles of the mixed gastric digesta from unheated and heated WPI-stabilised emulsions followed a fairly similar pattern. The rates of FFAs released from heated WPI-stabilised emulsion were slightly higher than those of unheated WPI-stabilised emulsion in the first 110 min. However, at late stage of digestion (after 110 min), the FFA release rate of the former was decreased and lower than the latter.

Because the mixed gastric digesta emptied from different dairy ingredient-stabilised emulsions varied with oil content (Table 6.2), the total amount of FFA released from per gram oil contained in various emulsions were calculated (Figure 6.13). MPC 4851-stabilised emulsion produced the greatest amount of FFA relative to the other four emulsions during the entire period of intestinal digestion. The amount of FFA released per gram of oil from MPC 4861-stabilised emulsion was much lower than that from the MPC 4851-stabilised emulsion. However, it still slightly higher than the other three emulsion samples.

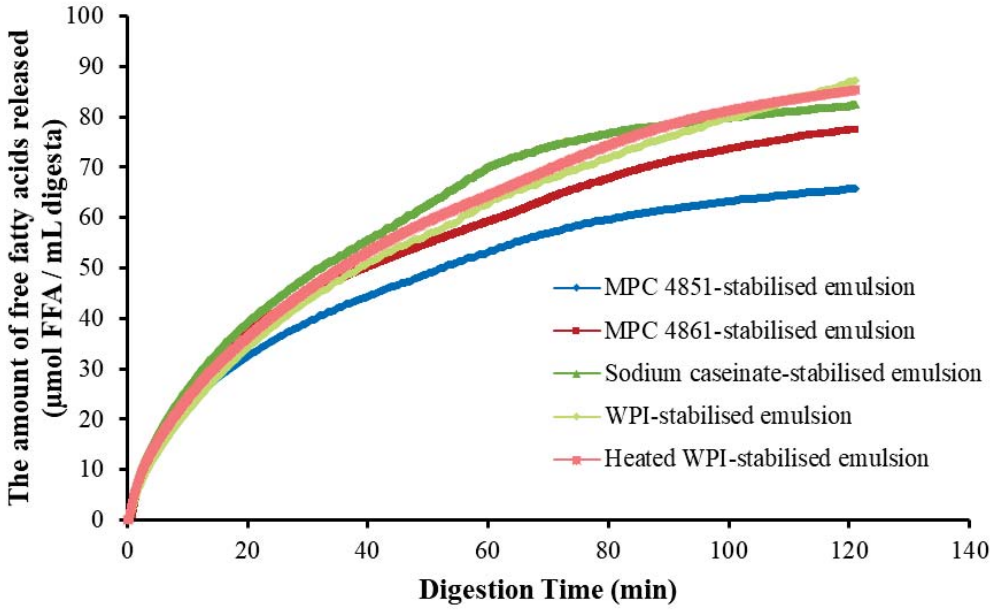


Figure 6.12. Amount of free fatty acids released (μmol FFA/mL mixed gastric digesta) from emulsions made with different dairy ingredients (titrated by 0.25 *M* NaOH) in pH-stat during 120 min intestinal digestion.

