Impact of calcium lactate and polysaccharides on skim milk gelation

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Abstract

Swallowing difficulty is a growing health issue in adults particularly in the elderly population throughout the world. Thickeners and/or stabilisers are often added to the fluids to make them easier to swallow. Calcium-fortified dairy products have also been recommended for adults and the elderly to minimise the development of osteoporosis. Texture-modified milk with added calcium and polysaccharides could benefit adults who require an adequate calcium intake and have swallowing difficulties. Therefore, this project aimed to investigate the effect of the addition of calcium lactate to skim milk with and without polysaccharides.

This project examined the calcium-added skim milk with and without polysaccharides for physical and rheological properties and microstructure. The first part of this project studied the effect of different heat treatments and the addition of different concentrations of calcium lactate on skim milk. The skim milk samples with added calcium lactate were heat treated at different temperatures (65^{o}C, 70^{o}C, 75^{o}C and 80^{o}C) and held at different periods of time (30 min or 60 min), followed by cooling to 20^{o}C. The second part of this project investigated the effect of the addition of calcium lactate and three polysaccharides (xanthan gum, high acyl gellan gum and guar gum) on skim milk with calcium lactate after heating and holding at 75^{o}C for 30 min. The physical properties of the skim milk samples were visually examined after heat treatment and the rheological properties were determined for different concentrations of polysaccharides using a rheometer. The microstructure of the samples was studied using a scanning electron microscope (SEM).

Increasing the concentration of calcium lactate promoted the gelation of skim milk along with high heating temperatures (>70^{o}C) and longer holding times. At the concentrations <10mM added calcium lactate, liquid skim milk samples with no gelation were observed. When the concentration of added calcium lactate was greater than 10mM, hard and firm gels were formed at all heat treatments. The final *G'* increased with increasing concentration of added calcium lactate and higher heating temperature and longer holding times. The highest final *G'* was achieved when the sample with 20mM added calcium lactate was heated at 80^{o}C for 60 min.

The addition of xanthan gum, high acyl gellan gum or guar gum demonstrated different interactions with calcium lactate in the milk system. At 10mM and 15mM calcium lactate, soft gels were formed when the xanthan concentration was less than 0.2% and viscous liquids were formed and became more viscous as the xanthan gum concentration increased above 0.2%. A dense and compact network with crosslinking was observed under SEM when 0.3% xanthan gum and 15mM calcium lactate were added to skim milk. With the addition of high acyl gellan gum, firmer gels were formed and the final *G'* with increasing concentration of high acyl gellan gum, while a fibrous network was observed at 0.3% added high acyl gellan gum. With the addition of guar gum, soft gels were produced with aggregates and phase separation was observed when 0.2%-0.3% guar gum was added. The final *G'* did not increase significantly ($p<0.05$) as the concentration of guar gum increased and a less compact structure was observed under SEM.

The major findings in this study may be used as a guideline for the potential development of thickened milk beverages. The mouthfeel of the skim milk with 10mM and 15mM calcium lactate was improved when the xanthan concentration increased above 0.1% and a smooth mouthfeel was perceived at 0.5% added xanthan. Guar gum was not recommended to be used as undesirable mouthfeel was perceived.

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Chapter 1 Introduction

1.1 Project background

Swallowing difficulty is a growing health concern in the population, particularly in aging adults and it can be caused by a number of factors such as neurological disorders, congenital and development conditions, obstruction and age-related diseases (Adams & Smith, 2012; Carucci & Turner, 2015; Sura et al., 2012; Waito et al., 2017). Swallowing difficulty can potentially lead to dysphagia which can be caused by functional or structural abnormalities of the oral cavity, pharynx, oesophagus and gastric cardia (Ala'A et al., 2015; Carucci & Turner, 2015). A growing population of elderly adults suffer from swallowing difficulties and it is highly prevalent to cause malnutrition, dehydration, aspiration and pneumonia (Jennifer & Mikoto, 2000; Vivanti et al., 2009). Current treatments including tube feeding, swallowing therapy and modification of foods and liquids (Cichero et al., 2013; Hadde, 2017). Texture modification of foods and liquids has had increasing attention for dysphagia treatment. Thickened liquids have been widely used and applied in the treatment of dysphagia by clinicians due to convenience, affordability and low-cost implementation (Mills, 2008). Some of current thickened products are not ready to drink (RTD) for elderly patients, and the preparation of the drink can often result in inconsistent thickness (Mills, 2008). Thickened RTD liquids often uses a thickening agent and/or a stabiliser to deliver a consistent texture and flavour (Nicholson et al., 2008). However, there are many factors that affect the consistent level of thickness and these factors are the type and concentration of thickening agents or stabilisers (Garcia et al., 2005; Sopade et al., 2008), solids content of the liquid and serving temperature (Garcia et al., 2005, 2008). The standards or criteria of thickened fluids have not been well-established and further studies are required to fill the gaps in this field (Atherton et al., 2007; Hadde, 2017). In addition, elderly people often suffer from osteoporosis as a result of the loss of bone mass and inadequacy of calcium intake (Heaney et al., 1982; Kucharska, 2017). The consumption of calcium-fortified dairy products has become popular and been recommended for elderly people (Caroli et al., 2011). The calcium-fortified milk is not only a good source of protein, but also provides adequate amount of calcium, reducing the risk of osteoporosis development (Daly et al., 2008).

A number of researchers have studied the calcium-added milk gels under different conditions and they have reported the addition of a calcium salt to milk can induce the formation of gels (Ju & Kilara, 1998a; Lin et al., 2020; Lin et al., 2018; Ramasubramanian et al., 2014; Vasbinder et al., 2003). The concentration and type of calcium salts with different solubility and bioavailability play an important role in the formation of milk gels (Lund et al., 2010; Weaver, 1998). The selection of calcium salts is important in this study as it requires good solubility and should not possess undesirable taste.

Texture-modified calcium-added milk with added polysaccharides could provide thickened beverages for dysphagia treatment in elderly patients who also require an adequate calcium intake. However, there is a lack of information and studies on rheological parameters and sensory characteristics of the thickened milk or gels with the addition of calcium salts and polysaccharides. The addition of polysaccharides could provide stabilisation to a dairy system under heat treatment and improve the mouthfeel of food products. It is still unknown the appropriate level of thickness of the milk or gels that can be used for adults with difficulty swallowing. Therefore, it is vital to investigate and understand the rheological and microscopic properties of the thickened milk or gels before sensory characterisation and before implementation clinical and commercial applications. It was also important to study how the properties of the skim milk changed from a liquid state to a solid state (gel), with varying concentrations of calcium lactate and/or polysaccharides.

It is hypothesised that the addition of a polysaccharide and a calcium salt can improve the texture and the mouthfeel of bovine milk. This project aimed to investigate the effect of additional polysaccharides on the rheological and physical properties of calcium-added milk gels, which would build a fundamental and pathway for further development of thickened milk drinks. This project studied rheological characterisation, physical and microscopic properties, to obtain information on how and when changes occur in the physical, microstructure and rheological properties of the thickened milk or gels.

1.2 Thesis objectives

The objectives of this project were:

- To investigate the rheological properties of skim milk solutions with added calcium lactate under different heat treatments.
- To characterise the rheological behaviour of calcium lactate-added skim milk solutions with added polysaccharides under heat treatment.
- To examine the microscopic structure and visual observation of skim milk solution with various concentrations of added calcium and polysaccharides under heat treatment.
- To investigate the correlations between rheological measurements, microscopic and visual observations.

1.3 Thesis outlines

Chapter 2 reviews the components of bovine milk, a variety of milk gels and their formation and common polysaccharides used in dairy systems. The interaction between the milk proteins and polysaccharides is investigated. The technical tests and measurements used in characterising of thickened fluids and gels was also studied. The current issues and potential application of thickened milk or milk gels is discussed.

Chapter 3 focuses on the experimental materials and methodology used throughout the project. It includes the preparation of the skim milk samples, the conditions of heat treatments and milk gelation, the parameters and procedures of rheological characterisation, calcium measurement in milk and the protocol of scanning electron microscope.

Chapter 4 studies the rheological properties of the milk gels with added calcium lactate under various heat treatments. It focuses on the mechanisms and changes in the final storage modulus of the milk gels during heating, holding and cooling periods. The milk gels were also prepared and visually observed.

Chapter 5 explores the properties of the milk gels with added calcium lactate and three different polysaccharides through visual observation and informal sensory assessment, rheological characterisation and scanning electron microscope imaging. The final storage modulus of each milk gel with various concentrations of polysaccharides was determined.

Chapter 6 discusses the main findings in this study. The results of this project were summarised, and major outcomes of this study were discussed. The last part of the chapter discusses the precautions need to be taken and improvement for the SEM gel preparation, and recommendations for potential development of thickened drinks.

Chapter 7 consists of conclusions of the project and recommendations for further work.

Chapter 2 Literature Review

2.1. Introduction

This literature review will provide an insight into the structure and composition of bovine milk and the formation of various milk gels as well as, the causes of milk gelation and how aggregation and gelation occur. The common polysaccharides used in food products and the interactions between polysaccharides and proteins in dairy systems will be discussed. This literature review also provides information on techniques to characterise milk gels from rheological, microscopy and particle analysis techniques. The potential applications of thickened milk beverages and current difficulties will also be discussed in the final section of the literature review.

2.2 Bovine Milk

Milk is a liquid food secreted by the glands of mammals, which contains the nutrients for the growth and development of the neonate. The major constitutes of milk are proteins, lipids, lactose and water (O'Mahony & Fox, 2014). Various milks are produced by mammals and their milk composition is different from one to another. Bovine milk is produced by cattle cows and contains a multitude of various minerals, vitamins and ions (Lu & Wang, 2017). The proximate composition of bovine, goat, sheep and human milks is shown in Table 2.1.

Table 2.1 Proximate composition (wt %) of bovine, goat, sheep and human milks (Jenness, 1974).

Component (wt %)	Bovine	Goat	Sheep	Human
Protein	3.4	2.9	5.5	1.0
- Casein	2.8	2.5	4.6	0.4
Fat	3.7	4.5	7.4	3.8
Lactose	4.6	4.1	4.8	7.0
Minerals	0.7	0.8	1.0	0.2
Water	88.6	87.7	81.3	88.0

2.2.1 Proteins

A good source of protein, bovine milk contains about 3.4% protein (Walstra & Jenness, 1984). Protein plays an important role in the growth and development of the neonates. The physical and chemical properties of milk are highly associated with the proteins present (Phillips & Williams, 2011). Milk proteins can be categorised into two principal

proteins casein and whey, there are also other minor proteins (Table 2.2). The isoelectric point of the protein is the pH at which the protein is the least soluble and precipitation occurs. Casein's isoelectric point is pH 4.6 and at this pH whey proteins remain in the serum phase (Phillips & Williams, 2011). The characteristics of each protein and the interaction between casein and whey proteins are different at different pH, temperatures and in the presence of other components in the system (Corredig & Dalgleish, 1996). During heat treatment, β-lactoglobulin began to aggregate and form larger denatured aggregates as heating temperature and/or heating time increased. Meanwhile, α-lactalbumin starts to denature and further form complexes with denatured β-lactoglobulin aggregates (Jang & Swaisgood, 1990). These complexes of denatured α-lactalbumin and β-lactoglobulin bind to the surface of casein micelles (Fox et al., 2015). Milk proteins undergo different degrees of denaturation when they are exposed to different heating conditions (Raikos, 2010). It was reported that the denaturation of the whey proteins and casein micelles and formation of their complexes occurred at a faster rate when the skim milk was heated at 90°C and at pH 5.8, comparing with the one heated at 75°C and at pH 6.8 (Corredig & Dalgleish, 1996). The rates and extent of the denaturation also changed when additional α-lactalbumin and β-lactoglobulin were added to the skim milk (Corredig & Dalgleish, 1996).

Table 2.2 Protein composition of bovine milk (Swaisgood, 2003; Walstra & Jenness, 1984).

Protein	g/kg	g/litre
Total protein	35.1	36
Total casein	28.6	29.5
Whey protein	6.1	6.3
α_{s1}Casein	11.5	11.9
α_{s2} Casein	3.0	3.1
β Casein	9.5	9.8
χ Casein	3.4	3.5
γ Casein	1.2	1.2
α Lactalbumin	1.2	1.2
β Lactoglobulin	3.1	3.2
Serum albumin	0.4	0.4
Immunoglobulin	0.8	0.8
Proteose-peptones	1.0	1.0

2.2.1.1 Casein

Caseins are heterogeneous phosphoproteins, which contains about 0.85% phosphorus. The phosphate groups bind with the calcium present in milk and forms calcium phosphates which play an important role in the characteristics of caseins (Fox et al., 2015). Casein micelles exist in colloidal form and they are held together by calcium ions and hydrophobic interactions. The micelles are reported to have a mean diameter of 90 – 300 nm (Damodaran & Parkin, 2017). Micelles are composed of 92% protein and 8% milk salts (primarily calcium phosphate, magnesium ions and citrate). One model for casein micelles postulates they are solid spheres with a coating of κ-casein on the surface and the coating provides steric stabilisation to the micelle (Damodaran & Parkin, 2017). Caseins show a sequence distribution of hydrophobic, polar and charged residues. The C-terminal of the κ-casein coating is hydrophilic and the polar domain of κ-casein is attached to many hydrophobic domains with many sites, which can interact with other caseins (Dalgleish, 1998; Damodaran & Parkin, 2017). Another model describing sub-casein micelles was described by Slattery (1976) and Slattery and Evard (1973) and the micelle consists of small spherical sub-units connecting with colloidal calcium phosphate. Figure 2.1 illustrates this sub-casein micelle model. The casein micelles aggregate via hydrophobic interaction into sub-casein and the sub-micelles which are rich in κ-casein congregate on the micelle surface, the submicelles deficient in κ-casein are located in the interior of the micelles (Slattery & Evard, 1973). Another model called the Holt model proposed is that the micellar calcium phosphate particle is equivalent to a nanocluster, which was observed in the transmission electron microscopy (De Kruif & Holt, 2003). De Kruif and Holt (2003) suggested that the micellar calcium phosphate is identical to the calcium phosphate nanocluster formed and the core of calcium phosphate is surrounded by 49 phosphoseryl cluster in casein molecules (Horne, 2006). The caseins are bound via their phosphoserines to the calcium phosphate cluster and they were linked together through hydrophobic and electrostatic interactions (McMahon & Oommen, 2008). The nanocluster of calcium phosphate connects with the proteins to form the big micelles (Figure 2.2). The α-caseins acts as bridge binding and crosslinking to different nanocluster and thereby the network arises. The β-caseins present surround the closed nanoclusters (De Kruif & Holt, 2003). It is reported that casein is heat stable and it will not coagulate at 100 ^{o}C and pH 6.7 for 24 hours or 140 ^{o}C for 20 min (Fox et al., 2015). The sub-micelle model relies on the known preference of the casein for self-association and yet it imposes a rigid framework of segregative assembly (Horne, 2006; Slattery &

Evard, 1973). While the Holt model describes the interaction of the phosphoserine cluster of the caseins with mineral calcium phosphate as the dominant path to micelle assembly (De Kruif & Holt, 2003; Horne, 2006). The submicelle model was the most popularly accepted model for many years but a homogneous network of casein polymers containing nanoclusters of calcium phosphate has become the preferred model with more studies conducted (De Kruif & Holt, 2003; Karlsson et al., 2007).

Figure 2.1 The schematic diagram of submicelle of the casein micelle (Horne, 2006).

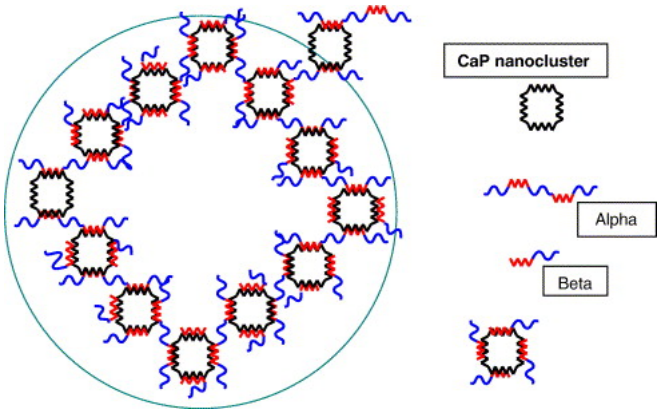

Figure 2.2 Schematic diagram of formation of the casein micelles in the Holt model (De Kruif & Holt, 2003).

The interaction between casein and polysaccharide can be different over a wide range of pH, ionic strength, charge distribution, pressure and temperature (Nayak & Singh, 2019; Syrbe et al., 1998). In the presence of polysaccharides, the behaviour of casein micelles can be evaluated in terms of adsorption or non-adsorption of polysaccharides (Nayak & Singh, 2019). It was reported that the ratio and concentration of protein to polysaccharide was crucial to the stability of the mixed system (Maroziene & De Kruif, 2000). The addition of κ-carrageenan to milk could lead to the electrostatic interaction with casein micelles and therefore stabilise dairy emulsions or milk gels (Benichou et al., 2002; Nayak & Singh, 2019). A casein-polysaccharide mixture could result in depletion

flocculation due to the difference in osmotic pressure between the bulk solvent containing the polysaccharide and solvent entrapped between casein micelles (Nayak & Singh, 2019). It was reported electrostatic interaction occurred between casein and carboxmethycellulose (CMC) and depletion flocculation was observed when excess amount of CMC was added (Nayak & Singh, 2019). The adequate amount of CMC added to milk can prevent milk flocculation in acidified milk drinks and stabilises emulsions (Nayak & Singh, 2019). The addition of polysaccharide to a system containing casein can result in the stabilisation of the system. The type and concentration of polysaccharide added to a system containing casein can be tailored depending on the functionality of the product.

2.2.1.2 Whey proteins

Bovine milk contains about 20% whey proteins which remain soluble in milk serum after precipitation of caseins at pH 4.6 and 20 °C (Fox et al., 2015). The two principal whey proteins present in bovine milk are β-lactoglobulin and α-lactalbumin. Figure 2.3 and 2.4 illustrate the structure of β-lactoglobulin and α-lactalbumin, respectively. Fox et al. (2015) reported that casein can undergo coagulation with the action of rennet or the interaction with calcium ions, while whey proteins do not undergo similar alternations and they are less sensitive and stable to the presence of rennet and ions. In addition, whey proteins are heat sensitive and they could denature under heat treatment with different denaturation degrees. The denaturation of whey proteins begins at 70 ^{o}C and the extent of whey denaturation increases with temperature and time (Parris et al., 1991). The denaturation degree of whey protein increased from 28.34% to 45.37% when the temperature of heat treatment time increased from 65 ^{o}C to 85 ^{o}C for 10 min (Qian et al., 2017). It has been reported that whey proteins are completely denatured by heating at 90^{o}C for 10 min (De Wit, 1990; Fox et al., 2015). β-lactoglobulin plays an important role in whey protein denaturation and it denatures when the temperature is above 70 ^{o}C. It was reported by Akkerman et al. (2016) that the denaturation degree was 94% for β-lactoglobulin when the skim milk was heated at 140 ^{o}C for 5 seconds using a plate heat exchanger (PHE), and over 90% of β-lactoglobulin was denatured when the temperature was greater than 95 ^{o}C. It was also found β-lactoglobulin had the highest denaturation degree compared with α -lactalbumin when the skim milk (Akkerman et al., 2016; O'Connell & Fox, 2011; Schokker et al., 2000). A number of researches have studied the mechanisms of whey protein denaturation (Damodaran & Parkin, 2017; De Kruif & Holt,

2003; Edwards et al., 2009; Wijayanti et al., 2014). The whey protein denaturation can be divided into two steps. The first step is unfolding of the globular proteins and the second step is aggregation. Whey denaturation is dependent on temperature, pH and heating time (Damodaran & Parkin, 2017; De Wit, 1990).

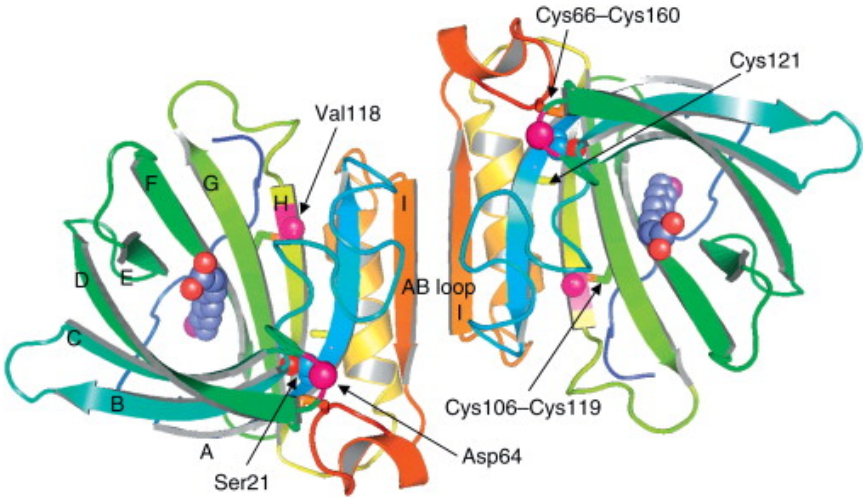

Figure 2.3 Diagram of the dimeric structure of β-lactoglobulin. The structure is rainbow coloured, beginning with blue at the N-terminus and ending with red at the C-terminus (Edwards et al., 2009).

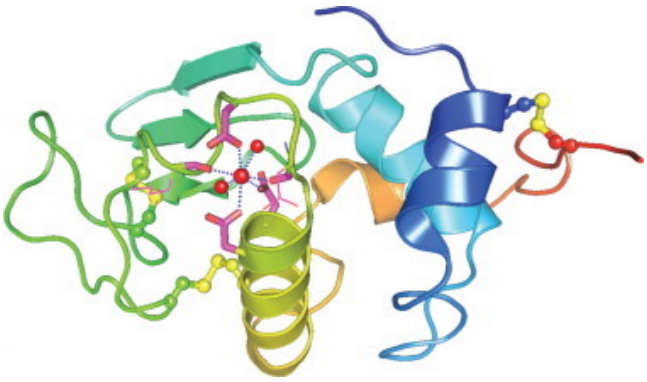

Figure 2.4 Structure of bovine α-lactalbumin showing the Ca2+ ion binding site. The peptide chain is rainbow coloured, beginning at the N-terminus in blue and progressing to the C-terminus in red, in order to show the assembly of the sub-domains (Edwards et al., 2009).

β-lactoglobulin is a globular protein which consists of 162 amino acids with a molecular weight of 18.3 kDa (Sawyer, 2003). Whey protein consists of approximately 50% β-lactoglobulin and it has the ability to bind small hydrophobic molecules into a hydrophobic cavity, hence it is proposed that β-lactoglobulin is used as a transport protein for retinoid species (Edwards et al., 2009; Papiz et al., 1986). The thermal properties of β-lactoglobulin are important for commercial processing as they can foul processing

equipment (Edwards et al., 2009). At neutral pH, the free thiol of Cys121 (Figure 2.3) and hydrophobic residues lead to the intermolecular association of covalent and hydrophobic bonds (Edwards et al., 2009). The interaction of whey proteins and casein in milk can also lead to aggregation. β-lactoglobulin from whey proteins interacts with κ-casein and form a β-lactoglobulin coating on the exterior of the casein micelle. In addition, the interactions of β-lactoglobulin and cysteine-containing serum caseins result in the formation of casein-whey complexes. (Vasbinder & De Kruif, 2003).

α-lactalbumin has a compact and globular structure and its molecular weight is about 14.2 kDa (Walstra & Jenness, 1984). It consists of 123 amino acids and four disulphide bridges (Figure 2.4). The isoelectric point of α-lactalbumin is between pH 4.2 and 4.5. The structural difference with β-lactoglobulin is the absence of a free thiol group in α-lactalbumin, which renders it better to heat stability (Edwards et al., 2009). α-lactalbumin itself does not form gel upon denaturation and acidification. The presence of calcium ion could confer stability to the tertiary structure of α-lactalbumin, which makes it more stable when exposed to thermal processing (Wehbi et al., 2005). It was reported that the addition of calcium ions could accelerate the refolding rate of α-lactalbumin and aids the formation of the disulphide linkages of the denatured reduced protein (Belloque et al., 2000).

2.2.2 Fat

Milk is an oil-in-water emulsion and the fat globules are dispersed in the continuous phase. Milk lipids serves as a source of energy for the young neonate and lipids has a marked influence on the flavour, texture and rheological properties of dairy products (Fox et al., 2015). The mean diameter of fat globules in raw milk is approximately 1 to 10 μm and it is reduced to 0.2 to 2 μm after homogenization (Fox et al., 2015).

2.2.3 Minerals

Bovine milk contains approximately 0.7 wt % of minerals which includes cations calcium, magnesium, sodium, potassium and anions phosphate, citrate and chloride (Gaucheron, 2005; Jenness, 1974). The minerals in milk are present in the form of salts, in which they interact with proteins. Calcium plays an important role in the structure and stability of the casein micelles. It was reported the calcium content of milk is on average 26 – 32 mM (Karlsson et al., 2019). The stability of the casein micelles is associated with the colloidal

calcium phosphate (CCP) and the formation of calcium bridges between negatively charged residues of the casein micelle and CCP (Gaucheron, 2005; Tsioulpas et al., 2007). Calcium phosphate is bound to the casein micelles and the dissociation of calcium phosphate can induce the changes in the structure of casein micelles, subsequently leading to changes in the properties of milk (Fox et al., 2015). The calcium equilibrium between calcium and the colloidal and serum phase is affected by pH, where the free calcium ion concentration in the serum increases as a result of a reduction in pH (Lewis et al., 2011). A number of studies have found the effect of various calcium salts on the milk pH, gelation time and stability of proteins (Omoarukhe et al., 2010; Singh et al., 2007).

In a system containing protein, polysaccharide and mineral ions, the components could interact with each other in several ways (Bernal et al., 1987). It was studied that the protein and polysaccharide could interact with or without the involvement of calcium ions (Antonovcan et al., 1985). However, the addition of calcium ions could form calcium bridges with the casein and casein-polysaccharide complexes, maximising the interaction between negatively charged molecules and improving the firmness and stability of a gel (Bernal et al., 1987).

2.2.4 Lactose

Bovine milk contains about 4.6% lactose and it is the predominant carbohydrate, which accounts for 50% of the total solids (Damodaran & Parkin, 2017). Lactose is a disaccharide consisting of a glucose and galactose molecule. Lactose not only provides some of the nutritive value of milk but also affects the flavour, colour and textural characteristics of heated milk products (Fox et al., 2015).

2.3 Milk Gels

Gels are solid-like soft materials, which are made from a three-dimensional network of cross-linked hydrophilic polymers or colloidal particles (Demirci & Khademhosseini, 2016). The formation of milk gels can be triggered by a change in pH, presence or addition of ions, heat, pressure and enzymes. The structure and properties of the milk gels vary under different conditions. A number of studies found that the formation of milk gels was due to the coagulation and denaturation of milk proteins (Parry, 1974; Schorsch et al., 2001). Hence, the formation of milk gels with various factors can be categorised into rennet, acid, heat or mineral-induced protein gels, which were described from Sections 2.3.2 to 2.3.5.

2.3.1 Gelation of milk

Milk gels are complex systems in which the presence of charged ions, lactose, whey and casein proteins, minerals and fat significantly affect the mechanisms of gelation (Ju & Kilara, 1998b; Koutina, Christensen, et al., 2015; Richardson & Ross-Murphy, 1981). The denaturation of milk proteins and interaction between proteins and charged ions are responsible for the formation of milk gels (Lucey, 2014). However, other factors such as pH, pressure, enzymes, ionic strength and the mode and degree of heat treatment also affect the formation of the gel network (Kinsella et al., 1994). One reported mechanism of milk gelation after heat treatment is divided into two steps. In the first step, β-lactoglobulin from whey proteins forms a complex with κ-casein of the casein micelles. Multiple anchor sites on κ- casein break down and this leads to the dissociation of the β-lactoglobulin and κ-casein complexes. In the second step, the complexes of β-lactoglobulin and κ-casein aggregate and form a three-dimensional matrix. In the gelation process, the complexes of β- lactoglobulin and κ-casein are released from the casein micelles and this leads to further crosslinking interactions between β- lactoglobulin and κ-casein (Datta & Deeth, 2001). The gelation of milk induced by rennet, acid, heat and the minerals will now be discussed.

