Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Pepsin-induced coagulation and *in vitro* dynamic gastric digestion of model infant formulae

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Food Technology

at Massey University, Manawatū, New Zealand

Cenyi Ouyang

2021

Abstract

The protein composition in infant formulae impacted on the protein digestion and the curd formation. This research was conducted to investigate the rheological properties of pepsin-induced coagulation and the digestion behaviours of a model infant formulae combined with different protein composition, with a focus on the effect of the casein to whey protein ratio on protein hydrolysis during gastric digestion.

Four infant formulae, Sample 0, Sample 40, Sample 60 and Sample 80 were used in current study. Their compositions were classified by the ratio of casein to whey protein: 0:100, 40:60, 60:40 and 80:20, respectively. By rheological measurement of all gels of Sample 0, G', G'' were slightly decreased around pH 4.0. During gastric digestion, the particle size of Sample 0 was increased, but the increasing rate was slightly decreased from 100min at pH 4.20±0.15 to 180 min at pH 2.61±0.20, which could be due to the reorganisation or partially collapse of its flocculates. At the end of gastric digestion, the pH of gastric digesta from Sample 0 reached the lowest value (pH 2.61±0.20), intact β-Lg in the digesta was detected by SDS-PAGE.

For infant formulae containing casein, the gels of Sample 40, 60 and 80 induced by pepsin were significantly impacted by the ratio of casein to whey protein. With the increasing content of caseins, the final G', $G^*$and breaking stress (σ_{max}) of the obtained gels gradually increased at pH 4.0. For Sample 40, the G^* was increased as function of pepsin concentration, the strongest gel was formed at 2.5U/mL of pepsin concentration. For Sample 60 and Sample 80, the $G^*$of gels reached the highest value at 1U/mL of pepsin concentration, along with the highest stiffness. Moreover, the gelation behaviour of Sample 40, 60 and 80 was examed at 0 U/mL and 2.5 U/mL of pepsin concentration, and the acidification was done by the addition of GDL. For Sample 40 and 60, the breaking stress (σ_{max}) of pepsin-induced gels has smaller value at 2.5U/mL in comparison with acid induced gels. However, the pepsin induced gels of Sample 80 obtained larger value of σ_{max} in comparison with acid induced gels. The results suggested that, Sample 40 and 60 obtained gels at an earlier stage than Sample 80, in both which the extensive hydrolysis resulted in the breakdown of gel structure in the following time. Consequently, the pepsin-induced gels of Sample 40 and 60 are more susceptible to rearrangement and fracture under large deformation than Sample 80.

Furthermore, the casein to whey protein ratio of infant formulae influenced the gelation time and gelation pH. At lower pepsin concentration (0-1U/mL), the infant

formulae with higher casein content obtained a relatively longer gelation time and lower gelation pH, which could be due to increased number of unhydrolyzed κ-casein. At higher pepsin concentration (2.5U/mL), the formation of coagulates was observed in Sample 40, 60 and 80 above pH 6.0, and the gelation time of them was below 5 min. By analysing the result of SDS-PAGE, the formation of para-κ-casein was started at 5 min at either lower pepsin concentration (1U/mL) or higher pepsin concentration (2.5U/mL). During gastric digestion, the coagulation of Sample 60 was observed at 20 min, but the coagulation of Sample 80 was occurred between 20 min and 60 min.

During gastric digestion, the ratio of casein to whey protein plays an important role in the coagulation behaviour and hydrolysis rate of Samples 40, 60 and 80. Comparing with Sample 0, smaller mean particle size of flocculates were observed in the digesta of Sample 40. It could be due to that the casein-covered oil droplets contributed to the high stability emulsion. The pH of Sample 40 reached pH 3.79±0.13 at the end of digestion, intact β-Lg and α-La was remained in the digesta, which could be due to that the pH value of it was much higher than the final pH of Sample 0 and the optimal pH of pepsin (pH 2.0). Comparing with Sample 40, Sample 60 present a greater coagulation during gastric digestion. At the end of digestion, caseins in the emptied digesta of Sample 60 was fully hydrolysed, no large curd was obtained at end of digestion. For Sample 80, large dense curds were formed and stayed in the stomach.

Overall, the rheological properties and gastric digestion behaviour of infant formulae were affected by its protein composition. The ratio of casein to whey protein impacted on the pH profile, coagulation behaviour and the rate of protein hydrolysis during gastric digestion. These results provide useful information for the design and development of infant formulae by allowing greater control over the manipulation of protein bioavailability.

Acknowledgment

First and foremost, I would like to express my deepest and sincere gratitude to my supervisor Associate Professor Aiqian Ye, who provide invaluable guidance throughout this research. He has taught me the methodology to carry out this research and provided me with encouragement, assistance, insightful comments and extensive personal and professional guidance throughout my Master study. His profound professional knowledge, rigorous academic attitude, excellence in the work style, keen insight on scientific research work is my lifelong learning model.

I also would like to express my appreciation to Ms Maggie Zou, Mr Warwick Johnson who provided me lab orientation, permitting me to use all required equipment and provide chemical materials to complete my research. I also would like to thank Mr. Chris Hall and Mr. Jian Cui, who provided me training and technical guidance in my overall lab experiments.

A special gratitude I give to Dr. Nan luo who gave me incredible support and guidance during the course of this research.

Furthermore, I also appreciate all the staffs and researchers at Riddet Institute for their help and encouragement. I am also grateful to my friends and flatmates who for their concern and encourage.

Last but foremost, I really appreciate my parents for their love, understanding, and financial support. I would also like to express my thanks to my grandma for her care, encouragement and prayers. Their love and support encourage me to overcome all difficulties in my life.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

GDL: Glucono-δ-lactone

α-La: α-lactalbumin

β-Lg: β-lactoglobulin

CCP: Colloidal calcium phosphate

HGS: Human gastric simulator

WPI: Whey protein isolate

SDS-PAGE: Sodium dodecyl sulfate-poly acrylamide electrophoresis

SGF: Simulated gastric fluid

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

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

p*I*: Isoelectric point

w/w: Weight/weight

w/v Weight/volume

GT Gelation time

Chapter 1: Introduction

Infant formulae are the substitutes of human milk, which provides sufficient nutrition for infants during the first few months of life. Historically, the formulation of infant formula was guided by the protein composition of human milk. The whey protein dominant infant formula (40:60, casein: whey protein) mimicked the casein to whey protein ratio in human milk (Packard, 1982). To meet the special nutritional needs, casein-dominant infant formulae (60:40 or 80:20, casein: whey protein) are developed, which could be more satisfying for hungry infants or older babies than whey-protein dominant infant formulae (Blanchard et al., 2013). Previous studies have shown the effect of casein to whey protein ratio on the gelation properties of acidified milk (Zhao et al., 2016), and the effect of protein composition on the physiological properties of infant formulae *in vivo* piglet digestion (Tari et al., 2018), while the research on the rheological properties of infant formula during acidification was limited, and the digestion behaviour of infant formulae made with different casein to whey protein ratios required further study.

Recently, previous research provided a novel insight into the rheological properties of goat milk and bovine milk under acidification (Wang et al., 2019). They proposed that the ratio of casein to whey protein could affect the gelation behaviour of infant formulae, the higher content of caseins in infant formulae led to the formation of stronger acid-gels. The mechanical properties were related to the rheological properties of infant formulae, the digestion behaviour of infant formulae could be consequently impacted.

The digestion behaviour of infant formulae made with goat milk and bovine milk with different casein to whey protein ratio was studied by Ye et al. (2019b). They indicated that the protein composition could affect the coagulation of protein and the flocculation of oil droplets. The higher ratio of casein to whey protein could cause the greater coagulation at an early stage, the digestion rate of casein could become slow.

The main objectives of the present study were as follow:

1) To explore the gelation properties of infant formulae with different casein to whey protein ratios induced by GDL and different pepsin concentration. The different ratios of casein to whey protein in infant formulae used in this study were: 0:100, 40:60, 60:40, and 20:80. The gelation time and pH

would be discussed and the physiological characteristics of milk gels would be analysed.

1) To understand the digestion behaviour of infant formulae with different ratios of casein to whey protein during *in vitro* dynamic digestion. The structure changes and the hydrolysis rate of protein in different infant formulae would be compared and investigated.

These objectives have been completed through two research chapters in this study. The pepsin-induced gelation behaviour of different infant formulae has been reported in Chapter. 4. The *in vitro* gastric digestion behaviour of different infant formulae has been studied in Chapter.5.

Chapter 2: Literature review

2.1 Infant formula

Infant formula is a substitute of human milk for under 12 months old infants. When mothers are not able to feed their children, infant formulae can provide abundant nutrition to meet the need for infant growth. Infant formula historically has a goal of imitating human milk to be an essential source of nutrition to infants. Recently, it has a more realistic goal to attempt to include the various functionalities of ingredients, which are proteins, lipids, carbohydrates, macronutrients and micronutrients (vitamin minerals), as well as some chemical entities which is not found in human milk (Callaghan et al., 2011; Lonnerdal, 2014). In this section, bovine milk and human milk were compared at the beginning, and the bovine milk-based infant formula was introduced.

2.1.1 Principal differences between bovine milk and human milk

The composition of significant micronutrition has been given below (Table 2.1). Bovine milk contains a higher level of protein than human milk. However, the fat content in them is similar from 3.5 to 4.5 % (w/w).

Table 2.1. The gross composition of bovine milk and human milk (%w/w) (Ballard et al., 2013; Jensen, 2002; Packard, 1982)

Component	Bovine milk	Human milk
Fat	3.7	1.3
Protein	3.3	1.06
Lactose	4.7	6.9
Water	87.6	87.4
Average pH	6.6	7.0

Moreover, not all nitrogen in milk is from protein, a group of nitrogen in milk is classified as nonprotein nitrogen (NPN). In bovine milk, the level of NPN is 5%, and the NPN level has been increased five folds in human milk (Packard, 1982).

In principle, based on the differences in needs between calves and baby, the protein compositions of bovine milk and human milk are different (Table 2.2.). About 80% of the protein in bovine milk consists of casein that could be precipitated from skim

milk at pH 4.6 and at temperature 20℃, which is mostly present in casein micelles at neutral pH at room temperature. The soluble protein remaining after precipitation of caseins in milk is whey protein, also called milk serum protein. The corresponding figures for breast milk are 40 % and 60% (McSweeney et al., 2013; Packard, 1982). Moreover, the fractions of casein and whey protein are varied between bovine dairy and breast milk. With respect to the casein fractions, α_{s1}-caseins dominate in bovine milk at 12-15g/L, while β-caseins have the highest concentration in human milk among other three casein fractions (Liao et al., 2017). For most mammals, β-Lg is the most abundant protein bovine in milk, which present 10% in total protein and 50% in total whey protein. However, in the case of human milk, β-Lg is ordinarily absent, and α-La is the dominant protein, around 36% in total whey protein. For lactoferrin, which can help baby absorb iron from food, one of the primary whey proteins second to α-La in human milk (12% of total protein), while it only constitutes some significant fractions in bovine milk. The concentration of predominant immunoglobulin fractions is low in human milk; the level of lgA content is the highest. However, in bovine milk lgG is the significant immunoglobulin fraction and the content of it is ten times the lgA content (Hernell, 2011; Walstra et al., 1984).

Table 2.2. The major components of protein in bovine milk and human milk(g/100mL) (Layman et al., 2018; Morris, 2002)

	Bovine milk	**Human milk**
Total protein	3.6g/100mL	0.95g/100mL
Caseins	2.800	0.380
α_{s1}-casein	1.232	0.046
α_{s2}-casein	0.308	0
β-casein	0.700	0.258
κ-casein	0.420	0.160
γ-casein	0.140	ND
Whey protein	0.800	0.570
β-lactoglobulin	0.416	0
α-lactalbumin	0.136	0.205
Serum albumin	0.040	0.034
Immunoglobulin (lgG, lgA, lgA)	0.080	0.097
Lactoferrin	0.012	0.143
Glycomacropeptide	0.096	ND
Other	0.020	0.057

2.1.2 Ingredients in infant formula

Normally, the rate of whey protein in infant formula accounts for 60%, followed by casein presented 40% (Blanchard et al., 2013). Moreover, there are some proteins, such as β-Lg and α_{s2}-casein, are not found in breast milk. Some proteins, like lactoferrin, β-casein and α-La, are attempted to be added into infant formula. Furthermore, fat globules membrane composition, the protein layer on droplet surface and micronutrient contents are different between human milk and bovine milk. Besides, the n-3 and n-6 long-chain polyunsaturated fatty acid are essential in human milk but absented in infant formula (Ballard et al., 2013; Jenness, 1979). Therefore, several novel technologies are applied to producing infant formula to mimic human milk and improving the quality of it. However, the current types of infant formula are continuedly being found deficient in essential nutrients (Callaghan et al., 2011).

Over the years, the composition of infant formula has been updated in response to scientific evidence of need. All of the alterations reflect the utilise of infant formula more nearly the design of breast milk. Generally, infant formula should provide fat at 30-54%

of calories, protein at 7-16% of calories (Koletzko et al., 2005). Because of the differences in composition between bovine milk and breast milk. Milk-based infant formula must consider the requirement to change the design of bovine milk. Firstly, protein content should be reduced. Secondly, the fat composition and mineral content should be adjusted. However, for decreasing the level of mineral and lactose, some new technologies find important implication. These technologies include electrodialysis (lowering mineral content), gel filtration (separate various protein fractions), ion exchange and ultrafiltration (Walstra et al., 1984).

For analysing the physical, chemical and quality of recombined dairy products, it is fundamental to study the ingredients for combination, which should include the controlled standard and adequate technical specifications with a system of rules for sampling and analysing (Federation et al., 1990).

2.1.2.1 Whey protein isolate

Typically, the whey-based products are whey protein concentration WPC35, WPC80, and whey protein isolate WPI. The proximate composition (dry basis) of them are summarised in Table 2.3. For meeting the requirement of high-protein content whey product (WPC 80), the process-facilitated reductions in lactose and ash are required. For producing WPI, the intervention of reducing lipid content associating with WPC 80 is necessary to increase the protein content to 90%. The most common commercially technology of whey-based ingredients production is ultrafiltration (UF) (Deeth et al., 2018).

Table 2.3. Proximate composition of commercially EPC35, WPC80 and WPI (Deeth et al., 2018)

Composition	WPC 35 (%)	WPC 80 (%)	WPI (%)
Protein	35	80	90
Fat	4	8	1
Lactose	60	3	1
Ash	6	3.5	4
Moisture	5	6	6

2.1.2.2 Skim milk powder

The milk powder used in the recombination process is skim milk powder (SMP), whole milk powder (WMP) and buttermilk powder. The composition of these protein ingredients is adjusted and designed for a specific application in manufacture. The typical arrangements for SMP and WMP are listed in Table 2.4.

Table 2.4. Approximate composition of SMP and WMP(Federation et al., 1990)

Composition	SMP (%)	WMP (%)
Protein	36-39	26-29
Fat	1	26-28
Lactose	49-54	37-40
Ash	7.4-7.8	5.3-6.3
Moisture	4	2.5

Physical properties of SMP and WMP recommended for recombination, and the heating temperature requirements of milk powders for recombined liquid dairy products are listed in Table 2.5. Comparing two types of milk powders, SMP has higher solubility and larger particle size in solution, but it requires a lower heating temperature than WMP in the recombination process(Federation et al., 1990).

Table 2.5. Physical properties and processing requirements of SMP and WMP(Federation et al., 1990)

	SMP (%)	WMP (%)
Heating temperature requirements	Low	Medium
Solubility (ml)	0.25	0.1
Particle size (μ)	<100	<200

2.1.2.3 Lipid and soy lecithin

Based on the food and agriculture organisation/world health organisation allowance for emulsifier in infant formula, the content of soy lecithin in infant formula is 0.5g/100ml (FAO) or 0.4g/100ml (WHO)(Walstra et al., 1984).

The recommend lipid content in infant formula is 4.4-6.0g/100kcal, which is similar to the value found (40-54% of energy content) in breast milk (Koletzko et al.,

2005). In terms of matching the oil content of breastmilk, the mixture of vegetable oil is recommended to match the total fatty acid profile of human milk. However, the limitations are the structural difference in triglycerides between milk fat and vegetable fat.

2.1.3 The processing technology for all kinds of infant formula

There are two processing lines for producing powdered infant formula, which are dry blending process and wet mixing-spray drying process. For making liquid infant formula, the technological procedures like recombination and standardisation and oil addition, sterilisation and homogenisation are adopted. Generally, the production of infant formula involves several steps: the mix of milk proteins and their hydrolysates, lipids, carbohydrates, vegetable oil, vitamin and minerals, followed by pasteurisation, homogenisation, the next is standardisation (Bhandari, 2013). The final steps of wet mixing-spray drying process are evaporation and spray drying. However, those procedures have typical disadvantages. For example, the high quality of raw materials is demanded in the dry mixing process due to the incorporation oil and undermixing problems. The quality of water is important to avoid microbiological contamination (Bhandari, 2013).

2.1.3.1 Pasteurisation

During processing, infant formulae are heat-treated to maintain the bacteriological quality of the product, extending the shelf-life of milk products (Lonnerdal et al., 1998). However, the heat treatment of infant formula might change the digestibility of milk protein on infants (Callaghan et al., 2011). Changes caused by heat treatment may be reversible or irreversible depending on the temperature with temperatures below 60°C generating reversible changes. Heat treatments used in the dairy industry are summarized in Table 2.6.

Standard pasteurization of 72.5°C 15 s does not cause any damage to the milk, apart from some small effects on vitamins, but inactivates most microorganisms and some enzymes. To suppress lipase activity in homogenized milk, more intense heating (75°C for 20 s) is required.

High temperature is more intense, the temperature is around 90℃ for 15s, which kills most vegetative microorganism and most enzymes, but it leads a part of whey protein to be insoluble and causes -SH group to be exposed. Sterilisation (20min at

118℃) and UHT (ultra-high temperature) heating (145℃ for few seconds) are normally applied on dairy process, but both of them will cause numerous chemical changes, such as browning reactions occurred and formic acid is formed. (Walstra et al., 1984).

Table 2.6. Major heat treatments applied in dairy industry (Deeth, 2017; Walstra et al., 1984)

Heat treatment	Temperature and time	Bacteria destroyed	Advantages	Disadvantages
Thermization	57-68℃/5s-30min	Psychrotrophic bacteria and some non-spore forming pathogens	No other irreversible changes	Products may not be phosphates negative and not ready for drink.
Low pasteurisation	63℃ 30min/72℃ 15s	all pathogenic organisms and some vegetative microorganisms	Very little other irreversible changes	Long time heating would lead to a receptive change of flavour
High pasteurisation	85-100℃ 20s	All vegetative microorganisms	Bacteriostatic system is largely inactivated	A cooked flavour arises
Sterilisation	130-145℃/ 1-30s	All microorganism and bacterial spores	Most enzymes are inactivated	The colour and flavour are changes the nutritive value is diminished

2.1.3.2 Homogenisation

Milk is an oil in water emulsion, and the oil droplets disperse in the continuous serum phase. However, the larger fat globules would rise and form a cream layer if raw milk were left for one hour without any treatments. The primary purpose of homogenisation is disrupting oil droplets into small globules, which slows down creaming. It also enlarges the fat layer and then produces denuded fat. The denuded fat is

covered largely by plasma protein, and partly casein micelles and whey protein. The figure of denuded fat has been shown in Figure. 2.1. For decreasing the interfacial tension, emulsifiers such as soy lecithin are generally added into dairy before homogenisation(Latreille, 1990).

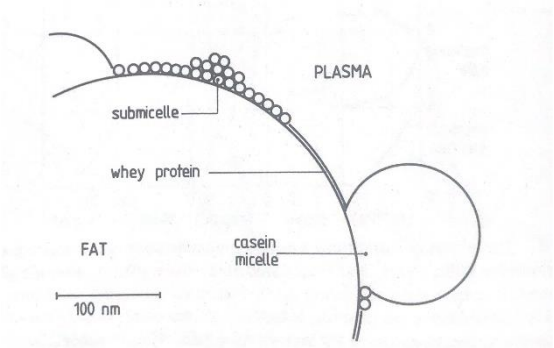


Figure 2.1. The schematic figure of fat globules (Walstra, 1995)

As mentioned in lipid section, the particle size of oil droplets of infant formula are that, mode diameter= 0.4±0.0μm; $d_{4,3}$ = 2.3±0.0μm; $d_{3,2}$=0.4±0.0μm.

Commonly, two stages of homogenisation is applied in the milk industry. The first stage is for reducing the size of fat globules; there is a tendency for clustering of oil droplets. The second stage separates those clusters into individual small oil droplets. Before homogenisation, the milk should be reheated to 60℃, for increasing the stability of homogenised milk. Reconstituted model infant formulas were homogenised at 140 and 40 bar pressure for the first and second stages in Toikkanen et al. (2018)

2.2 Milk protein

2.2.1 Chemistry of milk protein

The primary structure of the protein are polypeptides consists of amino acid sequence connected by a series of peptide bonds. Folding linear amino acid sequence into a more compact structure could be considered to have resulted from hydrogen bonding between amino groups and interaction of carboxyl groups with the amino group in the neighbouring regions. The example of them is the stable folding pattern alpha helices and beta sheets, which develops the secondary structure of proteins (Walstra et al., 1984). The ensemble of further folding and coiling polypeptides constitute a complex three-dimension shape called the protein tertiary structure. One polypeptide adopts tertiary

structure individually called subunit, and multiple subunits assemble and compactly fold into spherical or ellipsoidal units is referred to as the quaternary structure of a protein (Walstra et al., 1984).

2.2.2 Milk protein composition

Casein and whey proten both are the assembly of amino acids, and they are the most important protein source at biological value. The physical properties between them provided in Table 2.7.

Table 2.7. Comparison of the physical properties between whey protein and casein in bovine milk(Fox, 2003)

	Casein	**Whey protein**
Structure	Coil structure	Well-defined tertiary and quaternary structure
Amino acid composition	Low in sulfur-containing amino acids. High in proline	High in sulfur-containing amino acids but low in proline
Physical state	Large colloidal aggregates (casein micelles	Globular proteins in the form of monomer-octamers, depending on pH
Solubility at pH 4.6	Precipitated at pH 4.6	Soluble at pH 4.6
Heat stability	Very heat-stable	Heat-labile (completely denatured at temperature 90℃)
Coagulation by proteolysis or ethanol	Coagulated by chymosin (rennet or pepsin) and ethanol	Not sensitive to enzymes or ethanol

2.2.3 Whey protein

When caseins are precipitated at pH 4.6, several proteins are left in the supernatant, which is collectively called whey protein. The major whey proteins in bovine milk include β-Lg, α-La, bovine serum albumin (BSA), immunoglobulin, lactoferrin and protease peptone. Whey protein is a normally globular protein with compact structure, subject to heat denaturation. And it is tightly coiled and folded into a somewhat spherical

shape, and β-Lg could be unfolded when the temperature is above 60℃. Since the temperature is increased, the solubility of it is also reduced. The content of β-Lg and α-La is different between bovine milk and human milk, which has been compared in protein composition section. And the information about them was shown below (Morand et al., 2012; Morr et al., 1993).

2.2.3.1 β-lactoglobulin

β-lactoglobulin (β-Lg) carries 162 amino acids, and the molecular weight of it is 18.3 kDa. It belongs to the lipocalin family of protein because it can bind small hydrophobic molecules into a hydrophobic cavity. Consequently, the β-Lg functions as a transport protein, especially for vitamin A and other retinoid species(Edwards et al., 2008).

There are several genetic variants of β-Lg. The most abundant genetic variants are β-Lg A and β-Lg B. In Figure 2.2, β-Lg in bovine milk exists as a dimer of two monomeric subunits noncovalently linked. When at least two genetic variants of it are present, the hybrid dimers are formed. The structure of monomeric subunits and dimers are highly depended on the temperature, pH and ionic strength in solution (Edwards et al., 2008).

The thermal properties of β-Lg are crucial. At natural pH, when the temperature of dairy is around 70℃, the protein dimer dissociates, and the unfolding transition of β-Lg occurs. The free thiol Cys121 located at the end of H-strand in the C-terminal would have the inter-molecular association with hydrophobic residues. A patch of disulfide-bonded polymers would be formed by ensuing disulphide interchange reactions. Furthermore, the reversibility and the rate of β-Lg denaturation are highly depended on pH, ionic environment, protein concentration, genetic environment, the addition of ligands and its genetic variant. For example, at low pH with the presence of ligands, protein in milk resists to heat-induced denaturation(Edwards et al., 2008).

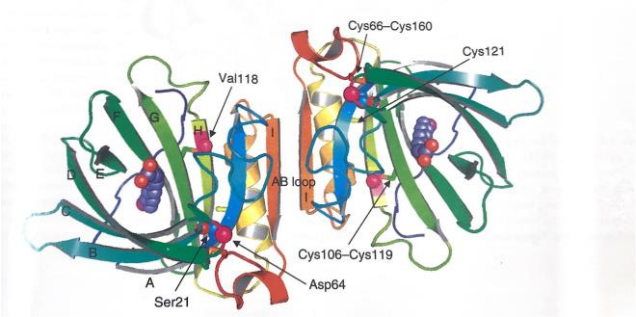


Figure 2.2. The three-dimensional structure of bovine β-Lg (Edwards et al., 2008)

2.2.3.2 α-lactalbumin

α-lactalbumin (α-La) carries 123 amino acids, and the molecular weight of it is 14.2 kDa. It is a calcium metalloprotein, and X-ray crystallography shows that the structure of it shares 72% homology with lysozymes (Fenelon et al., 2019). There are two domains in native α-La: α-helical domain and β-sheet domain, two domains are connected by Ca^{2+} ion binding loop. In Figure 2.3, four ball-dashed line-stick presentations indicate four disulphide bonds. Calcium ion co-ordinates with carboxylate oxygen atoms and water molecules in the ball-dashed line-ball presentation. Two of them linking the helical domain and calcium ion binding loop to the β-sheet domain are on the left of the figure(Brew, 2013; Edwards et al., 2008).

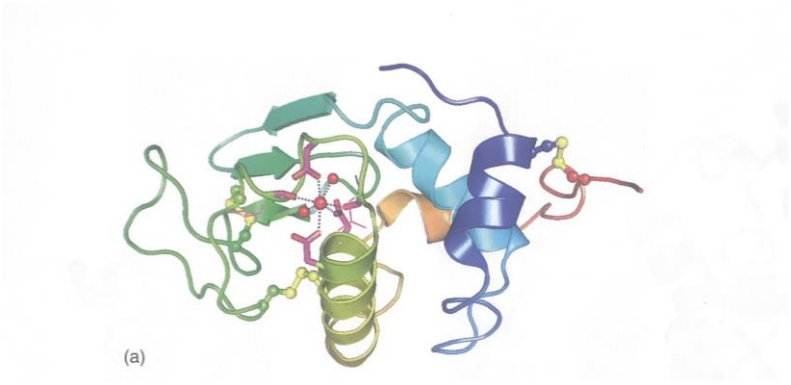


Figure 2.3. The structure of bovine α-lactoglobulin (N-terminus is in blue, C-terminus is in red)(Boland et al., 2020; Edwards et al., 2008)

Commercially, α-La is normally used in infant formula because the structure and composition of it are similar to the protein in human milk. Sandström et al. (2008) assumed that for decreasing the protein content in infant formula and narrowing the gap between infant formula and human milk, the level of α-La could be increased as an amino acid composition. Additionally, the thermal stability of α-La is relatively lower than β-Lg,

and the gelling capacity of it is accordingly insufficient. The denaturation of it is depended on temperature, pressure and metal ion concentration(Edwards et al., 2008).

2.2.4 Casein

Principle casein fraction in bovine milk is α_{s1}-casein (30-40%), α_{s2}-casein (10-15%), β-casein (25-40%) and κ-casein10-15%. The properties of them were shown in Table 2.8.

Table 2.8. Properties of caseins(Huppertz, 2013; McSweeney et al., 2013; Morris, 2002; Schmidt, 1982; Walstra et al., 1984)

	α_{s1}-casein	**α_{s2}-casein**	**β-casein**	**κ-casein**
Molecular Weight (Da)	23000	25000	23983	19023
Proline residues	17	10	35	20
Cysteine residues (-SH)	0	2	0	2
Phosphoseryl residue	8	11	5	1
Calcium-binding (calcium sensitivity)	Strong	Strong	Strong	Weak
precipitates at	>7mM Ca^{+2}	>7mM Ca^{+2}	>0.4M Ca^{+2}	Soluble
Carbohydrates	0	0	0	5%
Ionic strength (M)	0.01	0.2		>0.1

Casein presents four major gene products like α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein (Huppertz et al., 2018) and some minor derivative forms like γ-casein, which is the hydrolysate of β-casein by plasmin. Those genetic variants are resulted from the phosphorylation of casein at seryl and occasional at threonyl residues (McMahon et al., 2013). α_{s1}-casein and β-casein both contain no cysteine residues and 17% proline residues. α_{s2}-casein and κ-casein have two cysteine residues and 5% and 12% proline residues of their total residues. Because of the high proline content of four kinds of caseins, the α-helix and β-sheet structure are not significant in them, and the secondary and tertiary structures are hard to be organised(Walstra et al., 1984). Hence, there is very little structure to fold in them, the thermostability of them is high, and they are not easy to be denatured by heat. Moreover, β-casein possess the highest hydrophobicity among other

three main caseins. α_{s2}-casein obtain the highest charge frequency and the lowest hydrophobicity(Horne, 2008).

Furthermore, all caseins contain the unique ester phosphate groups except κ-casein. Because of the role of phosphate groups in calcium sensitivity, caseins divided into two different groups. One is calcium-sensitive casein such as α_{s1}-casein, α_{s2}-casein and β-casein, and another one is calcium-insensitive casein like κ-casein. All calcium-sensitive caseins are responsible for binding phosphorus and calcium, while κ-casein at neutral pH is for stabilising the micelle structure of casein(Aoki et al., 1985).

2.2.4.1 Casein micelles

Casein in bovine milk mostly present in casein micelles (95% of total casein) with supramolecular structure McMahon et al. (2008). The biological function of casein micelles is to provide a high value of micellar calcium phosphate(CCP). CCP, called colloidal calcium phosphate, is utilised to mammalian baby and form a curd in babies' stomach and suppling the nutrients for the development of infants' bones and teeth. Besides the water (72%) in casein micelles, the dry basis of micelles includes 92% protein, calcium phosphate and a small amount of citrate, minor ions (magnesium, sodium, potassium), lipase and plasmin enzymes, and entrapped milk serum. The approximate composition of casein micelles on a dry basis has been summarised in Table 2.9 (Huppertz et al., 2018; McMahon et al., 1984a).

Table 2.9. The gross composition of casein micelles (McMahon et al., 1984a; Schmidt, 1968, 1982)

Components	Content (%w/w)
α_{s1}-casein	35.6
α_{s2}-casein	9.9
β-casein	33.6
κ-casein	11.9
Minor casein	2.3
Calcium	2.9
Phosphate	2.9
Magnesium	0.1
Sodium	0.1
Potassium	0.3

Components	Content (%w/w)
Citrate	0.4
Sialic acid	0.3
Galactose	0.2
Galactosamide	0.2

2.2.4.2 The structure of casein micelles

Casein micelles are highly hydrated and sponge-like; the hydrophobic interaction provides stability to casein micelles. Surface charge and steric repulsions opposing the van der Waals forces by the κ-caseins plays a vital role in stabilising casein micelles suspension(Dalgleish et al., 2004).

A possible structure of casein micelle has been shown below (Figure. 2.4). Based on scanning electron micrograph, it suggests a near-spherical shape of the particle the diameter, the range of micelles is from 80 to 300 nm. (Dalgleish et al., 2012; Dalgleish et al., 2004; McMahon et al., 1984a).

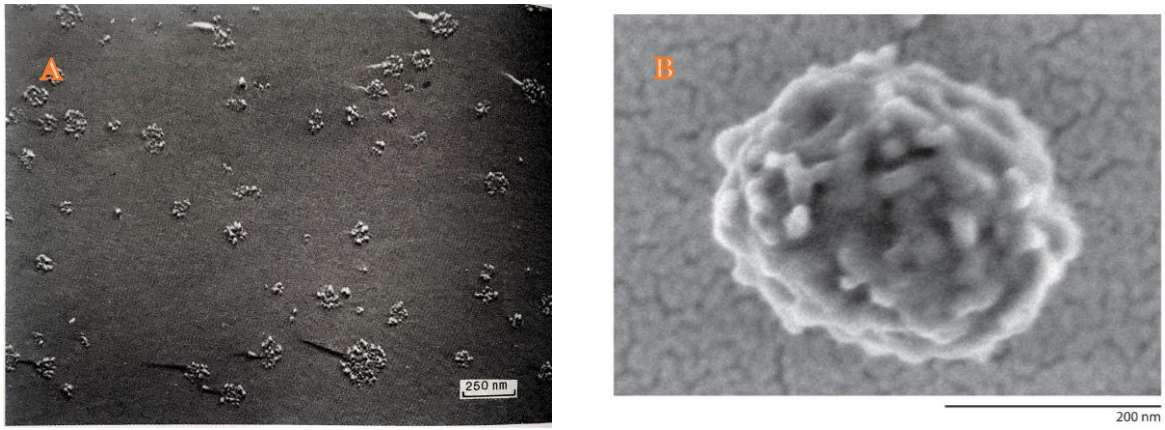


Figure 2.4. Field emission scanning electronic micrograph image of casein micelles (Dalgleish et al., 2012; Walstra et al., 1984)

The structure of casein micelles is assumed to be a complex of several discrete subunits. The characteristics of caseins' properties are provided in Table 2.10.

