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Comparison of Simulated Gastric Digestion Behaviour of Commercial Infant Formulae Made with Cow, Goat and Sheep Milk

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Abstract

Infant formula is used as a supplement or substitutes to provide primary nutrition for infant growth and development when breastfeeding is not available. The different protein compositions from different animal milk and the process such as heat treatment during infant formula manufacture could impact protein digestion. This study compared the effects of protein composition and heat treatment on simulated gastric digestion behaviour of commercial infant formulae made with cow, goat, and sheep milk using an infant human gastric simulator (IHGS).

Cow infant formula (CIF), goat infant formula (GIF) and sheep infant formula (SIF) were investigated in the present study. The results of the SDS-PAGE analysis indicated that the protein compositions, especially the casein compositions in three infant formulae were different. During the simulated dynamic gastric digestion, GIF chyme had an earlier initial aggregate compared to CIF and SIF, and the microstructures of GIF chyme showed fragmented and porous structures. On the contrary, CIF chyme formed dense protein networks that trapped oil droplets, whereas SIF chyme exhibited the structure of smooth oil droplets surrounded by fewer protein networks. The different aggregation behaviour and aggregate structures of three infant formula chyme could be related to the different protein compositions, especially the different casein compositions.

Furthermore, the open friable structure of GIF aggregates could lead to pepsin being easier to access and hydrolyse protein. This is supported by the results of the SDS-PAGE pattern of emptied digesta obtain from CIF, GIF and SIF. However, the pH, particle size, protein, and fat contents of three infant formulae's empty digesta only showed the difference at the late stage of the digestion. This indicated that the impact of different protein compositions of three infant formula on emptied digesta was subtle.

To compare with unheated samples during gastric digestion, heated (90°C for 5 mins) CIF and GIF chyme showed a loose and fragmented structure. This could be attributed to the high temperature-induced denatured whey protein associated with the casein micelles by disulphide bonds and with other unfolded denatured whey proteins at the early stage of digestion. The result was in a line with the observations of protein hydrolysis of emptied digesta obtained

from heated and unheated infant formulae. For heated samples of three infant formulae, both casein and whey protein were gradually hydrolysed by pepsin from the early stage of digestion. However, caseins of unheated samples were not hydrolysed until 100 min of digestion when the pH dropped to ~4.6. This suggested the hydrolysis rate was different between unheated and heated samples.

Overall, the different protein compositions of infant formulae made with cow, goat and sheep milk affect the formation of structured coagulum. The initial coagulation times and the aggregate structures were affected by the protein compositions, especially the composition of casein, and the heat treatment. These different aggregation behaviours impacted the infant's gastric emptying and the rate of protein hydrolysis. These results provide useful information for developing and designing healthier infant formulae by allowing greater control over the manipulation of protein bioavailability.

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List of Abbreviations

CW: Cow milk

CIF: Cow milk infant formula

GIF: Goat milk infant formula

SIF: Sheep milk infant formula

α-LA: α-lactalbumin

β-LG: β-lactoglobulin

$d_{4,3}$: Average volume-weighted diameter

$d_{3,2}$: Average surface-weighted diameter

FA: Fatty acid

GDL: Glucono-δ-lactone

IHGS: Infant human gastric simulator

MFG: Milk fat globule

MFGM: Milk fat globule membrane

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate-poly acrylamide electrophoresis

SGF: Simulated gastric fluid

v/v Volume/volume

w/v Weight/volume

w/w Weight/weight

Chapter 1: Introduction

Infant formula is a substitute for human milk to provide the nutrients for new-borns growth and development when breastfeeding is not available (Ahern et al., 2019). Compares to human milk, animal milk contains different quantities and compositions of protein, lactose, and minerals, such as higher casein/whey protein ratios (Ahern et al., 2019; Barłowska et al., 2011; Jandal, 1996; Park, 2009). Commercial infant formulae are simulated the biological and compositional properties of breastmilk to provide high-quality nutrition for infants. The infant formula digestion and coagulation behaviour play an important role in determining the infant gastric emptying rate, the nutrients releasement, and compositions delivery into the small intestine (Brulé et al., 2000; Ye et al., 2017). Understanding the compositions and physicochemical of milk from different species could help to better understand how milk and milk product nutrition absorbed. However, there are insufficient studies that have investigated the effect of protein composition in coagulation and gastric digestion behaviour of infant formulae from different animal spices.

Dairy products are widely consumed. There are various products, such as liquid milk (flavoured milk, fortified milk), cultured products (buttermilk, yoghurt, cheese), frozen products (ice cream), dried milk products, condensed milk, etc. Milk from all mammalian species is a good source of nutrition for young's growth and development (Park et al., 2006; Pietrzak. Fiećko & Kamelska. Sadowska, 2020). Dairy cattle and noncattle such as sheep and goat milk and their milk products have been consumed over the past centuries (Alichanidis et al., 2016; Clark & García, 2017). Recently, researchers have shown an increasing interest in comparing compositions, physicochemical properties, and digestibility of cattle and noncattle milk (Ingham et al., 2018; Nayak et al., 2020; Raynal. Ljutovac et al., 2008; Roy et al., 2021). Noncattle milk, especially goat and sheep milk, has increased in popularity among consumers due to its better digestion and health performance (Balthazar et al., 2017; Park et al., 2007; Wendorff & Haenlein, 2017). However, there is still not enough research for consumers to choose which milk is a better source of protein.

In recent years, there are a wide range of commercial infant formulae in the market made with milk from different species, such as goat and sheep milk. The milk from different species is known as having various compositions, casein micelle characteristics, and physicochemical

properties (Barłowska et al., 2011; Claeys et al., 2014; Park et al., 2007). Compared to cow milk, goat milk contains lower α_{S1}-casein content and larger casein micelles, while sheep milk has higher total solid and mineral contents (Park et al., 2007; Raynal. Ljutovac et al., 2008; Storry et al., 1983). However, the milk compositions of different milk are affected by many factors, such as feeding strategies, physiological factors, genetic factors and environmental conditions (Claeys et al., 2014; Morand. Fehr et al., 2007). The different protein compositions lead to different enzymatic or acid gels properties and coagulation behaviour (Roy et al., 2020b; Roy et al., 2021). Goat milk is considered to be easier to digest due to the rough and soft coagulation formed in the gastric condition (Lucey et al., 2000; Storry et al., 1983). On the contrary, sheep milk formed a firm gel due to higher minerals and protein content (Domagała, 2009).

Milk protein digestion behaviour is an increasingly important area, it has been studied in both *vivo* and *vitro* models (Brodkorb et al., 2019; Gallier et al., 2013; Mulet. Cabero et al., 2017; Wang et al., 2018; Ye, 2021; Ye et al., 2020). Most of the previous studies have focused on comparing the different compositions and physicochemical properties of cow, goat and sheep milk, as well as the different components that affect gastric digestion behaviour. The studies of gastric digestion behaviour of infant formulae made from different animal species are limited. Therefore, this research aimed to compare the coagulation, gelation and digestion behaviours of commercial infant formulae made from cow, goat and sheep milk. The objectives of this study are as follows:

1. To overview the knowledge of comparing the compositions and physicochemical properties of milk and the infant formulae from cow, goat, and sheep.
2. To understand the digestion behaviours of cow, goat and sheep milk based commercial infant formulae during in *vitro* simulated dynamic digestion.
3. To investigate and compare the physical characteristic changes, the hydrolysis rate of protein and the structural changes in the different infant formulae.
4. To investigate the effects of the manufacturing process, especially the heat treatment on the gastric digestion behaviours (e.g., pH profiles, protein and fat contents and particle size) of cow, goat and sheep infant formulae.
5. To explore the GDL and different pepsin concentration-induced gelation properties of commercial infant formulae made from sheep, goat, and cow milk. This study

investigated the gelation time, pH and physiological characteristics of milk gels related to the protein composition.

The chapter layout of this thesis is presented in Figure 1.1.

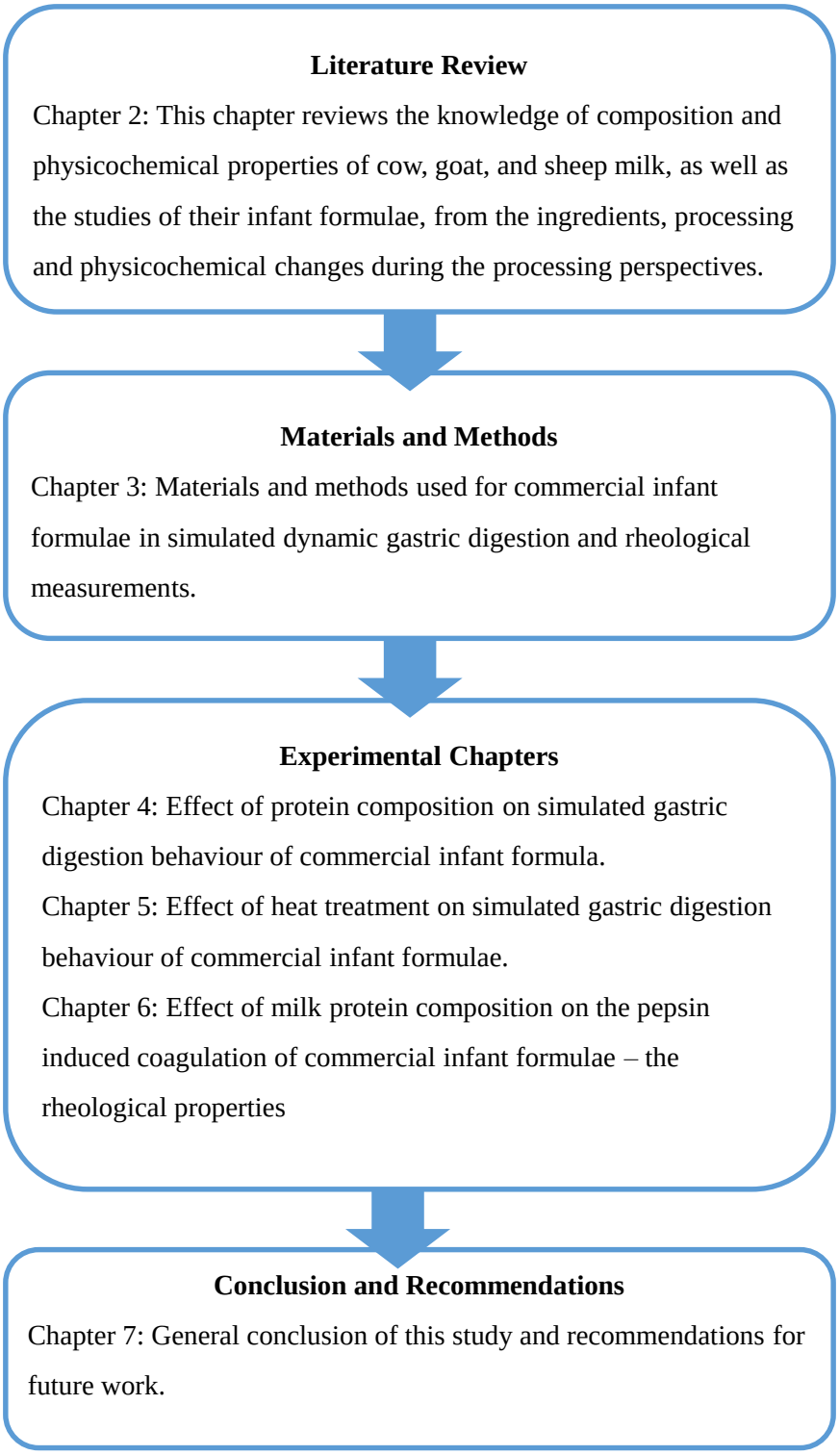


Figure 1.1. The schematic chapter layout of this thesis

Chapter 2: Literature Review

This chapter presents an overview of the literature related to cow's milk, goat's milk and sheep's milk, as well as their infant formulae. It is divided into three sections: the first section provides details of the compositions and physicochemical properties of cow, goat, sheep, and human milk; the second section looks at studies of infant formulae, from the ingredients, processing, and physicochemical changes during the processing perspectives. The third section covers protein digestion, infant gastric reviews, and the gastric behaviour of infant formulae in the current work of literature. Although the main components in ruminant milk and human milk are the same, the properties of each milk and their infant formula may differ, not only in the compositional but also in the physicochemical characteristics.

2.1 Comparison of cow, goat, and sheep milk

The literature notes that cow milk with 85% worldwide production is the most widely consumed dairy source, due to its awareness and availability with a large yield (Alichanidis et al., 2016). Goat milk is the most common non-bovine milk around the world. It has been consumed and produced in India, Pakistan, and Bangladesh. Sheep milk and its dairy products place a significant role in countries where cow milk is not sufficient or affordable, due to the high protein and calcium content. There is a large quantity of dairy sheep farming in the developed countries and Mediterranean areas, such as Greece, French, and Spain (Haenlein, 2001). In developing countries, primarily sheep rearing was established to meet the demand for mutton and some wool. Sheep and their products are relatively new in those countries. India has the third largest world sheep population (65 million), and their sheep are mostly used for meat and wool (Mohapatra et al., 2019).

2.1.1 Basic composition of cow, goat, and sheep milk

The majority of studies say that milk from all species contains the same basic composition – protein, fat, carbohydrates, minerals, and vitamins (Alichanidis et al., 2016; Ballard & Morrow, 2013; Engfer et al., 2000; Meena et al., 2014; Pietrzak. Fiećko & Kamelska. Sadowska, 2020). As Table 2.1 shows, the nutritional values of different mammals are different. Different articles have elucidated that the values of milk compositions were susceptible to many factors, such as

feeding strategies, farming systems, physiological factors, genetic factors, and environmental conditions (Claeys et al., 2014; Morand. Fehr et al., 2007; Raynal. Ljutovac et al., 2008). Even the individuals among the same species may vary considerably. In general, the total protein content in human milk is lower than that in animal milk, approximately 0.8 – 1.9% of human milk, 2.9 – 5.0%, 2.5 – 5.2%, and 4.5 – 7.2% of cow, goat, and sheep milk respectively. This indicated that undiluted animal milk is not recommended to feed, as it may cause infants to excessive intake of amino acids (Kapadiya et al., 2016; Räihä et al., 2002). In addition, the lactose content in human milk is higher than that in cow, goat, and sheep milk. Compared to cow milk and goat milk, sheep milk contains higher total solids, fat, proteins, and total ash. The high-fat content in sheep milk brings a high product yield, this revealed the most usage of sheep milk in fermented products, such as cheese and yoghurt (Moatsou & Sakkas, 2019; Mohapatra et al., 2019).

Table 2.1. Comparative composition of milk of different species from various research

Component	Human	Cow	Goat	Sheep
Water content (% Wet basis)	86.8 – 90.5	87.8	87.8	81.6
Solid-non-fat (%)	8.90	9.02	8.68	10.33
Dry matter (g/L)	107 – 129	105 – 137	119 – 163	152 – 200
Fat (%)	2.2 – 6.0	2.5 – 6.0	2.5 – 7.8	5.1 – 9.3
Protein (%)	0.8 – 1.9	2.9 – 5.0	2.5 – 5.2	4.5 – 7.2
Lactose (%)	6.0 – 9.0	3.6 – 5.6	3.9 – 6.3	3.7 – 5.5
Ash (%)	0.2 – 0.3	0.6 – 0.9	0.7 – 1.1	0.7 – 1.0

Sources are based on minimal and maximal values found in the literature – Alichanidis et al., 2016; Claeys et al., 2014; Kapadiya et al., 2016; Nayak et al., 2020; Park et al., 2007; Raynal. Ljutovac et al., 2008

2.1.1.1 Proteins

Studies noted that casein and whey protein are two distinguished groups of milk protein (Carr & Golding, 2016; Huppertz & Chia, 2021; Nakai & Li-Chan, 1987). Cow, goat, and sheep milk, as ruminant milk, are defined as *casein* milk, which contains higher casein than non-ruminant milk (horse, donkey). The casein factions are approximately 50% to 80% of the total protein

(Claeys et al., 2014; Park et al., 2007). As can be seen from Table 2.2, the protein components in different animal milk are different. The total casein content in sheep milk is the highest compared to goat, cow, and human milk. The casein value in goat and cow milk is in a similar range, while human milk contains the lowest total casein. In general, the α_{S1}-casein and β-casein values in sheep milk were higher than in cow milk and goat milk. Goat milk contains lower α_{S1}-casein than the other two milk (Raynal. Ljutovac et al., 2008). The lower α_{S1}-casein in goat milk leads to more hydrated pores in its casein micelles, which result in larger casein micelle size than the other milk (Ingham et al., 2018; Park et al., 2007).

In addition, the levels of casein in sheep milk and goat milk vary widely, and the casein fractions in the literature are contradictory (Mayer & Fiechter, 2012; Michaelidou, 2008; Moatsou & Sakkas, 2019; Wendorff & Haenlein, 2017). The percentage of each protein component in goat and sheep milk varies by breed. Studies by Jandal (1996) and Clark and García (2017) shows that goat and sheep milk of different genotypes contain variable amounts of α_{S1}-casein. In contrast, the α_{S1}-casein content in cow milk is more consistent (Ceballos et al., 2009; Korhonen, 2009). As such, the casein contents of goat and sheep milk exhibited widely range from research to research.

In terms of whey protein, the literature suggests that human milk is a whey protein-based milk, which consists of approximately 60% whey proteins (Ballard & Morrow, 2013; Pietrzak. Fiećko & Kamelska. Sadowska, 2020). α-lactalbumin is the most abundant whey protein in breast milk, accounting for 20% - 25% of the total whey proteins (Golinelli et al., 2014; Jenness, 1979). In contrast, cow, goat, and sheep milk have higher casein/ whey protein ratios. β-Lactoglobulin is the major whey protein found in animal milk, which is not present in human milk (De Wit, 1998; Gaye et al., 1986; Liao et al., 2017).

Table 2.2. Comparative protein fractions (g/L) in natural milk from human, cow, goat and sheep

Component	Human	Cow	Goat	Sheep
Total casein	2.4 – 4.2	24.6 – 30.2	23.3 – 46.3	41.0 – 66.0
α_{S1}-Casein	0.8 – 1.6	8.0 – 10.7	0 – 13.0	13.9 – 26.3
α_{S2}-Casein	-	2.8 – 3.4	2.3 – 11.6	4.9 – 16.4

β-Casein	1.2 - 3.9	8.6 – 9.3	0 – 29.6	15.2 – 40.7
κ-Casein	0.14	2.3 – 3.3	2.8 – 13.4	3.2 – 4.3
γ-Casein	-	0.8 – 2.2	-	-
Total whey protein	6.2 – 8.3	5.0 – 7.0	3.0 – 12.0	8.0 – 16.0
β-Lactoglobulin	-	3.2 – 3.3	1.5 – 5.0	6.5 – 8.5
α-Lactalbumin	1.9 – 3.4	1.2 – 1.3	0.7 – 2.3	1 – 1.9
Serum albumin	0.4 – 0.5	0.3 – 0.4	-	0.4 – 0.6
Lactoferrin	1.5 – 2.0	0.02 – 0.5	0.02 – 0.3	0.7 – 0.9
Casein/whey ratio	0.4 – 0.7	4.5 – 4.7	3.5 – 4.0	3.1 – 4.4
Casein micelle (nm)	64 – 80	150 – 182	206 – 260	180 -210

Sources are based on minimal and maximal values found in the literature – Alichanidis et al., 2016; Barłowska et al., 2011; Claeys et al., 2014; Jandal, 1996; Jenness, 1979; Korhonen, 2009; Mayer & Fiechter, 2012; Michaelidou, 2008; Moatsou et al., 2004; Odle et al., 1996; Park et al., 2007; Park, 2009; Uniacke-Lowe, 2011

The research noted that amino acids are the units of building up protein molecules (Gerchev et al., 2005; Guo et al., 2007; Park et al., 2007). The amino acid compositions in goat and sheep milk are encompassed all essential amino acids required by humans, and the contents are significantly higher than that in cow milk. The contents of amino acids in different species of milk are shown in Table 2.3. The type and the level of essential amino acids could affect the nutritional value of milk (Claeys et al., 2014; Kamal et al., 2007). Taurine is an essential nutrient for infant digestion and absorption, it can be found in goat and sheep milk (Mohapatra et al., 2019).

Table 2.3. Amino acids contents (g/100g protein) in human, cow, goat and sheep milk

Amino acids	Human	Cow	Goat	Sheep
Essential amino acids				
Histidine	2.3	3.0	5.0	NA
Isoleucine	5.8	4.2	7.1	4.6
Leucine	10.1	8.7	8.2	9.7 – 9.9
Lysine	6.2	8.1	8.2	7.7 – 7.8
Methionine	1.8	1.8	3.5	2.7

Phenylalanine	4.4	4.8	6.0	4.2 – 4.3
Threonine	4.6	4.5	5.7	4.2 – 4.4
Trypotophan	1.8	1.5	NA	NA
Valine	6.0	4.8	5.7	6.2 – 6.4
Non-essential amino acids				
Alanine	4.0	3.0	3.6	NA
Arginine	4.0	3.3	2.9	NA
Aspartate	8.3	7.8	7.4	NA
Cysteine	1.7	0.6	0.6	0.8 – 0.9
Glycine	2.6	1.8	2.1	NA
Glutamate	17.8	23.2	19.3	NA
Proline	8.6	9.6	14.6	NA
Serine	5.1	4.8	5.2	NA
Tyrosine	4.7	4.5	4.8	3.7 – 3.8

Sources are based on minimal and maximal values found in the literature – Barłowska et al., 2011; Gerchev et al., 2005; Guo et al., 2007; Haenlein, 2001; Kamal et al., 2007; Nayak et al., 2020

2.1.1.2 Fat

The research suggested that milk fat globule (MFG) is the most important microstructure that dispersed in the milk serum. The average diameter of MFG is 3 – 4 μm. MFG is enclosed in a milk fat globule membrane (MFGM), which protects the fat from coalescence and fusion, as well as lipase action. In general, MFGM is a lipoprotein membrane comprised of proteins, glycoproteins, phospholipids, neutral lipids, and other minor compositions (Alichanidis et al., 2016). There is quantitatively and qualitatively difference in fat content of milk due to the different breed, genotype, feeding strategy, seasons and stage of location (Claeys et al., 2014; Raynal. Ljutovac et al., 2008; Talpur et al., 2008). The review of Alichanidis et al. (2016) revealed that human milk fat globule has a larger average diameter (4.0 μm) than ruminant MFG. Goat milk fat globule is the smallest size on average, which was in the range of 2.5 – 3 μm. In contrast, sheep and cow milk have larger MFG size, which are 3 – 3.8 μm and 3 – 4 μm respectively. The smaller fat globules provide the highest fat digestibility of goat milk (Meena et al., 2014; Pietrzak. Fiećko & Kamelska. Sadowska, 2020). In addition, due to lacking agglutinin, goat and sheep MFG do not naturally aggregate under cooling storage (Alichanidis et al., 2016).

Fatty acids play a significant role in nutrition and it is essential for newborn health and growth development (Haenlein, 2001). Ruminant milk supplies a higher amount of essential fatty acids, especially mono-unsaturated and medium chain fatty acids are higher than cow milk. The study by Goudjil et al. (2004) indicates that sheep milk contains the highest content of conjugated linoleic acid (CLA) in ruminant milk. According to Table 2.4, sheep and goat milk contain more caprylic and capric fatty acid, which provides a special taste and aroma to the milk. Especially in goat milk fat, C6 - C10 fatty acid is about twice higher than cow milk, which is 16% - 18% and 6% - 8% respectively, and 11% - 12% in sheep milk (Nayak et al., 2020; Raynal. Ljutovac et al., 2008). The medium fatty acids, especially capric, caprylic and caplic have been widely used for treatments, such as intestinal disorders, premature infant nutrition, and gallstone problems. This is attributed to the unique metabolic ability – inhibition and dissolving cholesterol deposits while providing energy (Jandal, 1996; Odle et al., 1996). Goat milk contains the lowest level of polyunsaturated fatty acids (2.97%), followed by sheep milk (3.86%) and cow milk (5.25%). The total poly-unsaturated FA was higher than ruminant milk, and the total saturated FA was lower than ruminant milk, which indicated a good nutritional source of fatty acids (Pietrzak. Fiećko & Kamelska. Sadowska, 2020).

Table 2.4. Average fatty acid contents (g/100g milk) in human, cow, goat and sheep milk

Fatty acids	Human	Cow	Goat	Sheep
Butyric acid (C4:0)	0.02 ± 0.03	3.14 ± 0.27	2.56 ± 0.21	2.81 ± 0.14
Caproic acid (C6:0)	0.09 ± 0.05	2.17 ± 0.25	2.79 ± 0.04	2.54 ± 0.13
Caprylic acid (C8:0)	0.19 ± 0.09	1.41 ± 0.17	3.32 ± 0.51	2.60 ± 0.13
Capric acid (C10:0)	1.46 ± 0.56	3.25 ± 0.54	11.28 ± 0.69	9.88 ± 0.43
Lauric acid (C12:0)	5.53 ± 2.33	3.63 ± 0.50	5.62 ± 0.77	6.76 ± 0.26
Myristic acid (C14:0)	6.40 ± 2.79	11.62 ± 1.15	11.35 ± 0.90	14.96 ± 0.37
Palmitic acid (C16:0)	25.40 ± 3.95	24.90 ± 1.40	27.69 ± 1.39	29.79 ± 0.46
Palmitoleic acid (C16:1)	2.24 ± 0.81	1.03 ± 0.24	1.18 ± 0.09	2.06 ± 0.01
Oleic acid (C18:1)	40.25 ± 8.45	24.81 ± 3.81	19.77 ± 0.56	15.73 ± 1.39
Linoleic acid (C18:2)	8.84 ± 3.68	2.81 ± 0.42	2.32 ± 0.19	1.97 ± 0.55
Linolenic acid (C18:3)	0.78 ± 0.48	0.86 ± 0.09	0.23 ± 0.08	0.76 ± 0.05

Total saturated fatty acid (C4-18)	46.60 ± 7.88	67.73 ± 5.33	75.50 ± 0.96	77.50 ± 0.92
Total mono-unsaturated fatty acid (C16:1-22:1)	43.55 ± 8.33	27.30 ± 4.22	21.83 ± 0.52	19.01 ± 1.35
Total poly-unsaturated fatty acids (C18:2-18:3)	9.85 ± 4.13	5.25 ± 1.41	2.97 ± 0.33	3.86 ± 0.49

Sources are based on minimal and maximal values found in the literature – Nayak et al., 2020; Pietrzak-Fiećko & Kamelska-Sadowska, 2020; Talpur et al., 2008

2.1.1.3 Carbohydrates

Studies note that lactose is the major carbohydrate in milk (Storry et al., 1983). It is a disaccharide carbohydrate, comprised of galactose and glucose. As Table 2.1 shows, the content of lactose in ruminant milk is lower than that in human milk. It is about the same level in cow, goat, and sheep milk. The lactose in milk can be fermented to lactic acid. Also, lactose dissolves in aqueous phase, which can be removed from casein during draining. As a result, there is less lactose in yoghurt from any milk sourcing, and from hard cheese (Wendorff & Haenlein, 2017). The Oligosaccharide content is lower in animal milk than that in human milk (12 – 13g/L), it plays an important role in gastrointestinal health and newborns' brain development. The review of Giorgio et al. (2018) exhibited that goat milk oligosaccharides had a similar structure to human milk, and the level is higher than that in sheep milk and cow milk. The oligosaccharide level in caprine, ovine, and bovine milk is 0.25 – 0.30 g/L, 0.03 – 0.06 g/L and 0.02 – 0.04 g/L respectively.

2.1.1.4 Vitamins and Minerals

The vitamin contents in milk are variable due to the feeding regime and vitamin status (Claeys et al., 2014; Nayak et al., 2020). In addition, the water-soluble vitamins (B vitamins and C) are heavier influenced by feeding than the fat-soluble vitamins (A and E). The studies of Medhammar et al. (2012) and Jandal (1996) compared the vitamin concentration in human and animal milk, and found out breast milk contains a higher concentration of vitamin A, C and E, and the other vitamins are lower than animal milk on average. Goat and sheep milk contain higher levels of B vitamins, especially niacin, which plays an important role in the central nervous system (CNS) healthy (Fricker et al., 2018; Raynal. Ljutovac et al., 2008). Also, goats and sheep can convert carotene to retinol, as a result there are higher Vitamin A levels in goat

and sheep milk compared to cow milk. The lower carotene in goat and sheep milk gives whiter colour compared to cow milk (Jandal, 1996; Park et al., 2007; Raynal. Ljutovac et al., 2008).

The literature suggests that milk is a good source of mineral substances, including calcium, phosphorus, potassium, magnesium, sodium, chloride etc. (Gaucheron, 2005). The average mineral concentration in goat and sheep milk is higher than that in cow milk, and higher in ruminant milk than in human milk (Table 2.1). In general, the mineral contents of goat and sheep milk are mostly higher than those in cow milk, except for K and Na. However, the values vary due to the feeding regime and time (Claeys et al., 2014; Recio et al., 2009; Wendorff & Haenlein, 2017). Calcium plays a significant role in bone growth and it could be bound to casein and is readily released during digestion (Guéguen & Pointillart, 2000). Thus, the casein concentration is highly related to calcium bioavailability (Gaucheron, 2005). The content of calcium is as followed: sheep > goat > cow > human milk (Barłowska et al., 2011; Park et al., 2007).

2.1.2 Physicochemical properties of cow, goat and sheep milk

The majority of research suggests that the different compositions of cow, goat and sheep milk are reflected in various physicochemical properties (Bornaz et al., 2009; Park et al., 2007). Sheep milk is higher in viscosity, specific gravity and acidity compares to goat and cow milk (Mohapatra et al., 2019; Wendorff & Haenlein, 2017). The highest viscosity could be ascribed to higher water-binding capacity in the sheep milk protein (Labropoulos et al., 1984). Goat milk has the highest surface tension and conductivity, while cow milk has the highest refractive index and average pH (Mohapatra et al., 2019; Wendorff & Haenlein, 2017). In addition, it also has been reported that sheep milk is perishable and fragile, and is easy to be spoilt by unsuitable handling (Park et al., 2006).

The casein micelle characteristics are different in types of milk (Bornaz et al., 2009). There are more calcium and inorganic phosphorus in sheep and goat casein micelles. Thus, their casein micelles are less solvated, easy to lose β-casein and have more heat instability than cow casein micelles (Park, 2007; Remeuf & Lenoir, 1986). Goat and sheep milk are less hydrated than cow milk, due to the inverse relationship between the mineralization and hydration of micelle (Remeuf & Lenoir, 1986). In addition, the protein compositions and micelle structure also determine the renneting properties, which have a significant influence on coagulation time and

rate, and the firmness of gel (Roginski et al., 2003). The short renneting time and weak gel consistency of goat milk provide ordinary cheese suitability. However, the high protein concentration and total solids predestinate sheep milk as an excellent cheese raw material (Moatsou et al., 2004). Moreover, the study of Lara. Villoslada et al. (2005) revealed that the casein/whey ratio plays an important role in milk allergology. The lower ratio in goat and sheep milk could reduce the allergenic capacity.

2.2 Studies of infant formula

The optimal food for new-born is human breast milk, which could coordinate both the physical and mental health of infants, as well as mothers (Lueamsaisuk et al., 2014; Warren & Phillipi, 2012). Infant formula as a supplement or replacement when breastfeeding is not available, supplies the primary nutrition for infant growth. Apart from the specialty formulae for particular requirements of infants (lactose intolerance, anti-regurgitation, vegan diet preference, etc.), the most common infant formula in the market is iron-fortified cow milk with varietal protein proportions, fat sources, and different supplements (Ballard & Morrow, 2013; Montagne et al., 2009). Although there is a wide range of infant formula products in the market, the quality and composition of the ingredients, and the processing conditions are strictly in compliance to the worldwide standards and legislation. This is due to infant formula being the only nutrition source for babies, it is crucial to provide healthy and safe foods (Nasirpour et al., 2006). The component and bioactive properties of animal milk and human milk are different. There are different quantities and compositions of protein, fat, lactose and minerals (Jiang & Guo, 2021). As such, it is necessary to adjust the animal milk component to meet the specific target of infant formula to be suitable for infant consumption.

2.2.1 Composition

The literature suggests that the essential compositions of infant formula are proteins, lipids, carbohydrates, minerals, and vitamins (Martin et al., 2016). In general, skimmed or whole milk, casein, whey protein, lactose, vegetable oils, emulsifiers, minerals salts and micro-nutrients such as vitamins, and amino acids are the basic raw materials of infant formulae. Protein as the necessary parameter in infant formula provides amino acids and nitrogen for new-born maintenance and growth. The quantity of essential and semi-essential amino acids in infant formula should at least equal to that of human milk in an equal energy value (Koletzko et al.,

2005). To achieve that, the adaptations include protein level reduction and fractionation, enriching milk with whey proteins, decreasing the casein fraction, and enrichment with certain amino acids (Montagne et al., 2009). In the market, the protein of most infant formulae is sourced from cow milk, followed by goat and sheep milk (Ahern et al., 2019). Soy protein is also widely used in lactose-free products (Smith et al., 2011). Due to the difference in protein contents and proportions between human milk and animal milk, it is necessary to modify animal milk to closely resemble breast milk. Niers et al. (2007) revealed that apart from the immunoglobulin (IgA) antibodies in human milk cannot be reproduced, other nutritional constitutions can be mimicked in infant formula. For example, whey proteins can be fractionated to achieve a high proportion of α-lactalbumin, as β-lactoglobulin is the main allergenic composition to infants in animal milk, which is absent in human milk (Kelly & Fox, 2016).

Lipids are the dominant energy source in infant formula, supplying the dietary fat for new-born development (Carey & Hernell, 1992). Apart from providing energy value, it is also the source of essential fatty acids and fat-soluble vitamins, as well as the flavour carriers. Infant formula is usually recombined with vegetable oils, such as soy oil, sunflower oil and rapeseed oil to achieve a high unsaturated degree as human milk. Only a low level of milk fat is used to manufacture infant formulae (Montagne et al., 2009). ARA and DHA with the function of developing brain and visual acuity of babies, are the common long-chain poly-unsaturated fatty acid supplementations to enrich the oils (Guo & Ahmad, 2014). However, as the oils are susceptible to rancidity and oxidation, worldwide standards or regulatory have quality criteria for lipids. ANZ standards 2.9.1 requests the ratio of Linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3) must within 5:1 to 15:1, this is due to the synthesis of these two essential fatty acids are sharing enzyme systems (Montagne et al., 2009).