2.3.2 Rennet-induced gels

The cheese-making process is one of the applications of rennet-induced gels in which the coagulation of milk proteins occurs (Hyslop, 2003). The formation of the gel is caused by the addition of renneting enzymes and the process is divided into two stages. In the primary stage, κ-casein is cleaved by rennet at the Phe_{105}-Met_{106} bond, resulting in the hydrolysis of C-terminal part of the κ-casein molecules and a reduction in the steric and electrostatic repulsion of casein micelles. The rennet-altered micelles become more susceptible to aggregation. In the second stage, casein micelles start to aggregate and eventually a three-dimensional gel network is formed. The stability of casein micelles is associated with their net negative charge and steric repulsion by the macro-peptide region of κ-casein, calcium ions, hydrogen bonding and hydrophobic interaction. Caseinomacropeptide (CMP) is diffused from the casein micelles and this results in a decrease in the zeta potential, leading to a loss of electrostatic and steric stabilisation (Dalgleish, 1993; Hyslop, 2003; Lucey, 2011).

Temperature, pH, enzyme concentration and calcium ions affect the formation of rennet-induced gels. It was reported that the optimum temperature for milk gels that are induced by calf rennet at pH 6.6 was 45ºC, however the optimum temperature for different types of rennet varies at different pH (Lucey, 2011). During the cheese-making process, it was reported that the optimum temperature to achieve the desired firmness of rennet-induced gels is 30 to 35ºC (Lucey, 2011). Decreasing the pH of milk resulted in reduced rennet coagulation time and gel firmness could be increased at a faster rate. It was found the optimum pH for rennet-induced milk was between pH 6.0 and 6.3 (Lucey, 2011). In addition, rennet enzyme concentration influences the coagulation time of milk and it is described by the Holter equation (Equation 2.1) (Castillo et al., 2000; Ferreira, 2011).

$$\mathrm{CT} = \frac{K}{[E]} + A \qquad (2.1)$$

Where CT is the coagulation time, K and A are constants and E is enzyme concentration. This equation suggests that an increase in enzyme concentration results in a reduction in coagulation time. The addition of calcium salts reduces the pH of milk and this accelerates the hydrolysis reaction. It was reported that the addition of calcium salts in milk at a constant pH leads to a reduction in coagulation time (Gastaldi et al., 1994).

2.3.3 Acid-induced gels

Acid-induced gels can be induced by the addition of bacterial cultures, chemical acids or glucono-δ-lactone (GDL). With the addition of bacterial cultures, lactic acids are produced from the fermentation of lactose. The three methods listed results in a reduction in pH, however the rate of acidification varies depending on the type of bacterial cultures or GDL used (Lucey & Singh, 1997). During the acidification process, casein micelles become unstable and form aggregates due to charge neutralisation, this results in the formation of a three-dimensional gel network. Casein micelles undergo extensive particle rearrangements at its isoelectric point (pH at 4.6) and this leads to the formation of clusters of casein aggregates and further aggregation results in gel formation.

Heat treatment is one of the important processes required to produce a commercial acid milk gel such as yoghurt. Many studies suggested that heat treatment exerts an influence on the textural and rheological properties of yoghurt (Dannenberg & Kessler, 1988; Lucey et al., 1999; Mulvihill & Grufferty, 1995). The firmness and viscosity of yoghurt

gels were affected by the degree of protein denaturation during heat treatment. With heat treatment the gelation time was decreased and increasing pH affected the gelation point. In addition, the inoculation and gelation temperatures were also important for the formation of a gel. The optimum incubation temperature for yoghurt is between 40 to 45°C and the gel is cooled to below 10°C once the pH of yoghurt is at or just below 4.6 (Lucey & Singh, 1997). However, high incubation temperature may cause poor gel formation and defects in the gel. It was suggested that gel firmness and viscosity can be improved by lowering the incubation temperature to 38°C (Kosikowski & Mistry, 1977). In acid-induced milk gelation, it was found that the gelation of skim milk occurred at the isoelectric point of casein (pH 4.6) (Kruif, 1996; Van Vliet & Keetels, 1995). The higher the pH the higher the temperature required for gelation (Kruif, 1996). The acidification of milk results in a reduction of pH, which leads to the destabilisation of casein micelles as the pH approaches the isoelectric point of casein. The steric stabilisation of the casein micelles declines when pH is being decreased (Phadungath, 2005).

2.3.4 Heat-induced gels

The formation of heat-induced gels is primarily caused by the denaturation of whey proteins, the type of gelation can be divided into two stages. In the first stage, the globular structure of whey proteins is partially unfolded, and this denaturation process involves dissociation of intermolecular bonds such as non-covalent and disulphide bonds. In the second stage, new disulphide bonds between protein molecules are formed and this leads to intermolecular aggregation (Fitzsimons et al., 2007). As mentioned earlier, β-lactoglobulin can also interact with κ-casein and forms a coating on the exterior of the casein micelles. In addition, the β-lactoglobulin with cysteine-containing whey proteins will form whey-casein complex aggregates (Dalgleish et al., 1997; Pesic et al., 2012). The formation of heat-induced gels is influenced by the pH, temperature of heat treatment, the concentration of milk salts and presence of enzymes (Langton & Hermansson, 1992; Wang et al., 2012). It was reported that the serum protein aggregates vary with the intensity of heat treatment, and acid gels which were processed under heat treatments of 80 °C and 90 °C had better textural quality (Wang et al., 2012).

Cold-set gelation is an alternative to heat-induce gelation, which suits the products with low thermal stability (Bolder et al., 2006). Cold-set gels are a type of gel formed after being heat treated and cooled. The protein denaturation and aggregation were firstly

prepared by heating a solution of native proteins at a pH above the isoelectric point of the protein and in the absence of salt (Alting et al., 2003). In the heating step, the proteins were denatured and form aggregates. The gelation then occurs during the cooling process and intact gels are formed after cooling (Alting et al., 2003). It was reported that cold-setting gels were formed from 100g kg^{-1} whey protein isolate solution without addition of salt at pH 7 after being heated to 90 ^{o}C for 30 min and cooled to 20 ^{o}C (McClements & Keogh, 1995). In addition, it was found that the rate of gelation increased upon cooling with additional 0.2 mol kg^{-1} NaCl and the rigidity of the gels reduced with decreasing temperature (McClements & Keogh, 1995). In another study, it was reported that cold-set gels were formed with the addition of calcium salt and adjustment of pH (Bolder et al., 2006). The protein aggregates with long linear fibrils were formed from heating a β-Lactoglobulin solution at pH 2. The solution was then cooled to 20 ^{o}C with adjusted pH at 7. Cold-set gels were formed by adding calcium chloride at pH 7 and at 20 ^{o}C. The cross-linking of the fibrils was induced by the addition of calcium chloride (Bolder et al., 2006).

2.3.5 Mineral-induced gels

2.3.5.1 Mechanism of calcium-induced milk coagulation

There are many minerals which are present in milk. Calcium, phosphorus, potassium, citrate and chloride are the principal minerals in milk, and they exist in the form of salts in milk. Minor minerals such as selenium and zinc are low in concentration in milk (Gaucheron, 2005). There is approximately 30mM calcium in milk and the calcium is distributed in the ionic, soluble and colloidal phases. The ionic calcium at natural pH of milk makes up about 10% of total calcium (Lewis, 2011). The addition of soluble calcium salts in milk increases the amount of calcium in the three phases. The addition of ionic calcium not only increases the concentration of calcium in milk but also results in a reduction in pH of milk. The mechanism can be expressed by Equation 2.2, when more calcium ions are added the equilibrium favours the right hand side of the equation and more hydrogen ions are released (Lin et al., 2006; Omoarukhe et al., 2010).

$$3Ca^{2+} + 2HPO_4^- \leftrightarrow Ca_3PO_4 + 2H^+ \quad (2.2)$$

Hence, the concentration of hydrogen ions increases, and the pH of milk is reduced with excess calcium ions. Milk proteins coagulate and precipitation is observed when the isoelectric point (IP) of the proteins is reached. The heat stability of calcium-induced milk

coagulation is strongly associated with the calcium ion concentration and the acidity of the milk (Jeurnink & De Kruif, 1995). In addition, heat treatment, pH reduction and addition of calcium salts can alter the distribution of calcium in milk, thereby affecting the stability of milk proteins (Tsioulpas et al., 2007). Ramasubramanian et al. (2012) reported that the effect of temperature, concentration of calcium ions and pH on the coagulation of milk proteins have not been extensively investigated due to inconsistent milk types, heat coagulation methods in determining heat coagulation time. Koutina, Knudsen, et al. (2015) studied the effect of pH on calcium and phosphorus distribution in calcium-enriched skim milk. It was found that the added calcium from calcium lactobionate distributed between the micellar and serum phase between pH 6.0 and 6.6, whilst it existed primarily in the serum phase at pH 5.4-5.7 (Koutina, Knudsen, et al., 2015). The concentration of phosphorus was increased in the micellar phase and decreased in the serum phase at pH 6.0-6.6. In addition, it was reported that the total protein content and caseins in milk serum phase decreased after the addition of calcium in milk (Koutina, Knudsen, et al., 2015).

2.3.5.2 Studies on calcium-induced milk gels

The effects of calcium salts on the formation of milk gels have been extensively studied (Lin et al., 2018; Ramasubramanian et al., 2014; Siamand et al., 2014). The addition of calcium has an impact on rheological, textural and sensory properties of milk gels. It was reported that the strength and water holding capacity of the milk gel increased with addition of calcium. The storage modulus (*G'*) increased with the addition of calcium salts which provided a higher calcium activity (Lin et al., 2018). Additionally, sensory studies showed that the milk gels were acceptable with addition of calcium concentrations from 7 to 13.5 mM (Siamand et al., 2014). A number of studies have investigated the properties of milk gels with the additional of calcium (Table 2.4). It was found that different calcium salts exhibited various calcium activity and pH changes in milk. Furthermore, pre-heat treatment had a significant influence on the formation and strength of calcium-induced milk gels. Ramasubramanian et al. (2014) reported that strong milk gels were obtained after undergoing the pre-heating at 70 ^{o}C for 60 min followed by cooling to 20 ^{o}C, with 20 mM calcium chloride added. It was found that the combination of heat treatment and addition of calcium could provide a fine and dense milk gel at pH higher than 4.6 but lower than 5.6 (Koutina, Christensen, et al., 2015). With the addition of calcium, the gelation time and gelation temperature became independent of pH except

for pH 4.6 (Koutina, Christensen, et al., 2015). Both pH and the addition of calcium chloride influenced the formation of milk gels (Koutina, 2016; Koutina, Christensen, et al., 2015).

Ramasubramanian et al. (2012) investigated the coagulation of whole milk caused by the addition of calcium chloride and heat treatment. It was found that the pre-heat treatment at 90 ^{o}C for 10 min could make milk proteins more sensitive to coagulation and significant coagulation was found when 50 mM added calcium chloride was added to the whole milk solution and heated at 70 ^{o}C for 5 min. Furthermore, Hongsprabhas and Barbut (1996) investigated the effects of pre-heat treatment on calcium-induced whey protein and they reported that the water holding capacities were affected by the addition of calcium chloride when pre-heating was over 80 ^{o}C. The additional of calcium chloride had an influence on gel strength and high pre-heating temperature resulted in high water capacity and gel strength (Hongsprabhas & Barbut, 1996). It was reported the addition of soluble calcium salts such as calcium chloride, calcium lactate and calcium gluconate reduced milk pH, increased ionic calcium and led to the milk coagulation (Omoarukhe et al., 2010; Ramasubramanian et al., 2012). The addition of calcium chloride had the largest destabilising effect on milk, followed by calcium lactate and calcium gluconate (Omoarukhe et al., 2010). Moreover, Lin et al. (2018) reported that the calcium activity was increased in skim milk with the addition of calcium salts and the sample with added calcium chloride was the highest, followed by calcium lactate, calcium gluconate and calcium lactobionate.

Table 2.3 Summary of previous studies on the calcium-induced milk gels.

Type of calcium salt	**Concentration of calcium salt (mM)**	**Type of milk used**	**Treatment**	**Mechanism and properties of gels with change in pH**	**References**
Calcium chloride	10, 12.5, 15, 17.5, 20	Whole milk with 3.2% protein and 3.5% fat	Pre-heat treatment of milk at 90 °C for 10 min and then cooled to 20±2 °C. Calcium-added milk was heated from 20 to 70 °C and held at 70 °C for 60 min.	pH decreased with increasing concentration of calcium chloride from 6.57 to 5.95.	(Ramasubramanian et al., 2014)
Calcium chloride hydrate	30	Skim milk powder with 36% milk protein, 1.25% fat and 8% minerals.	Pre-heat treatment of milk at 90 °C for 10 min and then stored overnight at 22 °C.	The gelation occurs at a faster rate from 13±0.1min to 5.5±0.1 min when pH is lower. The pH was from 6.6 to 4.6.	(Koutina et al., 2016)
Calcium chloride	7, 10, 13.5, 17, 20	Skim milk	Skim milk was pre-heated at 85 °C for 20 min and cooled to 22 °C. Calcium-fortified milk was heated to 85 °C for 20 min to produce a gel.	pH was not measured. Sensory evaluation was conducted, and additional 13.5 mM calcium chloride provided the most palatable milk gel.	(Siamand et al., 2014)
Calcium chloride, calcium lactate, calcium gluconate, calcium lactobionate	5, 10, 12.5, 15, 20	Skim milk powder with 32.9% protein, 0.9% fat and 7.9% minerals	Skim milk was pre-heated from 20 to 90 °C in 5 min and held at 90±2 °C for 10 min, then cooled to 20±2 °C.	The addition of different calcium salts resulted in change in ionic activity and pH. At the same concentration of calcium salt, higher calcium ionic activity resulted in gels with higher G'.	(Lin et al., 2018)
Calcium chloride	20, 30, 40, 50, 100, 200	Whole milk with 3.2% protein and 3.4% fat	Pre-heat treatment at 90 °C for 10 min and cooled to 22 °C. The milk with the addition of calcium was heated to 70 °C and held for 5 min to produce a milk gel.	The addition of ionic calcium over 20 mM led to the milk coagulation at normal pH of milk. Combining with heat treatment at 70 °C with the addition of calcium resulted in a calcium-induced milk gel.	(Ramasubramanian et al., 2012)

2.4 Polysaccharides

Polysaccharides, gums and thickeners are known as food hydrocolloids. They play an important role in food formulations and they have been extensively used as stabilisers, emulsifiers, thickening and gelling agents (Stephen et al., 2006; Williams & Phillips, 2009). The use of polysaccharides in food products can improve the shelf life and quality attributes of food products. A myriad of polysaccharides that have been discovered, isolated, purified and added to food systems, to achieve the desirable textural properties and taste in various products (Saha & Bhattacharya, 2010; Stephen et al., 2006). The behaviours and properties of polysaccharides have been widely studied and Table 2.5 summarises the characteristics and rheological properties of the common polysaccharides used in food applications.

2.4.1 Non-ionic Polysaccharides

Guar gum and locust bean gum are non-ionic polysaccharides which have weak interaction with ions present in a solution. Guar gum is produced from the seeds of a plant *Cyamopsis tetragonoloba* and it has been widely used in food products such as breads, yoghurts, cake, sausages and ice creams (Mudgil et al., 2014). Guar gum used as a stabiliser in dairy product due to its water binding properties. For instance, guar gum has been used in ice cream to prevent ice crystal growth and for textural improvement (Phillips & Williams, 2009). The properties of guar gum favours high temperature short time processes as guar gum can hydrate fully in a short period of time (Phillips & Williams, 2009). The concentration of guar gum used in food products varies from 0.1 to 2 wt % depending on the desirable rheological and textural properties (Mudgil et al., 2014).

Locust bean gum is derived from the seeds of the locust bean tree *Ceratonia siliqua*. Locust bean gum is an efficient thickener and it can achieve high viscosity in products at low concentration (Barak & Mudgil, 2014). Locust bean gum is often mixed with other hydrocolloids to form a gel. The mixture of xanthan gum and locust bean gum produces an elastic and thermos-reversible gel. In addition, its interaction with κ-carrageenan can improve the strength of a gel and form an elastic gel (Barak & Mudgil, 2014). Locust bean gum is used as a thickener and stabiliser in food products, however it often requires the addition of other hydrocolloids to provide a desirable texture and stability in a product (Phillips & Williams, 2009).

Starch consists of amylose and amylopectin. Starch undergoes gelatinisation when starch granules are heated in excess water, which leads to the break-up of the amylopectin double helical structure and the swelling of granules (Jenkins & Donald, 1998). The amylose eventually leaches into the surrounding water. The gelatinisation temperature of starch varies depending on the source of the starch, pH, concentration of salts, sugar, fat, protein, and the amount of water in a system. In native starches, the gelatinisation temperature ranges from 60 to 80 ^{o}C (Belitz et al., 2009). Retrogradation occurs when the gelatinised starch is cooled for a long period of time, after which the molecules in starch re-form a crystalline structure and aggregate to form a gel (Wang et al., 2015). The highly viscous swollen starch granules often exhibit clear appearance and they can be mixed rapidly and efficiently with water. The use of starch has been applied in dairy products such as yoghurt and thickened milk. It was suggested that 1-3% starch can provide pouring consistencies in product and 4-6% starch gives a thick texture to a product (Phillips & Williams, 2009).

Carboxymethyl cellulose (CMC) is a non-ionic polymer and it can form complexes with proteins. It reacts with proteins in milk to form complexes below pH 3 or above pH 6. Stable complexes are formed when pH is between 3.0 and 5.5 (Phillips & Williams, 2009). The CMC and casein complexes are also heat stable and the casein is denatured to a much smaller extent with the presence of CMC (Phillips & Williams, 2009). CMC is used as a stabiliser to prevent the flocculation of milk protein in acidified milk (Du et al., 2007). It was found that steric forces were the driving force for stabilisation of acidified milk drinks, which was caused by the anchor of CMC onto the surface of casein micelles (Du et al., 2007). It was suggested that electro-sorption may be the driving force for adsorption of CMC on the casein micelles when the pH is below 5.2 (Du et al., 2007).

2.4.2 Ionic Polysaccharides

Gum arabic, pectin, gellan gum, carrageenan, xanthan gum, starch and modified starch are ionic polysaccharides and they are likely to interact with ions in a food matrix. Gum arabic is derived from the branches of *Acacia senegal* tree and contains calcium, magnesium and potassium salts. The colour of solution with the addition of gum arabic ranges from pale yellow to orange-brown with the pH up to 4.5 (Phillips & Williams, 2009). Higher concentrations of gum arabic are required to achieve a high viscosity compared to xanthan gum. It was suggested that 30% gum arabic solutions still provided

a lower viscosity than 1% xanthan gum at low shear rates (Phillips & Williams, 2009). However, gum arabic is widely used as an emulsifier for oils and flavours in confectionary and beverages. It is able to inhibit flocculation and coalescence of the oil droplets in a food system (Phillips & Williams, 2009).

Pectin is present in fruits in variable amounts and the major sources of pectin is extracted from citrus peel, a by-product of citrus juice and oil (Phillips & Williams, 2009). Pectin is widely used in the production of jams and jellies. Pectin is an ionic hydrocolloid, used as a gelling agent. It is sensitive to pH change and the presence of cations in the system (Thakur et al., 1997). It was reported high methyl ester pectin forms a gel at pH 3.4 and the gel strength and setting temperature increases when pH decreases (Phillips & Williams, 2009). On the other hand, the gelation of low methyl ester pectin is mainly driven by the interaction between calcium ions and pectin. The amount of calcium ions present in the system is important, however the amount of soluble solids, pH and the level of sequestrant can also affect the gelation mechanism of pectin (Phillips & Williams, 2009).

Gellan gum is secreted by the microorganism *Sphingomonas elodea* and the gelation of gellan gum is promoted by the cooling of a hot solution and the presence of cations in the system (Moritaka et al., 1995; Phillips & Williams, 2009). It can form either soft, elastic gels or hard, brittle gels at low concentration depending on the degree of substitution. There are two types of gellan gum low acyl (LA) gellan gum and high acyl (HA) gellan gum. Table 2.6 summarises the properties of two gellan gums. Gellan gum has been used in dairy products such as milk beverages and yoghurt. HA gellan gum at low concentration can produce a thickened milk fluid with weak gel network in the system. It was suggested 0.1 – 0.12% HA gellan gum can provide a long-term suspension of cocoa in chocolate milk (Phillips & Williams, 2009). In yoghurt products, LA gellan gum is widely used with concentration of 0.04% and the setting temperature of LA gellan gum in skim milk is approximately 41^{o}C (Phillips & Williams, 2009).

Table 2.4 Characteristics and rheological properties of the common polysaccharides used in food products.

Polysaccharides	Characteristics	Rheological properties	References
Guar gum	• It is neutral and water soluble. Dissolution and viscosity development increase with decreasing particle size, pH and increasing temperature. • Temperature significantly affects hydration rate and viscosity. Temperature from 25 to 40 °C is desirable for maximum viscosities.	• The loss modulus G'' dominates over storage modulus G' at lower frequency range for 1% guar gum in aqueous solution.	Mudgil et al. (2014)
Locust bean gum (LBG)	• A neutral polysaccharide and partially soluble in water. • Less viscous than guar gum and tara gum. • Heating above 80 °C achieves good dissolution of LBG.	• 0.5–2.0% LBG in aqueous solution shows loss modulus (G'') dominates over storage modulus (G') at lower frequency range. • Ability to hydrate in hot water. The viscosity of LBG increases with increasing concentration.	Barak and Mudgil (2014)
Starch	• Consists of two non-ionic polysaccharides amylose and amylopectin. The ratio of two polysaccharides in starch is important for gelatinisation, gelation and retrogradation. The gelatinisation temperature varies from 60 to 80 °C depending on the type of starch.	• The use of starch in a system can achieve high viscosity. • Different types of starch exhibit various rheological properties.	(Phillips & Williams, 2009)
Gum Arabic	• Negatively charged. • Readily dissolves in water and gives a pale yellow to orange-brown colour in a solution with a pH of 4.5.	• Exhibits Newtonian behaviour and its viscosity is shear rate independent.	Phillips and Williams (2009)
Pectin	• Ionic hydrocolloid and is sensitive to pH change. The cations present in solvent affect the gelation. High methyl ester pectin forms gel at up to pH 3.4.	• The interaction between low methyl ester pectin and calcium ions can significantly affect the gelation of the pectin.	Phillips and Williams (2009)
Xanthan gum	• Highly negative charged and stable to acids, alkalis and enzymes. • Temperature insensitivity and salt compatibility. Thermal transition occurs above 90 °C.	• High viscosity at low shear rates (suspension stabilising properties). • High viscosity at low concentrations and high elastic modulus. Viscosity is sensitive to change when pH is below 4.	(Butler, 2016); Phillips and Williams (2009)
Gellan gum	• Negatively charged • Low acyl (LA) gellan gum requires gelling ions to form a gel and setting temperature is from 25 to 60 °C. Transparent appearance, and firm and brittle texture. Thermal stability can be improved by the addition of calcium. • High acyl (HA) gellan gum requires a higher setting temperature 70 - 80 °C. Forms an opaque gel and soft and elastic texture.	• LA gellan gum provides a low viscosity. • HA gellan gum generates a high viscous gel. • Gellan gum exhibits excellent gelling properties when pH is at 6 – 10.	(Moritaka et al., 1995; Phillips & Williams, 2009)
Carrageenan	• Negatively charged and soluble in cold water, only kappa and iota carrageenan are soluble in cold water. • All carrageenans hydrate at high temperature and kappa and iota carrageenans exhibit a low fluid viscosity. Carrageenans form a range of gels on cooling between 40 and 70 °C. They lose viscosity and gel strength under heating with pH below 4.3.	• Lambda carrageenan develops viscosity in milk and viscosity increases under heating and cooling. • It has shear-thinning property and is widely used in dairy products to give creamy texture.	(Pereira, 2016); Phillips and Williams (2009)
Carboxymethyl cellulose	• Hydroxypropyl cellulose (HPC) is soluble in cold water but becomes insoluble when temperature is above 45 °C. • Methylethyl cellulose (MC) is soluble in cold water and forms weak gels on heating. • Carboxymethyl cellulose (CMC) is negatively charged and soluble in cold and hot water.	• Viscosity reduction is reversible on heating. • The CMC and casein complex is sensitive to shear force and viscosity decreases under agitation. • The degree of substitution between 0.6 to 0.95 tends to exhibit pseudoplasticity.	Phillips and Williams (2009)

Table 2.5 The characteristics of LA gellan gum and HA gellan gum (Phillips & Williams, 2009).

Characteristics	**LA gellan gum**	**HA gellan gum**
Hydration	Temperatures above 80 °C	Temperatures above 70 °C
Viscosity	Forms low viscous solution	Forms high viscous solution
Gelling ions	Required presence of ions	Ions not required
Setting and melting temperature	Gel forms at 25 – 60 °C Only thermal reversible in milk or low ionic strength solutions when melting temperature is above 100°C	Setting temperature from 70 to 80°C. The gel is thermal reversible.
Clarity	Clear, transparent	Opaque
Texture	Firm, brittle	Soft, elastic

Carrageenan is derived from the red algae plant *Rhodophyceae* and it is a high molecular weight linear polysaccharide (Phillips & Williams, 2009). There are three main types of carrageenan, kappa, lambda and iota. They can be extracted and purified using selective extraction techniques (Bemiller, 2018; Phillips & Williams, 2009). Carrageenan is used as a thickening and gelling agent in food products, however the properties of the three types of carrageenan are different. Iota carrageenan forms a soft and elastic gel with the addition of calcium ion, however lambda carrageenan does not interact with salts in the system (Phillips & Williams, 2009). It was found that all carrageenans are soluble in hot water and lambda carrageenan provides high viscosity if the solution is heated and cooled. Temperature and pH are important for the formation of gel with carrageenans and the gelation occurs when carrageenans are cooled from 70 °C and 40 °C. The viscosity and gel strength in carrageenan solutions decrease when the pH is below 4.3. Decreasing the pH increases the electrostatic interactions, which leads to the formation of aggregates (Phillips & Williams, 2009). The use of carrageenan provides a creamy and firm gel on cooling in dairy products such as milk and milk-based puddings. However, carrageenan is not a suitable stabiliser used in cheese and yoghurt as it is not compatible in dairy systems (Phillips & Williams, 2009).

Xanthan gum is produced by the microorganism *Xanthomonas capestris*. It exhibits an excellent stability to pH, temperature changes as well as enzymatic reactions (Phillips & Williams, 2009). Xanthan solutions lose their viscosity when the pH is below 4, however the viscosity can be recovered once the solution is neutralised. In addition, the thermal transition temperature of xanthan gum is above 90 °C, at which the viscosity decreases

rapidly (Phillips & Williams, 2009). As xanthan gum possesses good stability to pH and temperature changes, it has been extensively used in various food products such as sauces, dressings, baked and dairy products (Phillips & Williams, 2009; Sutherland, 2001). It was suggested that the addition of 0.2-0.4% xanthan gum can stabilise sauces and dressings depending on the oil content in products (Phillips & Williams, 2009).

2.4.3 Protein-Polysaccharide Interactions

Polysaccharides and proteins play an important role in the formation of structure and texture in a food product. The interaction between polysaccharides and proteins can have significant impacts on the properties of the final products, thus the concentration of polysaccharides used in a food matrix have to be considered (Corredig et al., 2011). The interaction between polysaccharide and proteins can be beneficial, at which the overall stability of a food system is improved. It also can result in macroscopic destabilisation. Their interactions are dependent on the type and concentration of the polysaccharide used in a food system as well as conditions such as temperature, pH and ionic strength (Doublier et al., 2000). A number of studies suggested that the interaction could improve colloidal stability, promote the formation of bridges or aggregation, or exert thermodynamic incompatibility (Doublier et al., 2000; Grinberg & Tolstoguzov, 1997; Turgeon et al., 2007).

The interaction between polysaccharides and proteins can lead to separation or association (Doublier et al., 2000). Separation is caused by the addition of non-interacting polysaccharides. This usually leads to depletion flocculation of the casein micelles, which is caused by the incompatibility between the polysaccharides and proteins. With the influence of an osmotic pressure gradient, a depleted layer around the casein micelles forms and this causes the casein micelles to attract to one another (Tuinier et al., 2003). Electrostatic attraction is the main driving force of associative interaction, at which oppositely charged parts of polysaccharides and proteins attract to each other. In addition, hydrophobic interactions and hydrogen bridging also affect the stabilisation of protein-polysaccharide complexes.