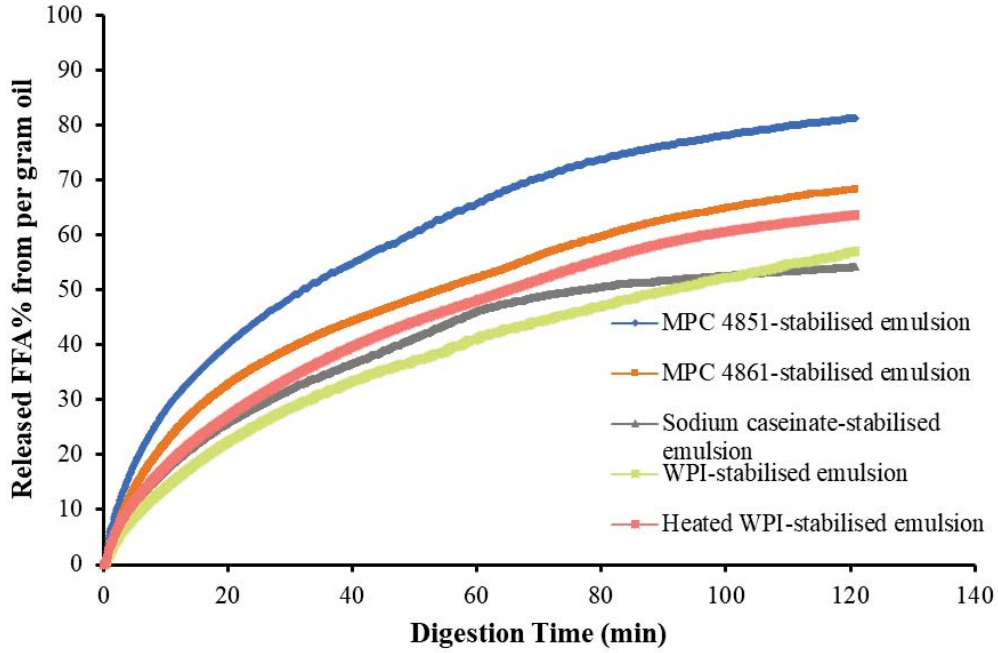


Figure 6. 13. Amount of free fatty acids (%) released from per gram oil contained in the mixed gastric digesta from emulsions made with different dairy ingredients (titrated by 0.25*M* NaOH) in pH-stat during 120 min intestinal digestion.

6.4 Discussion

6.4.1 The digestion behaviours of various dairy ingredients-stabilised emulsions during gastric digestion

During the gastric digestion, there was a consistent decrease in pH of the emptied digesta amongst all emulsion samples with an increase in digestion time (Figure 6.1). However, the decrease rates were different among the various samples. This difference in pH profile might be explained in part by the formation of aggregates with a range of different sizes in the emulsions stabilised by the various dairy ingredients. Hence, the diffusions rate of ions and molecules in and out of the aggregates were different, which is similar to as suggested by Ye et al. (2016b). Furthermore, it appears to be related to the different buffer capacities of the different milk protein ingredients. For example, there existed a more rapid decrease in pH of the emptied digesta from sodium caseinate-stabilised emulsion than those of MPC 4851 and MPC 4861-stabilised emulsions. This can be due to the phosphate contained in MPCs, whereas caseinate possesses reduced phosphate levels. Micellar phosphate levels may increase buffering capacity due to the formation of dihydrogenphosphate during acid solubilisation of colloidal phosphate calcium (Carr & Golding, 2016; Ferreira et al., 2003). The unheated and heated WPI-stabilised emulsions exhibited a more rapid decrease in pH, in particular at the early stage of digestion, probably because whey proteins possess a maximum buffering capacity at a lower pH region (pH 3-4) (Kailasapathy, Supriadi, & Hourigan, 1996; Srilaorkul, Ozimek, Wolfe, & Dziuba, 1989). The changes in pH may influence the activity of pepsin, whereby pepsin activity was relatively low in the early period of digestion due to the high pH (pH > 6). The activity of pepsin may increase with the decrease of pH in the stomach at a longer digestion time.

In MPC 4851-stabilised emulsion, a considerable aggregation was observed after 5 min of gastric digestion. As a result, the emptied digesta contained the lowest oil content (Figure 6.5) and protein level (Figure 6.8) at 20 min among all samples. The aggregates remained in the stomach with a relatively large size and did not disappear during the whole gastric digestion. This considerable amount of aggregates was first observed at pH ~ 6.5, which is far above the condition required for acid-induced aggregation (i.e., the pI of caseins, pI~4.6). Further, the κ-casein band in the emptied digesta was very faint at 5

min digestion [Figure 6.8(A)]. The emptied digesta almost contained no caseins bands, in particular for κ-casein band at 20 min. The initial hydrolysis rate of κ-casein by pepsin is known to be faster than other caseins at pH 6.0 in *in vitro* digestion (Tam & Whitaker, 1972). These observations indicate that the initial destabilisation (aggregation) of MPC 4851-stabilised emulsion was probably induced by the results of the proteolytic action of pepsin on micellar κ-casein (Ye et al., 2016b). κ-Casein is known to be the stabilising agent for casein micelles (Dalgleish & Corredig, 2012). Its destruction destabilised the casein micelles and it is likely that this induced the aggregation of emulsion. The aggregation catalysed by pepsin at the early stage of digestion (pH > c6.5) is probably attributed to the existence of micellar structure of caseins in MPC 4851-stabilised emulsion. It was known in the MPC, caseins exist in a micellar form, similar to that found in the fresh milk (Ye, 2011). Casein micelles are known to be coagulated both by the milk-clotting enzyme, pepsin (Tam & Whitaker, 1972) and acidic pH (Dalgleish & Corredig, 2012). The former may occur when pH is above 6, prior to the occurrence of acid-induced coagulation during the dynamic digestion. It is suggested that with increasing addition of SGF into the stomach, the micellar κ-caseins were hydrolysed by pepsin and so that casein micelles destabilised (Dalgleish & Corredig, 2012). As a result, a large amount of casein micelles in protein portion (both on the oil droplet surface and in the aqueous phase) of emulsion were coagulated and induced emulsion aggregation.