Table 2.10. Average characteristic of casein micelles (Huppertz et al., 2018; McMahon et al., 1984a)

Characteristic	Value
Diameter	130-160nm
Surface	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density	1.0632 g/cm^3
Mass	2.2×10^{-15}g
Water content	72%
Hydration	3.7 g H_2O/g protein
Voluminosity	4.4 $cm^3 \times 10^9$ daltons
Molecular weight (hydrated)	5×10^8 daltons
Molecular weight (dehydrated)	10^4
Number of peptide chains (MW:30000)	10^{14}-10^{16}
Whole surface of particle	5×10^4 cm^2/ml milk
Mean free distance	240nm

Furthermore, the particle size of casein micelles usually is less than 20 nm (80% of total micelles), but the volume of them is under 3% of the total casein micelles volume. Despite the micelle particles over 20 nm diameter, the average concentration of them is 1.2×10^{14}/ml milk. The mean diameter is about 65 nm, the volume of the particles at mean diameter is 104 nm, the volume of the micelle particles at median diameter is about 134 nm(McMahon et al., 1984a).

The variety of casein micelles models have been discussed over the years. By regular reviewing and appraising of those models, principal contenders have been classified into three categories: subunit or sub-micelle model(Slattery, 1976, 1979; Slattery et al., 1973); coat-core structure models (Dalgleish et al., 2012; Parry Jr et al., 1969; Payens, 1966; Phadungath, 2005); and dual-binding models (Barth et al., 1988; Boland et al., 2020; Horne, 2008); The unique functional properties and structure of casein micelles are related to the specific arrangement of proteins and minerals. The sub-micelle model of casein micelles has been illustrated below.

2.2.4.3 Coat core model

Based on the experiment data, the coat core model of caseins was proposed in the picture (Figure 2.5). In coat core model, α_{s1}-caseins are compactly folded to molecules, and the molecule attached to a loose network of β-caseins, which constitute the core of casein micelles. The surface of this model is a layer of κ-caseins.

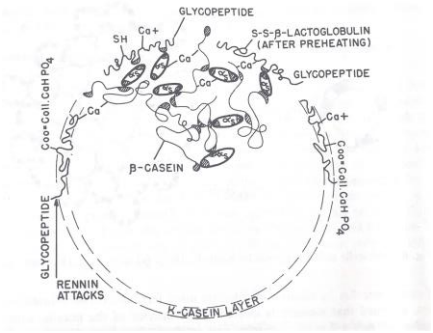


Figure 2.5. The coat core model of casein micelles (McMahon et al., 2013)

2.2.4.4 Dual binding model of casein micelles

Dual binding model of casein micelles is useful and necessary in providing mechanistic understanding of the acidification and gelation of milk protein, and it is also important in analysing the physical properties and micellar assembly of micellar suspensions (Boland et al., 2020). As the view of the dual binding model, the assembly of micellar is a polyfunctional condensation of bonding monomer caseins involving two distinct forms(Horne, 2002).

From recent studies, caseins exhibit strong tendency of self-association in solution, which has been revisited by de Kruif et al. (2012). Each casein molecules contains the structures of hydrophobic residues and hydrophilic residues. The hydrophobic regions, which has been detailed in Figure 2.6: the large circle displays the electrostatic repulsion arising from the negative charge centres, the small circle is the area of hydrophobic attraction in the trains. In the picture, α_{s1}-caseins and β-caseins are adsorbed on to a hydrophobic interface.

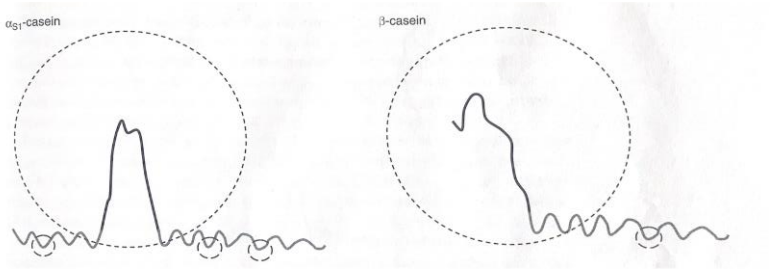


Figure 2.6. Self-consist-field calculation of the conformation of α_{s1}-casein and β-casein, which confirmed that α_{s1}-casein has a train-loop-train structure and β-casein shows a loop-train structure (Horne, 2008)

.

According to the dual binding model, the polymeric structures of α_{s2}-casein and κ-casein has been given in Figure 2.7. In the picture, α_{s2}-casein contains two hydrophobic regions and two phosphoseryl clusters (hydrophilic regions). And κ-casein only contains one hydrophobic region. Based on the self-consistent-field (SCF) calculation of protein, excluded κ-caseins (without clusters), the hydrophilic regions of caseins (consist of the phosphoseryl clusters) provides multiple functionality for crosslinking and increases protein hydrophobicity (Horne, 2011). Due to the interaction of α_{s1}-caseins and β-caseins' hydrophobic regions, the polymeric structure of them are generated (detailed in Figure 2.8).

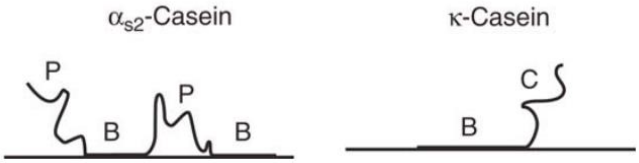


Figure 2.7. B, the hydrophobic regions of caseins; P, the hydrophilic regions which containing phosphoserine clusters; C, hydrophilic carboxyl-terminal (Horne, 2011)

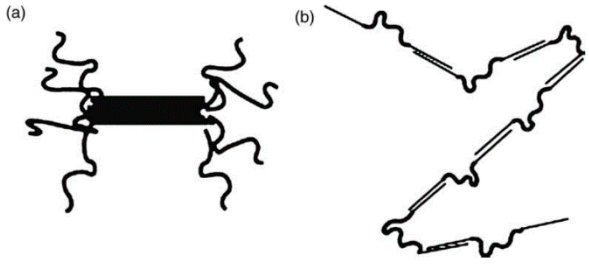


Figure 2.8. The polymeric structure of β-casein (A) and α_{s1}-caseins (B) (Horne, 2011).

In Figure 2.9, based on the hydrophobic interaction, β-caseins resemble a detergent molecule with a hydrophilic head and a hydrophobic tail, and α_{s1}-caseins self-associates in solution to form a wormlike chain polymer. The further growth is limited by the strong local electrostatic repulsion of the hydrophilic regions. Unless, the chain growth through phosphoseryl clusters cross-linkage to a positively charged calcium phosphate nanocluster, which provides the possibility of multiple proteins binding onto the surface of the cluster. The details about the chains have been presented in Figure 2.9, which attach to calcium-phosphate-nanocluster crosslink points to extend the network three-dimensionally (Horne, 2002).

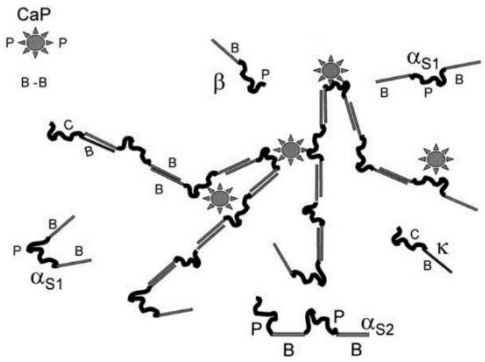


Figure 2.9. The schematic diagram of casein micelle assembly according to the dual-binding model. B, the hydrophobic regions. P, hydrophilic regions. C, the hydrophilic region of κ-caseins(Horne, 2003)

As to κ-caseins in the dual binding model, it contains hydrophobic N-terminal block which can link into growing chains, and hydrophilic C-terminal block which cannot sustain growth by linking hydrophobically to another casein molecules. Due to the lack of a phosphoserine cluster, κ-caseins cannot extend the polymer cluster through a calcium phosphate nanocluster link. Hence, wherever κ-caseins joins the chain, the growth of chain and network could be terminated immediately. κ-casein limited the process of self-association leading to stabilisation of casein micelles. Hence, the out layer of caseins in the network of κ-caseins has been illustrated earlier in this article (Barth et al., 1988; Boland et al., 2020; Horne, 2008).

2.2.4.5 Sub-micelle model

The first sub-micelle model was suggested by Morr, (1967). In the earliest model, the assembly of casein micelle is formed by casein particles which were glued by calcium

phosphate. The core and the surface of the sub-micelles consist of α-caseins and κ-caseins. Slattery et al. (1973) further declared that because the hydrophilic κ-caseins regions (C-terminal) are exposed in the solvent, the growth of casein micelles will be terminated when κ-caseins cover the surface of the micelles. Subsequently, calcium phosphates are responsible for the binding of sub-micelles. By binding Calcium-ions to the caseins, the negative charge of submicelles is reduced, and the hydrophobic interaction between submicelles has been modulated. The presence of inorganic phosphate provides electronic interaction between sub-micelles in the interior; the calcium phosphate bridges build up between the eater phosphate of adjacent casein micelles(Schmidt, 1982; Slattery, 1979). In Figure 2.10, An extension of the sub-micelle model described that there are two types of submicelles, the one is depleted in κ-caseins which is in the interior of the casein micelles (on the right), while the rest is rich in κ-caseins which occupies the surface position(on the left). It was, therefore, a straightforward hypothesis to postulate that the hairy layers (κ-caseins) contribute to prohibit the growth of casein micelles by local steric repulsion and electrostatic stabilisation (McMahon et al., 2008; Walstra et al., 1984).

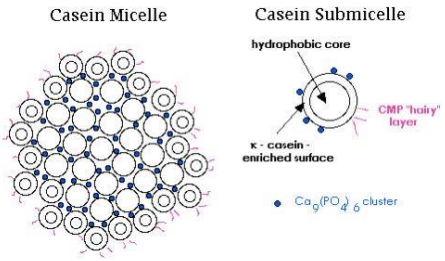


Figure 2.10. Sub-micelle model of casein micelles

2.2.4.6 The stability of casein micelles

K-casein is essential to stabilise casein micelles, which covers on the surface of the micelles, causing the micelles against coagulation, and protecting the Ca^{2+} sensitive caseins from precipitation. The layer of it appears to be estimated to be 5-10 nm thick(Dalgleish et al., 2012). The hairy layer provides the steric stabilisation on the micelle surface so that each particle cannot get too close. The hydrophilic C-terminal regions on the most casein micelles are sticking out from the micelle core into the solvent, as result of present hairy structure; while the hydrophobic N-terminus part is associating with the core of casein micelles. The stabilisation of casein micelles is achieved by

hydrophilic C-terminal regions protruding from micelles. Sub-micelles are held together by hydrophobic force and CCP in the interior of casein micelles. CCP is essential for the integrity of casein micelles. A large amount of amorphous CCP solubilise in the casein binds, and phosphoserine residues caseins clustered together, where is called the phosphate centre (Thorn et al., 2014).

Moreover, the stabilisation of casein micelles is also achieved by steric hindrance and the electrostatic repulsion of the κ-casein layer, the physical barrier and the charge repulsion prohibit the coagulation of casein micelles in milk(Gonzalez-Jordan et al., 2015). The C-terminal regions in the aqueous phase present a high level of Glu and Asp residues (with negative charge), and a low level of positive charge. In addition, the stability of casein micelles is enhanced by the glycosylation of Thr-residues, with sialic acid as part of the carbohydrate moieties, which provides solvency to κ-casein hairy structure. The colloidal stability of micelles is depended on the tendency and length of κ-casein hairy structure. Therefore, some process such as heat treatment, acidification, treatment with enzymes could change the stability of casein micelles. And the destabilisation process of casein micelles was illustrated below.

2.3 The destabilisation of milk proteins

Dynamic low-amplitude oscillatory rheology is used in the determination of viscous components and elastic components in emulsions. Accordingly, this measurement also applied to analyse the viscoelastic and rheological properties of milk gels. Parameters are normally determined as storage moduli (G'), loss moduli (G'') and loss tangent (tan δ). G' is the materials' ability of storing energy elastically; G'' characterises the deformation energy lost (dissipated) through internal friction when flowing; tan δ is the ratio of energy lost to energy stored during cyclic deformation. (Boland et al., 2020; Singh et al., 2008)

2.3.1 Acidification on casein micelles

Acidification of caseins is normally considered as the basis of developing yoghurt and other acid-coagulated milk products. The reduction of pH associating the properties of acid-induced gels has been illustrated at three stages.

Firstly, during acidification, the net charge of the casein micelles decreases from pH 6.7 (the natural pH in milk) to pH 4.6 (the isoelectric point of casein-micelles). The

normal size of casein micelles is 165nm at pH 6.6, and the size of it increased to 178 at pH 6.0, and the change of particle size is reversible (Sinaga et al., 2016). The electrostatic repulsion has been decreased because a part of the net negative charge in casein micelles are neutralised, which results in a relatively small amount of CCP removing from casein micelles, the colloidal stability of the micelles is relatively constant.

Secondly, decreasing pH from 6.0 to 5.0, the net negative charge in casein micelles are further decreased, and the electrostatic repulsion is accordingly reduced. As pH decreases, the steric stabilisation is reduced, and the colloidal stability of casein micelles is changed. The average size of the micelles approach the lowest value around 154nm at pH 5.5, and it slightly rises when pH decreases from 5.5 to 5.0 (Sinaga et al., 2016). The hairs of κ-caseins on the surface of the micelles are charged, the κ-casein brush (hairy structure) is progressively lost and collapsed. When pH is less than 5.3, organic and inorganic phosphate are progressively protonated, and colloidal calcium phosphate (CCP) are gradually dissolved from micelles into the aqueous phase (Gonzalez-Jordan et al., 2015). At pH 5.0, the colloidal calcium micelles inside casein micelles are completely dissolved. Due to the large aggregates of casein micelles is formed, the particle size of it become visible, the measured particle size is over 1.3μm (Sinaga et al., 2016).

Finally, since the pH is below 5.0 and getting close to PI (pH 4.6), the negative charge in casein micelles is neutralised, casein micelles aggregates as the isoelectric point of it is approached, in which the van der waals' forced and positive/negative charge interaction is increased. The hydrophobic interaction is stimulated as electrostatic repulsion is reduced. During acidification, the three-dimensional network gel is formed because of the linkage of chains and clusters. Normally Glucono-δ-lactose (GDL) as a direct acidification method is normally applied in analysing the rheological and structural properties of milk gel.

2.3.3 Rennet induced coagulation of caseins

For further analysis of rennet-induced casein micelles, loss tangent is a useful parameter in understanding the relaxation of bonds in the gel during deformation. During gelation, tan δ of rennet gel reduces from over 1 to less than one at the gelation point and then approaches a constant value around 0.35. The dynamic modulus increased relatively

fast at the beginning; after a period of time, it tends to plateaus. The range of typical plateaus value of rennet gel (32g protein/L) is from 100 to 200Pa (Singh et al., 2008).

2.3.3.1 Processing of rennet coagulation

Rennet (chymosin) is usually used in cheese, and rennet casein manufactures. The activity of it is similar to the pepsin. The difference between pepsin and rennet that is, pepsin is usually adopted from adults' stomach, and rennet only exists in the belly of infants or calves.

Rennet coagulation is normally divided into three essential stages. At the primary stage of coagulation, also called proteolysis process, rennet specifically attacks κ-caseins. Proteases (rennet or pepsin) specifically cleave the bond between the phenylalanine residue (105) and methionine residue of bovine κ-caseins in postion106, and/or the hydrophilic region part of caseinomacro-peptides. If highly glycosylated, glycol-macro-peptide (CMP or GMP) (residues 106-169) diffuses into the serum, the hydrophobic region (residues 1-105) of κ-casein has been left(Hallén, 2008). Once much of κ-caseins (70%) has been hydrolysed, the colloidal stability of casein micelles is lost, the coagulation of casein micelles occurs(Sandra et al., 2007). κ-caseins interact increasingly strongly with an increase in the extent of proteolysis. Meanwhile, the hydrophilic part of CMP or GMP released into whey protein, the negatively charged group is lost, and the steric stability is decreased. When nearly all of the κ-caseins has been hydrolysed, the micelles begin to aggregate (McMahon et al., 1984b), with the appearance of a space-filling gel. In the term of rheology, the loss tangent value of it is <1(Singh et al., 2008).

The secondary stage is the coagulation of para-κ-casein micelles which called coagulation. This stage coincides during the last phase of the enzymic reaction. Before the coagulation could be observed, 85% of κ-caseins have had to be hydrolysed(McMahon et al., 1984b). The coagulation of para-κ-casein micelles only occurs in the presence of free calcium ions. Molecular chains connect through hydrophobic interaction to form a three-dimensional network. By further solidification through a calcium-induced reaction between protein molecules, gelation of caseins is formed. As the rheological properties of rennet induced gel, completely hydrolysed caseins micelles form small linear chains initially, furthering aggregate to form clumps, clusters. Finally, a system-spanning network that has a fractal-like appearance is created. The viscosity of milk shows no

change until casein micelles aggregate completely under sustained pH and protein concentration.

Finally, in the third stage, the properties and structure of rennet clot are changed because whey protein is expelled from the network of casein micelles by syneresis(Hallén, 2008). The picture of rennet coagulation of milk gel under confocal laser scanning micrograph is provided in Figure 2.11. Two stages have been summarised in Figure 2.12.

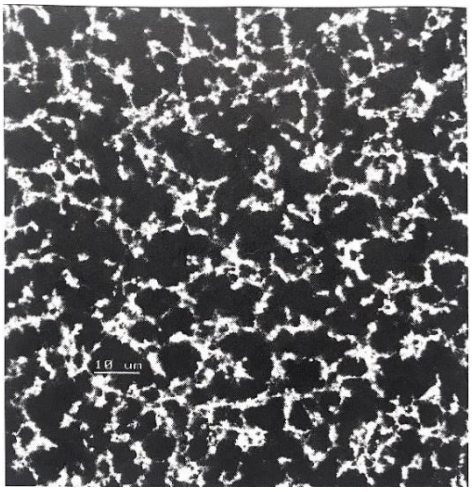


Figure 2.11 The rennet induced skim milk gel under confocal laser scanning micrograph. Scale bar is 10nm, and protein is white, water is black (Singh et al., 2008).

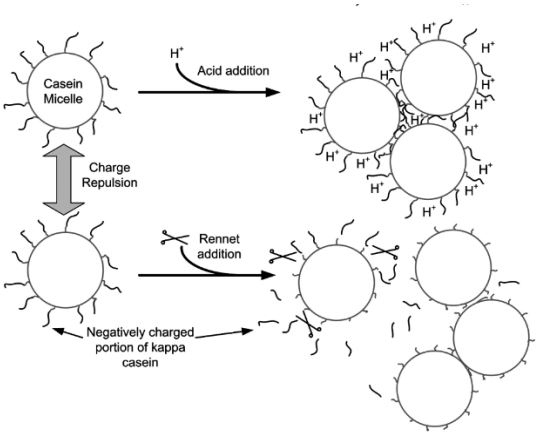


Figure 2.12. Flocculation of caseins and protease coagulation of caseins(Singh et al., 2008).

2.3.3.2 Factors influencing rennet coagulation of milk

It is known that the coagulation of dairy is affected by several factors such as temperature, protein concentration, pH, mineral content, ion strength, enzyme concentration, and the pre-treatment of dairy(Singh et al., 2008). In that case, for increasing the rate of proteolysed κ-caseins, the pH and the temperature of dairy should be manipulated to optimal values (Huppertz et al., 2018). For instance, skim bovine milk forms gel at pH between 5.19 to 6.21. In the view of rheological and microstructure properties of rennet-induced casein gels, the low value of pH and high concentration of NaCl in emulsion reduced the developing rate of elasticity of casein gels. Fracture strain and fracture stress of rennet induced gels reached the highest value at pH 5.8 and the lowest value at pH 6.29 during 48 hours after coagulation.

Moreover, the concentration of insoluble colloidal calcium phosphate (CCP) associating with casein micelles has an effect on the rheological properties of rennet induced casein gel. (Choi et al., 2007) suggested that adding EDTA as a calcium chelating agent in reconstituted skim milk for removing some colloidal calcium phosphate reduced the storage moduli of casein gel because the content of CCP crosslinking in the casein micelles is diminished. Removal of CCP before rennet coagulation leads to a higher tan δ, which indicates the greater mobility of bond in the emulsion. Furthermore, reducing pH (below 6.4) prior to rennet induced gelation results in higher storage than the casein gel made at pH 6.7 because the electrostatic repulsion in milk is reduced by reducing negative charges. Accordingly, because CCP crosslinking is slightly removed from casein micelles at pH 6.4, the storage moduli of casein reached the highest value at pH 6.4 and decreased when the pH of milk was reduced from 6.4 to 5.4(de Kruif et al., 2012).

2.3.4 Denaturation of whey protein in heat treatment

The heat treatment of milk is important in considering the thermal effects on milk protein, since heat treatment involved in the manufacture of all types of milk for extending the shelf-life of dairy. The temperature of heat treatment ranges from 65℃ to 142℃. When milk is heated, several competitive and independent reactions occur in milk, the denaturation of whey protein in milk happens. Due to the different thermal stability between α-La and β-Lg, α-La denatured at the first order, and the denaturation of β-Lg is second-order(Singh et al., 2008). Because of the highest percentage of β-Lg among other milk proteins, the coagulation of whey protein in bovine milk is normally driven by β-Lg.

The interaction of denatured whey protein with other milk proteins (including casein micelles) has influences on the stability of milk.

The coagulation of whey protein in bovine milk involves two stages: the first stage of it is the irreversible unfolding of globular whey proteins. At room temperature, β-Lg exists in an equilibrium of two forms (dimers and monomer). When the temperature is increased over 65℃, the tertiary and secondary structure of it is almost lost, dissociation of β-Lg occurs, dimers transform to monomers, the free-SH group hidden in the interior of β-Lg becomes exposing in the solvent. When heating on α-La, the S-S linked polymers is ruptured into monomers, and the S-H groups are formed, which are absent in native α-La. The involving of thiol-disulfide exchange reaction in milk leads to coagulation and polymerisation(Singh et al., 2008). The denaturation of whey protein is also affected by pH. When the pH of milk is high (from 6 to 9), the denaturation reaction of whey is enhanced. But at the low pH, the denaturation of β-Lg is retarded. Meanwhile, increasing the solid content with constant protein concentration in milk, the denaturation of both β-Lg and α-La is retarded(Singh et al., 2008).

The second stage is interactions between denatured whey proteins and κ-caseins at casein micelles surface. This stage happens at the temperature above 70℃, denatured whey proteins associate with κ-caseins via sulphur bridges, and the β-Lg/κ-casein complexes are formed at micelles surface. The interaction rate is mostly depended on the denaturation rate of β-Lg, once the S-H groups in β-Lg exposed to κ-casein, the interaction occurs(Singh et al., 2008). Moreover, the interaction between whey protein and caseins is more likely happened under low temperature, especially when the milk is heated in a water bath or indirect heat-exchangers. Under prolong heating time, the active monomers and small aggregated species of whey can penetrate the hairy layer of κ-casein and associate with it more readily. If heating milk rapidly in direct heat-exchangers, β-Lg and α-La aggregate in the serum phase and large aggregated species of whey are formed(Singh et al., 2008). As a result, shot time high-temperature heating on milk is not good for the interaction of whey protein and casein. (Singh et al., 2008)also suggested that the denatured whey proteins also interact with α_{s2}-casein via S-S bond. However, the activity of α_{s2}-casein is much less than κ-casein because its location inside casein micelles and κ-casein are dominant at the surface. The model of heat-denatured β-Lg and its coagulation is provided in Figure 2.13.

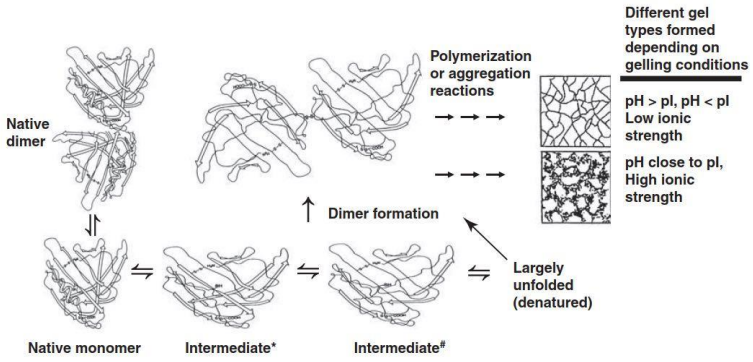


Figure 2.13. The formation of whey protein gel by heat treatment (Singh et al., 2008)

2.3.5 Cold gelation of whey protein

The cold gelation of whey protein involves adjusting pH, the addition of salts and manipulating temperature in milk production (increasing ionic strength) (Britten et al., 2001; Ju et al., 1998; Kharlamova et al., 2018). The proteolysis of whey protein also plays a minor role on modifying the hydrogen bonding, electrostatic interactions and hydrophobic interactions(Doucet et al., 2003; Otte et al., 1996; Otte et al., 1999).

In yoghurt production, casein micelles are precipitated at pH 4.6, and whey protein is dissolved in the aqueous phase, the homogenisation process is normally used to decrease the separation of whey protein and increase the consistency of products during storage. Further study by Morand et al. (2012) heated whey protein samples (different ratio of β-Lg and α-La) at 80 ℃, samples are acidified with GDL to decrease pH from 6.0 to 5.4, the gel was formed at room temperature.

2.4 Digestion behaviour of infant formula in the infant's stomach

For daily consumption, the food (infant formula) enters the baby's mouth and passes into the diaper; the digestion occurs along the way. However, for neonates who exclusively consume liquid infant formula, due to the liquid food is ordinarily move fast than solid food, the transit time of food through mouth, pharynx and oesophagus is short (10-15s) (Nguyen et al., 2015a). Hence, the digestion of food is rarely happened at the oral phase and mostly occurred in the infant's stomach. The stomach plays an essential role in storing swallowed food, combining and disintegrating food, and regulating the excretion of the stomach contents into the duodenum.

The process of protein digestion, gastric secretion and gastric empty has been widely discussed. Gastric secretion plays a vital role in protein digestion. Dairy proteins are hydrolysed by gastric protease in stomach fluid. Naturally, stomach fluid (gastric secretion) can be stimulated by a series act of eating and swallowing. The gastric secretion includes water, hydrochloric acid, mucus, enzymes, intrinsic factor and bicarbonate(Dumont et al., 1994; Leung, 2014; Schultz, 1989). By stomach emptying, stomach content is moved into duodenum gradually during digestion.

Stomach emptying is affected by several factors: muscle tone, pyloric sphincter tone, presence of amniotic fluid, the type of food, the level of hormone and mucus. Comparing the gastric motility of between infant and adults, the emptying time of new-borns are delayed, the gastric capability of infants is around 6mL per Kg of body weight (Nguyen et al., 2015a).

2.4.1 Infant gastric environment

Comparing to adults, the digestion and absorption of protein are different between infants and adults. The gastric proteolysis in infant stage is slower than adults. For example, 15% denatured whey protein could be hydrolysis in an infant, but this percentage increases to 90% in an adult(Menard et al., 2018). The gastric pH in an adult is close to the pepsin optimal pH (1.0-4.0), which leads to at least 70% of pepsin's maximal activity. On the contrary, the gastric pH in infant is 5.3, with only 10% of its maximal activity (Menard et al., 2018).

The comparison of the activity of enzymes between human adult and infants has been illustrated in Table 2.11. Low activity of pepsin is caused by the high gastric pH, which restricts the pepsin secretion in the stomach. The high gastric pH is due to the low value of gastric acid in infant stomach. The details about the value of stomach content and the physiologic factors of infant digestion have been described in Table 2.12, and the implication has been illustrated as well. The different physiologic limitation of infant digestion causes inadequate protein hydrolysis, and the proteolysis rate is even low for pre-term infants.

Table 2.11. The proteolytic enzymes in infant gastrointestinal tract (Nguyen et al., 2015a)

Enzymes	Activity (% of adults)	Effects on infant digestion
Pepsin	<10	Low
Trypsin	10-60	Adequate
Chymotrypsin	10-60	Adequate
Elastases	Na	Low
Carboxypeptidase	Na	Adequate

Table 2.12. The limited digestion and absorption of protein in infant gastrointestinal tract (Blackburn, 2007)

Stomach content	Value (% of adults)	Effects on infant digestion
Gastric acid	50	Increased gastric pH Decreased pepsin activity Decreased proteolysis
Pepsinogen/pepsin	50	Decreased proteolysis
Trypsin	Near adult level	Decreased proteolysis
Intestinal mucosal dipeptidase	Adequate for infants	Promote protein digestion
Amino acid absorption	Adequate for infants	Enough absorption of amino acid

2.4.1.1 Physical conditions

Infants have a much lower output of pepsin and higher postprandial pH in the stomach, comparing to adults, which consequently restrict the digestibility of milk protein. The postprandial pH of the infant under 28 days is between 3.0 to 4.0(Nguyen et al., 2015a). As to infants from 28 to 41 weeks, the pH is assumed to range from 4.93 to 6.25, the average pH is 5.59, and the pH could be decreased to pH1.5-3.0 when babies are 12 months old (Nguyen et al., 2015a; Poquet et al., 2016). Mason, (1962) estimated the mean pH value of 25 full-term infants (from 5-13 days) of stomach content. The pH in infant stomach content increased from 3,5 to 6.4 for 30 minutes feeding time, after 180 minutes gastric digestion the pH decreased to above 3.0 (Nguyen et al., 2015a). The curve of that is plotted in Figure 2.14.

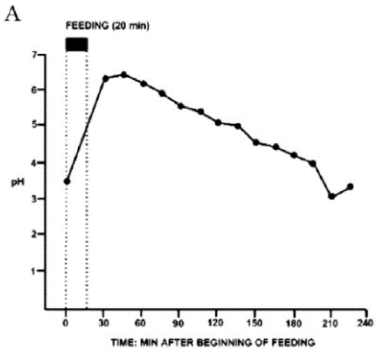


Figure 2.14. The gastric pH of infant during feeding and gastric digestion(Mason, 1962; Nguyen et al., 2015a)

Moreover, the details about the changes in gastric pH with the increasing age of infants has been shown in Table 2.13

Table 2.13. The changes in gastric pH in infant (T. T. Nguyen et al., 2015)

Age of infants	pH range	References
2-3 months	4.6-5.2	(Nguyen et al., 2015a)
4-6 months	3.5-5.5	(Nguyen et al., 2015a)
7-9 months	4.0-5.2	(Nguyen et al., 2015a)
12 months	1.5-3.0	(Poquet et al., 2016)

2.4.1.2 Enzymes

In infants' stomach secretion, there are proteolytic and lipolytic enzymes, trypsin and steapsin. Two main enzymes secreted from the gastric mucosa are pepsin and gastric lipase. Pepsin is an endopeptidase(Gupta, 2019). The catalytic activity of it in stomach is at an optimal pH 2.0, and the gastric lipase has the maximum activity at pH range 3.0 – 5.0. The optimal pH is maintained by hydrochloric acid in human stomach. The activity of pepsin and gastric lipase is different between full-term infants (26 weeks old) and adults. The basal gastric lipase activity in adults is from 15.6 to 64, and the mean activity of pepsin is 942-1333. After one hour digestion, the activity of lipase and pepsin is decreased to 13.1-25 and 718-1042, respectively. Healthy infant tends to have gastric lipase activity ranging from 15.3 to 76, and the level of gastric lipase has been decreased to 1-20 during the consumption of the meal. The details have been shown in Table 2.14.

Table 2.14. The biochemical condition of neonates and adults(Poquet et al., 2016)

	Meal	Gastric lipase	Pepsin	Calcium content
		Mean activity (U/ml)	**Mean activity (U/ml)**	**(mM)**
Infant	Fasted	15.3-76	65-119	-
	Feed	1-20	-	4.5-24.5
Adult	Fasted	15.6-64	942-1333	0.3-1.2
	Feed (0-60min)	13.1-25	718-1042	-

Some proteolytic enzymes in stomach fluid normally present in an inactive form. Pepsinogen is a zymogen and could be converted into pepsin by hydrochloric acid (H^+ ions) in the stomach(Gupta, 2018). For activating pepsin, the peptide bond between 42 and 43 residues of pepsinogen is hydrolysed, and a 42 amino acid segment is released from N-terminus of protein(Blanco et al., 2017). The increasing concentration of active pepsin further potentiates the activity of its own zymogen (pepsinogen), which is known as autocatalysis. The equation has been shown below(Gupta, 2018).

$$Pepsinogen\ (zymogen) \xrightarrow{HCl\ (H^+)} pepsin\ (active\ form)$$

$$pepsinogen \xleftrightarrow{pepsin\ (autocatalysis)} pepsin$$

Rennin is a proteolytic enzyme and normally exists in infant stomach. The optimum pH of it is 2.0, and the activity of it is similar to pepsin. During proteolysis action in the stomach, rennin acts on casein micelles and converts it into para-κ-caseins. Further study about that has been provided in casein section.

The digestion of protein firstly happens in the stomach, and pepsin is the protease responsible for digestion of protein in the stomach. The gastric digestion of milk proteins is described in the following part. (van der Sman et al., 2020).

2.5 *in vitro* infant gastric digestion models

In vitro digestion models mimic the digestion process in human digestive system, the method has been widely used in studying the digestive behaviour in oral phase, gastrointestinal phase, and occasionally intestinal fermentation (Minekus et al., 2014).

Introducing dairy protein into infant body is not easy and requires making sure that these proteins will not resist digestive process in the stomach (Dupont et al., 2015). *in vitro* digestion models are developed to simulate the physical conditions *in vivo*, taking into account the pH, digestion time, the concentration of all kinds of enzymes, salt contents, and other factors. The advantage of *in vitro* digestion models is more rapid, without any ethical restriction, requiring less labours, and low cost. Moreover, this method allows more samples to be studied in parallel for screening purpose, which makes mechanistic studies and hypothesis building of the research easier (Minekus et al., 2014). Following sections clarified the static models and the dynamic models.