The interaction between κ-carrageenan and casein micelles occurs at a specific positively charged patch of κ-casein between residue 97 and 112 (Snoeren et al., 1976). This associative interaction prevents phase separation and destabilisation of mixtures which

contains non-interacting polysaccharides (Thaiudom & Goff, 2003). Pectin is another common polysaccharide used in acid milk beverages. It was found that pectin with high degree of esterification more effectively stabilised acidified milk at pH greater than 4, compared to pectins with a low degree of esterification (Liu et al., 2006). The adsorption of pectin molecules onto the casein micelles contributes to the stabilisation of acid caseins as well as the formation of a gel (Tromp et al., 2004). Furthermore, the addition of starch or modified starch in milk can increase the strength of the continuous network of casein formed. It was reported that the addition of 2.5% modified waxy maize starch resulted in the gelation at higher pH and a shorter time of gelation (Azim et al., 2010). The concentration of polysaccharides used in dairy systems varies depending on different products and appropriate concentration can effectively prevent phase separation and stabilise the proteins in the dairy matrix as well as providing desirable textural properties.

2.5 Characterisation of Thickened Fluids and Gels

The characteristics of thickened fluids and gels can be studied from their rheological properties, sensory characteristics and microscopic structures. A study of the rheological characterisation of thickened fluids and gels can provide fundamentally rheological parameters and measurements to develop a safe and appropriate thickened beverage. Microscopic studies can observe the structural changes in thickened fluids or gels. In addition, sensory evaluation assists to determine whether the thickened fluids or gels are palatable and acceptable for end consumers.

2.5.1 Rheological Characterisation

By definition, rheology is the study of fluid deformation and flow of matter (Macosko & Larson, 1994). The science of rheology plays an important role in understanding the characteristics of materials and is beneficial for understanding industrial applications (Rao & Lopes da Silva, 2007).

Many studies have been conducted to investigate the rheological properties and behaviours of thickened fluids and many flow models have been developed to describe rheological data. The fluid flow behaviour can be described by means of shear rate versus shear stress, which can be categorised into Newtonian and non-Newtonian fluids (Figure 2.5). Non-Newtonian fluids can either have shear-thickening or shear-thinning properties, in which the viscosity changes at different shear rates. Newtonian fluids have constant viscosity at all shear rates, however thickened fluids and beverages are non-Newtonian

and they exhibit shear-thinning properties (Cho et al., 2012; Cichero et al., 2000; Garcia et al., 2005). Apparent viscosity is a useful rheological parameter in characterising fluid foods. Apparent viscosity (η) is defined by Equation 2.3 where τ is shear stress and γ is shear rate(Rao & Lopes da Silva, 2007).

$$\eta = \frac{\tau}{\gamma} \tag{2.3}$$

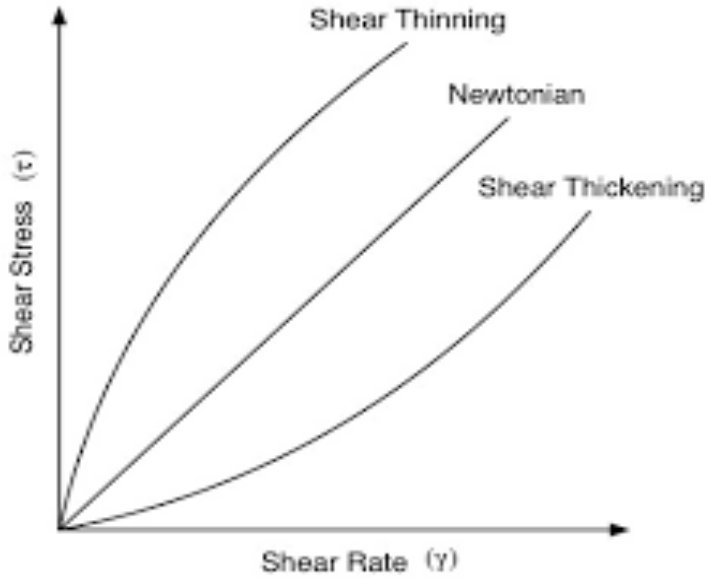

Figure 2.5 Schematic diagram of Newtonian, shear-thickening and shear-thinning fluids (Neutrium, 2012).

The power law model has been used by many researchers and it describes the characteristics of shear-thinning and shear-thickening fluids. Equation 2.4 describes the power law model, where K is the consistency coefficient, γ is shear rate, τ is shear stress and n is the flow behaviour index (Rao & Lopes da Silva, 2007). The power law model shows how the viscosity changes with various shear rates. The value n determines the properties of fluids. For $n \leq 1$ the fluid is shear-thinning, and for $n \geq 1$ the fluid is shear-thickening and Newtonian fluids have n=1 (Macosko & Larson, 1994).

$$\tau = K\gamma^n \tag{2.4}$$

Dynamic rheological experiments are used to determine the viscoelastic characteristics of fluid foods (Rao & Lopes da Silva, 2007). In dynamic rheological experiments, a food sample is subjected to sinusoidal deformation and the shear stress is measured. The viscoelastic properties of a material can be described by a complex modulus (G^*), at

which is calculated from a storage modulus (*G'*) and a loss modulus (*G''*) (Equation 2.5) (Rao & Lopes da Silva, 2007). It is suggested that the material behaves like a solid and the deformation is elastic and recoverable if *G'* is much greater than *G''*. The behaviour of the material is liquid like and the deformation is dissipated and viscous if *G'* is much smaller than *G''* (Rao & Lopes da Silva, 2007). Many studies suggested that the crossover point of *G'* and *G''* may be the gel point for a material at a given frequency (Djabourov et al., 1988; Stading & Hermansson, 1990, 1993). This can be an important parameter to observe the structural change in a material. In the mechanism of gelation, a polymer undergoes a sol-gel transition where a liquid becomes a gel. Based on gelation mechanism, there are cold-setting and heat-setting gel forming biopolymers. The cold-setting gelation is induced by cooling where biopolymer gels occur spontaneously and the heat-setting gelation involves denaturation of biopolymers (Rao & Lopes da Silva, 2007). Time and temperature are critical to determine a gel point. The relaxation time increases rapidly near the gel point and it diverges to infinity at the gel point (Rao & Lopes da Silva, 2007).

$$|G^*| = \sqrt{(G')^2 + (G'')^2} \tag{2.5}$$

However, it is important to determine the linear range of deformation of a material before measuring the viscoelastic properties of a material, as it is the range where *G'* and *G''* are independent of strain. In the linear viscoelastic region, the structure of a material is not disturbed, and a strain can be selected within the range to perform the test (Macosko & Larson, 1994; Rao & Lopes da Silva, 2007). The complex viscosity (η^*) of a fluid can be determined using Equation 2.6, where ω is the frequency of oscillation (Rao & Lopes da Silva, 2007).

$$\eta^* = \frac{G^*}{\omega} \tag{2.6}$$

The Cox-Merz rule is an empirical relationship which describes the dependence of the steady shear viscosity on the shear rate and frequency dependence of complex viscosity of polymer melts can be estimated from the dynamic viscosity complex viscosity as a function of frequency (Cox & Merz, 1958; Winter, 2009). The Cox-Merz rule is shown in Equation 2.7, where η^* is the complex viscosity, η is the dynamic viscosity, ω is the frequency of oscillation and $\dot{\gamma}$ is the shear rate (Cox & Merz, 1958). The Cox-Merz rule

is a measure of the degree of structure in fluid which breaks down under amplitude shear. This rule is often valid if the structure of the fluid does not change with deformation, but it may not suit for solid materials (Cox & Merz, 1958). The Cox-Merz rule is obeyed if apparent viscosity of the fluid is equal to the complex viscosity. (Cox & Merz, 1958). A number of studies have used Cox-Merz rule to investigate the rheological properties of different fluid materials (Mackley et al., 2013; Macosko & Larson, 1994). Mackley et al. (2013) reported the Cox-Merz rule is only obeyed for thickened solutions with added starch and yet it does not suit for xanthan gum solution.

$$|\eta^*(\omega)| = \eta(\dot{\gamma}) \tag{2.7}$$

2.5.2 Rheological Tests

Once the linear viscoelastic region is established, three types of dynamic rheological tests in oscillatory mode can be conducted to obtain useful characteristics of fluids (Rao & Lopes da Silva, 2007).

A frequency sweep test determines *G'* and *G''* values as a function of frequency (ω) at a fixed temperature over time. Also, the change of shear stress at different shear rates can be determined from this test.

A temperature sweep test studies the *G'* and *G''* values are a function of temperature at fixed frequency ω. This test is suitable for studying the gel formation of proteins and gel formation during cooling of a heated dispersion (Owen et al., 1992; Rao & Cooley, 1993; Rao & Lopes da Silva, 2007).

A time sweep test determines the *G'* and *G''* as a function of time at fixed frequency and temperature. This test provides information on how the macro or micro-structure of a material changes and it is well suited for studying the structural development of physical gels (Mazzeo, 2008). Apparent viscosity is an important parameter in studying the properties of shear-thinning fluids. The apparent viscosity and shear stress can be determined by using steady shear sweep tests.

2.6 Microscopic Techniques

2.6.1 Scanning Electron Microscope

A scanning electron microscope (SEM) is an electron microscope that can scan the surface with a focused beam of electrons and produce images of a sample (Reimer, 2013). The information on surface topography, crystalline structure, chemical composition and electrical behaviour of the top 1μm of a specimen can be obtained using SEM (Vernon-Parry, 2000). The interaction of the electron beam with at various depths in a sample generates various signals which include secondary electrons (SE), backscattered electrons (BSE), electron beam induced current (EBIC), characteristic X-rays, transmitted electrons and cathodoluminescence (CL) (Vernon-Parry, 2000). SEM has been used in characterising the microstructure and structural change in foods, particularly in dry food products and food ingredients. The images produced by SEM can provide information on the degree of association of proteins and a polysaccharide, which relates to the textural properties of foods (Webb & Holgate, 2003).

2.6.2 Confocal Laser Scanning Microscope

Confocal laser scanning microscope (CLSM) is an important tool to provide insights of the microstructure of a food material (Loren et al., 2007). It is used to observe the structural changes of food products under the microscope under dynamic conditions and it enables the reconstruction of three-dimensional structures in a material. Figure 2.6 shows the main components of a CLSM. The images are obtained from an optical section in a CLSM (Loren et al., 2007). The laser beam penetrates into the bulk of samples and the sample structure can be determined at different depths. Three-dimensional microstructures can be constructed by adding stacks of images from adjacent focal planes. Figure 2.6 illustrates the main components in a CLSM. The laser delivers various wavelengths onto an objective and the detector detects the local spatial distribution of different food ingredients such as fat, proteins and polysaccharides simultaneously in a specimen (Pawley, 2010). A CLSM equipped with a temperature stage can be used to observe and analyse temperature-induced changes in the microstructure of complex foods (Loren et al., 2007). It was reported that CLSM was used in observing the proteins and fats in the microstructure of yoghurt and cheese (Cardona et al., 2013; Loren et al., 2007). Hence, the CLSM can be a useful tool to observe the microstructure of food products.

2.6.3 Particle Size Analysis

Particle size is an important parameter of powders, suspensions and emulsions. The particle size in solution affects the rheological and textural properties as well as overall stability in a food product (Barbosa-Cánovas et al., 2005; Muresan, 2018). Particle size analysis is used to determine the size range and mean size of the particles in a food powder or a liquid sample. There are various techniques that can be used to characterise particles in foods. Static light scattering (SLS) is technique that measures the intensity of the scattered light at various angles to calculate and obtain the average molecular weight of a polymer or a protein molecule in solution (Minton, 2007; Stacey, 1956). SLS has been used to measure the size of particle suspensions in food system such as mayonnaises and analyse protein-protein interactions and macromolecular interactions (Minton, 2007; Stacey, 1956). Dynamic light scattering (DLC) is also a technique which measures a range of particle size and it is a useful tool to determine particle aggregation and monitor colloidal stability in a food system (Stetefeld et al., 2016). In addition, it can be used in studying the homogeneity and interactions of proteins, nucleic acids and their complexes (Stetefeld et al., 2016). Laser diffraction spectroscopy (LDS) is another technique that utilises a laser beam passing through an object at different angles and measures the dimension of a particle ranging from nanometres to millimetres in size (Merkus, 2009). LDS is well suited for analysing the dispersed dry powders, suspension and emulsions.

2.7 Applications of Thickened Fluids and Gels in Dysphagic Patients

Swallowing difficulty is a growing health concern in aging population and it could potentially lead to the development of dysphagia which is an extreme disorder of swallowing. Dysphagia is a medical term for patients who have difficulty swallowing foods or liquids via the oesophagus (Groher, 1997). Dysphagia often causes painful blockage, coughing and choking when patients eat and drink (Adams & Smith, 2012). Consequently, dysphagia patients may suffer from dehydration, malnutrition and other related diseases, resulting from inadequate and insufficient intake of nutrients (Whelan, 2001). The majority of dysphagic patients are elderly people and appropriate treatments are important to reduce the risk of dysphagia associated illness (Adams & Smith, 2012).

Figure 2.6 A diagram of the main components in a CLSM (Loren et al., 2007).

2.8 Current Treatments and Problems

Many treatments have been developed to tackle dysphagia, which include swallowing therapy, dietary changes, feeding tubes and thickened fluids (Ekberg, 2019; O'Gara, 1990; Zhong et al., 2018). With clinical treatment, thickened fluids have become one of the recommended methods to provide nutrients for dysphagic patients (Mills, 2008). However, the thickness of fluids is a critical factor to provide proper coordination from oral, pharyngeal to oesophagus, allowing a safer swallowing for patients (Reimers-Neils et al., 1994). The RTD thickened fluids and pre-mixed powders are the two most common products that have been used for people who suffer from swallowing difficulty (Adams & Smith, 2012; Ekberg, 2019; Mills, 2008). Both of these products use thickeners and/or stabilisers to improve the texture and mouthfeel of the fluids. Xanthan gum, guar gum, gellan gum, pectin, modified starch have been used as thickeners in the products, and some products may contain a mixture of multiple thickeners (Hadde, 2017; Mills, 2008). However, lack of standards in the thickness level and definition of thickened fluids cause difficulties for dysphagia patients to appropriately consume thickened fluids. Inappropriate use of thickened fluid products may result in serious aspiration problems such pneumonia or pharyngeal stasis (Hadde, 2017; Hind et al., 2012). In addition, the

lack of development of affordable and economical measuring devices is a constraint for clinicians and dysphagia sufferers to properly use thickened fluids.

The current tools such as the Bostwick consistometer, the Line spread test (LST) and the International Dysphagia Diet Standardisation Initiative (IDDSI) flow test used for measuring the thickness of the fluids for foods to be given to patients with swallowing difficulty do not reliably measure the rheological properties of thickened fluids (Hadde, 2017). The Bostwick consistometer is a tool which measures the thickness consistency of fluids (Germain et al., 2006). This method allows fluid to flow using gravity once the gate is opened (Figure 2.7). The distance of the fluid moves down is recorded after 30 seconds and it is used to compare the flow rate of other samples. It has been used by several thickener manufactures to assess the flow ability of thickened fluids (Germain et al., 2006). The LST is used to evaluate the thickness of fluids and it consists of a large sheet of paper with 1 cm interval of concentric circles (Kim, 2007; Nicosia & Robbins, 2007). A cylindrical tube is often placed in the centre of the circles (Figure 2.8). A fluid is poured in the tube and then the cylindrical tube is lifted, the fluid spreads out along the quadrants. The measurement in centimetres along the four lines connecting zone A, B, C and D is recorded and averaged after 60 seconds (Figure 2.8). The IDDSI flow test is a tool which uses a syringe to measure the thickness of a sample (International Dysphagia Diet Standardisation Initiative, 2017). This method allows a 10 mL of sample to flow through a syringe and records the amount of the sample left in each zone after 10 seconds (Figure 2.9). This method has been widely recognised for measuring the thickness of thickened fluids for dysphagic patients (Hanson, 2016).

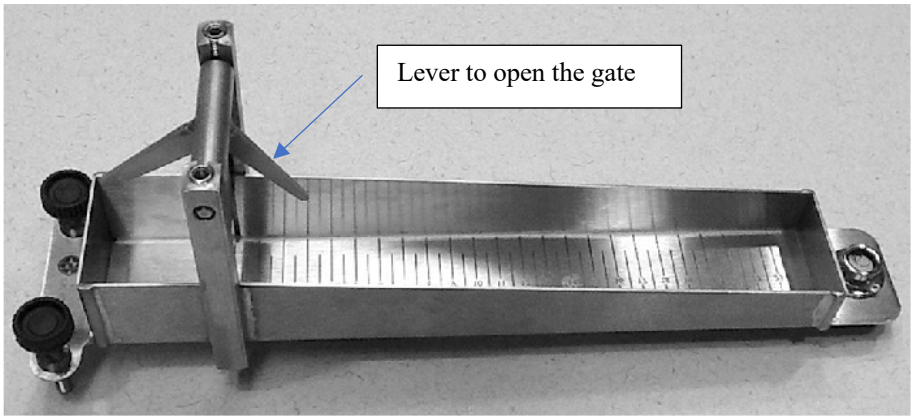

Figure 2.7 Bostwick consistometer (Steele et al., 2014).

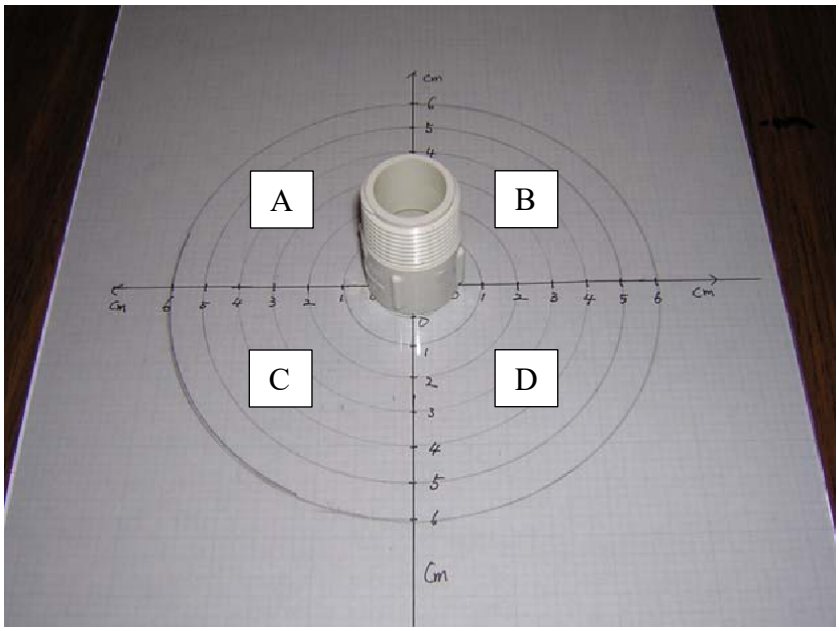

Figure 2.8 Line spread test (Kim, 2007).

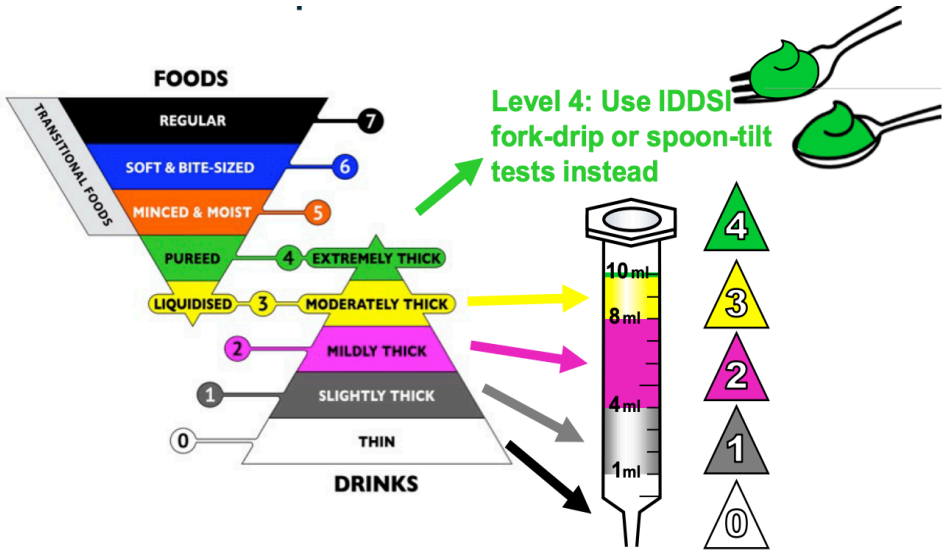

Figure 2.9 IDDSI flow test (International Dysphagia Diet Standardisation Initiative, 2017).

2.9 Conclusions

Casein and whey proteins as a component of milk are directly associated with in milk gelation. The formation of milk gels can be induced by pH, rennet, acid, minerals such as calcium salts and heat under different conditions. Protein denaturation and aggregation are the primary driving force of milk gelation. With different properties of polysaccharides, they can be functioned as thickeners, stabilisers and gelling agents. The addition of polysaccharides in milk systems could interact with milk proteins and mechanisms of such interaction would be investigated in this study. The techniques have been used to investigate the milk gelation include rheological characterisation, microstructural imaging (SEM and CLSM) and particle analysis. A number of researches

have studied the calcium-added milk gels and yet the effect of added calcium salts and polysaccharides to the milk gelation have not been explored. Therefore, this research will study the mechanisms of milk gelation with added polysaccharides.

Chapter 3 Materials and Methods

3.1 Materials

Low heat skim milk powder was acquired from Fonterra Co-operative Group (Auckland, New Zealand) and the specification of the skim milk powder can be seen in Appendix A1. Calcium L-lactate pentahydrate powder (≥ 98.0%, Acros Organics) was purchased from Thermo Fisher Scientific (Auckland, New Zealand). Three commercial polysaccharides were screen and selected. The Food Chemical Codex (FCC) grade xanthan gum with 80 mesh (NovaXan 80, 174910) was supplied by Archer Daniels Midland Company (Illinois, USA), food grade high acyl gellan gum (StabiL01, HWASTB101) was supplied by Hawkins Watts (Auckland, New Zealand). and FCC grade guar gum (Edicol 60-70) was supplied from Lucid Colloids Limited (Mumbai, India).

3.2 Skim milk solution and preheat treatment

A skim milk solution with 25 wt % total solids was made from the skim milk powder and reverse osmosis (RO) water. The skim milk solution was then mixed with a magnetic stir bar for 15 min and left for 10 – 12 hours at 20±2 ^{o}C to allow for full hydration. For pre-heat treatment of the skim milk, the hot water bath was set at 93.5 ^{o}C and the skim milk solution in a glass beaker was preheated to 90±2 ^{o}C in 300 mL batches and held at this temperature for 10 min. It took approximately 10 min for the skim milk to reach 90^{o}C. After the 10 min holding period, the skim milk solution was cooled to 20±2 ^{o}C within 10 min in a 4±2 ^{o}C water bath.

3.3 Calcium lactate solution

The concentration of a calcium lactate solution is dependent on its solubility in water and its concentration was calculated from its molar mass 308.29 g/mol. It was reported by (Vavrusova et al., 2014) that the solubility of calcium lactate is 5.8 g/100 ml and the concentration of its solution is 188 mmol/L(mM). An experiment was conducted to determine the actual solubility of calcium lactate pentahydrate at 20±2 ^{o}C. Four calcium lactate solutions were made in 50 mL in glass beakers according to their concentrations in Table 3.1 and each solution was mixed with a magnetic stir for 15 min, each solution was prepared in duplicate. The visual observation of the solutions was recorded.

Table 3.1 Visual observation and pH of four calcium lactate solutions.

Calcium lactate concentration (mM)	Mass of calcium lactate pentahydrate powder required (g)	Solubility observation
200	3.08	Pale white precipitation and powders are not dissolved
190	2.93	Small precipitation and powders are not fully dissolved
180	2.77	No precipitation and powders are fully dissolved
160	2.47	No precipitation and powders are fully dissolved

The maximum concentration of calcium lactate pentahydrate ensuring complete solubility in water was determined to be 180 mM. The solubility of calcium lactate in milk was expected to be the same as the solubility in water as no precipitates were shown when the same amount of calcium lactate added to the milk sample. The mass of calcium lactate pentahydrate was weighed to make a required volume of calcium lactate solution with the concentration of 180 mM. The solution was then mixed with a magnetic stir bar for at least 15 min until the original white cloudy solution became transparent. The 180 mM calcium lactate solution was prepared every 3 days and stored at 20±2 ºC.

3.4 Calcium-added skim milk solution

Skim milk solutions with at 25 wt % total solids were prepared and pre-heat. Previously prepared calcium lactate solution was added to the pre-heated skim milk to achieve 10 w/v % total solids concentration and the desired calcium addition in the calcium added skim milk solutions (Table 3.2). Each solution was made up to 100 mL and mixed well with a magnetic stir bar for 5 min. A flow diagram can be followed in Figure 3.1. The solution was prepared and used immediately after hydration and new batch was made each time before experiments. Note sodium azide was not was added to the samples as a sensory test was carried out on the mouthfeel of the milk samples.

Table 3.2 Required volume of skim milk, calcium lactate solution and distilled water to make 100mL final solution with 10 wt % total solids.

Concentration of added calcium lactate (mM)	Volume of 25 wt % skim milk (mL)	Volume of 180mM calcium lactate solution (mL)	Volume of distilled water (mL)
5	41.6	2.8	55.6
10	41.6	5.6	52.8
15	41.6	8.3	50.1
20	41.6	11.1	47.3

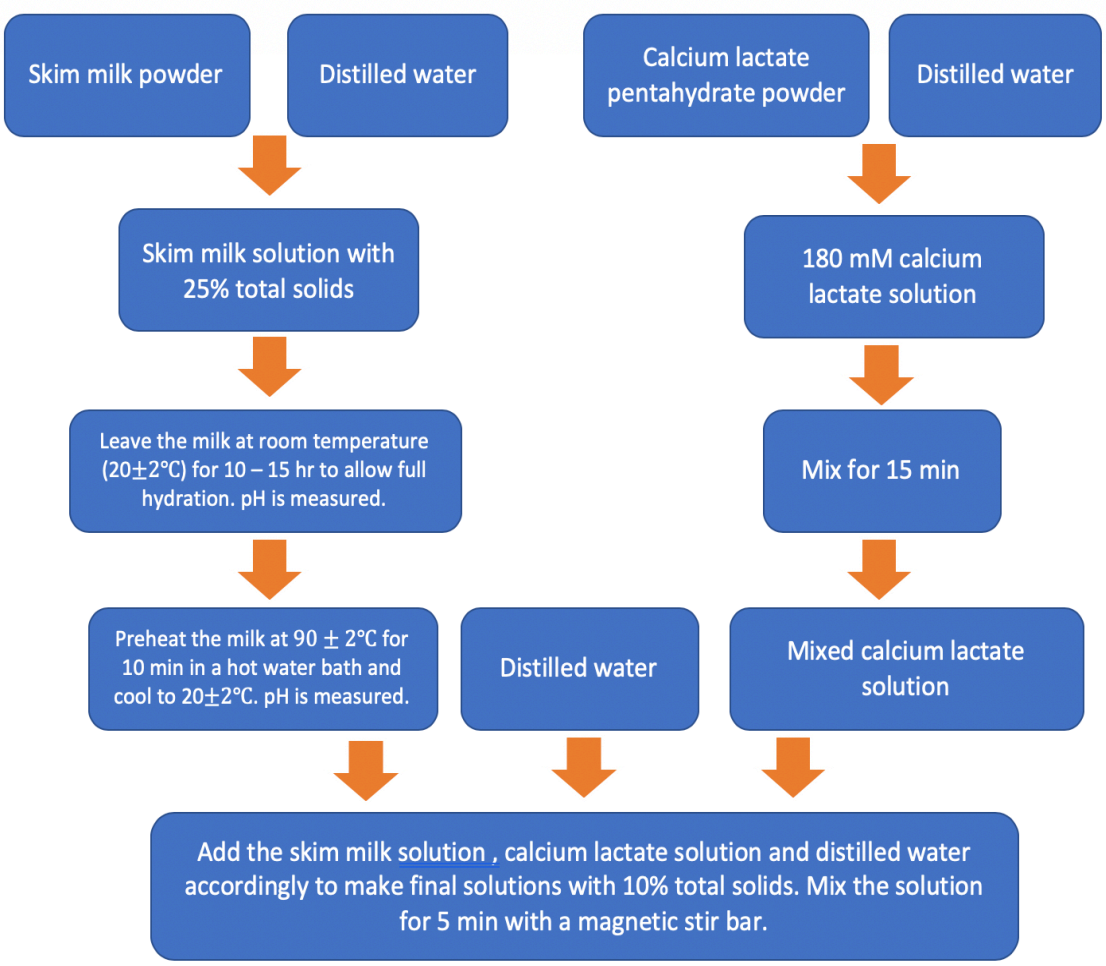

Figure 3.1 A flow diagram of the preparation of calcium-added skim milk solutions.