During the whole gastric digestion, the aggregates in MPC 4851-stabilised emulsion did not disintegrate completely, although the amount and the size of aggregates gradually became smaller with increasing digestion time. In contrast, no aggregated emulsion would be collected in the other four emulsions at the end of gastric digestion. This is probably due to a larger size and denser structure of the aggregates generated in MPC 4851-stabilised emulsion, compared to the other dairy ingredient-stabilised emulsions. This may have led to a longer time required to hydrolyse of all of the aggregated emulsion, completely. Previous work in our laboratory has demonstrated that the clot formed in skim milk was only degraded on its surface by pepsin because of the difficulty of diffusion into the clot (Ye et al., 2016b). It appears that the hydrolysis behaviour of pepsin on aggregate was diffusion-dependent, which started from the surface to interior of the clot/aggregate with the further effect of the diffusion of SGF. When a clot has a fragmented and crumbled structure, the clot with a larger size may be

hydrolysed by pepsin at a slower rate than that with a smaller size, which has been shown in Chapter 5. Therefore, the hydrolysis by pepsin of the aggregates with a larger size probably takes longer time than that with a smaller size.

The oil content in the emptied digesta represented the amount of oil that was delivered into the small intestine (Figure 6.5). At the early stage of digestion (20 min), the emptied digesta from MPC 4851-stabilised emulsion contained the lowest oil content, which was far lower than that of the other four emulsion samples ($P < 0.05$). This could be attributed to the generation of aggregates in MPC 4851-stabilised emulsion within this time period, in which most oil droplets were captured and remained in the stomach. With further digestion, the oil amount into the small intestine increased slightly. It is probable that the oil droplets were released from the aggregated emulsion matrix caused by the gradual hydrolysis by pepsin.

For MPC 4861- and sodium caseinate-stabilised emulsions, a considerable amount of aggregation was visible at around 40 min, which was later than the occurrence of aggregation in MPC 4851-stabilised emulsion. At 40 min, the pHs of emptied digesta were ~pH 5.6, and ~ 5.3 for emulsions made with MPC 4861 and sodium caseinate, respectively. Compared to the pH at which aggregation in MPC 4851-stabilised emulsion occurred (pH~6.5), these pH values were much lower and closer to the pI of caseins (pI~4.6). These differences between MPC 4861-, sodium caseinate-stabilised emulsions and MPC 4851-stabilised emulsion can be attributed to a lack of intact casein micelles in MPC 4861 and sodium caseinate. Coagulation of caseins in individual molecular form which occurs at around its pI. The principal portion of casein micelle in MPC 4861 has been dissociated by removing the calcium compared to MPC 4851 (Ye, 2011). Casein is coagulated only by acidic pH, not by pepsin, while casein micelles can be coagulated not only by low pH (Dalgleish & Corredig, 2012), but also by pepsin (Tam & Whitaker, 1972). This phenomenon has been reported to occur in the milk protein ingredient solutions under gastric digestion (Chapter 4). In emulsion, MPC 4861 and caseinate adsorbed on the oil droplet surface had similar behaviours to those which were observed in solution.

After 220 min of the gastric digestion, no aggregate was collected from emulsion made with MPC 4861 and sodium caseinate. It is likely due to the relatively small size of the aggregates formed in these two emulsions. These aggregates were hydrolysed by

pepsin more rapid. However, the time needed to hydrolyse the aggregates completely in MPC 4861 and sodium caseinate-stabilised emulsions differed. Aggregates were not observed in MPC 4861-stabilised emulsion after 200 min of gastric digestion, whereas most aggregates in sodium caseinate-stabilised emulsion had been depleted after 100 min. This variance in the time of enzymatic hydrolysis may be caused by the different proteolysis rates, which were induced by various pH of the different samples during the gastric digestion (Figure 6.1). A more rapid decrease in pH of sodium caseinate-stabilised emulsion probably induced a faster hydrolysis by pepsin than those of MPC 4861-stabilised emulsion.