2.5.1 Static model or semi-dynamic model

Static or semi-dynamic models are the simplest methods to simulate the physiological conditions of the human infant gastrointestinal digestion(Alegría et al., 2015), in which the undigested food content and products remained in reaction vessels during digestive processes, and other factors, which could affect experiment results are not considered, such as shearing, mixing, absorbing and decrease of stomach pH. However, because of the simplicity of static digestion models, which use an unchanged pH for all digestive phase and the constant ratio of food to the enzyme, were wildly applied in analysing the *in vitro* digestion in human(Brodkorb et al., 2019).

2.5.1.1 Digestive conditions for in vitro static gastric digestion model

Based on the physical conditions of infant gastric digestion, the constant parameters, such as the ratio of food to enzyme and stomach pH, are determined at the occurring of gastric emptying half-time. For infant the gastric emptying, half time is reported at 78 minutes, the mean flow rate of stomach secretion is at 0.53ml/min, and 37% meal has been digested after the gastric emptying half-time (Bourlieu et al., 2014; Menard et al., 2018).

The most common static model is a beaker or flask warmed in a shaking water bath set up at 37℃ and 60-250rpm to simulate physiological processes in human gastric, and the pH should be manually controlled (Nguyen et al., 2015a). In Bourlieu et al. (2015), the decrease of pH and the level of dilution of the meal by gastric secretion were adjusted according to the *in vivo* data. By using pH-stat, three stages of gastric

acidification were established at pH 6 (0-60 minutes), pH 5 (60-120minutes), pH 4 (120-180minutes). The pH is adjusted by adding HCl (150mM), NaCl (110mM) and CaCl (5mM). The secretion resulted in a dilution of the meal by 30% v/v during the entire digestion. For analysing the proteolysis in gastric, porcine pepsin with the activity of 3100U/mg was utilised as a substitute of human gastric pepsin. In agreement with the postprandial enzyme activities detected in infant human gastric contents by (Armand et al., 1996; Roman et al., 2007), respectively 63U/ml of pepsin and 26U/ml lipase of initial gastric content per kg of infant body weight were added at the beginning of digestion.

2.5.2 Dynamic gastric models and human gastric simulator

For more realistic stimulation of *in vivo* digestion, several dynamic models have been developed in recent years, which addressed the demands for stimulating both the biochemical and mechanical aspects of gastric digestion in a realistic time-depending manner (Verhoeckx et al., 2015). The two popular models are TIM1 (TNO gastrointestinal model 1) and TIM2 (TNO gastrointestinal model 2). The key parameters about digestion were taken into account in TIM 1 such as temperature, pH change in stomach, stomach and pancreatic automatic secretion, stomach emptying and peristalsis movements and nutrient absorption in intestinal. Normally, TIM 1 is utilised to study digestion behaviour in human infants. Based on TIM 1, TIM 2 was added to imitate the microbiota and applied to stimulate the digestion in all human ages. The dynamic model of gastric digestion is displayed in Figure 2.15, and the physical condition in it was illustrated below.

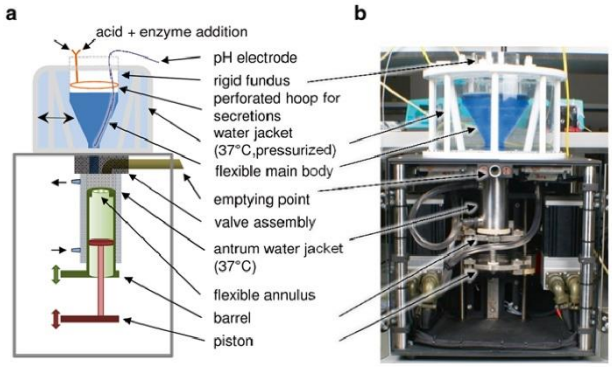


Figure 2.15. The dynamic gastric model(Verhoeckx et al., 2015)

Human gastric simulator (HGS) is developed by Kong et al. (2010b) for food digestion diagnosis in dynamic digestion model and making the gastric force as similar as *in vivo*, which is specially designed to reproduce the fluid mechanical conditions. In recent years, the human gastric simulator has been applied to analyse the digestion behaviour of different food and the physicochemical properties of food during digestion.

The stomach is the major compartment for food digestion in human, where both food particles reduction and biochemical reactions occur. The part of HGS simulating the stomach compartment is a cylindrical latex chamber, and the simulated digestion is operated inside an insulated chamber, the temperature is maintained at 37℃. The gastric compartment has been shown in Figure 2.16. In the process, a variable flow mini peristaltic pump delivers simulated gastric juice into the stomach chamber; the flow rate is adjusted.

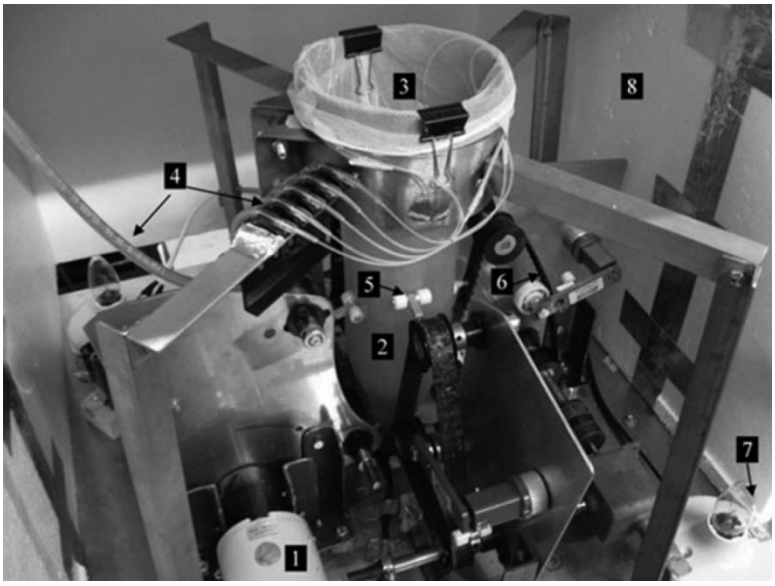


Figure 2.16. Human gastric simulator (Kong et al., 2010b).

2.5.2.1 Digestive conditions for in vitro dynamic gastric digestion models

At the beginning of gastric digestion, the stomach secretion plays an important role. The secretion rate of acid and enzyme relies on the composition and volume of food (Verhoeckx et al., 2015). According to the data from previous studies of *in vivo* adult and infant digestion, the pH of *in vitro* gastric condition in the dynamic model has been

summarised by Shani-Levi et al. (2013). In Table 2.15, the adult and infant pH was measured by pH-stat and only varied in pepsin levels.

In Ménard et al., (2014), during the gastrointestinal digestion of 210 minutes, the pH in the gastric phase after in fasting is varied from 5.4 to 3.09. Porcine gastric mucosa pepsin (EC 3.4.23.1, Sigma P6887) diluted at 1250 U/mL, and the constant flow rate is 0.25mL/min. More details about the *in vitro* infant gastric dynamic digestion is illustrated in Ye et al. (2019a), the pepsin from porcine gastric mucosa (EC 3.4.23.1; Merck 9001-75-6) is diluted at 16.8 FIP-units/L, the flow rate is fixed at 0.15 mL/min. In the gastric digestion time of 240min, emptied digesta (15mL) has been removed from the HGS every 20 minutes.

Table 2.15. The change of pH *in vitro* dynamic adult and infant digestion model (Shani-Levi et al., 2013)

Dynamic adult		**Dynamic infant**	
Time (min)	pH	Time (min)	pH
0	4.5	0	6.5
10	3.2	30	6.5
20	2.8	150	4.5
40	1.8	240	3.5
60	1.7		
120	1.5		

2.4.2 Digestion of infant formula

In this section, the factors influencing the digestion in infant formulae is discussed, the protein digestion *in vivo* and *in vitro* digestion model was compared.

2.4.2.1 in vivo digestion of infant formula

By reviewing the coagulation of casein micelles, casein was suggested to remain in the stomach longer and degrades slowly because of the combined behaviour of acid and enzyme. However, according to Bouzerzour et al. (2012), caseins more intend to be hydrolysed in the gastric phase, while β-Lg and α-La are both resistant to digestion *in vivo*. Moreover, the observation of ingesting skim milk powder in human by Mahe et al. (1991) established that 64% β-Lg and 44% α-La had been detected in the stomach, but casein was entirely hydrolysed.

Chatterton et al. (2004) analysed human milk proteins, by sampling gastric aspirates from human infants aged from 8 to 28 days. After 1 hr digestion, significant quantities of α-La, lactoferrin and secretion components were detected. Besides, β-caseins, marginally resistant proteins, was observed in 8 days old babies but was absent in 28 days old babies.

In Bouzerzour et al. (2012), the protein digestion kinetics of complex matrix-like infant formula has been exhaustively studied using the piglet as a model of the infant. The standard infant formula adapted to piglets contained a high level of protein (17.7%) and lipid (43.4) and low content of lactose (32.2). Before feeding piglets, the infant formula has been hydrated at 20% in milli-Q water. The piglet was fed by an automatic delivery system for 28 days, and slaughtered 30, 90 and 210 minutes after a meal. During gastric digestion, the content of digesta and the pH has been summarised in Table 2.16.

Table 2.16. Content, nitrogen content and residual protein in piglet stomach at 30,60,210 minutes after the last meal.

	30 minutes	**90 minutes**	**210 minutes**
Content pH	4.49	4.46	3.10
Total content (g)	157.00	108.27	33.48
Total nitrogen of ingested nitrogen (%)	79.00	39.24	10.05
β-lactoglobulin (%)	45.00	16	1.40
α-lactalbumin (%)	42.57	16.54	0.07
Casein (%)	23.76	6.16	0.18

For further analysing protein degradation in the gastrointestinal tract, Wada et al., (2017) utilised suckling rat pup model and conducted the identical peptides from major protein in human milk and infant formula. The major proteins in human milk are α-La and β-casein, whereas β-Lg and β-casein are dominant in infant formula. In their study, the specific peptides of major milk protein were observed at 30minutes, 60 minutes and 120 minutes of digestion. In the result, residuals 39-55 residual peptides of β-Lg (major protein in infant formula) resist to digestion in the gastrointestinal tract. Moreover, the acid clotting behaviour of β-caseins in human milk and infant formula differs in gastric phase, and the digestibility of them are varied because they have more than 50% sequence identity, and the differences are mostly in phosphorylation states. Accordingly, 70-92 of β-casein in human milk and 57-96 and 145-161 of β-caseins in infant formula are resistant to digestion *in vivo*. In the observing of the following digestion in proximal jejunum,

median jejunum and ileum, β-Lg and α-La remain intact after 60 minutes digestion (Bouzerzour et al., 2012).

Tari et al. (2018) investigated the effects of the protein composition of liquid model infant formula on the digestion behaviour *in vivo*. In the formula, the protein composition is 2.80% (w/v). Whey protein: casein ratio in infant formula is 60:40, two samples are based on this ratio but differed in the content of β-casein (12.5% (w/w) of protein and 17.1% (w/w) of protein), one sample only contained whey protein. Twenty-four new-born piglets were slaughtered at 60 and 120 minutes after the last meal. By observing the image of digesta formed during gastric digestion (Figure 2.17.), whey protein, only formula created a more fragment structure than whey protein-casein formula.

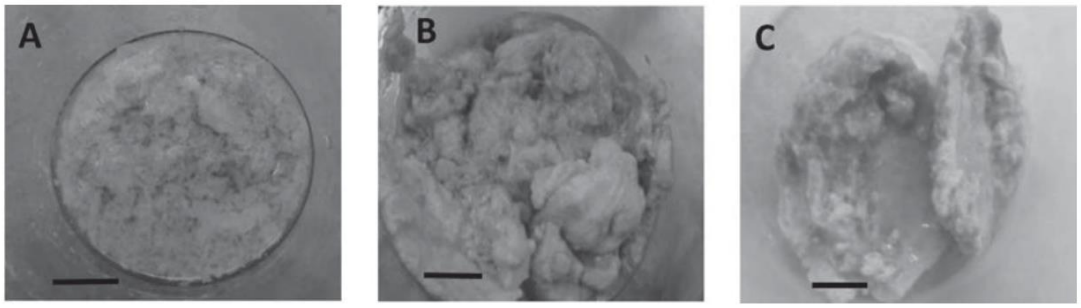


Figure 2.17. Images of digesta formed during the piglet gastric digestion. The samples were adopted after 60 min from the last meal. A, whey protein only formula; B, whey protein-casein formula (60:40), low β-casein; and C, whey protein-casein formula (60:40), high β-casein(Tari et al., 2018).

Figure 2.18 shows the microstructure of the clots obtained after *in vivo* gastric digestion of model formula. The result of rheological measurement of three digesta was shown in Table 2.18. The viscosity, storage moduli of the digesta from whey protein-casein formula is higher than whey protein only formula digesta, but whey protein digesta had more heterogeneous microstructure than whey protein and casein digesta. However, the level of β-casein has limited influence on the physicochemical properties of the stomach digesta (Tari et al., 2018).

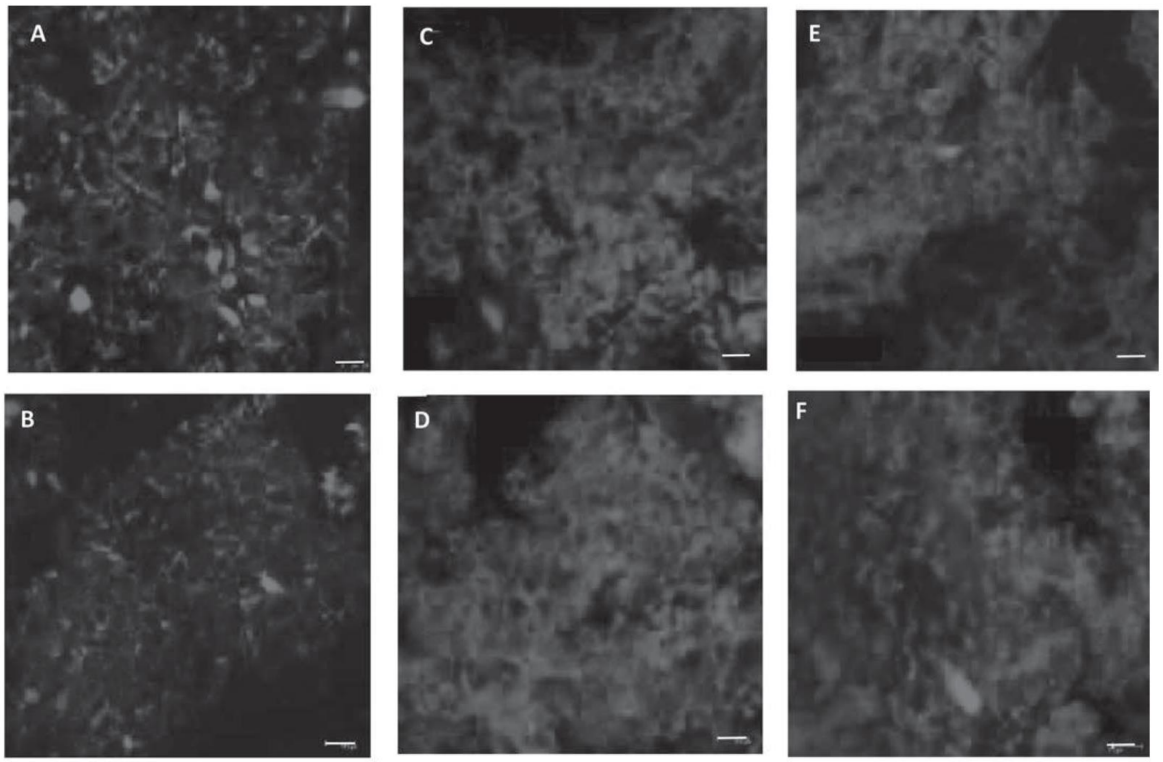


Figure 2.18. Confocal micrographs of the clots obtained after piglet gastric digestion of formula. Whey protein only formula was taken at 60 (A) and 120 min (B); Whey protein-casein formula (60:40), low β-casein was taken at 60 (C) and 120 min (D); Whey protein-casein formula (60:40), high β-casein taken at 60 (E) and 120 min (F) (Tari et al., 2018).

Table 2.17. Rheological properties of gastric digesta collected at postprandial times of 60 or 120 min (Tari et al., 2018)

	Viscosity (Pa.s)		**Storage modulus**	**Loss modulus at**
Infant Formula	60 min	120 min	**at120 minutes (Pa)**	**120 minutes (Pa)**
Whey protein only	0.16	0.08	18	5
Whey protein: casein (60:40) low β-casein	22.7	35.6	2622	1077
Whey protein: casein (60:40) high β-casein	19.0	27.4	2022	831

Moreover, the research supported the previous view about whey protein resist to the proteolysis in the stomach, although caseins form a clot during gastric digestion. The microstructure and viscoelastic property of digesta did not change with postprandial time. The pH between 60 minutes and 120 minutes is in the range 5.8-4.4, the gel in the piglet stomach is the mixture of acid gel and pepsin gel (Tari et al., 2018).

2.4.2.2 In vitro digestion of infant formula

Egger et al. (2019) analysed the digestion of SMP *in vivo* and *in vitro* digestion models. By comparing peptide patterns between dynamic and static *in vitro* gastric digestion with *in vivo* data, the protein hydrolysis showed similar kinetic behaviour in the static and dynamic digestion. In both systems, intact β-Lg was observed to be persistent in the gastric simulator, whereas intact caseins were disappeared at first 30 min in static digestion and at 60 min in dynamic digestion. The gastrointestinal endpoints of protein hydrolysis in static and dynamic digestion have been analysed, the kinetic in protein hydrolysis shows a good approximation to the *in vivo* pig digestion of the same SMP. Significantly, the release of free fatty acid in the gastric phase in the dynamic digestion protocol was closer to the *in vivo* situation, due to the constant addition of enzymes (Egger et al., 2019).

The *in vitro* infant gastrointestinal static digestion of commercial liquid infant formula was monitored by Menard et al. (2018). In Figure 2.19, by comparing the static digestion in the infant and adult gastric phase, casein has been significantly proteolysed in adult gastric digestion model during the first five minutes (G5). However, in infant gastric digestion model, casein has been hydrolysed up to a residual level of 10.9 ± 6.5% at 60 minutes (G60). In terms of β-Lg and α-La, both are hydrolysed dramatically in adult gastric digestion model during the first five minutes (Fig 2.19. 4C; 4D), and 11.5±3.3% β-Lg and 3.7±1.0% α-La remains at 60 minutes. On the contrary, the changes of the remaining of β-Lg and α-La are not obvious during gastric digestion in infants. 82.0±7.4% β-Lg and 86.7±6.8% α-La remains after 60 minutes digestion(Menard et al., 2018).

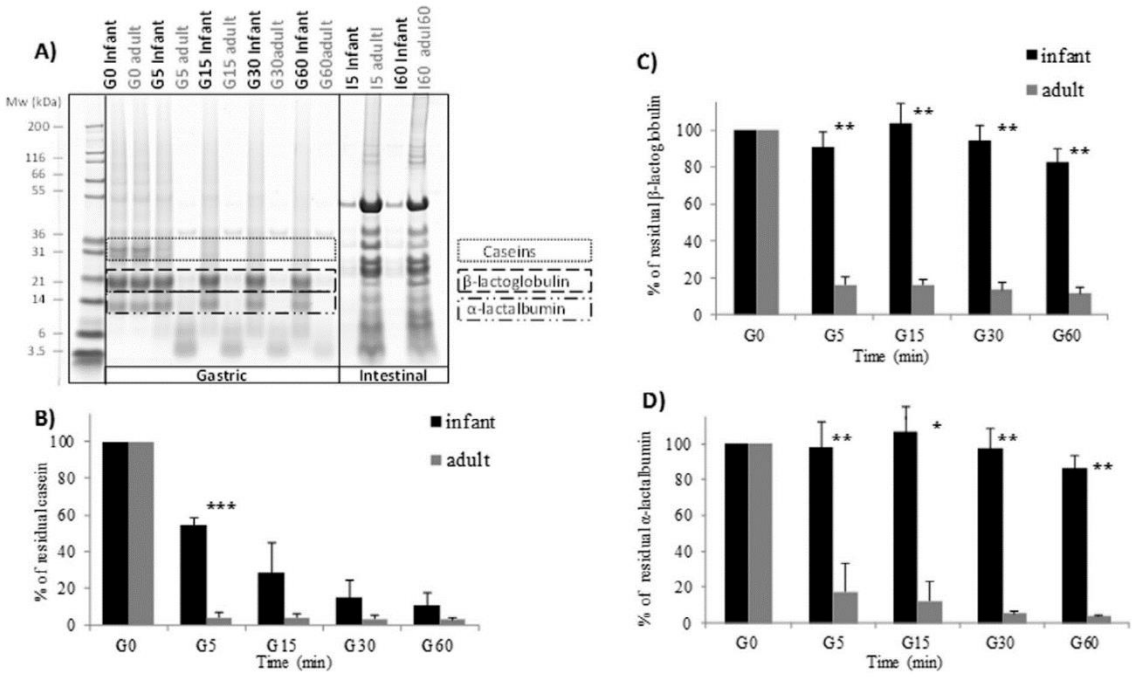


Figure 2.19. SDS-PAGE protein profile comparing the *in vitro* static digestion of an infant formula at the infant or adult stages (example of a gel, A) and calculated percentage (%) of the residual intact proteins for B) casein, C) β-lactoglobulin and D) α-lactoglobulin during gastric digestion at the infant (n = 3 digestions) or adult (n = 3 digestions) stage. (G: gastric phase, I: intestinal phase; the numbers represent the time in min after the start of the digestion)(Menard et al., 2018).

In previous studies, several specific proteins were suggested to be resistant to the effects of gastric proteases even at low pH. *in vitro* proteolysis with human gastric juice was investigated by Chatterton et al. (2004), 900µL of WPC or mature human milk was mixed with 10 µL human gastric juice, each sample was incubated for 1 hr, the pH was monitored from 6.5 to 2.0. In human milk, a small fragment of β-caseins in human milk arising from proteolysis was detected. Moreover, lactoferrin in bovine (WPC) is susceptible to be hydrolysed at pH 5, but lactoferrin in human milk is resistant to the effects of proteolysis at pH 4 and above. In comparison to human milk, whey protein in WPC is less resistant to gastric digestion than it in human milk at pH levels below 4. The intact whey proteins detected in gastric were β-Lg and a small quantity of α-La. Moreover, casein in commercial infant formula is resistant to digestion at the first hour at pH 5.0 but was cleaved below pH 4.5. It is in a similar manner to those in WPC.

Furthermore, the *in vitro* dynamic gastric digestion of 4 infant formulas in an infant HGS was researched by Ye et al. (2019a). Those infant formulas samples were

divided into two types, whey protein dominant infant formula (~60% whey protein) and casein (~80% casein) dominant infant formula. Each type of infant formula was made from cow milk and goat milk. In all infant formula, casein was digested faster than whey protein. In all digestas, the amount of whey protein is higher than that of casein. Moreover, goat milk-based infant formulas have faster protein digestion than cow milk-based infant formula. It suggested that the casein composition in goat milk plays a vital role in its coagulation. In goat milk, the α_{s1}-casein content is lower than that in cow milk, and the level of β-casein is relatively higher.

As reviewed above, the digestion of milk protein in infant formula has been attempted to understand. However, the information of protein digestion in infant gastric most studies are still not fully understood, most researches focus on the digestion behaviour of bovine milk employed a static gastric digestion model, and proteolysis in protein products were widely discussed, the information of the *in vitro* dynamic digestion of infant formula is still lack. The objective of this study was attempted to undertake a comparison of model infant formula with different protein composition, with a particular focus on digestion behaviour and rheological properties, to identify compositional features which may contribute to this different in simulated infant gastric environment.

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Dairy ingredients

In this research, two commercial dairy ingredients (low heat skim milk powder (SMP) and whey protein isolate (WPI 895)) were used, which were purchased from Fonterra Co-operative Group Ltd. (Auckland, New Zealand). The gross compositions (w/w%) of these dairy ingredients, as stated by the manufacturer, are given in Table 3.1.

Table 3.1. The composition (w/w%) of dairy ingredients

	Protein(N×6.38)	Moisture	Fat	Carbohydrate	Ash
SMP	32.9	3.8	0.9	54.5	7.9
WPI	93.0	4.8	1.0	0.3	1.7

3.1.2 Soy lecithin

Soy lecithin powder was purchased from ADM, Mount Maunganui, New Zealand.

3.1.3 Oil

Soybean oil was purchased from Davis Trading Company, Palmerston North, New Zealand. Coconut oil and Sunflower oil was purchased from blue coconut distribution Ltd, Hornby, New Zealand.

3.1.4 Lactose

Lactose was purchased from LD Carlson Company, Kent, U.S. state.

3.1.5 Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified and the reagents were made up in Milli Q water (Milli-Q apparatus; Millipore Corp., Bedford, MA, USA). All experiments have repeated at least twice.

3.1.6 Pepsin

Pepsin from porcine gastric mucosa (EC 3.4.23.1; CAS Number 9001-75-6, Sigma-Aldrich, USA) had a laboratory enzymatic activity of 425 U/mg protein, as stated by the manufacture.

3.1.7 Simulated gastric fluid

The preparation of SGF was modified based on the previous study of Minekus et al., (2014) with a slight modification. A solution of a fresh mixture of KCl (6.9 mmol/L), KH_2PO_4 (0.9 mmol/L), $NaHCO_3$ (25 mmol/L), NaCl (47.2 mmol/L), $MgCl_2$ $(H_2O)_6$ (0.1 mmol/L), and $(NH_4)_2CO_3$ (0.5 mmol/L) was prepared. The SGF (a final volume of 1L) was made up with deionised water to 800 mL. The addition of pepsin (4.8 g/L) and $CaCl_2$ (0.15 mmol/L) and water would result in the correct electrolyte concentration. The solution of a mixture of Pepsin and $CaCl_2$ was prepared priorly to use. The pH of the SGF was adjusted to 1.5 using 1 *M* HCl/NaOH.

3.2 Methods

3.2.1 Preparation of model infant formula emulsions

3.2.1.1 Emulsions preparation

There are four samples, which are different in the ratio of whey protein and casein, have been reconstituted in 100mL deionised water. The procedure for making infant formula has been illustrated by Walstra et al. (1984), 100ml of the liquid Sample comprises of 1.638g protein, 4.01g oil, 0.5g soy lecithin and 8.32g lactose.

The ratio of soybean oil, sunflower oil and coconut oil is 3:4:3, which is based on the research from (Damjanovic Desic et al., 2011; Sodhi et al., 2018). The lactose content and soy lecithin content are based on the literature (Cheong et al., 2018; Drapala et al., 2017; Walstra et al., 1984). And the composition of the mineral mixture is according to Sidnell et al. (2011). Sodium azide (0.2 wt%) was added as a preservative. The details of the components in model infant formula samples were listed in Table 3.3.

In this table, the ratio of WPI and SMP is based on the gross composition provided by the manufacturer. And the composition of protein ingredients added in emulsions was

provided in Table 3.2. In order to the different fat content and lactose content in dairy ingredients, lactose and oil mixture were added to standardised four model infant formula emulsions.

Table 3.2. The gross composition (g) of protein ingredients added in emulsions.

	Sample 0		Sample 40		Sample 60		Sample 80	
Items	**SMP**	**WPI**	**SMP**	**WPI**	**SMP**	**WPI**	**SMP**	**WPI**
Casein	0.000	0.000	0.655	0.000	0.983	0.000	1.310	0.000
Whey	0.000	1.638	0.164	0.819	0.246	0.021	0.328	0.000
Moisture	0.000	0.085	0.093	0.042	0.140	0.021	0.186	0.000
Fat	0.000	0.018	0.020	0.009	0.029	0.004	0.039	0.000
Carbohydrate	0.000	0.005	1.327	0.003	1.990	0.001	2.653	0.000
Ash	0.000	0.030	0.194	0.015	0.291	0.007	0.387	0.000
Solid Content	0.000	0.000	0.655	0.000	0.983	0.000	1.310	0.000

For preparing model infant formula emulsions, Sample 0 only has only whey protein isolate, Sample 40, 60, 80 contains 40%, 60% and 80% casein respectively. The addition of ingredients (w/v%) into four samples is shown in Table 3.3.

Table 3.3. The content in 500mL liquid infant formulae

Content	S0	S40	S60	S80
SMP	**0.00**	**10.64**	**15.96**	**24.52**
WPI	**8.81**	**3.76**	**1.88**	**0.00**
Soy lecithin	**2.50**	**2.50**	**2.50**	**2.50**
Lactose	**33.75**	**28.21**	**24.80**	**18.20**
Oil Mixture	**18.42**	**18.37**	**18.34**	**18.26**
Soybean oil	5.5716	5.5540	5.5453	5.5220
Coconut oil	5.3603	5.3434	5.3349	5.3125
Sunflower oil	7.4928	7.4692	7.4574	7.4261
Minerals	**3.11**	**3.11**	**3.11**	**3.11**
Disodium Phosphate (Na2HPO4)	0.2964	0.2964	0.2964	0.2964
Tri-calcium citrate (4H2O(TCC))	1.8196	1.8196	1.8196	1.8196
Calcium chloride (CaCl2)	0.2913	0.2913	0.2913	0.2913
Potassium chloride (KCl)	0.1212	0.1212	0.1212	0.1212
Potassium phosphate (KH2PO4)	0.5859	0.5859	0.5859	0.5859
Sodium azide	**0.10**	**0.10**	**0.10**	**0.10**
Total solid	**66.70**	**66.69**	**66.69**	**66.70**

SMP skim milk powder; WPI whey protein isolate
Potassium hydroxide (KOH) and Hydrochloric acid (HCl) were added to adjust pH

3.2.1.2 Processing of prepared emulsions

Blending and dissolving all ingredients in beaker for 30 min on a magnetic stirrer at room temperature. The final formulas were heated in shaking water bath at 72℃ for 15s. The pH of all model infant formula emulsions was adjusted at 6.8. The structure of the protein has little change at this stage. After that, a double-stage homogenisation was applied to the product with a two-stage homogeniser (Microfluidlc Co rp ., Boston, MA USA). All emulsions were placed in a water bath at 60℃, then, homogenised at 140 and 40 bar (Walstra, Jenness, & Badings, 1984; Toikkanen, Outinen, Malafronte, & Rojas, 2018). The particle size in the infant formulae has been analysed by the Malvern Mastersizer 2000 immediately. The surface-weight average diameter of fat globules $d_{[3,2]}$ is around 0.4 μm, $d_{[4,3]}$ is around 1.70 μm.

3.2.2 Rheological measurements (Chapter 4)

Sample 0, Sample 40, Sample 60 and Sample 80 were held for 15 min in a water bath at 37 ℃. Acidification of four different emulsions was done by the addition of GDL 2%(w/w). Moreover, five different levels of pepsin were added into emulsions in order. In order to adding a tiny amount of pepsin in samples, the pepsin was prepared in a 1mg/mL pepsin solution. The amount of GDL and pepsin are provided in Table 3.4.

Table 3.4. The content of GDL and pepsin added in each Sample

	Gelation time	**GDL concentration**	**Pepsin concentration**
1st	3hr	2.0 %(w/w)	0U/mL
2nd	3hr	2.0 %(w/w)	0.3U/mL
3rd	3hr	2.0 %(w/w)	1U/mL
4th	3hr	2.0 %(w/w)	1.7U/mL
5th	3hr	2.0 %(w/w)	2.5U/mL

Samples were stirred at 300rpm in a water bath for 1.5 min to allow total dissolution. The rheological measurement was started at 2 min of gelation. The GDL is gradually hydrolysed and released gluconic acid, which acidifies the solution. Samples for rheological measurements were taken and placed in the measuring equipment. The remaining of the Sample was used to monitor pH over the next 3 h.

The gelation of four dairy samples was determined using rotational Anton Paar MCR 301 stress-controlled rheometer (Anton Paar, Graz, Austria), C-CC27/T200 applied in this experiment. (1) During the time sweep measurement, the constant frequency was at 1HZ; the constant strain was at 1% to monitor gelation evolution. The storage moduli G′ and the loss modulus G″ were recorded every minute during 3 h; (2) The frequency sweep test was conducted at next; the strain was at 1% with the frequency between 0.1 and 10 HZ; (3) The amplitude-sweep was performed at the end with a fixed frequency at 1HZ, the strain varied from 0.1 to 1000%

3.2.3 Dynamic gastric digestion of model infant formula

Infant human gastric simulator (infant HGS) is utilised in *in vitro* dynamic digestion of infant formula, which is shown in Figure 3.1. This machine is located at the Riddet Instituted (Massey University, New Zealand), and the principle of it was from (Kong et al., 2010a)'s theory. In Figure 3.1, The driving system consists of 12 rollers, four belts, driving shafts, and a pulley system to stimulate peristaltic contractions in the latex stomach vessel (Champer). Three rollers are screwed each belt, and four belts are distributed along the four equality spaced sides of the stomach. The belt is driven by a motor, and the driver is set to create three contractions per minute, mimicking the actual stomach frequency of 3 cycles per minute. A latex stomach chamber was placed inside of the simulator, a plastic tube has connected the bottom of the chamber to the outside, for controlling the gastric emptying.