3.5 Calcium-added skim milk solution with stabilisers

The calcium-added skim milk solution was prepared prior to the addition of polysaccharides. The stabilisers were obtained in a powder form and various concentrations (wt %) of the three polysaccharides were added into calcium-added skim milk solutions. To ensure the polysaccharide was thoroughly mixed within the skim milk solution. The final skim milk solution was mixed in a Silverson L5M-A high shear mixer (Silverson Machines Ltd, Chesham, England) at 6000 rpm for 90 seconds once the polysaccharide was added. The calcium-added skim milk solution with polysaccharides were made prior to the rheological measurements and visual and microscopic observation of milk gelation. Time was an important factor in rheological characterisation, the time interval between the end of mixing the calcium-added skim milk solution and the start of the measurements was constant at 2 min. The samples were transferred into the sample

cup in the rheometer and tests were started after 2 min. This was also applied in the experiment for visual observations of milk gelation (from the end of mixing the skim milk solutions to the start of heating of the samples).

3.6 Experiment to determine the effect of heat treatment on calcium-added skim milk

3.6.1 Visual observation of calcium-added milk gelation after various heat treatments

The calcium-added skim milk solutions were prepared as per Section 3.4. Four samples with four different concentrations of added calcium lactate (5mM, 10mM, 15mM and 20mM) were prepared and 10ml of each sample was carefully filled into 15mL glass tubes with screwcap lids (Interlab, Auckland, New Zealand). The samples were prepared in duplicate and capped firmly prior to heat treatment. An additional sample with 5mM added calcium lactate was also prepared to monitor the temperature change in the sample. All the samples were placed and secured in a holder. A hot water bath and an ice-water bath were used to heat and cool the samples to induce milk gelation. The temperature of the hot water bath was set at a required temperature and cooling down to 20 ^{o}C was carried out in an ice-water bath in 15 min. The holding time began when the centre of the samples reached the required temperature and they were placed in the ice-water bath immediately after each heat treatment. Once the samples were cooled down to 20 ^{o}C, the surface of the glass tubes were dried and the samples were placed on a flat table with black background for visual observation and photography.

3.6.2 Rheological measurements of calcium-added skim milk under various heat treatments

The skim milk solutions with various concentrations of added calcium were prepared (Section 3.3) prior to the rheological measurements. The rheological properties of the calcium-added skim milk solutions were analysed using a DHR-3 rheometer (TA Instrument, New Castle, Delaware, USA). The rheometer was fitted with a stainless-steel vane rotor (28 mm diameter, 42 mm length) and a stainless-steel single gap cylinder cup with a diameter of 30.36 mm and was used to hold the samples for analysis. For every measurement, 50ml of the sample was filled in the single gap cylinder cup. The calcium-added skim milk solutions were heated to a required temperature in the rheometer and

held for a period of time (30 min or 60 min), followed by cooling to 20 °C. A flow diagram in Figure 3.2 can be followed to replicate the experiment.

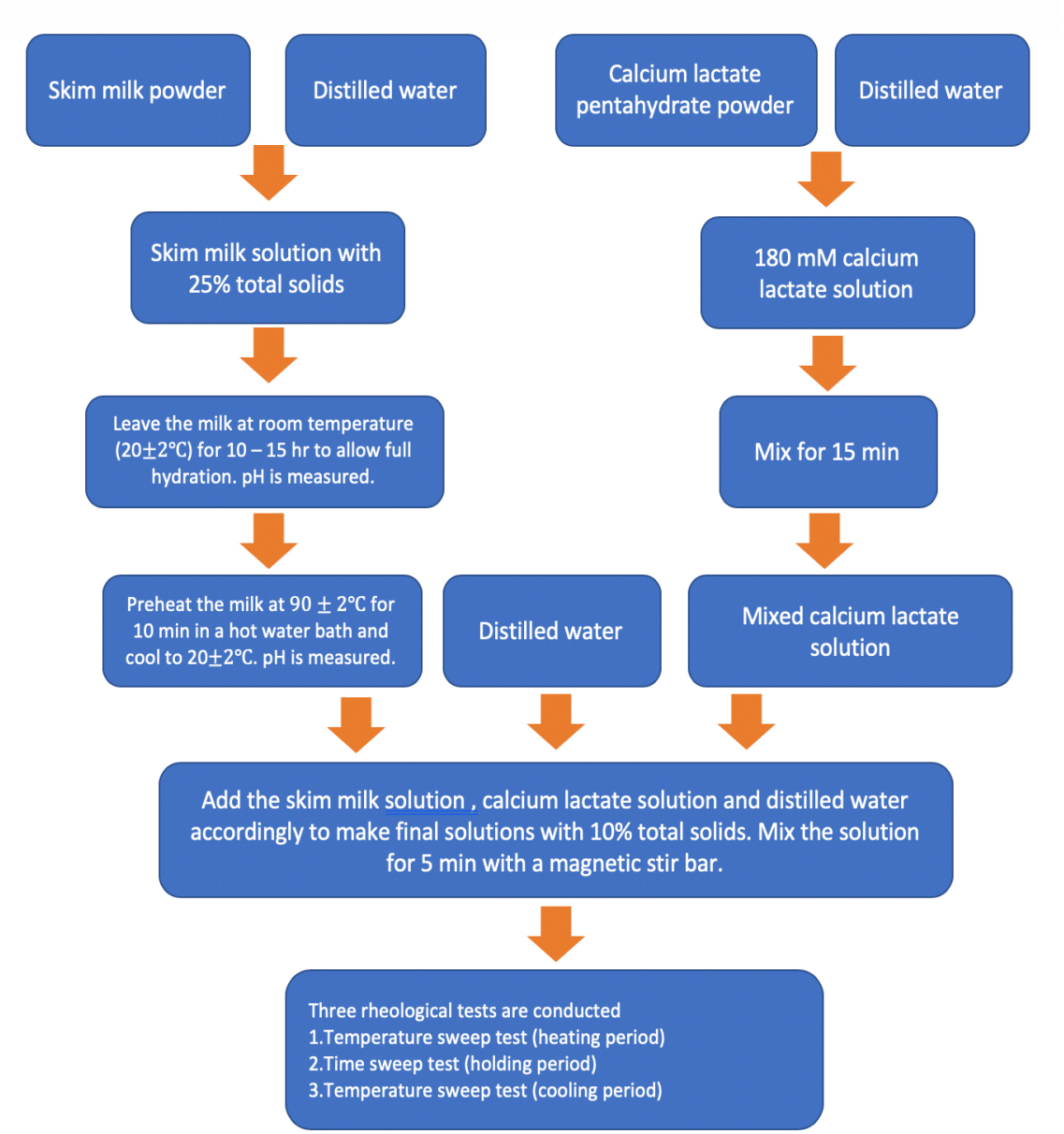

Figure 3.2 A flow diagram of the experimental of rheological characterisation of calcium-added skim milk.

The temperature sweep test was performed first when the sample was heated from 20 °C to the required temperature, which was to determine the change in G' and G'' with increasing temperatures. Then the time sweep test was followed to measure the change in G' and G'' with time. The time sweep test was performed for a total of 30 min or 60 min, with a recording of data every 150 seconds. During the cooling period, the temperature sweep test was performed to determine the final G' for each sample at the end of the cooling period. To minimise the evaporation of sample during heating, canola oil was used to cover the surface of the sample which was covered also with a solvent trap cover.

The tests were performed at the constant strain amplitude of 0.05% selected and a constant frequency of 0.1 Hz. The heating rate and cooling rate were set at 1.5 ºC per min and 2 ºC per min, respectively. The measurements of different samples were conducted in duplicate.

3.7 Experimental to determine the effect of addition of selected polysaccharides to calcium-added skim milk after heat treatment

3.7.1 Visual observation of the gelation of calcium-added milk with additional polysaccharides after heat treatment

In this experiment, the appearance and informal sensory assessment of calcium-added skim milk with polysaccharides was investigated. Only two concentrations of calcium lactate (10mM and 15mM) were added to the skim milk samples. The properties of a range of selected polysaccharides were investigated and covered in the literature review chapter. Xanthan gum, high acyl gellan gum and guar gum were screened and selected from the food polysaccharides. A range of selected polysaccharides concentrations in the calcium-added milk samples was suggested by the literature and only one polysaccharide was added in the sample at a time.

The calcium-added milk was prepared prior to the heat treatment following procedures outlined in Section 3.4. Three polysaccharides (xanthan gum, high acyl gellan gum and guar gum) were added to the calcium-added skim milk solutions as described in Section 3.5. The concentration of calcium lactate and each polysaccharide added in the skim milk solution is shown in Tables 3.3. Control samples with no added polysaccharides were also prepared for the two calcium lactate concentrations tested (10 and 15mM). Once all the samples were prepared, 10ml of each sample was transferred into 4cm×2.5cm×2cm rectangular shaped moulds (Storage Box, Auckland, New Zealand) and heat treated for 30 min in a hot water bath. The initial temperature of the samples was at 20±2 ºC and the temperature of the hot water bath was set at 75 ºC. The holding time began when the centre temperature of samples was 75±2 ºC. The samples were then transferred into an ice-water bath for cooling after heat treatment and the samples were removed from the bath when the sample temperature was 20 ºC. Figure 3.3 shows a typical heating profile of the sample with added calcium lactate and polysaccharides. The samples were then carefully inverted to remove any gels from the moulds and transferred onto petri dishes for visual observation. The appearance of each sample was recorded. An informal

assessment of the texture and mouthfeel of the gels was carried out. Note samples prepared did not contain sodium azide and samples were assessed once full hydrated and not kept for further analysis. Fresh samples were prepared for each replicate.

Table 3.3. Calcium-added milk samples with xanthan gum (XG), high acyl gellan gum (HAGG), guar gum (GG).

Experiment	Concentration of added calcium lactate(mM)	Concentration of polysaccharides (wt %)
XG1	10	0.1
XG2	10	0.3
XG3	10	0.5
XG4	15	0.1
XG5	15	0.3
XG6	15	0.5
HAGG1	10	0.1
HAGG2	10	0.2
HAGG3	10	0.3
HAGG4	15	0.1
HAGG5	15	0.2
HAGG6	15	0.3
GG1	10	0.1
GG2	10	0.2
GG3	10	0.3
GG4	15	0.1
GG5	15	0.2
GG6	15	0.3

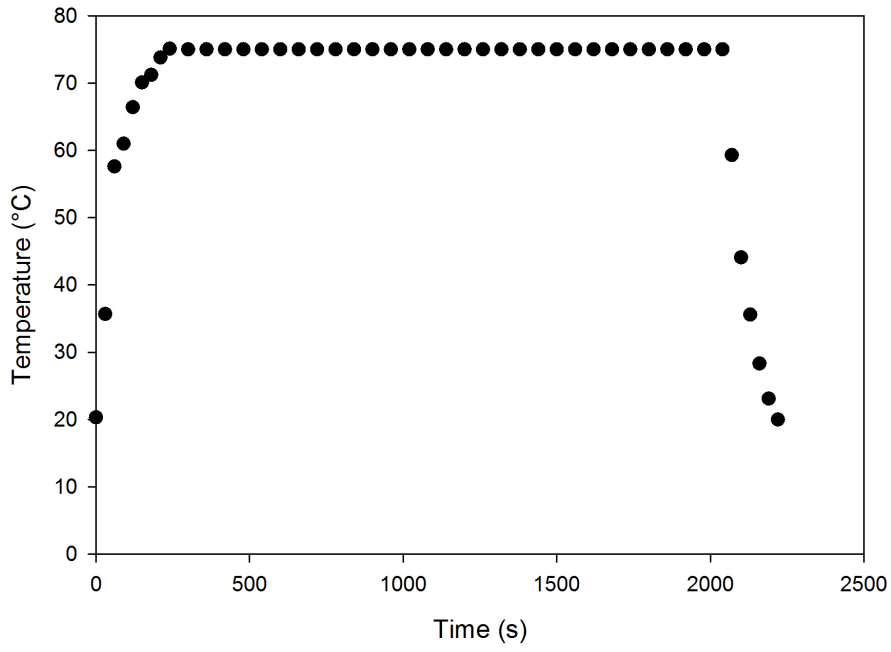

Figure 3.3 A typical heating profile of skim milk samples with added calcium lactate and the polysaccharides being heated and held at 75 °C for 30 min and cooled to 20 °C in a hot water bath.

3.7.2 Rheological characterisation of calcium-added skim milk with polysaccharides under heat treatment

The calcium-added skim milk solutions with added polysaccharides were prepared according to Section 3.5. The rheological properties of samples were analysed using a DHR-3 with the same settings in Section 3.6. For every measurement, 50ml of the sample was filled into the single gap cylinder cup and the surface of samples was layered with canola oil to prevent evaporation during heating. The heating of samples in the rheometer began at 20 °C and the samples were heated to 75±0.5 ºC and held for 30 min. Cooling was then followed after the holding period to 20 ºC. The temperature sweep test was performed during the heating and cooling period to observe the change in *G'* with increasing and decreasing temperature, time sweep test was conducted during the holding period. In the temperature and time sweep test, the strain and frequency were set at 0.05 % and 0.1 Hz, respectively. A flow sweep test was followed after the second temperature sweep test, which was to see whether the samples exhibited Newtonian or non-Newtonian properties. The change in shear stress with shear rate was determined in the flow sweep test. In the flow sweep test, the range of shear rate was set from 1 to 100 s^{-1}. Figure 3.4 shows the rheological tests carried out and Figure 3.4 shows a typical heating profile of the samples with added calcium lactate and polysaccharides in the rheometer. The design of this rheological tests was modified from previous studies (Hadde, 2017; Lin et al., 2018; Ramasubramanian et al., 2014).

3.7.3 Microscopic observation of calcium-added skim milk with polysaccharides using scanning electron microscope (SEM)

SEM (FEI Quanta 200, Thermo Fisher Scientific, Oregon, USA) was used to examine the microstructure of the formed gels. The milk gels were made prior to the SEM imaging. The calcium-added skim milk with polysaccharides were firstly prepared (Section 3.5) and the samples underwent heat treatment at 75±2 ºC in a hot water bath for 30 min and cooled to 20±2 ºC in an ice-water bath. The imaging preparation of the milk gels was followed by a standard protocol and was carried out by the Manawatu Microscopy and Imaging Centre, School of Fundamental Sciences, Massey University, Palmerston North.

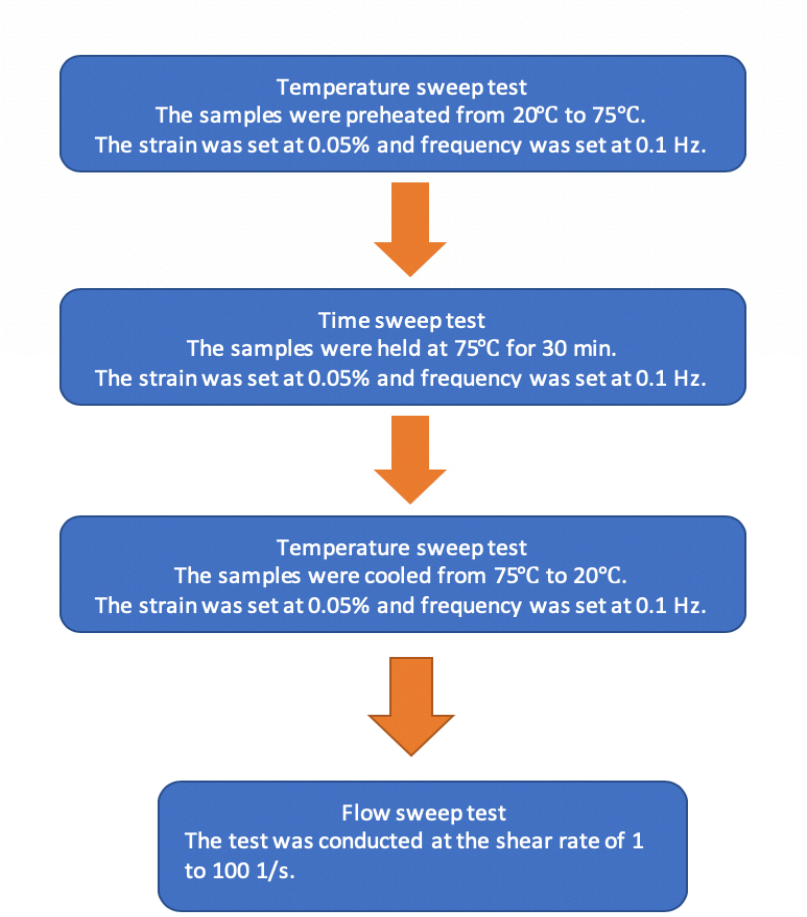

Figure 3.4 A flow diagram of rheological tests performed in calcium-added skim milk with polysaccharides.

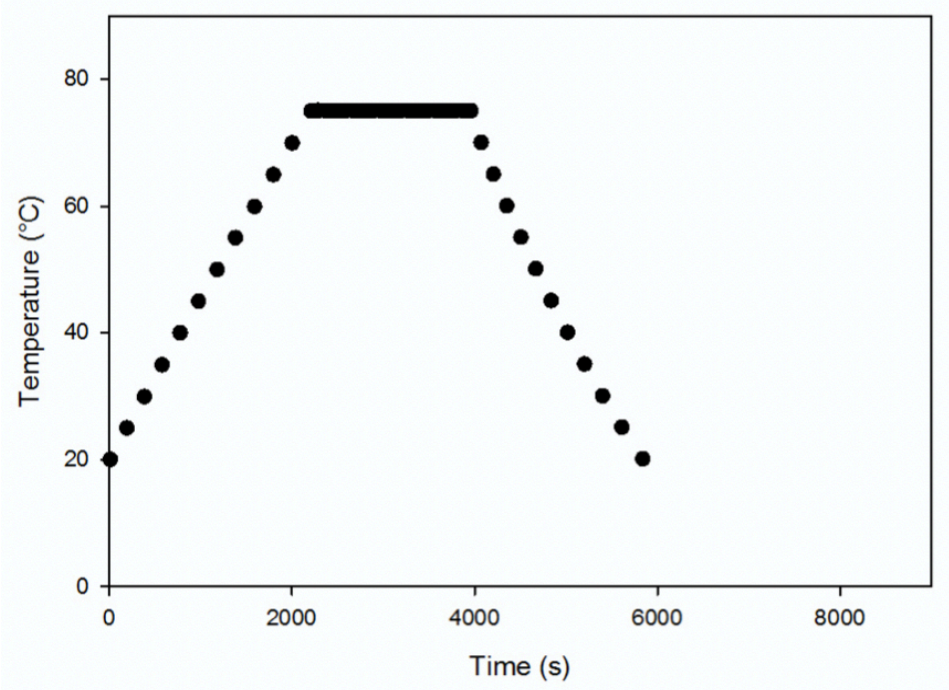

Figure 3.5 A typical heating profile of skim milk samples with added calcium lactate and the polysaccharides being heated and held at 75 °C for 30 min and cooled to 20 °C in rheometer.

The protocol followed fixing, drying, mounting and coating steps. Due to the nature of the milk gels, they were not rigid enough to be fixed so the samples were firstly embedded in 3% agarose. In the fixing step, the surface of the gel sample was wetted with a 1.4% glutaraldehyde solution and sliced into 3mm×3mm sections and sliced less than 1mm thick sections. The sections were fixed in an excess of the glutaraldehyde solution for 12 hours at 20±2 ºC and then washed thoroughly with distilled water. The excess water was drained off with filter paper. The samples were dehydrated in ascending concentrations of aqueous ethanol solutions (50, 75, 80 and 90%) and absolute ethanol (5min for each concentration). Then, the samples were transferred to a drying chamber until they reached its critical point of drying. In the mounting and coating stage, the samples were mounted one SEM holders. The samples were then coated using sputter coater with 300V, 10mA of sputtered gold (6nm). The coated samples were placed in the SEM for imaging. Two different areas at three magnification (10 μm, 20 μm and 200 μm) of each sample were scanned and imaged.

3.8 Calcium concentration determination in serum and sediment phase of skim milk using EDTA titration

The calcium concentration in the serum phase of skim milk was determined using the ethylenediaminetetraacetic acid (EDTA) titration method with Patton and Reeder's indicator described by Patton and Reeder (1956) and Pearce (1977). Before the titration, the skim milk samples were firstly centrifuged to separate serum and sediment phases. The skim milk samples (1.4 g) were weighed into 1.5 mL Eppendorf tubes and placed in a high-speed centrifuge (Himac CT15RE, Hitachi, Tokyo, Japan). The samples were centrifuged at 15,000 rpm and 21,500×g for 90 min at 20±0.5 ºC. After the centrifugation, the supernatant milk serum was pipetted out from the tube and weighed. Once the serum samples were separated from the sediment, a standard curve of mass of calcium versus volume of EDTA was prepared with a series concentration of calcium chloride solutions ranging from 5mM to 20mM (Figure 3.6). One gram of the serum sample was pipetted into a conical flask and 50 mL of distilled water was added. A NaOH solution (8 mol/L) was prepared and 3 mL of the solution added in the conical flask to increase the pH of the sample. The sample solution was allowed to stand for five min with occasional swirling, which was to allow any magnesium ions to precipitate (Pearce, 1977). A couple of drops of 0.5 w/v % Patton-Reeder indicator (Sigma Aldrich, Auckland, New Zealand) was added in the sample solution prior to the titration. The sample was titrated with 0.01

mol/L EDTA until the colour of the sample changed from light pink to persistent blue. The mass of calcium per gram of the milk serum sample was determined using the standard curve (Figure 3.6). Three titrations were conducted for each sample.

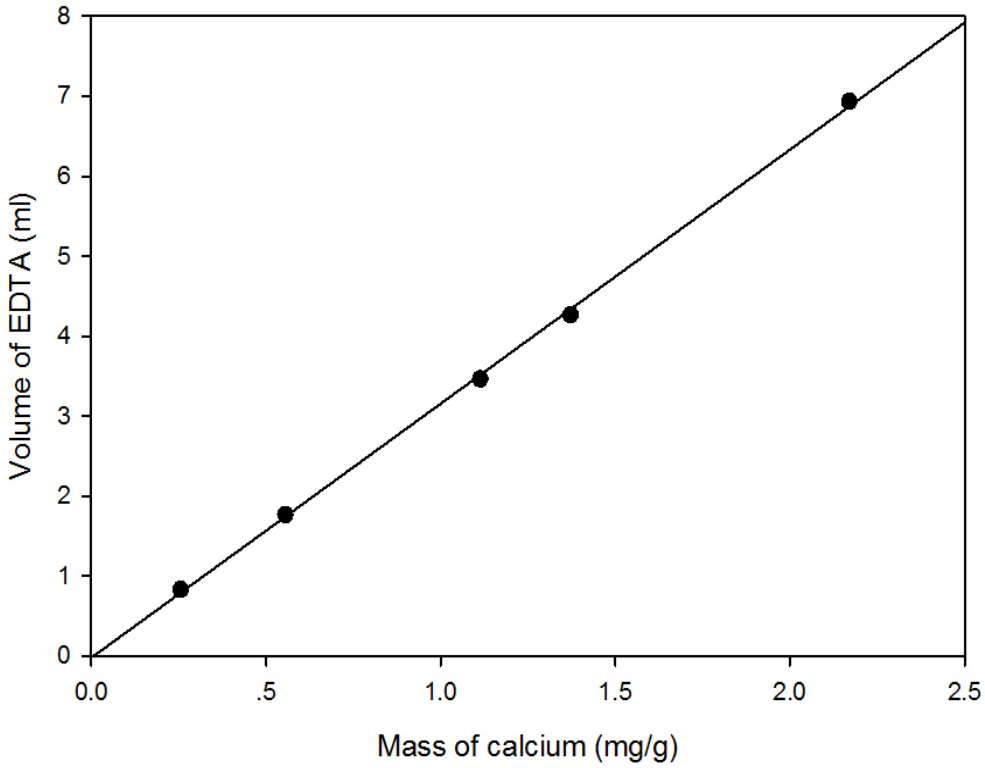

Figure 3.6 A typical standard curve of mass of calcium in a gram of milk serum versus volume of EDTA.

3.9 Statistical Analysis

All experiments were carried out in at least duplicates for sample treatments. The standard deviations were used to indicate the variability between repeated measurements. Sigma Plot (Systat Software Inc, California, USA) was used to plot graphs through this project. The significant difference between the means of data was determined using one-way ANOVA analysis in the SPSS (SPSS Inc, Chicago, USA).

Chapter 4 Effect of heat treatments and the addition of calcium lactate to skim milk

4.1 Introduction

Milk gelation can be induced by heat, acid, rennet and the addition of calcium salts; gelation is a result of the destabilisation of the milk proteins in the system (Dalgleish, 1993; Donato et al., 2007; Hongsprabhas & Barbut, 1996). The combination of heat and the addition of calcium salts can lead to an alteration of the milk proteins stability and formation of milk gels (Koutina et al., 2016). The degree of heat treatment, the type of and the concentration of calcium salt added can result in different types of milk gels in terms of rheological characteristic (Lin et al., 2018).

Heating has a significant effect on destabilising milk proteins. Whey proteins in milk begin to denature above 70^{o}C while casein is mostly heat stable (Anema et al., 1993). A higher temperature of heating accelerates the destabilisation of proteins particularly whey in the milk and the longer period of heating allows continuous denaturation of milk proteins, hence various heating conditions can affect the effectiveness and rate of the milk gelation (Lucey et al., 1999; Mahmoudi et al., 2007; Qian et al., 2017). In addition, the effects of the addition of calcium salt on milk gelation has also been studied (Ju & Kilara, 1998b; Koutina et al., 2016; Lin et al., 2018). It has been suggested that the stability of milk proteins is associated with the concentration of calcium salts, the type of calcium salt and their solubility (Lund et al., 2010; Weaver, 1998). It has been reported that calcium lactate, calcium chloride and calcium gluconate possess relatively higher solubility and calcium bioavailability compared to other organic calcium salts such as calcium acetate (Singh et al., 2007; Skibsted, 2016). Calcium lactate has greater solubility compared to calcium gluconate and it does not render undesirable taste in solutions while calcium chloride often gives the bitterness (Gaby, 2010; Ueda & Taira, 2013). In this study, calcium lactate was selected and used as the calcium fortifying agent through the entire project. Therefore, the objective of this study was to investigate the effects of various concentrations of calcium lactate to skim milk and the effect of the temperature of heating by evaluating the physical and rheological properties of the milk gels formed.

4.2 Materials

Low heat skim milk powder was acquired from Fonterra Co-operative Group (Auckland, New Zealand) and the specification of the skim milk powder can be seen in Appendix A1. Calcium lactate pentahydrate powder (≥ 98.0%) was purchased from Acros Organics (Thermo Fisher Scientific, Auckland, New Zealand).

4.3 Methods

4.3.1 Sample Preparation

The preparation of skim milk solutions, calcium lactate solutions and calcium-added skim milk solutions were followed as reported in Section 3.2, 3.3 and 3.4. The skim milk solutions with 25 wt % final solids were prepared along with the calcium lactate solution prior to addition, mixing and rheological measurements. A flow diagram showing the procedure used is in Figure 4.1. When the concentration of total solids was less than 20%, the liquid exhibited Newtonian behaviour. However, the liquid showed shear-thinning behaviour when the concentration of total solids was over 30% (Morison et al., 2013). The milks were well agitated during preparation.

4.3.2 Heat treatment of calcium-added skim milk and milk gel observations

To evaluate the effect of the addition of calcium lactate at different concentrations and with different heating temperatures and times. Calcium lactate at different concentrations was added to the skim milk solutions. After addition of the calcium salt, the solutions were placed into a hot water bath and held for a period of time, followed by cooling in an ice-water bath. The procedure and method can be found in Section 3.6.1. The conditions evaluated are shown in Table 4.1.

4.3.3 Rheological Characterisation of Skim Milk with Added Calcium Lactate

The rheological characterisation of the calcium added skim milk solutions was carried out using a Discovery HR-3 rheometer (DHR-3, TA Instrument, USA) under different conditions. The procedure and method can be found in Section 3.6.2. The samples were placed into a stainless-steel single gap cylinder cup and were heated to a specified temperature, held at that temperature for a period of time then cooled to 20±2 ^{o}C. Table 4.1 shows the various conditions used in the rheometer.