It is worth mentioning that creaming and flocculation were observed in the original sodium caseinate-stabilised emulsion. This could be attributed to depletion flocculation of oil droplets, which caused by self-association of unbound proteins in the aqueous phase when protein concentration is relative high (4.0%, w/w in this case) (Dickinson & Golding, 1997) [Figure 6.7 (sodium caseinate-0 min)]. However, this flocculation was weak and reversible (Dickinson & Golding, 1997), and the flocculation was observed to disappear from emulsion with further digestion,.

There were no aggregates obtained after 220 min of the gastric digestion from WPI-stabilised emulsions both with and without heating. In addition, no significant difference ($P > 0.05$) in the oil content of the emptied digesta between heated and unheated WPI-stabilised emulsions, with exception of that at 20 min (Figure 6.5). Both them had a faster oil delivery than that of MPC 4851, MPC 4861 and sodium caseinate-stabilised emulsions. In unheated and heated WPI-stabilised emulsions, aggregations were observed to occur at the same time (around 40 min), when the pH was ~4.8, which closed to the pI of whey proteins. Nevertheless, there was a difference in aggregation level between unheated and heated WPI-stabilised emulsions. Aggregation formed was of in a smaller size and extent and had disappeared by 80 min of digestion in unheated WPI-stabilised emulsion. In contrast, a considerably greater amount of aggregates with a larger size was observed until 100 min of digestion in heated WPI-stabilised emulsion. This formation of relatively mild aggregation in unheated WPI-stabilised emulsion may be because a portion of the hydrophobic groups at core of the native whey protein molecule become exposed on the surface due to the unfolding of molecules during emulsification (Dickinson, 1992). When pH decreased with the addition of SGF to the pI

of whey proteins, unheated WPI-stabilised emulsion underwent aggregations induced by increased hydrophobic interactions. However, the aggregation level is far lower than that formed in heated WPI-stabilised emulsion. This difference could be partly attributed to the full unfolding and exposure of the hydrophobic residues of whey protein induced by heating at above 80 °C in heated WPI emulsion (Kyros Demetriades & McClements, 1998). However, this pH-dependent aggregation could no longer be observed when the pH was far away from the pI of whey proteins.

The oil contents of the emptied digesta from unheated and heated WPI-stabilised emulsions were much higher than those of other three emulsions at 60 min, which indicates a higher delivery rate of oil into the small intestine in both WPI-stabilised emulsions. This difference could be attributed to the different level of aggregation in the different emulsions. In both unheated and heated WPI-stabilised emulsions, the aggregation levels were much lower than those of MPC 4851, MPC 4861 and sodium caseinate-stabilised emulsions. The oil droplets were thus delivered to the small intestine from unheated and heated WPI-stabilised emulsions at a more rapid rate with the gastric emptying.

There was an essential difference in the hydrolysis pathway between unheated and heated WPI-stabilised emulsions during the whole gastric digestion [Figure 6.8(D) and (E)]. In unheated WPI-stabilised emulsion, β-Lg remained relatively intact without degradation during whole digestion period. Native β-Lg is resistant to hydrolysis by pepsin because of its compact globular structure (Miranda & Pelissier, 1983; Reddy et al., 1988b). It should be noted that β-Lg adsorbed at the oil-water interface during emulsification can be hydrolysed by pepsin during the *in vitro* gastric digestion, due to its unfolding and conformational rearrangement on the oil droplet surface, leading to the exposure of its peptic cleavage sites (Sarkar et al., 2009). However, only a small portion of β-Lg has been reported to be denatured, while some intact native β-Lg are still present in the aqueous phase and are resistant to hydrolysis (Nik et al., 2010b). Therefore, during the gastric digestion, some denatured β-Lgs might be partially hydrolysed by pepsin, although almost no peptide bands can be observed in the SDS-PAGE pattern of emptied digesta from unheated WPI-stabilised emulsion in this study. It may be that the amount of the peptides was far less than that of native β-Lg, thus the degradation products of denatured β-Lg were not shown along this β-Lg band.