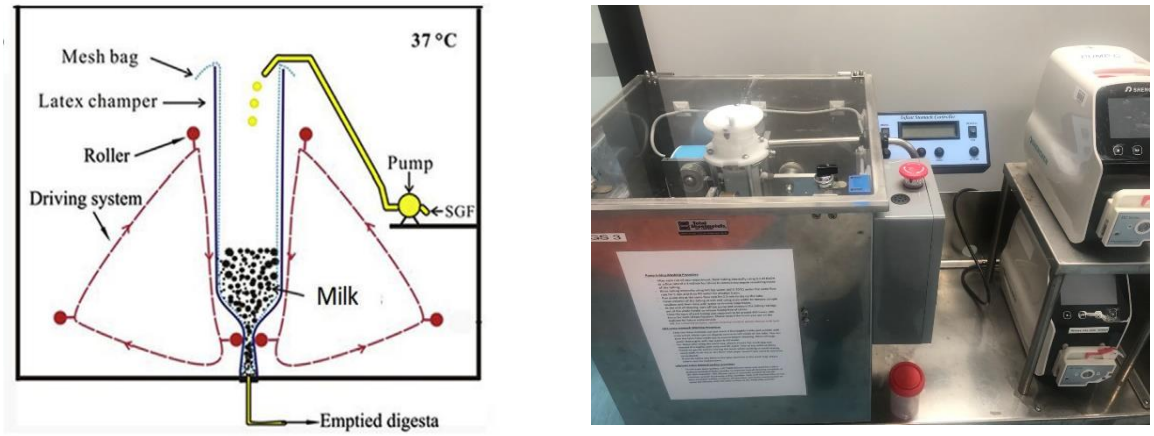


Figure 3.1. Infant human gastric simulator (right) and schematic illustration of a latex stomach chamber (left)

All dairy samples (100mL for each dispersion sample) are reheated in 37℃ water baths with stirring for 20 min. Each 100mL dairy sample was mixed with 9.6 mL of SGF and 1.95 mL of pepsin solution(3.2mg/mL) in an infant HGS. The SGF and pepsin solution was pumped into the infant HGS separately at flow rates of 0.6 and 0.15mL/minutes. For simulating the empty gastric rate, digesta samples (15mL) were removed from infant HGS every 20 minutes. For simulating the contraction of the stomach, the frequency was set to 3 times/minute. The temperature of the digestion environment was fixed at 37℃ by a heater and thermostat.

3.2.4 pH measurement

The initial pH of all samples is between 6.7 and 6.9. The pH has been measured in 180 min during all process.

Method (Chapter 4)

The pH of emulsions was lowered by the addition of GDL. The pH has been measured at every minute by utilising pH-stat. The pH of emulsions in rheometer was represented by the pH in pH-stat.

Method (Chapter 5)

With ingestion of SGF (0.75 mL/min) and gastric emptying (0.75 mL/min), the pH in the infant HGS at different times was represented by the pH of emptied digesta, because of the gastric contraction (roller movement) prevented the easy access into the infant HGS.

3.2.5 Measurement of the solid content of emptied gastric digesta

The emptied digesta were through a sieve (with a pore size ~1 mm), and adopted at 40, 80, 120, 160 and 180 min of digestion time. Original dairy sample (15mL) and the emptied digesta (15mL) were dried in a vacuum oven at 105℃ for overnight, and the mass of dry ingredients was determined.

3.2.6 Particle size distribution

The particle size distribution of these four emulsions was measured by the Malvern Mastersizer 2000 (2000S, Malvern Instruments, Malvern, Worcestershire, England) after all treatments. The refractive index value used for the dispersed phase and aqueous phase were set as 1.5 and 1.33. Mean value of $d_{[3,2]}$, $d_{[4,3]}$, $d_{[0,9]}$and $d_{[0,1]}$were recorded. The mean value was determined as the average of three times measurements, and all samples were measured in duplicates. The $d_{[3,2]}$ and $d_{[4,3]}$ are the surface weight mean and volume weight mean of oil droplets, which were calculated according to the equation at below.

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

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

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

The particle size distribution of gastric digesta emptied from the infant HGS at 20, 60, 100, 140, 180 min were through a sieve (with a pore size ~1 mm). Gastric digesta were mixed with 2%(w/w) SDS solution at the ratio of 1:1 to dissolve the protein coagulation, stabilised for one hour before the measurement.

3.2.7 Confocal laser scanning microscopy

The microstructure of gastric digesta, original Sample and clot in Sample 80 was studied using confocal laser scanning microscopy (Leica SP5 DM6000B, Leica microsystems, Heidelberg, Germany). The gastric digesta was collected at 20, 60, 100, 140, 180 min. The fluorescent fast green (1.0%, w/v) was used to stain the protein (He–Ne laser with an excitation line at 633 nm) (Ye et al., 2019a), A fluorescent dye Nile red, dissolved in acetone (0.1%, w/v), was added the Sample to stain the oil phase (Argon laser with an excitation line of 488 nm). Stained samples were placed on a concave confocal microscope slide (Sail; Sailing Medical-Lab Industries Co. Ltd, Suzhou, China), and were then covered with a coverslip. The confocal images acquired using a digital image processing software (Leica LASAF) consisted of 1024×1024 pixels. Image analysis was performed using the Image J software. All samples were observed using the ×63 oil immersion lens.

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

The protein composition of hydrolysed samples was determined by SDS-PAGE. After 180 min hydrolysis, the protein gelled by 1U/mL pepsin and 2.5U/mL pepsin at different gelation time was analysed (Chapter 4). After 180 min of *in vitro* gastric digestion, the protein composition of emptied digesta at a different time was observed (Chapter 5).

3.2.8.1 Preparation of stock solutions

（1） Acrylamide/Bisacrylamide 37.5:1 (30%T, 2.6%C) solution

30g Acrylamide/Bisacrylamide premixed powder was added into 100mL Milli-Q water and then mixed well. The solution was preserved under 4℃ in a dark bottle.

（2） 10% Sodium Dodecyl Sulfate (SDS)

10g Sodium Dodecyl Sulfate powder was dissolved in 100 mL Milli-Q water. The solution was stored at the room temperature.

（3） 1.5M Tris-HCl Buffer (resolving gel buffer)

18.15 g Tris was dissolved in 60 ml milli-Q water, the pH of the solution was adjusted to 6.8 using 6 M HCl, and Make up the volume to 100ml. The gel buffer was stored at 4℃.

（4） 0.5M Tris-HCl Buffer (stacking gel buffer)

6.05g Tris was dissolved in 60ml milli-Q water and mix well. 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℃.

（5） 0.1% Bromophenol blue solution

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

（6） 10% Ammonium Persulphate (APS)

0.1 g of APS was dissolved in 1 mL of Milli-Q water and mix well. The solution was freshly prepared before use.

（7） Coomassie Brilliant Blue Solution (0.3%) – stain solution

3.00 g of Coomassie Brilliant Blue R-250 was dissolved in a mixture of 700mL Milli-Q water, 200 ml of Isoproponol and 100 ml of glacial acetic acid, and filtered the solution through Whatman Filter Paper (Grade 4). The solution was stored in a dark bottle.

（8） Destaining Solution

It was adding 100 ml of Isoproponol and 100 ml of glacial acetic acid into 800 ml of water. The solution was freshly prepared before use.

（9） 5X Electrode Buffer/Tank Buffer

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

（10） Sample Buffer (SDS Reducing buffer)

The mixture of 1.25 g of glycerol, 7.5mL, 0.5M Tris-HCl buffer, 12mL 10% SDS and 5mg bromophenol blue powder was prepared and made up to 60mL with Milli-Q water. The solution was stored at 4℃.

3.2.8.2 Gel preparation

（1） Resolving gel (16%)

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

（2） Stacking gel (4%)

Stacking gel is prepared by mixing 2.5 mL of 0.5 M Tris-HCl buffer, 100 µL of 10% SDS, 1.3mL of acrylamide/bisacrylamide solution, and 6.1 mL of Milli-Q water. The mixture was stirred and then degassed for 15 min. Before gel casting, 10 µL of TEMED and 50 µL of 10% APS was added into the mixture.

3.2.8.3 Digested sample preparation

Method (Chapter 4)

（1） After 5 minutes proteolysis time and 120 minutes proteolysis time, 1 mL aliquots were adopted from all samples. The gelation process in samples was stopped by adding 7 mL Sample buffer (include 10%SDS). The protein content of the aliquot is fixed at 2mg/mL. Next, aliquots were added with 350μL β-Mercaptoethanol, and heated in water bath kettle at 95℃ for 10 minutes.

（2） For dissolving para-κ-casein curd in the aqueous phase, all aliquots were stirred at room temperature for overnight.

（3） For removing the oil phase of all aliquots, petroleum ether was added, and those aliquots were centrifuged at 14kPa in centrifugation.

（4） All aliquots have been stored in -20℃ freezer.

（5） The protein composition of all aliquots was determined by SDS-PAGE.

Method (Chapter 5)

Chapter 1: 200μL emptied digesta of all samples was collected at 20, 60, 100, 140, 180 min. The original samples as comparisons were also adopted at the beginning. Additionally, a small quantity of curd was collected in Sample 80 after 3 hr digestion.

Chapter 2: For all digesta, the digestion process was stopped by adding 800μL Sample buffer (include 10%SDS). Next, 40μL β-Mercaptoethanol was added, all solutions were heated in a water bath kettle at 95℃ for 10 minutes. For the curd, 0.75mg curd was dissolved in 750μL sample buffer, and 37.5μL β-Mercaptoethanol was added, the following is the same heating process.

Chapter 3: The composition of these samples was determined by SDS-PAGE.

3.2.8.4 Running of electrophoresis, staining and destaining

At the beginning, the gel was prepared on a Mini PROTEIN II system (Bio-Rad Laboratories, Richmond, CA, USA). Next, 8μL of Sample was loaded onto the resolving gel. The electrophoresis analysis was conducted at 120 V for approximately 90 min. after running, the gel was stained for 60 min with a Coomassie Brilliant Blue R-250 solution (0.003% (w/v) in 10% acetic acid (BDH, Poole, England) and 20% isopropanol

(Merck)). Then the gel was destained with a solution of 10% acetic acid and 10% isopropanol for 1h and then soaking the gel in a fresh destaining solution for overnight. At the end, the gel was scanned using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories).

Chapter 4: Effect of milk protein composition on the pepsin-induced coagulation of model infant formulae: the rheological properties.

4.1 Abstract

The objective of this study was to investigate the gelation behaviour of infant formula induced by GDL in the presence and absence of pepsin. There are four model infant formulas named Sample 0, Sample 40, Sample 60 and Sample 80, which are ranked by the level of casein content. Changing the ratio of whey protein to casein significantly affect the rheological properties of the gels of infant formulae. The gelation of infant formula was induced by the action of GDL and/or milk-clotting enzyme (pepsin). For Sample 0, it showed limited influence by the addition of pepsin because native whey protein is not sensible to pepsin hydrolysis. Moreover, the gel strength of Sample 0 was slightly decreased between 70 and 100min. When the pH was decreased from 4.01 to 3.97, the aggregates of whey protein were collapsed.

For Sample 40, 60 and 80, as the casein content increased, the stiffness of the obtained gels was increased. Since the pepsin concentration was 1U/mL, the G^* of Sample 60 and 80 reached the highest value, along with the highest stiffness. However, the stiffness of Sample 40 decreased from 0U/mL to 2.5U/mL of the pepsin concentration. Moreover, the increasing content of caseins in infant formulae contributed to the enhance of breaking stress σ_{max} of formula gels. At 2.5U/mL, sample 40 and 60 concentration demonstrated a smaller value of σ_{max} in comparison with the gels formed in acidification. In contrast, the gel of Sample 80 at 2.5U/mL exhibited a larger value of σ_{max} in comparison with the acid-induced gels. This finding suggested that the pepsin induced gels of Sample 40 and Sample 60 are more susceptible to rearrangement and fracture under large deformation. By analysing the result of SDS-PAGE, at either lower pepsin concentration (1U/mL) or higher pepsin concentration (2.5U/mL), para-κ-casein bands were detected in Sample 40, 60 and 80 at 5 min. At lower pepsin concentration (0-1U/mL), the infant formulae contained lower casein content exhibited shorter gelation time and higher gelation pH. At higher pepsin concentration (2.5U/mL), all casein contained infant formulae could form gel above pH 6.0 within 5 min. The observed differences in the rheological properties and gelation behaviour in different infant formulae may impact digestive behaviour.

4.2 Introduction

The pH of milk is an important parameter, which influences the gelation characteristics of dairy proteins. For reducing the pH, hydrochloric acid and GDL are commonly utilised. Comparing with adding hydrochloric acid, adding GDL can reduce the pH on solution without stirring and form homogeneous gels. Hydrolysing GDL in water could reach lower pH values than adding HCL, the final pH is reached slowly. Moreover, because it releases one proton per molecule in water, the pH of the emulsion could be reduced progressively (Kharlamova et al., 2018), the homogenous gel could be firmed at around pH 4 in 3 hours (Mession et al., 2017).

There are two primary milk protein ingredients (whey protein isolates and skim milk powder) utilised in this experiment, the expected coagulation behaviour of those ingredients has been provided by reviewing previous reports. Firstly, whey protein isolate solution is expected to be gelled in the region of pH 5.0-5.2, where the turbidity and the particle size of whey protein reach the maximum value (Ju et al., 1998; Rabiey et al., 2009). The major whey proteins are β-Lg (around 50% of total whey protein) and α-La (about 20% of whey protein). β-Lg, as a globular protein, exists as a dimer at high and neutral pH, aggregates in solution at its iso-electric point (p$I \approx 5.1$). Between pH 4.8 and 5.2, the net charge of β-Lg is really low and this causes the formation of larger aggregates. The pH continuous to decline in the gel, the turbidity of β-Lg gel decreases below pH 4.5 due to the increased electrostatic repulsion and soluble positively charged coagulation (Alting et al., 2003). At the pH below 3, it forms a mixture of monomers and dimers before dissociating (Macierzanka et al., 2012). In terms of α-La, it is a smaller globular protein with p*I* around 4.8. As the rest, bovine serum albumin is typically gelled at its p*I* (pH4.7-4.9) (Nicolai et al., 2011). Secondly, unheated skim bovine milk generally aggregates at pH 4.8 (Li et al., 2020). Bovine milk commonly contains around 80% caseins and 20% whey proteins. Casein micelles become disintegrated when dairy is acidified to pH 5, the layer on the surface collapses but still remains intact. When the pH of dairy reached to pH 4.6 (the p*I* of casein micelles), casein micelles become coagulated (Vasbinder et al., 2003). In simulated gastric fluid (SGF), casein could form acid-induced gel at about pH 5. If employing pepsin on the coagulation process, the gelation time could be shorter and the gelation pH could be higher (pH>6) due to the action of pepsin on casein micelles (Ye et al., 2019a).

Chapter 4: Effect of milk protein composition on pepsin-induced gelation of model infant formulae: the rheological properties.

There are several factors, which could affect the rheological properties of dairy samples. For example, at any given pH, increasing the milk concentration from 10 to 20% of total solids resulted in about a five-fold increase in gel stiffness (Lakemond et al., 2008). Moreover, the temperature on pre-treatment and the salt content also has influences on the gelation of diary samples. However, the information on the physicochemical characteristic of infant formula during pepsin-induced and acid-induced gelation is limited. In current study, the objective was to compare the physiological properties and gelation behaviours of model infant formula made with different protein composition.

4.3 Results and Discussion

4.3.1 pH profile

The changes in the pH of the samples obtained from the coagulation process is shown in Figures 4.1. Principally, the initial pHs of all samples were fixed at 6.8. Since the pH decreased as the function of gelation time, the final pH of all gels was at the range of 3.8 to 4.3. There were some discrepancies among the four dairy samples in pH curves.

In the case of acid-induced coagulation (Figure 4,1A), at the first 10 min, the pH profile of four model infant formulae shows no difference. In terms of pepsin induced coagulation (Figure 4.1B), At the first 10 minutes, the pH in Sample 0 reached pH 4.5 earlier than other three samples, it was assumed that the pH-induced coagulation in whey protein was relatively faster.

Comparing the acid-induced curves and pepsin-induced curves (Figure 4.1), acid-induced samples have faster decrease in pH, decreasing to pH 5 within the first 20 minutes and close to pH 4 at 180minute. Pepsin addition lightly increased the final pH of all casein-contained samples, the influences on the final pH of Sample 0 was not obvious. It is assumed that the addition of pepsin has no change in the gelation behaviour of whey protein, whey protein aggregates were resistant to pepsinolysis because of the hidden intermolecular β-sheet (Macierzanka et al., 2012).

Comparing the final pH of Sample 80 with other two casein-contained samples, Sample 80 reached a final pH at 4.04, Sample 40 and Sample 60 reached a final pH both at 4.20. Hence, when the ratio of casein to whey protein increased from 40:60 to 80:20, the final pH of model infant formula presented a slight drop of final pH. It is assumed that the final pH was slightly decreased because of the reduction of free calcium ions in serum phase. The presence of free calcium ions promotes the aggregation of para-κ-casein micelles. By further solidification, calcium-induced reaction happens between protein molecules. The quantity of free calcium ions was decreased, a system-spanning network that has a fractal-like appearance is formed. The higher content of caseins contributed to higher consumption of calcium ions, the final pH of Sample 80 was consequently mildly lower than Sample 40 and Sample 60.

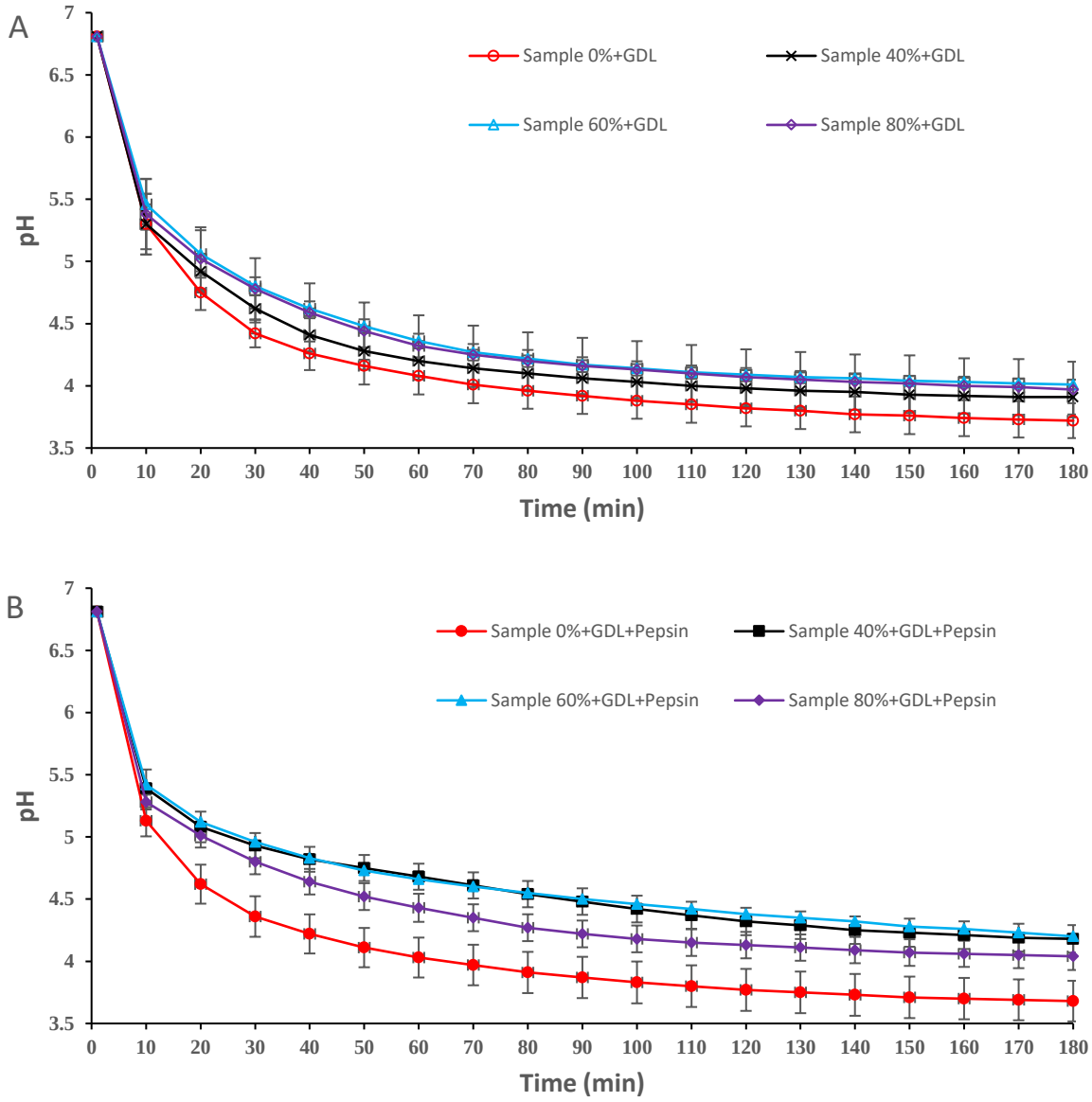


Figure 4.1. The changes in pH over time for samples with different whey protein/casein ratios: (A) acidification with the absence of pepsin; (B) acidification with the presence of pepsin: sample 0 (whey/casein: 100/0), sample 40 (whey/casein: 60/40), sample 60 (whey/casein: 40/60) and sample 80 (whey/casein: 20/80).

4.3.2 Changes in elastic modulus of infant formula gels

The coagulation of dairy protein-based emulsions (model infant formula) has been illustrated by elastic modulus (G') and loss modulus (G''), the changes in G' and pH as a function of gelation time in model infant formula are presented in Figure 4.2. The G' are important to be applied to indicate the rheological property of gel firmness. The rheological measurement started at 2 min of gelation.

The acidification process of all model infant formulas was shown in Figure 4.2A, 2% (w/w) Glucono-δ-lactone (GDL), progressive acidification using agents, has been employed in acid gelation of Sample 0, 40, 60, 80. During the process, the final G' of sample 40 was slightly lower than sample 60 and Sample 80. As the result, the absence of casein content in Sample 0 resulted in a lower gel hardness which agrees with the results of Tari et al., (2018).

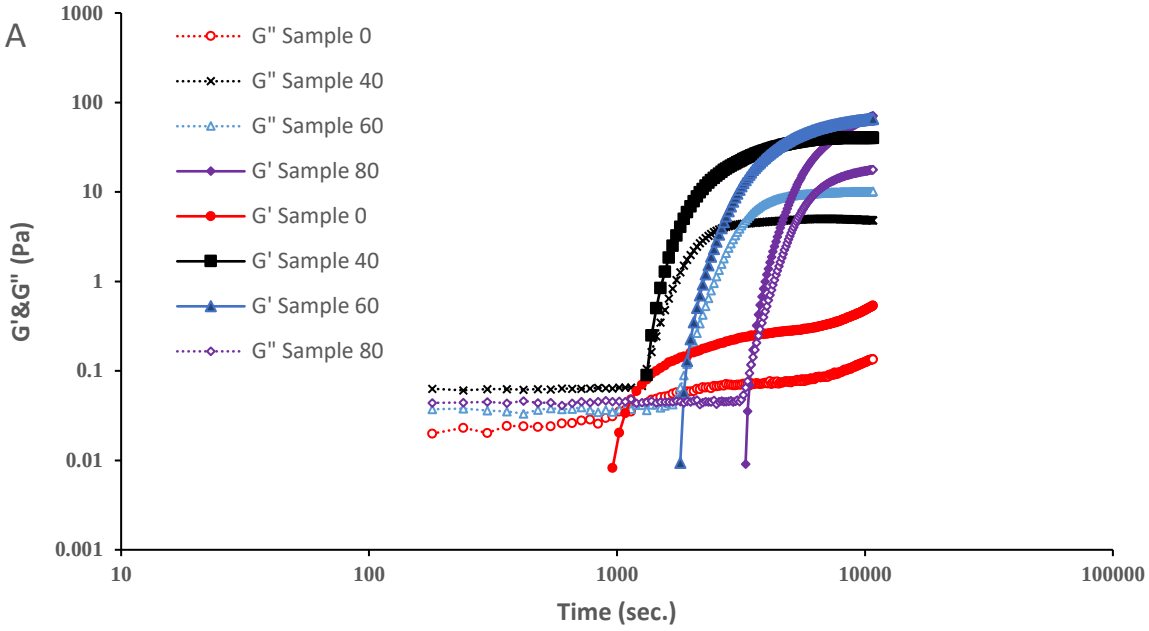
For casein contained Samples, obtaining higher gel hardness relied on the greater number of interactions between casein micelles at low pH. The decrease pH led to solubilisation of colloidal calcium phosphate and a decreased net negative charge of casein micelles, the electrostatic repulsion was reduced and the hydrophobic attraction between proteins was increased (Nguyen et al., 2018). Since the presence of casein content would cause a greater number of 'crosslinks' (interactions) between casein micelles during acidification, therefore leading to a stronger acidified milk gel network, resulting in the increase in the G' (Li et al., 2018; Nguyen et al., 2018; Wang et al., 2019). However, in terms of the G' of Sample 40, Sample 60 and Sample 80, the G' among them was present similar values. Particularly, the initial increase of G' of Sample 0 occurs at the earliest, the G' of Sample 40 increased at next, followed by the Sample 60. The growing of G' of Sample 80 started at the last. The initial increase of G' could be impacted by the level of whey protein, indicating a rapid process of acid-induced whey protein coagulation, which might have influence on the gelation pH in acid-induced reaction.

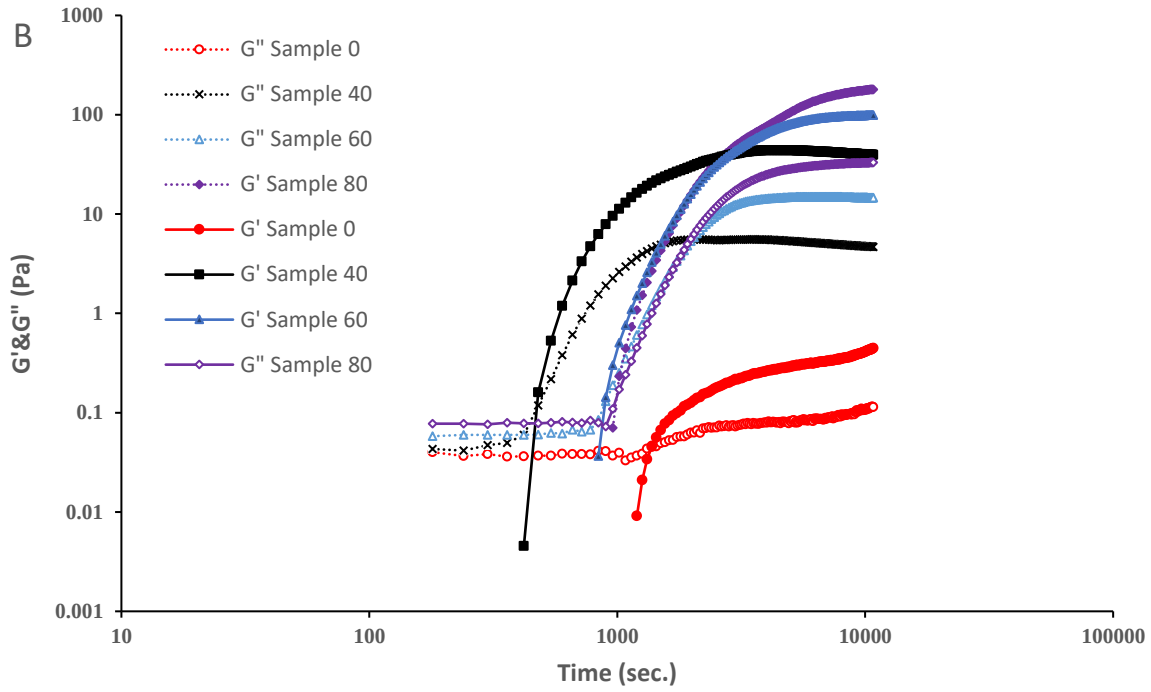
In Figure 4.2 (B,C,D,E), 2%(w/w) GDL were added in those samples, and 4 different level of pepsin (0.3U/mL; 1U/mL; 1.7U/mL; 2.5U/mL) were employed in 4 dairy samples in orders. For Sample 0, the G' and $G^{"}$curves were presented at the lowest part in all pictures, which presented a more fragment structure than whey-casein mixed infant formulas. The gel of Sample 0 was fragile because native whey proteins are resistant to the proteolysis (Macierzanka et al., 2012), and the gelation of it was highly depended on the loss of steric repulsion during acidification. (Sadeghi et al., 2014). When the different level of pepsin added into Sample 0, the G' profiles showed inapparent changes. Interestingly, in Figures 4.2A, G' of Sample 0 showed a trend of increase at the beginning. When the pH was decreased between 4.4 and 4.0, the G' profile of it began dropping; it suggested that a break of gels structure happened in the middle of coagulation. Further decreasing pH from 4.0 to 3.5, the G' however returned to growth.

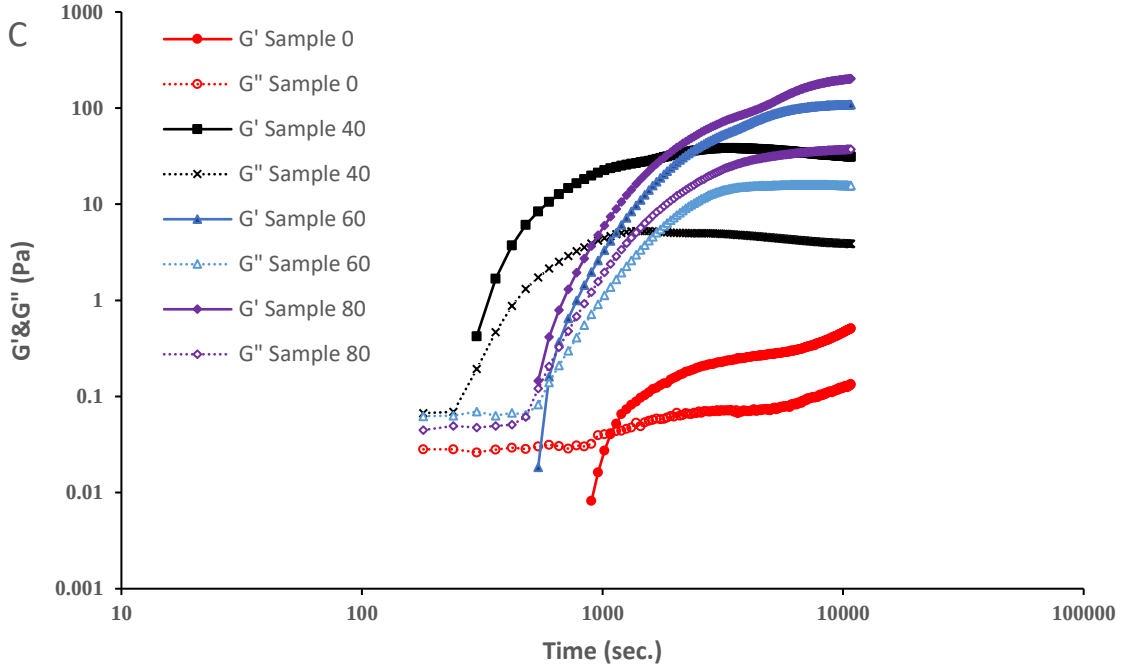
In terms of casein contained infant formulae, since a slight amount of pepsin (0.3U/mL) added into those samples, Sample 80 shows higher final G' than Sample 60 and sample 40. When 0.3U/mL-1.7U/mL pepsin employed in these samples, the G' profiles of sample 60 and sample 80 showed similar trend but differed in final G'. Further increasing the pepsin concentration to 2.5U/mL, the G' curves of sample 80 is higher than sample 60 during the whole coagulating time. By further analysis, the viscosity of milk shows no change until casein micelles aggregate completely under sustained pH (Donald J. McMahon & Brown, 1984b). The result indicates that the infant formula with high level of casein is more sensitive to the concentration of pepsin. The addition of pepsin could enhance the gel hardness of caseins because of the unique structure and functional properties. (Devle et al., 2012; Sandra et al., 2007). During acidification, the steric stabilisation is reduced, colloidal calcium phosphate (CCP) are gradually dissolved from micelles into the aqueous phase (Gonzalez-Jordan et al., 2015). The hairs of κ-caseins on the surface of the micelles are charged, the κ-casein brush was progressively lost and collapsed. At the isoelectric point of caseins (pH 4.6), aggregates were formed via hydrophobic interaction as electrostatic repulsion was reduced (Sinaga et al., 2016). The addition of pepsin stimulated the action of proteolysis of caseins, κ-caseins were mostly hydrolysed and the colloidal stability of casein micelles was lost, the aggregation of casein micelles occurred in the presence of free calcium ions (Douglas G et al., 2004). Molecular chains connect through hydrophobic interaction to form a three-dimensional network. By further solidification through a calcium-induced reaction between protein molecules, aggregates of caseins is formed.

Additionally, since the higher pepsin concentration employed, the initial increase of G' of Sample 40 started at first, followed by Sample 60 and Sample 80. At 2.5U/mL of pepsin concentration, the initial increase of G' of all casein contained samples occurred at the same time. it assumed that the addition of pepsin increased the coagulation rate of caseins in these samples. For Sample 40, the higher pepsin concentration the shorter time casein contained infant formula consumed to reach plateau, which was consistent with Hemar et al., (2004), the addition of proteolytic enzymes led to the coagulation of casein micelles, when the casein micelles were fully aggregated to form a particulate gels, the plateau value is consequently reached. The initial decrease in G' occurs after these samples reached plateaus, indicating an initiating weakening of hydrophobic bonds(Lucey et al., 1997). For analysing the extensive hydrolysis in four infant formula, the final

complex modulus (G^*) of all samples at different pepsin concentration were further researched.