Carbohydrates accounted for a large proportion of infant formula, generally around 55g per 100g per powder (Martin et al., 2016). This is to mimic the carbohydrate fraction in breast milk. Lactose is the major digestible carbohydrate in human milk, usually around 55 – 70 g per litre. The other carbohydrates, such as maltose, maltodextrins, and starch, are also being used in infant formula manufacturing to increase the thickness and satiety (Montagne et al., 2009). The digestible carbohydrates play an important role in providing energy and synthesising glycoproteins and glycolipids. The diet disaccharides and polysaccharides hydrolysed to

monosaccharides, then absorbed in the upper small intestine and transferred to glucose in the liver (Engfer et al., 2000). Apart from digestible carbohydrates, there is a complicated mixture of oligosaccharides in human milk. As Kunz et al. (2000) reported, they are not digestible by enzymes in the gastrointestinal. They could operate as rival receptors on the cell surface to protect breastfed infant cells from adhering to pathogens and bacteria.

Minerals and vitamins also play a significant role in the nutrition of infant formula (Montagne et al., 2009). Minerals include six elements with content in mg per 100 kcal – Ca, Cl, K, Mg, Na and P, and trace elements which are in μg per 100 kcal – Cu, Fe, I, Mn, and Zn. Same as breast milk, there are water- and fat-soluble vitamins in infant formula, the former is easier to eliminated if excess intaken (Montagne et al., 2009). In general, animal milk has higher mineral contents than breast milk. The minerals in infant formula could be reduced by membrane filtration or electrodialysis to match the lower content in human milk (Kelly & Fox, 2016). On the contrary, some vitamins in animal milk are inadequate for infants, such as folate, vitamin D and vitamin C (Turck, 2013). Thus, it is important to modulate the mineral and vitamin levels to meet infant dietary requirements.

2.2.2 Process of infant formula manufacture

The general process of infant formula powder is either *dry blending* or *wet mixing-spray drying* two types of process (Jiang & Guo, 2021; Montagne et al., 2009). The dry blending is a low-cost process, as it requires less equipment with lower energy, less space and maintenance (Montagne et al., 2009). The ingredients for dry blending are in powdered form and mixed in large batches until macro- and micronutrients are uniformly distributed (Caric, 1994). Then the blender is transferred through a sifter to remove extraneous material and oversize particles. After sifted, the blender is placed in a filler hopper to be packed into inert gas flushed cans (Jiang & Guo, 2021). Due to no water involved in the blending, it is less expectation of microbiological growth. However, since no heat treatment in the process, the microbiological quality of the completed product relies on the raw materials. The risk of *Salmonella* and *Coliforms* may exist in the dry blending powders (Jiang & Guo, 2021). In addition, the physical quality such as solubility and wettability of the product is limited by the single ingredients. The final products have the potential to be inhomogeneous during transportation due to the different densities of different compositions (Masum et al., 2020). The dry blending process also had to incorporate oils (Montagne et al., 2009).

Infant formula is a highly controlled and regulated product, the wet mixing – spray drying process is generally being used in the manufacture to improve the microbiological, physical, and chemical properties of the final product. The wet mixing process also can be combined the dry blending process by adding some nutrients (carbohydrates, vitamins etc.) after the wet mixing and spray drying of the other ingredients (Proudy et al., 2008). As Figure 2.1 indicated, the wet mixing – spray drying process of infant formula manufacture is starting with preparation of the mix, followed by evaporation, and then ends up by drying in a spray dryer (Montagne et al., 2009).

Preparation of the mix can be based per batch or continuously, it includes water-soluble and oil-soluble parts (Montagne et al., 2009). The water-soluble ingredients are blended into milk or water in a high-shear mixer. Skim milk powder or liquid skim milk as the base of the mix, adjusting the lactose content and the concentration of casein and whey protein by adding lactose, demineralised whey or whey protein concentrate (WPC) (Kelly & Fox, 2016). The pH of the mixer can be adjusted by alkali or citric acid solution (Bylund, 1995). Then the recombination mix is added to preheating line under a temperature ~60 – 70°C (Guo & Ahmad, 2014). Oils and emulsifiers are added after preheating to protect the intact function, such as long poly-unsaturated oils (Montagne et al., 2009). The supply pipes and tanks for preparation should be compressed air flushed and the mix line should be cleaning-in-place (CIP) every day (Montagne et al., 2009).

After the preparation of the mix, the blending is transferred through the homogenisation, heating and evaporation process (Montagne et al., 2009). The heating process is performed by either indirect heat exchanger of temperature from 5 – 80°C or direct heat by stream injection to 90 – 120°C for 5 – 30s (Montagne et al., 2009; Murphy et al., 2013). The mix then cooled to ~78°C before being fed into the evaporator, such as a falling film or multi-stage vacuum evaporator (Kelly & Fox, 2016). Evaporation is an essential step to remove water and concentrate the mix for the spray drying at the process in a minimized energy cost way (Hui, 2007). The milk powder after the evaporation process has larger particle sizes with less amount of air and better quality with long shelf life (Jiang & Guo, 2021).

The concentrated mix from the evaporation process is conveyed to a drying chamber/ tower to

produce a good quality infant formula powder. Spray drying is commonly used in infant formula manufacture due to the higher microbiological quality and the final products have higher water solubility compared to roller drying (Jiang & Guo, 2021). The evaporated milk is transferred to a rotary atomizer or high-pressure nozzles for the atomization to produce individual powder particles (Kelly & Fox, 2016). The particle size is determined by the milk feed pressure. The dying process could be completed in single, or several stages with the various types of the spray-drying chamber (Kelly & Fox, 2016).

In general, infant formula is pumped into the drying chamber for the first stage of drying with heaters and filters, then is transferred to an integrated fluid bed with lower temperature and dry air for the second stage of drying (Walstra, 1999). The third stage is occasionally necessary and completed in an external fluid bed with the cooling process (Jiang & Guo, 2021). The completed product from the spray dry process is either transferred to a storage silo by positive pressure and dense phase transportation or falls by gravity into a hopper for direct bagging (Montagne et al., 2009). The final infant formula is required to be packed in an N_2 atmosphere to avoid milk fat and polyunsaturated fatty acid oxidation (Montagne et al., 2009). The flow chart of the infant formula process is shown in Figure 2.1.

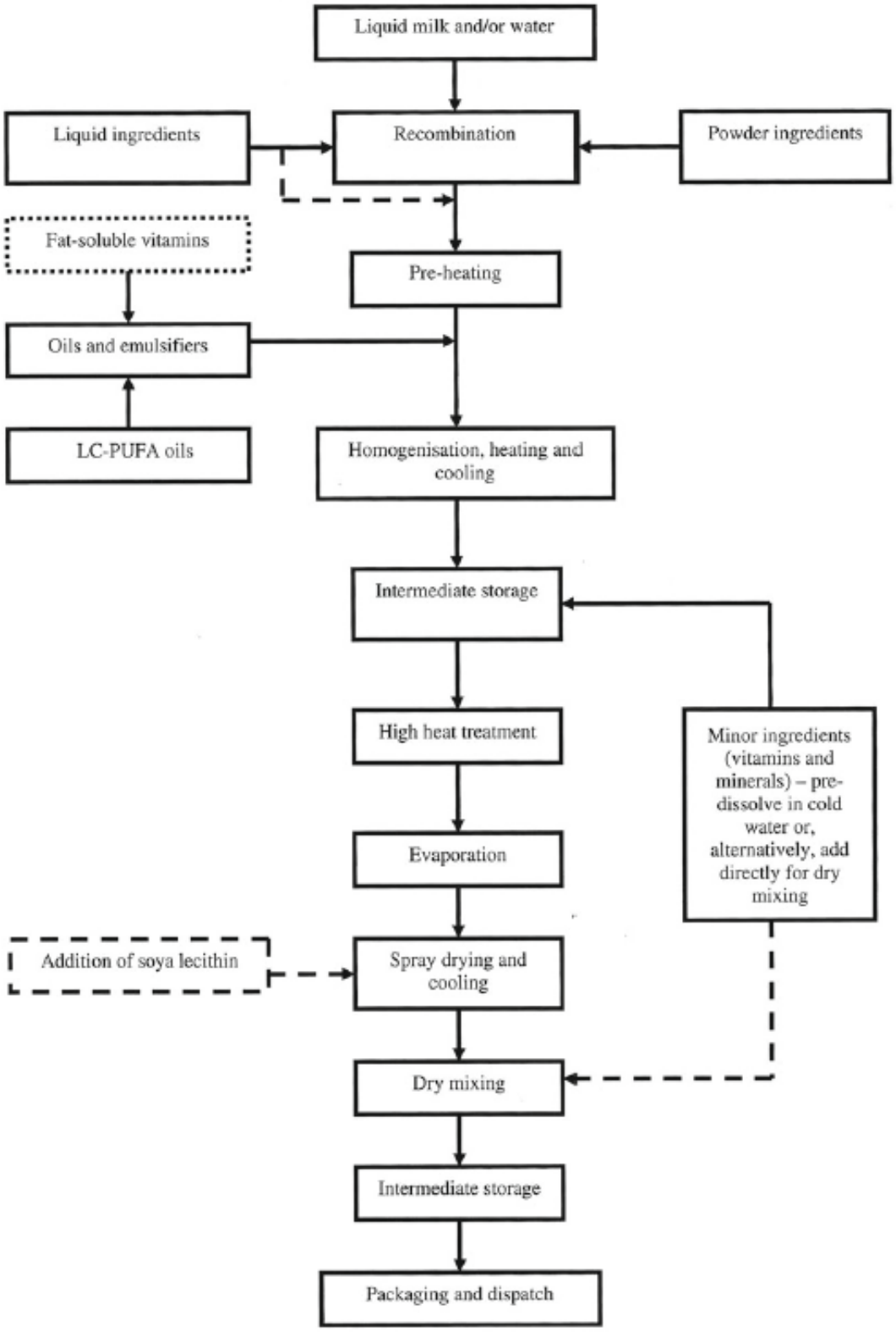


Figure 2.1. The flowchart of infant formulae manufacturing (Dotted lined are alternative processing route) (Montagne et al., 2009)

2.2.3 Physicochemical changes during infant formula processing

Some studies note that in the evaporation and drying process of infant formula manufacturing, the high temperature could influence the physicochemical properties of infant formula (Kelly & Fox, 2016; Singh & Havea, 2003). The heating process highly affected the whey proteins, but only has a minor impact on casein (Kitabatake & Kinekawa, 1998). The study by Murphy et al. (2013) revealed infant formula as a whey dominated product, the heat treatment plays a significant role during manufacturing. The high-solids (60% w/w) steam injection process led to a lower whey protein denaturation level compared to indirect tubular heat exchanger treated control formulations (30% w/w).

β-lactoglobulin is the most sensitive to the temperature, when the temperature is above 75°C at a pH of around 6.5, the secondary and tertiary structure of β-lactoglobulin changed significantly (Corredig & Dalgleish, 1999; Noh & Richardson, 1989). The native structure of β-LG is a compact globular, which hides the cleavage sites (He & Giuseppin, 2014; Ye, Liu, et al., 2019). With the temperature rising, the interactions of whey proteins occur. When the temperature reaches 90°C, the unfolded structure exposes the cleavage sites, and a disulphide bond of denatured β-LG and κ-casein forms at the micelle surface, an extended interaction of β-lactoglobulin and α-lactalbumin occurs (Donato et al., 2007; Fairise et al., 1999). The conformational changes induce whey proteins to be more susceptible to pepsin hydrolysis, which improved its digestibility (Inglingstad et al., 2010). In addition, the thermal unfolding of the whey protein globular structure during evaporation could influence the bulk density of milk powder (McSweeney & Fox, 2013).

The heating process not only changes the structure of whey proteins but also to the structure of the fat globule interface (Claeys et al., 2014). There is a protective layer coated on fat globules that gastric lipases must go through the fat globule interface to access the triacylglycerols. Human milk fat globules covered branched oligosaccharide structure, while cow milk fat globules contain proteins and phospholipids (Malacarne et al., 2002). The study of Armand et al. (1996) and Michalski and Januel (2006) revealed that the heating treatment, as well as the homogenization process, could change the physicochemical properties of the fat globules, and produce finer casein coated fat globules, that consequently improving the fat digestibility.

In addition, the presence of the minerals in infant formula and the quantities could affect the physical stability of the products. For instance, Ca and Mg are easy to aggregate with protein during heat processing when they are in bivalent soluble cations and tend to get sediment in their insoluble phosphate or citrate form. As such, it is a challenge to find heat stable salts with no sedimentation (Montagne et al., 2009). Furthermore, avoiding the oxidation of high bioavailability salts with fatty acid is important.

2.3 Digestion

In recent years, there has been an increasing interest in the study of milk digestion. A large amount of research has worked on the milk protein digestion behaviour, dynamic *in vitro* digestion as one of the most common methods have been widely accepted (Mulet. Cabero et al., 2020; Ye, Cui, et al., 2019). The different protein compositions in milk from different species highly influence digestibility (Barłowska et al., 2011; Claeys et al., 2014; Roy et al., 2020a). In addition, understanding the protein digestive properties in infant formula is important, as the digestive environment and the capacity of the new-born is different to adults (Bourlieu et al., 2014). As the previous section discussed, the physicochemical properties of infant formula could be changed during infant formula manufacturing, especially by the heat treatment (McSweeney & Fox, 2013).

2.3.1 Digestion of milk

The protein compositions in different mammal milk vary. In the previous section the differences including the casein concentration, casein micelle size, β-casein-to- α_S-casein ratio etc. is reviewed. The different protein compositions in milk are highly influencing the food matrix structure formation, thus affecting the protein digestibility. The review of Tari et al. (2018) indicated that the lower casein content provides a lower hardness of the coagulation. As the previous section reviewed, human milk contains lower casein content than ruminant milk, which produces a loose and softer coagulum in the stomach (Barłowska et al., 2011; Hodgkinson et al., 2018; Uniacke-Lowe, 2011). On the contrary, higher casein, fat and calcium in sheep milk, result in rapid clot and firmer curd (Roy et al., 2021). In addition, the casein ratio also affects milk coagulation. According to Roberto Ambrosoli (1988), the higher α_{S1}-casein milk produces firmer curd, which is the advance of increasing cheese yield. Also, the larger micelle diameter of sheep milk compares to cow milk, provided lower hydration and

colloidal stability (Park et al., 2007). Furthermore, a higher β-casein-to-α_S-casein ratio in human and goat milk could relate to more friable aggregates (Li & Nakai, 1988; Roy et al., 2020a).

The different protein compositions related to food matric structure influences protein digestibility. The study by Lucey et al. (2000) and Park et al. (2007) revealed goat milk forms a loose and open structured coagulate compared to cow and sheep milk, which allows greater diffusion of pepsin. As the result, goat milk is easier to be hydrolysed by pepsin, and thus improves the digestibility compared to cow and sheep milk. In addition, the review of Roy et al. (2020a) found that the non-cattle milk form relatively softer curds compared to cattle milk during gastric digestion. The casein micelle properties, such as the micelle size, casein distribution and mineralization, all play an essential role in protein digestion (Claeys et al., 2014).

The different digestibility of milk is also attributed to the whey protein from different milk having different digestibility. For instance, the studies of Michaelidou (2008) and Uniacke-Lowe (2011) revealed that the β-LG in goat and sheep milk is faster digested than β-LG in cow milk, whereas the other whey protein such as lactoferrin and serum albumin in cow and goat milk is harder to digest compared to human and horse milk (Inglingstad et al., 2010). The same found in the study of Amatayakul et al. (2006) that the β-LG in goat milk is easier to digest than cattle milk β-LG in both gastric and intestinal digestion. In addition, El-Zahar et al. (2005) compared the pepsin hydrolysis of isolated β-LG between cattle milk and sheep milk and determined that due to the higher surface hydrophobicity and different tertiary structure of sheep milk β-LG, it shows a faster hydrolysis. However, α-LA in all species has lower digestibility (Inglingstad et al., 2010).

2.3.2 Infant digestive conditions

The first infant formula digestion occurs in the gastric phase mainly, as new-borns (0 – 6 months of age) are fed on liquid-based milk meal, which reduces the oral cavity activity (Bourlieu et al., 2014). Compared to adults, infant digestion condition was different, which has higher gastric pH and lower enzyme activity. The pH of the infant gastric phase is usually higher than adults (Cavell, 1979; Henderson et al., 2001; Rødbro et al., 1967). In general, the

pH is in the range of 3.2 – 3.5 before the meal, then directly increase to 6.0 – 6.5 after eating and remains higher than 5.0 for 50 minutes.

There are two gastric enzymes in the gastric phase – human gastric lipase (HGL) and pepsin (Bourlieu et al., 2014). The development of two enzymes in infant gastric is nonparallel. The review of Lindquist and Hernell (2010) is indicative of the lipase appearing in the 11th week of gestation and is well developed in preterm infants. The HGL activity is only little susceptible to the fat content and the age of infants (Armand et al., 1996; Hamosh et al., 1981; Roman et al., 2007). However, the development of pepsins in infant's stomach is slow (DiPalma et al., 1991; Henderson et al., 2001; Ménard et al., 2018). As Figure 2.2 shows, pepsins start to develop at 14th weeks gestation, but only reach 18% of adult activity after 4 weeks of birth (McClean & Weaver, 1993). Pepsins are small basic peptides the active from pepsinogens by selective cleavage. It is the autocatalytic activation in the pH of 1.0 – 2.5. Due to the higher after meal pH (> 5.0) in the infant gastric environment, the pepsinogens to pepsins conversion is in low level and low proteases activity (Bourlieu et al., 2014).

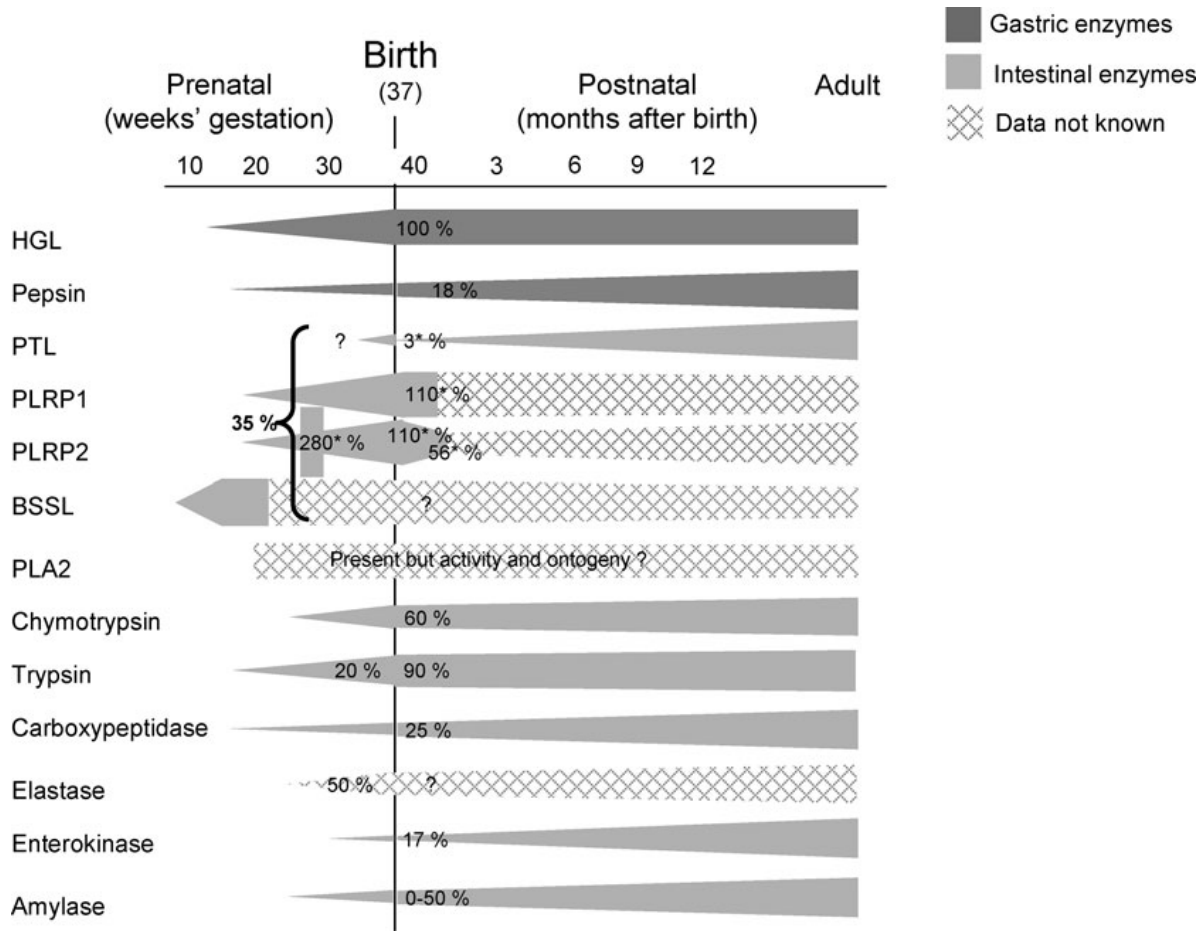


Figure 2.2. The level of childhood enzyme development (Bourlieu et al., 2014)

2.3.3 Digestion of infant formula

The typical infant formula with addition of whey proteins to mimic the whey to casein ratio of human milk (60:40) (Heird, 2007; Ménard et al., 2014). However, the whey protein composition of human milk and animal milk are different. The whey proteins of human milk contain α-lactalbumin, lactoferrin and immunoglobulins, but no β-LG (Kelly & Fox, 2016; Koletzko et al., 2005; Liao et al., 2017). On the contrary, the β-LG is the main component in cow, goat and sheep milk, and the value of α-lactalbumin, lactoferrin and immunoglobulins are much lower than that in human milk (Park et al., 2007; Roncada et al., 2012). The different protein types and the content could affect the digestibility. Bourlieu et al. (2014) has reported the gastric half emptying time ($t_{1/2}$) of the infants fed with cow's milk is longer than those fed with breast milk, which is 80 min and 50 min, respectively.

The previous studies elucidated that compared to cow and sheep infant formula, goat infant formula has faster protein digestibility as it formed smaller protein aggregation (Hodgkinson et al., 2018; Ye, Cui, et al., 2019). On the contrary, Maathuis et al. (2017) reported that there is no significant difference in digested protein quality in different types of infant formulae and human milk. Although the protein digestion kinetics of goat infant formula is similar to human milk. However, the comparisons of different species milk digestion were mostly carried out on raw milk, with only limited academic research and few facts on infant formula gastrointestinal digestion.

As the previous section mentioned, heat treatment as a common process included in infant formula manufacturing plays an important role in the gastrointestinal digestion of infant formula. The high temperature (above 70 °C) could induce whey protein denaturation and initially the unfolding globular structure forms disulphide bond aggregations between adjacent whey proteins and between casein micelles and whey proteins (Kelly & Fox, 2016). As figure 2.3 shows, compared to unheated gastric coagulation, heat-treatment induced to an open structure clot, which allowed pepsin access to the surface easier (Li et al., 2021; Ye et al., 2017).

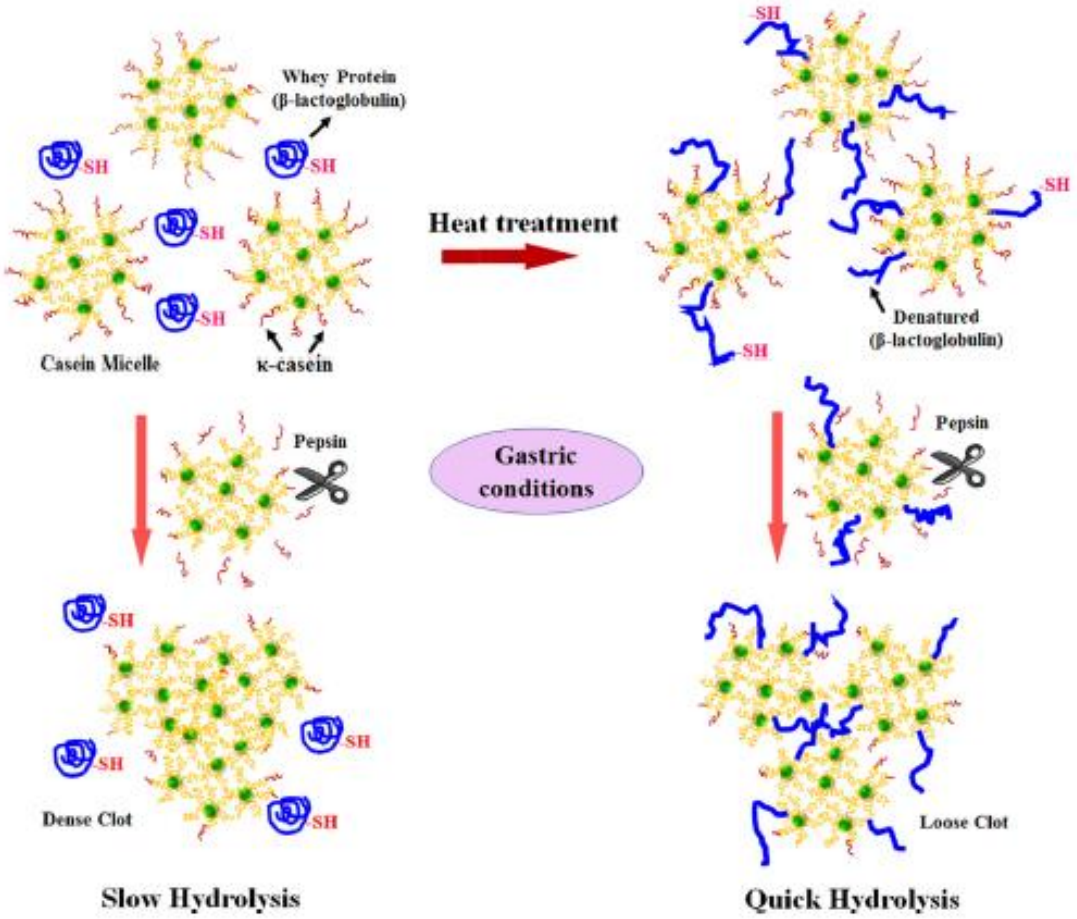


Figure 2.3. The possible mechanism schematic diagram of unheated and heated milk protein hydrolysis under the gastric environment. (Ye, Liu, et al., 2019)

However, the study by Wada and Lönnerdal (2015) revealed that heat treatment might reduce the digestibility due to whey-enhanced infant formula being more susceptible to glycation (Prosser et al., 2019). The influence is more manifested in milk-based formulas than in non-modified milk. In addition, ingestion of glycation products could affect the gut microbiota (Seiquer et al., 2014). The present research is more focused on the studies of raw milk protein digestion, the studies on the effect of heat treatment on the infant formula from different species are limited. More research is needed to better understand the effects of different protein compositions and the heat treatment on simulated gastric digestion behaviour of infant formulae.

2.4 Conclusion

As the review above, most of the previous studies have been focused on the comparison of raw

milk from different species. The coagulation behaviour and the matrix structure change during dynamic gastric digestion of infant formulae made from cow, goat and sheep have been attempted to understand. However, infant formula undergoes several treatments during manufacturing processes including homogenisation, heating, evaporation, spray drying etc. The effect of protein compositions from different species and the manufacturing process on infant formula digestion behaviour under dynamic gastric conditions are still not fully understood. Therefore, this research aimed to investigate the comparative dynamic simulated gastric digestion behaviour of cow, goat and sheep infant formulae using the IHGS, to explore the influence of protein composition and heat treatment on coagulation and gastric digestion behaviour.

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Infant formula

Three whey protein dominant commercial infant formulae (CIF, GIF and SIF) were purchased from local supermarket, which were made from cow milk, goat milk and sheep milk, respectively. The nutrition information of these products is stated on label, listed in Table 3.1.

Table 3.1. The nutrition information of infant formulae

Average per 100g	CIF	GIF	SIF
Energy (kJ)	2043	2073	2045
Protein (g)	15.30 ± 0.17	13.13 ± 0.06	12.76 ± 0.10
Fat (g)	24.43 ± 2.16	27.43 ± 2.64	22.64 ± 1.52
Carbohydrate (g)	52	54	52

3.1.2 Pepsin

Pepsin from porcine gastric mucosa (EC 3.4.23.1; catalogue no. 9001-75-6), was purchased from Sigma-Aldrich, USA. As manufacturer stated, it had a laboratory enzymatic activity of 541 Units/mg solids.

3.1.3 Simulated gastric fluid (SGF)

The preparation of simulated gastric fluid (SGF) was based on the salt composition suggested by a previous study (Mulet. Cabero et al., 2020) with a slight modification. A mixture of the chemicals in Table 3.2 hydrated in Milli-Q water with stirring for 30min. 1.25x concentrate 1L SGF was made by adding 800 ml water, then adjusted the pH to 2.0 by using 1M HCL and 1M NaOH. 0.15 mmol/L $CaCl_2$ and 3.2g/L pepsin were added into SGF prior to use – adjust with water to correct electrolyte concentration.

Table 3.2. The Chemicals of Simulated gastric fluid (SGF)

Chemicals	Concentration (mmol/L)	Molar Mass (g/mol)	Volume (ml)	m (g)
Potassium chloride (KCl)	6.9	74.5513	2000	1.02881
Potassium dihydrogen phosphate (KH_2PO4)	0.9	136.086	2000	0.24495
Sodium bicarbonate ($NaHCO_3$)	25	84.007	2000	4.20035
Sodium chloride (NaCl)	47.2	58.4428	2000	5.51700
Magnesium chloride hexahydrate ($MgCl_2(H2O)_6$)	0.1	203.3	2000	0.04066
Ammonium carbonate ($(NH_4)_2CO_3$)	0.5	96.09	2000	0.09609
Calcium chloride ($CaCl_2$)	0.15	110.98	150	0.00250

3.1.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stock solution

Acrylamide/Bisacrylamide 37.5:1 (30%T, 2.6%C) – 30g Acrylamide/Bisacrylamide premixed powder was dissolved into 100mL Milli-Q water and mixed well. The solution was stored under 4°C in a dark bottle.

10% Sodium Dodecyl Sulfate (SDS) – 10g Sodium Dodecyl Sulfate powder was dissolved in 100 mL Milli-Q water with gentle stirring. The solution was stored at the room temperature.

1.5M Tris-HCl Buffer (resolving gel buffer), pH 8.8 – 18.15 g Tris was dissolved in 60 ml milli-Q water. The pH of the solution was adjusted to 6.8 with 6 M HCl and made up the volume to 100ml. The gel buffer was stored at 4°C.

0.5M Tris-HCl Buffer (stacking gel buffer), pH 6.8 – 6.05g Tris was dissolved in 60ml milli-Q water. The pH was adjusted to 6.8 with 6 M HCl and make up the volume to 100ml. The gel buffer was stored at 4°C.

10% Ammonium Persulphate (APS) – 0.1 g of APS was dissolved in 1 mL of Milli-Q water and mixed well. The solution was freshly prepared before use.

0.1% Bromophenol blue solution – 0.0025 g of Bromophenol blue was dissolved in approx. 0.0109 mL 0.1 M NaOH solution, and the volume was made up to 2.5 mL with milli-Q water.

Sample Buffer (SDS Reducing buffer) – The mixture of 7.875 g of glycerol (25%), 3.125 mL 0.5M Tris-HCl buffer (62.5mM), 5mL 10% SDS (2%), 2.5 ml0.1% bromophenol blue and 6.875 mLmilli-Q water was made up to 23.75 ml. The solution was stored at 4°C. Added 5%

β-Mercaptoethanol to sample buffer prior to use.

5X Electrode Buffer/Tank Buffer – 7.5 g Tris base, 36 g Glycine and 2.5 g SDS was dissolved in Milli-Q water and made up to 500 mL. Diluted 70 mL of concentrated electrode buffer with 280 mL Milli-Q water before use (4 parts of water).

Coomassie Brilliant Blue Solution (0.3%) (stain solution) – 3.00 g Coomassie Brilliant Blue R was dissolved in a mixture of 700 mL Milli-Q water, 200 ml of Isoproponol and 100 ml of glacial ac etic acid. The solution was filtered through Whatman Filter Paper (Grade 4) and stored in a dark bottle.

Destaining Solution – A mixture of 100 ml of Isoproponol, 100 ml of glacial acetic acid and 800 ml water, stored in a well closed container.

All solutions were prepared from analytical-grade chemicals and deionised water, which was purified by treatment with Milli–Q apparatus (Millipore Corp., Bedford, MA, USA).

3.2 Methods

3.2.1 Sample preparation

Three infant formula powders were dissolved in water, warmed in 50 °C water bath with a magnetic stirring for 30 mins to make up a dispersion sample at a target level of 1.565% (w/w) protein. 100 ml dispersion sample was warmed in 37 °C then used for *in vitro* dynamic gastric digestion (chapter 4). To exam the influence of heat treatment (chapter 5), 100 ml dispersion sample was heated at 90 °C in a water bath, holding for 5 min then immersed in ice water to cool down to 37 °C then used for *in vitro* dynamic gastric digestion.

3.2.2 *In vitro* dynamic gastric digestion in an infant human gastric simulator (IHGS)

Infant human gastric simulator (IHGS) was designed by Kong and Singh (2010) and developed by Riddet Instituted (Massey University, New Zealand). As Figure 3.1 shows, the machine consists of a latex stomach chamber, drive system, gastric secretion, and temperature control. The driving system stimulates 3 cycles per minute contractions on the stomach vessel by driving pulleys, 12 rollers and 4 belts. A heater and thermostat maintain the temperature at 37 °C. Two variable flow pumps simulate the secretion of gastric juice into the gastric vessel through plastic pipes. With connection of a plastic tube from the chamber bottom to the outside

for gastric emptying.