Table 4.1 The conditions of heat treatment, holding and cooling temperature and time for skim milk with added calcium lactate used on the DHR-3.

Experiment	Heating and holding temperature (°C)	Holding time (min)	Cooling temperature (°C)
1	65±0.5	60	20±0.5
2	70±0.5	60	20±0.5
3	75±0.5	30	20±0.5
4	75±0.5	60	20±0.5
5	80±0.5	60	20±0.5

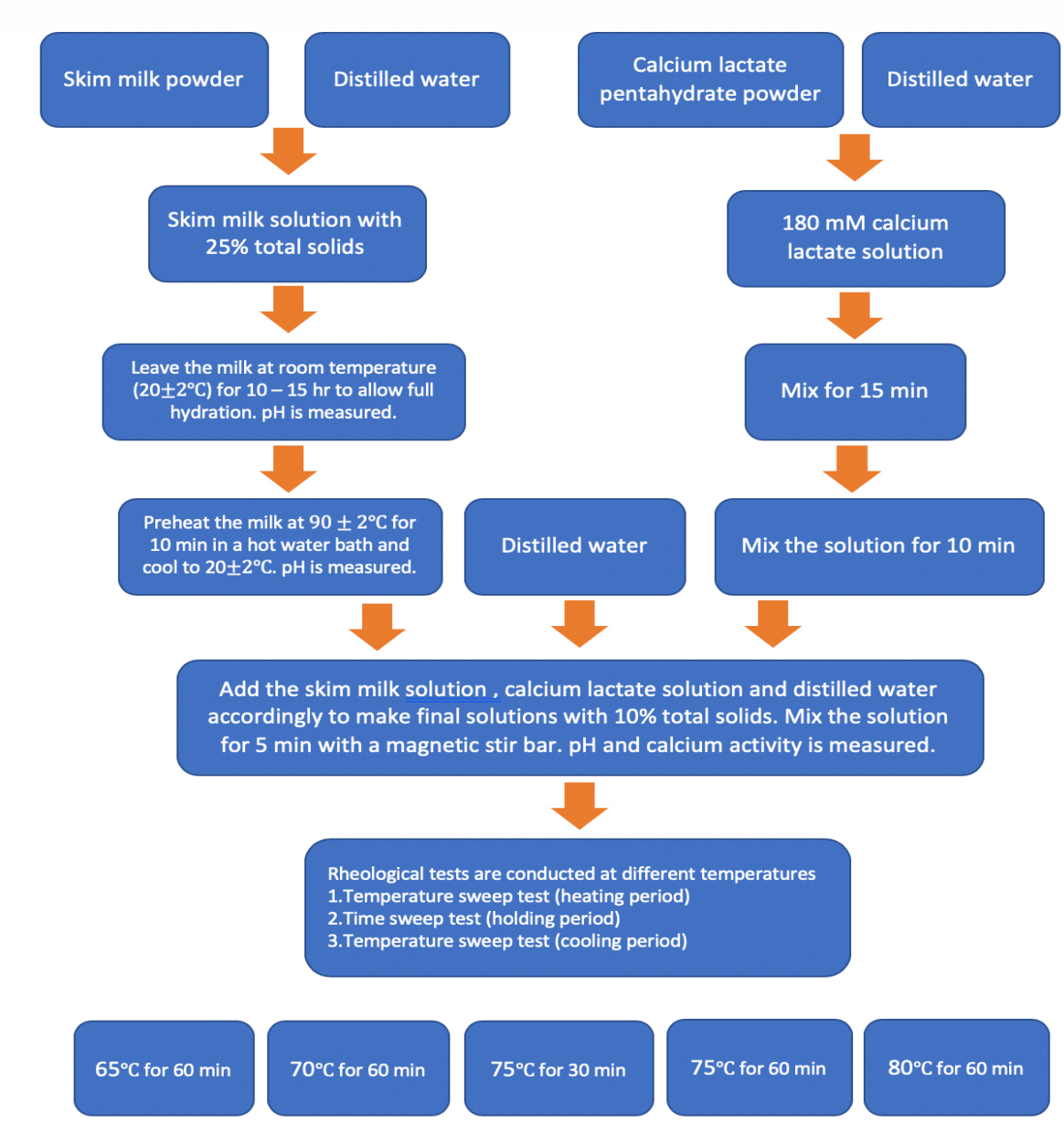

Figure 4.1 Flow diagram for sample preparation of skim milk with added calcium lactate.

4.4 Results and Discussion

4.4.1 Visual observation of skim milk solutions with added calcium lactate

The skim milk solutions with four different concentrations of added calcium lactate were heat treated under five different conditions. The pH of the calcium lactate solutions recorded and are presented in Table 4.2. pH is an important factor in a dairy system and did not significantly change ($p \leq 0.05$) over the range of concentration of calcium lactate used in this study. The selected polysaccharides (xanthan gum, high acyl gellan gum and guar gum) were stable in acidic condition (pH 6.18 – 6.57).

The solutions were visually observed after being cooled to 20±2 ^{o}C, all glass tubes were inverted after reaching 20 ^{o}C to assess if the sample had formed a soft or hard gel or had not gelled at all. If a gel formed and remained at the top of the test tube after inversion, this was deemed to be a firm gel. If the gel formed moved down the tube either partially or all the way to the bottom, this was deemed a soft gel. If no gel was formed, this was also recorded. The visual results of various gels were presented in Figures 4.2 to 4.6. The key observations for all the skim milk solutions after different heat treatments and concentrations of calcium lactate are summarised in Table 4.3.

Table 4.2 pH of the samples with different concentrations of calcium lactate.

Concentration of added calcium lactate (mM)	pH
0	6.57±0.03
5	6.44±0.04
10	6.32±0.01
15	6.23±0.01
20	6.18±0.02

Table 4.3 Key results from visual observation of the skim milk solutions after being heat treatment and cooling.

Heat treatments	Added calcium lactate (mM of skim milk)			
	5	10	15	20
65^{o}C for 60 min	No gel. Remained in liquid form.	No gel. Remained in liquid form.	Partially formed soft gel	Firm gel
70^{o}C for 60 min	No gel	Gel	Firm gel	Firm gel
75^{o}C for 30 min	No gel	Gel	Firm gel	Firm gel
75^{o}C for 60 min	No gel	Gel	Firm gel	Firm gel
80^{o}C for 60 min	No gel	Gel	Firm gel	Firm gel

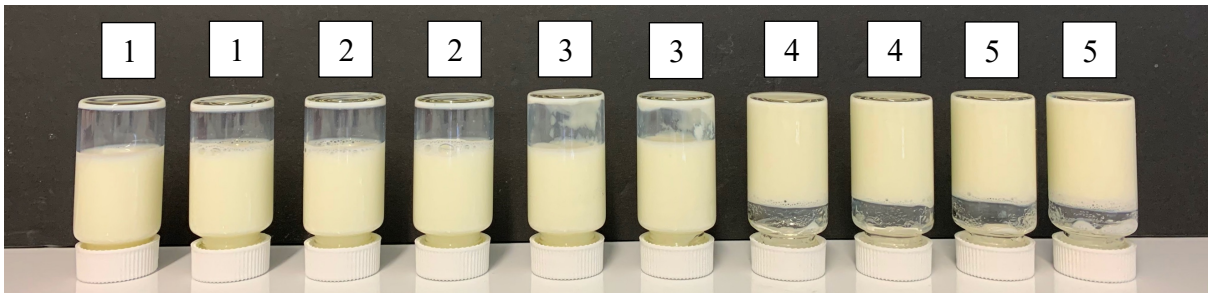

Figure 4.2 Skim milk solutions after being heat treated at 65 °C for 60 min. (1) 0mM added calcium lactate; (2) 5mM added calcium lactate; (3) 10mM added calcium lactate; (4) 15mM added calcium lactate; (5) 20mM added calcium lactate.

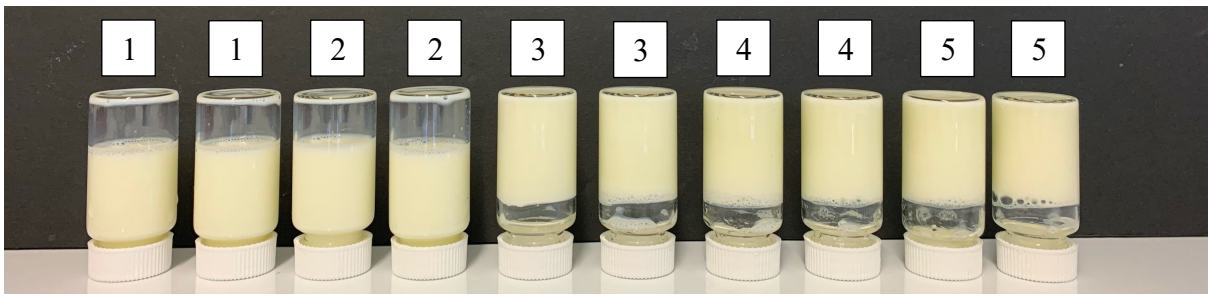

Figure 4.3 Skim milk solutions after being heat treated at 70 °C for 60 min. (1) 0mM added calcium lactate; (2) 5mM added calcium lactate; (3) 10mM added calcium lactate; (4) 15mM added calcium lactate; (5) 20mM added calcium lactate.

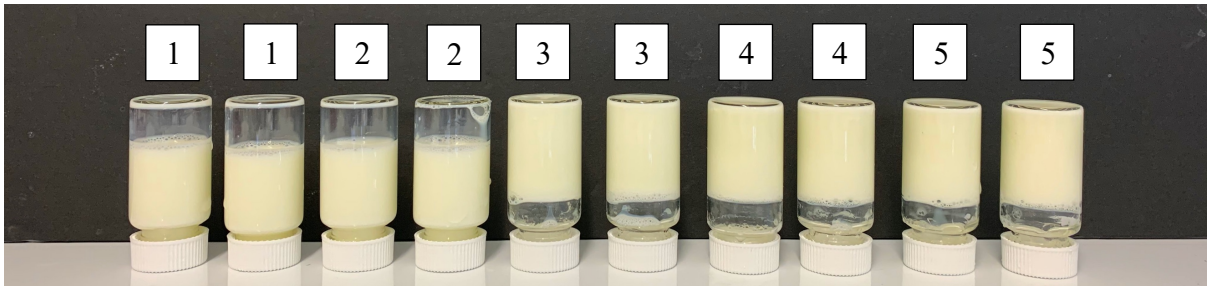

Figure 4.4 Skim milk solutions after being heat treated at 75 °C for 30 min. (1) 0mM added calcium lactate; (2) 5mM added calcium lactate; (3) 10mM added calcium lactate; (4) 15mM added calcium lactate; (5) 20mM added calcium lactate.

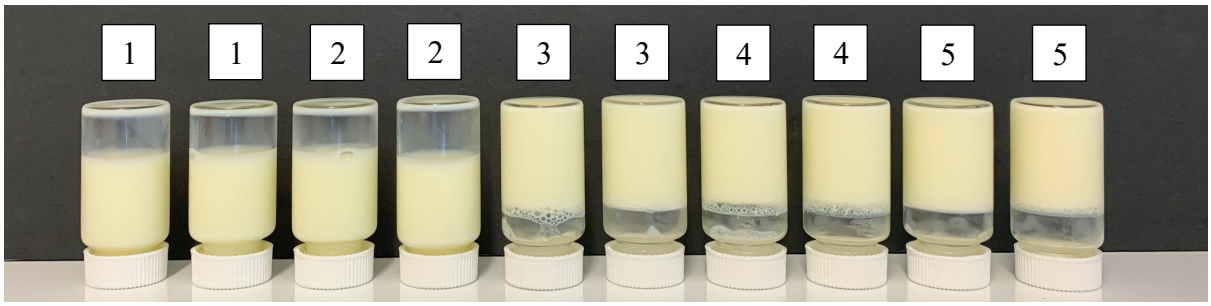

Figure 4.5 Skim milk solutions after being heat treated at 75 °C for 60 min. (1) 0mM added calcium lactate; (2) 5mM added calcium lactate; (3) 10mM added calcium lactate; (4) 15mM added calcium lactate; (5) 20mM added calcium lactate.

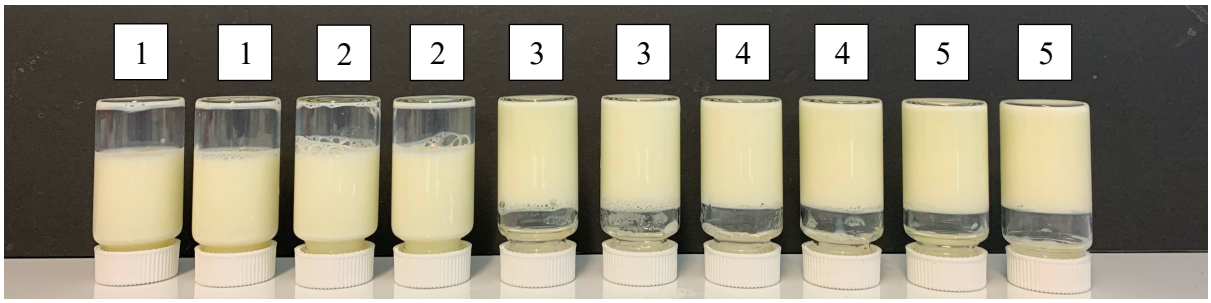

Figure 4.6 Skim milk solutions after being heat treated at 80 °C for 60 min. (1) 0mM added calcium lactate; (2) 5mM added calcium lactate; (3) 10mM added calcium lactate; (4) 15mM added calcium lactate; (5) 20mM added calcium lactate.

No gel formation was observed when 5mM added calcium lactate was added for all heat treatment conditions. Soft gels were formed when heating temperature was above 65 ºC and 10mM calcium lactated were added to the skim milk solutions. The addition of 15mM calcium lactate to the skim milk solutions resulted in the formation of gels under all heat treatments. Firm gels were observed after the heat treatments in which the skim milks were heated to 70 ºC and above. A soft gel was partially formed when the skim milk solutions with 15mM calcium lactate were heat treated at 65 ºC for 60 min. With the addition of 20mM calcium lactate, firm gels were observed after all heat treatments.

Overall, the apparent physical changes were not observed in the skim milk solutions with 5mM added calcium lactate after heat treatments, while the addition of 15mM and 20mM calcium lactate induced the formation of milk gels under all heat treatments. However, the samples with added calcium lactate were gritty and the gels broke easily.

4.4.2 Rheological Characterisation

Rheological measurements of the milk solutions under various heat treatments were carried out to determine changes in *G'* during heating, holding and cooling. The samples with various concentrations of added calcium lactate were heat treated with different heating profiles. The gelation point was defined as the point *G'* is greater than 1 Pa (Ramasubramanian et al., 2014). The heating profiles on the rheometer for five different heat treatments are shown in Figure 4.7.

4.4.2.1 Heating period

During the heating period, the *G'* of all samples did not change significantly and their *G'* values were less than 1 Pa (Figure 4.8). Hence, no gelation was observed during this period. The *G'* values remained approximately constant when the skim milk solution was heated from 20 ºC to 65 ºC for all calcium concentrations. However, *G'* values of the

samples with 15mM and 20mM added calcium lactate rapidly increased when the heating temperature was above 70 ^{o}C (Figure 4.8 (c)(d)(e)). A sharp increase in *G'* was observed in the samples with 15mM and 20mM added calcium lactate once they reached 80^{o}C (Figure 4.8 (e)).

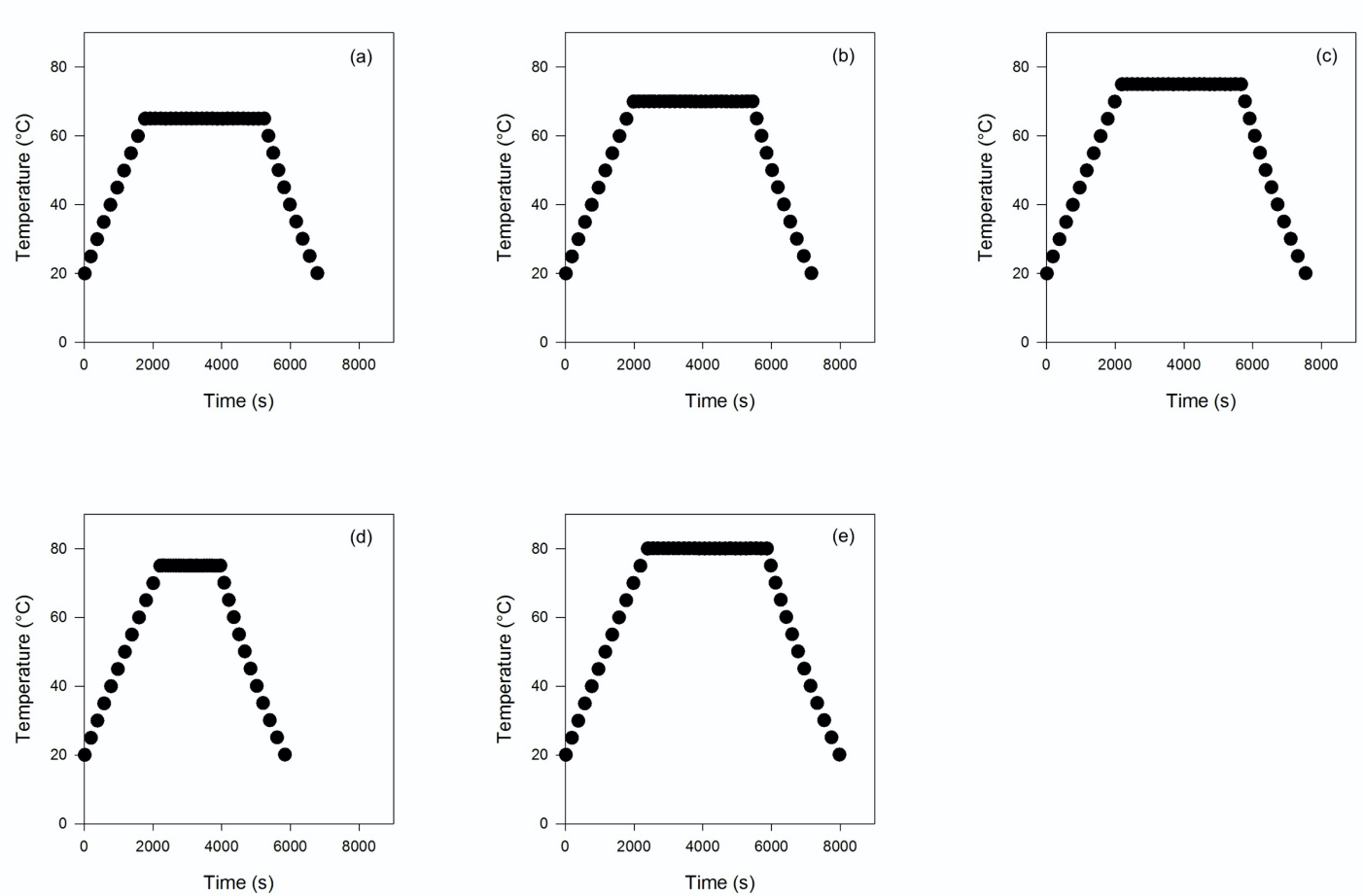

Figure 4.7 Heating profile from 20 °C of changes in sample temperatures with time under different heat treatments. (a). 65 °C for 60 min; (b). 70 °C for 60 min; (c). 75 °C for 60 min; (d). 75 °C for 30 min; (e). 80 °C for 60 min.

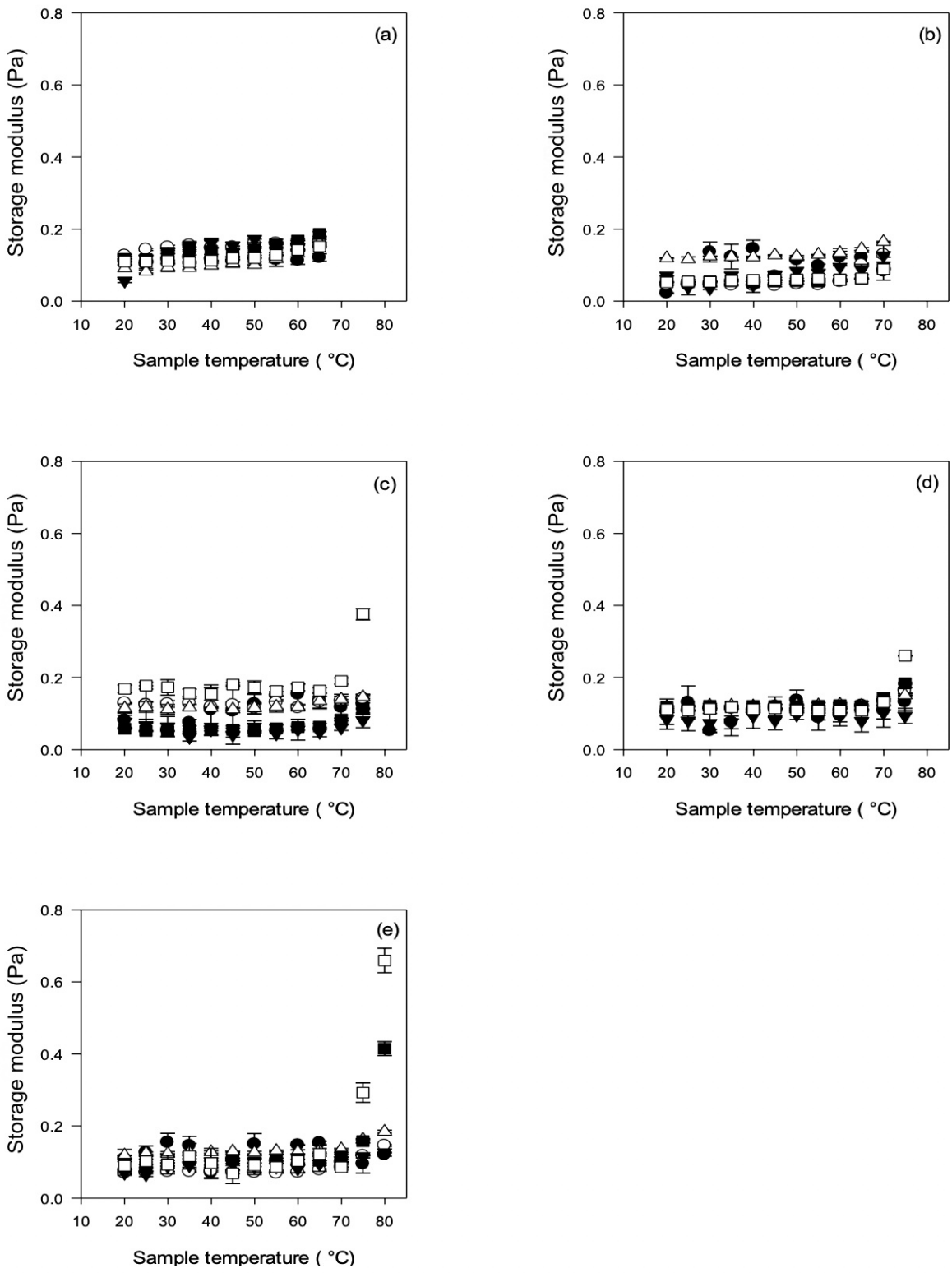

Figure 4.8 The change in *G'* of the skim milk solutions during heating with added calcium lactate. ● 0mM; ○ 5mM; ▼ 7.5mM; △ 10mM; ■ 15mM; □ 20mM. (a) 65 ºC for 60 min; (b) 70 ºC for 60 min; (c) 75 ºC for 30 min; (d) 75 ºC for 60 min; (e) 80 ºC for 60 min. The values of *G'* is carried out as a mean ± standard deviation, where n is 2.

When no calcium lactate was added, the *G'* remained unchanged during the heating period. The *G'* values of the samples with 15mM and 20mM added calcium lactate at 80ºC was found to be 0.42 Pa and 0.65 Pa respectively, where the *G'* values of other samples remained below 0.2 Pa. A similar trend can be seen in Figures 4.8 (c) and (d), the *G'* values of the samples with 20mM calcium lactate increased at 75 ºC. With 5mM and 10mM added calcium lactate in the skim milk samples under all heat treatments, there was a slow increase in *G'* from 20 ºC to 80 ºC.

4.4.2.2 Holding period

During the holding period, there was no apparent change in *G'* of the samples with 5mM and 7.5mM added calcium lactate under all heat treatments for the entire holding period and their *G'* values were less than 1 Pa. Hence, no gelation occurred at these low concentrations of added calcium lactate.

Gelation in skim milks was observed during the holding period in skim milk with 10, 15 and 20mM added calcium lactate. This agreed with results in Section 4.4.1. With the addition of 10mM calcium lactate, gels were observed after heating at 70 ºC for 60 min (Section 4.4.1) and yet observations on the rheometer showed that any gels formed had a *G'* over 9 Pa after heating (Figure 4.9(b)). For the 10mM calcium lactate solutions heated to 75 ºC for 60 min and 80 ºC for 60 min, the *G'* of these solutions reached 1.95±0.04 Pa and 13.53±0.24 Pa after heating, respectively (Figure 4.9(d) and (e))

It was found that *G'* of the samples with the same concentration of added calcium lactate increased moderately at 65 ºC or 70 ºC for 60 min and 75 ºC for 30 min (Figure 4.9 (a), (b) and (c)). The *G'* of the samples heated to 75 ºC held for 60 min was higher than held for 60 min, showing that holding time was important for gel formation. The addition of calcium lactate also exerted an important impact on *G'* of the skim milk samples, *G'* values increased as the concentration of added calcium increased. Overall, holding temperature and the concentration of added calcium lactate play an important role in the change in *G'* and therefore the formation of gels.

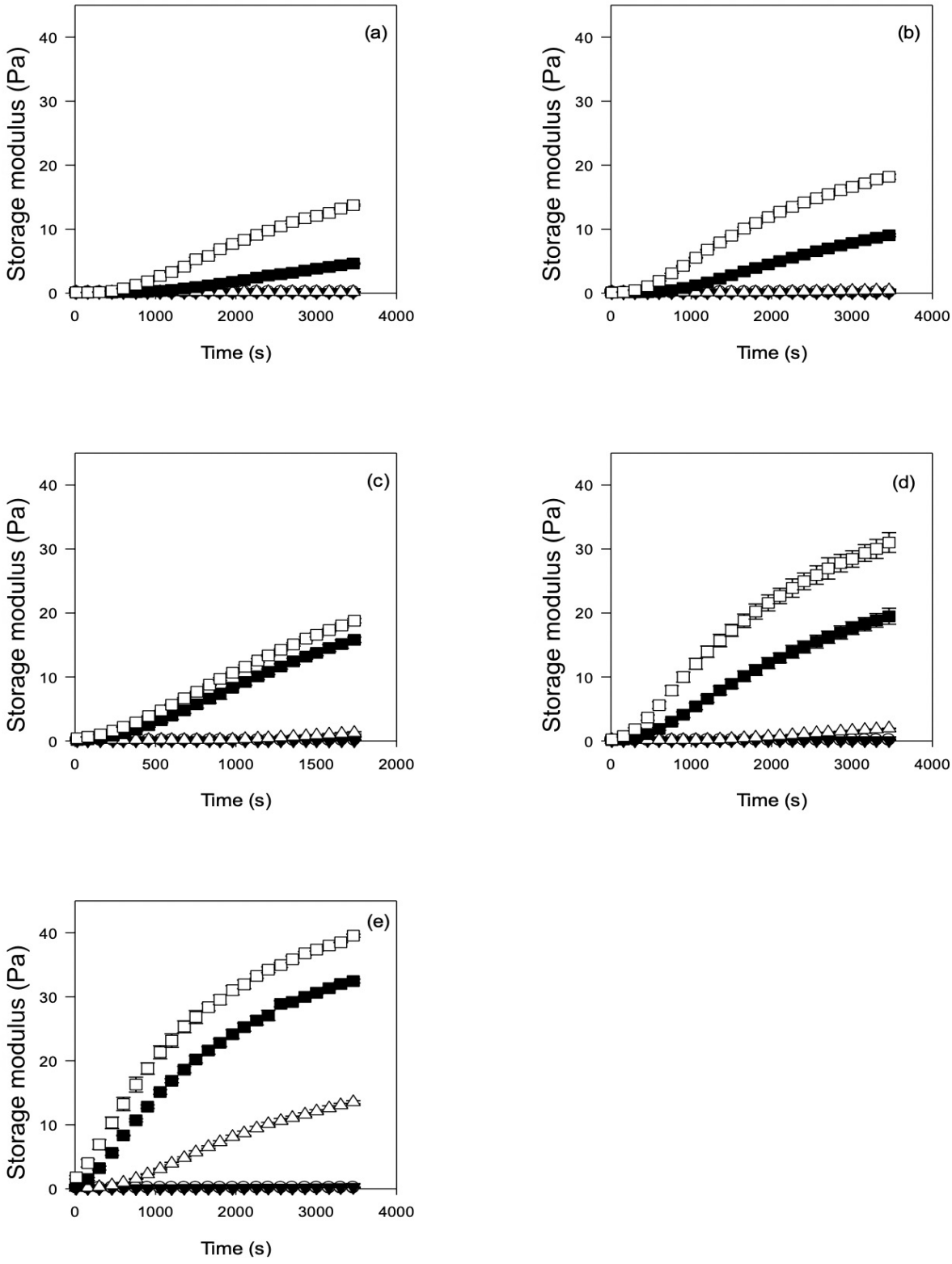

Figure 4.9 The change in *G'* of the skim milk solutions during holding with added calcium lactate. ● 0mM; ○5mM; ▼ 7.5mM; △ 10mM; ■ 15mM; □ 20mM. (a) 65 ºC for 60 min; (b) 70 ºC for 60 min; (c) 75 ºC for 30 min; (d) 75 ºC for 60 min; (e) 80 ºCfor 60 min. The values of *G'* is carried out as a mean ± standard deviation, where n is 2.