B
G" Sample 0
G" Sample 40
G" Sample 60
G' Sample 80
G' Sample 0
G' Sample 40
G' Sample 60
G" Sample 80
G'&G" (Pa)
1000
100
10
1
0.1
0.01
0.001
10
100
1000
10000
100000
Time (sec.)


C
G' Sample 0
G" Sample 0
G' Sample 40
G" Sample 40
G' Sample 60
G" Sample 60
G' Sample 80
G" Sample 80
G'&G" (Pa)
1000
100
10
1
0.1
0.01
0.001
10
100
1000
10000
100000
Time (sec.)

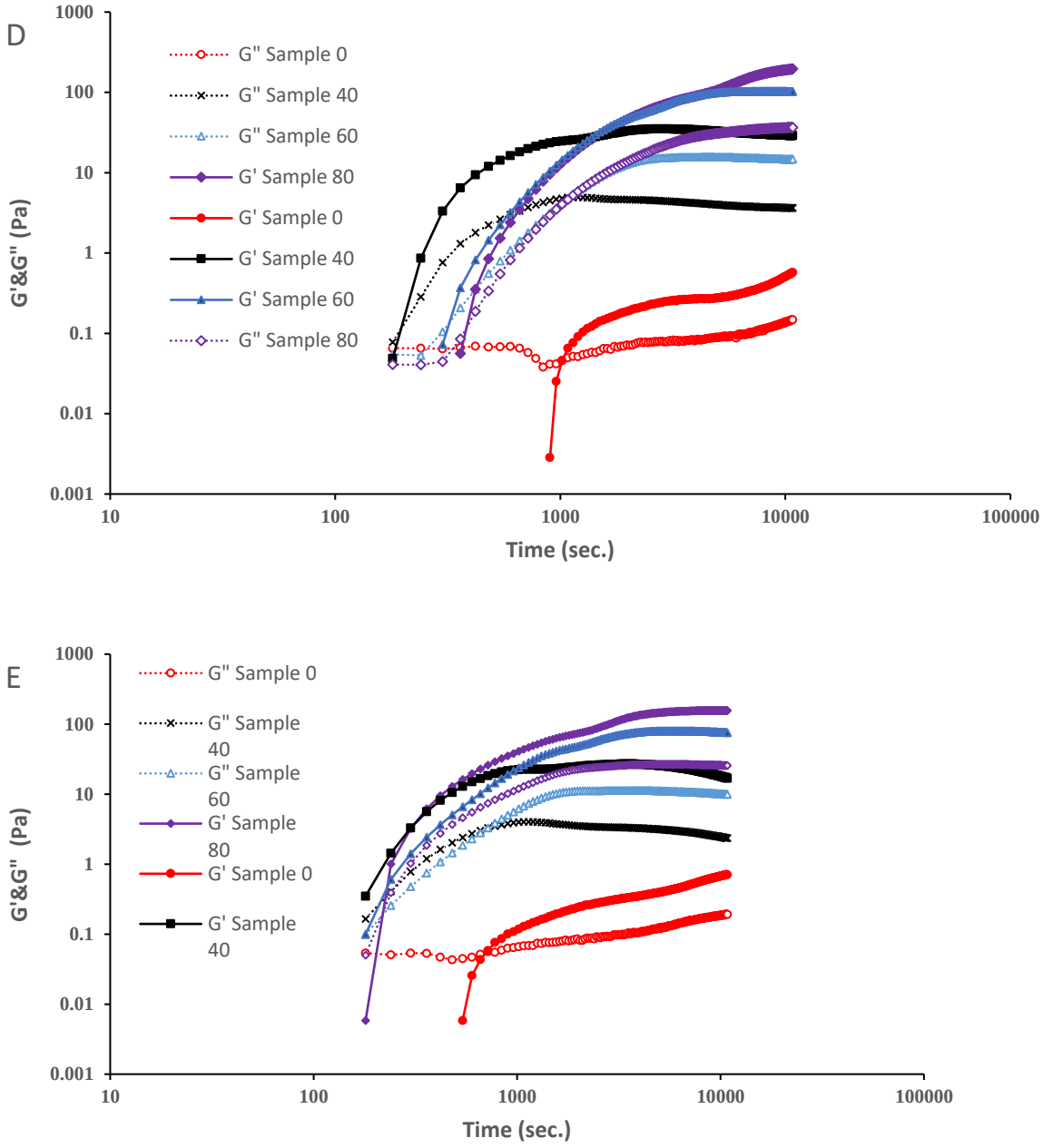


Figure 4.2. Elastic modulus G' and loss modulus G'' as a function of time (min). Change in G' of the model infant formulae: -●-, Sample 0; -■-, Sample 40; -▲-, Sample 60; -◆-, Sample 80. Change in G'' of the model infant formulae: -○-, Sample 0; -×-, Sample 40; -△-, Sample 60; -◊-, Sample 80. (A) Acid coagulation curve of dairy samples, 2% (w/w) Glucono-delta-lactone (GDL) was employed in process; (B) Coagulation curve of dairy samples, 2%(w/w) GDL and 0.3U/mL pepsin was employed in process; (C) Coagulation curve of dairy samples, 2%(w/w) GDL and 1U/mL pepsin was employed in process; (D) Coagulation curve of dairy samples, 2%(w/w) GDL and 1.7U/mL pepsin was employed in process; (E) Coagulation curve of dairy samples, 2%(w/w) GDL and 2.5U/mL pepsin was employed in process. sample 0 (whey/casein: 100/0), sample 40 (whey/casein: 60/40), sample 60 (whey/casein: 40/60) and sample 80 (whey/casein: 20/80).

4.3.3 Course of complex modulus at the end of time sweep

Figure 4.3 showed how final complex modulus (G^*), a measure of viscoelastic properties of the gel, changed in the pepsin concentration of model infant formula. The final $G^* = \sqrt{G'^2 + G"^2}$ is taken into considering the G' and $G"$, as a function of pepsin concentration for model infant formulas.

During the acidification of infant formulas, casein contained samples (sample 40, sample 60 and sample 80) formed stiffer gel (reach higher final G^*) compared to whey protein-based samples (sample 0), Fox, (1993) indicated that the increase in the value of stiffness is due to the increase in the strength and number of casein gel network. By comparing the final G^* of Sample 40, Sample 60 and Sample 80, the value showed small differences. It suggested that the effect of casein to whey ratio did not have remarkable influences on the final stiffness of acid-induced casein gels, which is consistent to (Sadeghi et al., 2014; Sinaga et al., 2016).

In terms of casein-contained samples, as Figure 4.3 demonstrated, the stiffness of them was varied at different concentration of pepsin. Without pepsin addition, the G^* value of Sample 40, Sample 60 and Sample 80 was between 40.3 Pa and 70.2 Pa. Since the pepsin concentration increased to 1U/mL, the G^* curves of them gradually reached the peak at 110Pa and 206Pa respectively. It consisted to Devle et al. (2012), the gastric enzyme (Pepsin) could enhance the firmness of infant formula gels by forming casein coagulation.

Moreover, the final G^* values of infant formulae were impacted by differences in the content of caseins. For example, G^* of sample 80 increased much faster than that of sample 60, the G^*value of sample 80 has almost been tripled, and the G^* value of sample 60 increased over 1.5 times. However, when the pepsin concentration became higher, there was a gentle decrease of their G^*. In the range of 1U/mL -2.5U/mL, the G^* of sample 80 has been decreased by 23%, and that of sample 60 has been reduced by 30%. The G^* curve of sample 40 was below that of sample 60 and sample 80, which was keeping down throughout the whole time. The decreases of G^* reflects the breakdown in the gel structure after the formation of casein gels. Under 0.3U/mL of pepsin, the extensive hydrolysis could start in Sample 40. In the range of 1U/mL -2.5U/mL, the extensive hydrolysis occurred in sample 60 and sample 80.

For whey protein-based sample, the stiffness of the gel of Sample 0 is below 1Pa. The G^* showed no significant change since the pepsin concentration was increased. The

result consists to Mat et al., (2018), the major protein in whey protein (β-Lg) is resistance to be hydrolysed by enzyme, the free S-H groups were hidden in the interior of its global structure (Boland et al., 2020).

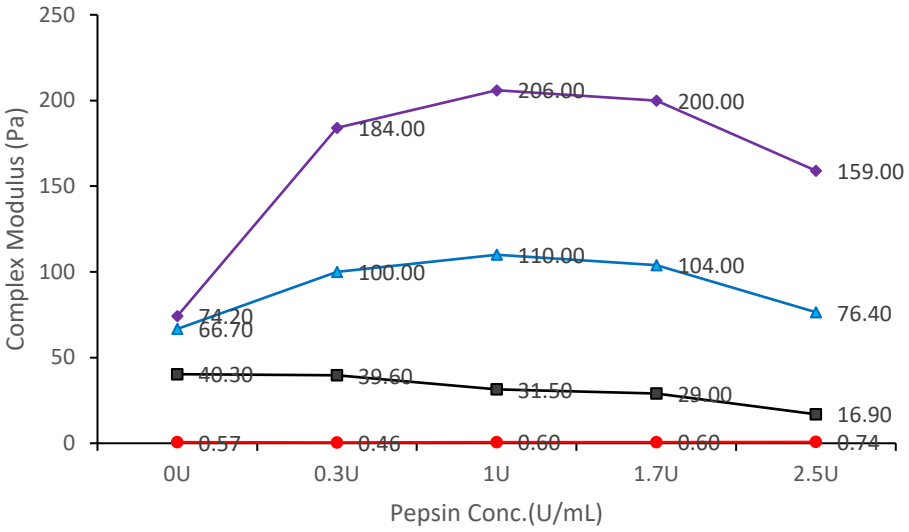


Figure 4.3. Variation of Final G^* for the different mixtures: sample 0 (whey/casein: 100/0), sample 40 (whey/casein: 60/40), sample 60 (whey/casein: 40/60) and sample 80 (whey/casein: 20/80).

4.3.4 Frequency sweep measurement and strain sweep measurement

For investigating the viscoelastic behaviour of infant formula gels, a frequency sweep was performed at 1% strain at the end of time sweep. Figure 4.4 A and B demonstrated the frequency sweep tests for different infant formula. For all casein-contained gels, in the whole range of frequency, G' was almost parallel with G'' and both have the trend of increasing with the increase of frequency, proposing a weak solid gel characteristic (Wang et al., 2019). For Sample 0, with the combination effects of pepsin and GDL, the G' decreased moderately and crossed with G'' between 24-20 Hz.

To compare between the different infant formula gels, the value of breaking stress (σ_{max}) and maximum strain (γ_{max}) (in Figure 4.4 C) were studied. In the case of Sample 0, the profile of shear stress was below 2Pa throughout the strain sweep, which indicated that Sample 0 presented in a liquid form in the gelation process. For all casein contained infant formula, the shear stress profile exhibit linear elasticity in the low strain region. after the linear viscoelastic region, the plateau of shear stress was carried out, which indicated a gel-hardening system in these gels. After the non-linear region, the shear stress was decreased as the function of strain, it assumed that the breakdown of gels

occurred (Groot et al., 1996). For Sample 40, the value of σ_{max} is 5.90±0.1 Pa and the value of γ_{max} is 10% in acidification, and the value of σ_{max} is 1.18±0.1 Pa and the value of γ_{max} is 20% in pepsin coagulation. For Sample 60, the value of σ_{max} is 14.1±0.1 Pa and the value of γ_{max} is 32% in acidification, and the value of σ_{max} is 9.57±0.1 Pa and the value of γ_{max} is 16% in pepsin coagulation. For Sample 80, the value of σ_{max} is 16.90±0.1 Pa and the value of γ_{max} is 40% in acidification, and the value of σ_{max} is 29.10±0.1 Pa and the value of γ_{max} is 25% in pepsin coagulation. According to the data obtained by the time sweep measurement and frequency sweep measurement, the Sample with higher level of casein obtained the higher value of σ_{max} and γ_{max}, indicating the formation of more elastic gels. In agreement with (Lucey et al., 1997), the shear stress was reduced in gels with the increasing content of whey protein. Moreover, for sample 40 and sample 60, the gel made with pepsin is more fragile than the acid-gel, which assumed the extensive hydrolysis of casein by pepsin in lower casein content samples. Compared the maximum strain between acid-gels and pepsin-gels, the maximum strain of pepsin aggregates was lower than that of acid-induced aggregates. It could due to the demineralization during acidification. At low pH, CCP could be released to serum phase. For enzyme-induced gels, the structure would become fragile. For acid-induced gels, the syneresis could happen and the structure would become dense (van Vliet, 2000; Nogueira et al., 2020).

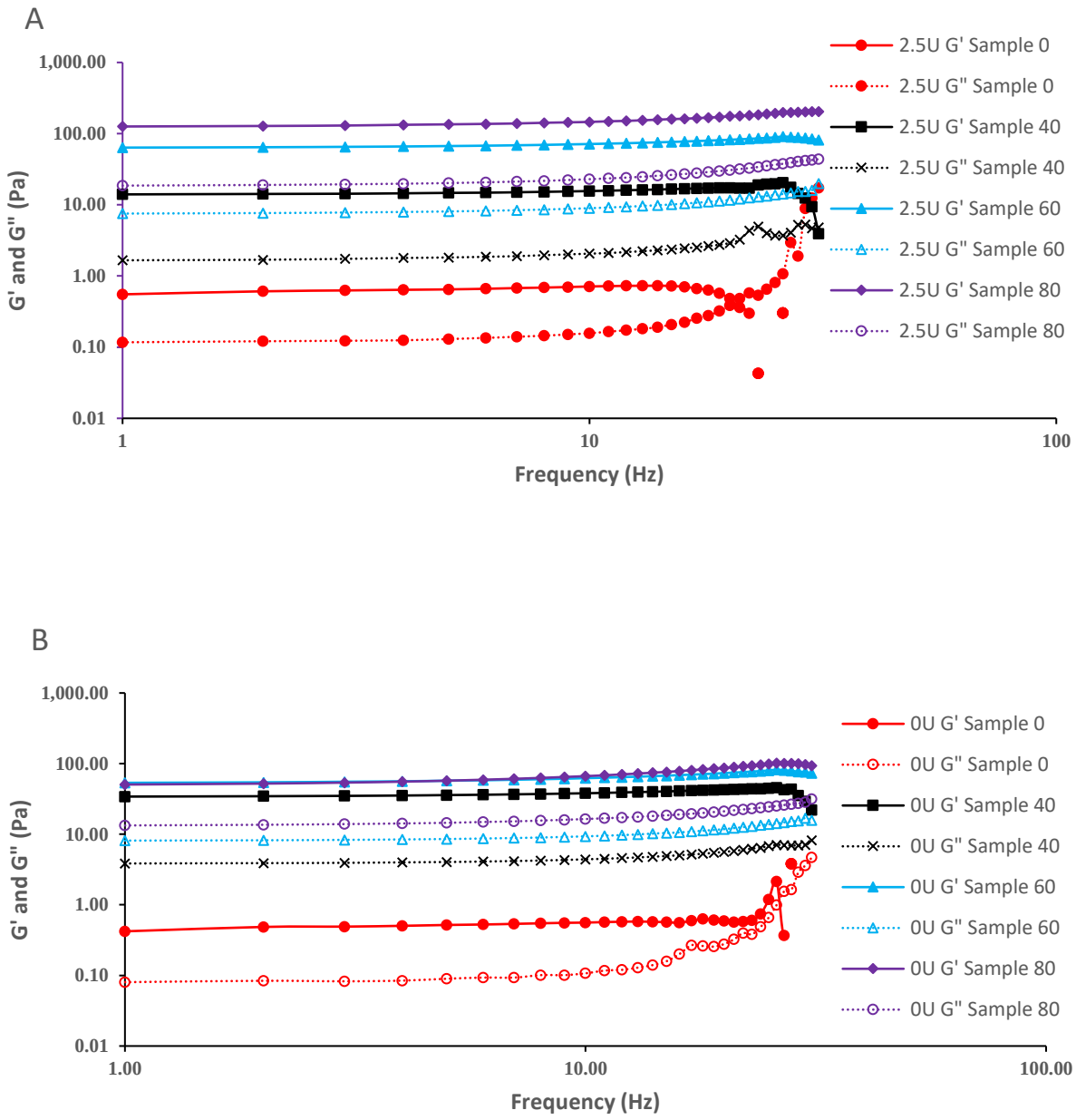


Figure 4.4. Storage modulus (G') and loss modulus (G'') as a function of frequency. A showed the coagulation of samples with 2%(w/w) GDL and 2.5U/mL pepsin. B showed the acid-induced coagulation of samples with 2% (w/w) GDL. sample 0 (whey/casein: 100/0), sample 40 (whey/casein: 60/40), sample 60 (whey/casein: 40/60) and sample 80 (whey/casein: 20/80).

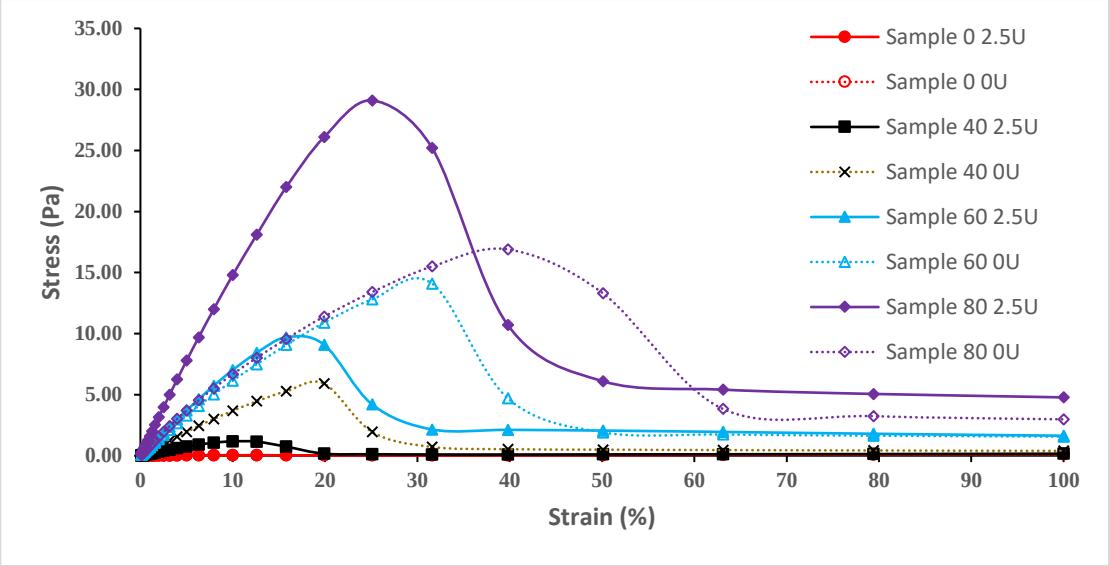


Figure 4.5. The stress-strain curves of Sample acidified with 2% (w/w) GDL and/or 2.5U/mL pepsin. Protein composition (whey/ casein) are 100/0 (sample 0); 60/40 (sample 40); 40/60 (sample 60); 20/80 (sample 80).

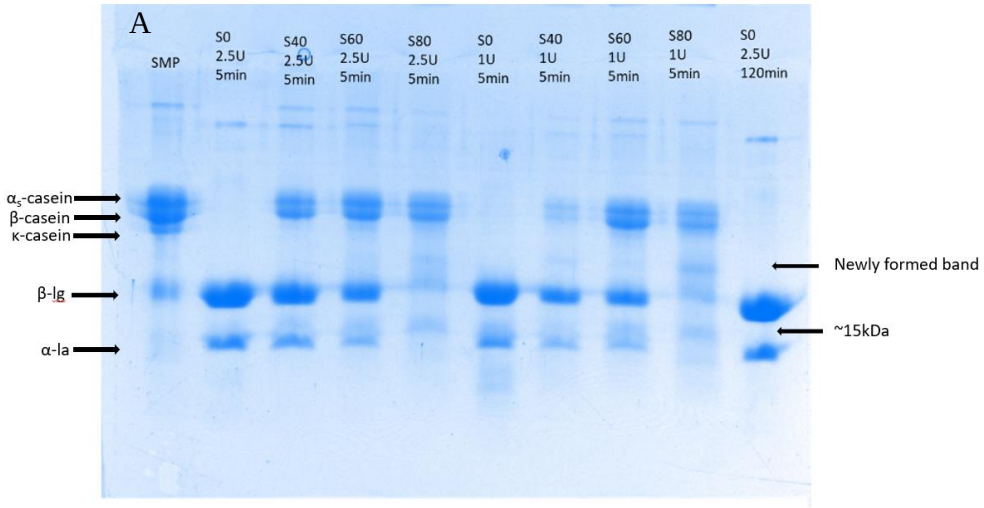
4.3.5 Protein hydrolysis

The protein hydrolysis of dairy samples during the 120 minutes gelation phase is shown in Figure 4.6. The major protein composition of all samples at 5 minutes and 120 minutes of digestion time was determined by SDS-PAGE.

In general, the intensities of α_s-casein, β-casein, β-Lg and α-La did not appear to change during the whole time. In agreement with Ye et al., (2016b), α_s-casein, β-casein, β-Lg and α-La were resist *in vitro* gastric digestion of skim milk. For β-Lg and α-La, the level of pepsin content and the gelation time did not appear to affect the amount of residual. The hydrolysis of Sample 0 showed the similarity in behaviour of both β-Lg and α-La in whey protein isolate.

Figure 4.6 showed the protein hydrolysis at 5 min. Under the pepsin concentration at 1U/mL, the κ-casein band was appeared in Sample 60and Sample 80, the κ-casein in Sample 40 was hydrolysed. Moreover, 15 kDa band could be observed in sample 40, 60 and 80, which reports that the formation of para-κ-casein were occurred in all casein contained samples at 5 minutes of digestion. Under the pepsin concentration at 2.5U/mL, the κ-casein band could be also observed in Sample 40, 60 and 80, the 15kDa band showed apparently. Additionally, the SDS-PAGE pattern showed that the newly formed band at ~20kDa was also observed in all casein content samples at 5 minutes.

After 120 min, the coagulation in casein-contained samples was confirmed. The intensities of 15 kDa band was increased as the function of time. However, the intensities of 15 kDa band in 1U/mL is higher than that in 2.5U/mL of pepsin concentration. By analysing the curves of G', $G^{"}$ and G^*, when 1U/mL pepsin induced casein in 180 minutes, the G' is growing with the increasing gelation time, κ-casein was consumed, and para-κ-casein was produced after 120minutes of digestion time. In contrast, 2.5U/mL pepsin induced casein in 180 minutes, the G' is dropping at the early stage because of the higher enzyme concentration. Furthermore, the G^* value of the sample under 2.5U/mL pepsin is lower than the sample under 1U/mL. Hence, the band of 15kDa showed limited changes because the para-κ-casein was mildly hydrolysed by pepsin before the end of digestion. Moreover, the κ-casein bands of 2.5U/mL pepsin induced samples showed apparently, it assumed that the hairs of κ-casein were released to the serum phase during fast hydrolysis process.



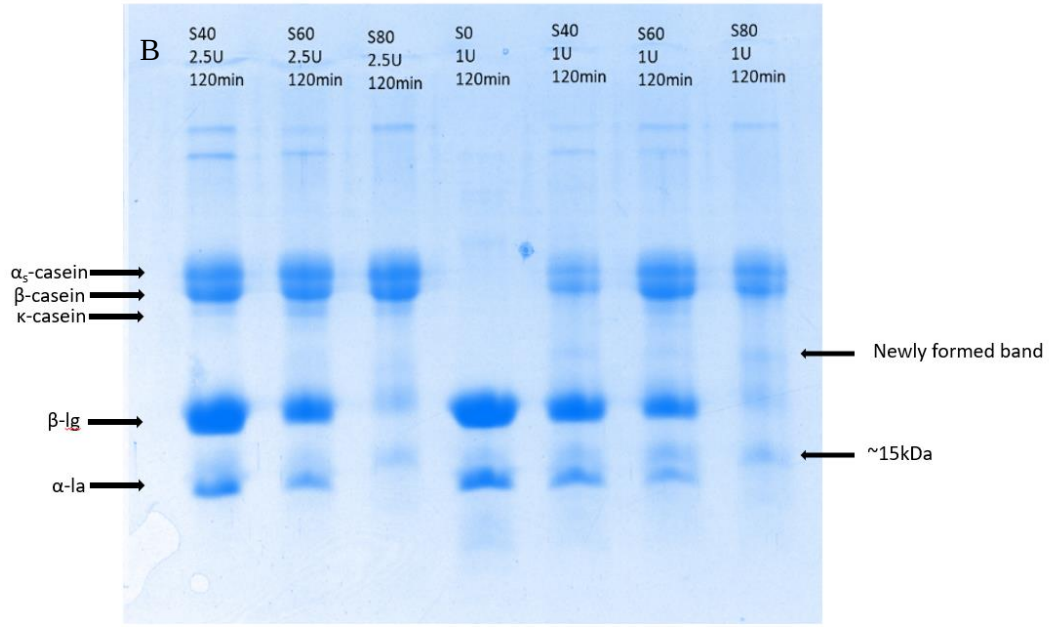


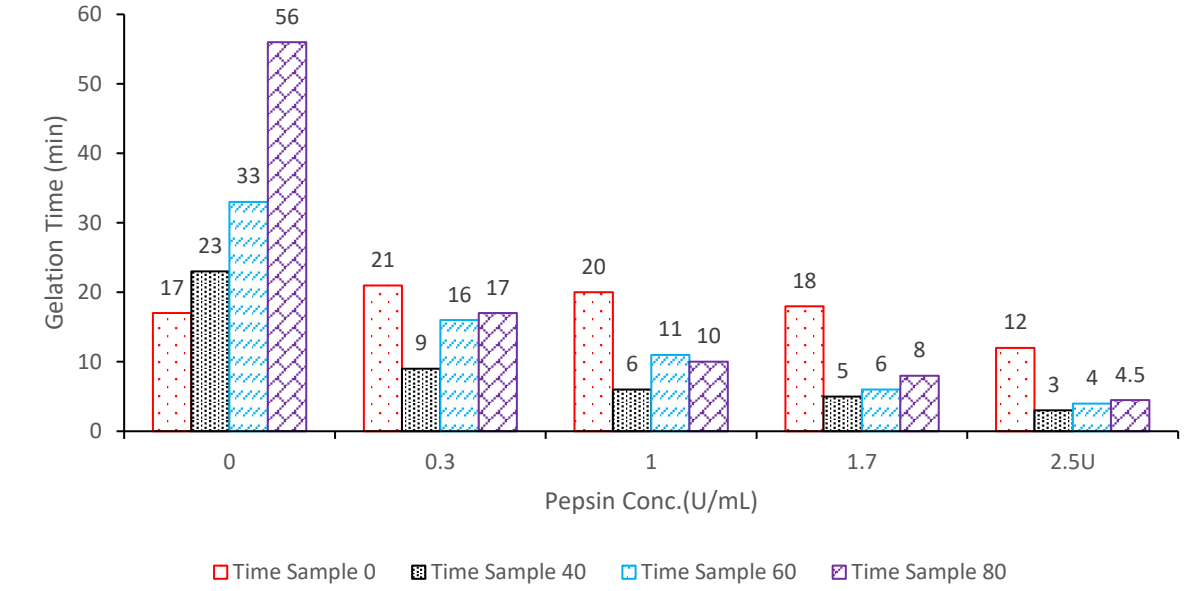
Figure 4.6. SDS-PAGE patterns under reducing condition of Sample 0 (S0), Sample 40 (S40), Sample 60 (S60), Sample 80 (S80); 2.5U, pepsin concentration at 2.5U/mL; 1U, pepsin concentration at 1U/mL. A showed all samples at 5min of gelation time; B showed all samples at 120 min of gelation time.

4.3.6 The gelation pH and gelation time

Figure 4.7 showed the effect of protein composition on the coagulation of milk protein mixture, meanwhile, the pepsin-induced gelation influenced by varying pepsin concentration was studied. Table 4.1. displayed the critical data that allocated from Figure 4.7. The influences on coagulation could be observed on the varying gelation time and gelation pH, those two factors have been described in the following paragraphs. The gelation pH is the milk pH at the critical point where G'' crossed G'. The gelation time, defined here as the time at which the coagulation in milk started.

Table 4.1. Gelation time (GT) and pH of each sample.

	0U		**0.3U**		**1U**		**1.7U**		**2.5U**	
	pH	GT	pH	GT	pH	GT	pH	GT	pH	GT
Sample 0	5.10	17	4.90	21	4.94	20	5.02	18	5.01	12
Sample 40	4.97	23	5.49	9	5.71	6	5.96	5	6.28	3
Sample 60	4.93	33	5.43	16	5.87	11	6.09	6	6.28	4
Sample 80	4.41	56	5.21	17	5.63	10	5.95	8	6.30	4.5



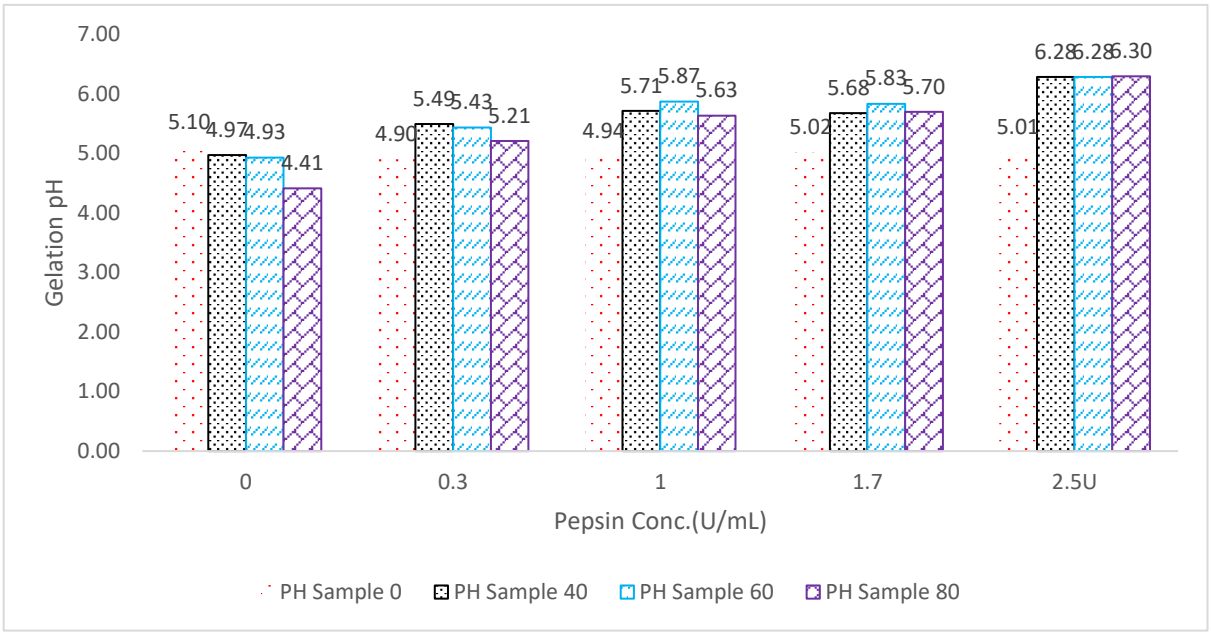


Figure 4.7. Variation of gelation time and gelation pH for the different mixtures. Whey protein/caseins 100/0 (sample 0), 60/40 (sample 40), 40/60(sample 60), 20/80 (sample 80) for 1.638% (w/w) protein.

4.3.6.1 Coagulation behaviour of whey-protein based infant formula

In terms of whey protein based infant formula, Sample 0 had the shortest gelation time during the acidification. In agreement with Macierzanka et al., (2012), since the pH of sample 0 got close to its p*I* (4.8-5.2), the net charge of β-Lg is very low resulted in the formation of large aggregates, the formation had been completed in a short time. β-Lg is the major whey protein in bovine milk, accounting for approximately 60% of all. it is a well folded-globular protein and has a unique structural feature, which leads it to be resistant to pepsin and other proteases at low pH (Nicolai et al., 2011; Ye et al., 2016b; Zhuang et al., 2018).

Based on the result in Table 4.1., When the pH of whey protein solution reached 4.9-5.1, where is close to its p*I* (pH 5.5), the gels were formed. This result was in consistent with the previous studies from (Ju et al., 1998). In that study, the whey protein mechanism has been provided in Figure 4.8. At the first stage, the protein aggregates are dominated by physical interaction. At the second stage, the thiol-disulfide interchange reactions is occurring, which attribute to the formation of gel network and the increase of gel hardness (Nicolai et al., 2011).