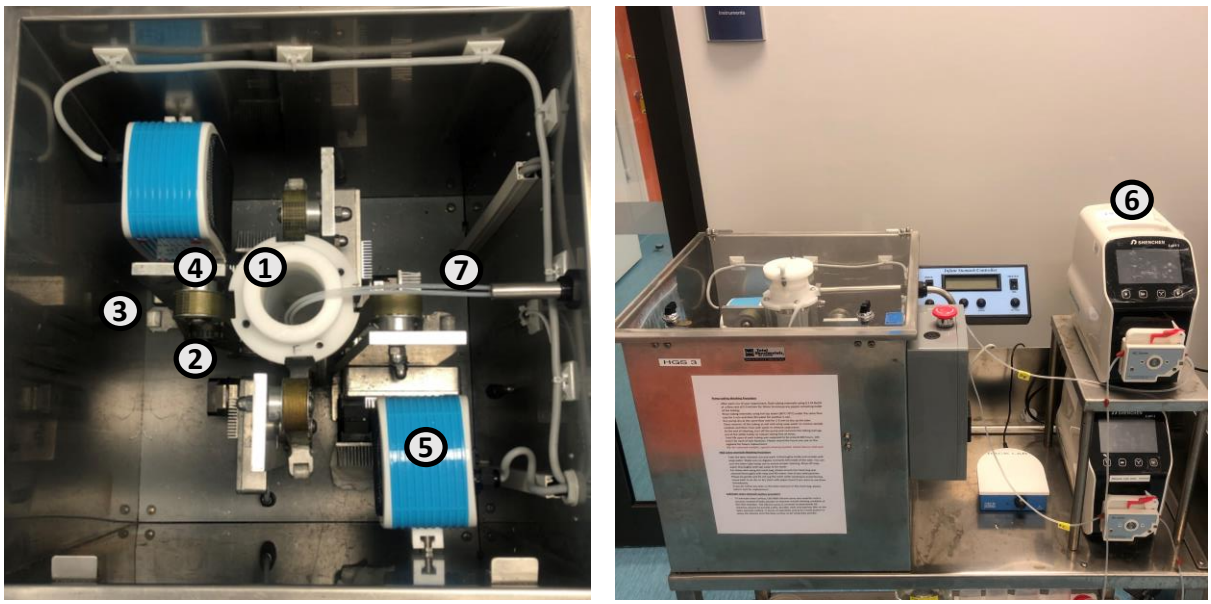


Figure 3.1. Image of an infant human gastric simulator (IHGS).
(1) Latex stomach chamber; (2) pulley; (3) roller; (4) belt; (5) fan heater (6) pump; (7) plastic tubs for secretion.

100 ml 37 °C dispersion sample (1.565% w/w protein) was mixed with fasting simulated gastric fluid (Mix of 7.2 ml Simulated gastric fluid (SGF) and 1.8 ml pepsin solution) in the IHGS. The SGF and pepsin solution were pumped into the IHGS separately at flow rate of 0.4 and 0.1 ml per minute. 22 ml digesta samples were removed from the infant HGS every 20 minutes for simulating the empty gastric rate.

3.2.3 pH measurement

The pH of each infant formula sample and their digesta at each time point were measured using a pH meter (pH mV Temp PL-700PV). The initial pH in the IHGS was defined as the pH of the infant formula sample in fasting gastric fluid. During the 180 min digestion, the emptied digesta at each time point was represented the pH in IHGS, as the roller movement of IHGS prevented easy access into latex stomach chamber.

3.2.4 Particle size distribution

The particle size distribution and the mean particle size of the infant formula and their digested

samples were measured by Mastersizer (2000S, Malvern Instruments, Malvern, Worcestershire, England). Refractive indices of dispersed phase and aqueous phase were 1.46 and 1.33. The particle size of samples was delineated using the volume-surface average diameter $d_{3,2}$ (μm) and the volume-weighted average diameter $d_{4,3,}$ (μm), calculated according to the equations below:

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

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

Wheren_i is the number of particulars with diameter d_i

Digested samples were mixed with 2% (w/w) SDS (sodium dodecyl sulfate) in 50mM EDTA (ethylenediamenetetraacetic acid)) at the ratio around 1:4 to dissolve the protein aggregation, stabilised at least for one hour before the measurement.

3.2.5 Confocal laser scanning microscopy

The microstructures of the infant formula and the gastric chymes during the gastric digestion were observed using confocal laser scanning microscopy (Leica ZEISS LSM 900 with Airyscan 2, Leica microsystems, Heidelberg, Germany) at the Manawatu Microscopy and Imaging Centre (Massey University, New Zealand). The chyme samples were collected at 80, 100 and 180min. Sample were not further treated, such as adjusting pH or heating. They were placed in the ice bath to inactive pepsin before the analysis.

The fluorescent dye Nile Red, 0.1% (w/v) dissolved in acetone, was used to stain oil phase (Argon laser with an excitation line of 561 nm). Fast Green (1.0% w/v) was used to stain the protein (He– Ne laser with an excitation line at 640 nm). 400 μL liquid samples were mixed with 20 μL 0.1% (w/v) Nile Red and 10 μL 1.0% (w/v) Fast Green in an Eppendorf, stained at least 5 min. Placed the stained samples on a concave confocal microscope slide, and covered with coverslips. Following this, the microstructure of the samples was observed using 63x magnification oil immersion lenses. The confocal images acquired and performed using a digital image processing software (ZEISS Zen) consisted of 1024x1024 pixels. Each sample was prepared in duplicate and was taken at least 5 images.

3.2.6 Chemical composition analysis

The total protein (total nitrogen x 6.38) contents of the infant formula and their emptied digesta were determined using the Kjeldahl method, and fat contents were determined using Mojonnier ether extraction method (Horwitz & Latimer, 2010). The emptied digesta collected from 20, 40, 80, 100, 120 and 160 min were determined for protein content, and digesta obtained from 60, 140 and 180 min were determined for fat content.

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

The time-dependent hydrolysed protein in infant formula and their emptied digesta were determined by SDS-PAGE. Samples collected from 20, 40, 80, 100, 120 and 160 min were observed.

Gel preparation

Stacking gel was prepared by a mixture of 0.5M Tris-HCl buffer, pH 6.8, 10% SDS, 30% acrylamide/bisacrylamide solution, TEMED, 10% APS and Milli-Q water. Resolving gel was made up of 1.5M Tris-HCl buffer, pH 8.8, 10% SDS, 30% acrylamide/bisacrylamide solution, TEMED, 10% APS and Milli-Q water. The gel was prepared on a Mini PROTEIN II system (Bio-Rad Laboratories, Richmond, CA, USA). Depends on the quantity of gel, different amount solutions were added and stirring while degassing.

Sample preparation

Different amount samples mixed with 800 µl sample buffer (25% glycerol, 0.5M Tris-HCl, pH 6.8, 10% SDS, 0.1% Bromophenol Blue, 5% β-mercaptoethanol) to achieve equal protein content (0.1%). All solutions were heated in a 90 ℃ water bath for 10 minutes, then cooled to room temperature and load 10 µl into a resolving gel.

Running of electrophoresis, staining, and detaining

The electrophoresis analysis was carried out on a constant voltage of 125V for approximately 90 min until the bromophenol blue dye line reached the bottom of the gel. Stained the gel for 40 min with a Coomassie Brilliant Blue R-250 solution, then destained with the destaining solution overnight. Then scanned the gel using a Bio-Rad Molecular Imager Gel Doc XR+

imaging system. Analysed and quantified the protein compositions by using Bio-Rad Image Lab software version 6.1.

3.2.8 Gelation of infant formulae using glucono-δ-lactone and pepsin

Three infant formula samples have been reconstituted in Milli-Q water to make 1.565% (w/w) protein milk. Added 1.0% (w/v) GDL and different level of pepsin concentration to induce acidification. The amount of GDL and pepsin concentration listed in Table 3.3. Whole cow milk with 1.0% (w/v) GDL alone, and together with different pepsin concentrations (0.3u/ml, 1.0u/ml, 2.5u/ml and 10u/ml) as a control.

Table 3.3. The content of GDL and pepsin concentration in each sample

	GDL Concentration	Pepsin Concentration
Sample 1	1.0% (w/v)	0 Unit/mL
Sample 2	1.0% (w/v)	2.5 Unit/mL
Sample 3	1.0% (w/v)	10 Unit/mL

3.2.8.1 Gel formation and rheology measurements

For each measurement, milk samples were warmed in 37℃ for 30min. After added GDL and pepsin, stirred at 300 rpm for 30 seconds to allow GDL gradually hydrolysed and released gluconic acid to acidify the solution. Then 20ml samples were loaded into the rheometer cup-and-bob geometry (cup diameter of 30.36mm, bob diameter 27.93mm, length 42.09mm) for measurement. The remaining samples were placed into pH-stat to monitor pH in every minute for three hours. The elastic modulus G' and the viscous modulus G" were monitored using oscillatory rheology in Alphatech AR-G2 rheometer at Riddet Instituted (Massey University, New Zealand). A time sweep measurement constantly monitored gelation evolution at 1HZ frequency and 1% strain. The storage modulus G' and the loss modulus G" were recorded every minute for 3 hours. The measurements were carried out at 37°C in duplicate.

3.2.8.2 Confocal scanning laser microscopy of gel

The microstructures of the gel were observed using confocal laser scanning microscopy (Leica ZEISS LSM 900 with Airyscan 2, Leica microsystems, Heidelberg, Germany). After gel were formed in three hours, took out a 400ml placed into ice bath to inactive pepsin before analysis.

Each sample was mixed with 20 mL 0.1% (w/v) Nile Red and 10 mL 1.0% (w/v) Fast Green in an Eppendorf, stained at least 10 min. Placed the stained samples on a concave confocal microscope slide, and covered with coverslips. Following this, the microstructure of the samples was observed using 63x magnification oil immersion lenses. The confocal images acquired and performed using a digital image processing software (ZEISS Zen) consisted of 1024x1024 pixels. Each sample was prepared in duplicate and was taken at least 5 images.

3.2.9 Statistical analysis

All experiments have a minimum of twice repeats using freshly prepared samples. A repeated-measures two-factor ANOVA model with the *in vitro* replication as the experimental unit was performed for the PH, particle size, protein, and fat content of infant formula and their empty digesta using the MIXED model procedure of the statistical software SAS (SAS/STAT version 9.4; SAS Institute Inc.). The statistical linear mixed model included sample number (1,2or 3), time (0 to 180min), and their interaction as fixed effect, whereas replication as a random effect. The most appropriate covariance structure for the mixed models was selected after fitting the models by the Restricted Maximum Likelihood method and comparing them by using the log-likelihood ratio test.

Chapter 4: Effect of Protein Composition in Simulated Gastric Digestion Behaviour of Commercial Infant Formulae

4.1 Introduction

Infant formula as an alternative food for new-borns provides required nutrition when breastfeeding is not available. Cow milk is usually regarded as the most common source of infant formulae. In recent years, consumers have shown an increased interest in non-bovine milk infant formula, such as sheep milk and goat milk infant formulae. Compared to cow milk, sheep milk contains higher total solid content and minerals (Domagała, 2009; Raynal. Ljutovac et al., 2008), while goat milk contains larger casein micelles and lacking in α_{S1}-casein (Park et al., 2007; Storry et al., 1983). The different physicochemical characteristics lead to different properties of enzymatic or acid gels. Goat milk forms a softer and weaker gel while sheep milk forms a firmer gel compared to cow milk (Lucey et al., 2000). The observations reflect goat milk may be easier to digest as the rough and soft coagulation formed in the human stomach (Park et al., 2007).

In recent years, there has been an increasing interest in understanding the gastric digestion behaviour of milk from different species. Roy et al. (2021) compared the structure changes in sheep, goat and cow skim milk using a dynamic *in vitro* gastric digestion model. The clot of sheep skim milk at the end of the digestion was firmer than the other two skim milk. Furthermore, studies were carried out under infant *in vitro* digestion conditions. The observations from Hodgkinson et al. (2018) elucidated that casein from goat milk is considered digested more efficiently than cow milk in infant and young children *in vitro* digestion conditions. Additionally, Ye, Cui, et al. (2019) compared the digestion behaviour of infant formulae made with cow milk and goat milk in a dynamic gastric digestion model. The study found out goat milk infant formula has faster protein digestion ability compared to cow milk infant formula, as it formed smaller protein aggregation and oil droplet flocculation. Another study by Maathuis et al. (2017) reported that compared to cow infant formula, the protein digestion kinetics of goat infant formula is more corresponding to human milk. However, there

is no significant protein quality difference between different types of infant formula and human milk. This was indicated new-borns who fed by cow or goat infant formula were grown in the same way.

Infant formula is the only nutrition source for neonates, it is important to understand the macronutrients delivery and digestion from the stomach to the small intestine. A large amount of research has demonstrated that the food structure change during the gastric phase plays an important role in protein digestion and amino acid release (Guo et al., 2017; Mulet. Cabero et al., 2017). The coagulation behaviour is highly influenced by protein composition and food processing (Gan et al., 2018; Ye, 2021). The protein composition could be the protein sourcing from different animal species, different concentrations, or ratios et al. So far, most of the previous research conduction the milk from different species were carried out using fresh raw milk. There has been little discussion about the comparison of gastric digestion behaviour of infant formula made from different species.

This present study investigated the aggregation behaviour of commercial infant formula made by sheep, goat, and cow milk during *in vitro* gastric digestion by using a dynamic digestion model (infant human gastric simulator). The infant human gastric simulator (HGS) mimics the infant gastric environment by continuous pumping in fresh simulated gastric fluid (SGF) with pepsin and imitating gastric emptying. The evaluation of pH, protein, and fat contents of the empty digesta as function of time, and the microstructure of chyme, the protein hydrolysis of empty digesta, as well as the particle sizes of both chyme and empty digesta were examined. The results obtained from this experiment contribute to the understanding of the influence of protein compositions on digestion behaviour.

4.2 Results

4.2.1 Protein composition of infant formula samples

The protein composition of cow, goat and sheep infant formula samples were analysed by SDS-PAGE under reducing conditions. Figure 4.1 clearly shows that three infant formulas contained different protein compositions, especially the proportions of casein. GIF had a clear α_{S2}-casein band and invisible α_{S1}-casein band, while β-casein took the highest proportion in GIF casein.

On the contrary, the α_{S1}-casein band was clearer in CIF, and it took a similar portion as β-casein, whereas α_{S2}-casein was very faint in CIF. β-casein was the most visible band in SIF, both α_{S1}-casein and α_{S2}-casein brands were not observed. The κ-casein brands were shown similar intensity in all samples. The SDS-PAGE pattern of caseins in the three samples was aligned with the previous findings that there is a lower level α_{S1}-casein in goat milk than in other ruminants' milk, while cow milk contains a higher level of α_{S1}-casein (Raynal. Ljutovac et al., 2008; Wendorff & Haenlein, 2017). Also, Mohapatra et al. (2019) has reviewed the studies of sheep milk casein fractions and found out the findings were contradictory and deficient. The α_{S1}-casein were ranged from 5.3% to 39.9% of the total casein (Mohapatra et al., 2019). It is also reported that the casein concentrations were varied between different sheep breeds (Park et al., 2007). Compared to whey protein brands (β-LG and α-LA), GIF and CIF were shown slightly more intense than that in SIF. The whey/casein ratio of GIF and CIF agree with the label claim that containing 60% whey protein, which is whey-protein-dominate infant formula.

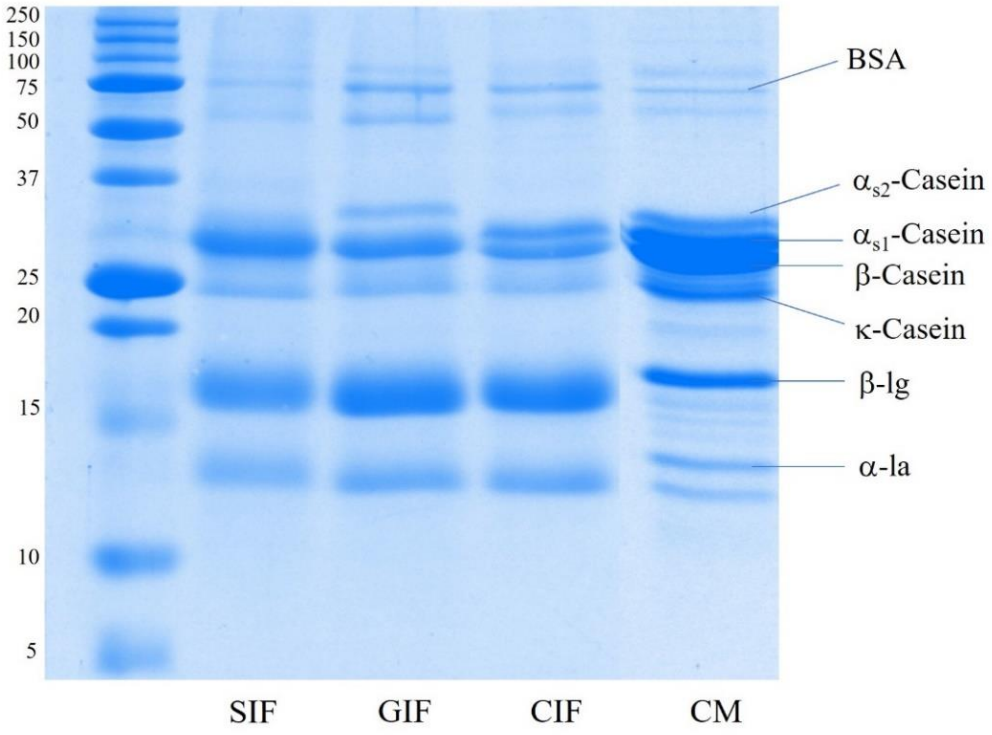


Figure 4.1. SDS-PAGE pattern under reducing condition of the sheep, goat, and cow infant formulae

4.2.2 pH profiles in *in vitro* dynamic gastric digestion

The pH profile of empty digesta from sheep milk, goat milk and cow milk infant formulae (SIF, GIF and CIF) were monitored during 180 min *in vitro* dynamic gastric digestion. There was no significant difference ($P > 0.05$) among the three samples in pH profile change with the digestion time. As it can be seen from Figure 4.2, the pH profile of three samples all experienced a decreasing trend with the constant secretion of SGF in three hours. The initial pH of SIF, GIF and CIF with fasting SGF was 6.42 ± 0.07, 6.54 ± 0.02 and 6.74 ± 0.02 respectively, where the protein contents were 1.565% (w/w) as per prepare instruction shown on the labels.

In the early 60 min of digestion, the pH remained steady with a slight decrease. From 60 min to 160 min, there were sharp declines in all three empty digests. The pH of SIF was decreased from 6.41 ± 0.03 to 2.71 ± 0.12, GIF was from 6.48 ± 0.07 reduced to 2.76 ± 0.15, and CIF was a decline from 6.63 ± 0.09 to 2.71 ± 0.19, respectively. From then on, the pH stayed the same level till the end of the digestion, the end pH was 2.59 ± 0.23, 2.67 ± 0.16 and 2.66 ± 0.15 respectively of SIF, GIF and CIF. The pH profile is designed to follow the study of Bourlieu et al. (2014), where the infant gastric pH increased to 6.0-6.5 immediately after feeding and remained higher than 5.0 more than 50 minutes. Then the pH decreased to ~3 after intaking the meal, which is higher than the adult gastric pH.

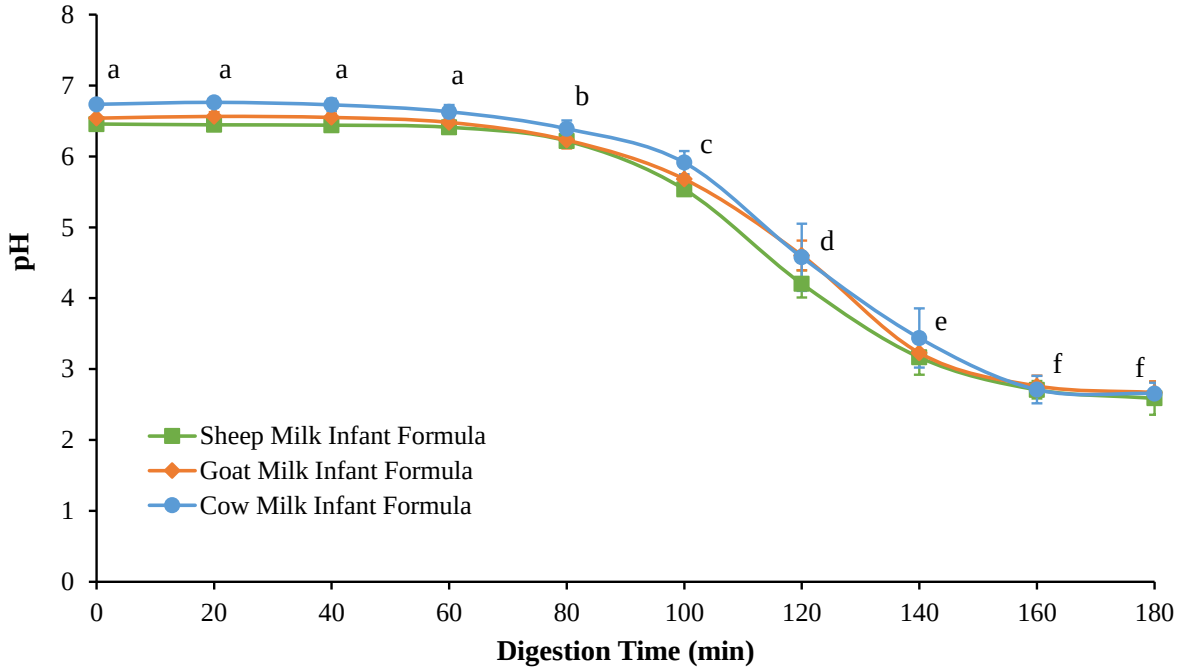


Figure 4.2. pH profiles change during the gastric digestion of sheep, goat, and cow milk infant formula with 1.565% (w/w) protein

4.2.3 Digestion behaviour

As Figure 4.3 shows, the significant difference among the infant formulae made with three types of milk was the trends of flocculation that led to phase separation. GIF primarily showed visual phase separation at 40 minutes, while SIF and CIF were seen phase separation at 60 minutes. Also, compared with SIF and CIF, GIF presented a larger and fragile flocculate. Only creaming and phase separation were observed in three samples, no firm clot was formed during digestion. The phase separation may be induced by the coagulation of milk protein under gastric conditions. The coagulation could be induced by pepsin. As reported in Ye et al. (2016a) study, milk protein precipitation and aggregation were due to low pH and proteolytic enzyme present. In the present study, the pH of initial aggregations was around 6.4 – 6.6, which was well above the acidic coagulation pH of the milk. This indicated that the aggregations of three infant formulae were pepsin induced. In addition, the aggregation and creaming may affect each other. The aggregation that occurs could enhance the creaming rate, whereas creaming also could increase the aggregation rate as creaming could bring the droplets closer to each other.

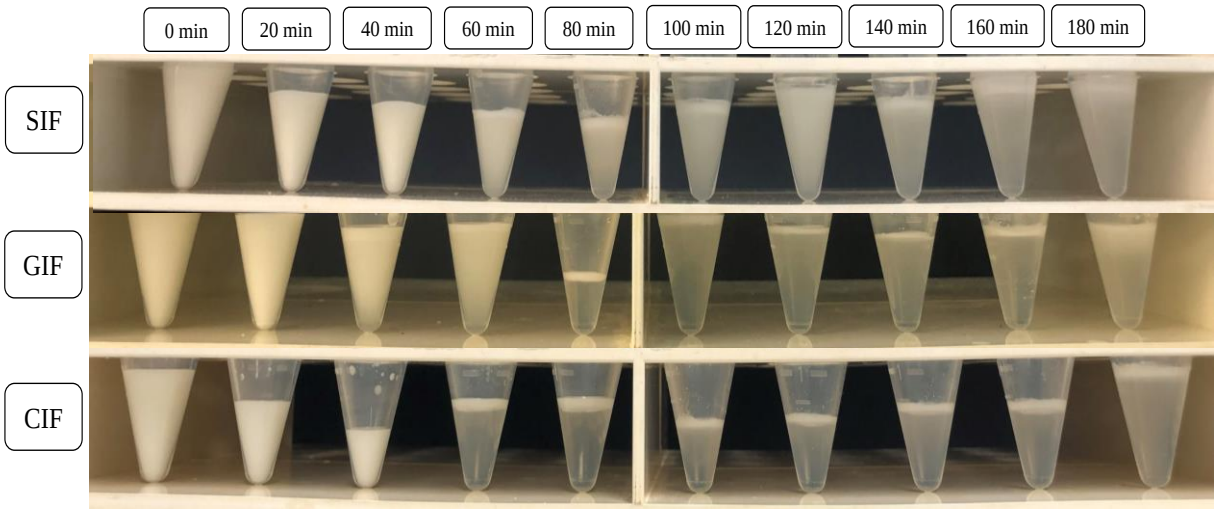


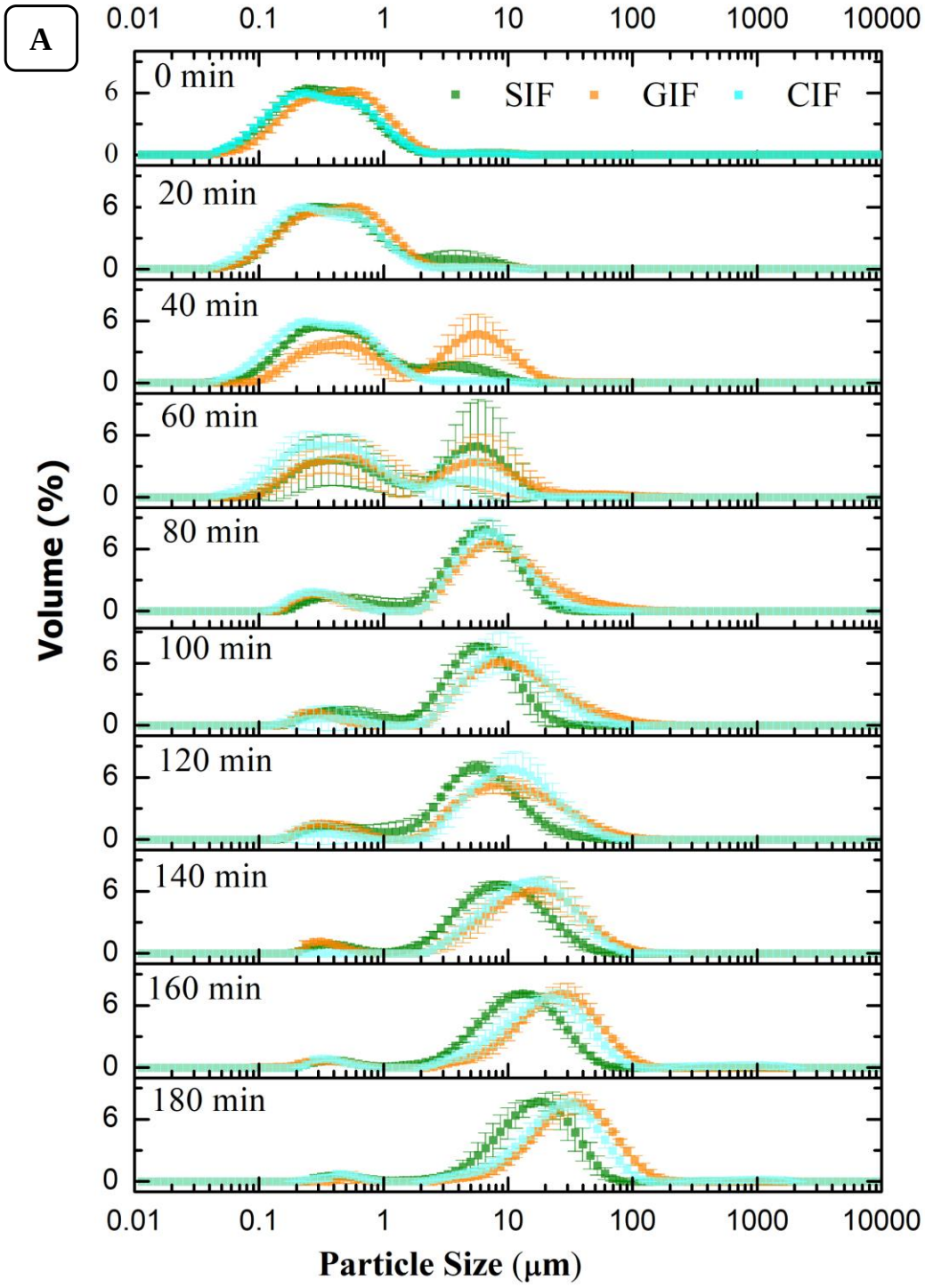
Figure 4.3. Chyme of sheep, goat, and cow infant formula during digestion

4.2.4 Changes in particle size of chyme and emptied digesta

The digestion behaviour observations were aligned with the results of chyme particle size distribution. Figure 4.4 A shows the changes in the particle size distribution of the chyme. The initial models of three samples showed a monomodal pattern with a peak between 0.1-1 µm. At 40 min of digestion, GIF showed a bimodal pattern with peaks near 0.5 µm and 6 µm, whereas SIF showed a bimodal pattern with a major peak near 0.5 µm and a small tail in the range of 1-10 µm. However, CIF remained as a monomodal at 40 min. This indicated that the initial coalescence started earlier in GIF, followed by SIF and CIF. The observation is consistent with the study of Ye, Cui, et al. (2019). From 60 to 80 min, the particle size distribution of three samples all shifted to the right with bimodal patterns. With further digestion, the peaks slightly shifted to the right.

The changes in the particle size distributions of three sample chyme dispersions in SDS and EDTA solution are shown in Figure 4.4 B, which described the size of casein micelles and flocculated oil droplets are divided into individuals. Combining the trends of both figures, it can be elucidated the digestion behaviour of three samples. At the early digestion time, the increase in the particle size may mainly cause by protein aggregation. At 40 min of digestion when the initial aggregations occurred, there was little size increase of oil droplets. From 60 min, the particle size increased of infant formula samples may be attributed to both protein aggregation and oil coalescence, as the peaks of both models gradually shifted to the large size

region with the time increase. The greatest oil droplets increase was found in sheep milk chyme. However, the overall particle size of aggregations was GIF higher than CIF, and SIF was in the smallest size.



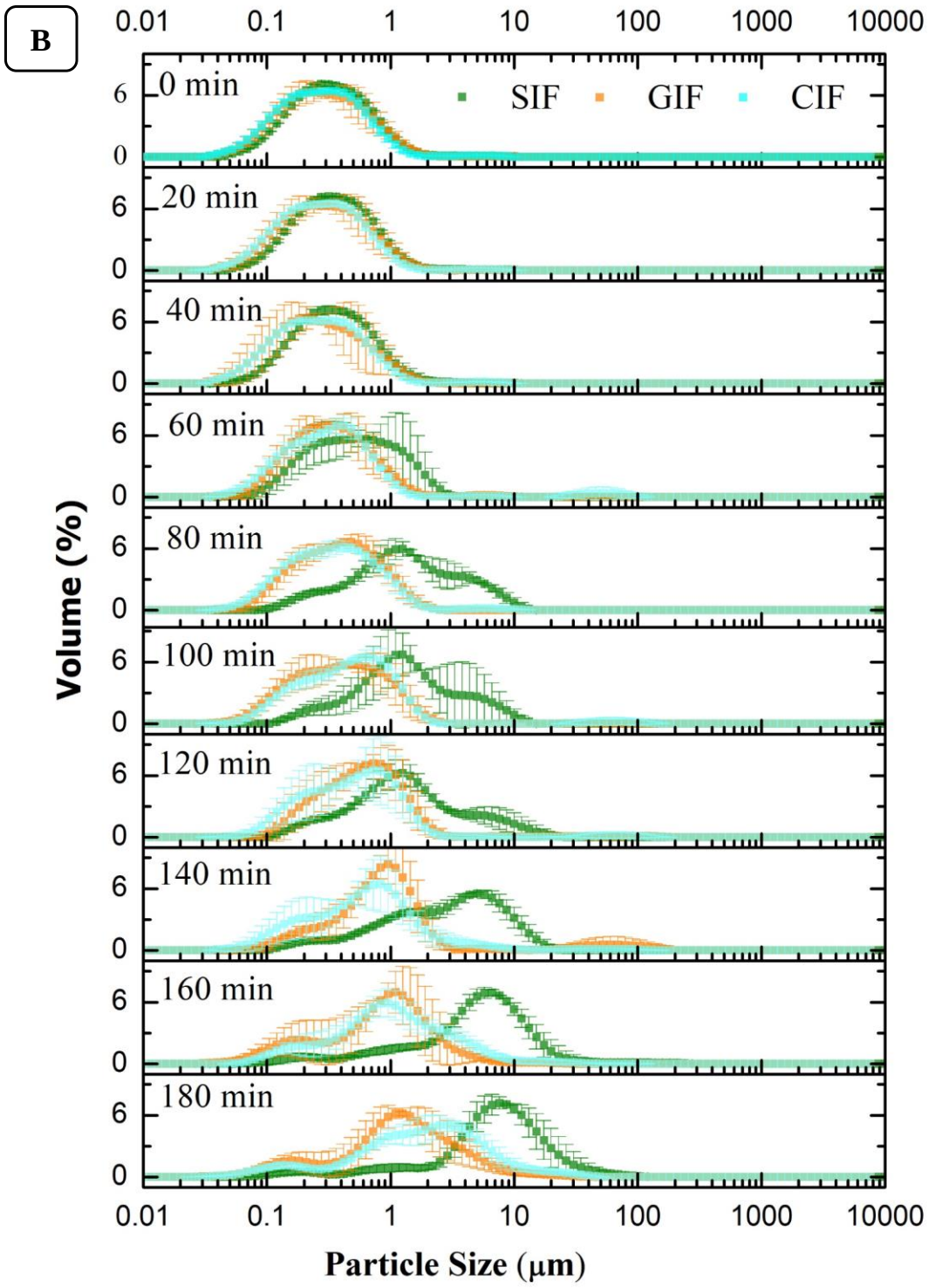
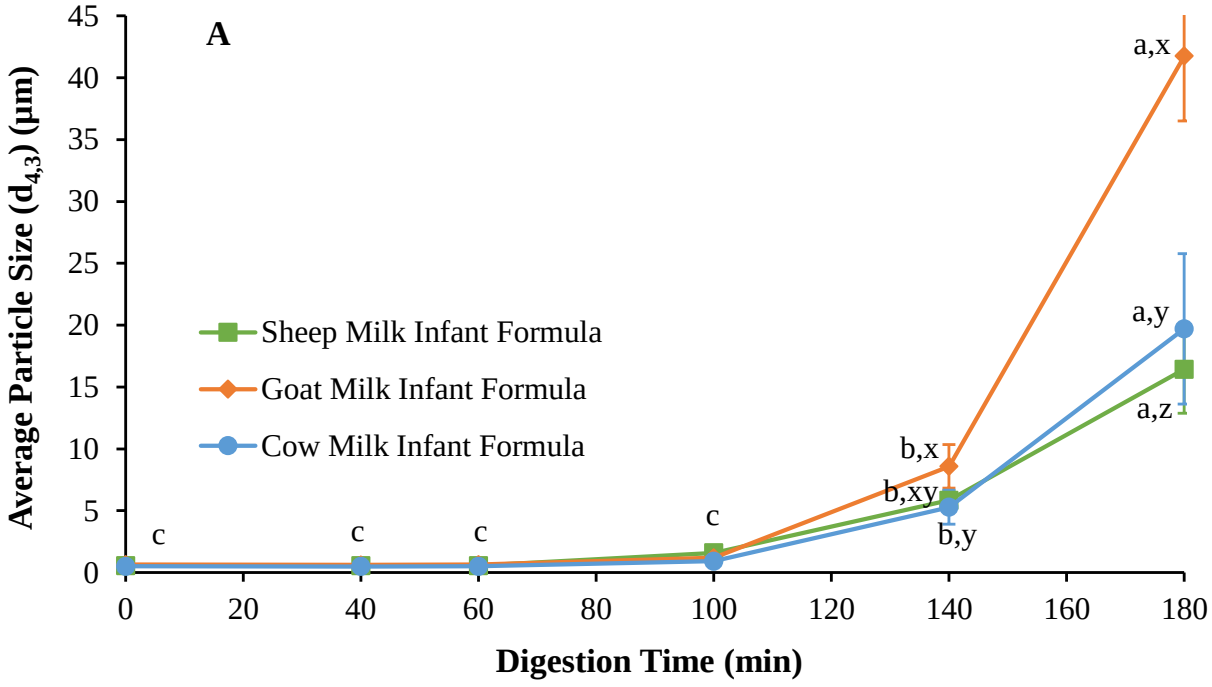


Figure 4.4. The particle size distributions of SIF, GIF and CIF chyme (A) and chyme in SDS and EDTA solution (B)

The particle size distribution of the infant formula chyme showed increasing trends from 40 min of the digestion, and three samples' chyme had slightly different digestion behaviour as shown in Figure 4.4. However, the changes in the average particle size of the emptied digesta have shown different performances. As shown in Figure 4.5, the average diameters of three emptied digesta (A) were not changed until 60 min ($P > 0.05$). Then increased slightly at 100 min. At 140 min of digestion, GIF emptied digesta increased higher than the other two samples, then increased dramatically at the end of the digestion. The $d_{4,3}$ value of GIF increased from 8.59 ± 1.77 µm at 140 min to 41.75 ± 5.25 µm at 180 min. Whereas, the emptied digesta of CIF raised from 5.28 ± 1.37 µm to 19.71 ± 6.08 µm and SIF increased from 5.85 ± 0.75 µm to 16.43 ± 3.54 µm respectively at the same period. Overall, similar trends were found in emptied digesta of three samples. However, the increased rate of emptied digesta particle size was in the following order: goat> cow> sheep ($P < 0.05$). Regarding the average diameters of oil droplets shown in Figure 4.5 (B), there was no significant difference among the three samples ($P > 0.05$). The sizes all remained at the same level for the 100 min of digestion, then gradually increased till the end of the digestion.