4.4.2.3 Change in *G'* in cooling period

After the holding period, the skim milk samples were cooled from their holding temperature to 20±2 ºC (Figure 4.10). The storage modulus of samples exhibited various behaviours, which was strongly associated with the concentration of added calcium lactate and the heating temperatures. The samples with lower concentrations of calcium lactate (5mM) did not show increases in *G'* by the end of cooling. The final *G'* value of all the skim milk solution exceeded 1 Pa except for those with 5mM added calcium lactate where no gelation was observed at any heat treatments. The samples with 10mM added calcium lactate heated at 70ºC and held for 60 min did not show a significant increase in *G'* at the end of cooling though soft gels were observed in Section 4.4.1 and the final *G'* was 1.35±0.02 Pa. The storage modulus of the samples with 15mM and 20mM added calcium lactate increased gradually with the lower-temperature heat treatments (65 ºC and 70 ºC) and rose exponentially with higher-temperature heat treatments (75 ºC and 80 ºC) after they were cooled below 40 ºC (Figure 4.10).

The final *G'* of the samples with calcium lactate concentrations less than 10mM were found to achieve of final *G'* of less than 1 Pa, which was also influenced by the heating temperature and holding times. As the samples were cooled from 65 ºC to 20 ºC, the *G'* values remained approximately unchanged for the skim milk solutions with 5mM added calcium lactate. After heating to 65 ºC for 60 min, and then cooling to 20 ºC, the *G'* values of the skim milk solutions with 20mM and 15mM added calcium lactate were 28.34±0.90 Pa and 10.81±0.05 Pa, respectively.

For the samples cooled from 75 ºC to 20 ºC, *G'* values showed a similar trend with the samples cooled from 80 ºC (Figure 4.10(c) and (d)). As the samples heated to 75 ºC for 30 min or 60 mins achieved different values after heating their final *G'* values after cooling did not achieve the same values. Heating for 60 mins results in the final *G'* of the cooled gel higher than heating for 30 min. With a longer holding time (60 min at 75 ºC), the skim milk solution had a final *G'* of 63.21±2.99 Pa, 41.13±2.93 Pa and 5.23±0.04 Pa for 20, 15 and 10 mM, respectively. For the samples heated to 75 ºC and heated for 30 min the final *G'* after cooling was 44.33±0.71 Pa, 20.07±3.03 Pa and 3.61±0.01 Pa for 20, 15 and 10 mM, respectively.

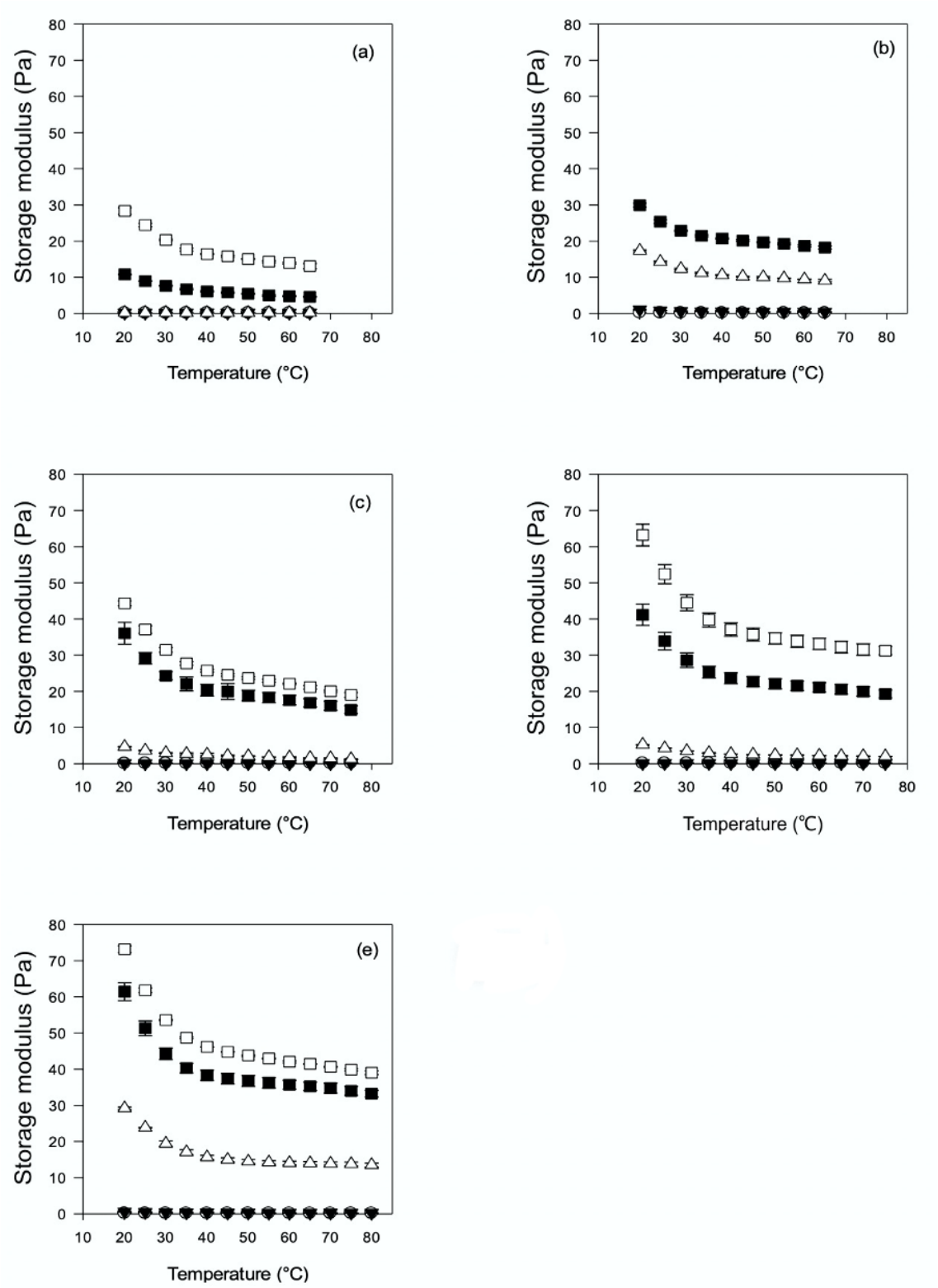

Figure 4.10 The change in *G'* of the skim milk solutions during cooling with added calcium lactate. ● 0mM; ○ 5mM; ▼ 7.5mM; △ 10mM; ■ 15mM; □ 20mM. (a) 65 ºC for 60 min; (b) 70 ºC for 60 min; (c) 75 ºC for 30 min; (d) 75 ºC for 60 min; (e) 80 ºC for 30 min. The values of *G'* is carried out as a mean ± standard deviation, where n is 2.

After heating at 80 ºC for 60 min, as the samples were cooled from 80 ºC to 20ºC, the *G'* values increased for the samples with 10mM, 15mM and 20mM added calcium lactate when they were cooled to 20 ºC (Figure 4.10 (e)). The skim milk solution with 20mM added calcium had the highest final *G'* which was 73.47±0.93 Pa, followed by the final *G'* value of 61.4±1.33 Pa and 29.18±0.36 Pa for the samples with 15mM and 10mM added calcium lactate, respectively.

The final *G*' of the samples with various concentrations of added calcium lactate under different heat treatments were obtained at the end of the cooling period and a plot of the change in final *G'*s with various concentration of added calcium lactate is shown in Figure 4.11. Overall, the final *G'*s in the skim milk solutions with 10% total solids increased with increasing concentration of added calcium under all heat treatments. The significance of the change in final *G'* with various concentrations of calcium lactate under different heat treatment were statistically analysed and presented in Appendix 2.

The higher the holding temperature and the longer the holding time had the greatest influence on the final *G'* of the samples with the same concentration of added calcium lactate. The effect of calcium lactate concentration was found to have a significant effect on final *G'* ($p<0.05$) (Appendix 2 shows the results for the ANOVA). When the calcium lactate concentration increased from 0 to 7.5mM there was no significant difference in final *G'* for all the heat treatments ($p>0.05$).

With 10mM added calcium lactate there was no significant difference in final *G'* for 65 ºC 60min and 70 ºC 60min ($p>0.05$) and no significant difference between 75 ºC for 30min and 75 ºC for 60 min ($p>0.05$). For 15mM and 20 mM added calcium lactate all heat treatments results in significantly different final *G'* values with final values increasing with increasing heating temperature or time ($p<0.05$).

With the addition of 20mM calcium lactate, the higher holding temperature and longer holding time had a significant impact on the final *G'*, in which the final *G'* changed significantly at different heat treatments ($p<0.05$). When the concentration of added calcium lactate was greater than 7.5mM, the final *G'* changed significantly as the samples were heated and held at 80ºC for 60 min ($p<0.05$).

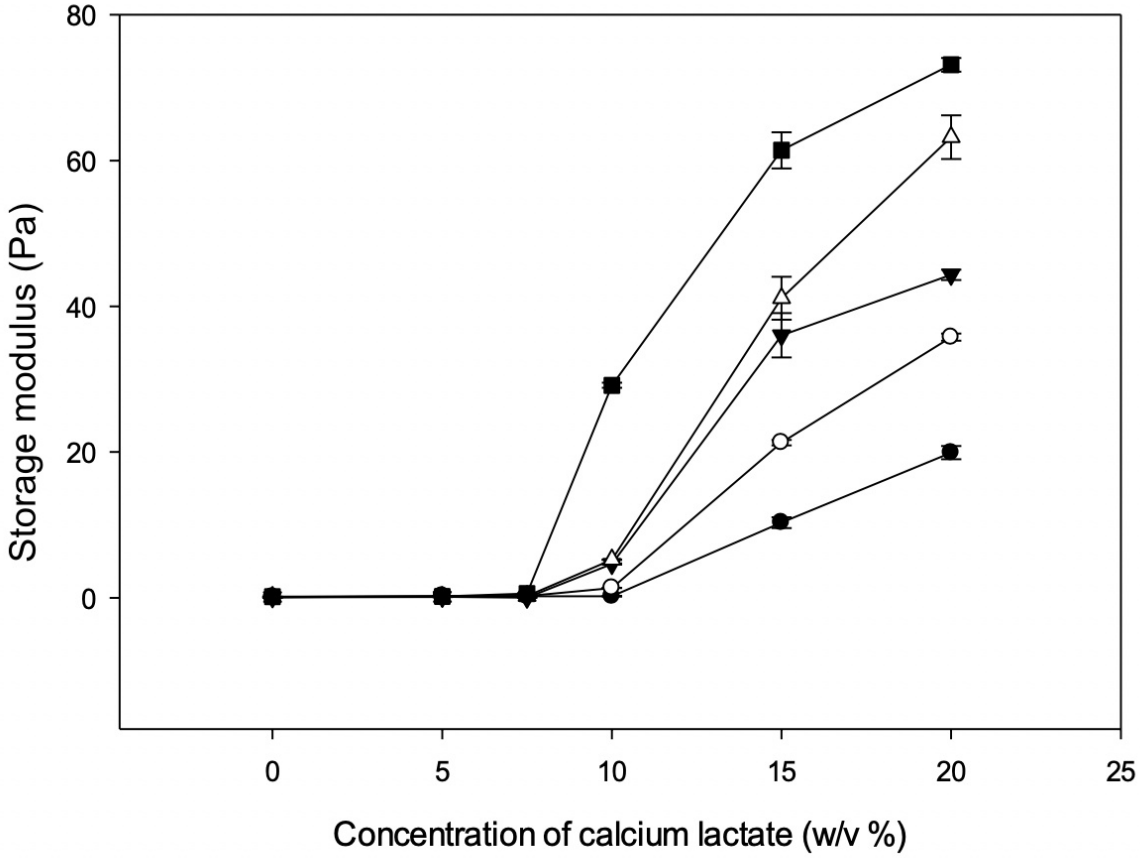

Figure 4.11 Final *G'*s of the samples with various concentration of added calcium lactate under different heat treatments. ● 65 ºC for 60 min; ○ 70 ºC for 60 min; ▼ 75 ºC for 30 min; △ 75 ºC for 60 min; ■ 80ºC for 60 min.

Two studies investigated the storage modulus change of the skim milk with different added calcium salts when the samples were heat treated. (Lin et al., 2020; Lin et al., 2018). It was found the pattern of change in *G'* of the skim milk with added calcium lactate agreed with the results obtained when the samples were heated and held at 70 ºC for 60 min. Although Lin et al. (2018) reported the final *G'* of the samples with 15mM and 20mM was approximately 12 Pa and 25 Pa, respectively. These values were lower than the final *G'* obtained in this study as this study found final *G'* values of 21.29±0.38 Pa and 35.72±0.49 Pa for 15mM and 20mM calcium lactate, respectively. This may be caused by the selection of rheometer cup, Lin et al. (2018) used a hard-anodised aluminium cup to heat the samples, while the stainless steel cup was used in this study. The steel cup heats up faster than the aluminium cup and it takes shorter time to heat up to desired temperature. Therefore, the samples in this study heated faster and resulted in higher final *G'* values.

4.4.3 Calcium concentration determination in skim milk

The concentration of calcium in the serum and sediment of the calcium added skim milk samples were also determined in order to investigate where the added calcium was present in the skim milk sample. Skim milk with various concentrations of added calcium lactate were separated into serum phase and sediment after centrifugation. The calcium concentration in each phase was determined by the EDTA method and the results were shown in Table 4.4. It was found that the calcium concentration in the milk serum increased significantly ($p<0.05$) with increasing concentration of added calcium lactate from 5mM to 20mM, while the calcium concentration in the sediment decreased when more was added. However, the overall concentration of calcium in the sediment was higher than in the serum.

This postulates that the added calcium almost remained in the serum of skim milk and this finding was in agreement with a number of studies (Philippe et al., 2003; Sievanen et al., 2008; Udabage et al., 2000). It was reported by Walstra (1999) that the increasing concentration of calcium ions in milk would result in the ion activity product of calcium phosphate exceeding its solubility product as milk is supersaturated. This will lead to the precipitation of calcium phosphate. A study reported that the addition of calcium chloride in milk led to the concentration of calcium in the serum increased and yet the concentration of phosphate in the serum decreased (Philippe et al., 2003). This may also occur in this study, in which calcium lactate was added into the skim milk. The milk serum could accommodate more calcium ions as a result of the precipitation of calcium phosphate. Therefore, with the addition of calcium lactate to skim milk, the serum had relatively higher concentration of calcium than in sediment. The results in this study agreed with the findings from Lin et al. (2018). It was also found the mass of calcium in the serum increased significantly as the amount of added calcium was increased from 5 to 40 mmol L^{-1} (Lin et al., 2018).

Table 4.4 Concentration of calcium in serum and sediment phase of skim milk with different concentration of added calcium lactate.

Concentration of added calcium lactate in skim milk (mM)	Calcium concentration in serum phase (mg g^{-1})	Calcium concentration in sediment (mg g^{-1})
5	0.32^{d}	1.19^{d}
10	0.46^{c}	1.09^{c}
15	0.54^{b}	1.05^{b}
20	0.84^{a}	0.85^{a}

Average of mean values and pooled standard deviation±0.02 mg g^{-1} (n = 3 replicates)
Means in the same column followed by different letters are significantly different ($p < 0.05$)

4.5 General discussion

The results in this study demonstrated the impact of heat treatment and concentration of calcium lactate on the change in physical structure and storage modulus of the skim milk samples. Firstly, the milk pH of all samples was measured and pH was above the isoelectric point (pH 4.6), hence the milk proteins possess a net negative charge (O'Kennedy, 2011). The interaction of calcium ions and casein started with the dissolution of calcium lactate in the system. The chemical structure of calcium lactate was shown in Figure 4.12 and an equilibrium relationship of the dissolution of calcium lactate is shown in equation 4.1, where L^- is a lactate anion (Croguennec et al., 2016; Vavrusova et al., 2014). The addition of ionic calcium ions to milk could interact with the proteins through electrostatic interaction, at which the calcium ions bind to the milk proteins (Dalgleish et al., 1997; Totosaus et al., 2002).

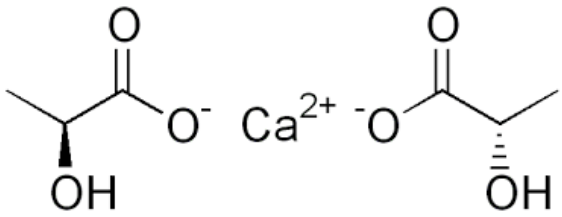

Figure 4.12 Chemical structure of calcium lactate.

$$CaL_2 = CaL^+ + L^-$$
$$CaL^+ = Ca^{2+} + L^- \quad (4.1)$$

The increasing concentration of calcium salts in milk will lead to more available calcium ions present in the system. The free calcium ions are likely to interact with hydrogen phosphate ions and thereby form calcium hydrogen phosphate. The equilibrium shifts to form calcium phosphate (Croguennec et al., 2016; Lewis, 2011). The reduced number of hydrogen phosphate ions leads to the breakdown of dihydrogen phosphate in the system, which results in the release of hydrogen ions (equation 4.2). As a result, the presence of increasing number of hydrogen ions causes a reduction of milk pH and destabilisation of the proteins with the effects of heat (Croguennec et al., 2016).

$$Ca^{2+} + HPO_4^{2-} \rightleftharpoons CaHPO_4$$
$$H_2PO_4^- \rightleftharpoons {HPO_4}^{2-} + H^+ \quad (4.2)$$

A number of studies revealed the effect of heat treatment on whey proteins and gel properties, and they found that milk protein denaturation occurs at a faster rate when heating temperature rises (Dissanayake et al., 2013; Hashizume & Sato, 1988; Lucey et al., 1998; Qian et al., 2017).

Qian et al. (2017) reported that the final *G'* of the skim milk samples with the same concentration of added calcium lactate increased with increasing heating temperature. A similar study was reported that the calcium-induced milk gel structures were associated with the combination of heat treatment and the addition of calcium salts (Koutina et al., 2016). In addition, it was also found that a fine and dense gel network was formed by addition of 30mM calcium chloride to skim milk with pH adjustment to 6.6 and 4.6 (Koutina et al., 2016). The pH of the samples decreased with increasing concentrations of added calcium lactate, where it reached the lowest pH when 20mM calcium lactate was added in the milk solution. The addition of ionic calcium salts in milk not only increase the concentration of free calcium ions in milk but also reduce the pH of milk (Croguennec et al., 2016; Ramasubramanian et al., 2012).

Several studies were found that the milk gelation is influenced by many factors such as milk composition, pH, mineral distribution and temperature, particularly, temperature is an important factor in inducing milk gelation and causing the final structure of milk gels (Anema, 2009; Heertje et al., 1985). The caseins in milk have a higher calcium binding capacity compared to whey. It was found that applying heat on the casein at 80 ^{o}C for 60 min or 95 ^{o}C for 30 min, caused conformation changes of the caseins, which resulted in increasing calcium binding ability and interactions between the caseins and calcium ions (Pappas & Rothwell, 1991). Upon heating, the casein micelles dissociated into κ-casein-whey protein complexes and calcium-sensitive κ-casein-depleted casein micelles (Dumpler et al., 2017). The micelles became increasingly loose in structure and became larger and stronger hydrated compared to its native structure as the cleavage of the linkage between caseins and colloidal calcium phosphate increased (Aoki et al., 1990; Dumpler et al., 2017). This leads to an increase in hydrodynamic radius and facilitates the dissociation of κ-casein, which increases the attractive forces between micelles as larger calcium-sensitive contact areas become depleted in κ-casein are exposed (Dumpler et al., 2017). Therefore, the rate of aggregation into large protein structures increased.

The addition of calcium ions could screen charges and reduce electrostatic repulsion, which favoured hydrophobic interactions between the proteins. It was reported that increasing temperature resulted in stronger hydrophobic interactions and increased in whey and casein gel strength (Dalgleish, 1998; Horne, 1998; McClements & Keogh, 1995). The binding of calcium ions to caseins at various temperatures also plays a role in hydrophobic interactions, and this increases with increasing temperature (Dalgleish & Parker, 1980; Pappas & Rothwell, 1991). This was likely caused by an increasing number of calcium binding sites from the unfolding of the proteins at high temperatures (Pappas & Rothwell, 1991). Therefore, hydrophobic interactions increase and electrostatic repulsion reduced with increasing temperature, as a result of increased binding of calcium to proteins occurs (Horne & Lucey, 2014). The rheological results in this study also showed the final *G'* increased significantly as the concentration of calcium lactate was greater than 10mM ($p<0.05$). This agreed with the visual observation, at which firm gels were obtained when heating temperature was greater than 70 ^{o}C and the concentration of calcium lactate was 15mM or 20mM.

4.6 Conclusions

This study shows the addition of various concentrations of calcium lactate and/or temperature and time of heat treatment leads to changes in apparent physical structure and *G'* of the skim milk solutions. With increasing concentration of calcium lactate added, it promoted the formation of gels with higher final *G'* under the same heat treatment conditions. The formation of firm gels was observed when the concentration of calcium lactated was 15mM and 20mM under all heat treatment conditions. An increase in heating temperature and time led to a rise in final *G'* of the skim milk samples with the same concentration of added calcium lactate. In conclusion, increasing temperature and/or the addition of calcium lactate promoted and induced the formation of stronger milk gels.

Chapter 5 Effect of the addition of calcium lactate and polysaccharides to skim milk

5.1 Introduction

Chapter 4 presented the visual observation and storage modulus of the skim milk solutions with various concentrations of added calcium lactate under different heat treatments. Firmer gels were observed when the concentration of calcium lactate was above 10mM and the heating temperature was greater than 75 ºC. While no gels were formed at calcium lactate concentrations less than 10mM. This chapter focused on the effect of two concentrations of calcium lactate (10mM and 15mM) and on the properties of the skim milk solutions with added polysaccharides. Previously it was found that heat treatment at 75 ºC for 30 min produce calcium gels at these two concentrations and hence will be the concentrations used in this chapter.

Calcium lactate is more bioavailable than other calcium salts such as calcium acetate, calcium gluconate and calcium carbonate (Gaby, 2010; Guéguen & Pointillart, 2000). Adding calcium lactate to skim milk to form gels could provide a useful matrix for delivery of calcium in a dairy system. However, the texture of the calcium-induced gels with added calcium lactate was undesirable and therefore it is important to study the ways to improve the perceived texture. Polysaccharides have been widely used in dairy products to obtain desirable properties such as stability and improved texture (Corredig et al., 2011; Phillips & Williams, 2009; Stephen et al., 2006).

Three polysacchardies were selected, xanthan gum, guar gum and high acyl gellan gum. Xanthan gum is temperature insensitive and salt compatible, and it does not alter its properties and structure easily under heat treatment. It has been used as a stabiliser and thickener in dairy systems (Butler, 2016; Phillips & Williams, 2009). The use of xanthan gum in liquid food could improve the mouthfeel of the products. Guar gum is a non-ionic polysaccharide which would have little interaction with ions present in a solution. Guar gum is used as a stabiliser in dairy products due to its water binding properties and it can hydrate fully in a short period of time (Mudgil et al., 2014; Tuinier et al., 2000). High acyl gellan gum has been used a gelling agent in dairy systems. It can generate a high viscous gel with soft and elastic texture (Moritaka et al., 1995; Sworn, 2009). Those three gums have been used in different dairy systems and applications. Considering the type of

heat treatment was used in this study and it is a milk system. Xanthan gum, guar gum and high acyl gellan gum were therefore selected. It is hypothesised that the addition of polysaccharides to the calcium lactate-milk system will enhance the mouthfeel of the skim milk gels. Therefore, it is important to investigate the rheological properties and other characteristics of the calcium-induced gels with added polysaccharides.

The texture and appearance of the skim milk solutions with various concentrations of added calcium lactate was firstly assessed in Chapter 4 with visual observation of the gels and informal assessment of the mouthfeel. However, it was found that the samples were gritty, and the gels appeared to break easily. Thus, the next step was to investigate the addition of polysaccharides. A number of polysaccharides were considered based on their gelling properties and compatibility with milk proteins and calcium ions. Xanthan gum is a negatively charged gum with heat durability and it does not alter or lose its properties at high temperatures (Dickinson, 2005; Phillips & Williams, 2009). Xanthan gum also possesses good thickening and stabilising properties, which is used to enhance the texture of food products. High acyl gellan gum is an anionic polysaccharide. It is used in milk beverages such as chocolate milk to form a fluid gel with improved texture and the gels can be formed after cooling a hot solution from 70 ^{o}C and 80 ^{o}C (Phillips & Williams, 2009). Guar gum is neutral and it has good solubility in hot and cold solutions and exhibits excellent stabilising and gelling properties (Mudgil et al., 2014). Therefore, those three polysaccharides were selected and investigated to determine their impact on the calcium-induced milk gels as produced in Chapter 4. It is hypothesised that the addition of the selected gums would provide stabilisation in a calcium-added skim milk system and improve the mouthfeel of milk gels. Various concentrations of the three polysaccharides were added in the calcium-added skim milk samples and a number of tests were performed to study the properties of the skim milk gels.

The objectives of this chapter were to study the physical, rheological and microscopic properties of the skim milk samples with the added of calcium lactate and selected polysaccharides.

5.2 Materials & Methods

The procedures and experimental plans of this study are summarised in a flow diagram as presented in Figure 5.1.

5.2.1 Sample preparation

The skim milk solutions were prepared prior to the addition of polysaccharides. The three polysaccharides were in a powder form and they were weighed accordingly at various concentrations. The concentration of each polysaccharide was measured in wt %. Once each polysaccharide was weighed, it was directly added in each skim milk solution and continuously mixed with a high shear mixer. The procedure is outlined in Sections 3.4 and 3.5.

5.2.2 Preheat treatment and heat treatment to induce milk gels

The skim milk samples were prepared prior to the heat treatment. All samples were pre heated at 90±2 ºC for 10 min. For the heat treatment, the samples at 20±2 ºC were heated to 75±2 ºC and held for 30 min and rapidly cooled to 20±2 ºC. Samples were evaluated visually and also evaluated informally for their mouthfeel and texture as described in Section 3.7.1.