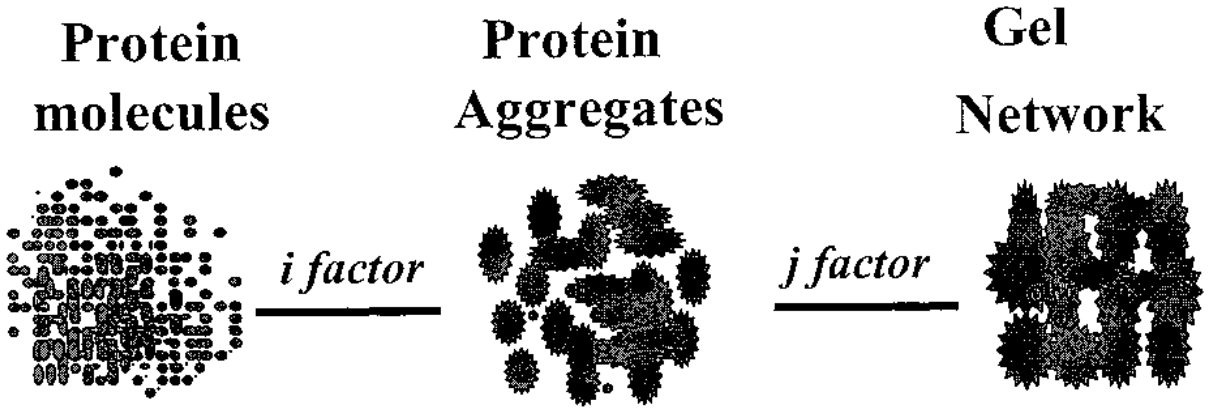


Figure 4.8. Protein gelation mechanism. *i* and *j* factors are environmental factors, which could be acid, salt, enzyme and heat. Those factors produce the coagulation of whey protein and the gel formation, which present at two separating stages. (Ju et al., 1998)

Analysing the G' in Figures 4.2, G' of all gelation samples began decreasing around 70-100 min when pH is between 4.01±0.15 and 3.88±0.15 and between 3.97±0.17 and 3.83±0.17. Further decreasing pH from 4.0, the G' however retuned to growth. It is suggested that the gel stiffness decreased between pH 4.3-4.0, which was also assumed by Donato et al., (2011), Kharlamova et al., (2018) and Rabiey et al., (2009). In their

researches, the gel formed by acidification of the mixture of whey protein microgels (R_h=270nm) and soluble aggregates (R_h=100nm), the stiffness of it was decreased below pH 4.3. (Ju et al., 1998) suggested that since the pH decreased from pH 6 to pH 5.2 of whey protein, the repulsive electrostatic interaction between aggregates decreased simultaneously. Further lowering pH from pH 5.2 to 4.0, the stiffness of gel presented a slight decrease, which could be due to that the gels formed at pH 5.2 were gradually disaggregated at pH 5.2.

4.3.6.2 The gelation behaviour of casein-whey based infant formulae

During the gelation of infant formula, the gelation behaviour of infant formulae was impacted by the ratio of casein to whey protein. Moreover, the pepsin concentration could influence their gelation pH and gelation time.

Firstly, the four different infant formulae were compared. In terms of the gelation without pepsin, Sample 80 formed gel at pH 4.41, and Sample 40 & 60 started gelation at the pH of 4.97 and 4.93 respectively. It is known that the electrostatic repulsion in unheated milk is largely depended on the stability of casein micelles, the p*I* of micelles is between 4.0 and 4.5 (Morand et al., 2012). The acid-induced coagulation was dominant in this process.

With the increasing concentration of pepsin from 0U/mL to 2.5U/mL, the gelation pH of sample 80 was increased dramatically from 4.41 to 6.30, and the gelation time was decreased from 56 to 4.5, minimised of 92%. For sample 40 and 60, the gelation pH was increased from around 4.95 to 6.28, and the gelation time was dropped of 86% and 88% respectively. As a result, the gelation pH of casein contained samples grew rapidly since increase the pepsin to 1UmL, and then the increase rate of gelation pH was declined with the further addition of pepsin. In the case of gelation time, the higher content of casein in dairy samples, the more influence shown on the gelation time of casein contained samples. It could be due to increased hydrolysis rate of κ-casein arising from the addition of pepsin. (Gastaldi et al., 2003; Koutina et al., 2015).

With the combined action of acid and pepsin, at pepsin concentration 1U/mL, the gelation time of sample 60 is slightly longer than sample 40, leading to a higher gelation pH of sample 60. The shorter gelation time in Sample 40 could be related to the higher hydrolysis rate of κ-casein (Koutina et al., 2015; Nogueira et al., 2020). Sinaga et al., (2017) indicated that two phases reactions occurred during coagulation, which are

enzymatic and non-enzymatic (pH) coagulation. During enzymatic reaction, pepsin hydrolysed κ-casein led to unstable structure of casein micelles. Since most κ-caseins were hydrolysed and most macro-pepide forming hairs were removed, pH coagulation started. Under low pepsin concentration, the hydrolysis rate of κ-caseins was slower. The sample with lower casein content obtained the higher degree of κ-casein hydrolysis in a shorter time, which is in agreement with McMahon et al., (1984b), the addition of caseins could increase the number of unhydrolyzed κ-casein and the lag time of coagulation would be extended. Hence, in current study, the sample contained 40% caseins formed gel earlier than Sample 60 and Sample 80 at pH<6.

With the increasing pepsin concentration to 1.7U/mL, the gelation time in samples with higher casein content decreased rapidly, and the gelation pH of all Samples were around pH 6. It could be related to the fast hydrolysis rate of κ-casein at pH 6.0, the breakdown of κ-casein is known to destabilise casein micelles led to coagulation (Ye et al., 2016b). when pepsin concentration increased to 2.5U/mL, all infant formula samples were gelled above pH 6.2, and the gap of the gelation time among them became narrow.

4.4 Conclusion

This study provides an insight into the physical properties and hydrolysis behaviour of proteins in different infant formulae under different pepsin concentration during acidification.

The different infant formulae with different ratio of casein to whey protein were aggregated by the action of pepsin and GDL. For Sample 0, the stiffness of it was slightly decreased between its p*I* and pH 4.0, which could be due to the partial collapse of its aggregates. The stiffness of Sample 0 shows limited influence by the addition of pepsin because whey protein is resistant to pepsinolysis. For Sample 40, 60 and 80, the formation of gels in infant formulae with the different casein to whey protein ratios caused the variation of gel stiffness. The higher casein content in infant formulae led to the higher stiffness in formed gels after 180 min gelation. The stiffness of Sample 40 was increased with increasing the casein content.

Besides, the gels of Sample 40 and Sample 60 formed with the addition of pepsin demonstrated a smaller value of σ_{max} in comparison with the gels formed without the addition of pepsin. This finding suggested that the pepsin induced gels of Sample 40 and Sample 60 are more susceptible to rearrangement and fracture under large deformation. Moreover, the gelation time and gelation pH were impacted by different pepsin concentration, which was resulted by different hydrolysis rate of κ-casein. Under lower pepsin concentration (below 1 U/mL), the infant formula samples with lower casein content obtained higher degree of κ-casein hydrolysis in a shorter time, which led to the higher gelation pH. With the increase in pepsin concentration, all casein contained infant formulae could form gel above pH 6.0, the gelation time among all those samples exhibited limited differences. These results indicated the ratio of casein to whey protein have different gelation properties induced by acid and pepsin, which may suggest that the infant formulae may have different digestion behaviours under gastric conditions due to these various gelation properties. This assumption will be investigated in following chapter.

Chapter 5: Simulated gastric digestion behaviour of model infant formulae: Effect of protein composition

5.1 Abstract

The objective of this study is to analyse the effect of protein composition on the *in vitro* gastric digestion of model infant formulae. Four infant formulae were classified by the casein to whey protein ratios, which were 0:100, 40:60, 60:40 and 80:20. The protein composition plays an important role in the digestion behaviour of infant formulae. The coagulation observed in four different infant formulae were affected by the casein to whey protein ratios. At the end of digestion, Sample 0, obtained large and dense flocculates and reached the lowest pH, and α-La was hydrolysed but intact β-Lg was detected. For Sample 40, small and fragile flocculates was formed during the gastric digestion. The casein aggregates covered oil droplets contributed to the long-term stability emulsion, the mean particle size of the digesta of Sample 40 was consequently smaller than Sample 0 at the end of digestion. After 180 min of digestion, all caseins in the digesta of Sample 40 were fully digested, but intact β-Lg and α-La was detected in the emptied digestion. Comparing with Sample 40, Sample 60 present a greater coagulation during gastric digestion. Small quantity of coagula was formed in Sample 60 within 20 min, and then the coagulation was occurred in Sample 80 within 60 min. At the end of digestion, caseins in the emptied digesta of Sample 60 was fully hydrolysed, no large curd was obtained at end of digestion. For Sample 80, large casein curds were firmed and remained in the stomach, the bands of whey proteins were hardly observed. After 180 min of digestion, small quantity of firm curds were only adopted in the stomach content.

5.2 Introduction

Breast milk is believed to be the best food for neonates; however, infant formula can be a substitute when breast-feeding is not an option. Infant formula can supply human infants with high quality nutrition for their growth and development (Packard, 1982; Sidnell et al., 2011). The requirement of protein and essential amino acid for human infant is higher than adult (Zhang et al., 2014). The composition, physical characteristic and structural properties of protein can affect its digestion behaviours(Nguyen et al., 2015b; Ye et al., 2016a; Ye et al., 2017). In particular, the kinetic of protein degradation during gastric digestion can be influenced by protein composition (Ballard et al., 2013; Gan et al., 2018; Zhao et al., 2016). Previous studies have demonstrated that the release of peptides and amino acid and the coagulation behaviour are influenced by the protein composition during gastric digestion (Wada et al., 2017; Wang et al., 2018). The structure of protein during coagulation largely depends on the protein composition and the manufacturing technique of infant formulae (Tari et al., 2018; Ye et al., 2019a; Ye et al., 2017).

The different casein to whey protein ratio influenced the kinetic behaviour of digestion, physicochemical properties, and impacted on the extent of pepsinolysis. This is resulted by the difference in peptic hydrolysis rate between caseins and whey protein(Dupont et al., 2010; Shani-Levi et al., 2013; Wang et al., 2018; Zhao et al., 2016). The proteolysis resistance and the coagulation behaviour of casein and whey protein under adult and infant's gastrointestinal conditions are different (Dupont et al., 2010). The understanding of protein digestion in infant gastric is important for the development of infant food. In previous studies, the digestion of variant protein ingredients under infant gastric condition was widely discussed, but the research on the *in vitro* infant gastric digestion of infant formula with different protein composition is limited. The objective of this study was to compare the gastric digestion behaviour of model infant formula made with different ratio of casein to whey protein, using dynamic gastric digestion model.

5.3 Result and discussion

5.3.1 pH profile

The pH profiles of the emptied digesta of four model infant formulae during *in vitro* dynamic gastric digestion are shown in Figure 5.1. In general, with the constant secretion of SGF, the pH of emptied gastric digesta from all model infant formulae decreased as a function of digestion time. The initial pH of all samples was around 6.8; the protein content was fixed at 1.638% (w/v). For casein-contained samples (i.e. sample 40, 60 and 80), the pH of gastric digesta from these samples dropped to 5.57±0.11, 5.46±0.13 and 5.42±0.05 in the order at 60 min. At the end of digestion, the pH of those samples decreased to 3.79±0.13, 3.52±0.16 and 3.32±0.00 respectively. Gastric digesta from sample 0 showed the fastest decrease in pH, the pH decreased to 5.32±0.09 at 40 min and 2.61±0.20 at 180 min. The digestion behaviour in infant HGS was produced to mimic the gastric phase of human infants. In the previous study (Mason, 1962), specimens of stomach contents from breast-fed infants were adopted at interval by feeding infants with plastic gastric tubes, the final pH of infant stomach contents is above 3 after 180 min of gastric digestion. Based on Yu et al., (2014), the gastric pH of infants under the fasted state could be strongly acidic with a pH value below 3. Hence, the stomach content could influence the pH in stomach. According to Dupont et al., (2015), the buffering capability of food could enhance the pH in stomach contents. Milk constituents contribute differently to its buffering capacity, soluble phosphate, colloidal calcium phosphate, and the protein profile could impact on the buffering capability (Salaün et al., 2005). Based on (Srilaorkul et al., 1989), the contribution of milk components to its buffering capability can be approximately estimated at 35%, 5%, 40% and 20% for caseins, whey proteins, soluble minerals and colloidal calcium phosphate, respectively. Based on Figure 5.1., the casein content in model infant formula samples was slightly increase the pH in stomach contents after digestion. Hence, the final pH of the emptied digesta of Sample 0 (whey protein infant formula) is lower than Sample 40, 60 and 80 (casein-contained infant formula), and the final pH of Sample 0 was consequently lower than 3.

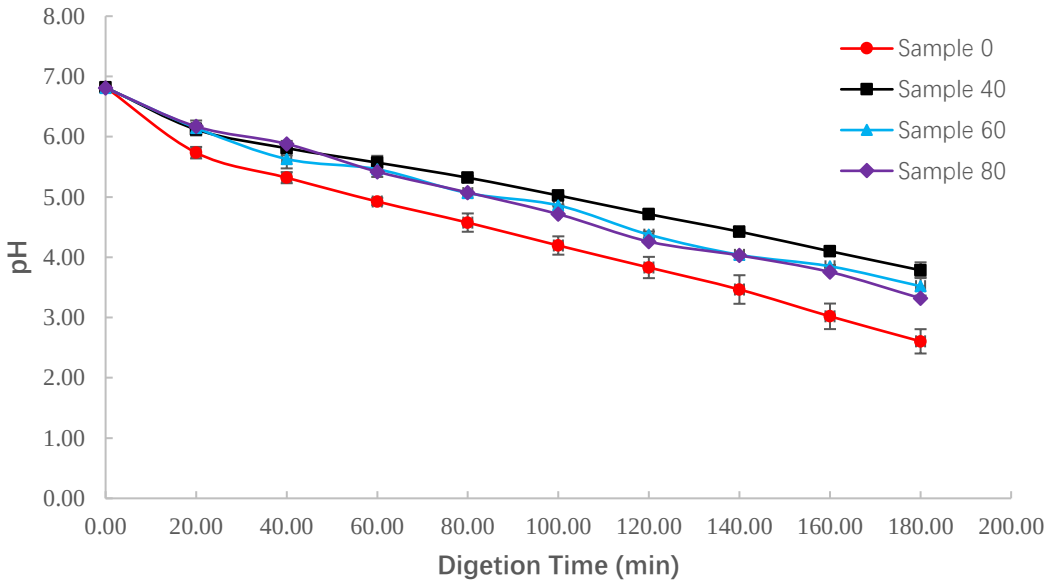


Figure 5.1. Reduction in pH during *in vitro* gastric digestion of the four model infant formulations: whey protein/caseins: sample 0 (whey/casein: 100/0), sample 40 (whey/casein: 60/40), sample 60 (whey/casein: 40/60) and sample 80 (whey/casein: 80/20).

5.3.2 Clotting behaviour of four infant formulations during gastric digestion

By observing the digestion process of all samples, the digestion behaviours of them were worth to be discussed. For Sample 60 and Sample 80, protein coagulation occurred in the first 20 min of digestion. At about 40 min, a mass of fragile flocculates and a few small protein pieces was formed, and then the flocculates and small protein piece were dissolved in the stomach within 80 min, which means the casein micelles have been hydrolysed by pepsin. The flocculates and protein coagula were dissolved in the stomach after 80 min and emptied out. In terms of Sample 0 and Sample 40, there was no observed formation of flocculates formed during digestion process, which could be due to the relatively lower casein content in the two samples. The gastric contents in the infant HGS gradually became clear with increasing digestion time. After 180 min of digestion, in Sample 0, 40 and 60, no structural coagulum was observed inside the infant HGS. For samples 80, a few small protein coagula were observed and collected at the end of digestion.

Figure 5.2 shows the dry matter content of emptied gastric digesta from four samples as a function of digestion time. At 40 min of digestion, the dry matter weight of Sample 60 was lower than Sample 0, Sample 40 and Sample 80. At this stage, the formation of aggregates was visibly observed in Sample 60 and Sample 80. While, at similar pepsin concentration, sample 60 had more coagulates remained in stomach, which led to the lower protein content in its emptied digesta. At the primary stage of coagulation, over 70% κ-casein should be hydrolysed, and then the second stage of coagulation started. According to McMahon et al., (1984b), the addition of caseins could increase the number of unhydrolyzed κ-casein, the lag time of coagulation would be extended. Hence, at 40 min, the quantity of coagulates in Sample 60 is higher than Sample 80. With further digestion, caseins in sample 80 were mostly hydrolysed, a mass of coagulates was remained in the stomach. Hence, the dry matter weight of Sample 80 is lower than Sample 0, Sample 40 and Sample 60 during 80-120 min of the digestion time.

For Sample 0 and Sample 40, the aggregates could not be observed in emptied digesta or stomach content. The dry matter weight of Sample 0 decreased steadily because whey protein was resistant to proteolysis and its structure was stable at low pH. The decreasing dry matter weight of Sample 0 could be due to the dilution of the emptied digesta. The dry matter weight of Sample 40 was higher than Sample 60 and 80 between 40 min and 160 min. For Sample 40, tiny aggregates of caseins could be formed and finely suspended in stomach content. When the emptied digesta was collected, higher amount of casein aggregates was obtained. Hence, the dry matter weight of it was increased. With further digestion, the dry matter weight of the emptied digesta of Sample 40 were decreased sharply, casein aggregates would be extensively hydrolysed in stomach by the addition of pepsin.

At 160 min of digestion, the dry matter weight of Sample 0 reached the lowest value. The difference could be due to the retention of caseins in casein-contained samples in stomach. At the end of digestion, the dry matter weight of all samples showed the close values, which could be due to that most of the aggregates have been extensively hydrolysed at higher pepsin concentration after longer digestion time.

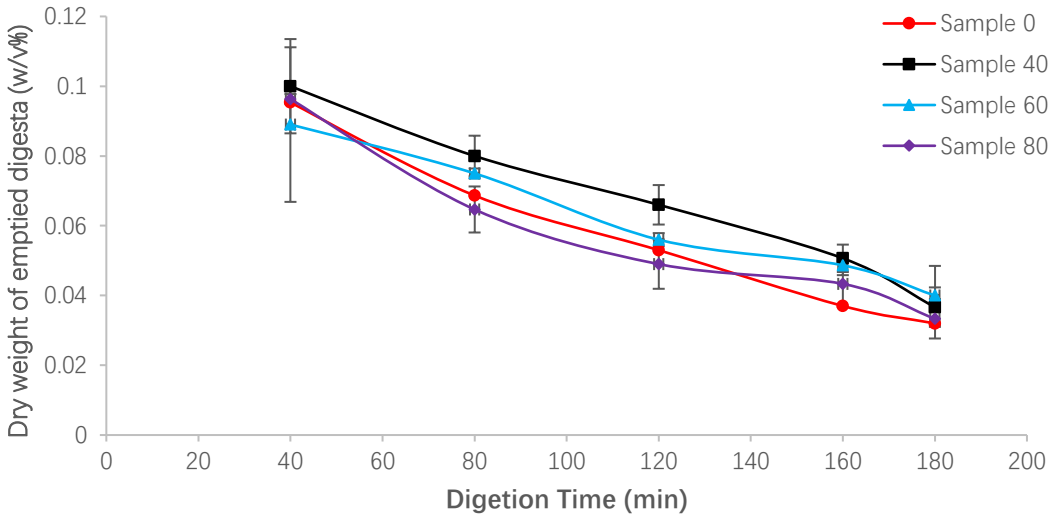


Figure 5.2. Dry weight curve of dairy samples. sample 0 (whey/casein: 100/0), sample 40 (whey/casein: 60/40), sample 60 (whey/casein: 40/60) and sample 80 (whey/casein: 80/20).

5.3.3 The physical characteristic of emptied gastric digesta

The average weight-to-volume diameter ($d_{4,3}$) of four model infant formulae was between 0.72±0.12 and 2.95±0.04 µm, and the average volume-to-surface diameter ($d_{3,2}$) was between 0.38±0.00 and 0.44±0.04 (Table 5.1).

Table 5.1. The average volume-to-surface diameter ($\boldsymbol{d_{3,2}}$, µm) and weight-to-volume diameter ($\boldsymbol{d_{4,3}}$, µm) of four model infant formulae in emptied digesta (1.638 w/w% protein, 4.0 w/w% oil). Results are shown as mean ± standard deviation.

	Sample 0	**Sample 40**	**Sample 60**	**Sample 80**
$\boldsymbol{d_{4,3}}$ (µ$\boldsymbol{m}$)	1.46 ± 0.03	2.95 ± 0.04	0.72 ± 0.12	1.67 ± 0.02
$\boldsymbol{d_{3,2}}$ (µ$\boldsymbol{m}$)	0.38 ± 0.00	0.44 ± 0.04	0.39 ± 0.02	0.44 ± 0.02

The changes in the average weight-to-volume diameter ($d_{4,3}$) of all samples as a function of time in the infant HGS are shown in Figure 5.3. In general, the average particle size changed remarkably in all samples during the *in vitro* digestion process.

For Sample 0 and Sample 40 (Figure 5.3), the particle size ($d_{4,3}$) of the emptied digesta gradually increased with increasing digestion time. For sample 0, the average particle size increased to 49.54±3.69 μm at the end of digestion, while, the average particle size of Sample 40 reached 16.81±1.60 μm at the end of digestion.

The size of Sample 60 (Figure 5.3) steadily increased to 20.22±3.46 μm within 60 min, followed by a decrease to 15.03±1.96 μm at 140 min of digestion. With further digestion, the average size increased to 23.89±2.36 μm at the end of digestion.

In terms of Sample 80 (Figure 5.3), the average size dramatically increased to 82.22±3.65 μm at 60 min, and then remarkedly dropped to 24.51±5.30 μm at 140 min. At 180 min, the particle size slightly rebound to 29.21±7.27 μm.

As shown in Figure 5.3. At 40 min of digestion time, a mass of aggregates was observed in Sample 80 and Sample 60. In Figure 5.3., the particle size of Sample 80 and Sample 60 reached peak at 60 min, and the particle size of Sample 80 was four times higher than that of Sample 60, which could be due to the formation of larger aggregates in Sample 80. With further digestion, the mean particle size of aggregates in Sample 60 and 80 were decreased until 140 min of digestion time. At the end of digestion, larger clots (>1mm) were adopted in the stomach content of Sample 80, fragile flocculates were observed in Sample 60, and the average particle size of Sample 60 and Sample 80 were increased at the end of digestion.

For Sample 0 and Sample 40, the formation of aggregates was not visibly observed during the digestion, the particle size was increased steadily in Sample 40 and ascended significantly in Sample 0. At the end of digestion, the droplet size of Sample 0 is nearly three times higher than that of Sample 40, and a foam layer was especially observed in the stomach content of Sample 0. The result is consistent to the data in confocal micrographs.

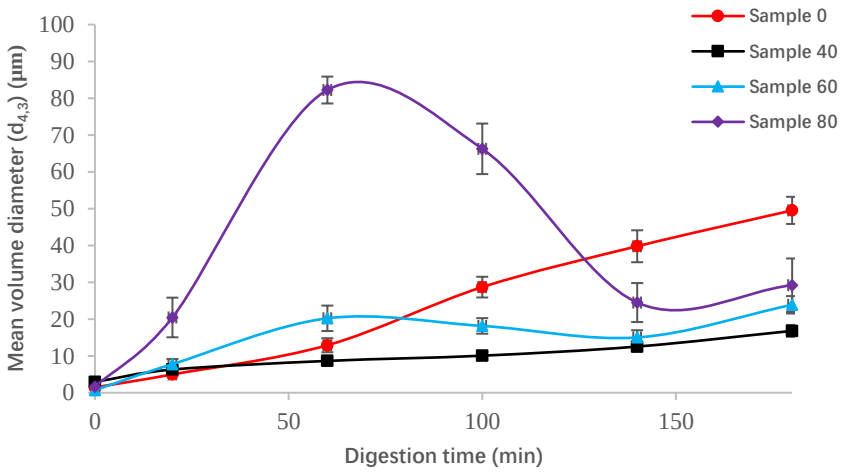


Figure 5.3. Changes in average particle size ($\boldsymbol{d_{4,3}}$) of emptied gastric digesta during 180 min of gastric digestion in the infant HGS from four model infant formulae. sample 0 (whey/casein: 100/0), sample 40 (whey/casein: 60/40), sample 60 (whey/casein: 40/60) and sample 80 (whey/casein: 20/80).

5.3.4 Particle size distribution

For Sample 0 (Figure 5.4A), the initial model infant formula showed a bimodal distribution with a major peak near 0.1 µm, and a small tail in the range of 0.4 to 1 µm. At 20 min of digestion, the peaks shifted to the right indicating the increase in particle size, the digesta still showed a bimodal distribution with a new and major peak appearing near 4 µm and a smaller peak near 0.4 µm. At this stage, the pH of sample 0 is 5.74±0.10, the increasing of particle size could be due to the partially pepsin hydrolysis of whey protein adsorbed on emulsion, and the flocculation and coalescence of the emulsion droplets occurred during the gastric digestion. The small peak could be the emulsion droplets remained stable during digestion. With increasing digestion time from 30 min to 180 min, the peaks continued to shift to the right indicating the gradual increased in the particle size of flocculates. At the end of gastric digestion, the digesta had a major peak near 45.78 µm, with a small tail in the range of 100 to 1000 µm. The stomach content was transparent with a foam layer. The pH was decreased to 2.61±0.20, the 45.78µm population was assigned as the formation of flocculates, and the particle greater than 100µm was assumed to be an indication of foaming. Additionally, when the digestion time increased to 100min, the pH of Sample 0 is 4.20±0.15, the increasing rate of particle size of its flocculates was slowed down. According to (Ju et al., 1998), since the pH of

whey protein was lowering from its p*I* to pH 4.0, the surface charge was changed and the reorganisation or partially collapse of the flocculates was occurred. Hence, the increasing of particle size of flocculates was limited.

For sample 40 (Figure 5.4B), the particle size distribution of the initial model infant formula showed a bimodal pattern with a major peak near 0.1 µm and a smaller peak near 1.47 µm. At 20 min of digestion, the pH was 5.81±0.12, the distribution shifted to a larger size region, a larger peak near 6.77 µm and a small peak in the 0.1-1 µm region. This indicates that, the flocculation and coalescence of emulsion droplets could be occurred, and a small part of emulsion droplets with small sizes remained stable. With further digestion, the peak became monomodal and gradually shifted to the right. The particle size of emulsion droplets increased. At the end of digestion, the pH was 3.79±0.13, the digesta showed a major peak near 11.07 µm. According to the micrograph in Figure 5.5, the major peak could be due to the formation of flocculates.

For Sample 60 (Figure 5.4C), the particle size distribution peak of the initial model infant formula appeared to be composed of two overlapping peaks in 0.01-0.4 µm region, and the a small peak near 1 µm. At 20 min of digestion, the pH was 5.63±0.16, the particle size distribution became monomodal, with a peak near 11.51 µm. It indicates that the flocculation of emulsion droplets has occurred. With further digestion, the peaks slightly shifted to a smaller size region indicating a mildly decreased in the particle size of flocculates. At the end of digestion, the digesta had a major peak near 9.07 µm with a small tail in the region of 0.1 to 1 µm. According to Figure 5.5., the flocculates in Sample 60 were varied in particle size at the end of digestion.

Sample 80 exhibited a multimodal distribution, with a small wide peak in 0.01-0.1 µm region, one larger narrow peak in 0.1-1 µm region and one small wide peak near 1 µm (Figure 5.4D). The particle size distribution of the digesta at 20 min of digestion had a narrow peak near 10 µm. At this stage, a mass of curds and flocculates remained on the 1 mm sieve when the emptied digesta was adopted. At 60 min, large curds still could be visibly observed on the sieve, and the peak became boarder and shifted to the right near 6.63 µm with tails on both sides. With increasing digestion time, the peak shifted to a smaller size region, and the volume of the curds on the sieve was decreased as a function of digestion time. At 140 min, the particle size distribution of the digesta exhibited a trimodal pattern, with a peak near 17.38 µm, a peak in the range of 1-100 µm, and a peak in the range of 100-1000 µm. At 180 min of digestion, the peak in the range of 0.1-1 µm disappeared and the digesta showed a bimodal distribution with a peak near 10 µm and a

tail in the range of 100-1000 μm. From 60min to 180 min of digestion time, the volume of particles at the size of 100-1000μm was decreased, and the volume of particles at the size of 1-10μm was increased. It could be due to the retention of large curds in infant stomach, the curds at the size of 100-1000μm was further hydrolysed in stomach, and the hydrolysate at the size of 1-10μm was increased in the emptied digesta.

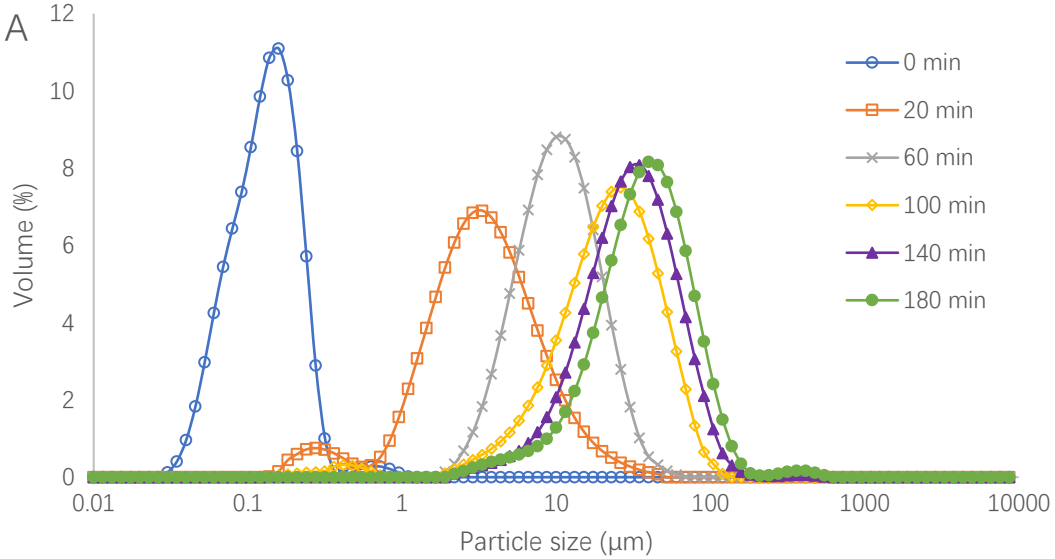
The difference on the distribution of particle size between all samples at a constant time was worth to be discussed. Initially, the particle size distribution of all samples showed a main peak near 0.1μm, which was consistent to the mean particle size of all samples.

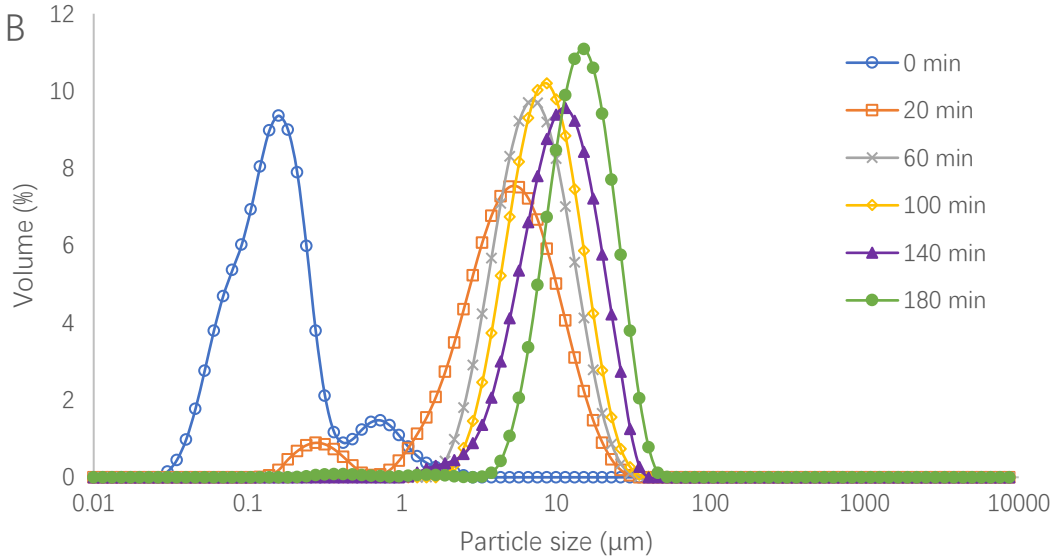
At 20 min, only Sample 0 and Sample 40 had small peaks in the region of 0.1-1μm, at pH 5.5-6. The increase in particle size could be due to the oil droplet coalescence via the hydrolysis of adsorbed whey proteins. However, a small amount of oil droplets remained stable at the size in 0.1-1μm region, which would be caused by two factors: Firstly, the whey protein could be partially hydrolysed at high protein concentration (1.638%w/w). As present in Malaki Nik et al., (2010), the effect of pepsin digestion was smaller at a high protein concentration (>1.5%w/w) solution, whey protein could be partially digested, led to less coalescence after proteolysis, the increase of particle size of emulsion droplets could be limited. Secondly, α-La was resistant to be pepsin digestion, and α-La stabilized oil droplets remained stable. In previous studies, whey protein is resistant to pepsin hydrolysis, presumably because its β-sheets were buried inside the folded globular structure (Nicolai et al., 2011). While, as present in Singh et al., (2013), β-Lg hydrolysis rate would be increased when it was present as the adsorbed layer. It could be attributed to the partially unfolding and reorganization of its structure, which exposes the peptic cleavage sites for proteolysis. In contract, α-La could be more resistant to pepsin hydrolysis when it adsorbed at the interface (Singh et al., 2013). Hence, during acidification, small quantity of α-La and absorbed at the interface were resistant to be hydrolysed during digestion. On the other hand, oil droplets with smaller size (located in the 0.1-1μm region) have relatively slower rate to coalescence to large size, comparing with large oil droplets. Hence, a small apart of oil droplets in Sample 0 and Sample 40 remained in the original size between 0 and 20 min,

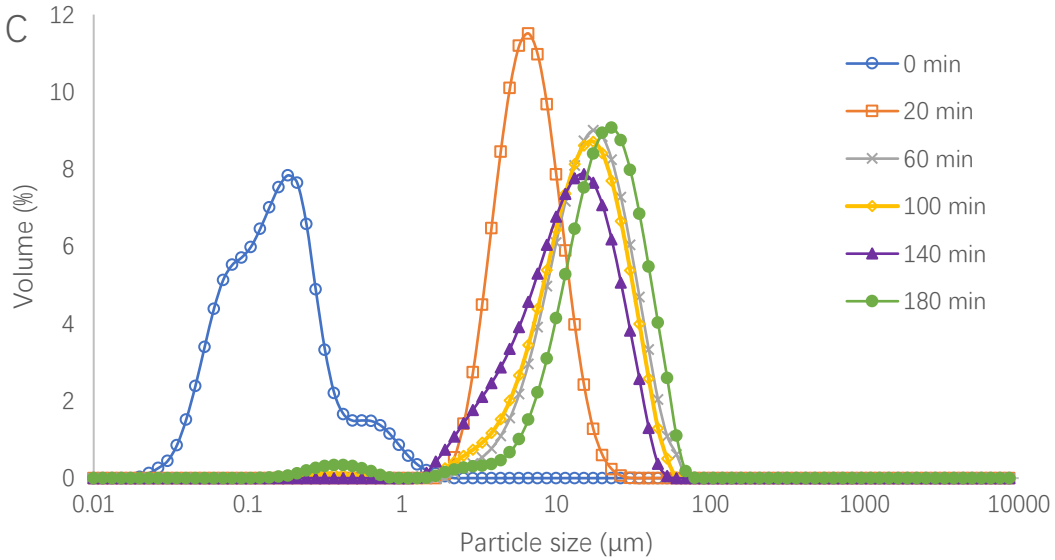
With further digestion, the particle size of Sample 0 and Sample 40 was increased as a function of time, and the pH of both samples was $\leqslant$ 5.5. For Sample 0, 40 and 60, the increasing rate of particle size became slower with the decreasing of the ratio of whey protein. For Sample 80, the peak was remarkably shifted to the small particle size region.