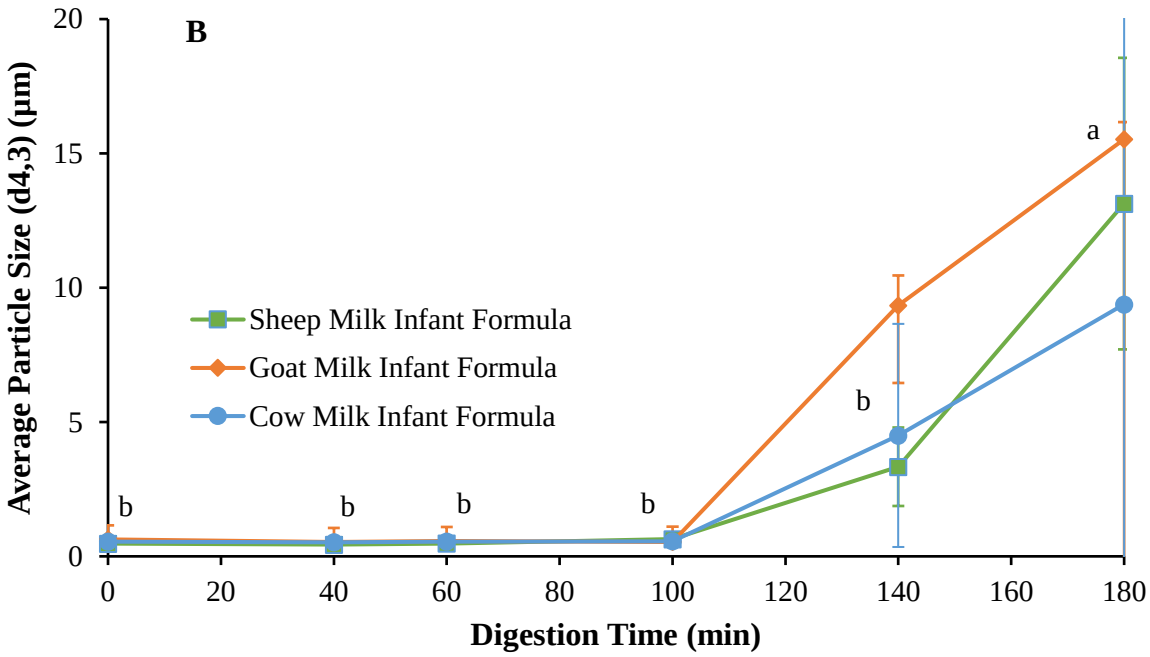


Figure 4.5. Changes in volume-weighted average diameter ($d_{4,3}$, µm) of SIF, GIF and CIF emptied digesta (A) and the emptied digesta in SDS and EDTA (B)

4.2.5 Microstructure of chyme

The microstructure of three infant formulae and their chyme at 80 min and 100 min and the residuum at end of the digestion were observed by confocal laser scanning microscopy (CLSM). The fat was stained as red colour by Nile Red and protein was stained as green colour by Fast Green. As shown in Figure 4.6, three infant formulae at 0 min showed uniformly dispersed oil droplets of similar size, without any coagulation or flocculation. At 80 min of digestion, where pH was around 6.2 – 6.3, protein coagulation was observed. For GIF and CIF, the oil droplets were embedded in the protein coagulation, the primary oil droplet sizes were similar to the ones at 0 min.

SIF was shown a slightly different matrix that the oil droplets of sheep milk were coalesced, which showed larger size than the ones at 0 min. The aggregate particles increased slightly at 100 min when pH was around 5.5 – 5.9. The largest flocs of three samples were all seen at 180 min (pH ~ 2.6). It is clearly shown in figure 4.6, both oil flocculation and protein coagulation were observed. In SIF, there were larger extent of coalescence than aggregations occurred, and

the individual oil droplets were more consistent in size. In contrast, GIF showed dense, coarse and porous structure aggregates. The oil droplets were of various sizes and incorporated into the coagula. For CIF, there were more protein aggregates were observed, along with various size oil droplets trapped in the aggregates. The extensive protein aggregation and oil coalescence could be attributed to the decrease of pH to ~ 2.6, which was well below the casein isoelectric point 4.6 (McSweeney & Fox, 2013). Overall, SIF trended to form smooth oil droplets with fewer protein networks. CIF exhibited dense protein networks trap oil droplets. Oppositely, GIF formed irregular aggregates with porous and coarse textures.

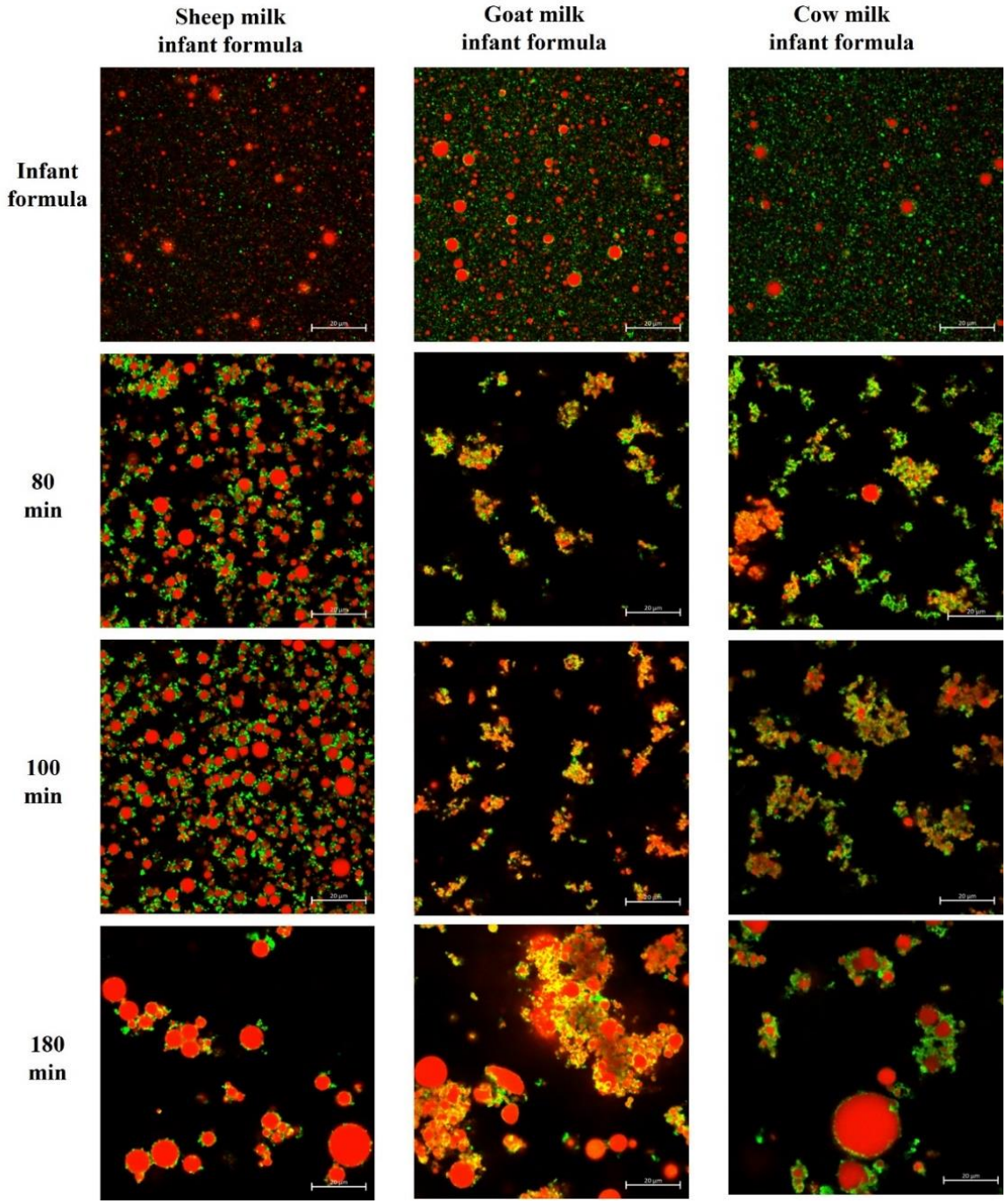


Figure 4.6. Confocal laser scanning microscopy (CLSM) images of sheep, goat and cow milk infant formula chyme. Scale bars represent 20μm

The observations of confocal laser scanning microscopy images (Figure 4.6) were supported by the chyme particle size ($d_{4,3}$, μm) values. Table 4.1. listed the chyme particle size or that of chyme in SDS and EDTA solution. The results are shown as mean ± standard deviation. At 0 min, the $d_{4,3}$ values of SIF and CIF were the same (~ 0.49 ± 0.02 μm), and GIF was slightly

bigger (~ 0.61 ± 0.01 μm). At 80 mins of digestion, the average diameters of all samples were rapidly increased, then slightly increased at 100 mins. GIF showed the largest aggregation compared to SIF and CIF, it was 10.98 ± 1.72 μm at 80 min and increased to 14.78 ± 1.79 μm at 100 min. Followed by CIF, particle size has grown from 7.26 ± 0.64 μm at 80 min to 11.22 ± 2.67 μm at 100 min. On the contrary, SIF remained at a similar size (6.0 ± 0.67 μm at 80 min and 5.83 ± 1.01 μm at 100 min). At the end of digestion, the sizes of the three samples all increased remarkably. CIF was the largest (40.76 ± 20.65 μm), which similar to GIF (37.23 ± 0.85 μm), and SIF was in the smallest size (17.23 ± 2.24 μm).

The average particle size of samples in SDS and EDTA solution was measured to show the oil droplet size. According to Table 4.1, the fat droplet size of three samples was similar at 0 mins (~ 0.5μm), then slightly increased by the time of digestion and remain the similar size at 80 min and 100 min, which were ~ 1.9 μm, ~ 0.6 μm, and ~ 0.65 μm respectively of SFI, GIF and CIF. At end of digestion, the oil droplets of SIF increased remarkably, from 0.43 ± 0.01 μm at 0 min to 9.25 ± 2.03 μm at 180 min. Followed by CIF, was from 0.52 ± 0.02 μm at 0 min to 4.16 ± 0.42 μm at 180 min. There was little increase of GIF oil droplet, was from 0.51 ± 0.00 μm at 0 min to 2.55 ± 0.74 μm at 180 min. The results also indicated there was extensive oil droplet coagulation occurred in SIF but formed the smallest aggregates. In opposite, GIF and CIF formed the larger size aggregates with the smaller oil droplets. The observations of the chyme structure and their average weight-to-volume diameters indicated that the different protein compositions from different species had an impact on the chyme digestion behaviour.

Table 4.1. The volume-weighted average diameter ($d_{4,3,}$ μm) of sheep, goat, and cow infant formula

	0 min	80 min	100 min	180 min
Sheep Milk Infant Formula	0.49 ± 0.02	6.00 ± 0.67	5.83 ± 1.01	17.23 ± 2.24
SIF in SDS and EDTA solution	0.43 ± 0.01	1.92 ± 0.04	1.93 ± 0.67	9.25 ± 2.03
Goat Milk Infant Formula	0.61 ± 0.01	10.98 ± 1.72	14.78 ± 1.79	37.23 ± 0.85
GIF in SDS and EDTA solution	0.51 ± 0.00	0.58 ± 0.08	0.63 ± 0.13	2.55 ± 0.74

Cow Milk Infant Formula	0.49 ± 0.02	7.26 ± 0.64	11.22 ± 2.67	40.76 ± 20.65
CIF in SDS and EDTA solution	0.52 ± 0.02	0.66 ± 0.05	0.64 ± 0.00	4.16 ± 0.42

4.2.6 The physical characteristic of gastric emptied digesta

The protein contents of emptied digesta derived from different milk source infant formula as a function of digestion time were present in Figure 4.7. Three samples all went through a similar trend ($P > 0.05$). In the early 20 minutes of digestion, the protein contents of three samples all decreased slightly following the trend of the dilution line. From 40 min to 100 min, the protein contents gradually declined but the contents were higher than the dilution values. After 100 min till the end of the digestion, the protein contents kept decreasing to slightly lower than the dilution line. The protein contents above the dilution line in the 40 – 100 min could reflect that there was flocculation that occurred in the samples, which lead to phase separation and protein precipitation. After 120 min, where pH reached isoelectric point ~ 4.6, the protein contents in the digesta were lower than the dilution value. This could be ascribed to the extent aggregations occurred in the stomach induced by both pepsin and low pH.

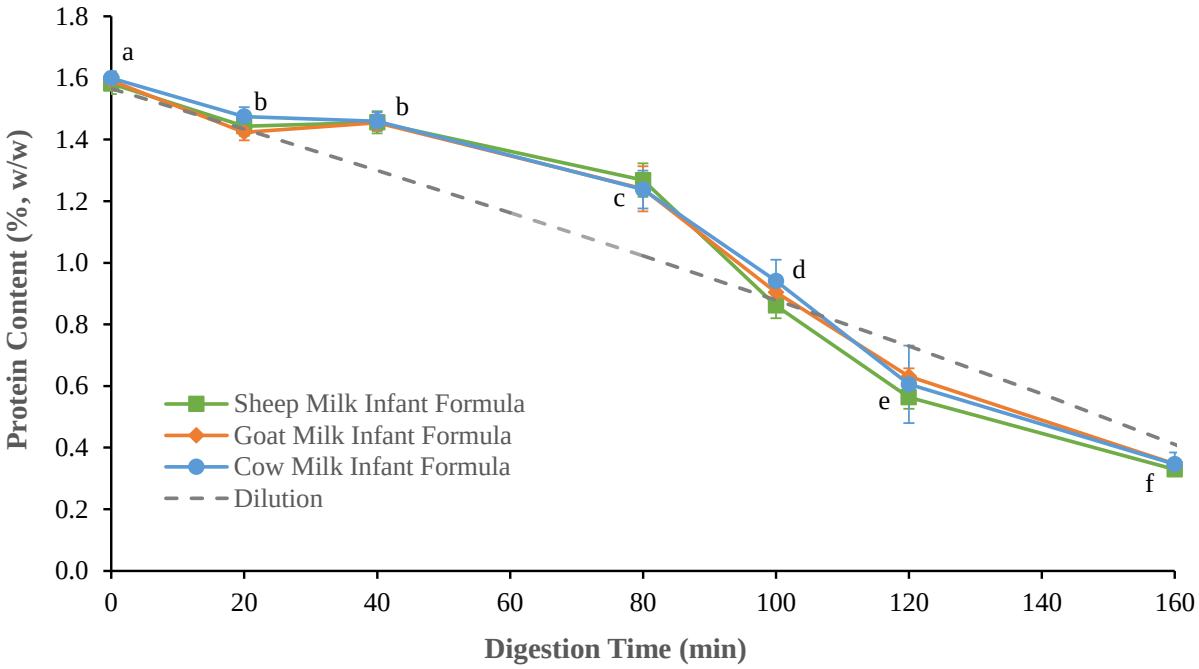


Figure 4.7. The protein content of the emptied digesta during gastric digestion in the IHGS of sheep, goat, and cow milk infant formula

The fat percentages in emptied digesta of three samples were shown in Figure 4.8. In the early 60 min of digestion, the fat contents decreased mostly following the dilution trend. Then sharply dropped under the dilution line till 140 min. This could be attributed to the extended aggregations that occurred that trapped more fat globous in the stomach. At the end of digestion, the fat content of GIF considerable increased, while the other two samples had slightly increased. In conclusion, the overall fat contents of three emptied digesta were all followed a similar trend, though GIF showed the highest fat content at the end of digestion, followed by CIF. SIF emptied digesta had the lowest fat content at 180 min of digestion.

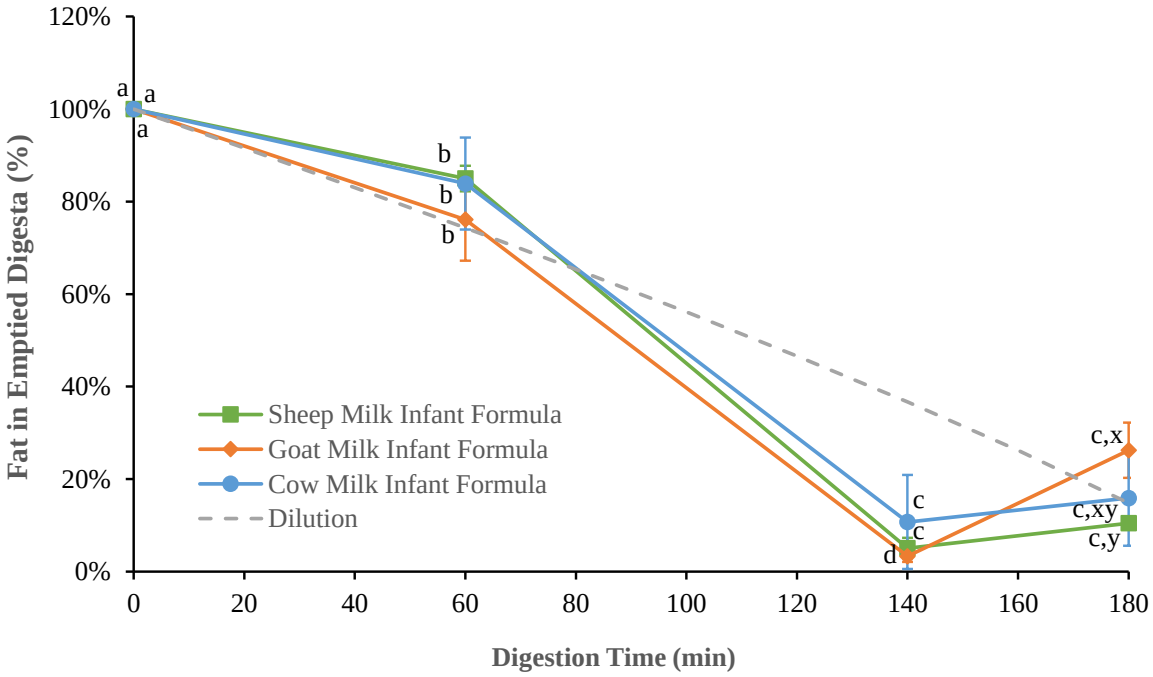


Figure 4.8. The fat in emptied digesta during gastric digestion in the IHGS of sheep, goat, and cow milk infant formula

4.2.7 Hydrolysis of protein in empty digesta

The protein composition of sheep, goat and cow infant formula emptied digesta were analysed by SDS-PAGE under reducing conditions. Analysis of the cow milk infant formula (Figure 4.9, CIF) showed that the κ-casein band disappeared at 20 min of the digestion, where pH was around 6.76 ± 0.02. Whereas the other protein bands (especially α_{S1}-casein, α_{S2}-casein, β-

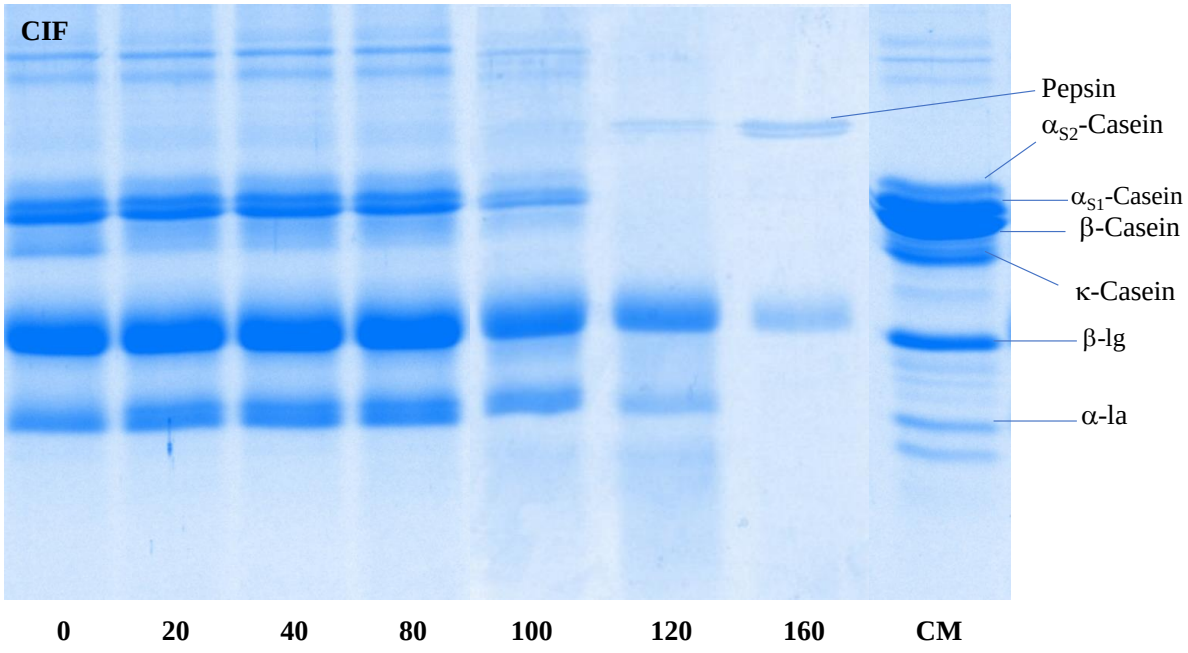
casein, β-lactoglobulin and α-lactalbumin) remained similar intensities at 0, 20, 40 and 80 mins. The result was in a line with the previous studies that κ-casein could be hydrolysed by pepsin and was faster than the hydrolysis of other caseins (Ye, Cui, et al., 2019; Ye et al., 2017). Also, a 15-kDa band was observed at 20 – 100 min, indicating that κ-casein could be hydrolysed and formed para-κ-casein.

The observation supported by the review of Ye (2021) that milk aggregating by proteolytic enzymes followed enzymatic hydrolysis and milk micelle aggregation two stages. In the beginning, pepsin causes peptide bone cleavage of κ-casein, and the casein micelles lose electrostatic and steric repulsion. The destabilisation of casein micelles induced the decreasing of zeta potential, which may increase the sensibility to the environment and aggregation occurs when the zeta potential decrease to a critical point. The zeta point of casein is also affected by pH, temperature and the Ca^{2+} or other ionic concentrations (Carr & Golding, 2016). The other casein bands of CIF emptied digesta decreased in intensity at 100 min, then almost invisible at 120 min and beyond. The intensity of whey protein started getting faint from 120 min (pH 4.2 ± 0.19), especially α-LA. At 160 min of digestion, α-LA was hydrolysed and the β-LG band decreased in intensity. This is aligned with the findings in the study of Ye, Liu, et al. (2019) that pepsin has higher activity on casein and α-LA at low pH. The pepsin band can be observed at 120 min and increased the intensity at 160 min.

Goat milk infant formula gastric digesta exhibited a similar pattern as CIF digesta (Fig 4.9, GIF). Same as CIF, the κ-casein band cannot be detected at 20 min, where pH was around 6.57 ± 0.06. However, the para-κ-casein band was more obvious in GIF at 20, 40 and 80 min, also two new brands appeared at 20 -100 min (~19 kDa and ~ 16.5 kDa). This indicated that GIF protein may have greater hydrolysis than CIF protein. The result was in a line with the previous observations on pasteurized milk clots from cow and goat milk (Roy et al., 2021). Moreover, casein bands of GIF almost disappeared at 100 min, but casein brands of CIF were still observed at 100 min. The observation could indicate that the protein hydrolysis in GIF was faster. This is in agreement with the previous studies that GIF formed porous and soft coagulations, which is easier to digest in the stomach (Hodgkinson et al., 2018; Maathuis et al., 2017; Ye, Cui, et al., 2019). The whey protein band intensities showed a similar trend in GIF

and CIF. The pepsin band was only detected in the digesta from 160 min of digestion.

Regarding the SDS-PAGE pattern of SIF emptied digesta, it displayed a similar trend to CIF and GIF, with a few exceptions at the late stage of digestion. At 100 min of digestion, no intact casein brands can be observed, and whey protein brands were getting faint. At 120 min, the pepsin band appeared and increased the intensity at 160 min. None of the casein or whey protein bands could be observed at 160 min of digestion. It is well known that whey protein in the native structure is less sensitive to pepsin, especially β-LG (Li et al., 2021). β-LG resists to be hydrolysed by pepsin and remains soluble in the stomach then pass into the intestine. Only denaturing the whey proteins by pre-treatment such as heating treatment could lead to aggregations and hydrolyse by pepsin (Brodkorb et al., 2016). As a result, the different protein hydrolysis patterns between sheep infant formula and the other two formulae could be due to the milk source of three samples having been treated in different processes, which lead to denaturation of the whey proteins in sheep infant formula.



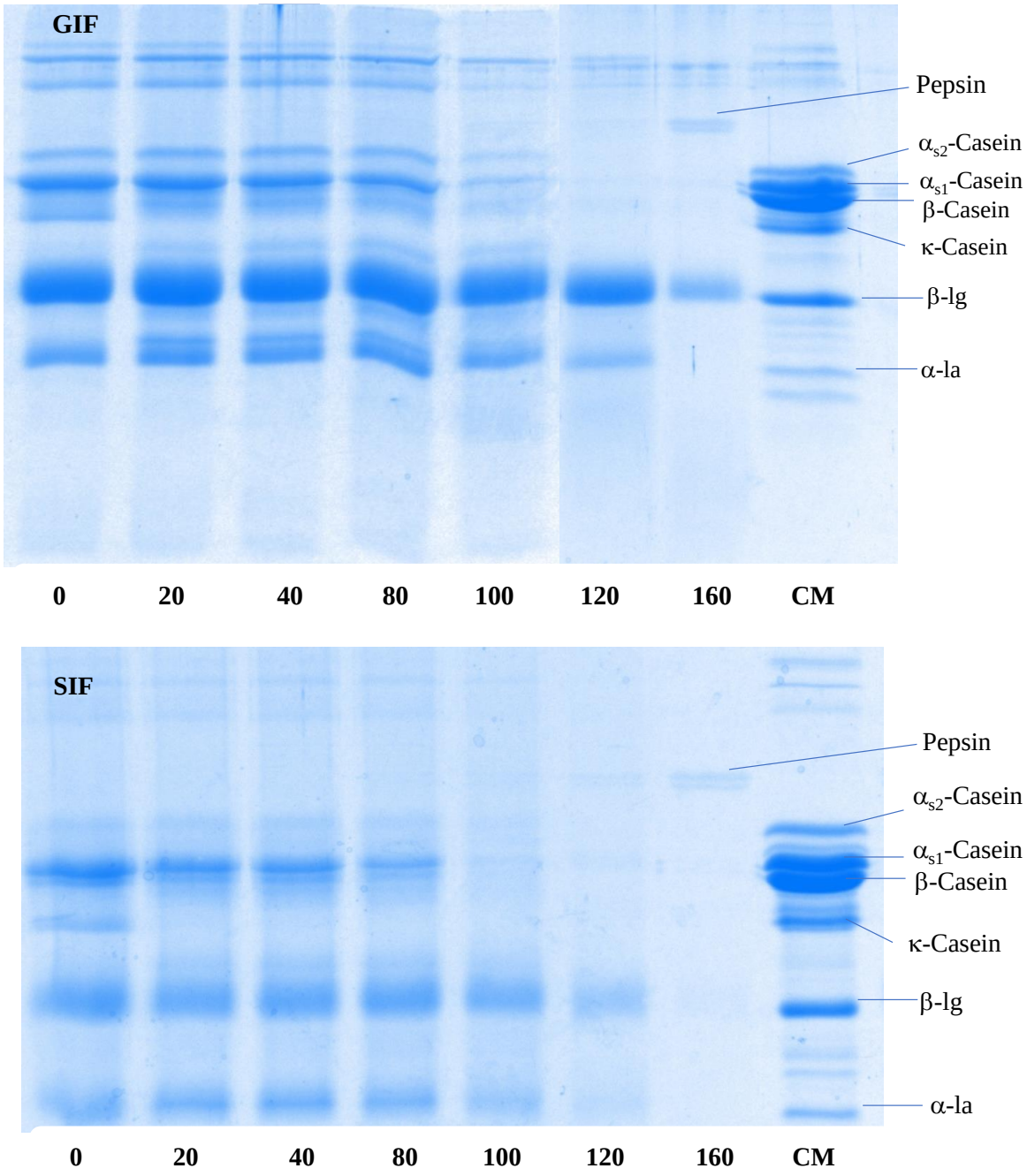


Figure 4.9. SDS-PAGE pattern under reducing conditions of emptied digesta obtained from CIF, GIF, and SIF during gastric digestion (SGF with pepsin) in HGS at different times

4.3 Discussion

The observation of the present experiment showed that there was no firm clot formed in sheep, goat, or cow infant formulae during the gastric digestion in the infant human gastric simulator.

However, the different structures of aggregates in chyme were observed. SIF trended to form smooth oil droplets with fewer protein networks. GIF formed irregular aggregates with porous and coarse textures, whereas CIF exhibited dense protein networks. The different structures among three infant formulae chyme could be attributed to the differences in their protein compositions, especially the different constituents of caseins. As the SDS-PAGE examined the protein compositions of three infant formulae, GIF and SIF contained higher β-casein and lower α_S-casein, while CIF contained a nearly similar amount of β-casein and α_S-casein. The SDS-PAGE patterns were in agreement with the previous studies of the comparative of milk from various species (Raynal. Ljutovac et al., 2008; Roy et al., 2021; Wendorff & Haenlein, 2017; Ye, Cui, et al., 2019).

According to the review of Roy et al. (2020a), the formation of the partial and friable aggregates could be related to the β-casein-to-α_S-casein ratio in milk. Li and Nakai (1988) modified casein by rennin to increase the β-casein fraction relative to α_{S1}-casein in bovine milk to match the casein in human milk. In the *vitro* study of acid coagulation of caseins, the results showed that the rennin-modified cattle milk, which contains a higher β-casein-to-α_S-casein ratio, had a similar coagulation behaviour to human milk. Under the conditions of pH 2 and pH 4, rennin-modified casein formed looser and less structured clumps, whereas the coagulum of bovine casein was large and dense. In addition, the previous studies of *in vitro* digestibility of skimmed cow, goat, and sheep milk reported that the non-cattle milk, especially goat milk proteins were higher susceptibility to pepsin due to the observation of faster hydrolysis and a faster generated amino group (Hodgkinson et al., 2018; Rutella et al., 2016; Tagliazucchi et al., 2018; Ye, Cui, et al., 2019). This was in a line with the findings of the present experiment in protein hydrolysis of three infant formulae. The observed of SDS-PAGE patterns indicated that the casein hydrolysis in GIF was faster than SIF and CIF. In conclusion, the different protein compositions of cow, goat and sheep infant formulae have highly related to the structure of aggregation formation in the stomach. The moderate and friable structure in aggregates could influence the rate of protein hydrolysis and digestion behaviour.

However, there were few differences in emptied digesta of three infant formulae have been detected. This indicate that the digestion of protein delivered to intestine could be similar. The result aligned with the study of Maathuis et al. (2017). They have reported that GIF and human

milk showed fasted protein digestion compared to CIF in the early stage of digestion but slower at the end stage, which may be due to their different protein composition related to different clotting behaviours in stomach led to different gastric emptying rate. However, by analysed the comparative of bioaccessible nitrogen and amino acids, the results suggested the protein qualities and digestinilities of GIF, CIF and human milk were similar. Another reason could be the infant formulae in the present study are whey dominated products, and the reconstituted milk were in low protein concentration by following the feeding instruction (1.565% w/w). The low protein concentration of whey-protein dominated infant formulae leads to a low absolute casein concentration under gastric conditions. In this case, the different aggregate structures may only be obtained in the chyme during digestion, and the differences of emptied digesta can only be observed in the late stage of digestion where the pH decreased to below the casein isoelectric point 4.6 (McSweeney & Fox, 2013).

4.4 Conclusion

This chapter was to investigate the influence of protein compositions on simulated gastric digestion behaviour. From the result of the SDS-PAGE analysis, three commercial infant formulae are whey protein dominate infant formulae. In addition, the protein compositions and ratios of cow, goat and sheep infant formulae were different, especially the casein proportions. There was little α_{S1}-casein in goat and sheep infant formulae, but it was abundant in CIF. α_{S2}-casein take a big portion in GIF but lacking in cow and sheep infant formulae. The contents of β-casein were higher in GIF and SIF, but lower in CIF. The different casein compositions were aligned with that in raw milk reported in the previous studies (Alichanidis et al., 2016; Balthazar et al., 2017; Claeys et al., 2014; Park et al., 2006; Raynal. Ljutovac et al., 2008; Wendorff & Haenlein, 2017).