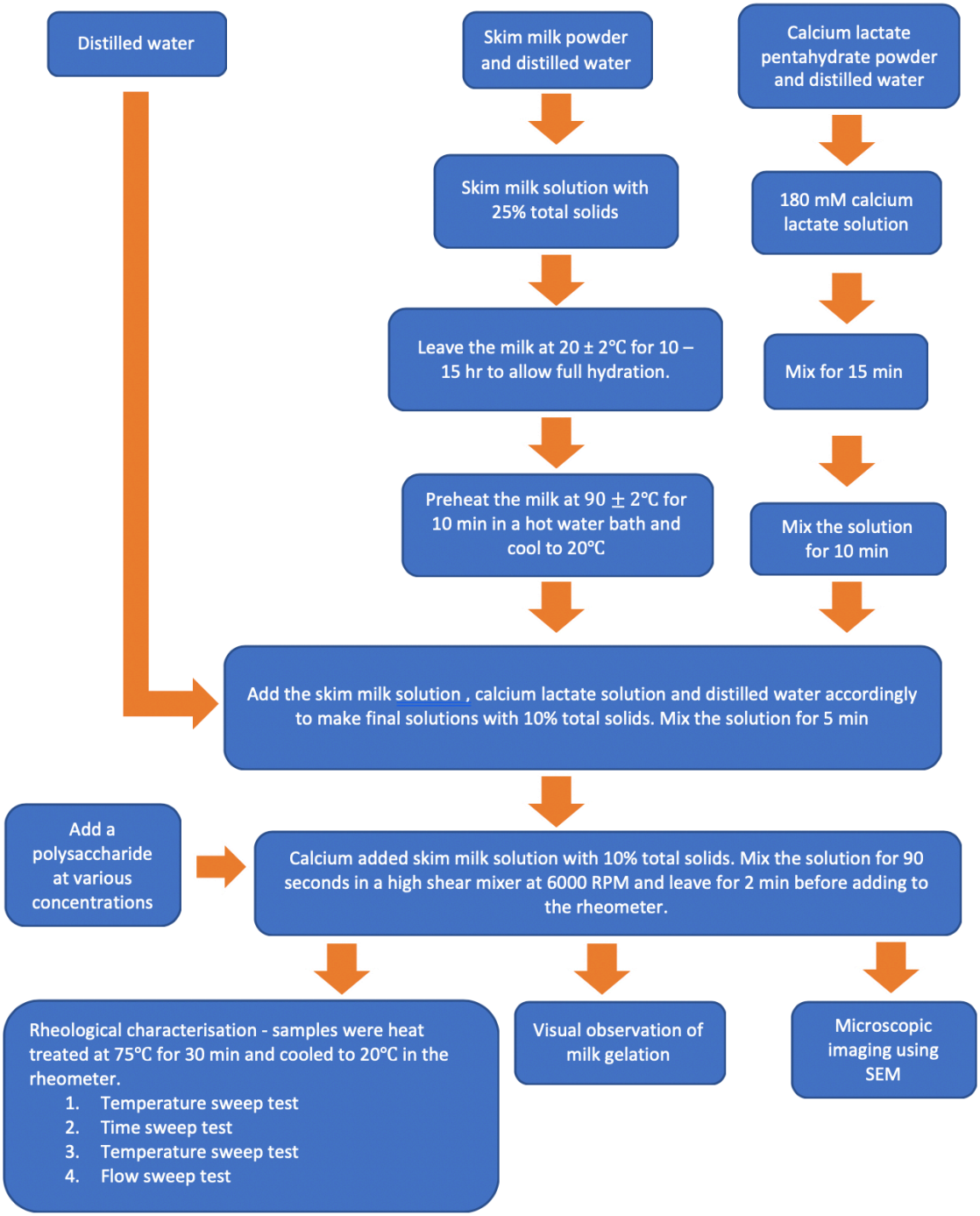

Figure 5.1 Flow diagram for sample preparation of skim milk with added calcium lactate and polysaccharides.

5.2.3 Rheological characterisation of calcium and polysaccharide skim milk gels

Rheological properties of calcium-added skim milk with polysaccharides were characterised by a rheometer. Various tests as outlined in Section 3.7.2. The temperature sweep test and time sweep test were carried out and flow sweep test were performed after the second temperature sweep test for skim milk solutions with added polysaccharides. Firstly, the gel properties were evaluated by determining the *G'* of solutions at different temperatures and times. Secondly, the flow properties and relationship between shear stress and shear rate was determined. The concentration of each polysaccharide used and the corresponding concentrations of calcium lactate of each sample is presented in Table 5.1. The rheology procedure can be followed in Section 3.7.2.

Table 5.1 Calcium-added milk samples with xanthan gum (XG), high acyl gellan gum (HAGG) and guar gum (GG).

Samples with polysaccharides	**Concentration of added calcium lactate(mM)**	**Concentration of polysaccharide (wt %)**
XG 1	10	0.1
XG 2	10	0.3
XG 3	10	0.5
XG 4	15	0.1
XG 5	15	0.3
XG 6	15	0.5
HAGG 1	10	0.1
HAGG 2	10	0.2
HAGG 3	10	0.3
HAGG 4	15	0.1
HAGG 5	15	0.2
HAGG 6	15	0.3
GG 1	10	0.1
GG 2	10	0.2
GG 3	10	0.3
GG 4	15	0.1
GG 5	15	0.2
GG 6	15	0.3

5.2.4 Microscopic evaluation of calcium and polysaccharide added skim milk gels

The microstructure of various milk gels was observed using SEM and the objective was to gain some insights into their microscopic properties in a three-dimensional matrix and investigate any correlations with physical and rheological properties. The type and concentration of each polysaccharide and concentration of calcium lactate added in samples investigated with SEM are presented in Table 5.2. The procedure and protocol of SEM gel preparation can be seen in Section 3.7.3.

Table 5.2 The concentration of each polysaccharide and added calcium lactate added in the SEM evaluated samples.

Sample	The concentration of added calcium lactate (mM)	The type of polysaccharide	The concentration of the polysaccharide (wt %)
1	10	N/A	N/A
2	15	N/A	N/A
3	15	Xanthan gum	0.1
4	15	Xanthan gum	0.3
5	15	Xanthan gum	0.5
6	15	High acyl gellan gum	0.3
7	15	Guar gum	0.3

5.3 Results and Discussion

5.3.1 The effect of xanthan gum on calcium-added skim milk

5.3.1.1 Visual observation of milk gels in vials and on petri dishes

The skim milk solutions in glass vials were heat to at 75±2 ^{o}C and held at this temperature for 30 min then cooled to 20±2 ^{o}C. Any physical changes were observed visually. If gelation occurred in the glass vials, after inversion the gel remained at the top of the vial (Figure 5.2). Results in Chapter 4 showed that the addition of 10mM and 15mM of calcium lactate to skim milk formed firm gels after heating to 75 ^{o}C and holding for 30 min. In this study, 10mM and 15mM calcium lactate were added in the skim milk samples with various concentrations of polysaccharides.

It was found earlier in Section 4.4.1 and 4.4.2 that at 10mM and 15mM added calcium lactate after heating at 75 ^{o}C for 30 min, a gel was observed and the final *G'* after cooling was 4.63±0.08 Pa and 36.03±3.03 Pa, respectively. Xanthan gum was added at 0 % to 0.5 wt % to calcium-added skim milk solutions. When 0.1 wt % xanthan gum was added to

the skim milk with 10mM and 15mM added calcium lactate, a gel was observed (Figure 5.2). When the xanthan gum concentration was increased to 0.3 wt % and 0.5 wt %, the skim milk samples even with added calcium lactate did not form a gel and remained in a liquid, and yet they appeared to be more viscous than the control with no added xanthan gum.

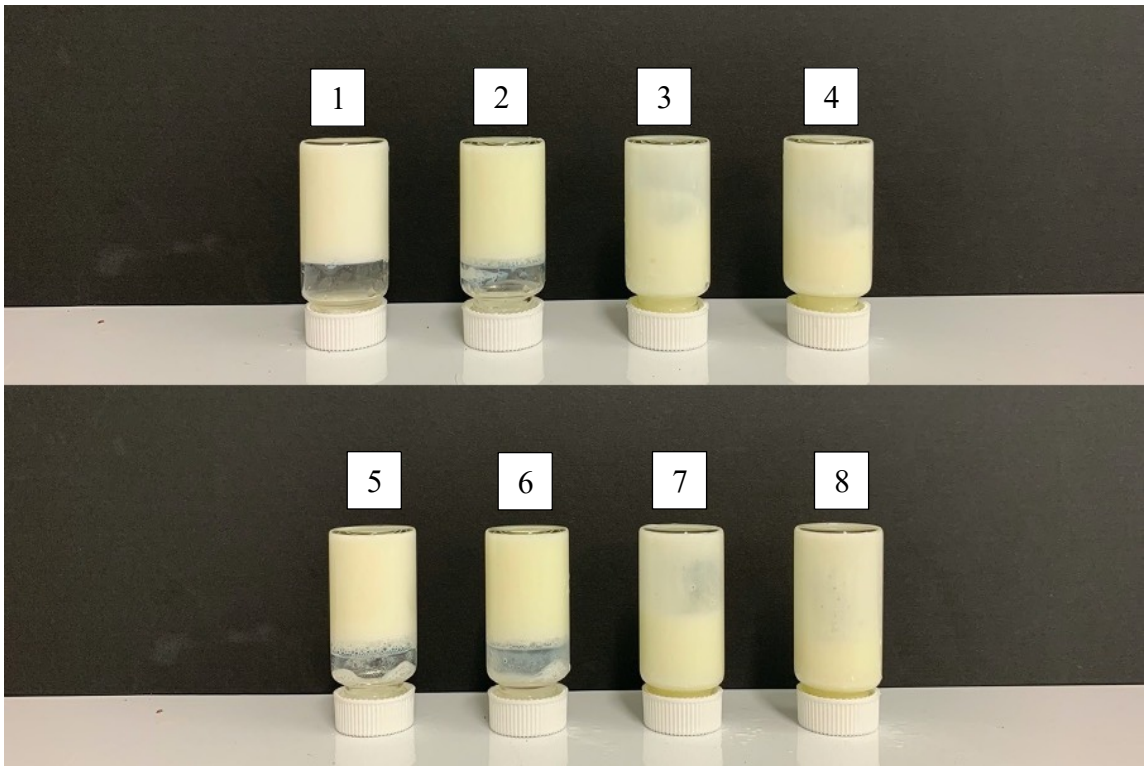

Figure 5.2 Skim milk samples with added calcium lactate and xanthan gum after heating at 75 ºC for 30 min. 1, 2, 3, 4 10mM calcium lactate; 5, 6, 7, 8 15mM calcium lactate. 1. no added xanthan gum. 2. 0.1% xanthan gum; 3. 0.3% xanthan gum; 4. 0.5% xanthan gum; 5. no added xanthan gum; 6. 0.1% xanthan gum; 7. 0.3% xanthan gum; 8. 0.5% xanthan gum.

To carry out further investigation of the texture of the gels and calcium-added skim milk samples with the xanthan gum were prepared in preformed plastic moulds and then visually observed when inverted into a plastic petri dish. The rectangular plastic moulds used are shown in Figure 5.3.

After heat treatment and cooling to 20 ºC, the skim milk samples with added calcium lactate and a polysaccharide were transferred to petri dishes for visual observation and informal evaluation of the mouthfeel. Table 5.3 provides definitions used to describe the solution or gels formed.

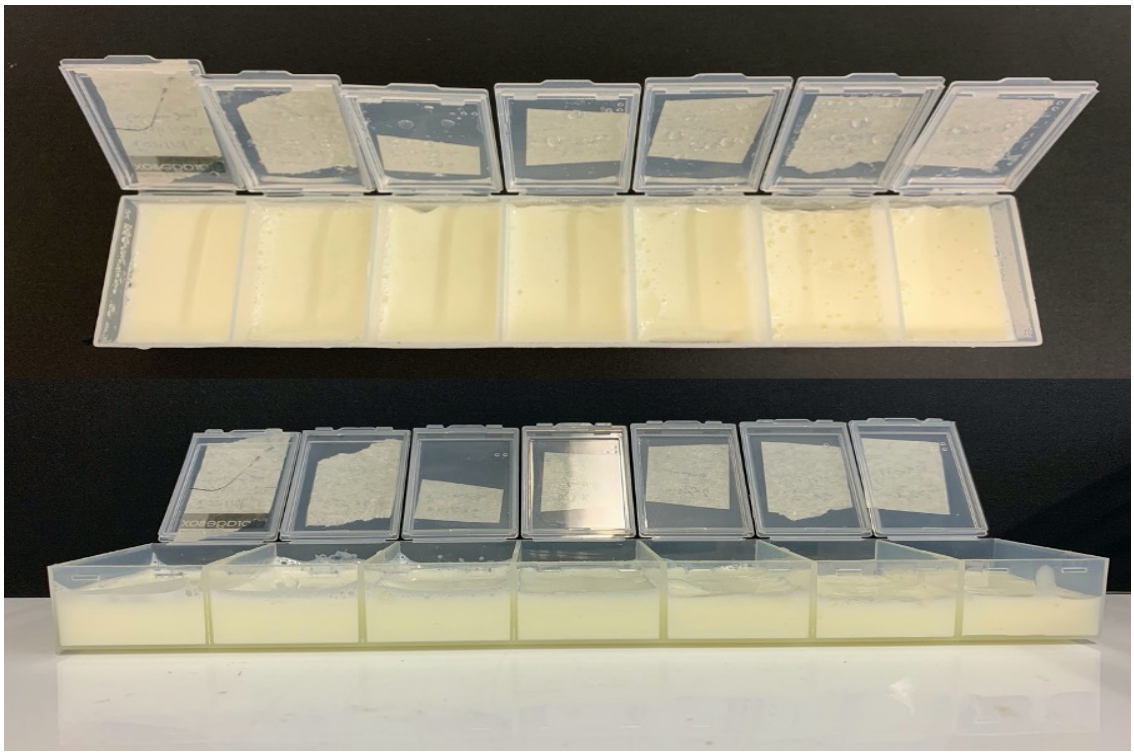

Figure 5.3 The rectangular plastic moulds used for heat treatment and evaluation of any gel formation in skim milk plus calcium lactate samples.

Table 5.3 Definition of gel types.

Gel type	**Definition**
Hard gel	The gel is firm but fractures easily.
Firm gel	The gel is firm but does not fracture easily
Soft gel	The gel is soft but syneresis is observed.
Semi-liquid gel	The solution remains mostly liquid with small gel aggregates present.
Liquid	The solution is liquid
N/A	Not available. This solution was not assessed.

Firm milk gels were formed when no xanthan gum was added to the skim milk with added calcium lactate (10mM and 15mM). With the addition of xanthan gum, the samples with 0.1% added xanthan gum formed a soft gel with 10mM calcium lactate, while a firm gel formed with 15mM added calcium lactate (Table 5.4, Figure 5.4). As the xanthan gum concentration was increased to 0.3%, the skim milk solution with added calcium lactate remained a liquid with no gel formation with 10mM calcium lactate (Table 5.4, Figure 5.4). However, the samples with 15mM added calcium lactate appeared to be more viscous when compared with the samples with 10mM added calcium lactate (Table 5.4,

Figure 5.4). After the addition of 0.5% xanthan gum added to the skim milk samples, for 10mM calcium lactate, a semi-liquid gel with small aggregates was observed (Table 5.4, Figure 5.4). A smooth mouthfeel was perceived when 0.5% xanthan gum added to the skim milk with 10mM and 15mM calcium lactate. The rational of the formation of the gels with the addition of xanthan gum was unknown, there may be interactions or competition between calcium ions, xanthan gum molecules and milk proteins (Baumgartner et al., 2008; Bergmann et al., 2008; Groves & Chaw, 2015).

Table 5.4 Description of the gels observed with added calcium lactate and xanthan gum after being heated and held at 75 ºC for 30 min and cooled to 20 ºC, based on visual observation and evaluation of mouthfeel.

Concentration of xanthan gum (wt %)	**10mM calcium lactate**	**15mM calcium lactate**
0	Hard gel	Hard gel
	Gritty and sandy mouthfeel	Gritty and sandy mouthfeel
0.05	N/A	Hard gel
		Sandy mouthfeel
0.1	Soft gel or semi-solid gel	Hard gel
	Smooth mouthfeel	Sandy mouthfeel
0.15	N/A	Semi-liquid gel with small aggregates
		Sandy and water mouthfeel
0.2	Liquid gel	Semi-liquid gel
	Smooth mouthfeel	Sandy and watery mouthfeel
0.3	Liquid gel	Semi-liquid gel
	Smooth and watery mouthfeel	Smooth mouthfeel with small lumps
0.4	N/A	Liquid and viscous gel
		Smooth mouthfeel
0.5	Liquid and viscous gel	Liquid and viscous gel
	Smooth mouthfeel	Smooth mouthfeel

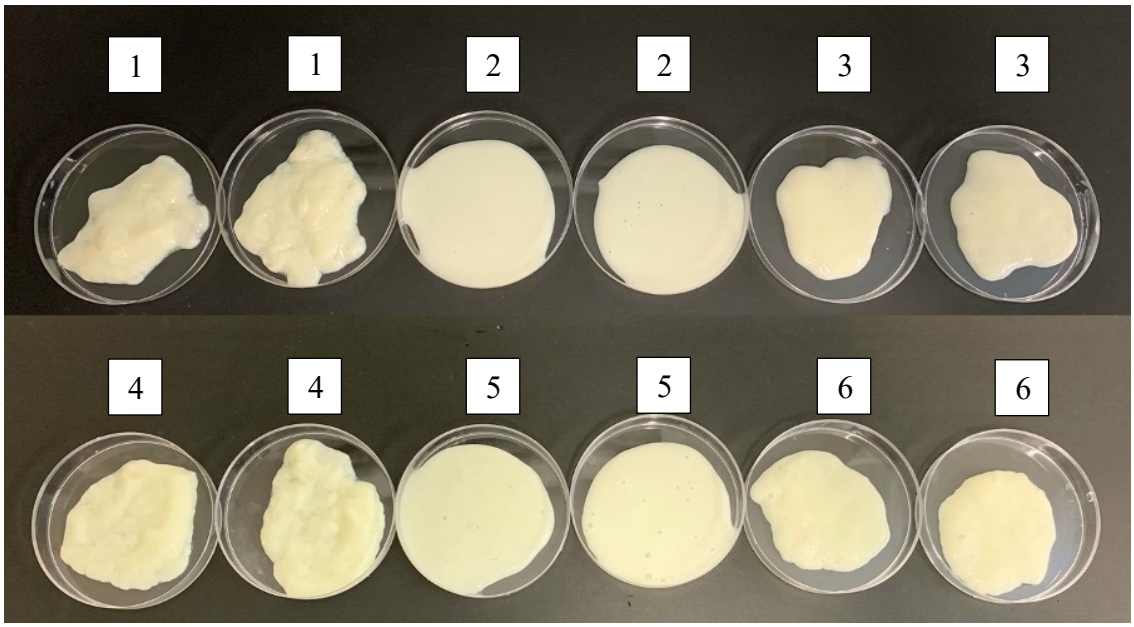

Figure 5.4 Skim milk samples with added xanthan gum and calcium lactate on petri dishes after heat treated at 75±2 ºC and held for 30 min and cooled to 20±2 ºC. 1. 10mM calcium lactate and 0.1% xanthan gum; 2. 10mM calcium lactate and 0.3% xanthan gum; 3. 10mM calcium lactate and 0.5% xanthan gum; 4. 15mM calcium lactate and 0.1% xanthan gum; 5. 15mM calcium lactate and 0.3% xanthan gum; 6. 15mM calcium lactate and 0.5% xanthan gum.

For further investigations on the effect of xanthan on the skim milk solutions, xanthan gum concentrations of (0%, 0.05%, 0.1%, 0.15%, 0.2%, 0.3%, 0.4%, 0.5 wt %) were added to skim milk solutions with 15mM calcium lactate and heat treated. The results from the visual observation of the formed gels are shown in Figure 5.5 and descriptions are provided in Table 5.4. Firm milk gels were formed when no xanthan gum was added to the skim milk solutions. When the concentration of xanthan gum added was 0.1%, soft gels were formed with lumps and a small amount of syneresis. Particularly, the milk solutions became more like liquid gels with small lumps when the concentration of xanthan gum was increased to 0.15%. On further increase of xanthan gum to the skim milk solutions, after heat treatment they remained in a viscous liquid state. The results may indicate that there were different phase transitions or gelation mechanisms taking place with increasing concentrations of added xanthan gum from 0% to 0.5%. The transition from semi-solid to viscous liquid occurred at approximately 0.15% xanthan gum. This would be further investigated from the rheological characterisation and microscopic imaging. It was observed that the gels at all concentrations appeared to have water separation and small amounts of syneresis after they were stored at 4 ºC for 12 hours (Figure 5.6). In particular, the solid gels with no xanthan and 0.05% added xanthan gum appeared to have more water separation.

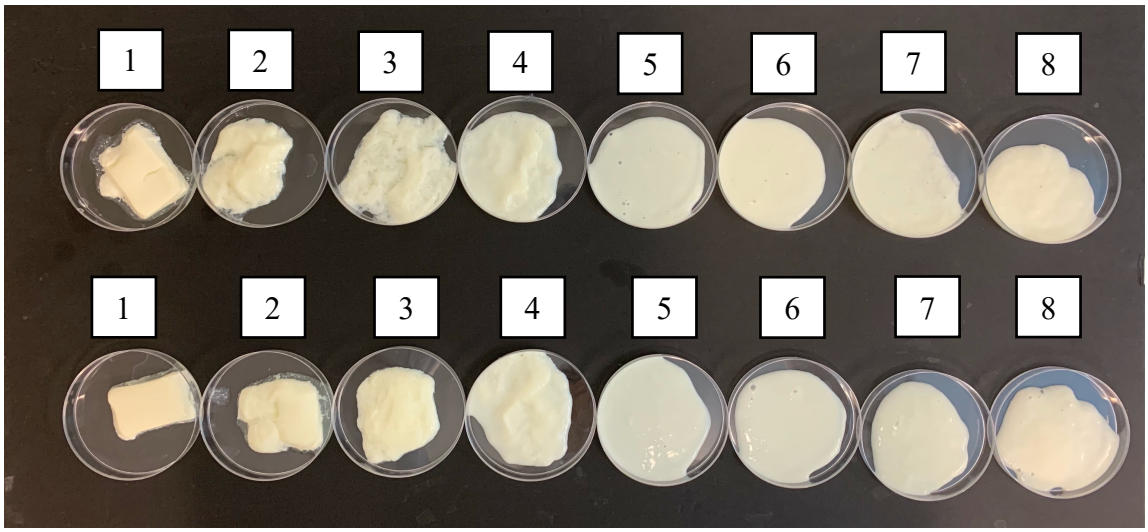

Figure 5.5 Skim milk samples with added xanthan gum and 15mM calcium lactate on petri dishes after heat treated at 75±2 ºCand held for 30 min and cooled to 20±2ºC. 1. no xanthan gum; 2. 0.05% xanthan gum; 3. 0.1% xanthan gum; 4. 0.15% xanthan gum; 5. 0.2% xanthan gum; 6. 0.3% xanthan gum. 7. 0.4% xanthan gum; 8. 0.5% xanthan gum.

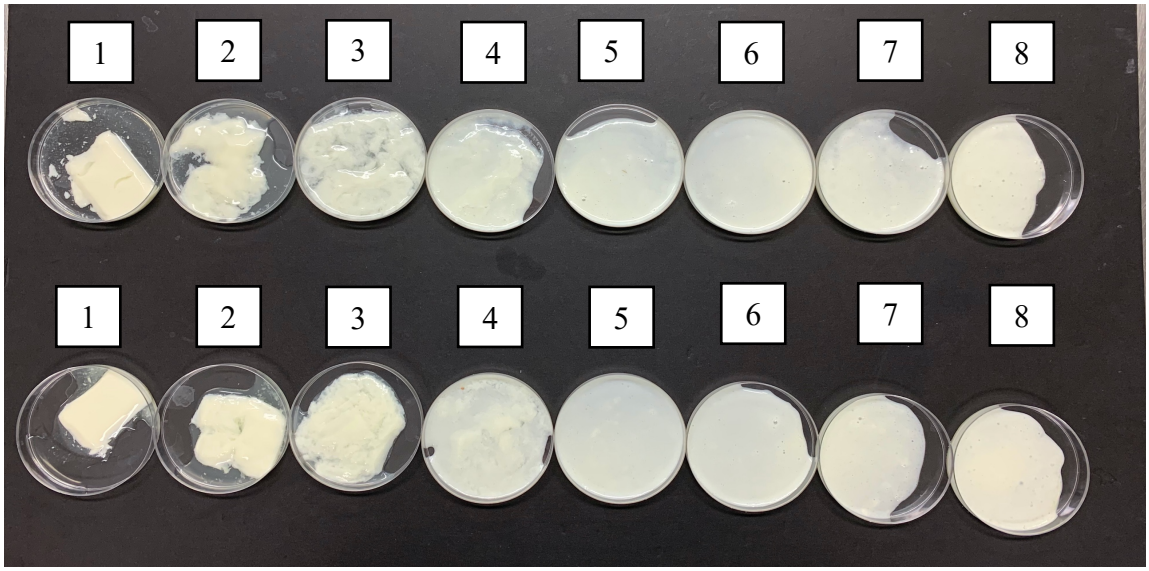

Figure 5.6 Heat treated skim milk samples with added xanthan gum and 15mM calcium lactate on petri dishes after being stored 4 ºC for 12 hours. 1. no xanthan gum; 2. 0.05% xanthan gum; 3. 0.1% xanthan gum; 4. 0.15% xanthan gum; 5. 0.2% xanthan gum; 6. 0.3% xanthan gum. 7. 0.4% xanthan gum; 8. 0.5% xanthan gum.

5.3.1.2 Rheological characterisation of calcium-added skim milk with xanthan gum

Skim milk solutions containing 10mM or 15mM calcium lactate were mixed with xanthan gum and immediately placed in the rheometer geometry before heating to 75 ºC held at 75±0.5 ºC for 30 min and then cooled to 20±0.5 ºC. The storage modulus was monitored with temperature sweeps and a time sweep tests during heating, holding and cooling period.

5.3.1.2.1 Heating period and holding period

The temperature sweeps for the heating period for skim milk samples with 10mM and 15mM calcium lactate and added xanthan gum are shown Figure 5.7. For both 10mM and 15mM added calcium lactate, the *G'* of the samples with no xanthan gum and with 0.1% xanthan gum remained unchanged for the entire heating period and the *G'* values were less than 1Pa, hence gels were not yet formed during the heating period (Figure 5.7). However, the *G'* of the samples with 0.5% xanthan gum decreased with increasing temperature.

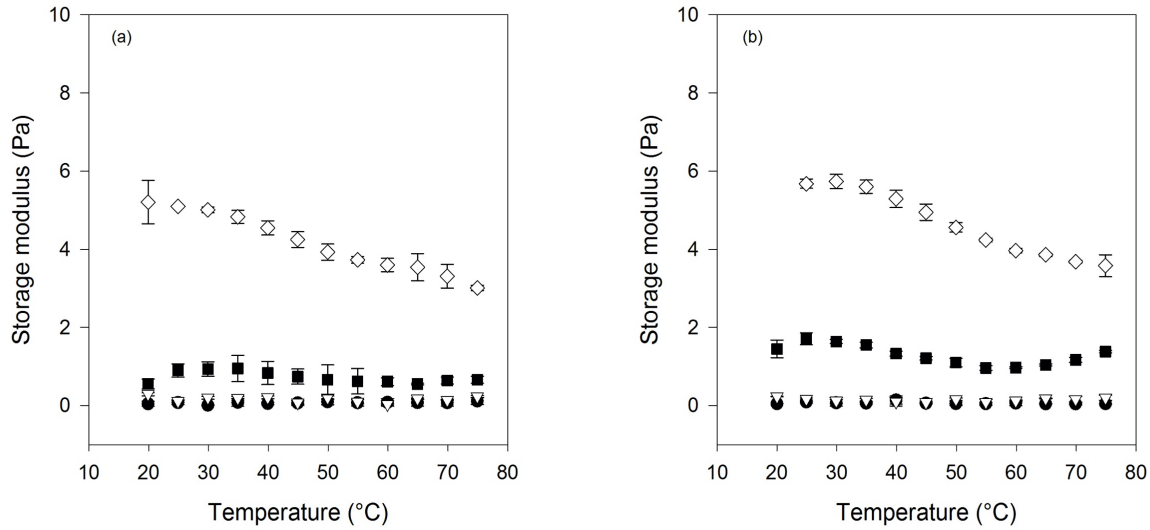

Figure 5.7 Changes in *G'* during heating of the samples with added calcium lactate and xanthan gum heated to 75±0.5 ºC. (a) 10mM added calcium lactate; (b) 15mM added calcium lactate. ● without xanthan gum; ▽ 0.1% xanthan gum; ■ 0.3% xanthan gum; ◇0.5% xanthan gum. Each data point is a mean ± standard deviation (n=2 replicates).

During the holding period, the samples were held at 75±0.5 ºC for 30 min to observe the change in *G'* of the samples with the added xanthan gum, shown in Figures 5.8. During the holding time, with 10mM added calcium lactate and no added xanthan gum, there was small change in *G'* but not significant ($p>0.05$). This agreed with the holding time results shown in Chapter 4 (Section 4.4.2.2).