For Sample 0 and Sample 40, the coagulation of whey protein was pH-dependent. At neutral pH, β-Lg exhibited as dimers; during acidification, the dimers dissociated into monomers, and then the monomers would form aggregates around the p*I* (below pH 5.5) of whey protein. It was also suggested by Demetriades et al. (1997) that emulsion droplet flocculation occurred near the p*I* of whey protein, leading to the decrease in emulsion stability and causing creaming. Hence, when the pH of Samples decreased below pH 5.5 in 60 min, the oil droplets flocculation occurred, the sample with higher amount of whey proteins (Sample 0) obtained the faster increased in the particle size comparing to Sample 40, and the result is consistent to Ju et al. (1998).

For Sample 60 and Sample 80, which contained higher quantities of caseins. Compared with whey protein, caseins had rather flexible structure and lack regular secondary and tertiary structures, which could unfold rapidly at the emulsion interface (Singh et al., 2013). During the gastric digestion, aggregated caseins also adsorbed on to the emulsion droplets surface. Since the protein aggregates covered emulsion droplets, layers were formed, the layer formed by casein aggregates was thicker and denser than whey protein formed layer, which contributed to the long-term stability of emulsions against coalescence (Singh et al., 2013).


A
Volume (%)
Particle size (μm)
0 min
20 min
60 min
100 min
140 min
180 min


B
Volume (%)
Particle size (μm)
0 min
20 min
60 min
100 min
140 min
180 min


C
Volume (%)
Particle size (μm)
0 min
20 min
60 min
100 min
140 min
180 min

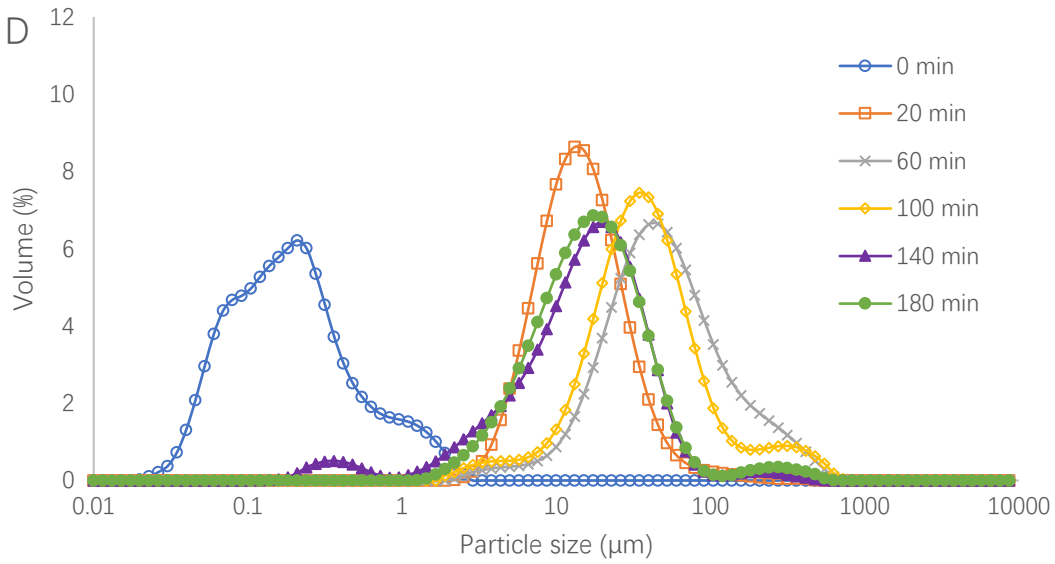


Figure 5.4. Particle size distribution of emptied digesta obtained from 180 min gastric digestion of four model infant formulae (4.0% oil content and 1.638 % protein, w/v) in the infant HGS: A sample 0 (whey/casein: 100/0), B sample 40 (whey/casein: 60/40), C sample 60 (whey/casein: 40/60) and D sample 80 (whey/casein: 20/80).

5.3.5 Microstructure of emptied digesta in the infant HGS

Figure 5.5 shows the confocal laser scanning microscopy (CLSM) images of digesta and clot of four different model infant formula samples during gastric digestion. Initially, all infant formula samples obtained small uniformly dispersed oil droplets, although a few larger oil droplets were observed.

At 40 min of digestion, the pH of the digesta of Sample 0 was 5.32±0.09. In the micrograph, small protein aggregates were observed, the oil droplets were embedded within the coagulation matrix. Since the pH of Sample 0 decreased below pH 5.5, the coagulation of whey protein could be occurred. The observed aggregates in Sample 0 could be β-Lg bridged flocculates. In agreement with Ju et al., (1998), during acidification, β-Lg molecules formed aggregates at the pH near the p*I* of β-Lg (around pH 5.1). With the addition of enzymes, small quantities of β-Lg with the exposed β-sheets was acted by enzymatic hydrolysis, and the gel network was induced from larger β-Lg aggregates. For the digesta of Sample 40, dense structure with larger protein aggregates were observed, larger oil droplets in varied size were embedded in protein matrix. For Sample 60, since the casein content was increased from 40% to 60%, the protein aggregate particles became larger. Compared with Sample 40 and 60, a closely knitted network with numerous aqueous pores was observed in Sample 80, a few oil droplets

filled in the protein particles and some separated free oil were dispersed in serum phase. The oil droplets in Sample 80 had smaller size, comparing with other three Samples. It was suggested that the layer formed by casein aggregates was more stable than whey protein formed layer.

At 180 min of digestion, the pH of all samples was between 2.61 and 3.79. for Sample 0, only one protein aggregate was observed, along with some larger size oil droplets trapped in the aggregates. The aggregates in digesta showed a porous structure and was varied in different size. It is in agreement with Langton et al., (1992), the aggregates of β-Lg at low pH is regular with dense and porous structure. During gastric digestion, a small quantity of whey protein could be unfolded, which contributed to the formation of aggregates.

For Sample 40, a few aggregates with dense structure were observed, and few oil droplets appeared to be incorporated in the aggregates. The aggregates in Sample 40 could be constitute with caseins. Those aggregates are smaller than the aggregates in Sample 60 and 80 because of the lower ratio of caseins in Sample 40.

For Sample 60, larger protein particles were observed with tiny oil droplets embedded in the aggregates, and the protein aggregates showed a porous stucture. For Sample 80, the aggregates exhibited a dense and pores structure, with tiny oil droplets trapped in aggregates. Firm clot (>1 mm) was obtained at the end of digestion.

Clots and flocculates of Sample 80 were observed at 20 min of digestion time, but the amount of them have increased in 40 min of digestion time, and then deceased steadily as a function of digestion time. According to Figure 5.3, the mean particle size increased at beginning, and decreased between 60 and 140 min of digestion time. Between 140 and 180 min, the mean particle size slightly increased. However, in Figure 5.4, the major peak of it had been keeping shifted to become smaller clots between 60 and 180 min. indicating that, a number of clots was hydrolysed in stomach, and small quantity of larger particles was dissolved in emptied digesta at the end of digestion.

At the end of digestion, larger clots (>1mm) were collected in the stomach content of Sample 80. By observing Figure 5.5, the clot exhibited a large aggregated structure with no aqueous pores, and the closely knitted network appeared to fuse into a smooth block after long digestion time.

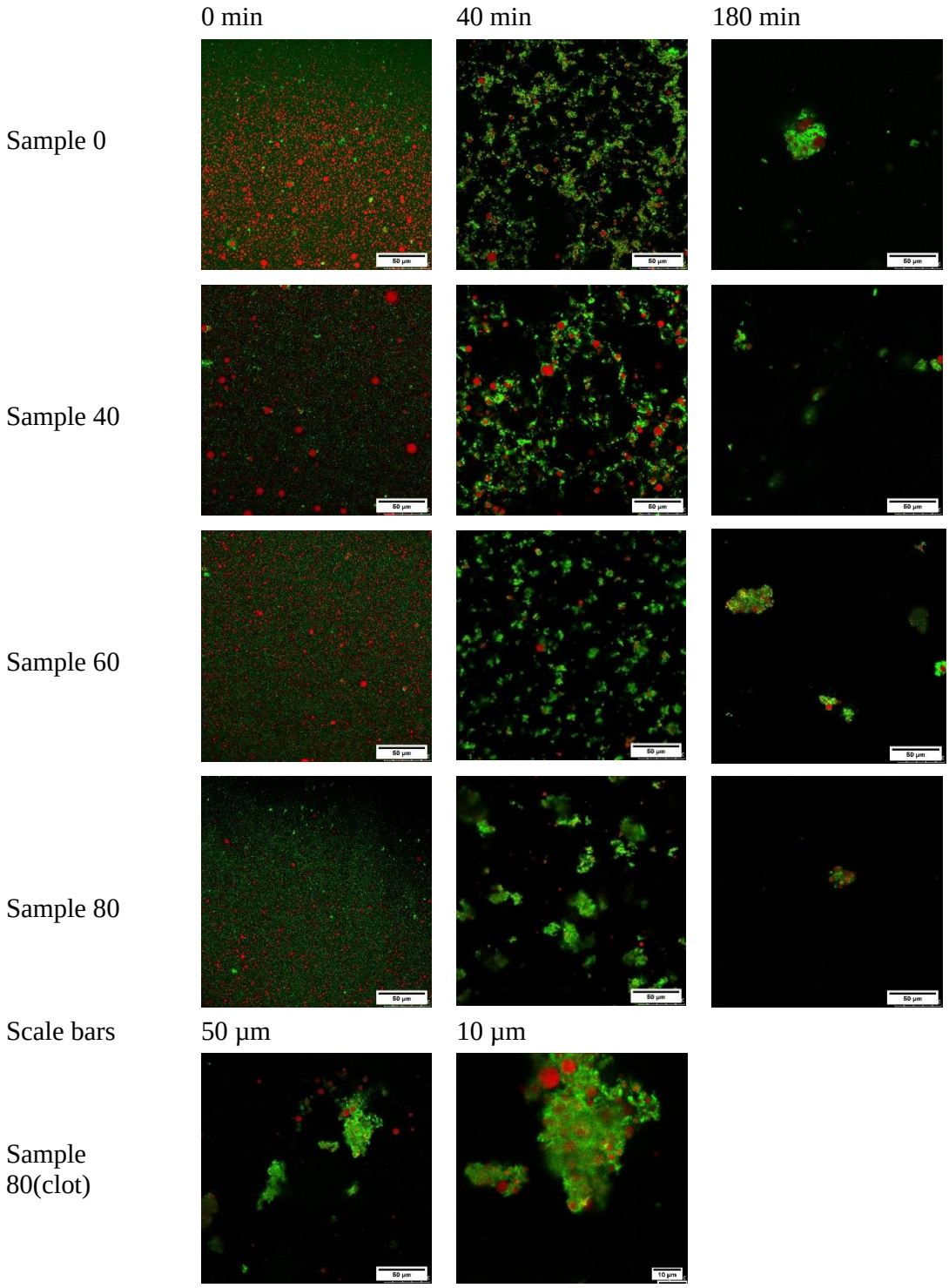


Figure 5.5. CLSM images of the digesta and clot of infant formulae during gastric digestion in the infant HGS at 40 and 180 min of digestion. Scale bars represent 50 µm.

5.3.6 Hydrolysis of protein

In the SDS-PAGE pattern of Sample 0 (Figure 5.6A), the initial sample exhibited clear β-Lg and α-La bands. The intensity of these two bands decreases with increasing digestion time. It could be caused by the dilution with SGF and hydrolysis of whey proteins by the action of pepsin. At 100 min, the β-Lg and α-La bands became less dense, bands with smaller molecular weight (<10 kDa) could be observed. Ghosh et al. (2017) indicated the appearance of these smaller bands was caused by the enzymatic hydrolysis of whey protein. With further digestion, α-La band is not visibly observed at 180 min.

For sample 40 (Figure 5.6B), intact β-Lg, α-La, α_s-casein, β-casein and κ-casein bands were observed in the initial sample. At 20 min of digestion time, there was a moderate decrease in band intensity of α_s-casein, β-casein, and κ-casein band was disappeared, a new band ~ 15 kDa was observed, indicating that para-κ-casein could be formed, via the hydrolysis of micellar κ-casein. Between 60 and 100 min, the intensity of α_s-casein, β-casein bands gradually decreased, para-κ-casein band cannot be detected at 100 min, and all casein bands were not observed at 140 min. It could be caused by the dilution of digesta and the continuing action of pepsin, and para-κ-casein could be hydrolysed in stomach. The bands of β-Lg and α-La were observed at the end of digestion, although the intensities of whey proteins decreased steadily as a function of digestion time. The decreased in intensities of whey proteins could be caused by the dilution effect of emptied digesta during sample collected overtime.

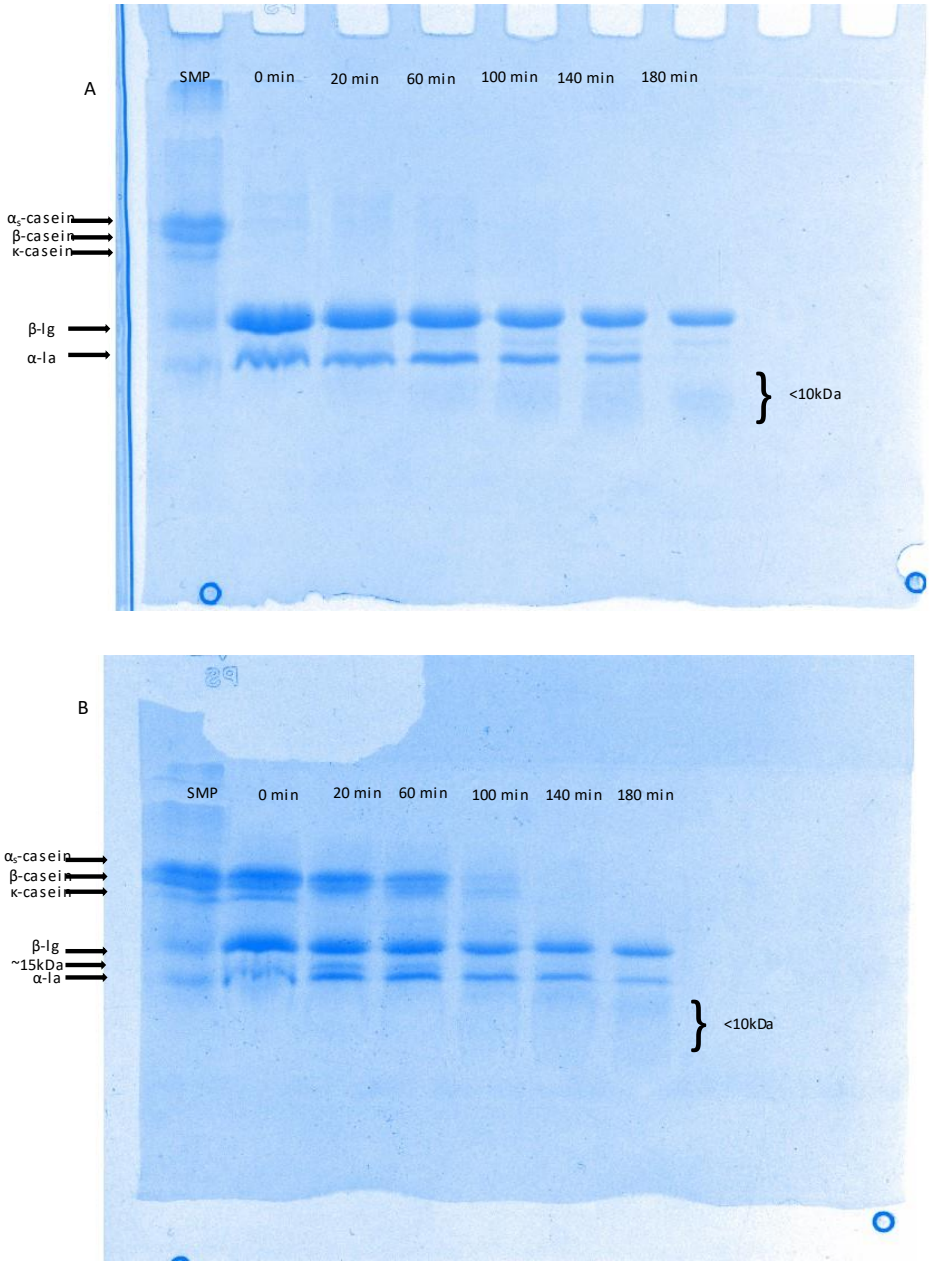
At the end of digestion, the pH of Sample 40 was 3.79±0.13. the α-La band of Sample 40 was observable, the reason could be that α-La at low pH is more easily to be adsorbed at the interface, and become more resistant to be hydrolysed than β-Lg. When the oil-in-water emulsions (0.5-2.5%w/w WPI) were acidified from pH 7 to pH 3, the surface composition was changed, and the emulsions were least stable at pH5.5 (Hunt et al., 1994). Below pH 6, α-La became a form of a molten globule (the A-conformer), the flexibility has consequently increased because it lost the tertiary structure. Moreover, β-Lg was dissociated into monomers, and the monomers, compared with dimers and octamers, are more likely to facilitate compositional and structural changes. At neutral pH, α-La and β-Lg were adsorbed in percentage to their ratio in the emulsion. At pH 3.0, the adsorbed protein composition was gradually changed, more α-La adsorbed and β-Lg was displaced (Hunt et al., 1994). Based on Malaki Nik et al., (2010), adsorbed β-Lg is more susceptible to be hydrolysed by pepsin, while, adsorbed α-La would become more resistant to pepsinolysis.

For Sample 0, the α-La band was disappeared at 180min. After 180 min of digestion, the pH reached to a low value. Based on the study about the rheological properties of pepsin-induced whey protein during acidification, the oil droplet size could be tripled after 180 min. It assumed that α-la was adsorbed at the interface of large oil droplets at low pH, large oil droplets with α-la floated upon the stomach content. Hence, when emptied digesta was collected from the bottom of stomach simulator at 180min, the of α-la band was not detected.

β-Lg, α-La, α_s-casein, β-casein and κ-casein bands were observed in the initial Sample 60 (Figure 5.6C). At 20 min of digestion, the κ-casein band disappeared while para-κ-casein band was appeared. The intensities of casein bands displayed a considerable reduction at this time point. With further digestion, the newly formed para-κ-casein band was not observed at 100 min, and all casein bands were not observed at 140 min of digestion, which could be due to the formation of larger clots during digestion time. The content of caseins could not be detected in emptied digesta because larger clots remained in stomach. The β-Lg band was visible at each point of digestion and a steady reduction in the intensities of band was observed as a function of digestion time, and α-La band was observed until 180 min. The low intensity of α-La could be due to the lower level of whey protein in Sample 60, the decreased in the intensities of whey protein could be due to the dilution of gastric digesta.

In the SDS-PAGE pattern of Sample 80 (Figure 5.6D), the initial sample contains clear α_s-casein, β-casein, β-Lg and κ-casein bands. The κ-casein band disappeared at 20 min of digestion. After 60 min of digestion, protein bands can hardly be observed in the gastric digesta. At this time point, all caseins were coagulated, larger clots formed and remained in stomach content.

At the end of digestion, firmed clot could only be collected in the stomach content of Sample 80. By observing the band pattern of clot, bands of α_s-casein, β-casein, κ-casein, para-κ-casein (~15kDa), α-la and small peptides (< 10 kDa) was detected, and newly formed bands appeared above β-Lg band.


A
SMP
0 min
20 min
60 min
100 min
140 min
180 min
αs-casein
β-casein
κ-casein
β-lg
α-la
<10kDa
B
SMP
0 min
20 min
60 min
100 min
140 min
180 min
αs-casein
β-casein
κ-casein
β-lg
~15kDa
α-la
<10kDa

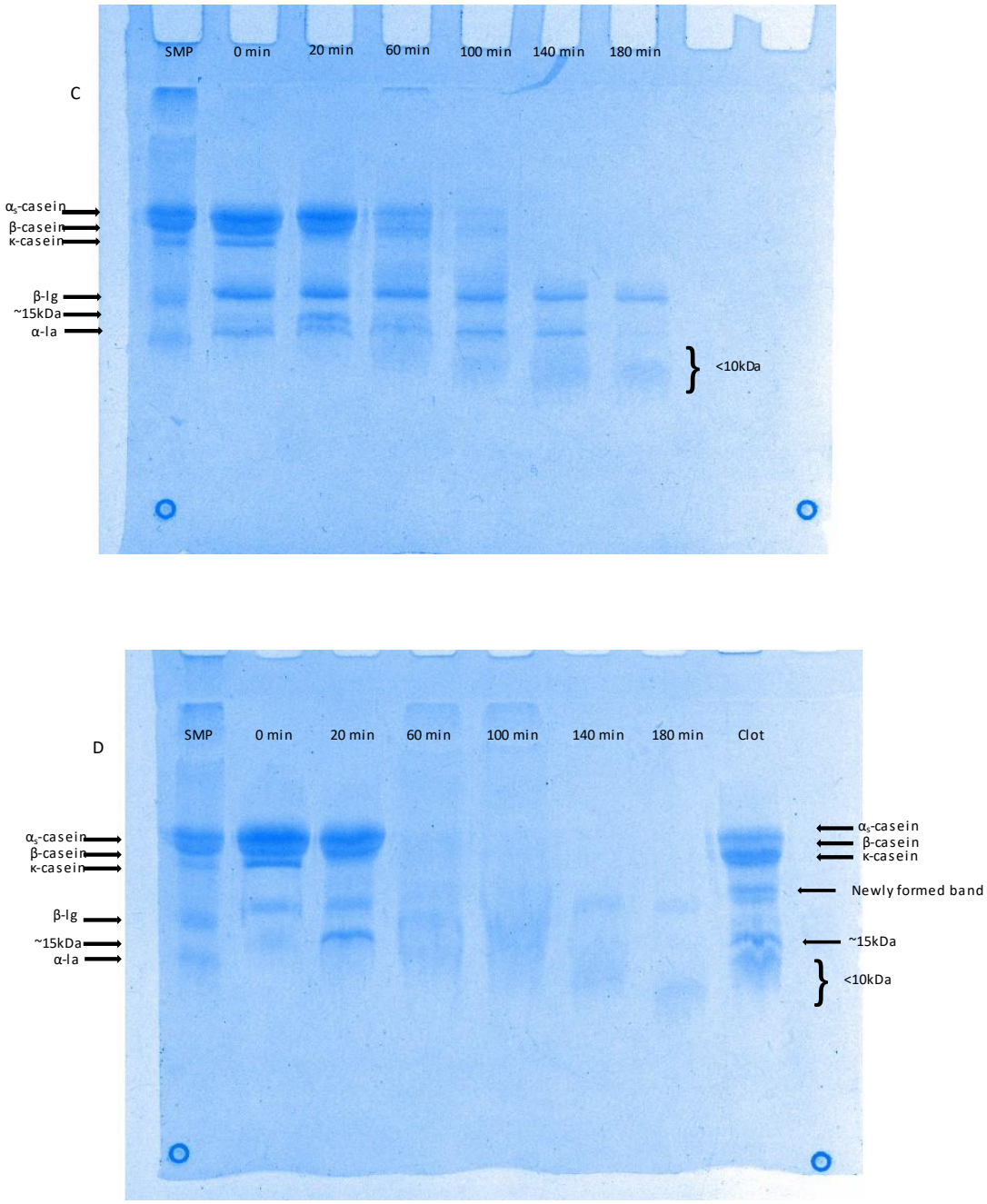


Figure 5.6. SDS-PAGE patterns under reducing conditions of the digesta obtained from the model infant formulae during the gastric digestion (simulated gastric fluid and pepsin) in infant human gastric simulator of Sample 0 (A), Sample 40 (B), Sample 60(C), Sample 80 (D).

5.3.7 The digestion behaviour of four model infant formula during gastric digestion

During the gastric digestion process, the pH of the emptied digesta of all model infant formula samples decreased as a function of digestion time (Figure.5.1). However, the decreasing rates were different between the samples with and without casein. The pH decrease was faster in the sample that contains only whey protein than the samples that contain both casein and whey protein, this could be related to the different buffering capacities of the different milk protein ingredients. This difference can be attributed to the lower buffering capability of whey protein than casein. Micellar phosphate may increase buffering capacity due to the formation of dihydrogenphosphate during acid solubilisation of colloidal calcium phosphate.

Since the pH of Sample 0 was decreased to whey protein's p*I* (below pH 5.5), the particle size was remarkably increased, which was showed in Figure 5.3A and 5.4A. The disintegration of aggregates occurred, the particle size should have decreased (Ju et al., 1998). However, the activity of pepsin during the gastric digestion caused the reduction of positive charge, and led to the decrease of the thickness of the adsorbed protein layer. The β-Lg adsorbed on the interfacial layer of oil-in-water emulsion droplets could be rapidly hydrolysed by pepsin (Sarkar et al., 2009), as shown in Figure 5.6A. The interfacial layer stabilised by small peptides were unable to provide sufficient steric stabilization and electrostatic repulsion. Hence, the emulsion is unstable and prone to flocculation or coalescence.

For whey protein-dominant infant formula, Sample 40, the flocculates were not visible during the whole gastric digestion. For casein dominant infant formula, Sample 60, a number of aggregates was observed at 20 min. Sample 60 present a greater extent of coagulation than Sample 40, which was attributed to the higher ratio of caseins in Sample 60, and consequently its higher sensitivity to coagulation via low pH and pepsin reaction. It was also suggested by Ye et al. (2019a) that whey protein is less sensitive to acid-induced gelation and more resistant to pepsin. Hence, the extent of coagulation was lower in Sample 40 than Sample 60.

For both Sample 40 and Sample 60, the aggregates could be observed at 60 min on confocal images in Figure 5.5. In Figure 5.6, at 20 min of digestion, the pH of the digesta of them were 6.12±0.04 and 6.15±0.12 respectively, κ-casein was rapidly hydrolysed, the bands of α-caseins and β-caseins were clear, this could be the reason that the hydrolysis rate of κ-casein was faster than other caseins above pH 6.0 in *in vitro* digestion(Ye et al.,

2016a). The pepsin hydrolysis of κ-casein was indicated by the formation of para-κ-casein at early digestion time (Ye et al., 2016b). During 20-100 min of digestion, an intermediate band (above β-Lg band) was observed, suggesting the presence of intermediate casein peptides. The pepsin hydrolysis of caseins is in agreement with an *in vivo* piglet gastric digestion study of infant formula (Tari et al., 2018). In their research, soluble phase of the gastric digesta from the model formula was collected. The 2 main whey proteins, β-LG and α-LA, were the major undigested proteins in the soluble fraction, even in gastric samples collected 120 min postprandial.

There was no curd obtained after 180 min of the gastric digestion from both whey protein dominant infant formula (Sample 40) and casein dominant infant formula (Sample 60). The hydrolysis rate of casein was faster, compared with whey protein. In Figure 5.6, the bands of caseins in Sample 40 and Sample 60 were disappeared at 140 min of digestion, this could be caused by the retention of aggregates and the dilution with SGF in the stomach; the aggregates were small in size and extensively hydrolysed by pepsin (Ye et al., 2017). At the end of digestion, β-Lg was still observed in both samples and α-La was detected in Sample 40. This is in agreement with a previous study done by Ye et al. (2019b) that β-Lg was resistant to pepsin and stable at low pH, but α-La was hydrolysed by pepsin below pH 4. Overall, the caseins are hydrolysed faster than whey proteins during gastric phase. However, when caseins in clots, the hydrolysis rate of caseins would be decreased because of the larger aggregates size and denser structure.

In Sample 80, firm curd and flocculates were observed during the whole gastric digestion. The particle size of digesta remarkably increased, and gradually decreased after 60 min of digestion. The increase in particle size could be due to the coagulation of protein and the flocculation of oil droplets, which could be observed in Figure 5.5. The mean particle size decreased between 60 and 140 min of digestion time in Figure 5.4, and increased between 140 and 180 min. However, in Figure 5.5, between 60 and 180 min, the major peaks were shifted to small particle size region. The decrease in the particle size could be due to the breakdown of fragile aggregates by the extensive pepsinolysis. In Figure 5.6, the casein bands were disappeared at 60 min of digestion, which was earlier than other three samples. This probably due to the formation of curd with larger size and dense structure. At 60 min of digestion, the pH decreased to 5.42±0.05, the protein was coagulated not only by pepsin, but also by acid due to loss of electrostatic stability. The behaviour of this sample was similar to that observed for bovine milk based infant formula in gastric digestion (Ye et al., 2019a).

With further gastric digestion, both casein band and whey protein band disappeared at 60 min in Figure 5.6. In the pattern of clot, the bands of para-κ-casein(~15kDa) and other caseins were clear, and a newly formed band (above β-Lg band) was observed. It was in agreement with Ye et al. (2019a). The reduction of whey protein could be resulted from the dilution of protein and peptic hydrolysis. The caseins coagulated and remained in the stomach with size larger than 1 mm. The curds did not disappear at the end of digestion. A few firm curds were collected from inside the stomach after 180 min of gastric digestion. In figure 5.5, the aggregates of protein were formed at 40 min of digestion, the oil droplets were entrapped in the protein matrix. In the confocal image of curds, the oil droplets were evenly distributed in the matrix. Free oil droplets were observed in the aqueous phase, which indicated that oil droplets were separated from protein matrix at the end of digestion during the formation of curds (Ye et al., 2019b).

5.4 Conclusion

The pH, protein coagulation and protein hydrolysis in four different infant formulae were examined. The results indicated that the protein composition of infant formula present significantly influence on the decreasing rate of pH. The decreasing rate of pH in Sample 0 is faster. For Sample 0, at the end of digestion, it obtained larger and dense flocculates, intact β-Lg was detected by SDS-PAGE. For Sample 40, with the higher addition of casein content than Sample 0, the increasing rate of flocculates' particle size was slower. After 180 min of digestion, the pH was higher than Sample 0, and no aggregates could be visibly observed. The average particle size in its digesta was the smallest, intact β-Lg and α-La were detected by SDS-PAGE. For Sample 60, larger coagula were observed within 20 min of digestion time, and the coagulation in Sample 80 was detected within 60 min. With further digestion, the large coagula were remained in stomach and were hydrolysed as a function of time. The increasing rate of average particle size for the digesta of Sample 60 was limited between 60 and 180 min, and the average particle size was smaller than Sample 0 and Sample 80 at the end of digestion. After 180 min of digestion, all caseins and α-La were fully hydrolysed in Sample 60. Firm and large curds were only obtained in Sample 80. For Sample 80, dense and larger curds were formed during the gastric digestion. Large curds were remained in the stomach until the end of digestion, and the bands of whey proteins were hardly observed.

Chapter 6: Overall Summary and Recommendations

This project provides an insight into understanding of the effects of protein composition on the digestion behaviour of infant formula.

6.1 Summary

The protein compositions of infant formulae (Sample 0, Sample 40, Sample 60 and Sample 80) were classified by the ratio of casein to whey protein: 0:100, 40:60, 60:40 and 80:20. In the first part of this study, the rheological properties and gelation behaviour of the infant formulae with different protein composition were investigated at different pepsin concentration. In terms of whey protein based infant formula sample, the stiffness and gelation behaviour showed no significant change, because whey protein is not sensible to pepsin hydrolysis. Moreover, the gel strength of Sample 0 was slightly decreased around pH 4.0, which was attributed to the partially collapse of its aggregates. Changing the ratio of whey protein to casein combined with constant protein content could significantly affect the rheological and physical properties of the gels of infant formulae. With the increasing content of caseins, the final G', $G^*$and breaking stress (σ_{max}) of the obtained gels was increased. The G^* of casein gels was dependant on the pepsin concentration, when the pepsin concentration was 1U/mL, the G^* of Sample 60 and Sample 80 reached the highest value, along with the highest stiffness. However, the G^* of Sample 40 was decreased as a function of pepsin concentration due to the further pepsin hydrolysis. Furthermore, the pepsin-induced gels of Sample 40 and 60 obtained smaller value of breaking stress (σ_{max}) at 2.5U/mL in comparison with their acid-induced gels. In contrast, the gel of Sample 80 at 2.5U/mL exhibited a larger value of σ_{max} in comparison with the acid-induced gels, indicating that pepsin-induced gels of Sample 40 and 60 are more susceptible to rearrangement and fracture under large deformation.