An infant human gastric simulator (IHGS) was used to investigate the in vitro dynamic gastric digestion. During the simulated gastric digestion, enzymes and acidic gastric fluid were added to the IHGS. The chyme and empty digesta of each infant formulae at different times were collected and investigated. No firm curd formed in the three samples. However, the observations of confocal laser scanning microscopy (CLSM) images indicated that the aggregation behaviours of the three sample chyme were different in the stomach. CIF formed

dense protein networks trapped oil droplets, whereas GIF showed irregular aggregates in an open and porous structure. SIF chyme formed a different structure compared to CIF and GIF – smooth oil droplets were surrounded by fewer protein networks. The observations were supported by the particle size results of three sample chyme. Moreover, the particle size distributions indicated that the initial aggregation of GIF started earlier than CIF, followed by SIF. The different aggregation behaviour and the structures could be related to the different protein compositions in three infant formulae, especially the different proportions of casein (Inglingstad et al., 2010; Li & Nakai, 1988; Roy et al., 2020a).

The pH, particle sizes, protein and fat contents of three infant formulae's empty digesta were also investigated as functions of time in the first part of this study. The particle size results showed that in the early ~100 mins of digestion, there were no significant differences among the three samples ($P > 0.05$). From 140 min of the digestion, the $d_{4,3}$ value of GIF empty digesta increased faster than that of the other two samples. At the end of the digestion, the $d_{4,3}$ values of empty digesta were GIF> CIF> SIF. Similarly, the fat contents of three infant formulae followed the same trend till 140 min. At the end of the digestion, the fat content of GIF was higher than CIF, followed by SIF. Moreover, the pH and protein contents of three infant formulae followed similar trends during the 180 min digestion ($P > 0.05$). These results suggested that the impacts of different protein compositions of three infant formulae could be subtle on the emptied digesta, and influence only at the late stage of digestion. This could be ascribed to the milk samples in low protein concentrations by following the feeding instructions (1.565%w/w). The lower casein content contributes to lower protein aggregation.

Furthermore, the observations of three infant formula protein hydrolysis indicated that GIF and SIF casein were hydrolysed faster than CIF, and the whey protein of SIF showed a different pattern than the other samples. The results indicated that the different aggregation structures of infant formulae could relate to the different protein digestion behaviours. The friable and porous structure of GIF aggregates could lead pepsin easier to access and hydrolyse protein. This was in a line with the finding in the previous studies (Hodgkinson et al., 2018; Rutella et al., 2016; Tagliazucchi et al., 2018; Ye, Cui, et al., 2019). In conclusion, the most obvious finding to emerge from this chapter is that the different protein compositions in cow, goat and sheep infant formulae had an impact on the gastric aggregation behaviour, in such effect the

digestion behaviour. However, the digestion behaviour is not only affected by the protein compositions but also influenced by the processing such as heat treatment. Further studies on the effect of heat treatment on the digestion behaviour of IF are demonstrated in the following chapter.

Chapter 5: Effect of Heat Treatment in Simulated Gastric Digestion Behaviour of Commercial Infant Formulae

5.1 Introduction

In the previous chapter, the influence of protein compositions in cow, goat and sheep infant formulae on infant gastric digestion was investigated under a dynamic *in vitro* digestion model. It has been reported that the different manufacturing processing, especially the heat treatment of infant formula could influence the structure of whey protein, which could impact the aggregation behaviour. Therefore, the objective of the experiment in this chapter was to compare the gastric digestion behaviour of heated samples with unheated samples in regard to the pH, protein and fat contents, particle size and protein hydrolysis of the empty digest as functions of digestion time, as well as the particle sizes and microstructure of chyme in the three infant formulae.

Thermal treatment is the most common processing method in dairy manufacture, which includes pasteurization (typically 72 – 75°C, 15 – the 20s), UHT (135 – 140°C, a few seconds) and extensive thermal treatment (115 – 120°C, 20 – 30min) (Montagne et al., 2009). In infant formula manufacturing, the liquid milk undergoes spray drying, which is usually at 70 - 80°C (Montagne et al., 2009). Heating milk above 100°C could induce protein physicochemical changes such as micelle aggregation and casein dephosphorylation (Brodkorb et al., 2016; Li et al., 2021; Miranda & Pelissier, 1987). The heating process leads to the variation of milk protein structural and physicochemical properties, which impacts the digestion of proteins.

Milk protein is split into casein and whey protein, two categories of model proteins, which can be used to investigate the rate of protein digestion and absorption. Boirie et al. (1997) defined casein as a slow protein while whey protein as a fast protein due to their different gastric emptying speed. Casein aggregates to form clots under gastric conditions and has a slower gastric emptying process (He & Giuseppin, 2014). Individual caseins and the casein micelles have different coagulation behaviours. Under gastric conditions, the individual caseins or

sodium caseinate, which is a compound derived from casein, only aggregate by acidification, when pH drops under casein isoelectric point 4.6 (Fox & McSweeney, 2013). However, casein micelles could be coagulated by low pH and proteases such as pepsin or chymosin, and the previous treatments (e.g. heat treatment and homogenization) could impact the structure of coagulation (Ye et al., 2017). Casein micelles are described as colloidal hard spheres covered by κ-casein as a *hair layer* protecting micelles from aggregations by providing electrostatic and steric interactions. κ-Casein plays an important role in the casein micelle stabilization (Alexander et al., 2002; Ye, 2021).

On the contrary, whey protein is classified as a fast protein, which remains soluble in the stomach and rapidly pass into the small intestine as it is resistant to protein hydrolysis by pepsin (He & Giuseppin, 2014). It is well reported that heat treatment around 70 °C leads to whey protein denaturation (Brodkorb et al., 2016; Li et al., 2021; Ye, Liu, et al., 2019). In the native structure, β-lactoglobulin (β-LG) is a globular molecule that hides the pepsin hydrolysis cleavage sites. Heat treatment could induce β-LG unfolding and expose a free thiol group that initiate disulphide bond aggregations between adjacent whey proteins and casein micelles to whey proteins (McSweeney & Fox, 2013). The heat-induced conformational changes could lead to β-LG being more susceptible to hydrolysis by pepsin. α-lactalbumin can be hydrolysed by pepsin when the pH decreased under 4 (Kuwajima, 1977).

Several studies have investigated the impact of heat treatment on the denaturation and interactions of whey protein (Brodkorb et al., 2016; Ju & Kilara, 1998), and heat treatment inducted the different structures of clots formed during gastric digestion (Gallier et al., 2013; Miranda & Pelissier, 1987). Recently, Ye et al. (2016b) reported the clot of heated and unheated skim milk was observed in different structures under gastric digestion. The clots formed by heated milk showed more open knitted networks with larger pores. In addition, Pan et al. (2021) investigated the heated and homogenized sheep milk formed a more fragile and looser structure than the untreated milk.

However, the previous studies were carried out in fresh milk, there was insufficient experiments on infant formulae. The objective of this chapter was to compare the aggregation behaviour of heat treated (90°C for 5 min) cow, goat and sheep based infant formulae with their unheated

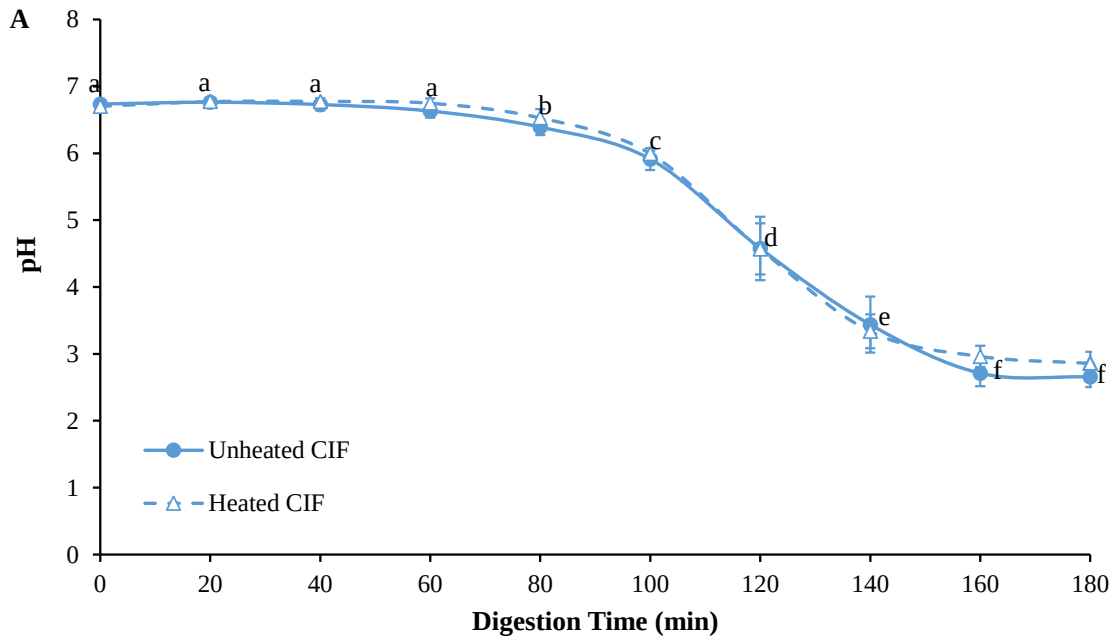
milk under gastric conditions. The results revealed the heat treatment highly impacted on the structure of aggregates in chyme, but little differences of empty digesta were observed at the end stage of the gastric digestion.

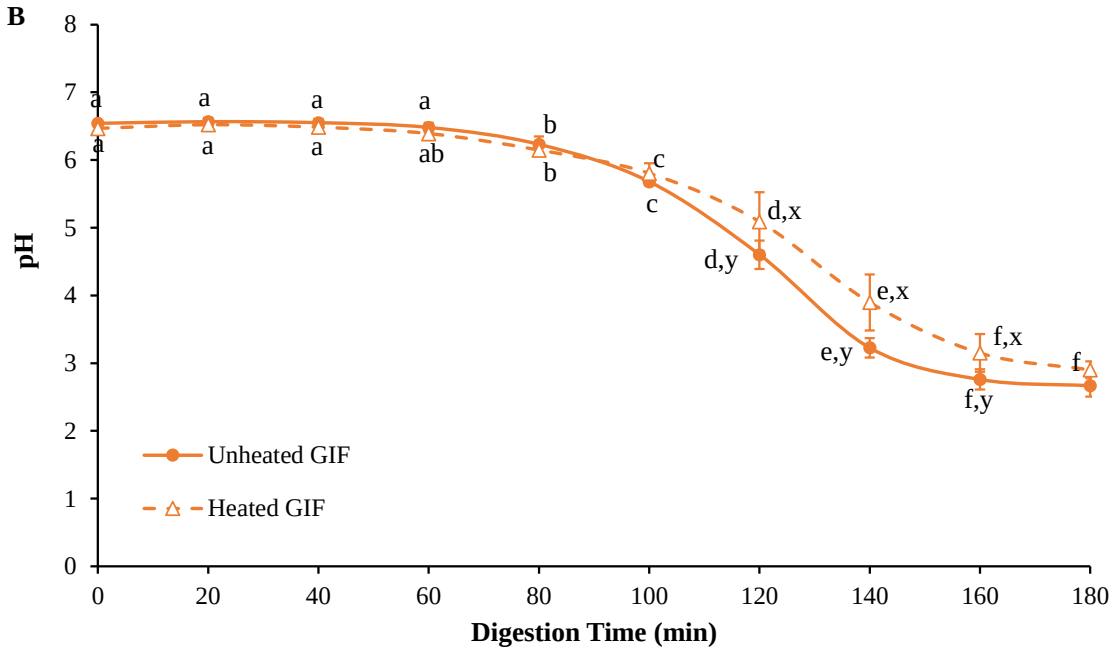
5.2 Results

5.2.1 pH profiles *in vitro* dynamic gastric digestion

Comparing the heat-treated (90°C, 5min) samples with unheated ones, from Figures 5.1 shows that the pH of heat-treated CIF had no significant difference to the unheated CIF ($P > 0.05$). However, heated GIF showed higher pH at 120 – 160 min than unheated GIF ($P < 0.05$) which indicated a slow decrease of heated GIF pH with digestion time. The heated sample was 5.80 ± 0.15 at 100 min gradual decreased to 2.90 ± 0.13 at end of the digestion, while the unheated sample declined from 5.68 ± 0.05 to 2.67 ± 0.16 in the same period.

Similarly, the pH profiles of heated SIF were higher than unheated SIF at 120 – 140 min ($P < 0.05$). In the period of 120 – 180 min, heated SIF dropped from 4.85 ± 0.11 to 2.87 ± 0.17, while unheated SIF decreased from 4.20 ± 0.19 to 2.59 ± 0.23. The overall pH profile of heated SIF was decrease slower than unheated SIF. This result was aligned with the study of comparing of sheep whole milk with heated homogenized sheep milk (Pan et al., 2021). The slow decrease in pH of heated milk could relate to the denature of whey proteins and formed more open structure aggregates (Ye et al., 2016b).


A
pH
Digestion Time (min)
Unheated CIF
Heated CIF
a
a
a
a
b
c
d
e
f
f


B
pH
Digestion Time (min)
Unheated GIF
Heated GIF
a
a
a
a
b
c
d,y
e,y
f,y
a
a
a
ab
b
c
d,x
e,x
f,x
f

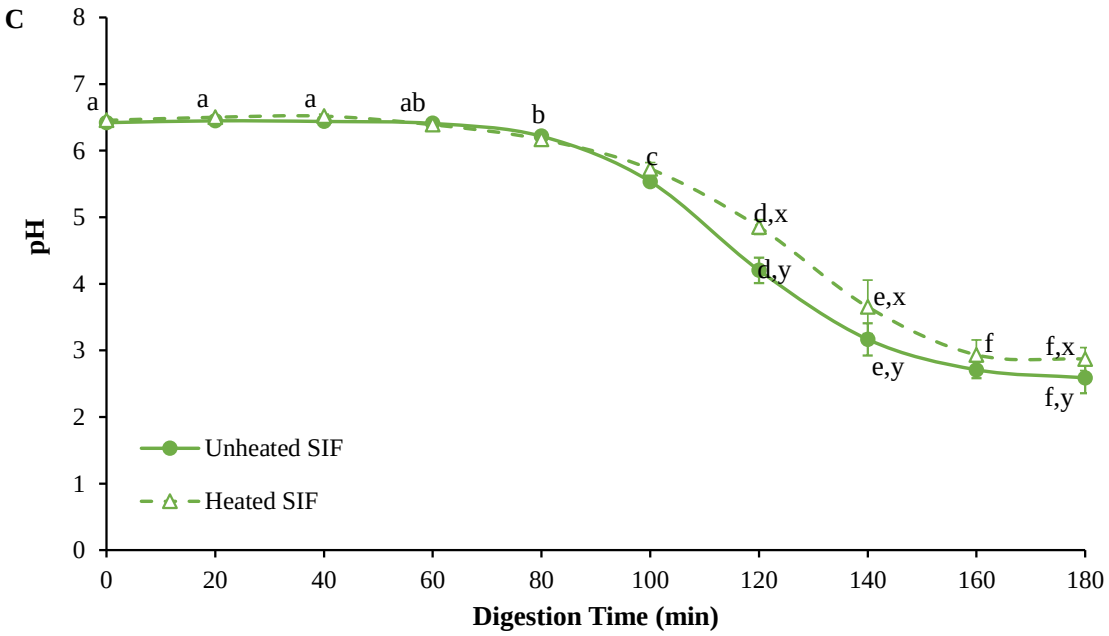


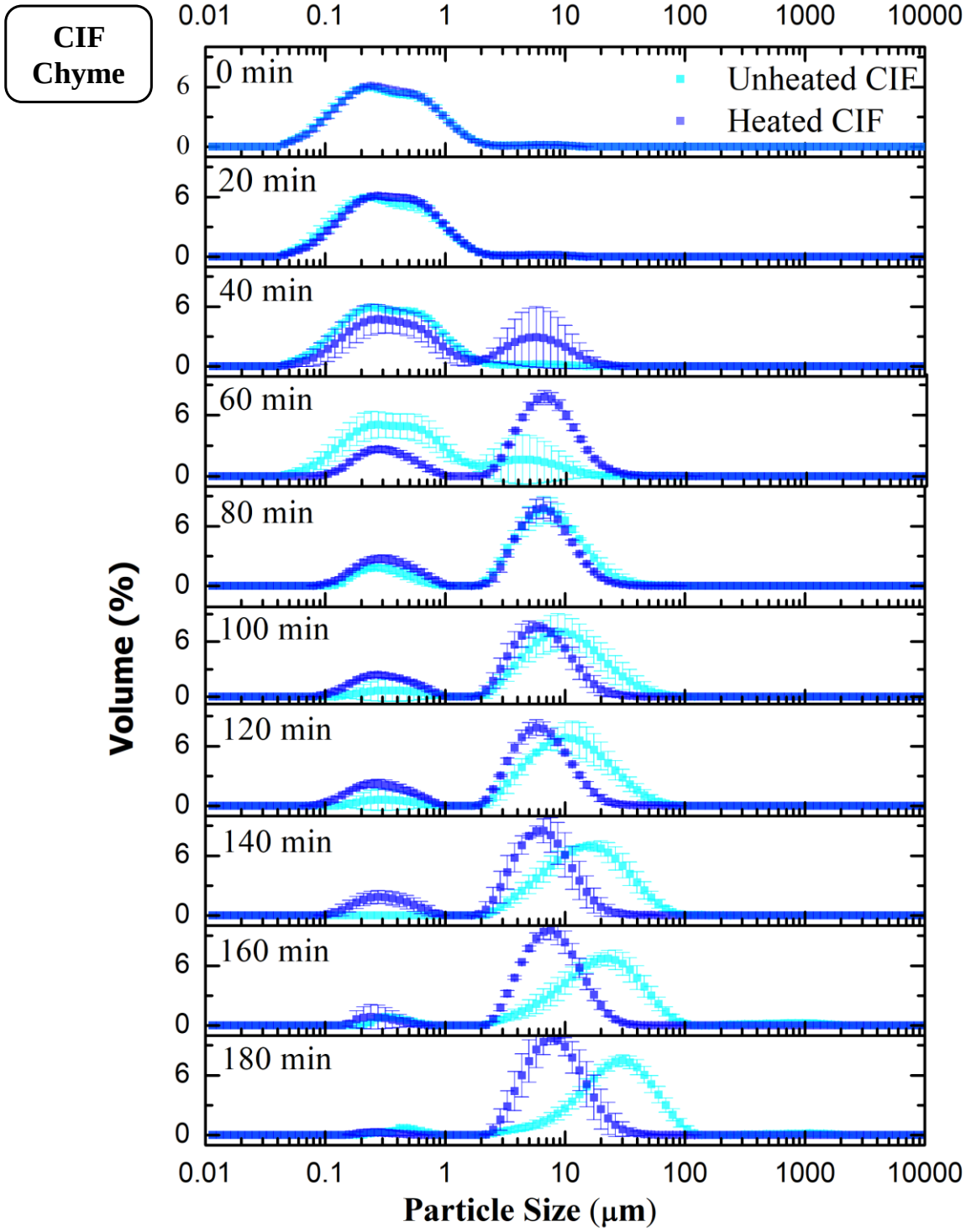
Figure 5.1. Comparation of pH profiles change during the gastric digestion of heated and unheated cow (A), goat (B), and sheep (C) milk infant formula with 1.565% (w/w) protein

5.2.2 Changes in particle size distribution of chyme

For cow milk infant formula chyme, both heated and unheated samples had a monomodal distribution of particle size and remained the same pattern in the first 20 min of digestion (Figure 5.2). However, the size distribution of the heated sample shifted to a larger size region at 40 min, while the unheated sample did not change significantly at the early stage. When the sample chyme was digested from 60 min to 180 min, both heated and unheated samples showed bimodal patterns. The pattern of the heated sample had a big peak near 5 μm and a small peak around 0.3 μm at 60 min, the big peak slightly increased while the small peak decreased with the time of digestion.

Regarding the unheated CIF chyme, at 60 min, it showed a bimodal pattern with a major peak near 0.3 μm and a tail in the range of 1 - 11 μm. Then the peak moved to the large size region, almost coinciding with the heated pattern at 80 min. After that, the distribution gradually shifted to the right till the end of the digestion. The different trends were also found in the distribution of chyme in SDS and EDTA solution (Figure 5.2). The particle size of oil droplets in heated CIF almost remained as a monomodal pattern with a peak in the range of 0.1 – 2 μm. By

contrast, the particle size of unheated chyme in SDS and EDTA solution shifted to the larger size range, which was ascribed to oil droplets coalescence. Overall, the observation indicated that heated CIF had an earlier initial aggregation than the unheated sample, and the particle size increased rapidly in the early stage of digestion. However, there were subtle changes been observed in heated CIF after 80 min till the end of digestion. The difference in the aggregation behaviour could be ascribed to the heat treatment induced by the denaturation of whey proteins (Anema & Li, 2003; Ye et al., 2017).


CIF
Chyme
0 min
20 min
40 min
60 min
80 min
100 min
120 min
140 min
160 min
180 min
Unheated CIF
Heated CIF
Volume (%)
Particle Size (μm)
0.01
0.1
1
10
100
1000
10000
0
6

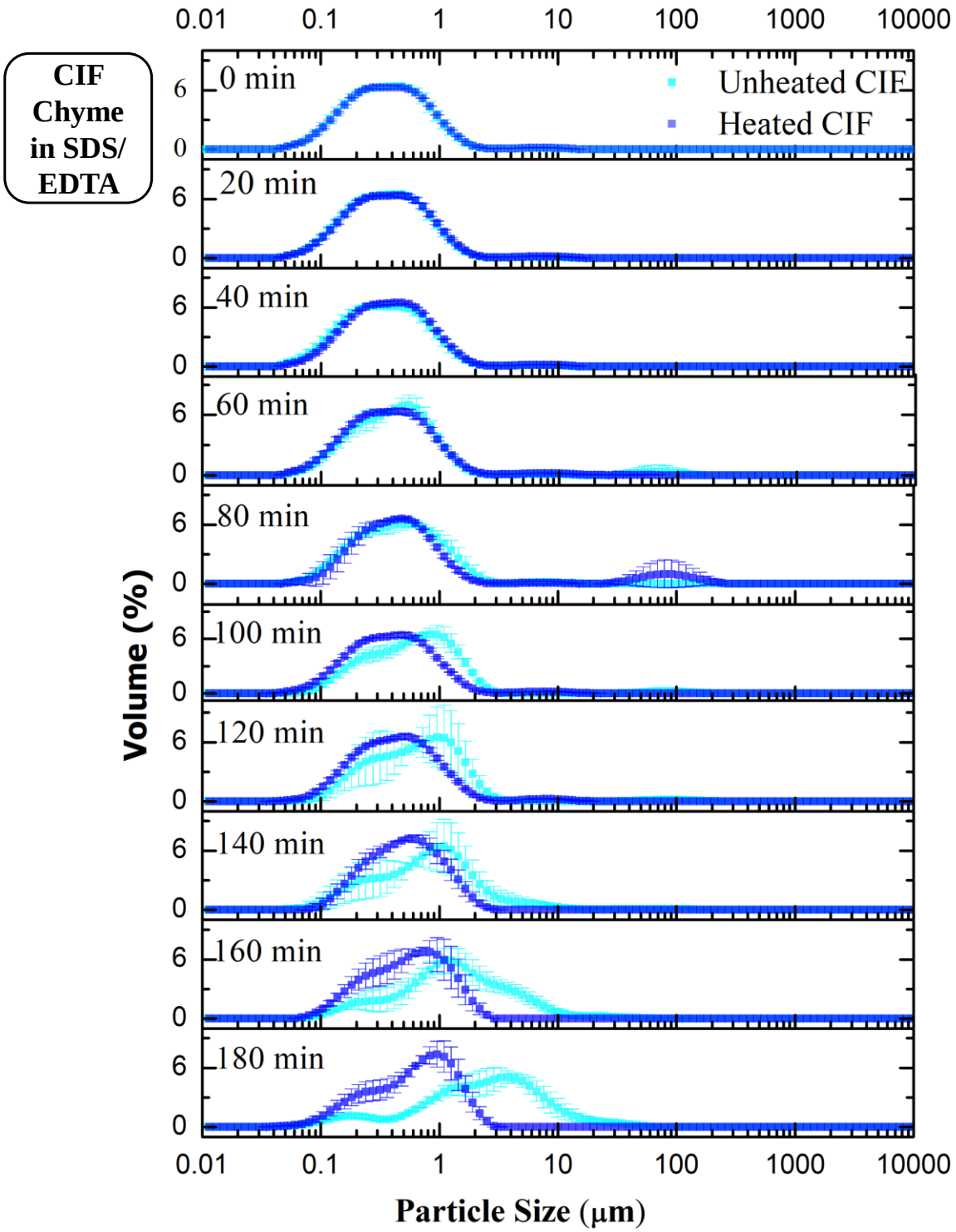
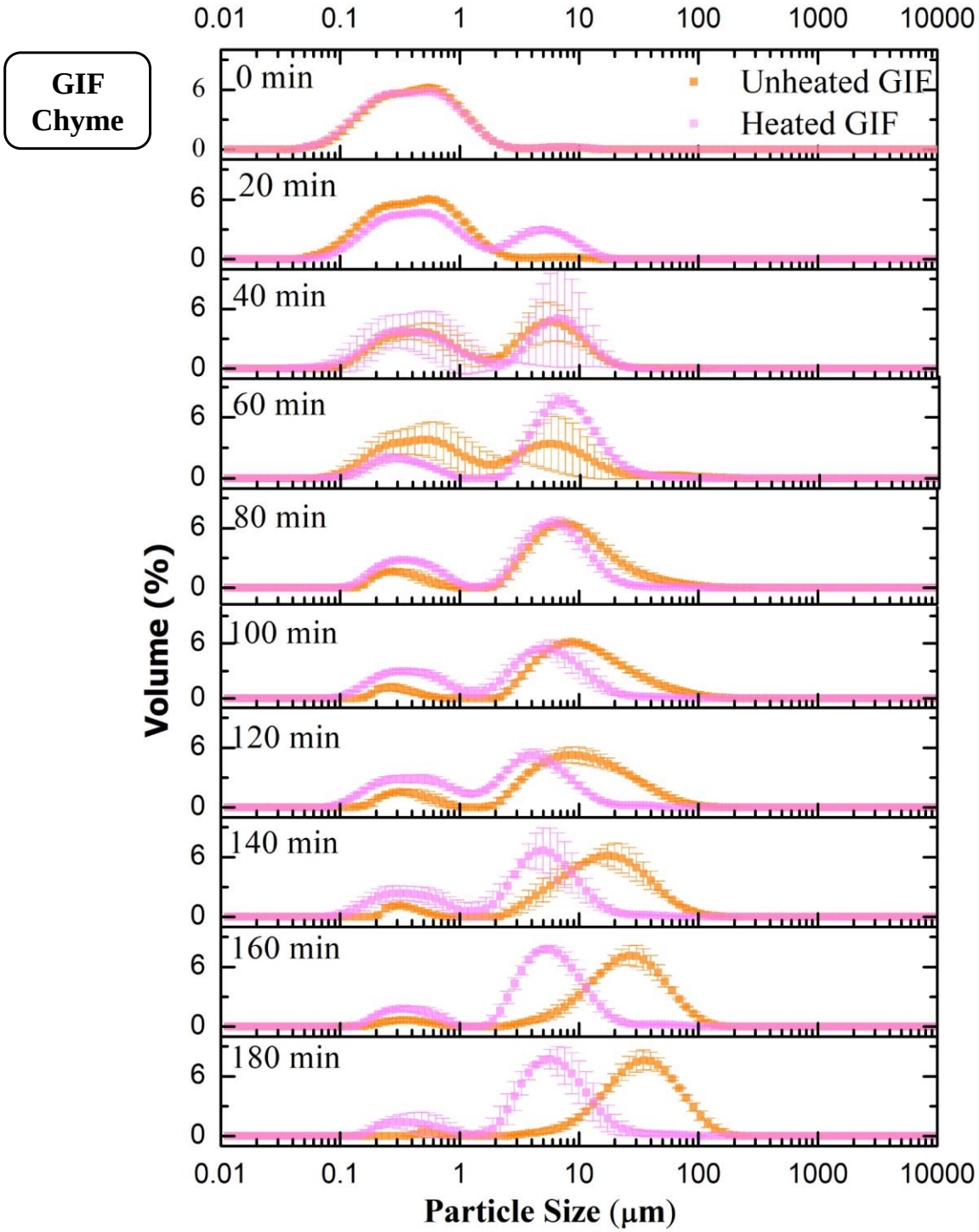


Figure 5.2. Comparation of the particle size distribution of heated and unheated cow milk infant formula chyme and the chyme in SDS and EDTA solution

As figure 5.3 shows, the comparative particle size distribution of heated and unheated goat milk infant formula showed similar trends as heated and unheated CIF with a few exceptions. The distribution of heated GIF changed to a bimodal pattern at 20 min with a major peak in the range of 0.1 – 1 μm, then the major park shifted to the range of 1.1 – 11 μm at 60 min and remained at the same level. The particle size distribution of the unheated chyme appeared in a bimodal pattern at 40 min, which almost overlapped with heated GIF chyme. After that, the big peak gradually shifted to the large range, located in the range of around 10 – 100 μm at the end of digestion.

The distributions of GIF chyme in the SDS and EDTA solution (Figure 5.3) showed similar patterns as CIF, in that the heated chyme in SDS and EDTA solution remained at the same ranges during digestion (0.1 – 2 μm). By contrast, unheated chyme in SDS and EDTA solution gradually shifted to the large size range with the time of digestion. The observation indicated that heated GIF had earlier initial aggregation than unheated GIF, but the aggregation increase rate was slower than the unheated GIF. Also, it was nearly no oil coalescent occurred in heated GIF, while obvious oil droplet size increased in unheated GIF.


GIF Chyme
0.01
0.1
1
10
100
1000
10000
0 min
20 min
40 min
60 min
80 min
100 min
120 min
140 min
160 min
180 min
Unheated GIF
Heated GIF
Volume (%)
Particle Size (μm)

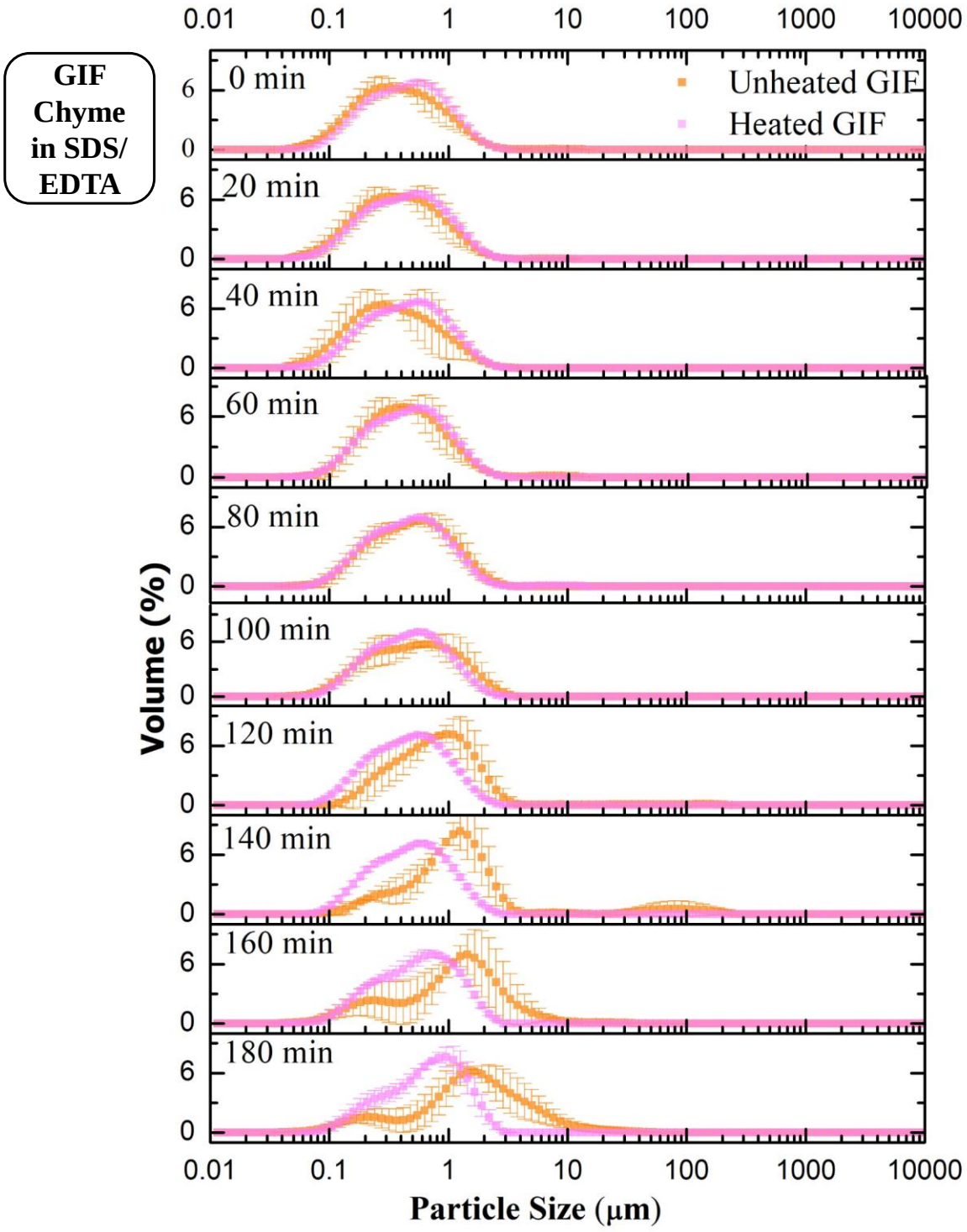
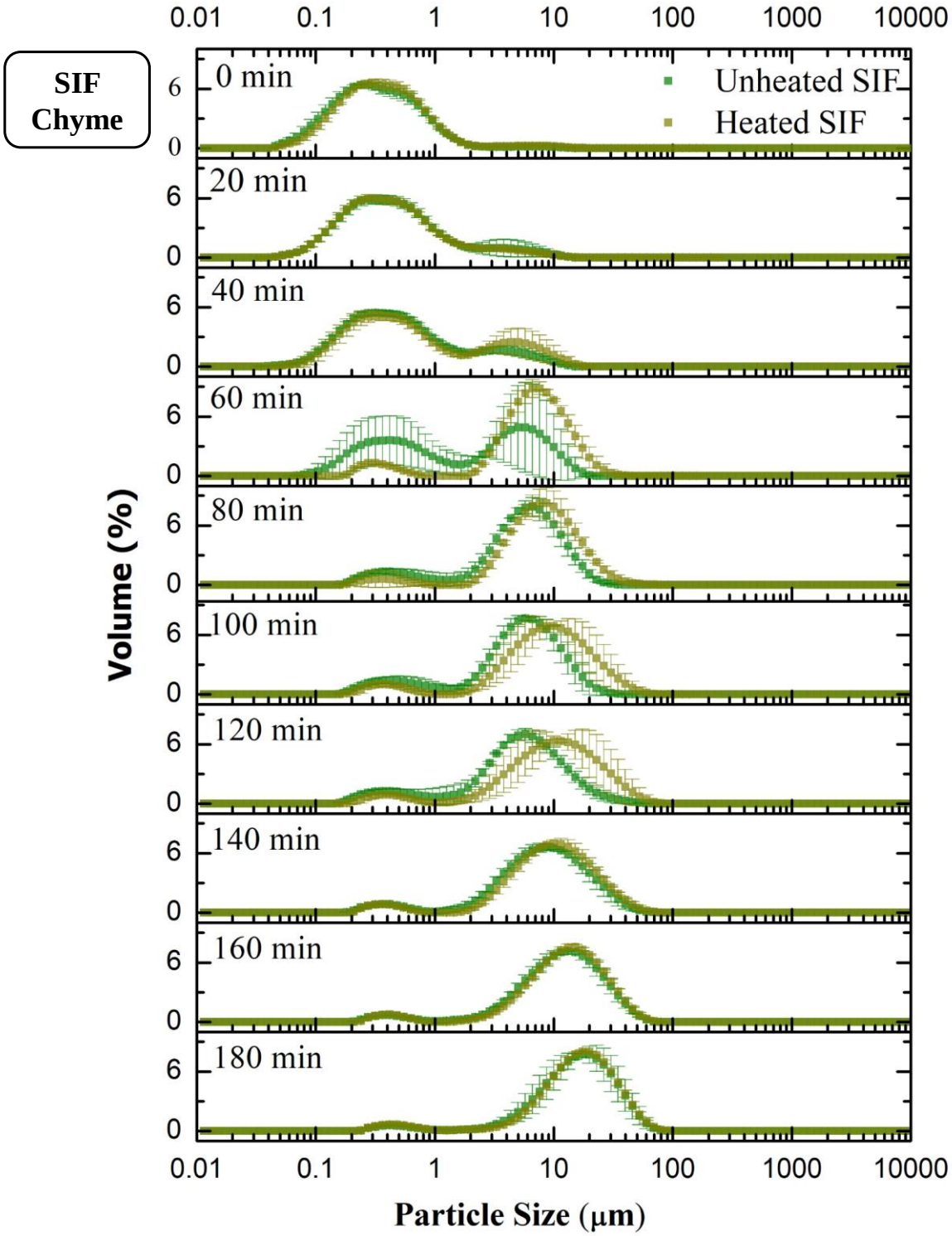


Figure 5.3. Comparation of the particle size distribution of heated and unheated goat milk infant formula chyme and the chyme in SDS and EDTA solution

In contrast with CIF and GIF, the compositions of the particle size distribution of heated and unheated SIF chyme and the chyme in SDS and EDTA solution exhibited different trends. As Figure 5.4 shows, both heated and unheated SIF started appearing in small tails in the range of 2 – 10 μm at 20 min, the peaks shifted to the right with the time of digestion. At 80 min, both distributions showed bimodal patterns with a major peak located around 8 μm, heated SIF moved toward big size range slightly faster than unheated SIF. With further digestion from 140 to 180 min, the distributions of both samples nearly overlapped. Likewise, both heated and unheated SIF chyme in SDS and EDTA solution showed monomodal, with peaks gradually shifting to the right side. However, unheated SIF is located in a slightly larger size range than heated SIF. The particle size increase of both heated and unheated SIF could be ascribed to the oil coalescence. The different coagulation behaviours of heated SIF compared to CIF and GIF may be attributed to different manufacturing processing of SIF, which may affect the oil droplets trapped within a different protein matrix.