In contrast, in heated WPI-stabilised emulsion, a large amount of enzymatic degradation products (i.e., peptides) in the evacuated digesta at 40 min of digestion were detected by electrophoretic analysis [Figure 6.8(E)]. This finding was in agreement with the previous report showing that heated WPI-stabilised emulsion containing an abundance of unadsorbed protein might increase the extent of protein hydrolysis (Nik et al., 2010b). This has been reported to improve digestibility partially, whereby heating of β-Lg above 80 °C can allow the conformation of β-Lg to fully unfold and expose its buried amino acid side groups to a reactive state, as a result, making it more susceptible to pepsin hydrolysis (Anema & Li, 2003a; Demetriades & McClements, 1998; Guo et al., 1995). This may also be caused by the increased adsorption of proteins at the interface due to heat-induced interactions, or because of the rearrangements at the interface induced by pepsin hydrolysis (Nik et al., 2010b).

It is worth mentioning that no intact protein bands were observed in the SDS-PAGE pattern at longer gastric digestion times (120 min-220 min) in heated WPI-stabilised emulsion, indicating that both unadsorbed and adsorbed whey protein were hydrolysed by pepsin [Figure 6.8 (E)]. Only peptides remained at the interface to stabilise the oil droplets. Moreover, at 220 min, some aggregates were observed on the confocal microscopy images of the emptied digesta and digestive residues of both heated and unheated emulsion stabilised by WPI [Figure 6.6 (WPI-220 min) and (WPI heated -220 min)]. This is most likely to have been caused by extensive digestion and leading to the destabilisation of the emulsions (Nik et al., 2010b). At the late stage of the digestion, emulsion was cationic as the pH was between ~1.7 and ~2.1, which was lower than the pI of whey proteins (pI~5.1). In a previous study, during the *in vitro* gastric digestion of a β-Lg-stabilised emulsion, a gradual loss of positive charge was caused by the pepsin hydrolysis on the droplet surface. This resulted in a reduction in the thickness of the adsorbed protein layer. The interfacial layer stabilised by peptides are unable to provide sufficient steric barriers and electrostatic repulsion. Thus, the emulsion is unstable and prone to flocculation or coalescence (Sarkar et al., 2009).

Aggregation behaviours of emulsion in the stomach play an important role in the release of oil droplets to the next digestion step. The emptied digesta from MPC 4851-stabilised emulsion contained a significantly smallest amount ($P < 0.05$) of oil droplets delivered into the small intestine compared to the other dairy ingredient-stabilised

emulsions at 20 min (Figure 6.5). The oil content only showed a slight increase at 120 min and remained unchanged until the end of digestion. This may be attributed to the earliest formation time and the largest size of the aggregates in MPC 4851-stabilised emulsion, in which most oil droplets have been enclosed and cannot be delivered to the small intestine. The larger size may have prevented the diffusion of SGF and pepsin into the aggregates and action on them. This slowed down proteolysis and dissociation of large particles, so that oil droplets were released from the protein matrix at a slower rate. The MPC 4851-stabilised emulsion thus had the slowest delivery of oil into the small intestine. In comparison to MPC 4851-stabilised emulsion, MPC 4861 and sodium caseinate-stabilised emulsions contained a significantly higher ($P < 0.05$) oil content in emptied digesta at 20 min. The oil contents showed a dramatic decrease ($P < 0.05$) to a very low level at 60 min, which was similar to that of MPC 4851-stabilised emulsion, and was not observed to increase until the end of gastric digestion. This may be attributed to the aggregation occurred at a later time (around 40 min) and with a smaller size in those two emulsions. Therefore, at early stage of digestion (before aggregation formed in the stomach), the oil droplets in those two emulsions were delivered directly into the small intestine with the gastric emptying. These suggest that MPC 4861 and sodium caseinate-stabilised emulsions had a more rapid oil delivery into the small intestine than that of the MPC 4851-stabilised emulsion. The emptied digesta from WPI-stabilised emulsions both with and without heating contained higher ($P < 0.05$) oil contents than those of MPC 4851-stabilised emulsion at 20 min, a significantly highest oil content ($P < 0.05$) among all emulsions at 60 min. This is probably because of aggregation occurred in the smallest extent, with a smallest size in both WPI-stabilised emulsions. Both heated and unheated WPI-stabilised emulsions thus had the most rapid delivery of oil droplets into the small intestine than other three emulsions. These differences in the delivery rates of oil droplets into the small intestine can be attributed to the different aggregation behaviours in different dairy ingredient-stabilised emulsions.