Moreover, the gelation time and gelation pH were impacted by different pepsin concentration, which was resulted by different hydrolysis degree of κ-casein. In the result of SDS-PAGE, with the addition of 1U/mL or 2.5U/mL of pepsin concentration, the para-κ-casein bands in all casein-contained infant formulae were detected at 5 min. At lower pepsin concentration (0-1U/mL), the sample with higher casein content obtained higher degree of unhydrolyzed κ-casein and the lag time of coagulation would be extended, the gelation time was consequently increased, and the gelation pH was accordingly lowered. Since the pepsin concentration was increased to 2.5U/mL, the coagulation of four infant formula was occurred

above pH 6.0 within 5 minutes. The gelation time and gelation pH of four infant formulae present limited difference.

In the second part of this study, the digestion behaviour impacted by the protein composition of infant formulae was studied. The results indicated that protein composition could impact on the extent of protein coagulation and the oil droplet flocculation. During gastric digestion, the mean particle size of the digesta of Sample 0 and Sample 40 was keeping increased, which could be due to the formation of flocculates. For Sample 0, its particle size distribution is consistent to the previous conclusion: when the pH was below 4.20±0.15, the reorganisation and partially collapse of the flocculates of whey proteins were occurred. Besides, small quantity of coagula formed in Sample 60 at 20 min, and then the coagulation was occurred in Sample 80 within 60 min. The result agrees to the first part of this study, the increasing content of casein could increase the number of unhydrolyzed κ-casein and the lag time of coagulation would be extended. The coagulation in high casein content infant formulae occurred later than low casein content infant formulae.

At the end of digestion, the mean particle size of the digesta of Sample 0 was higher than Sample 40, because casein aggregates covered emulsion droplets had long-term stability, the increase of mean particle size was consequently limited. After 180 min of digestion, the pH of Sample 0 reached 2.61±0.20 close to the optimal pH of pepsin. However, the final pH of Sample 40 was slightly higher than Sample 0 at 3.79±0.13. Hence, intact β-Lg and α-La was detected in the digesta of Sample 40, but α-La was fully hydrolysed in Sample 0. Comparing between Sample 60 and Sample 80 at 180 min, caseins in the emptied digesta of Sample 60 was fully hydrolysed, no large curd was obtained by 1mm sieve. The infant formulae containing higher amount of casein would form stronger gel network by the action of acid and pepsin, and only Sample 80 was observed the formation of firm curd.

Overall, the protein composition plays an important role in both physiochemistry properties and the digestion behaviour of infant formulae. The amount of caseins relative to whey protein affected the formation of curd and the rate of protein hydrolysis. the information obtained from this study will be useful for a better understanding of digestion behaviours of different infant formulae in stomach. It, as well, provides aids for a better design of the commercial infant formulae by suitably optimizing the ratio of casein to whey protein, regulating digestion of protein in the infant stomach.

6.2 Recommendation for future work

Based on current study, some recommendations for future work were listed at below:

- Understanding of protein digestion in infant stomach of extensively hydrolysed casein-based formula.

In current study, the higher amount of casein to whey protein ratio resulted in the longer lag time of coagulation and the formation of large firm curd. The further study of coagulation behaviours of the extensively hydrolysed casein-based formula could be carried out to understand the effect of hydrolysis time and enzyme specificity on casein hydrolysate in infant formulae.

- The effect of fat composition of infant formulae on the gastric digestion.

The lipolysis in gastric phase is important for infants. Lingual lipase and gastric lipase is known to be present in rodent infant, which is different to adult. The gastric lipolysis of infant formulae could be carried out to further study the effect of fat composition on the infant gastric digestion.

- The effect of protein composition of infant formula on the gastrointestinal digestion: *in vivo* piglet digestion to confirm the findings in the present in vitro study

The present study analysed the protein digestion *in vitro* dynamic digestion. For further observing the digestion behaviour, structure changes, and the protein hydrolysis under infant gastric, the in vivo study is required.

Reference

Alegría, A., Garcia-Llatas, G., & Cilla, A. (2015). Static digestion models: General introduction. In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, A. Mackie, H. Wichers, T. Lea, D. Swiatecka, & T. Requena (Eds.), *The impact of food bioactives on health* (pp. 3-12). Springer.

Alting, A. C., Hamer, R. J., de Kruif, C. G., de Jongh, H. H., Simons, J., & Visschers, R. W. (2003). Physical and chemical interactions in pH-induced aggregation and gelation of whey proteins. *Food colloids, biopolymers and materials, 284*, 49-57.

Aoki, T., Toyooka, K., & Kako, Y. (1985). Role of Phosphate Groups in the Calcium Sensitivity of αs2-Casein. *Journal of Dairy Science, 68*(7), 1624-1629.

Armand, M., Hamosh, M., Mehta, N. R., Angelus, P. A., Philpott, J. R., Henderson, T. R., Dwyer, N. K., Lairon, D., & Hamosh, P. (1996). Effect of human milk or formula on gastric function and fat digestion in the premature infant. *Pediatric research, 40*(3), 429-437.

Ballard, O., & Morrow, A. L. (2013). Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am, 60*(1), 49-74.

Barth, C., & Schlimme, E. (1988). *Milk proteins*. Springer.

Bhandari, B. (2013). *Handbook of Food Powders : Processes and Properties*. Cambridge, UK. Woodhead Publishing.

Blackburn, S. T. (2007). *Maternal, fetal, & neonatal physiology : a clinical perspective* (3rd ed ed.). Elsevier Saunders.

Blanchard, E., Zhu, P., & Schuck, P. (2013). Infant formula powders. In B. Bhandari, N. BANSAL, M. Zhang, & P. Schuck (Eds.), *Handbook of Food Powders* (pp. 465-483). Elsevier Science.

Blanco, A., & Blanco, G. (2017). In A. Blanco & G. Blanco (Eds.), *Medical Biochemistry* (pp. 251-273). Academic Press.

Boland, M., & Singh, H. (2020). *Milk proteins : from expression to food* (3rd ed ed.). Academic Press, an imprint of Elsevier.

Bourlieu, C., Ménard, O., Bouzerzour, K., Mandalari, G., Macierzanka, A., Mackie, A. R., & Dupont, D. (2014). Specificity of infant digestive conditions: some clues for developing relevant in vitro models. *Critical reviews in food science and nutrition, 54*(11), 1427-1457.

Bourlieu, C., Ménard, O., De La Chevasnerie, A., Sams, L., Rousseau, F., Madec, M.-N., Robert, B., Deglaire, A., Pezennec, S., Bouhallab, S., Carrière, F., & Dupont, D. (2015). The structure of infant formulas impacts their lipolysis, proteolysis and disintegration during in vitro gastric digestion. *Food Chemistry, 182*, 224-235.

Bouzerzour, K., Morgan, F., Cuinet, I., Bonhomme, C., Jardin, J., Le Huërou-Luron, I., & Dupont, D. (2012). In vivo digestion of infant formula in piglets: protein digestion kinetics and release of bioactive peptides. *British Journal of Nutrition, 108*(12), 2105-2114.

Brew, K. (2013). α-Lactalbumin. In P. L. H. McSweeney & P. F. Fox (Eds.), *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects, 4th Edition* (pp. 261-273). Springer US.

Britten, M., & Giroux, H. J. (2001). Acid-induced gelation of whey protein polymers: effects of pH and calcium concentration during polymerization. *Food Hydrocolloids, 15*(4-6), 609-617.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., & Carrière, F. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature protocols, 14*(4), 991-1014.

Reference

Chatterton, D. E. W., Rasmussen, J. T., Heegaard, C. W., Sørensen, E. S., & Petersen, T. E. (2004). In vitro digestion of novel milk protein ingredients for use in infant formulas: Research on biological functions. *Trends in Food Science & Technology, 15*(7-8), 373-383.

Cheong, L.-Z., Jiang, C., He, X., Song, S., & Lai, O.-M. (2018). Lipid Profiling, Particle Size Determination, and in Vitro Simulated Gastrointestinal Lipolysis of Mature Human Milk and Infant Formula. *Journal Of Agricultural And Food Chemistry, 66(45)*, 12042-12050.

Choi, J., Horne, D. S., & Lucey, J. A. (2007). Effect of Insoluble Calcium Concentration on Rennet Coagulation Properties of Milk. *Journal of Dairy Science, 90*(6), 2612-2623.

Dalgleish, D. G., & Corredig, M. (2012). The structure of the casein micelle of milk and its changes during processing. *Annu Rev Food Sci Technol, 3*, 449-467.

Dalgleish, D. G., Spagnuolo, P. A., & Douglas Goff, H. (2004). A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *International dairy journal, 14*(12), 1025-1031.

Damjanovic Desic, S., & Birlouez-Aragon, I. (2011). The FAST index – A highly sensitive indicator of the heat impact on infant formula model. *Food Chemistry, 124*(3), 1043-1049.

de Kruif, C. G., Huppertz, T., Urban, V. S., & Petukhov, A. V. (2012). Casein micelles and their internal structure. *Adv Colloid Interface Sci, 171-172*, 36-52.

Deeth, H., & Bansal, N. (2018). *Whey proteins : from milk to medicine*. Academic Press.

Deeth, H. C. a. L., M.J. . (2017). Heat Treatments of Milk – Thermisation and Pasteurisation. *High Temperature Processing of Milk and Milk Products*, 15-39.

Demetriades, K., Coupland, J. N., & McClements, D. (1997). Physical properties of whey protein stabilized emulsions as related to pH and NaCl. *Journal of Food Science, 62*(2), 342-347.

Devle, H., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G. E., Ekeberg, D., & Schüller, R. B. (2012). " Rheological characterization of milk during digestion with human gastric and duodenal enzymes. *Annual Transactions of The Nordic Rheology Society, 20*, 271-276.

Donato, L., Kolodziejcyk, E., & Rouvet, M. (2011). Mixtures of whey protein microgels and soluble aggregates as building blocks to control rheology and structure of acid induced cold-set gels. *Food Hydrocolloids, 25*(4), 734-742.

Doucet, D., Otter, D. E., Gauthier, S. F., & Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. *Journal Of Agricultural And Food Chemistry, 51*(21), 6300-6308.

Drapala, K. P., Auty, M. A. E., Mulvihill, D. M., & O'Mahony, J. A. (2017). Influence of emulsifier type on the spray-drying properties of model infant formula emulsions. *Food Hydrocolloids, 69*, 56-66.

Dumont, R., & Rudolph, C. D. (1994). Development of gastrointestinal motility in the infant and child. *Gastroenterology Clinics of North America, 23*(4), 655.

Dupont, D., & Mackie, A. R. (2015). Static and dynamic in vitro digestion models to study protein stability in the gastrointestinal tract. *Drug Discovery Today: Disease Models, 17-18*, 23-27.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Leonil, J., Faulks, R. M., Wickham, M. S., Mills, E. N., & Mackie, A. R. (2010). Comparative resistance of food proteins to adult and infant in vitro digestion models. *Mol Nutr Food Res, 54*(6), 767-780.

Edwards, P. B., Creamer, L. K., & Jameson, G. B. (2008). Structure and stability of whey proteins. In A. Thompson, M. Boland, & H. Singh (Eds.), *Milk Proteins:From Expression to Food* (pp. 163-203). Academic Press.

Reference

Egger, L., Menard, O., Baumann, C., Duerr, D., Schlegel, P., Stoll, P., Vergeres, G., Dupont, D., & Portmann, R. (2019). Digestion of milk proteins: Comparing static and dynamic in vitro digestion systems with in vivo data. *Food Res Int, 118*, 32-39.

Federation, I. D., & Ḥalab, J. (1990). *Recombination of milk and milk products : proceedings of a seminar organized by the International Dairy Federation and the University of Alexandria 12-16 November, 1988*. International Dairy Federation.

Fenelon, M. A., Hickey, R. M., Buggy, A., McCarthy, N., & Murphy, E. G. (2019). In H. C. Deeth & N. Bansal (Eds.), *Whey Proteins* (pp. 439-494). Academic Press.

Fox, P. (2003). Milk proteins: general and historical aspects. In P. Fox & P. McSweeney (Eds.), *Advanced dairy chemistry—1 proteins* (pp. 1-48). Springer.

Fox, P. F. (1993). *Cheese: Chemistry, Physics and Microbiology : Volume 1 General Aspects* (Second edition ed.). Springer US.

Gan, J., Bornhorst, G. M., Henrick, B. M., & German, J. B. (2018). Protein Digestion of Baby Foods: Study Approaches and Implications for Infant Health. *Mol Nutr Food Res, 62*(1)

Gastaldi, E., Trial, N., Guillaume, C., Bourret, E., Gontard, N., & Cuq, J. L. (2003). Effect of controlled kappa-casein hydrolysis on rheological properties of acid milk gels. *Dairy Science & Technology, 86*, 704-711.

Ghosh, B. C., Prasad, L. N., & Saha, N. P. (2017). Enzymatic hydrolysis of whey and its analysis. *J Food Sci Technol, 54*(6), 1476-1483.

Gonzalez-Jordan, A., Thomar, P., Nicolai, T., & Dittmer, J. (2015). The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution. *Food Hydrocolloids, 51*, 88-94.

Groot, R. D., Bot, A., & Agterof, W. G. M. (1996). Molecular theory of strain hardening of a polymer gel: Application to gelatin. *Journal of Chemical Physics, 104*(22), 9202.

Gupta, A. (2018). *Comprehensive Biochemistry for Dentistry: Textbook for Dental Students*. Springer.

Gupta, A. (2019). Digestion and Absorption of Proteins. In A. Gupta (Ed.), *Comprehensive Biochemistry for Dentistry* (pp. 367-375). Springer.

Hallén, E. (2008). *Coagulation properties of milk: Association with Milk Protein Composition and Genetic Polymorphism.* [Doctoral dissertation, Swedish University].

Hemar, Y., Singh, H., & Horne, D. S. (2004). Determination of early stages of rennet-induced aggregation of casein micelles by diffusing wave spectroscopy and rheological measurements. *Current Applied Physics, 4*(2-4), 362-365.

Hernell, O. (2011). Human milk vs. cow's milk and the evolution of infant formulas. *Nestle Nutrition workshop series. Paediatric programme, 67*, 17-28.

Horne, D. S. (2002). Casein structure, self-assembly and gelation. *Current Opinion in Colloid & Interface Science, 7*(5), 456-461.

Horne, D. S. (2003). Casein micelles as hard spheres: limitations of the model in acidified gel formation. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 213*(2), 255-263.

Horne, D. S. (2008). Casein micelle structure and stability. In A. Thompson, M. Boland, & H. Singh (Eds.), *Milk Proteins:From Expression to Food* (pp. 133-162). Academic Press.

Horne, D. S. (2011). Milk Proteins | Casein, Micellar Structure. *Encyclopedia of Dairy Sciences*, 772-779.

Reference

Hunt, J. A., & Dalgleish, D. G. (1994). Effect of pH on the stability and surface composition of emulsions made with whey protein isolate. *Journal Of Agricultural And Food Chemistry, 42*(10), 2131-2135.

Huppertz, T. (2013). Chemistry of the Caseins. In P. L. H. McSweeney & P. F. Fox (Eds.), *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects, 4th Edition* (pp. 135-160). Springer US.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2018). The caseins: Structure, stability, and functionality. *Proteins in Food Processing*, 49-92.

Jenness, R. (1979). *The composition of human milk.* Paper presented at the Seminars in perinatology.

Jensen, R. G. (2002). The Composition of Bovine Milk Lipids: January 1995 to December 2000. *Journal of Dairy Science, 85*(2), 295-350.

Ju, Z. Y., & Kilara, A. (1998). Gelation of pH-Aggregated Whey Protein Isolate Solution Induced by Heat, Protease, Calcium Salt, and Acidulant. *Journal Of Agricultural And Food Chemistry, 46*(5), 1830-1835.

Kharlamova, A., Chassenieux, C., & Nicolai, T. (2018). Acid-induced gelation of whey protein aggregates: Kinetics, gel structure and rheological properties. *Food Hydrocolloids, 81*, 263-272.

Koletzko, B., Baker, S., Cleghorn, G., Neto, U. F., Gopalan, S., Hernell, O., Hock, Q. S., Jirapinyo, P., Lonnerdal, B., Pencharz, P., Pzyrembel, H., Ramirez-Mayans, A., Shamir, T., Turck, D., Yamashiro, Y., & Ding, Z. Y. (2005). Global standard for the composition of infant formula: Recommendations of an ESPGHAN Coordinated International Expert Group. *41*, 584-599.

Kong, F., & Singh, R. P. (2010a). A Human Gastric Simulator (HGS) to Study Food Digestion in Human Stomach. In (Vol. 75, pp. E627-E635).

Kong, F., & Singh, R. P. (2010b). A human gastric simulator (HGS) to study food digestion in human stomach. *Journal of Food Science, 75*(9), E627-E635.

Koutina, G., Knudsen, J. C., Andersen, U., & Skibsted, L. H. (2015). Influence of colloidal calcium phosphate level on the microstructure and rheological properties of rennet-induced skim milk gels. *LWT - Food Science and Technology, 63*(1), 654-659.

Lakemond, C. M. M., & van Vliet, T. (2008). Acid skim milk gels: The gelation process as affected by preheating pH. *International dairy journal, 18*(5), 574-584.

Langton, M., & Hermansson, A.-M. (1992). Fine-stranded and particulate gels of β-lactoglobulin and whey protein at varying pH. *Food Hydrocolloids, 5*(6), 523-539.

Latreille, Y. P. M. B. B. (1990). Effect of High-Pressure Homogenization on a Sterilized Infant Formula: Microstructure and Age Gelation. *Food Structure, 9(1)*, 1-8.

Layman, D. K., Lönnerdal, B., & Fernstrom, J. D. (2018). Applications for α-lactalbumin in human nutrition. *Nutrition reviews, 76*(6), 444-460.

Leung, P. S. (2014). *The gastrointestinal system : gastrointestinal, nutritional and hepatobiliary physiology*. Springer.

Li, Y., & Corredig, M. (2020). Acid induced gelation behavior of skim milk concentrated by membrane filtration. *J Texture Stud, 51*(1), 101-110.

Li, Z., Yang, Z., Otter, D., Rehm, C., Li, N., Zhou, P., & Hemar, Y. (2018). Rheological and structural properties of coagulated milks reconstituted in D2O: Comparison between rennet and a tamarillo enzyme (tamarillin). *Food Hydrocolloids, 79*, 170-178.

Liao, Y. A.-O., Weber, D., Xu, W., Durbin-Johnson, B. P., Phinney, B. S., & Lönnerdal, B. (2017). Absolute Quantification of Human Milk Caseins and the Whey/Casein Ratio during the First Year of Lactation. (1535-3907)

Lonnerdal, B., & Hernell, O. (1998). Effects of feeding ultrahigh-temperature (UHT)-treated infant formula with different protein concentrations or powdered formula, as compared with breast-feeding, on plasma amino acids, hematology, and trace element status, 350.

Lucey, J. A., van Vliet, T., Grolle, K., Geurts, T., & Walstra, P. (1997). Properties of acid casein gels made by acidification with glucono-δ-lactone. 1. Rheological properties. *International dairy journal, 7*(6), 381-388.

Macierzanka, A., Böttger, F., Lansonneur, L., Groizard, R., Jean, A.-S., Rigby, N. M., Cross, K., Wellner, N., & Mackie, A. R. (2012). The effect of gel structure on the kinetics of simulated gastrointestinal digestion of bovine β-lactoglobulin. *Food Chemistry, 134*(4), 2156-2163.

Mahe, S., Messing, B., Thuillier, F., & Tome, D. (1991). Digestion of bovine milk proteins in patients with a high jejunostomy. *The American Journal Of Clinical Nutrition, 54*(3), 534-538.

Malaki Nik, A., Wright, A. J., & Corredig, M. (2010). Surface adsorption alters the susceptibility of whey proteins to pepsin-digestion. *Journal of Colloid and Interface Science, 344*(2), 372-381.

Mason, S. (1962). Some aspects of gastric function in the newborn. *Archives of Disease in Childhood, 37*(194), 387.

Mat, D. J. L., Cattenoz, T., Souchon, I., Michon, C., & Le Feunteun, S. (2018). Monitoring protein hydrolysis by pepsin using pH-stat: In vitro gastric digestions in static and dynamic pH conditions. *Food Chem, 239*, 268-275.

McMahon, D. J., & Brown, R. J. (1984a). Composition, Structure, and Integrity of Casein Micelles: A Review1. *Journal of Dairy Science, 67*(3), 499-512.

McMahon, D. J., & Brown, R. J. (1984b). Enzymic Coagulation of Casein Micelles: A Review1. *Journal of Dairy Science, 67*(5), 919-929.

McMahon, D. J., & Oommen, B. S. (2008). Supramolecular structure of the casein micelle. *J Dairy Sci, 91*(5), 1709-1721.

McMahon, D. J., & Oommen, B. S. (2013). Casein Micelle Structure, Functions, and Interactions. In P. L. H. McSweeney & P. F. Fox (Eds.), *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects, 4th Edition* (pp. 185-209). Springer US.

McSweeney, P. L. H., & Fox, P. F. (2013). *Advanced dairy chemistry. basic aspects* (4th ed ed.). Springer.

Menard, O., Bourlieu, C., De Oliveira, S. C., Dellarosa, N., Laghi, L., Carriere, F., Capozzi, F., Dupont, D., & Deglaire, A. (2018). A first step towards a consensus static in vitro model for simulating full-term infant digestion. *Food Chem, 240*, 338-345.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new in vitro dynamic system to simulate infant digestion. *Food Chemistry, 145*, 1039-1045.

Message, J.-L., Roustel, S., & Saurel, R. (2017). Interactions in casein micelle - Pea protein system (Part II): Mixture acid gelation with glucono-δ-lactone. *Food Hydrocolloids, 73*, 344-357.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D. J., Menard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S. J.,

Weitschies, W., & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *5*, 1113-1124.

Morand, M., Guyomarc'h, F., Legland, D., & Famelart, M.-H. (2012). Changing the isoelectric point of the heat-induced whey protein complexes affects the acid gelation of skim milk. *International dairy journal, 23*(1), 9-17.

Morr, C. V. (1967). Effect of Oxalate and Urea upon Ultracentrifugation Properties of Raw and Heated Skimmilk Casein Micelles1. *Journal of Dairy Science, 50*(11), 1744-1751.

Morr, C. V., & Ha, E. Y. W. (1993). Whey Protein Concentrates and Isolates: Processing and Functional Properties. *Critical reviews in food science and nutrition, 33*(6), 431-476.

Morris, G. A. (2002). The self-assembly and structure of caseins in solution. *Biotechnol Genet Eng Rev, 19*, 357-376.

Nguyen, H. T. H., Afsar, S., & Day, L. (2018). Differences in the microstructure and rheological properties of low-fat yoghurts from goat, sheep and cow milk. *Food Res Int, 108*, 423-429.

Nguyen, T. T., Bhandari, B., Cichero, J., & Prakash, S. (2015a). A comprehensive review on in vitro digestion of infant formula. *Food Research International, 76*, 373-386.

Nguyen, T. T. P., Bhandari, B., Cichero, J., & Prakash, S. (2015b). Gastrointestinal digestion of dairy and soy proteins in infant formulas: An in vitro study. *Food Res Int, 76*(Pt 3), 348-358.

Nicolai, T., Britten, M., & Schmitt, C. (2011). β-Lactoglobulin and WPI aggregates: Formation, structure and applications. *Food Hydrocolloids, 25*(8), 1945-1962.

Nogueira, M. H., Ben-Harb, S., Schmutz, M., Doumert, B., Nasser, S., Derensy, A., Karoui, R., Delaplace, G., & Peixoto, P. P. S. (2020). Multiscale quantitative characterization of demineralized casein micelles: How the partial excision of nano-clusters leads to the aggregation during rehydration. *Food Hydrocolloids, 105*

Otte, J., Ju, Z., Faergemand, M., Lomholt, S., & Qvist, K. (1996). Protease‐induced aggregation and gelation of whey proteins. *Journal of Food Science, 61*(5), 911-916.

Otte, J., Schumacher, E., Ipsen, R., Ju, Z., & Qvist, K. B. (1999). Protease-induced gelation of unheated and heated whey proteins: effects of pH, temperature, and concentrations of protein, enzyme and salts. *International dairy journal, 9*(11), 801-812.

Packard, V. S. (1982). *Human milk and infant formula*. Academic Press.

Parry Jr, R., & Carroll, R. (1969). Location of κ-casein in milk micelles. *Biochimica et Biophysica Acta (BBA)-Protein Structure, 194*(1), 138-150.

Payens, T. (1966). Association of Caseins and their Possible Relation to Structure of the Casein Micelle1. *Journal of Dairy Science, 49*(11), 1317-1324.

Phadungath, C. (2005). Casein micelle structure: a concise review. *Songklanakarin Journal of Science and Technology, 27*(1), 201-212.

Poquet, L., & Wooster, T. J. (2016). Infant digestion physiology and the relevance of in vitro biochemical models to test infant formula lipid digestion. *Molecular nutrition & food research, 60*(8), 1876-1895.

Rabiey, L., & Britten, M. (2009). Effect of protein composition on the rheological properties of acid-induced whey protein gels. *Food Hydrocolloids, 23*(3), 973-979.

Roman, C., Carriere, F., Villeneuve, P., Pina, M., Millet, V., Simeoni, U., & Sarles, J. (2007). Quantitative and qualitative study of gastric lipolysis in premature infants: do MCT-enriched infant formulas improve fat digestion? *Pediatric research, 61*(1), 83-88.

Reference

Sadeghi, M., Madadlou, A., Khosrowshahi, A., & Mohammadifar, M. (2014). Acid-induced gelation behavior of casein/whey protein solutions assessed by oscillatory rheology. *Journal of Food Science and Technology, 51*(9), 2113.

Salaün, F., Mietton, B., & Gaucheron, F. (2005). Buffering capacity of dairy products. *International dairy journal, 15*(2), 95-109.

Sandra, S., Alexander, M., & Dalgleish, D. G. (2007). The rennet coagulation mechanism of skim milk as observed by transmission diffusing wave spectroscopy. *Journal of Colloid and Interface Science, 308*(2), 364-373.

Sandström, O., Lönnerdal, B., Graverholt, G., & Hernell, O. (2008). Effects of α-lactalbumin–enriched formula containing different concentrations of glycomacropeptide on infant nutrition. *The American Journal Of Clinical Nutrition, 87*(4), 921-928.

Sarkar, A., Goh, K. K. T., Singh, R. P., & Singh, H. (2009). Behaviour of an oil-in-water emulsion stabilized by β-lactoglobulin in an in vitro gastric model. *Food Hydrocolloids, 23*(6), 1563-1569.

Schmidt, D. (1968). Further studies on the associating subunit of alpha S-1-casein. *Biochimica et biophysica acta, 154*(3), 613-614.

Schmidt, D. (1982). Association of caseins and casein micelle structure. *Developments in dairy chemistry*

Schultz, S. G. (1989). *The gastrointestinal system*. American Physiological Society.

Shani-Levi, C., Levi-Tal, S., & Lesmes, U. (2013). Comparative performance of milk proteins and their emulsions under dynamic in vitro adult and infant gastric digestion. *Food Hydrocolloids, 32*(2), 349-357.

Sidnell, A., & Greenstreet, E. (2011). Infant nutrition - review of lipid innovation in infant formula. *Nutrition Bulletin, 36*(3), 373-380.

Sinaga, H., Bansal, N., & Bhandari, B. (2016). Effects of milk pH alteration on casein micelle size and gelation properties of milk. *International Journal of Food Properties, 20*(1), 179-197.

Sinaga, H., Bansal, N., & Bhandari, B. (2017). Effects of milk pH alteration on casein micelle size and gelation properties of milk. *International Journal of Food Properties, 20*(1), 179-197.

Singh, H., & Ye, A. (2008). Interactions and functionality of milk proteins in food emulsions. In A. Thompson, M. Boland, & H. Singh (Eds.), *Milk Proteins:From Expression to Food* (pp. 321-345). Academic Press.

Singh, H., & Ye, A. (2013). Structural and biochemical factors affecting the digestion of protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 18*(4), 360-370.

Slattery, C. W. (1976). Model calculations of casein micelle size distributions. *Biophysical chemistry, 6*(1), 59-64.

Slattery, C. W. (1979). A phosphate-induced sub-micelle-micelle equilibrium in reconstituted casein micelle systems. *Journal of Dairy Research, 46*(2), 253-258.

Slattery, C. W., & Evard, R. (1973). A model for the formation and structure of casein micelles from subunits of variable composition. *Biochimica et Biophysica Acta (BBA)-Protein Structure, 317*(2), 529-538.

Sodhi, C. P., Fulton, W. B., Good, M., Vurma, M., Das, T., Lai, C.-S., Jia, H., Yamaguchi, Y., Lu, P., Prindle, T., Ozolek, J. A., & Hackam, D. J. (2018). Fat composition in infant formula contributes to the severity of necrotising enterocolitis. *The British Journal Of Nutrition, 120*(6), 665-680.

Reference

Srilaorkul, S., Ozimek, L., Wolfe, F., & Dziuba, J. (1989). The Effect of Ultrafiltration on Physicochemical Properties of Retentate. *Canadian Institute of Food Science and Technology Journal, 22*(1), 56-62.

Tari, N. R., Fan, M. Z., Archbold, T., Kristo, E., Guri, A., Arranz, E., & Corredig, M. (2018). Effect of milk protein composition of a model infant formula on the physicochemical properties of in vivo gastric digestates. *Journal of Dairy Science, 101*(4), 2851-2861.

Thorn, D. C., Ecroyd, H., & Carver, J. A. (2014). Polymorphism in Casein Protein Aggregation and Amyloid Fibril Formation. In V. N. Uversky & Y. L. Lyubchenko (Eds.), *Bio-nanoimaging* (pp. 323-331). Academic Press.

Toikkanen, O., Outinen, M., Malafronte, L., & Rojas, O. J. (2018). Formation and structure of insoluble particles in reconstituted model infant formula powders. *International dairy journal, 82*, 19-27.

van der Sman, R. G. M., Houlder, S., Cornet, S., & Janssen, A. (2020). Physical chemistry of gastric digestion of proteins gels. *Current Research in Food Science, 2*, 45-60.

van Vliet, T. (2000). Structure and rheology of gels formed by aggregated protein particles. In K. Nishinari (Ed.), *Hydrocolloids* (pp. 367-377). Elsevier Science.

Vasbinder, A. J., Rollema, H. S., De Kruif, C. G., & Bot, A. (2003). Gelation mechanism of milk as influenced by temperature and pH; studied by the use of transglutaminase cross-linked casein micelles. *Journal of Dairy Science, 86*(5), 1556-1563.

Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., & Wichers, H. (2015). *The impact of food bioactives on health: in vitro and ex vivo models*. Springer Nature.

Wada, Y., Phinney, B. S., Weber, D., & Lönnerdal, B. (2017). In vivo digestomics of milk proteins in human milk and infant formula using a suckling rat pup model. *Peptides, 88*, 18-31.

Walstra, P. (1995). Physical chemistry of milk fat globules. *Advanced dairy chemistry, 2*, 131-178.

Walstra, P., Jenness, R., & Badings, H. T. (1984). *Dairy chemistry and physics*. Wiley.

Wang, X., Ye, A., Lin, Q., Han, J., & Singh, H. (2018). Gastric digestion of milk protein ingredients: Study using an in vitro dynamic model. *Journal of Dairy Science, 101*(8), 6842-6852.

Wang, Y., Eastwood, B., Yang, Z., de Campo, L., Knott, R., Prosser, C., Carpenter, E., & Hemar, Y. (2019). Rheological and structural characterization of acidified skim milks and infant formulae made from cow and goat milk. *Food Hydrocolloids, 96*, 161-170.

Ye, A., Cui, J., Carpenter, E., Prosser, C., & Singh, H. (2019a). Dynamic in vitro gastric digestion of infant formulae made with goat milk and cow milk: Influence of protein composition. *International dairy journal, 97*, 76-85.

Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2016a). The formation and breakdown of structured clots from whole milk during gastric digestion. *Food Funct, 7*(10), 4259-4266.

Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2016b). Formation of a structured clot during the gastric digestion of milk: Impact on the rate of protein hydrolysis. *Food Hydrocolloids, 52*, 478-486.

Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2017). Effect of homogenization and heat treatment on the behavior of protein and fat globules during gastric digestion of milk. *Journal of Dairy Science, 100*(1), 36-47.

Ye, A., Liu, W., Cui, J., Kong, X., Roy, D., Kong, Y., Han, J., & Singh, H. (2019b). Coagulation behaviour of milk under gastric digestion: Effect of pasteurization and ultra-high temperature treatment. *Food Chemistry, 286*, 216-225.

Reference

Yu, G., Zheng, Q.-S., & Li, G.-F. (2014). Similarities and differences in gastrointestinal physiology between neonates and adults: a physiologically based pharmacokinetic modeling perspective. *The AAPS journal, 16*(6), 1162-1166.

Zhang, Q., Cundiff, J. K., Maria, S. D., McMahon, R. J., Wickham, M. S., Faulks, R. M., & van Tol, E. A. (2014). Differential digestion of human milk proteins in a simulated stomach model. *J Proteome Res, 13*(2), 1055-1064.

Zhao, L. L., Wang, X. L., Tian, Q., & Mao, X. Y. (2016). Effect of casein to whey protein ratios on the protein interactions and coagulation properties of low-fat yogurt. *J Dairy Sci, 99*(10), 7768-7775.

Zhuang, Y., Ueda, I., Kulozik, U., & Gebhardt, R. (2018). Influence of beta-lactoglobulin and calcium chloride on the molecular structure and interactions of casein micelles. *Int J Biol Macromol, 107*(Pt A), 560-566.