SIF Chyme
0.01
0.1
1
10
100
1000
10000
0 min
20 min
40 min
60 min
80 min
100 min
120 min
140 min
160 min
180 min
Unheated SIF
Heated SIF
Volume (%)
Particle Size (μm)

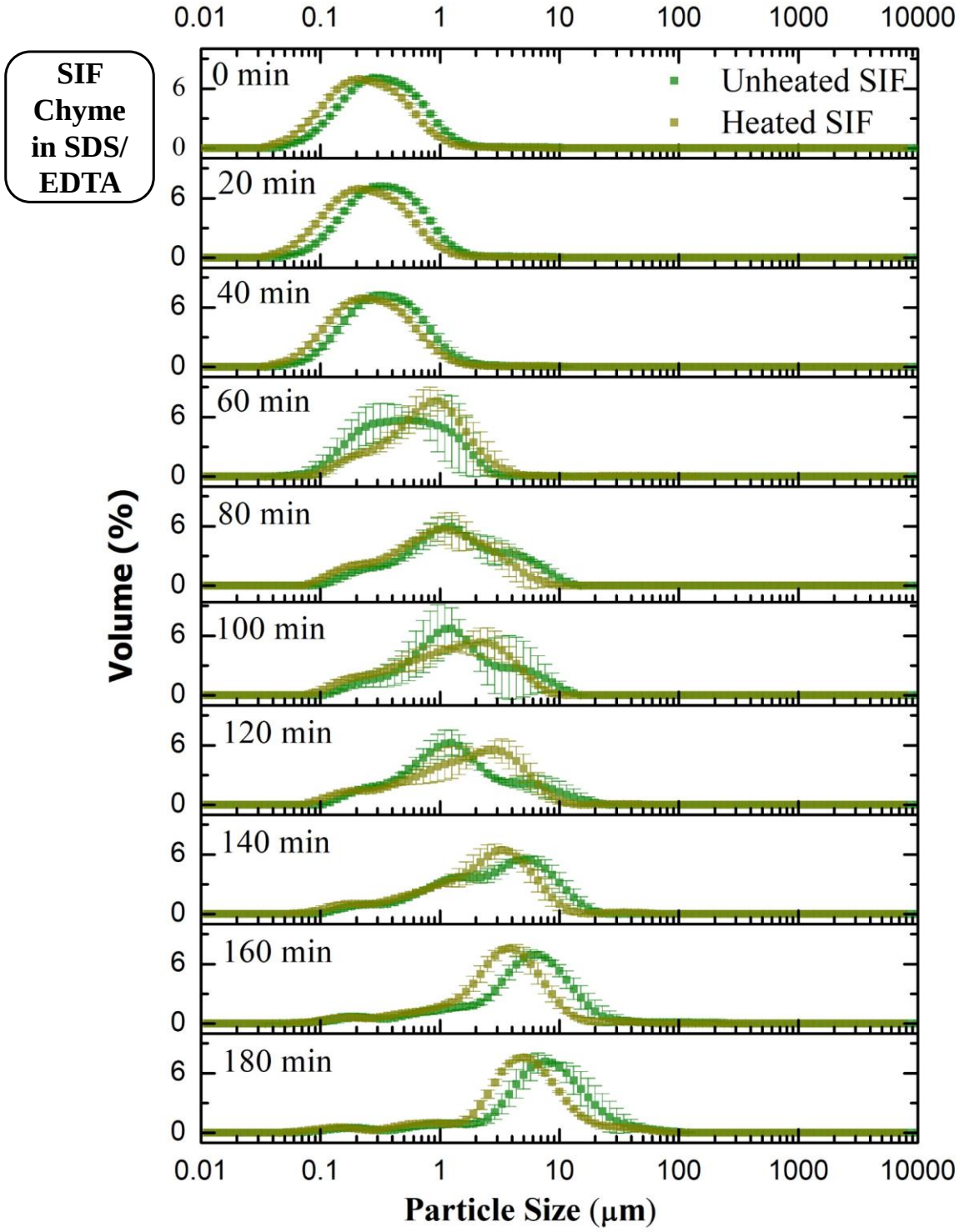


Figure 5.4. Comparation of the particle size distribution of heated and unheated sheep milk infant formula chyme and the chyme in SDS and EDTA solution

5.2.3 Microstructure of chyme

As shown in Figure 5.5, heat-treated cow and goat milk infant formulae have different structures compared to the unheated ones. There was a small number of aggregations observed in heated CIF and GIF at 80, 100 and 180 min with dense and porous structure, and the sizes of aggregates were smaller than unheated chyme. The more open structures in heated CIF and GIF were in a line with the previous study of whole bovine milk. Ye et al. (2017) found that the clot structures of the heated sample contained more open pores. Moreover, no oil droplet coalescence was observed in heated CIF or GIF. The observations of chyme microstructures were in line with the chyme particle size distributions (Figure 5.2 – 5.3). The particle size values of heated CIF and GIF went up from 0 min to 80 min. After that, there were slightly growth at 100 and 180 min. However, subtle increases in fat globules have been observed. Conversely, both particle size of aggregations and fat globules in unheated CIF and GIF chyme were increased. The difference between heat and unheated CIF and GIF could be indicative of the whey protein denaturation and unfolding explode the cleavage sites that allow whey protein participant in the aggregation (Anema & Li, 2003).

On the contrary, there was no significant difference observed between heat and unheated treated SIF. Both samples showed uniformly dispersed droplets at 0 min. Then the aggregations were observed at 80 minutes. The oil droplets merged into larger round shape droplets, embedded in the protein flocculation. At 100 min of digestion time, the sizes of oil droplets and protein aggregates increased and increased towards the largest size at the end of digestion. The observations were in agreement with the particle size distributions of heated and unheated SIF (Figure 5.4). Both patterns were moved to the large size range by the time of digestion. The aggregates of the unheated sample were showed smaller sizes at 80 and 100 min compared to the heated chyme, then reached similar sizes at 180 min. The fat globules particle size distributions of both heated and unheated SIF chyme followed the same trends. The size increased by the time of digestion and reached the largest size at 180 min.

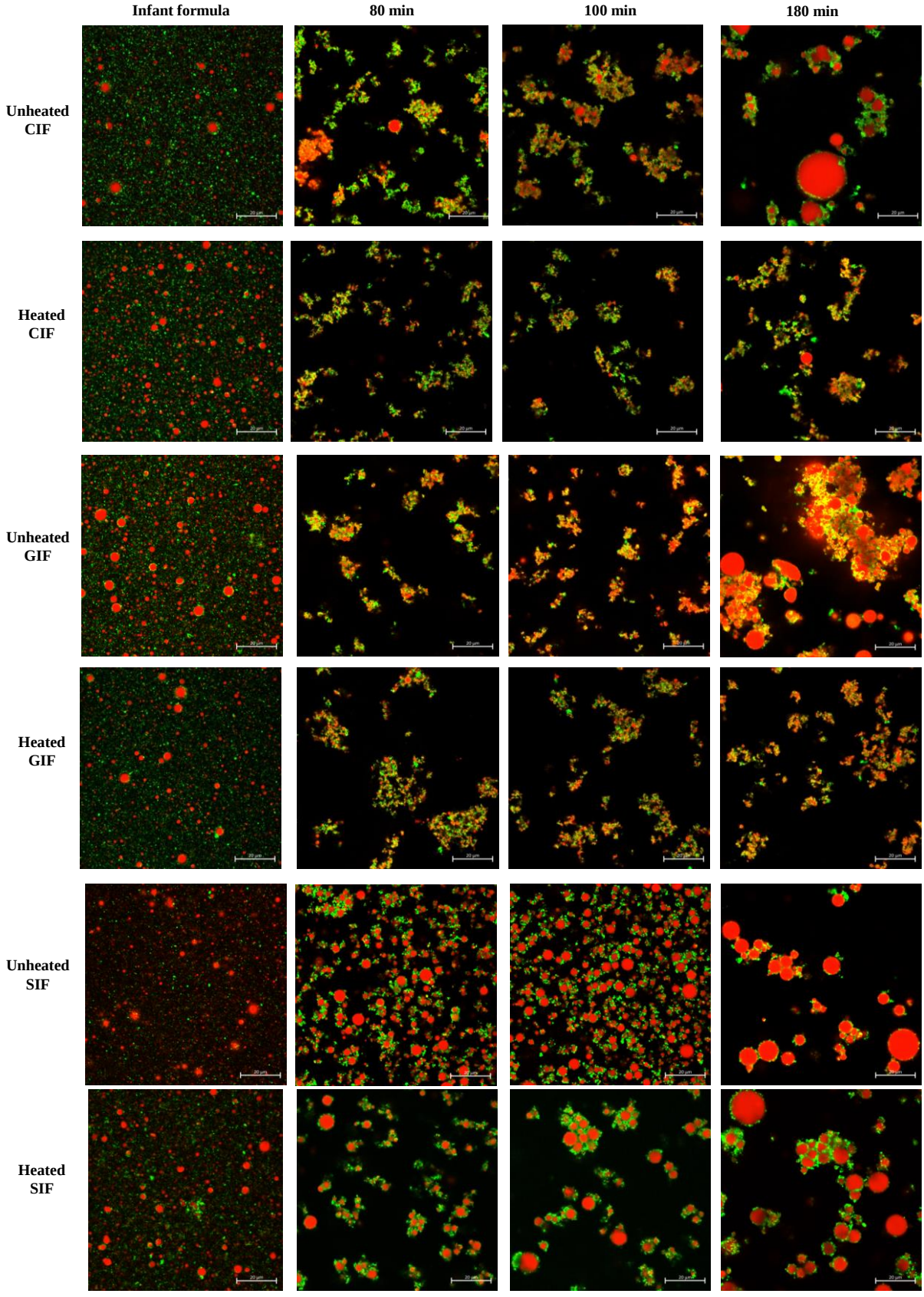
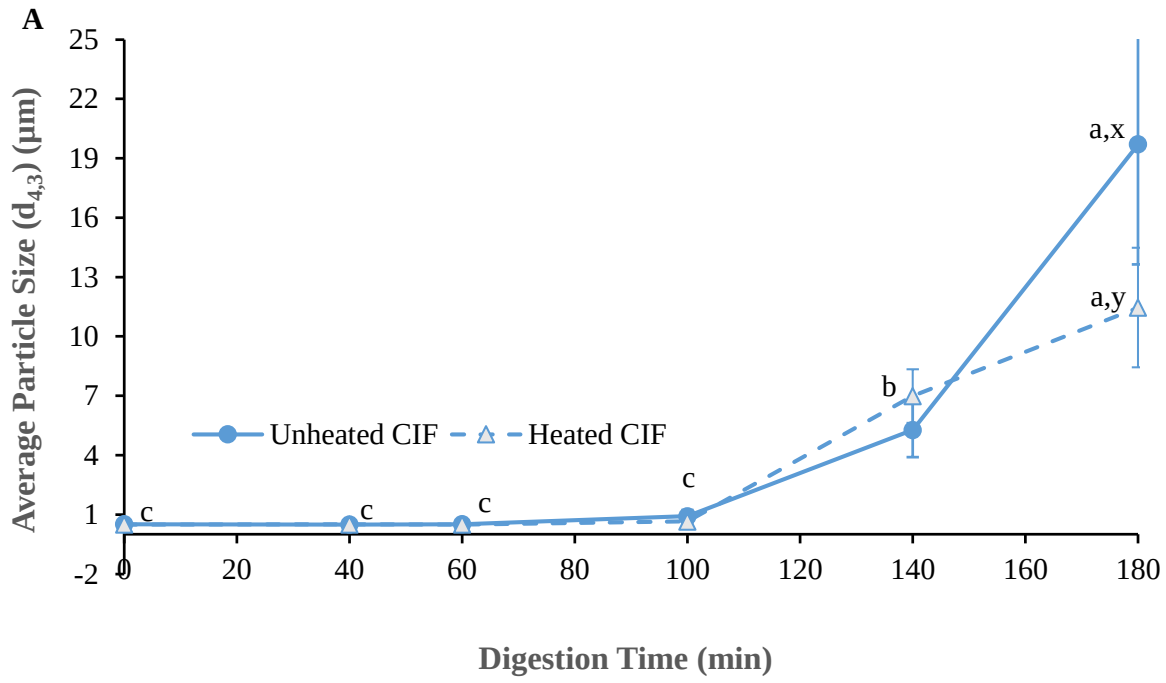


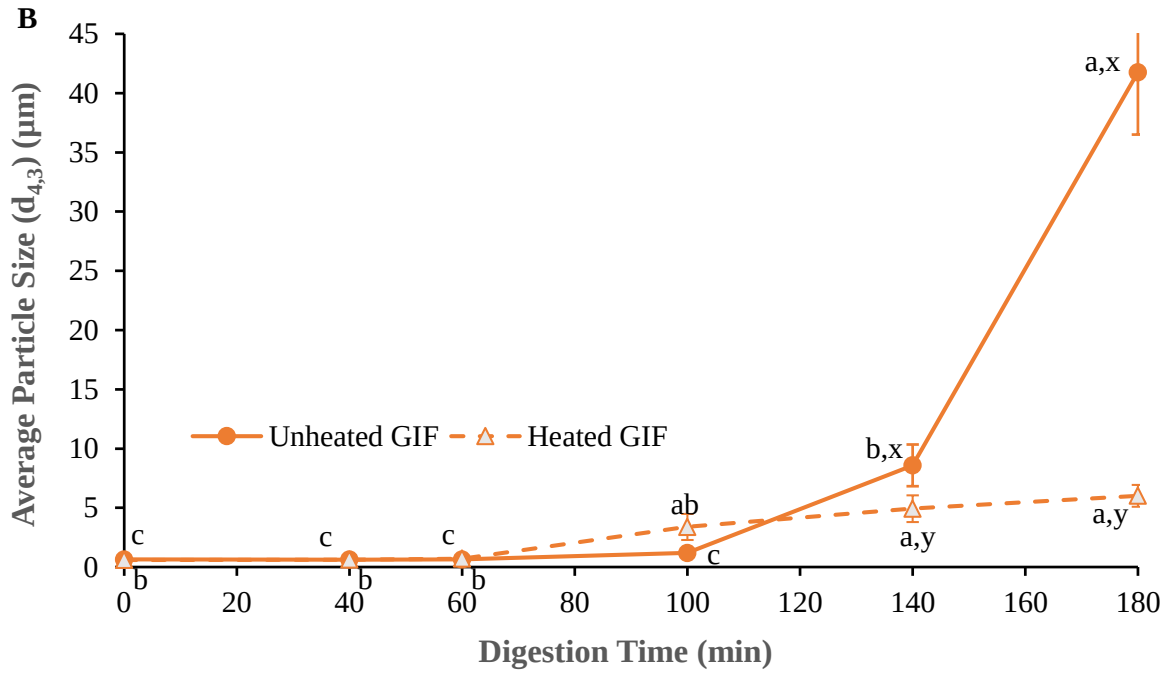
Figure 5.5. Comparison of Confocal laser scanning microscopy (CLSM) images of heated

and unheated CIF, GIF and SIF chyme. Scale bars represent 20μm.

5.2.4 The physical characteristic of gastric emptied digesta changes in particle size of emptied digesta

Unlike the chyme, there were only subtle differences in particle size between heat and unheated emptied digesta during digestion. As Figure 5.6 A shows the volume-weighted average diameter ($d_{4,3}$) of heated CIF emptied digesta followed the same trend as unheated CIF, except the unheated CIF increased sharply at 180 min ($P < 0.05$). The average size was 19.71 ± 6.08 μm versus 11.46 ± 3.01 μm for unheated and heated emptied digesta respectively. Similarly, the $d_{4,3}$ values of both heated and unheated emptied digesta of GIF stayed in the same levels before 100 min of digestion ($P > 0.05$). Then, the unheated GIF emptied digesta had a dramatic increase from 8.59 ± 1.77 μm at 140 min to 41.75 ± 5.25 μm at 180 min, whereas the heated GIF increased from 4.93 ± 1.14 μm at 140 min to 6.01 ± 0.91 μm at 180 min. On the contrary, the heated SIF emptied digesta was followed the same trend as unheated SIF ($P > 0.05$). As Figure 5.6 C shows, both average diameters of heated and unheated SIF emptied digesta stabilized at ~ 0.5 μm till 60 min, then both increased with the time of digestion. From 100 – 180 min of digestion, unheated SIF increased from 1.60 ± 0.13 μm to 16.43 ± 3.54 μm, while heated SIF raised from 1.29 ± 0.17 μm to 17.12 ± 3.54 μm.


A
Average Particle Size ($d_{4,3}$) (µm)
25
22
19
16
13
10
7
4
1
-2
0
20
40
60
80
100
120
140
160
180
Digestion Time (min)
Unheated CIF
Heated CIF
a,x
a,y
b
c
c
c
c


B
Average Particle Size ($d_{4,3}$) (µm)
45
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
160
180
Digestion Time (min)
Unheated GIF
Heated GIF
a,x
b,x
ab
a,y
a,y
c
c
c
c
b
b
b

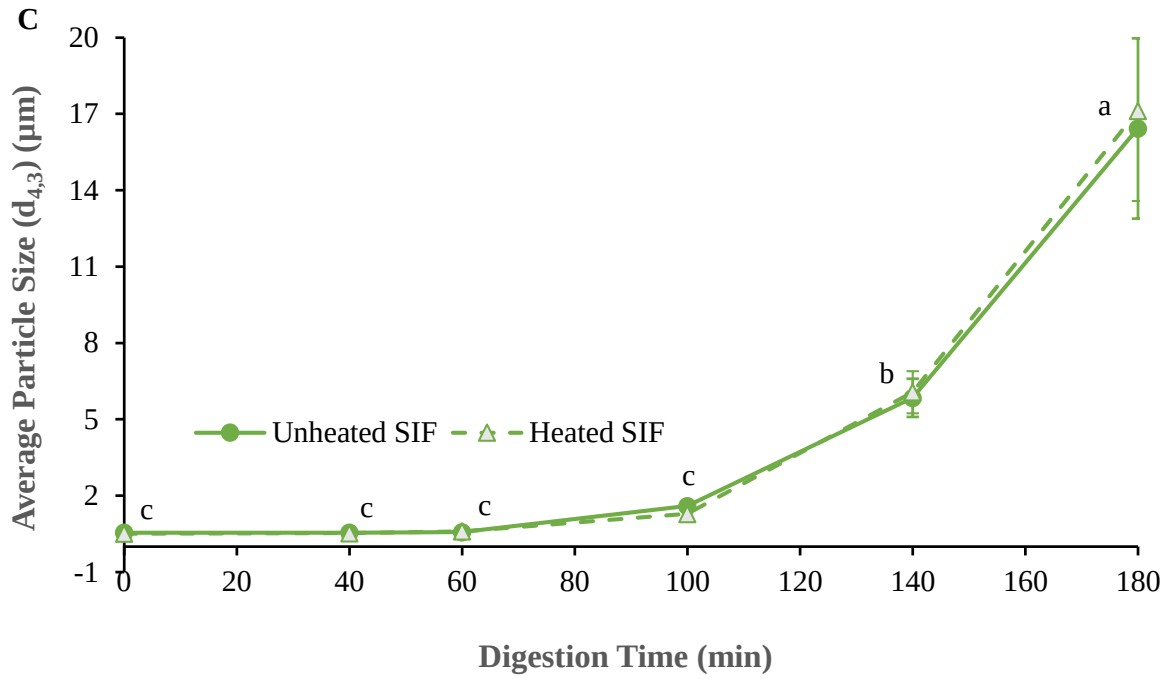
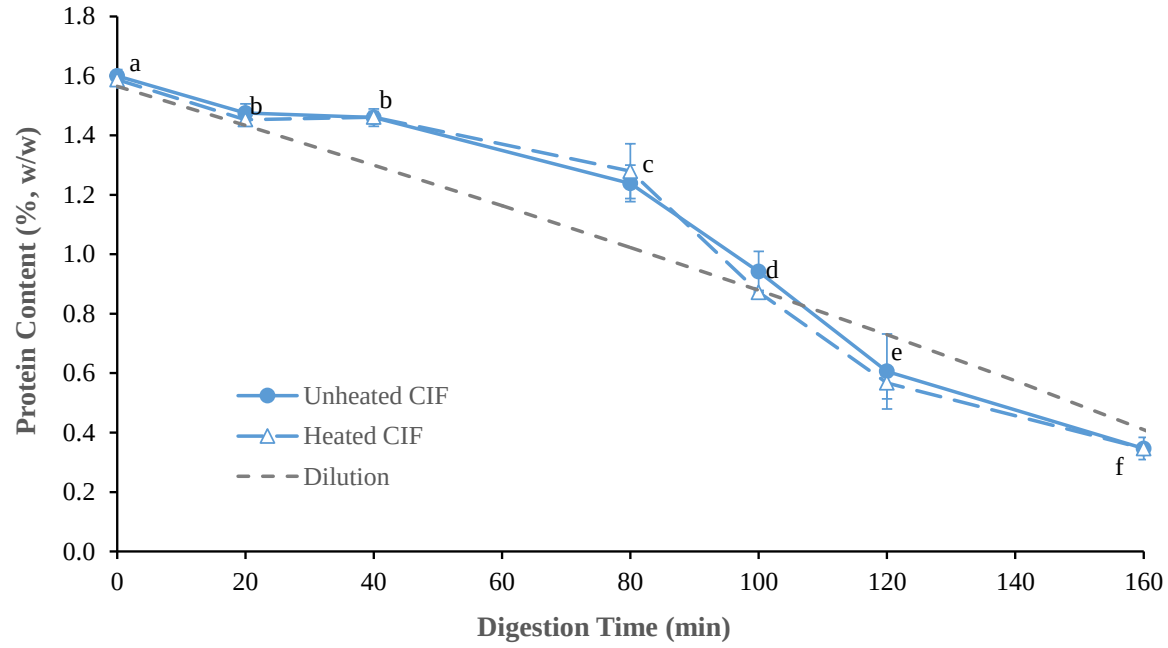


Figure 5.6. Changes in volume-weighted average diameter ($d_{4,3}$, μm) of heated and unheated CIF (A), GIF (B) and SIF (C) emptied digesta

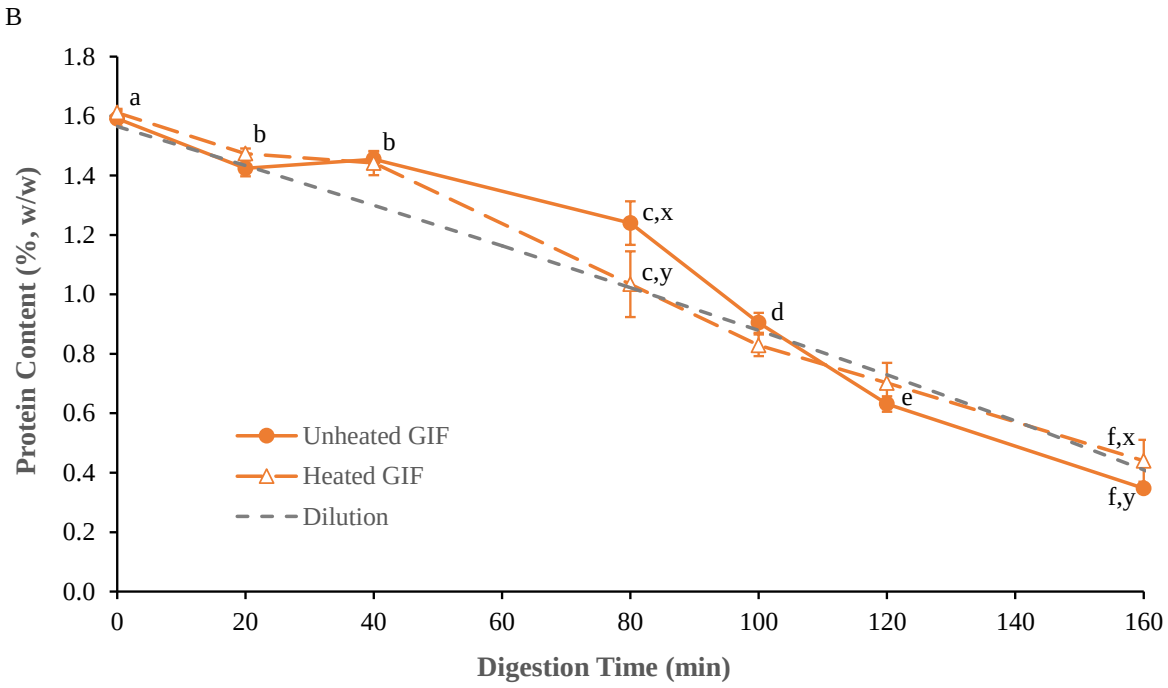
5.2.5 The contents of protein and fat in the gastric emptied digesta

The protein contents in the emptied digesta of heat and unheated infant formula were determined as a function of digestion time. As Figure 5.7 A shows, no significant difference ($P > 0.05$) was observed between heated and unheated CIF at each time point of digestion. Contrarily, GIF and SIF protein contents exhibited differences in certain time points as Figure 5.7 B and C shows. Compared to the protein content of heated GIF almost followed the dilution line excepted on 40 min, unheated GIF was higher than the dilution line at 40 and 80 min, then decreased slightly under the line at 120 and 160 min. There were significant differences between heated and unheated GIF protein contents at 80 and 160 min ($P < 0.05$). At 80 min, the unheated emptied digesta contained a higher protein content than the heated GIF but decreased to lower than that of the heated GIF at 160 min. Similar trends were found in heated and unheated SIF. The protein contents of both samples were higher than the dilution line and dropped to lower than it after 100 min. However, the protein contents of heated SIF were lower than that of unheated SIF before 100 min, then shifted higher than the unheated SIF till 160 min. The different contents were observed at 80 and 120 min ($P < 0.05$).

A


Protein Content (%, w/w)
Digestion Time (min)
Unheated CIF
Heated CIF
Dilution
a
b
b
c
d
e
f

B


Protein Content (%, w/w)
Digestion Time (min)
Unheated GIF
Heated GIF
Dilution
a
b
b
c,x
c,y
d
e
f,x
f,y

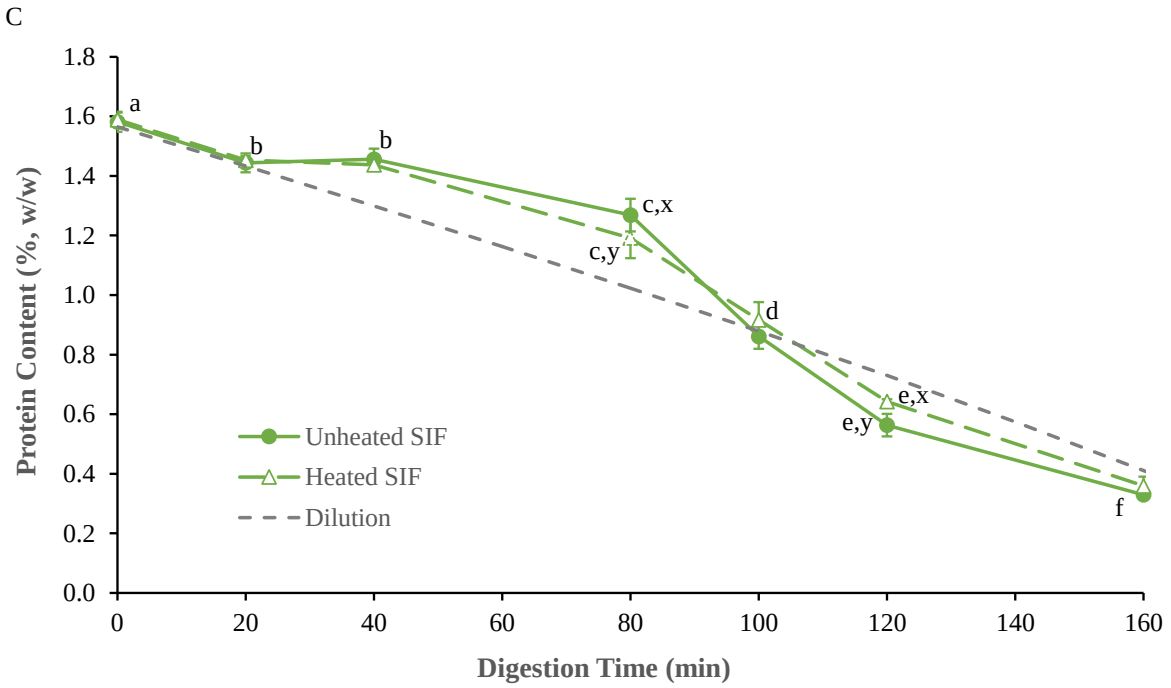
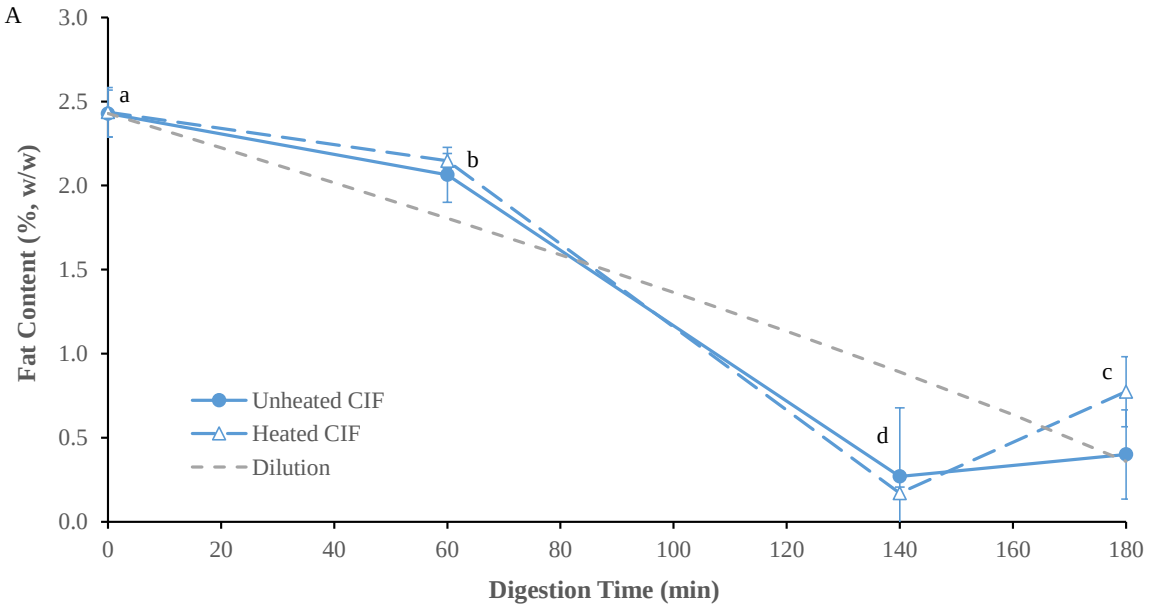
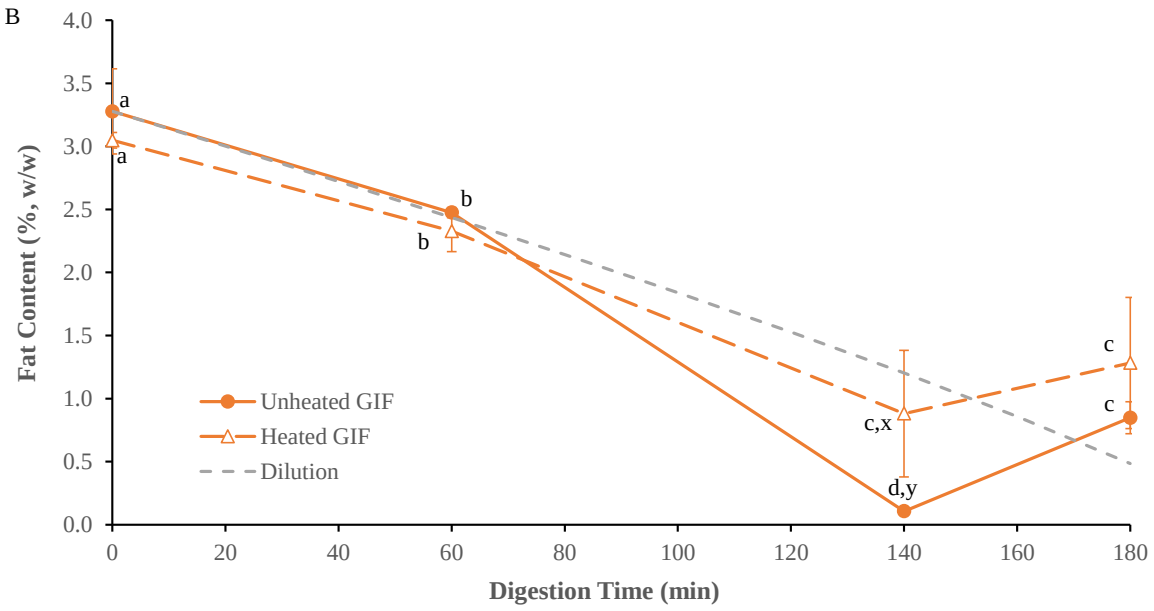


Figure 5.7. Comparation of protein contents in emptied digesta during gastric digestion in the HGS of heated and unheated CIF (A), GIF (B) and SIF (C)

The fat contents of emptied digesta derived from heated and unheated CIF, GIF and SIF as a function of digestion time were present in Figure 5.8. As Figure 5.8 A shows, the fat contents of heated and unheated CIF were above the dilution line, then sharply dropped under the dilution line around 80 min. At the end of the digestion, they both had an increase, and the fat content in the emptied digesta of heated sample was higher than the unheated one. Regarding the fat contents of GIF, the values of heated samples were higher than the unheated samples after 60 min of digestion. It was significantly higher than unheated emptied digesta at 140 min ($P < 0.05$). Similar trends were observed in the heated and unheated SIF emptied digesta fat contents, expected 180 min the value of the heated sample was higher than that of the unheated ($P < 0.05$). Overall, the fat contents in heated and unheated emptied digesta of three infant formulae were experienced similar trends, which had a decrease with the time of digestion and raised at the end of digestion. Also, the fat values of three heated emptied digesta were all higher than the unheated ones at 180 min.