6.4.2 The digestion behaviours of various dairy ingredients-stabilised emulsions during the small intestinal digestion

The changes in the particle size ($d_{4,3}$) of MPC 4851 and MPC 4861-stabilised emulsions showed a similar pattern, i.e., the particle size underwent a drastic decrease within 1 min of digestion, and was not observed to change with further digestion [Figures

6.11 (A) and (B)]. This noticeable reduction in $d_{4,3}$ value was observed after mixing the sample with pancreatic enzyme and bile salts, which was probably because the emulsion droplets dissociated from aggregates to form the individual droplets. This change may be caused by protein hydrolysis at the interface by pancreatic enzyme, or partly because of the rapid adsorption of the bile extract at the oil-water interface resulting in the displacement of surface protein at the oil-water interface (Fave, Coste, & Armand, 2004; Ivanova, Panaiotov, Bois, Gargouri, & Verger, 1990; Mun, Decker, & McClements, 2005; Wickham, Garrood, Leney, Wilson, & Fillery-Travis, 1998). In addition, in WPI-stabilised emulsions both with and without heating, the average droplet size ($d_{4,3}$) increased slightly at the end of the small intestinal digestion. This can be attributed to coalescence, promoted by pancreatic lipase, which occurred in the emulsions. Moreover, it is probably because of the formation of insoluble matter, such as calcium soaps with further lipid digestion (Scow, 1988).

A previous study has shown that the mean droplet size of emulsions may affect hydrolysis rate by pancreatic lipase in the small intestine. An emulsion with a larger particle size was hydrolysed slower than an emulsion with a smaller particle size (Borel et al., 1994). However, in our case, although the particle size of the mixed gastric digesta from different emulsion samples showed significant differences at 0 min, the large particle size in MPC 4851 and MPC 4861-stabilised emulsion samples underwent a considerable decrease to a smaller size similar to that of other samples after incubation in SIF for 1 min. Therefore, the particle size is considered to have a limited effect on the lipid hydrolysis during 120 min of the small intestinal digestion in the present study.

The extent of FFAs release from different samples (Figure 6.12) followed a similar pattern to that of oil content (Table 6.2): MPC 4851-stabilised emulsion < MPC 4861-stabilised emulsion < sodium caseinate-stabilised emulsion, WPI-stabilised emulsions both with and without heating. These suggest that the extent of lipid digestion by pancreatic lipase is mainly dependent upon the oil contents in the mixed gastric digesta emptied from different emulsions.

For WPI-stabilised emulsion with and without heating, it was observed that there was no difference in the total amount of FFAs release between those two samples (Figure 6.12). The FFAs released from heated WPI-stabilised emulsion were slightly faster than

unheated sample during the first 110 min of digestion. This slightly faster rate of lipid digestion of heated WPI-stabilised emulsion was probably due to more hydrolysis by pepsin during the gastric digestion than that occurring in unheated WPI-stabilised emulsion. These observations indicate that the extent of lipolysis of emulsified lipids would mainly be affected by the oil content; however, the rate of lipolysis could have been to some extent affected by proteolysis extent of emulsified droplets during pervious gastric digestion.

6.5 Conclusions

This study provides an insight into the lipid digestion in emulsions made with different commercial dairy protein ingredients as well as the physicochemical changes during the small intestinal digestion after the dynamic gastric digestion in a human gastric simulator (HGS).

The behaviours of emulsion in the stomach plays an important role in the protein hydrolysis and the lipid digestion in the small intestine. Aggregations induced by the action of milk-clotting enzyme pepsin and/or acidic pH formed in different dairy ingredient-stabilised emulsions during the dynamic gastric digestion. The formation time and the size of aggregates varied in the different emulsion samples. The oil droplets were incorporated into the aggregates by entrapment with the formation of aggregations in emulsions. They were subsequently released from the aggregated matrix with the hydrolysis of protein fraction of emulsions by pepsin. The aggregates of the emulsions with different structures and formation time were hydrolysed by pepsin to different levels in the stomach, which led to the different delivery rates of oil into the small intestine, resulting in a different extent of lipid digestion within the small intestinal environment.

Aggregation in MPC 4851-stabilised emulsion induced by pepsin formed at an earlier time in the HGS. The aggregates were of the largest size and remained in the stomach during whole gastric digestion. This led to a lower oil content in the emptied gastric digesta and the delayed delivery of oil droplets into the small intestine. The emulsions stabilised by MPC 4861 and sodium caseinate formed aggregates with a relatively small size at a later time in the stomach, and dissociated with further digestion in the HGS. This resulted in a more rapid delivery of oil droplets into the small intestine than MPC 4851-stabilised emulsion; however, it was slower than that of WPI-stabilised emulsions both with and without heating. In unheated and heated WPI-stabilised emulsions, the pH-dependent aggregation formed at a similar time to that of MPC 4861 and sodium caseinate-stabilised emulsions. Compared to other three emulsions, these aggregates were of the smallest size, and disintegrated quickly with further digestion in the HGS. Both WPI-stabilised emulsions with and without heating had a higher oil content in the emptied digesta at the early-stage of the gastric digestion and caused a more rapid delivery of oil droplets into the small intestine.

The aggregation behaviours of different emulsions in the stomach led to a difference in the amount of oil delivered into the small intestine. The oil content plays a significant role in the extent of lipid digestion by pancreatic lipase in the small intestine. The sample with a higher oil content released a greater amount of FFAs than the sample with a lower oil content. The extent of FFA release from different samples followed the following pattern: MPC 4851-stabilised emulsion < MPC 4861-stabilised emulsion < sodium caseinate-stabilised emulsion, WPI-stabilised emulsions both with and without heating.

Chapter 7: Overall Summary and Recommendations

This project provides an insight into understanding of the digestion behaviours of various commercial dairy ingredients in the *in vitro* dynamic gastric digestion and the lipid digestion in emulsions stabilised by these dairy ingredients under the simulated gastrointestinal (GI) conditions.

In the first part of this study, the digestion behaviours of a range of dairy ingredients were investigated under dynamic gastric conditions using a human gastric simulator (HGS). Caseins exist in a micellar form in SMP and MPC 4851. Casein micelles in these two ingredients were destabilised to form a dense ball-like clot with a closely knit structure at an earlier digestion time in the stomach. The clots did not breakdown during the whole digestion time. This was induced by the action of milk-clotting enzyme, pepsin. MPC 4861 and sodium caseinate formed a clot at longer digestion time, which was induced by acidic pH. At the end of digestion, the clots consisted of numerous small, loose, fragmented protein blocks. In contrast, no clot was observed at the end of digestion in WPI both with and without heating. Unheated WPI remained soluble in the stomach; β-Lg without being hydrolysed by pepsin went through to the intestine during the whole period of gastric digestion. While heated WPI was hydrolysed rapidly by pepsin and formed aggregations when the pH closed to the isoelectric point of whey proteins in the stomach. However, the aggregates were dissociated with further digestion. These different coagulation behaviours in the different dairy ingredients led to different protein compositions of the emptied digesta, different rates of gastric emptying and protein hydrolysis by pepsin under the gastric conditions. The dense structure of clot in SMP and MPC 4851 slowed the protein hydrolysis by difficulty of permeation into the clot. The earlier clotting time and slower proteolysis caused a slower gastric emptying of caseins in SMP and MPC 4851. The clotted casein in MPC 4861 and sodium caseinate showed a more rapid hydrolysis and gastric emptying than that in SMP and MPC 4851, because of a later clotting time and the more fragmented, loose structure of the curds. Both WPI with and without heating showed the most rapid gastric emptying of proteins into the small intestine among all ingredients due to no clot formation. These results suggest that the gastric emptying rates of different dairy ingredients are different because of the formation of clots with different time and structures. The formation of structured clots, the

differences in the gastric emptying and proteolysis are affected by the state of caseins, the protein composition and the pretreatment of dairy ingredients, which may be used to manipulate the bioavailability of proteins and to regulate amino acid and lipid metabolism.

Similar patterns were observed in oil-in-water emulsions made with different dairy ingredients during the gastrointestinal digestion. The behaviours of emulsion in the stomach plays an important role in protein hydrolysis and the lipid digestion in the small intestine. Aggregations were formed from different dairy ingredient-stabilised emulsions during the dynamic gastric digestion. The formation time and the size of aggregates varied in the different emulsion samples. The oil droplets were incorporated into the aggregates by entrapment with the formation of aggregations, and subsequently released from the protein matrix with the hydrolysis of protein fraction of emulsions by pepsin. The aggregates with a different structure and formation time were hydrolysed by pepsin to different levels in the stomach. The dynamic process of the capture and subsequent liberation of oil droplets led to different delivery rates of oil droplets into the small intestine, resulting in a different extent of lipid digestion in the small intestinal environment.