A
Fat Content (%, w/w)
Digestion Time (min)
Unheated CIF
Heated CIF
Dilution
a
b
c
d


B
Fat Content (%, w/w)
Digestion Time (min)
Unheated GIF
Heated GIF
Dilution
a
a
b
b
c
c
c,x
d,y

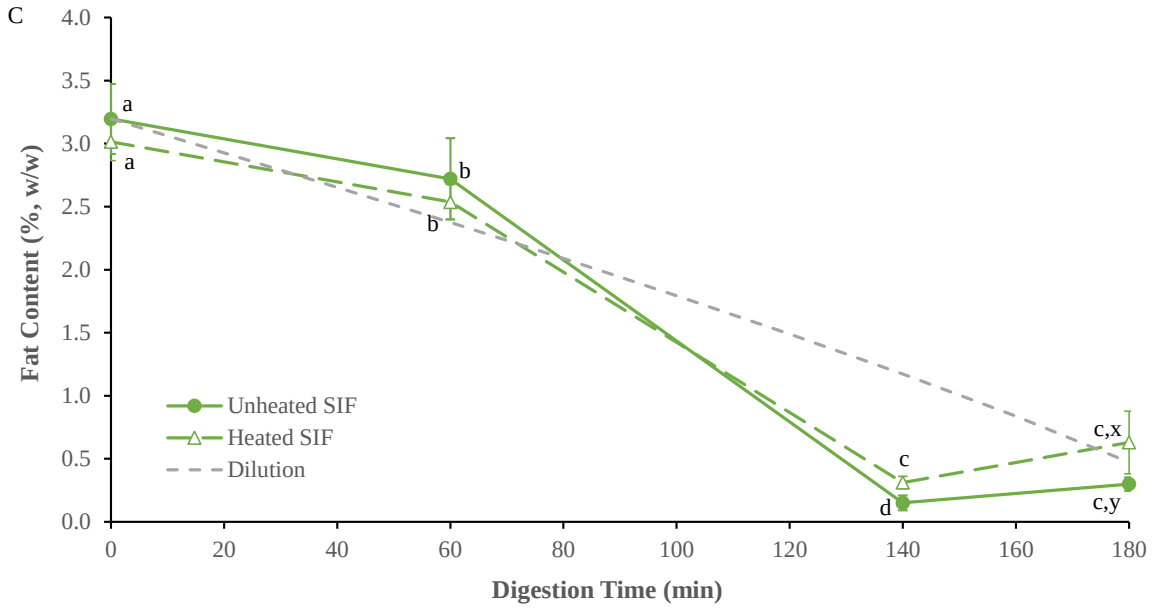


Figure 5.8. Comparation of fat content in emptied digesta during gastric digestion in the HGS of heated and unheated CIF (A), GIF (B) and SIF (C)

5.2.6 Hydrolysis of protein in empty digesta

As shown in Figure 5.9, compared to unheated samples (on the left-hand side), heated (on the right-hand side) goat and cow infant formulae were observed different patterns of their protein hydrolysis. In heated GIF and CIF, casein bands were seen gradually faint trends, till 120 min no casein band could be observed. However, the casein bands in the unheated CIF and GIF were shown similar intensities in 0 – 80 min, then suddenly decrease the intensity at 100 min, and disappeared at 120 min. This indicated casein hydrolysis started near 100 min, where pH was around 4.6, but casein hydrolysis of heated CIF and GIF were started earlier. The results were in agreement with the report of Miranda and Pelissier (1987) that heat treatment improved the casein hydrolysis and gastric emptying rate.

The differences of whey proteins between heated and unheated emptied digesta of CIF and GIF were more obvious. Both α-LA and β-LG bands in the heated sample showed decreased intensities with increasing time of digestion. At 160 min, no bands of the heated sample could be observed, while β-LG brands were still observed in unheated GIF and CIF. The observations of CIF and GIF were aligned with the study of Ye et al. (2017), both casein and whey protein were hydrolysis slower in unheated milk compared to heated milk. Both casein and whey

protein in heat whole milk were participated in clot formation, so small amounts of intact whey proteins in the emptied digesta.

On the contrary, both unheated and heat-treated SIF showed a similar trend. Casein bands in both heated and unheated SIF were getting faint from 100 min, while whey protein bands decreased intensity from 120 min. Casein bands could not be seen at 120 min and whey protein could not be seen at 160 min in both samples. At the end stage of digestion, there should be many peptides evacuated from the stomach, which was too small to be detected by SDS-PAGE.

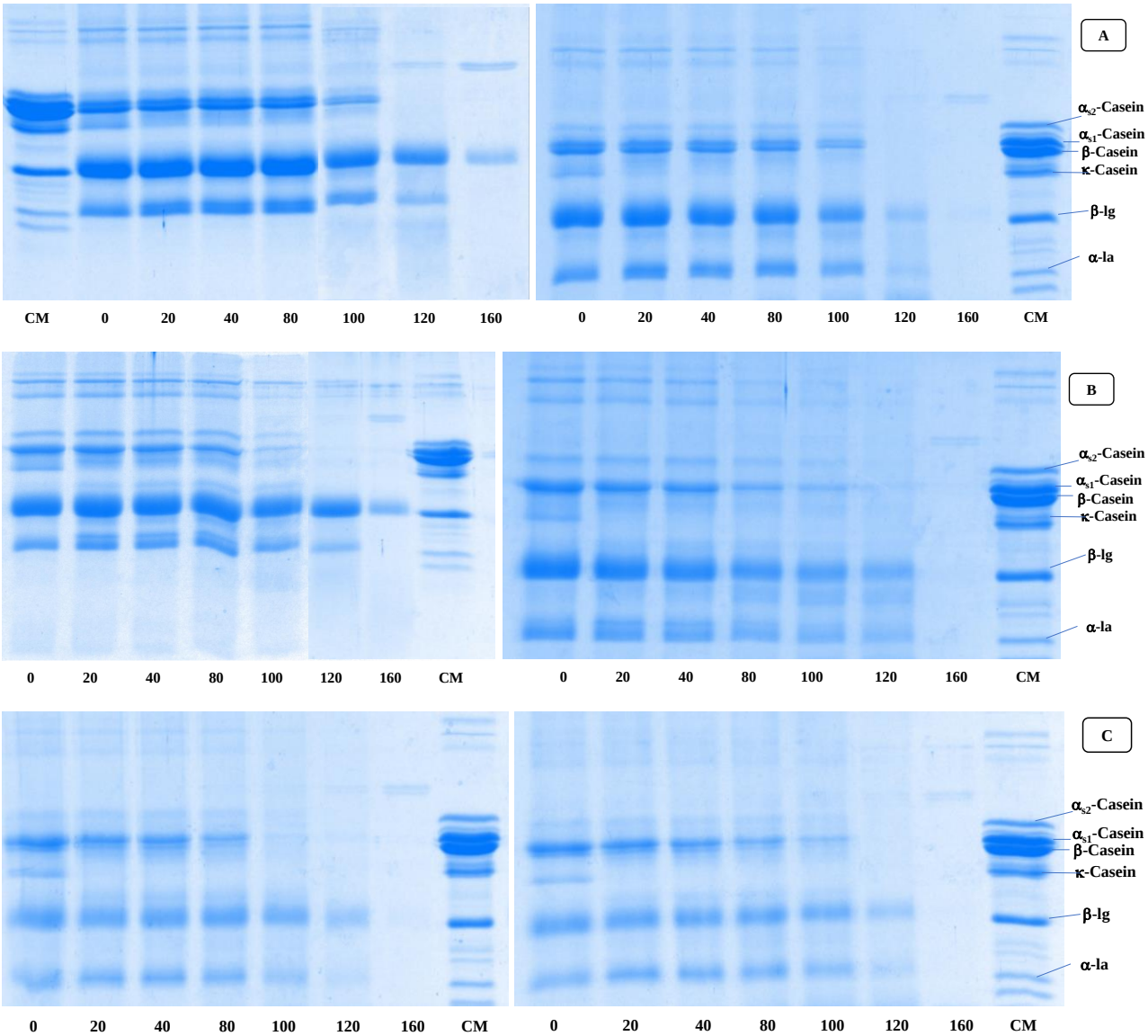


Figure 5.9. SDS-PAGE pattern under reducing conditions of emptied digesta obtained from heated and unheated CIF (A), GIF (B), and SIF (C) during gastric digestion (SGF with

pepsin) in HGS at different times

5.3 Discussion

The results indicated that the heat treatment could influence the aggregate formation by protein and fat globules during gastric digestion. The particle size distributions and microstructures of heated CIF and GIF were different to the unheated milk. Heated chyme microstructures showed less intact with more porous structures. The open structure of aggregates led to the slow increase in particle size observed during gastric digestion. Also, the heated IFs showed an earlier initial aggregation than the unheated IFs. In addition, the fat contents of heated IFs were higher than unheated IFs at the late stage of digestion, which could due to the faster protein hydrolysis during the digestion.

The different digestion behaviours could be ascribed to denaturation of whey proteins induced by heat treatment (Anema & Li, 2003; Li et al., 2021; Singh & Havea, 2003; Ye et al., 2017). Whey proteins are susceptible to heat treatment, which can be easily denatured and unfolded when heated up to 70 °C. Infant formulae in the present experiment were whey-dominant emulsions. In the native structure, β-LG is resistant to enzymatic hydrolysis as its compact globular structure hide the cleavage sites (He & Giuseppin, 2014; Ye, Liu, et al., 2019). According to Singh and Havea (2003), whey protein can be mostly denatured when heated up to 90 °C. The cleavage sites of unfolded whey protein are explored and associated with disulphide bond aggregations between adjacent whey proteins and casein micelles to whey proteins (Anema & Li, 2003; Donato et al., 2007; McSweeney & Fox, 2013). Wang et al. (2018) reported that no aggregation was observed in whey protein isolate (WPI) during gastric digestion, whereas the heated WPI aggregated in the early stage of digestion.

In addition, according to the previous studies, the denaturation of whey proteins led to whey proteins being involved in the whole milk clot formation and induced accelerated gastric emptying (Ye et al., 2016a, 2017). The heat-induced conformational changes could lead to β-LG being more susceptible to hydrolysis by pepsin. This was aligned with the observation of comparative SDS-PAGE pattern of heated and unheated infant formulae in the present experiment that both casein and whey protein bands in heated CIF and GIF showed lighter

intensities than unheated ones and have not been detected at 160 min of digestion.

5.4 Conclusion

In this chapter, the effect of heat treatment in simulated gastric digestion behaviour of commercial infant formulae was studied. The particle size distributions of CIF and GIF chyme indicated that heated IF had an earlier initial aggregation than unheated samples, the coagulations were in smaller sizes, and the coalescence of oil droplets decreased after heating. Similar observations were found in the confocal laser scanning microscopy (CLSM) images of heated and unheated CIF and GIF. Heated chyme formed less compact and fragmented aggregates. These observations could be attributed to the heat-induced whey protein denaturation and the association of denatured whey protein with casein micelles in the heated IFs. Denatured whey protein formed aggregations at the early stage of digestion and the associated whey proteins hinder the casein micelle coagulation to form the dense coagulum structure under gastric conditions.

Furthermore, heat-induced gastric restructuring could impact protein hydrolysis. This is supported by the results of the SDS-PAGE pattern under reducing conditions of emptied digesta. Both casein and whey protein of heated CIF and GIF were gradually hydrolysed by pepsin from the earlier stage of digestion, while unheated samples were started hydrolysis around 100 min when the pH dropped to ~4.6. However, the heated SIF showed a different coagulation behaviour compared to the other two infant formulae. The particle size distribution of heated SIF nearly overlapped with the unheated SIF. In addition, there was no significant difference between the microstructures and protein hydrolysis of heated and unheated SIF aggregates. This could be attributed to SIF having already undergone heat treatment during the manufacturing process. The denature whey protein coated the fat droplets after the homogenisation and formed a stable emulsion. A further investigation of the manufacturing process of the commercial infant formula is required.

Chapter 6: The Pepsin Induced Coagulation of Commercial Infant Formulae: The Rheological Properties

6.1 Introduction

The previous chapter has discussed the different protein compositions in cow, goat and sheep infant formulae impact on the digestion behaviour. The results indicated that the milk from different species contains various protein compositions, which could affect the different gastric coagulation behaviour. The aggregates of goat infant formula formed in the *in vitro* digestion model showed a porous and fragmented structure. The observations related to the previous studies of the gelation properties of the coagulation formed from various milk. The study by Gamble (1939) revealed goat milk-formed gel or curd was 31% to 54% softer than the gel from cow milk. In addition, goat yoghurt has a softer texture compared to cow milk yoghurt, with a more porous microstructure (Miocinovic et al., 2016).

In the gastric environment, gastric juice increases secreting when ingested milk, which leads to milk pH gradually decreasing and pepsin concentration increasing in the stomach (Minekus et al., 2014; Ye et al., 2016b). Previous studies have found that milk protein coagulation occurs at the early stage of digestion, even when the pH is still higher than the casein isoelectric point (pI 4.6) and low pepsin concentration. Coagulation is related to the presence of acid and pepsin in the stomach (Huppertz & Chia, 2021; Ye et al., 2016a). Roy et al. (2020b) compared the gelation properties of milk from different species in dynamic low-amplitude oscillatory rheology, induced by glucono-δ-lactone (GDL) alone or a combination of GDL and pepsin. The results indicated that sheep milk formed a stronger gel due to the higher total solids and protein content. In addition, with the addition of pepsin with GDL, a higher aggregation pH and strong gel strength were found. However, different milk responses vary to pepsin.

Although rheology properties of sheep, goat and cow skim milk, and the influence of pepsin have been studied, there has been little discussion about the coagulation properties of infant formula from different species. This chapter is aimed to compare the coagulation behaviours

of commercial CIF, GIF and SIF during gastric digestion to understand the different protein composition impact on the gastroic coagulation, which are induced by acid and pepsin. The chapter begins by comparing the pH and rheological properties of infant formula from cow, goat and sheep milk. Then looks at how the coagulation properties changed after different concentrations of pepsin were applied during gastric digestion. End by comparing the microstructure of coagulum formed by three infant formulae.

6.2 Results

6.2.1 pH profile of acid infant formula gel formation

The change in pH of three infant formulae samples (1.565% (w/w) protein) with 1.0% GDL during the gelation process showed in Figure 6.1. The initial pH profiles were 6.91 ± 0.01, 6.64 ± 0.08 and 6.61 ± 0.01 of CIF, GIF, and SIF, respectively. After adding 1.0% GDL, the pH rapidly decreased to around 5.3 in the first 20 minutes, and the pH decrease were followed: GIF > SIF > CIF. The pH gradually reduced with the time of gelation. The pH at 180 min were 4.51 ± 0.07, 4.24 ± 0.03 and 4.48 ± 0.03 for CIF, GIF and SIF, respectively.

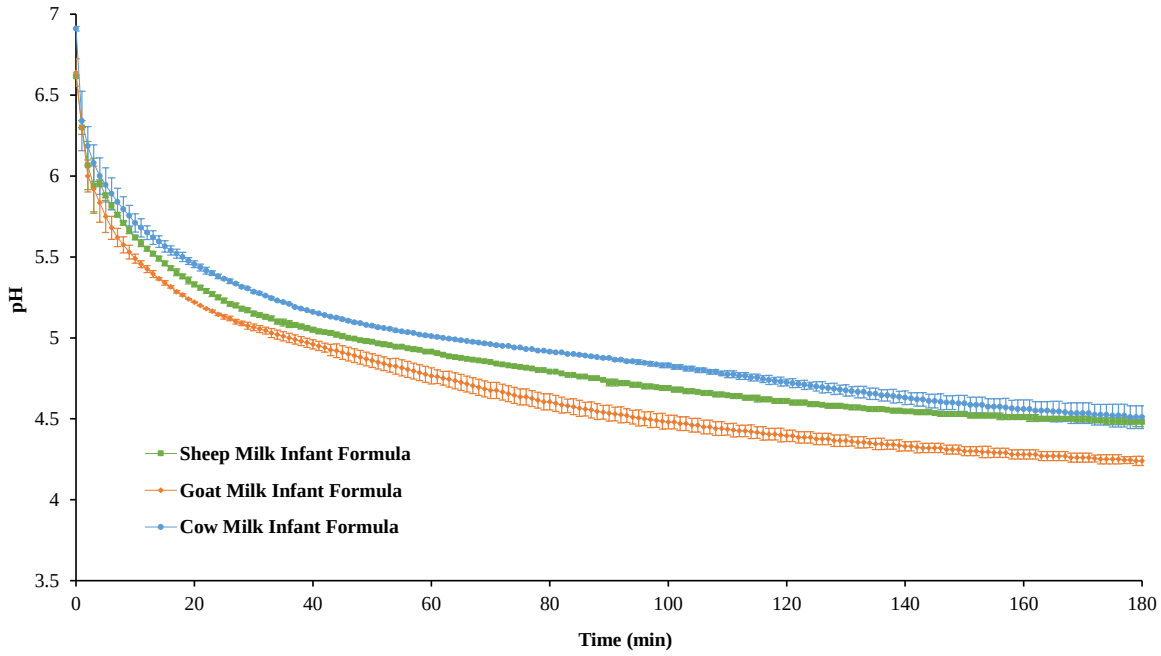


Figure 6.1. The change in pH of CIF, GIF and SIF (1.565% (w/w) protein) during the gelation process at 37°C

6.2.2 Acid infant formula gels formation

A time sweep measurement constantly monitored gelation evolution at 1 HZ frequency and 1% strain. The storage modulus (G') and the loss modulus (G") were recorded every minute for 3 hours of rheology measurement. Figure 6.2 shows the acidification process of sheep, goat and cow infant formulae with 1.0% (w/v) Glucono-δ-lactone (GDL). The value of G' indicated the firmness of each sample during pH reducing. It increased with the pH decreased in all three samples.

After GDL was added into the infant formulae, the initial sharp increase of G' was observed in around 16 min and 25 min of goat and cow infant formulae, then slowly increased with the pH decreased. However, the G' value of sheep infant formula was stably increased with the pH decreased. In the 3 hours measurements, the overall G' values were followed: GIF > CIF > SIF. The final G' of GIF was 9.822 ± 0.533 Pa, followed by GIF was 7.698 ± 0.258 Pa. SIF's final G' was 1.537 ± 0.410 Pa, which was much lower than the other two samples. Similar trends were found in the G" curves of three samples. The final G" values were 2.203 ± 0.023 Pa, 1.943 ± 0.027 Pa and 0.371 ± 0.119 Pa, respectively of GIF, CIF and SIF. The observation indicated that sheep infant formula formed a less strong acid-induced gel, while goat infant formula formed a firmer gel compared to the cow infant formula gel and sheep infant formula gel.

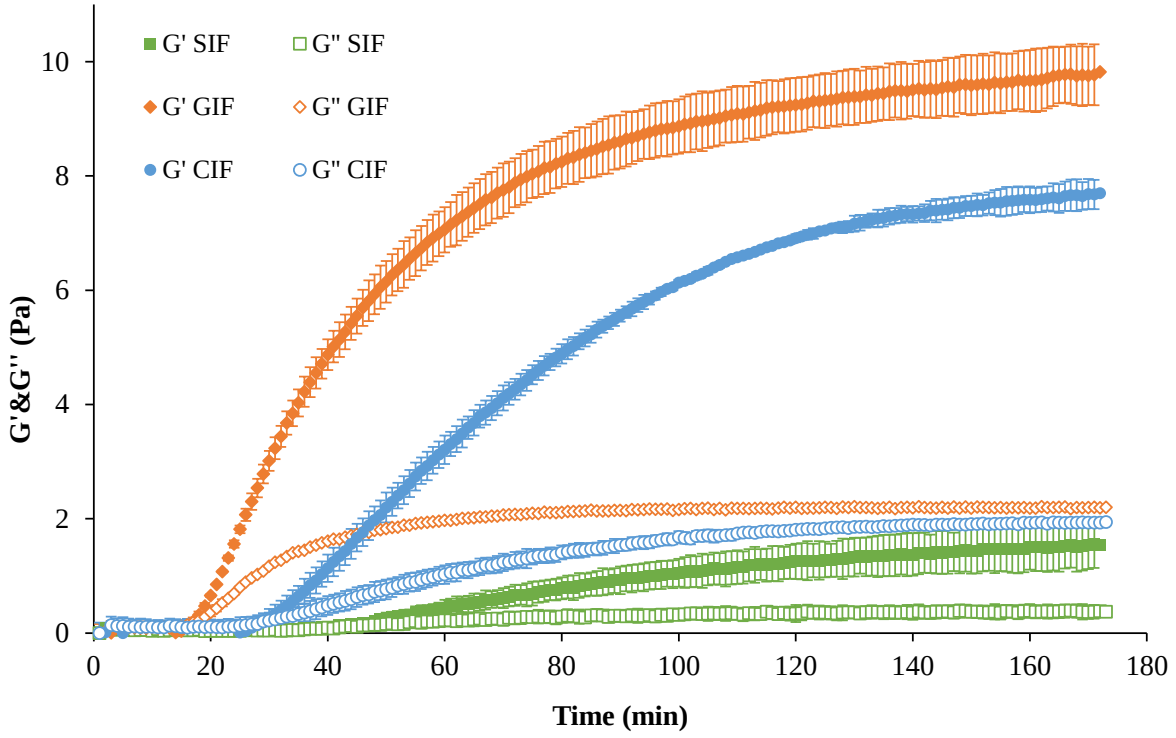


Figure 6.2. Changing in storage modulus G' and loss modulus G" as a function of time (min) 37°C

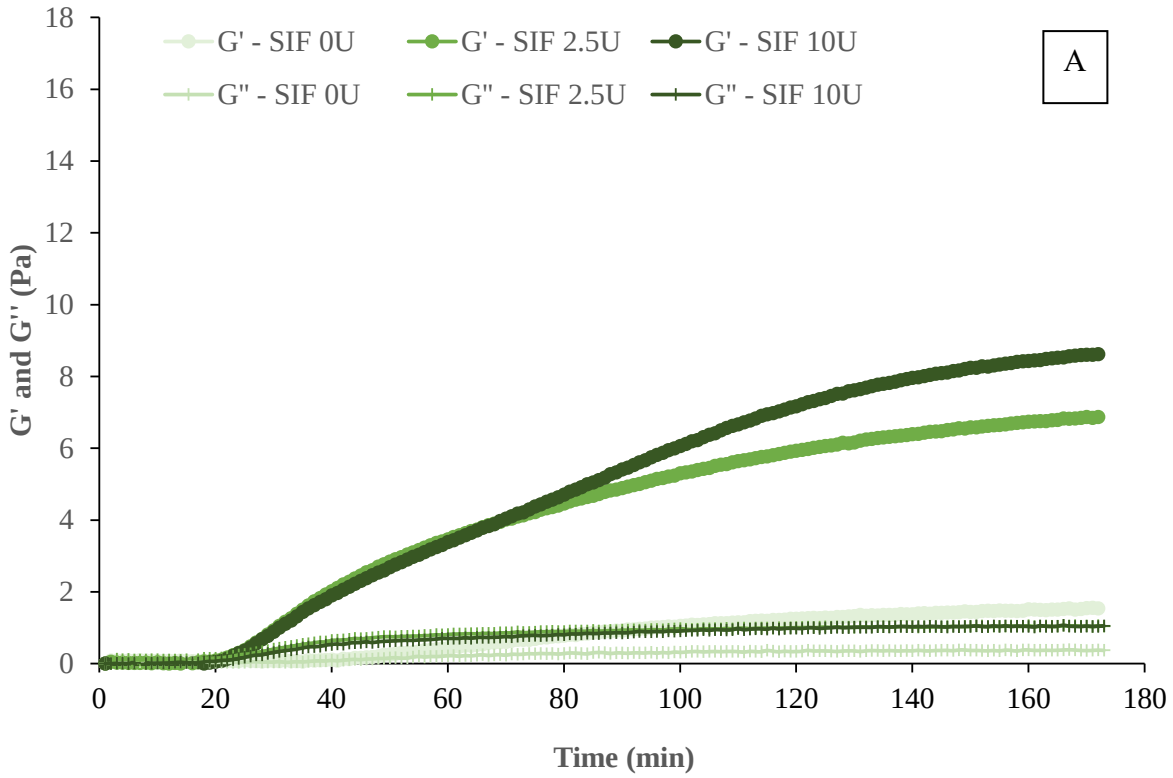
6.2.3 Changes in storage modulus of gels induced by a combination of GDL and pepsin

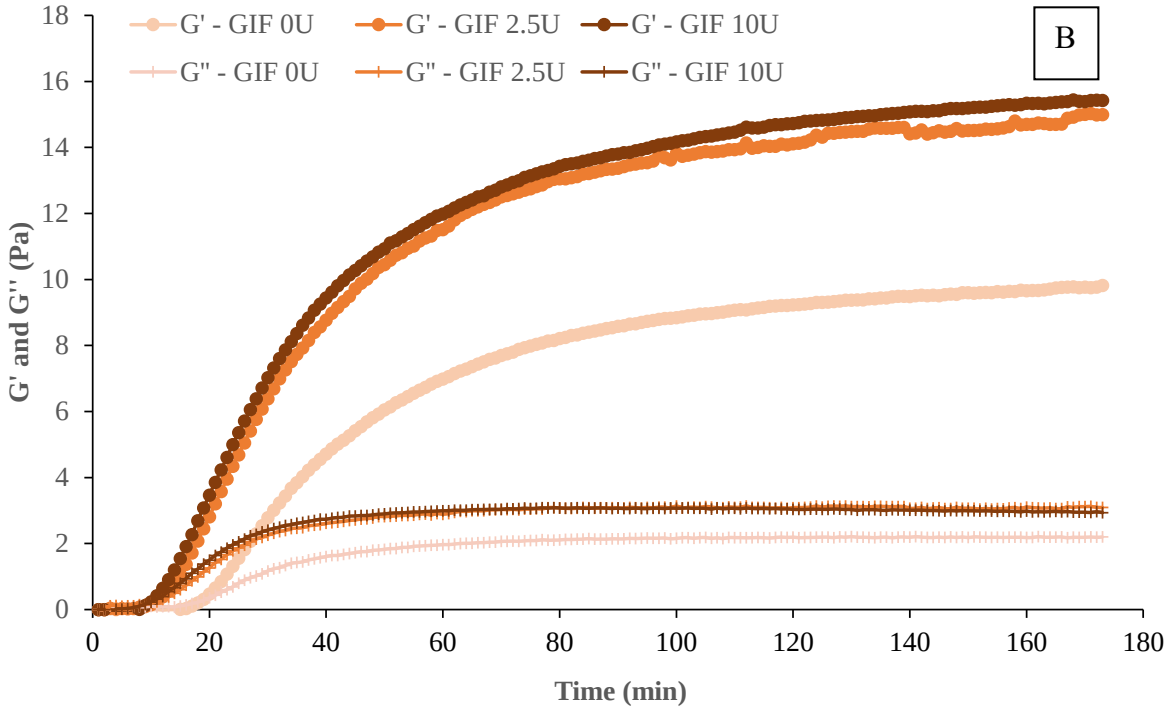
1.0% (w/v) Glucono-δ-lactone (GDL) with different concentration of pepsin (0, 2.5, and 10.0 units/ml) were added into three infant formulae (1.565% (w/w) protein). The G' and G" versus time curves show in Figures 6.3. For sheep infant formula (Figure 6.3 A), the G' and G" curves of the sample with GDL only were exhibited at the lowest part of the figure, which means the G' and G" value of GDL without pepsin were very low. After pepsin was added into SIF, the G' profiles increased significantly, while G" profiles showed inapparent increases. In the early 20 mins, G' values of GDL in sheep infant formula with and without pepsin remained steady at low values. Then the G' value of samples with 2.5 u/ml and 10 u/ml pepsin increased similarly. From around 75 min, the G' value of 10 u/ml pepsin started growing faster than the sample with 2.5 u/ml pepsin. The final G' was 1.537 ± 0.410 Pa, 6.865 ± 1.066 Pa, and 8.619 ± 1.243 Pa of sheep infant formula with GDL only, 2.5 u/ml and 10 u/ml pepsin, respectively. The observations of G' curve changes with the different pepsin concentrations indicated that

the sheep infant formula gel with pepsin is much firmer than the one without pepsin. In addition, a higher pepsin concentration could generate a firmer gel.

The G' and G" values of goat milk infant formula are shown in Figure 6.3 B. The initial increase of G' of goat infant formula with GDL started around 15 min, then crossed with G" curve approximately 20 min. When 2.5 u/ml and 10 u/ml pepsin were employed with GDL, the initial increase of G' started earlier and crossed to G' curve around 10 min. The G' and G" curves of both levels of pepsin were present in the higher part of the image during 180 min rheological measurement, which means the values are always higher than GIF with GDL only. The results indicated that the combination gels have stronger structures than the GLD induced gel, and G' values were higher in the higher pepsin concentration (10 u/ml). In addition, compared to SIF, the overall G' and G" values of GIF were much higher than SIF (Figure 6.3).

On the contrary, the gel properties of CIF were presented in a different trend compared to SIF and GIF (Figure 6.3 C). The initial increase storage modulus (G') was around 25 min of the measurement and crossed with G" curve around 32 min. When 2.5 u/ml pepsin was added into CIF, both G' and G" curves were reduced and crossed around 24 min. After more pepsin (10 u/ml) was applied, both G' and G" values decreased more. These results mean that unlike the G' and G" values of GIF and SIF increase with an increase in the pepsin concentration, the values of CIF decreased with increasing the pepsin concentration. The final G' of 10 u/ml pepsin with GDL in CIF was 6.358 ± 0.486 Pa, which was lower than the G' of 2.5 u/ml pepsin with GDL (6.620 ± 0.011 Pa) and GDL only (7.698 ± 0.258 Pa). The overall values of CIF were lower than GIF but higher than SIF in the same condition (measurement time and pepsin concentrations).


G' - SIF 0U
G' - SIF 2.5U
G' - SIF 10U
G" - SIF 0U
G" - SIF 2.5U
G" - SIF 10U
A
G' and G'' (Pa)
Time (min)
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
160
180


G' - GIF 0U
G' - GIF 2.5U
G' - GIF 10U
G" - GIF 0U
G" - GIF 2.5U
G" - GIF 10U
B
G' and G'' (Pa)
Time (min)
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
160
180

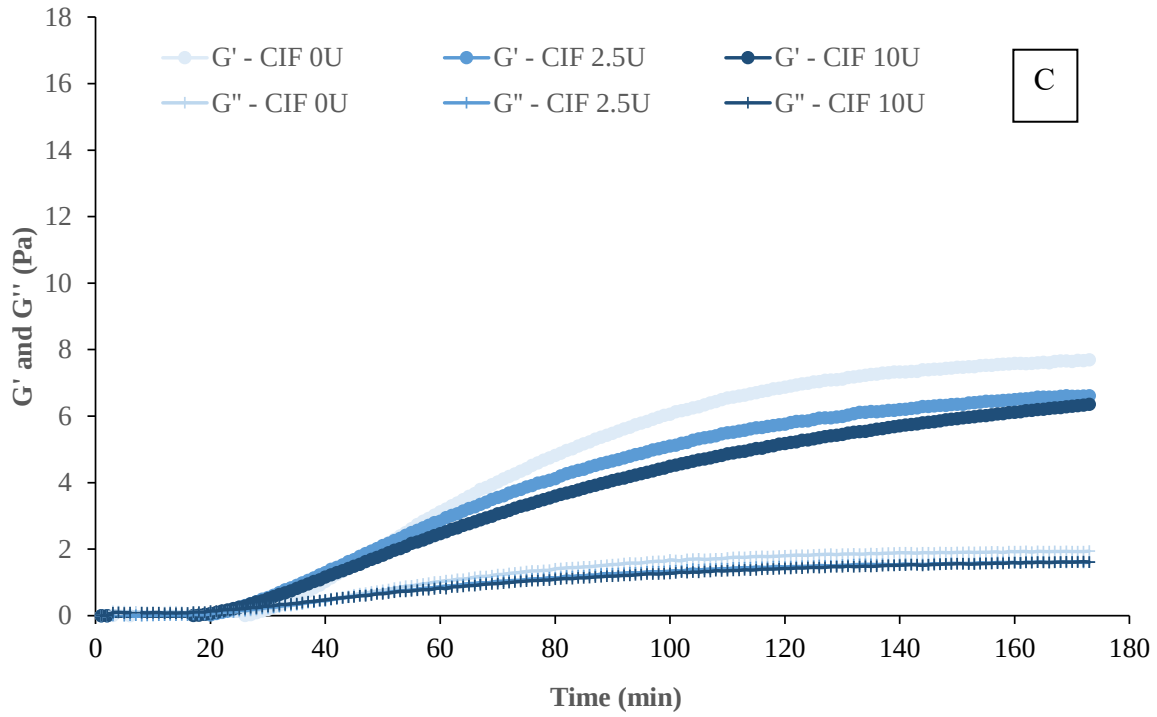


Figure 6.3. Changing in storage modulus G' and loss modulus G" as a function of time (min) 37°C of SIF (A), GIF (B), and CIF (C)

In terms of the control, the rheological properties of acid-induced and acid/pepsin induced whole cow milk gels were shown in Figure 6.4. The storage modulus (G') of acid-induced gel is presented in lower values, which started increasing at around 60 min till the end of the measurement. After the addition of 0.3 u/ml pepsin with GDL in the whole cow milk, the G' value increased dramatically in the early stage of the measurement and crossed to G" around 14 min. From ~40 – 62 min, the G' value experienced a dropping till the pH decreased to around 5.08. Similar trends were found in the G' curves of 1.0 u/ml and 2.5 u/ml pepsin employed in the whole cow milk. There were sharp increases at the beginning till ~29 min and ~18 min respectively. Then they both experienced a slight decrease till ~62 min when the pH dropped to ~5.08. After that, the G' curves returned to growth till the end of the measurement. The observations indicative of pepsin could induce coagulation and increasing the pepsin concentration could increase the coagulation rate. Overall, the firmness of the whole milk gels was much stronger compared to the gels from infant formulae, and the pepsin induced gelation was markedly in the whole cow milk.

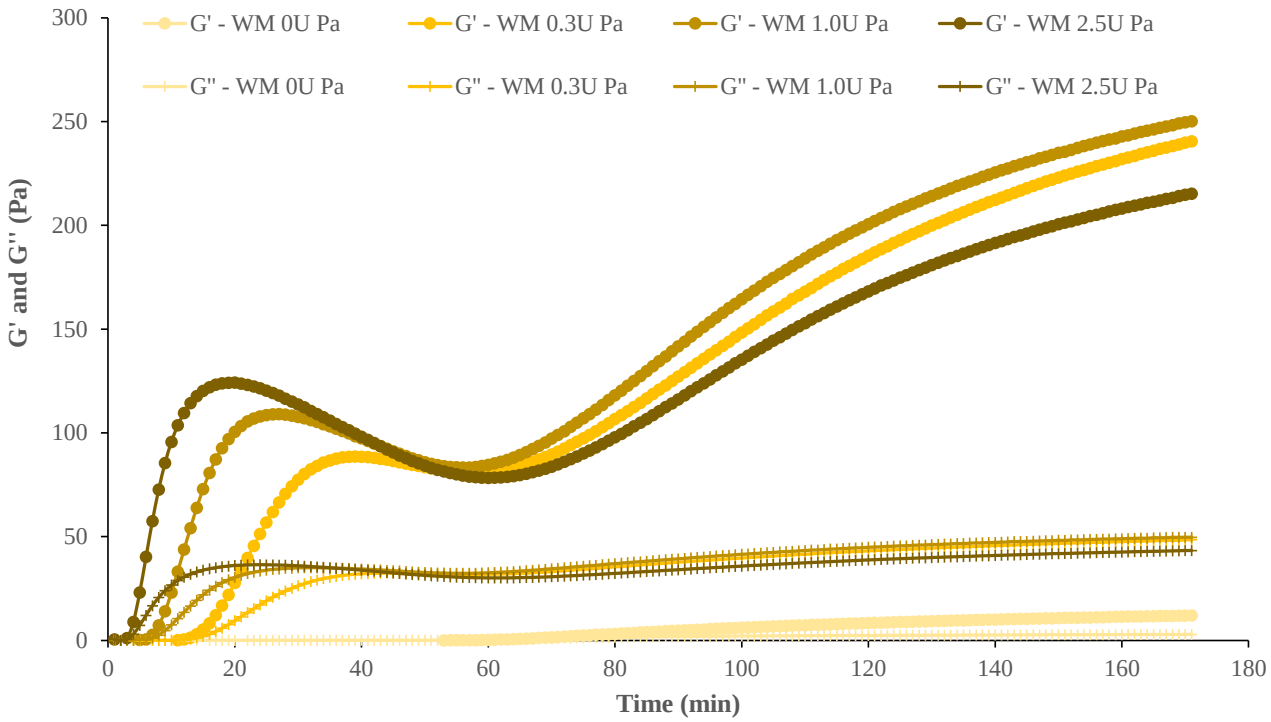


Figure 6.4. Changing in storage modulus G' and loss modulus G" as a function of time (min) 37°C of whole bovine milk

6.2.4 Effect of pepsin concentration on the gelation pH, gelation time and final storage modulus

The pepsin effect on the gelation pH and time on the whole bovine milk was obvious as a contral. The whole milk with GDL was gelled (G' = G") at 65 ± 3.0 min when the pH dropped to 5.04 ± 0.01. After employing pepsin at concentrations of 0.3 u/ml, 1.0 u/ml and 2.5 u/ml, the gelation times were ~14, 8, and 4 min, respectively. Also, the gelation pH increased with the addition of pepsin concentrations, which were ~5.63, 5.81, and 6.10. In addition, the final G' were increased markedly from ~12.0 Pa of GDL only to ~ 240.5, 250.1 and 215.2 Pa of pepsin concentrations of 0.3 u/ml, 1.0 u/ml and 2.5 u/ml, respectively. With increasing the pepsin level, the whole bovine milk gelled in a shorter time with a higher pH, and the gel were stronger.

Table 6.1. pH and gelation time (min) of Glucono-δ-lactone (GDL) and pepsin formed sheep, goat, and cow infant formulae gels at 37°C

	0 U/ml		2.5 U/ml		10 U/ml	
	pH	Gelation time (min)	pH	Gelation time (min)	pH	Gelation time (min)
SIF	4.99 ± 0.02	48 ± 1.41	5.15 ± 0.06	27.5 ± 0.71	5.23 ± 0.04	23 ± 1.41
GIF	5.26 ± 0.03	19 ± 0.00	5.58 ± 0.22	10 ± 3.46	5.54 ± 0.08	11 ± 0.00
CIF	5.27 ± 0.01	32 ± 0.00	5.44 ± 0.04	21.5 ± 0.71	5.39 ± 0.02	23.5 ± 0.71

Similar observations were also found in the infant formula gels (Table 6.1). For sheep infant formula, there was no gel occurred within 48 ± 1.41 min for the GDL induced gel. Addition of 2.5 u/ml pepsin, the gelation time decreased to 27.5 ± 0.71 min, and a further shorter gelation time at the pepsin concentration of 10 u/ml (Table 6.1 and Figure 6.5). The combination of goat milk gels also took shorter gelation times compared with GDL induced gel, which was decreased from 19 min to 10 ± 3.46 min of 2.5 u/ml pepsin and 11 ± 0.00 of 10 u/ml pepsin. The gelation time for GDL induced cow infant formula was 32 min, whereas it decreased to 21.5 ± 0.71 min after applying 2.5 u/ml pepsin, and 23.5 ± 0.71 min on the addition of 10 u/ml pepsin. Overall, the pepsin-induced gels of infant formulae had shorter gelation times compared to acid-induced gel, but the pepsin concentration only had little impact on the gelation time.

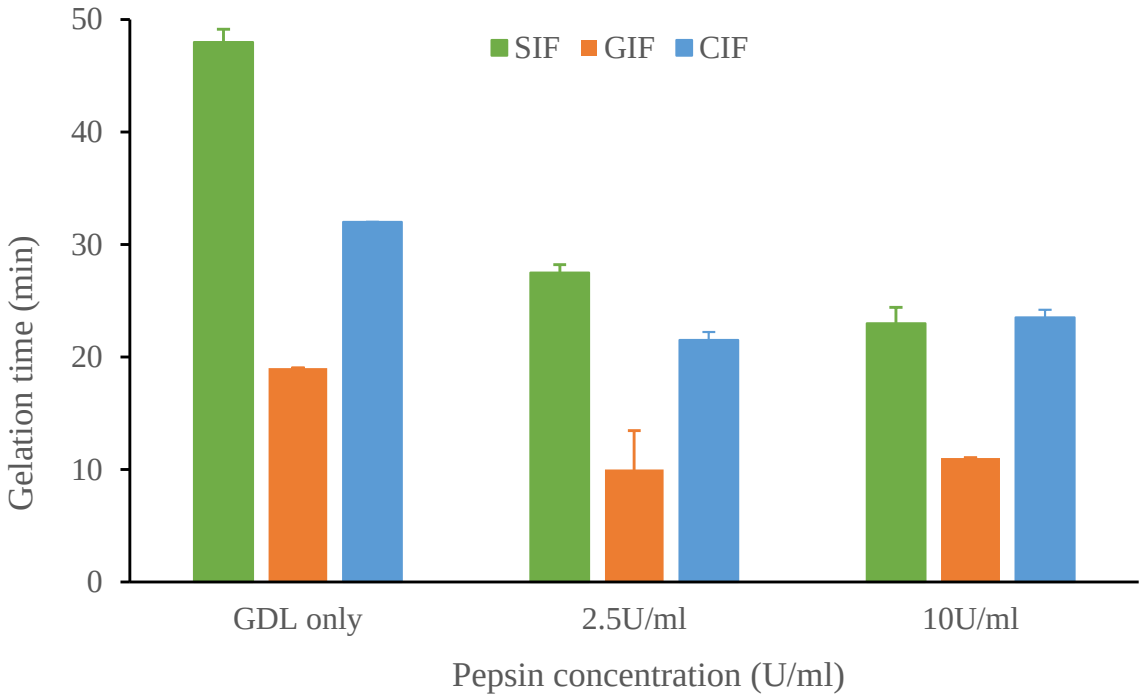


Figure 6.5. Effect of Glucono-δ-lactone (GDL) and pepsin on gelation time

Regarding the gelation pH, from Table 6.1and Figure 6.5, the gelation pH increased with the addition of pepsin. SIF was gelled at pH 4.99 ± 0.02 with GDL and increased to 5.15 ± 0.06 with pepsin employed (2.5 u/ml). Similar trends were found in GIF and CIF. After the addition of pepsin, the pH was increased from 5.26 ± 0.03 to 5.58 ± 0.22 and 5.27 ± 0.01 to 5.44 ± 0.04 respectively GIF and CIF. In terms of the effect of pepsin concentrations on the gelation pH, SIF gelation pH showed a slight increase with the pepsin concentration increase, which from 5.15 ± 0.06 at 2.5 u/ml to 5.23 ± 0.04 at 10 u/ml. On the contrary, GIF and CIF showed a negative correlation between pepsin concentration and gelation pH. After pepsin concentration increased from 2.5u/ml to 10u/ml, GIF gelation pH decreased from 5.58 ± 0.22 to 5.54 ± 0.08 and CIF dropped from 5.44 ± 0.04 to 5.39 ± 0.02.

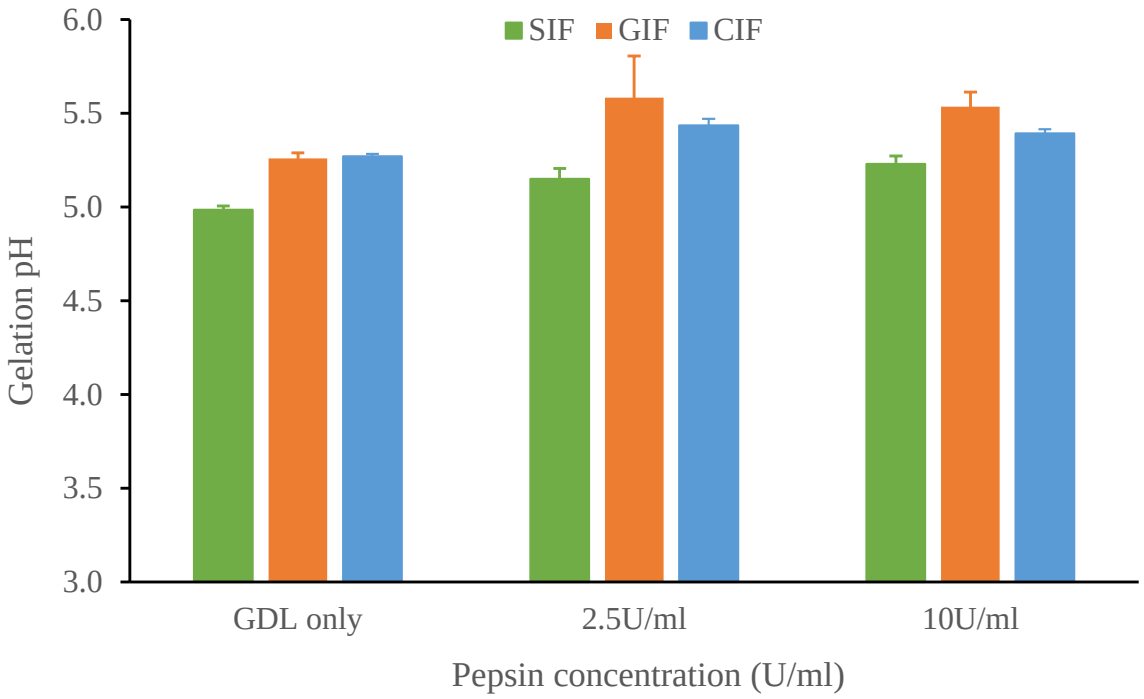


Figure 6.6. Effect of Glucono-δ-lactone (GDL) and pepsin on gelation pH

The effect of Glucono-δ-lactone (GDL) and pepsin on the final storage modulus (G') of three infant formulae were shown in Figure 6.7. For the final G' of SIF and GIF, there were marked increases in combination gels compared to the acid-induced gel. The final storage of GDL induced SIF gel was 1.537 ± 0.371, increased to 6.865 ± 1.045 with 2.5 u/ml pepsin applied and further increased to 8.619 ± 1.052 after pepsin increased to 10 u/ml. Similarly, the final G' of GDL induced GIF gel was 9.823 ± 2.203, increased to 15.000 ± 3.094 and 15.425 ± 2.940 respectively of employed pepsin in the concentration of 2.5 u/ml and 10 u/ml. On the contrary, the final storage modulus of CIF showed a different trend. It was 7.698 ± 1.943 in the GDL only gel and dropped to 6.620 ± 1.611 with 2.5 u/ml. Then further decreased to 6.358 ± 1.613 when the pepsin increased to 10 u/ml. The observation indicated that the application of pepsin and the concentrations could affect the gel stiffness, and the infant formulae from different species respond differently to the presence of pepsin.

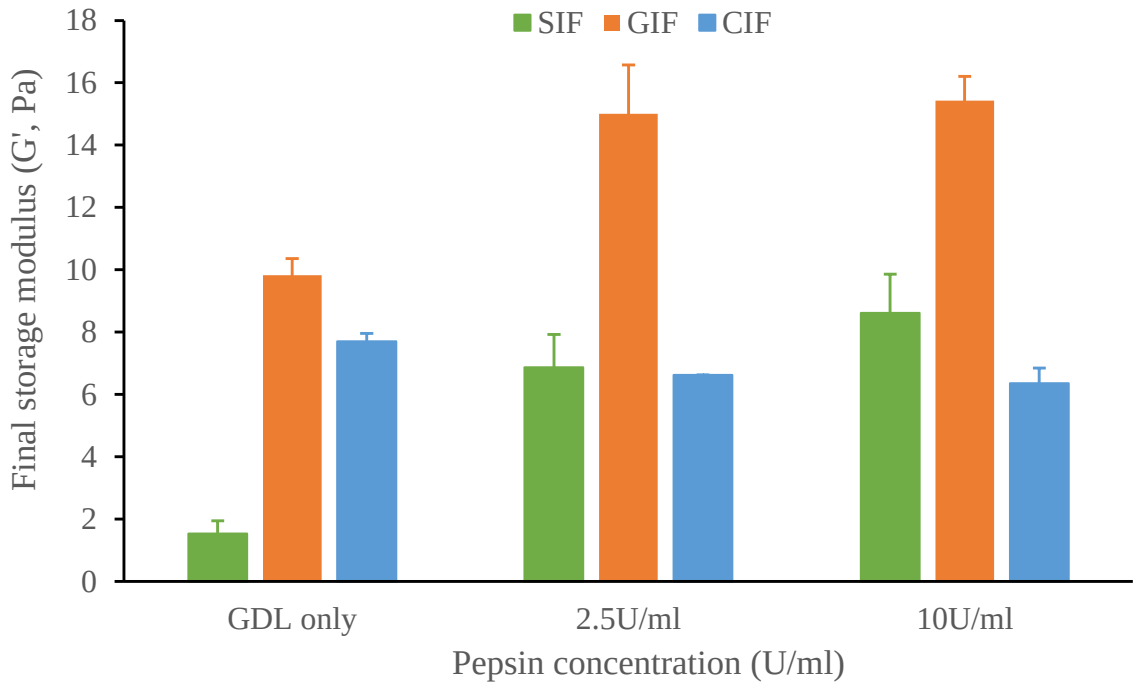


Figure 6.7. Effect of Glucono-δ-lactone (GDL) and pepsin on the final storage modulus

6.2.5 Microstructure of gels

Figure 6.8 shows confocal micrographs of GLD or combination of GDL and pepsin induced gel structures of whole bovine milk. The results are used as a control of the infant formula gel micrograph observations (Figure 6.9). The acid bovine milk gel was less compact compared to the combination gel (GDL and 0.3 u/ml pepsin induced gel). After the pepsin concentration increased to 2.5 u/ml, there were more dense clusters of protein aggregations in the gel structure. The observation of whole bovine milk gels indicated that pepsin and its concentrations could affect the gel microstructure same as effect on the gel strength. There were large, cross-linked aggregates in the higher pepsin concentration gel.

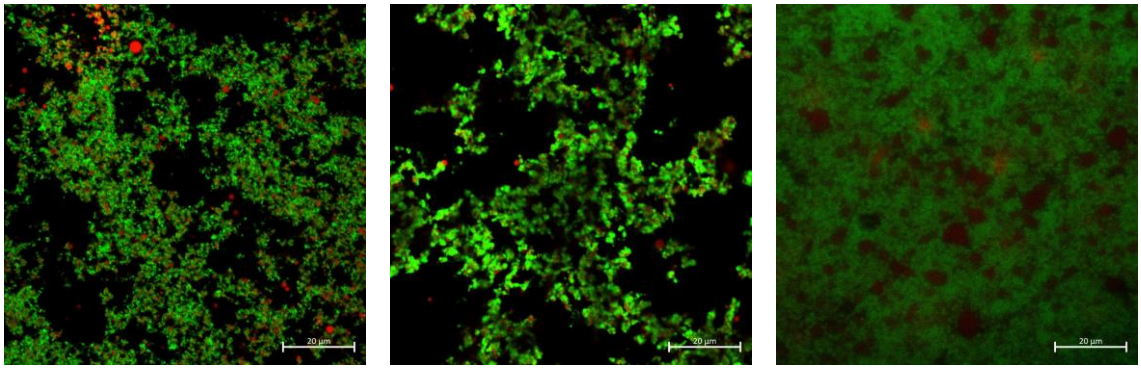


Figure 6.8. Confocal laser scanning microscopy (CLSM) of acid and combination (acid and pepsin) induced gels made from whole bovine milk. Scale bars represent 20 μm.

Figure 6.9 shows the confocal micrographs of acid and combination infant formula gels. There was no significant difference in different animal infant formula gels. Three sample gels all showed similar microstructures. There were some separated blocks of protein aggregates, and the oil droplets were trapped within the aggregated matrix. After pepsin was employed, at the concentration of 2.5 u/ml, the microstructures remain similar structures. The oil droplets were embraced by protein aggregates in an open and loose structure. Similar gel microstructures have been observed when the pepsin concentration increased to 10 u/ml. The observations illustrated that the microstructures of three infant formula gels were porous and fragmented, which indicated the gels were weak in strength. In addition, unlike the whole bovine milk, there was no significant influence of pepsin and its concentration on the gel structures of infant formulae.

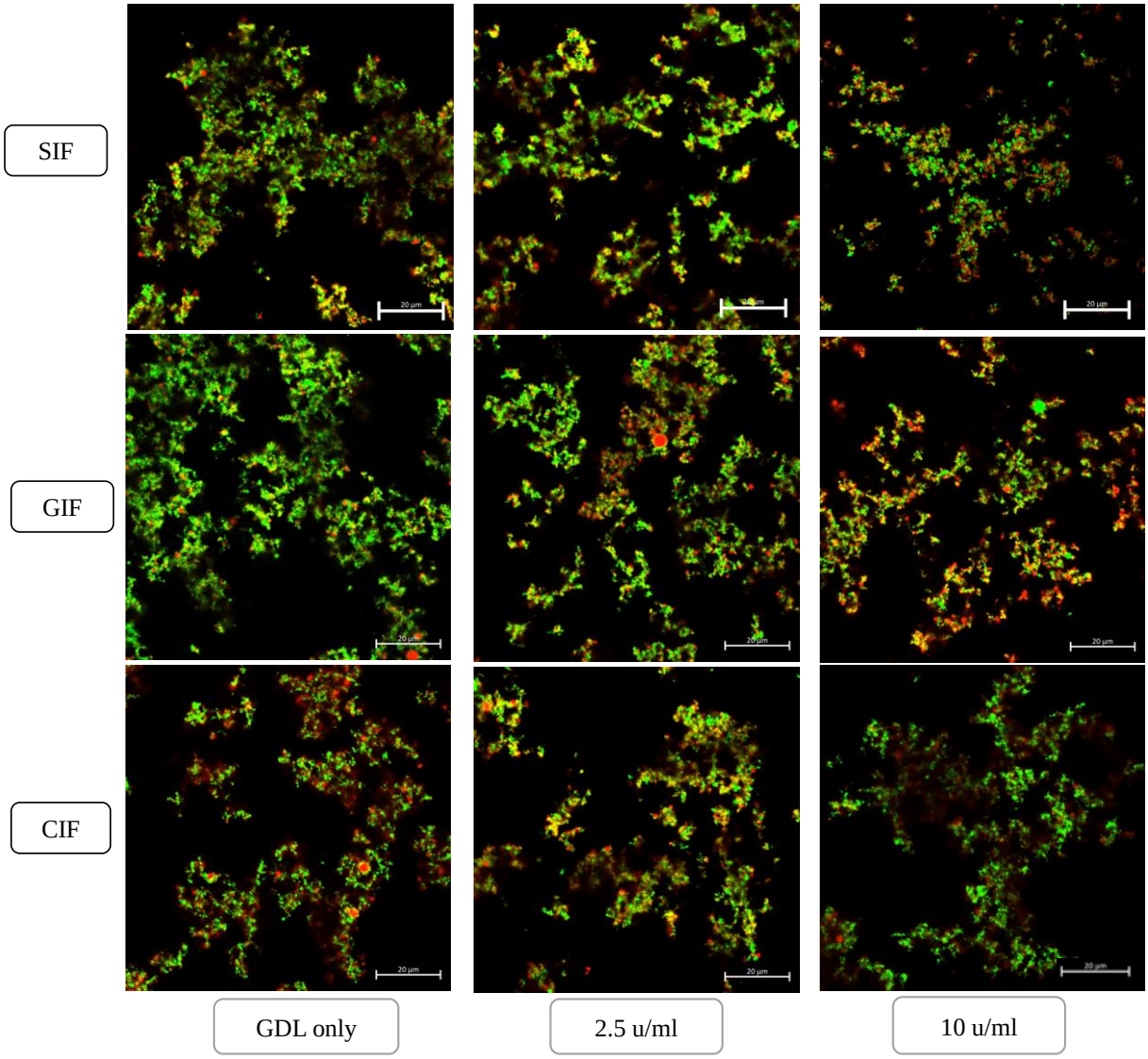


Figure 6.9. Confocal laser scanning microscopy (CLSM) of acid and pepsin induced gels made from SIF, GIF, and CIF. Scale bars represent 20 μm.

6.3 Discussion

The rheological characteristics of the acidified sheep, goat and cow infant formula were different. This could be ascribed to the different buffering capacities (Roy et al., 2020b). The gelation pH of acid CIF, GIF and SIF were 5.27 ± 0.01, 5.26 ± 0.03, and 4.99 ± 0.02 respectively (Table 6.1 and Figure 6.6), which were all higher than the isoelectric point of casein (pH 4.6) (McSweeney & Fox, 2013). This could be due to the samples in this experiment being whey-protein dominated infant formulae. There are different isoelectric points of casein and whey proteins. The isoelectric point of caseins is 4.6 (McSweeney & Fox, 2013). When the pH is

reduced under the isoelectric point, both individual caseins in sodium caseinate and casein micelles can be aggregated by hydrophobic interaction (Horne, 2020). However, milk-clotting enzymes such as pepsin could induce casein micelles coagulation in the gastric condition, but not the individual caseins (Wang et al., 2018; Ye, 2021). Whey in certain concentration is gelled at pH ~5.0 – 5.2 (Ju & Kilara, 1998; Kharlamova et al., 2018). The major whey proteins are β-lactoglobulin and α-lactalbumin. The isoelectric point of β-LG and α-LA are 5.1 and 4.8 respectively (Relkin & Mulvihill, 1996).

The storage modulus G' and loss modulus G" of three acid-induced infant formula gels gradually increased with time. The final G' followed GIF > CIF > SIF (Figure 6.2). The observation was opposite to the findings in the study of raw milk by Roy et al. (2020b), which reported the final storage modulus of acid-induced raw milk were sheep milk gel > cattle milk gel > goat milk gel. The higher final G' value in raw sheep milk gel was due to the higher total protein and total solids (Ould Eleya et al., 1998; Wang et al., 2019; Ye, Cui, et al., 2019). However, in the present study, infant formulae were at similar protein content and casein to whey protein ratio, and they could have been processed differently in manufacturing. The gelation properties of infant formulae have been changed compared to the raw milk, which could be related to many factors, such as the casein compositions, casein micelle conformation, heat treatment, salt contents etc (Anema, 2008; Gamble, 1939; Lakemond & van Vliet, 2008; Rabiey & Britten, 2009). According to Park (2007), heat treatment could reduce the viscosity, while promoting the coagulation point.

For gel induced by combined with GDL and pepsin, the results of three infant formulae all indicated that the gelation time was decreased after pepsin was applied along with GDL, and the gelation pH increased (Table 6.1 and Figure 6.7 – 6.8). However, the concentrations of pepsin have little impact on the gelation time and pH. In addition, the final storage modulus (G') of cow infant formula had different responses to the goat and sheep IF. With pepsin applied and increased the concentration, the final G' showed a decreased trend. On the contrary, the final G' value of GIF and SIF were increased with the pepsin content (Figure 6.9). However, there were only slight changes in infant formulae final G' compared to the control (whole cow milk). The shortened gelation time and higher gelation pH in the pepsin and GDL induced gel could be due to the action of pepsin on casein micelles, which were started with hydrolysing

the κ-CN at high pH of ~6 (Jasińska, 1995; Lucey et al., 2000; Yang et al., 2022). The different gelation properties could be susceptible to the different casein micelle sizes and structures, as well as the different mineral contents in the infant formulae (Roy et al., 2020b). However, the casein concentrations in infant formulae were low, in such the influence of pepsin concentrations was unobvious. This was also proved by the confocal laser scanning microscopy (CLSM) of acid and pepsin induced gels made from SIF, GIF and CIF. There was no significant discrepancy in structures among the gels formed by the three infant formulae.

6.4 Conclusions

The effect of milk protein composition on the acid and pepsin-induced coagulation of commercial infant formulae was examined using rheology measurement. This study provides insight into the different rheological properties of infant formulae from different species, which may be related to the different physicochemical properties, different protein compositions of milk from different species, and other factors such as the heat treatment, casein micelle sizes and structures, and the different mineral contents (Lakemond & van Vliet, 2008; Rabiey & Britten, 2009). Furthermore, the results of combined gel induced by Glucono-δ-lactone (GLD) and pepsin showed that the pH of the gel increased, and the gel time decreased after adding pepsin to the gel. These observations could be attributed to the action of pepsin hydrolysing the κ-CN and changing the casein micelle structure (Jasińska, 1995; Yang et al., 2022). However, different infant formulae respond differently to the pepsin levels.

Furthermore, all three infant formulae gels had low firmness, with no significant difference in structure compared to the whole bovine milk control. The observations could be due to the different casein compositions, Overall, the results from this experiment could be used to understand the coagulation and digestion behaviours of infant formulae made from cow, sheep and goat milk. A further investigation of infant formula manufacture processes and the combination gels of casein-dominated infant formulae would help to better understand the gelation properties in infant formulae from different species.

Chapter 7: Overall Conclusions and Recommendations

This study provides fundamental insight into the understanding of the comparative simulated gastric digestion behaviour and the rheological properties of commercial infant formulae made from cow, goat, and sheep milk. The overall discussions of the three parts of this study and the recommendations for further work are concluded in this chapter.

7.1 Overall conclusions

The first part of this study suggested that different protein compositions influenced the digestive behaviour of infant formula but only had little effect on emptied digesta. During simulated dynamic gastric digestion, goat infant formula chyme showed aggregates earlier than cow and sheep infant formulae, and the microstructure of aggregates existed as porous and fragmented structures. The fragile and porous structure makes pepsin more accessible and hydrolyses proteins faster. The results may be related to the different protein compositions of infant formulae, especially the different casein compositions. In contrast, no significant differences in protein and fat contents were observed in the emptied digesta of the three infant formulae. Only minor differences in particle size and proteolysis were observed at the late stage of digestion.

Gastric digestion is affected not only by the different protein compositions but also impacted by the different processing such as heat treatment. The second part of this study investigated the effect of heat treatment (90°C, 5 min) on the simulated gastric digestion behaviour of commercial infant formulae. Based on the observation of the microstructure and particle size distribution of chyme aggregates, the heated CIF and GIF exhibited loose, and fragmented structures compared to the unheated ones. However, there was no significant difference between heated and unheated SIF, which may be due to the manufacturing process of sheep infant formula having been already heat treated. Whey protein nitrogen index (WPNI) analysis was carried out at the Nutrition lab. The tests of sheep and goat infant formulae were unable to be completed due to a cloudy filtrate, and the WPNI of cow infant formulae was 5.3 mg/g.
The final part of this study was to use rheological measurements to determine the effect of milk protein composition on acid- and pepsin-induced coagulation in commercial infant formulae. The gelation pH and rheological properties differ among the three acidified formulations,

which may be due to different protein compositions in the three samples and other factors such as heat treatment. In addition, the results of the composite gel showed that the gelation time was shortened, and the gelation pH increased when pepsin was applied together with GDL in the three infant formulae. These observations can be attributed to the action of pepsin, which hydrolyzes κ-CN and alters the casein micelle structure (Jasińska, 1995; Yang et al., 2022). However, the differences in gel stiffness and structure between the three samples in this study are subtle.

7.2 Recommendations for future work

There are limitations in this study and further research is recommended, specifically focusing on the areas listed below:

- Understanding the raw material and manufacturing process of commercial infant formulae.

In the current study, infant formulae were purchased at the local market. The raw material and the manufacturing process of each product were unknown. The source of raw material and the manufacturing process of infant formulae significantly impact the result of the experiment. Therefore, it is important to understand the commercial manufacturing process and keep all samples in a consistent process.

- The effect of different casein to whey protein ratios in simulated gastric digestion behaviour of infant formulae from different species.

The samples of the present study were whey protein-dominated infant formulae. Due to casein and whey protein having different coagulation and digestion behaviour, a further study of casein-dominated infant formulae from different species could be carried out to understand the effect of different casein to whey protein ratios in gastric digestion behaviour.

- Different protein content in sample milk.

The total protein content of the sample milk in this study was 1.565% w/w by feeding instruction. A different protein level could be carried out in the gastric digestion and rheology analysis to fully understand the effect of the difference in infant formulae make with milk from different animals.

- Understand the role of other ingredients in infant formulae digestion.

There are added ingredients in the infant formulae to help newborn's health and growth development, such as vitamins, minerals and probiotics. It is important to understand the role of these ingredients in infant formula gastric digestion.

- The effect of fat composition on simulated gastric digestion behaviour of infant formulae from different species.

Gastric lipase (HGL) is another important enzyme in the gastric secretion which catalyzes the hydrolysis of lipids. The conditions of HGL in the infant are different to that in the adult gastric phase. As such, a further study of the effect of fat composition in gastric digestion of infant formulae from different species could be investigated.

- Quantification of the nutrition delivered to the intestine from empty digesta.

The methods in the current study are only qualitative methods. More sophisticated methods such as HPLC and Quantitative amino acid analysis (qAAA) may provide more details around the comparison of the nutrition in empty digesta delivered to the intestine.

- The effect of protein composition and heat treatment of infant formula on the *in vivo* gastrointestinal digestion.

The current study was carried out *in vitro* dynamic digestion. A further *in vivo* digestion experiment could be carried out to observe the digestion behaviours of cow, goat and sheep infant formulae to confirm the findings in the present study.

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