The aggregates were formed in MPC 4851-stabilised emulsion induced by pepsin at the earliest time and were of the largest size, remained in the stomach during whole gastric digestion. This led to a lower oil content and a delayed delivery of oil droplets into the small intestine. MPC 4861 and sodium caseinate-stabilised emulsions formed aggregates at longer digestion time with a smaller size, and dissociated with further digestion in the stomach. This resulted in a more rapid delivery of oil droplets into the small intestine compared to MPC 4851-stabilised emulsion; however, it was slower than that of WPI-stabilised emulsions both with and without heating. In unheated and heated WPI-stabilised emulsions, aggregation formed at a similar time to that in MPC 4861 and sodium caseinate-stabilised emulsions. These aggregates in WPI-stabilised emulsions were of the smallest size, and disintegrated with further digestion in the stomach. WPI-stabilised emulsions thus had the highest amount of oil delivered into the small intestine.

These differences in the aggregation behaviours of emulsions stabilised by different dairy ingredients led to a different amount of oil in the mixture of gastric digesta obtained from each digestion time point. This difference in oil content is an important

factor affecting the extent of lipid digestion by pancreatic lipase in the small intestine. The extent of free fatty acids (FFAs) release from different samples followed a similar pattern that of oil content: MPC 4851-stabilised emulsion < MPC 4861-stabilised emulsion < sodium caseinate-stabilised emulsion, WPI-stabilised emulsions both with and without heating.

These results suggest that different aggregation behaviours of emulsions in the stomach, and hence the dynamic changes in oil content of the emptied digesta, in the gastric emptying rate of protein and lipids, affect lipid digestion in the small intestine. These could be used to manipulate the bioavailability of oil and proteins to affect digestion and absorption of lipophilic functional/bioactive food components or other nutrients in food.

The impact of protein concentrate (0.5-5.0%, w/w) on the digestion behaviours of SMP and MPC 4851 was further evaluated under the gastric conditions. Protein concentration plays a significant role in the structure of clot formed in the gastric environment. The gastric hydrolysis of protein was greatly influenced by the clot structure. In both SMP and MPC 4851, the clot had a fragmented, loose and porous structure when protein concentration was lower than 2.0% (w/w). An intact ball-like clot had a more compact, dense structure observed when protein concentration varied from 2.0% to 5.0% (w/w). The hydrolysis rate followed the order: the sample with a protein concentration of 0.5% > 1.0% > 2.0%-5.0% (w/w). These results suggest that a clot with loose and fragmented structure causes a more rapid protein hydrolysis compared to a clot with a dense structure. A low protein content in protein solution can induce a more rapid proteolysis in the stomach.

Overall, the coagulation behaviours and the structure of clots formed in the stomach plays an important role in both gastric and small intestinal stages of dairy digestion. The structure of the clots affected the rate of both protein hydrolysis and oil droplets release, and further affected the extent of lipid digestion in small intestine. The structures of clot formed in various dairy ingredients under gastric conditions are determined by the state of casein, the processing prior to digestion and protein composition. The information obtained from this study will be useful for a better understanding of digestion behaviours of different commercial dairy ingredients in the GI

tract. It, as well, provides aids for a better design of the processed food products derived from these dairy ingredients by suitably optimizing the formulation or manipulating protein-based emulsions, regulating digestion of protein and lipids in the GI tract.

Based on this study, some recommendations about future work are list as follow:

•Coagulation behaviours of SMP, MPC 4851, MPC 4861 and sodium caseinate at different time points during *in vitro* gastric digestion

In this present study, the characteristics of curd were studied after 220 min of gastric digestion. The further study of coagulation behaviours of different dairy ingredients at different gastric digestion time points should be carried out to understand the dynamic changes in the clot during gastric digestion.

•Studying lipid digestion in the small intestine of oil-in-water emulsion emptied from different gastric digestion time point

Digestion and absorption in the human gastrointestinal tract is a dynamic process. Different aggregation behaviours of emulsions in the stomach cause significant difference in gastric emptying rate, protein composition and oil content in emptied digesta at different digestion time points. It can thus be inferred that lipid digestion may follow different patterns at these time points. Therefore, different gastric digestion time points should be selected to carry out subsequent experiment of lipid digestion.

•Understanding of the role of calcium in digestion behaviours of calcium reduced MPC

The present research suggests that the state of casein micelles determines the gastric digestion behaviours of MPC 4851 and MPC 4861. The casein micellar structure had been dissociated by removing calcium in MPC 4861. The digestion behaviours of MPC at different levels of calcium supply may be further studied to understand the role of calcium in the structure of clot.

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