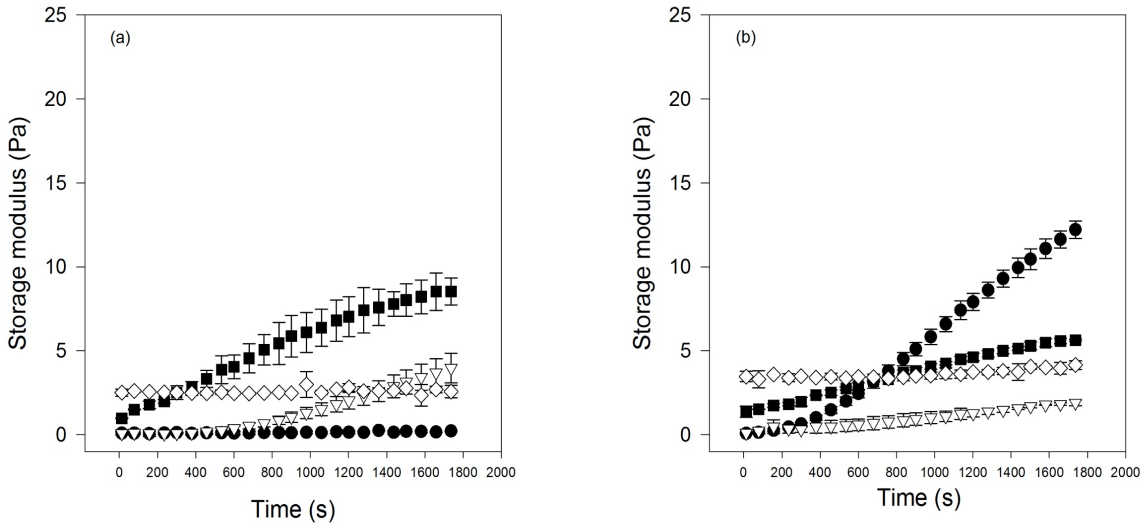

Figure 5.8 Changes in *G'* with holding time of the samples with added calcium lactate and xanthan gum held at 75±0.5 ºC for 30 min. (a) 10mM added calcium lactate; (b) 15mM added calcium lactate. ● without xanthan gum; ▽ 0.1% xanthan gum; ■ 0.3% xanthan gum; ◇0.5% xanthan gum. Each data point is a mean ± standard deviation (n=2 replicates).

The *G'* of the samples with 10mM added calcium lactate increased with holding time when 0.1% and 0.3% xanthan gum were added (Figure 5.8(a)). In contrast, the *G'* values remained almost unchanged in the sample with 0.5% xanthan gum. Similarly, with 15mM added calcium lactate, there was an increase in *G'* of the samples with increasing in concentration from 0% to 0.3% of xanthan gum (Figure 5.8(b)). The *G'* of the sample with 0.5% also showed no an apparent increase or drop, which did not match with the expectation and as the final *G'* was expected to increase with increasing concentration of xanthan gum. Hence, this sample with 0.5% xanthan gum was repeated four times on the rheometer and similar results were obtained. This indicated there may be interactions or competition between xanthan gum, calcium ions and proteins. The *G'* of the sample without xanthan gum increased with holding time when 15mM calcium lactate was added (Figure 5.8(b)). This suggested that the additional calcium ions influenced the gelling mechanisms in the skim milk and therefore impacting on the *G'*.

5.3.1.2.2 The change of *G'* in cooling period

The change in *G'* of samples with 0.1, 0.3 and 0.5% added xanthan gum is shown in Figure 5.9. The sample with no added xanthan gum on cooling, increased in *G'* to 13.69±2.25 Pa and 29.67±1.06 Pa for 10mM and 15mM added calcium lactate, respectively. The samples with 0.1% added xanthan gum increased to 2.64±1.25 Pa and 7.03±1.32 Pa for 10mM and 15mM calcium lactate, respectively. The samples with 0.3%

added xanthan gum increased to 18.96±1.85 Pa and 22.91±1.42 Pa for 10mM and 15mM calcium lactate, respectively. The samples with 0.5% added xanthan gum did not achieve the same *G'* as for 0.3% xanthan gum, their final *G'* was 8.96±0.60 Pa and 14.01±2.29 Pa for 10mM and 15mM calcium lactate, respectively.

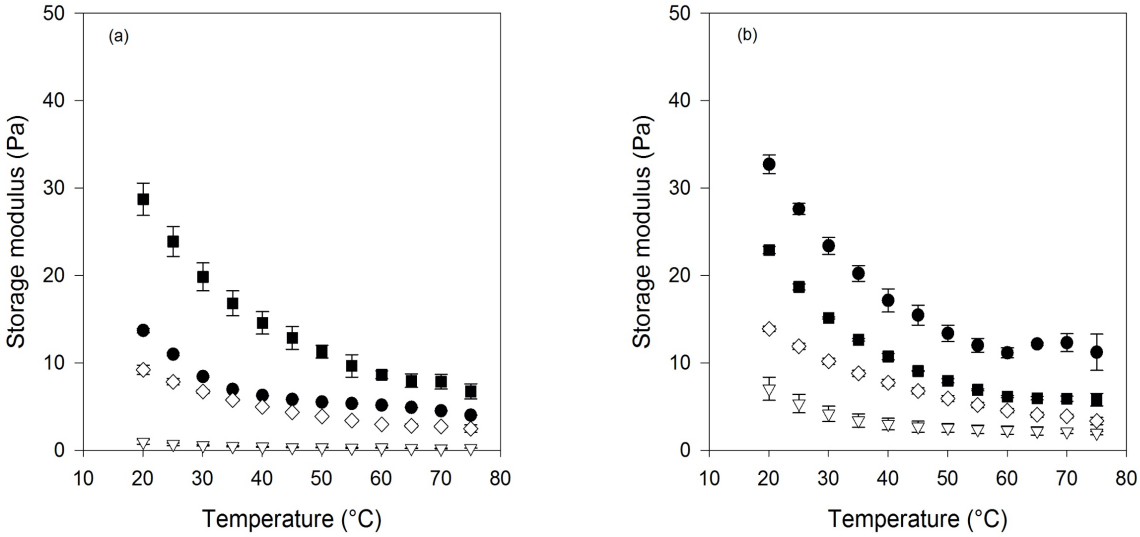

Figure 5.9 Changes in *G'* during cooling of the samples with added calcium lactate and xanthan gum cooled to 20±0.5 ºC. (a) 10mM added calcium lactate; (b) 15mM added calcium lactate. ● without xanthan gum; ▽ 0.1% xanthan gum; ■ 0.3% xanthan gum; ◇0.5% xanthan gum. Each data point is a mean ± standard deviation (n=2 replicates).

The final *G'* of the samples were further investigated with additional concentrations of added xanthan gum to samples with 15mM calcium lactate. The final *G'* of the samples with 0.1%, 0.15%, 0.2%, 0.25% and 0.4% xanthan gum were determined after the same heating, holding and cooling protocol, to further study the change in final *G'* at different concentrations of xanthan gum (Table 5.4). Samples were with no added calcium lactate at selected concentrations of xanthan were also evaluated.

The addition of xanthan gum in the skim milk samples with added calcium lactate resulted in an irregular change in final *G'* and the any significance differences in final *G'* were analysed by one-way ANOVA, results are summarised in Appendix 3. The final *G'* after cooling, all of samples with added xanthan gum, without calcium lactate and with 10mM and 15mM calcium lactate is shown in Figure 5.10.

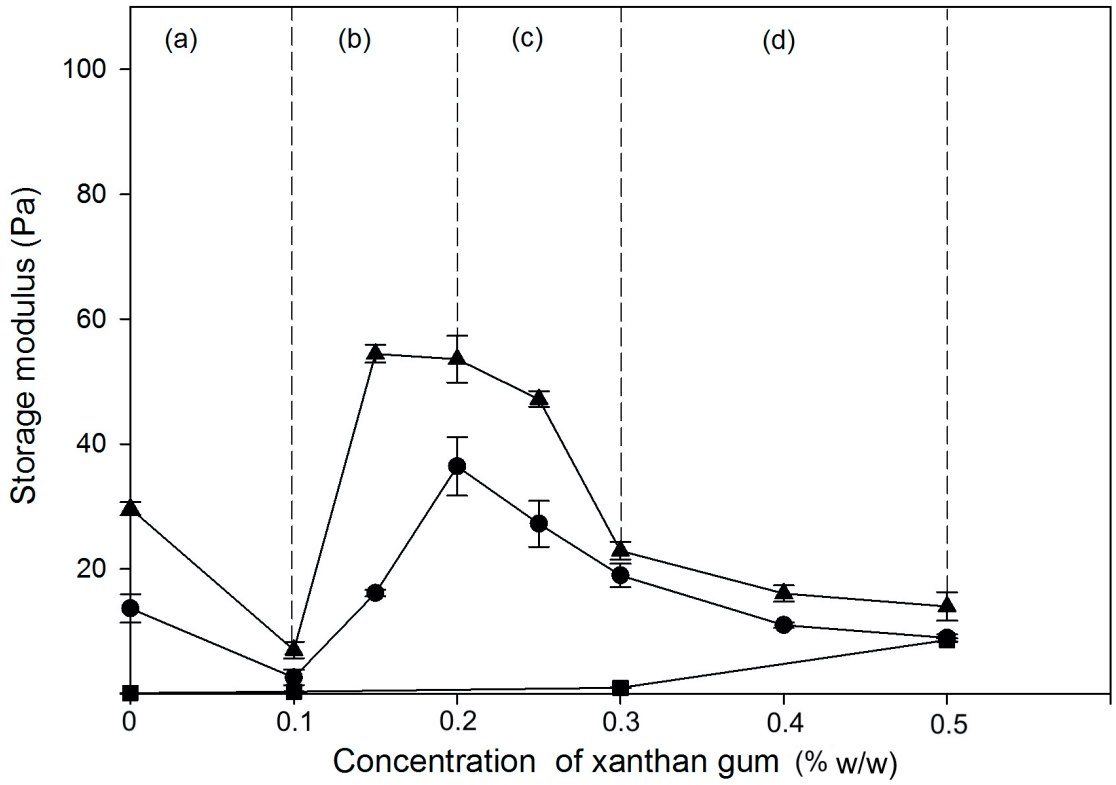

Figure 5.10 The final *G'* of the samples with various concentrations of xanthan gum. ■ without calcium lactate; ● 10mM added calcium lactate; ▲ 15mM added calcium lactate. Each data point mean ± standard deviation (n=2 replicates). (a), (b), (c) and (d) are different zones of change in final *G'* of the samples at different concentrations of xanthan gum.

In Figure 5.10, the plot was divided into four regions (a), (b), (c) and (d) and each region may involve a different mechanism. First, a drop in final *G'* in zone (a) where the concentration of added xanthan gum went from 0% to 0.1%. Any interaction may only occur between milk proteins and added calcium ions when no xanthan gum was added in the skim milk sample (Hongsprabhas & Barbut, 1996; Ju & Kilara, 1998a, 1998b; Lin et al., 2018). A greater reduction in *G'* occurred with 15mM calcium lactate as the final *G'* for a system with calcium only addition formed a higher final *G'* than a 10mM addition of calcium lactate. The drop in the final *G'* occurred at 0.1% added xanthan gum may have occurred because the xanthan gum chains bind with free calcium ions in the solution, yet the interactions were not enough to bind with all the free calcium ions (Mohammed et al., 2007). The effect of added calcium ions may be reduced as less free calcium ions were present in the serum as they interact with the xanthan chains present. It was reported that the addition of small counterions such as Ca^{2+} reduces the charge of the xanthan chain, this resulted in a reduction in the viscosity of xanthan gum solutions and an increase in ionic strength at low polymer content (Baumgartner et al., 2008; Zhong et al., 2013). The

molecular chains of xanthan gum in the presence of calcium ions forms ordered helical molecular conformations and this occupies a smaller hydrodynamic volume, which leads to decrease in viscosity (Zhong et al., 2013).

For 10mM added calcium lactate, the final *G'* increased as the concentration of xanthan gum increased from 0.1% to 0.2%, the final *G'* reached its highest point at 36.41±4.68 Pa with 0.2% added xanthan gum. And yet it began decreasing as the xanthan gum concentration increased from 0.2% to 0.5%. As a check skim milk samples with no added calcium lactate were tested with 0.1, 0.3 and 0.5% xanthan gum. Skim milk with 0.5% xanthan achieved a final *G'* of 8.62±0.26 Pa, significantly higher that achieved at 0.3% xanthan with no added calcium lactate ($p<0.05$). Several questions were raised from the results of *G'* in in sections (b), (c) and (d) in Figure 5.10, in samples with added calcium lactate. Firstly, the *G'* of the samples with increasing concentrations of xanthan gum exhibited unusual behaviours, at which *G'* did not increase with increasing concentration of xanthan gum. This confirms the visual observations in Section 5.3.1.1, where liquid milk samples were observed at the concentrations of xanthan gum was greater than 0.2%. With different concentrations of added calcium lactate, the *G'* had different responses to various concentration of xanthan gum. It is postulated that this pattern of the change in final *G'* of the samples with added calcium lactate and xanthan gum is the result from the interactions between milk proteins, calcium ions and xanthan gum molecules (Bergmann et al., 2008; Bryant & McClements, 2000; Groves & Chaw, 2015; Le & Turgeon, 2013; Mohammed et al., 2007).

For 15mM calcium lactate, the final *G'* decreased at concentrations greater than 0.15% added xanthan gum and only achieved a final *G'* of 14.01±2.29 Pa with 0.5% xanthan gum. With the addition of 10mM calcium lactate, the final *G'* of the samples followed a similar pattern as 15mM calcium lactate though the final *G'* achieved were significantly lower ($p<0.05$) except for concentrations of 0.3% and 0.4% added xanthan gum, they were not significantly different ($p>0.05$) (Appendix 3). The final *G'* of the samples with 10mM calcium lactate and 0.1% xanthan gum was significantly smaller ($p<0.05$) than the solutions with 0.15%, 0.2%, 0.25% and 0.3% xanthan gum.

In zone (b), an increase in final *G'* was observed for both 10mM and 15mm concentrations of calcium lactate at 0.15% and 0.2% xanthan gum, respectively. The final *G'* of the samples with 15mM added calcium lactate reached its peak with 0.15% xanthan gum, and the final *G'* of the sample with 10mM added calcium lactate reached its highest point with 0.2% added xanthan gum. This may be caused by the interaction between xanthan gum molecule, calcium ions and milk proteins (Groves & Chaw, 2015; Mohammed et al., 2007; Zhong et al., 2013). When the calcium ions approach the pyruvate unit and guluronic acid in xanthan gum molecules, the charge of the molecule chain reduces and this elongates the molecule chains through electrostatic interaction, leading to a decrease in viscosity (Zhong et al., 2013). When the final *G'* reaches the peak point, xanthan gum molecules may be bound to the free calcium ions and form inter-molecular or intra-molecular crosslinks of xanthan and calcium ions (Mohammed et al., 2007; Turgeon et al., 2007). As a result, the interaction of xanthan polymer chains was promoted and increased, which was likely to induce a more rigid structure of xanthan and calcium complex (Turgeon et al., 2007; Zhong et al., 2013). Hence, the final *G'* rise in zone (b).

In zone (c), it showed a steady decrease in final *G'* of the samples with 15mM added calcium lactate and the final *G'* of the sample with 10mM added calcium lactate began to decrease at 0.2% xanthan gum. The decrease in *G'* from 0.2% as a result of the addition of xanthan gum at higher concentrations and this may lead to excessive xanthan gum molecules which do not bond with calcium ions in the system. The xanthan gum molecules may begin acting strongly as a stabiliser and exhibited its thickening properties, in which it increased the viscosity and hence reducing the opportunities for any free calcium ions to bind with the xanthan gum molecules. This was in agreement with the visual observation of the milk gels. At higher concentration (above 0.2%) of xanthan gum in the skim milk samples, the samples became more viscous with increasing concentration of xanthan gum.

Lastly, the final *G'* continued to decrease with increasing concentration of xanthan gum in zone (d). It was visually observed the viscous solutions and they became more viscous as the concentration of xanthan gum increased from 0.2% to 0.5% (Figure 5.10). The property of xanthan gum may dominate the properties of the liquid samples and the relationship between shear stress and shear rate is important to study particularly in the

liquid samples. Therefore, the changes in shear stress of the samples at various shear rates (1–100 s^{-1}) were investigated and the change of shear stress at different shear rates in log scale for the samples with added xanthan gum is shown in Figure 5.11.

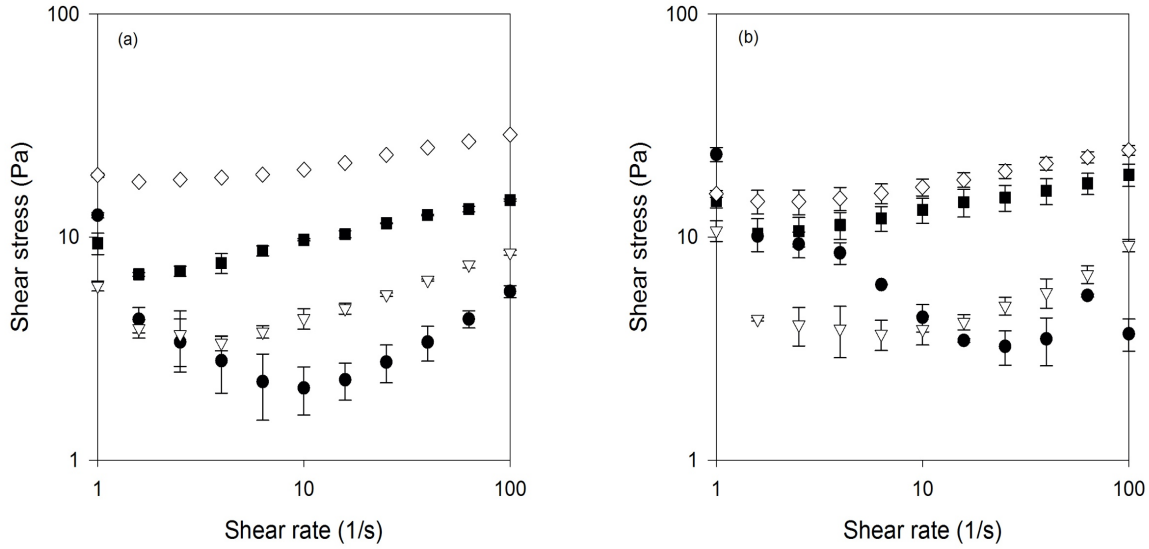

Figure 5.11 The change in shear stress of the samples with added xanthan gum and calcium lactate with increasing shear rates in log scale. (a) 10mM calcium lactate. (b) 15mM calcium lactate. ● no xanthan gum; ▽ 0.1% xanthan gum; ■ 0.3% xanthan gum; ◇0.5% xanthan gum.

With the addition of 10mM calcium lactate, the shear stress of the samples with no xanthan gum showed an unusual change, where the shear stress decreased from 1 to 10 s^{-1} and then increased from 10 to 100 s^{-1} (Figure 5.11 (a)). This suggested that the sample with no xanthan gum did not exhibit flow properties but formed a gel as observed after the temperature and time sweeps on the rheometer. The samples with 0.3% and 0.5% xanthan gum showed a steady increase in shear stress with increasing shear rate. These relationships were found to be linear with R^2 for 0.3% and 0.5% xanthan gum at 0.91 and 0.90, respectively. This showed at that 0.3% and 0.5% xanthan gum the skim milk solution exhibited non-Newtonian behaviour and can be classed as pseudoplastic (Figure 5.11(a)).

For the samples with 15mM added calcium lactate, it showed similar flow patterns to 10mM calcium lactate for the samples with 0.3% and 0.5% xanthan gum (Figure 5.11(b)). They also showed a linear relationship between shear stress and shear rate with R^2 values of 0.90 and 0.85, respectively. The samples with 0.3% and 0.5% xanthan also showed non-Newtonian properties. However, the samples with no xanthan gum and 0.1% did not

show any flow properties but a firm gel was observed at both concentrations of xanthan gum.

Overall, when the concentration of added xanthan gum was less than 0.1%, the addition of calcium lactate had a greater influence on the properties of the milk gels in the system. At concentrations of xanthan gum between 0.1 and 0.2%, the combination of calcium lactate and xanthan gum resulted in stronger gels, as shown by the higher final *G'* values. When the concentration of xanthan gum increased over 0.2%, the solid or firm gel structure did not form as xanthan gum increased the viscosity of the solution, reducing any interactions between calcium ions and xanthan gum or calcium and milk proteins. The excessive xanthan gum functioned as a thickening agent above 0.2%.

5.3.2 The effect of high acyl gum on calcium-added skim milk

5.3.2.1 Visual observation of milk gels in vials and on petri dishes

High acyl gellan gum at 0.1, 0.2 and 0.3 wt % were added to the skim milk with added calcium lactate at 10mM and 15mM. After heating the samples at 75 ^{o}C for 30 min, followed by cooling to 20^{o}C, all samples were observed to form firm gels (Figures 5.12).

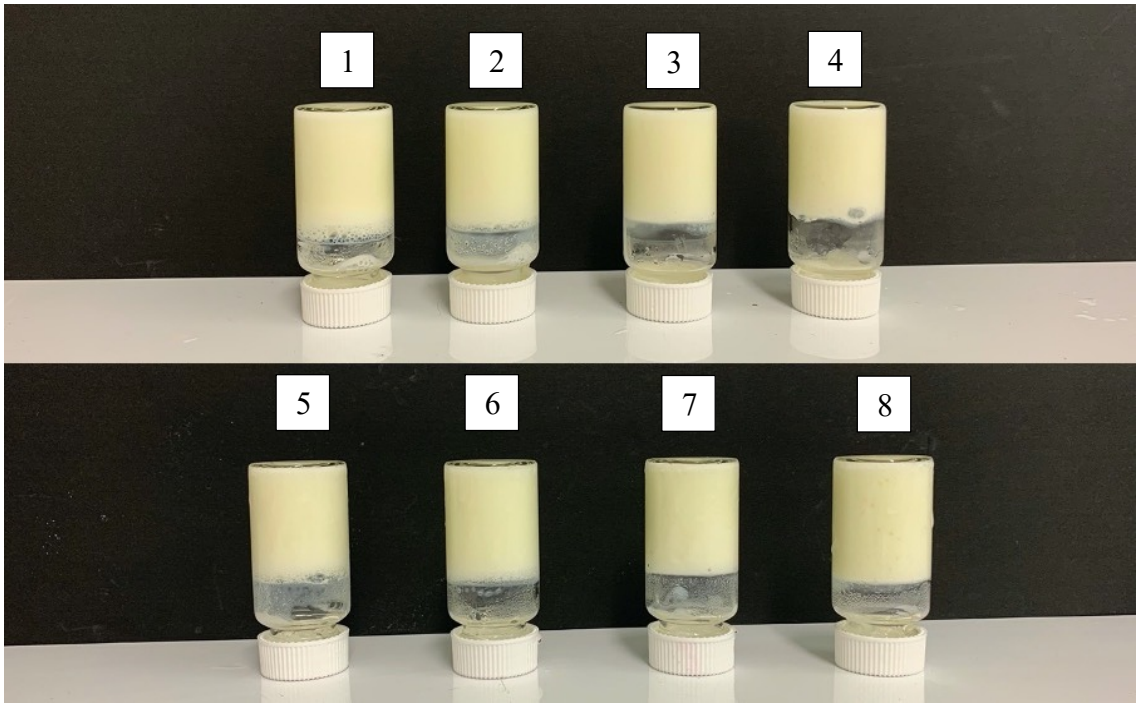

Figure 5.12 Skim milk samples with added calcium lactate and high acyl gellan gum after heating at 75 °C for 30 min. 1, 2, 3, 4 10mM calcium lactate; 5, 6, 7, 8 15mM calcium lactate. 1. no added high acyl gellan gum; 2. 0.1% high acyl gellan gum; 3. 0.2% high acyl gellan gum; 4. 0.3% high acyl gellan gum; 5. no added high acyl gellan gum; 6. 0.1% high acyl gellan gum; 7. 0.2% high acyl gellan gum; 8. 0.3% high acyl gellan gum.

With the addition of high acyl gellan gum at 0.1 to 0.3 wt % to skim milk solution with 10mM and 15mM calcium lactate, firm gels were formed at all concentrations and the firmness of gels appeared to increase with increasing concentrations of high acyl gellan gum (Figure 5.13). The gels with 15mM were less fragile and did not fracture, holding their shape, compared to the gels with 10mM added calcium lactate. However, the mouthfeel of the samples with high acyl gellan gum were found to be gritty and sandy, which did not change with increasing concentrations of high acyl gellan gum (Table 5.5).

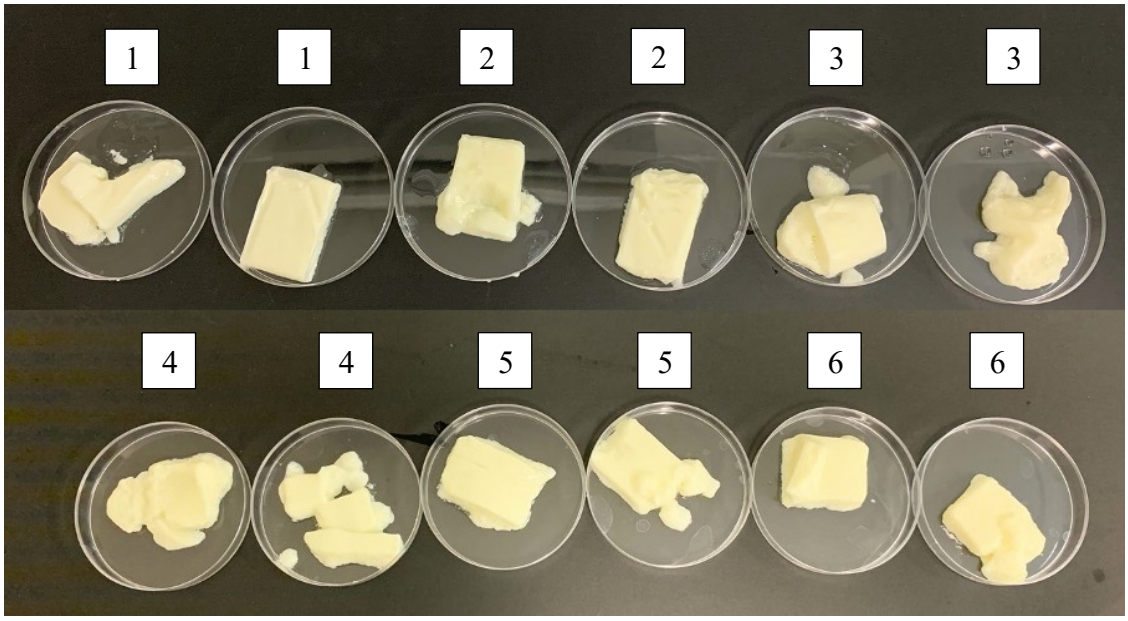

Figure 5.13 Skim milk samples with added high acyl gellan gum and 10mM calcium lactate on petri dishes after heat treated at 75±2 ºC and held for 30 min and cooled to 20±2 ºC. 1. 10mM calcium lactate and 0.1% high acyl gellan gum; 2. 10mM calcium lactate and 0.2% high acyl gellan gum; 3. 10mM calcium lactate and 0.3% high acyl gellan gum; 4. 15mM calcium lactate and 0.1% high acyl gellan gum; 5. 15mM calcium lactate and 0.2% high acyl gellan gum; 6. 15mM calcium lactate and 0.3% high acyl gellan gum.

Table 5.5 Description of the gels observed with added calcium lactate and high acyl gellan gum after being heated and held at 75 ºC for 30 min and cooled to 20 ºC, based on visual observation and evaluation of mouthfeel.

Concentration of high acyl gellan gum (wt **%)**	**10mM calcium lactate**	**15mM calcium lactate**
0	Hard gel	Hard gel
	Gritty and sandy mouthful	Gritty and sandy mouthful
0.1	Firm gel	Hard gel
	Gritty and sandy mouthful	Gritty and sandy mouthful
0.2	Firm gel;	Hard gel
	Gritty and sandy mouthful	Sandy mouthfeel
0.3	Firm gel	Firm gel
	Sandy mouthfeel	Smooth mouthfeel

5.3.2.2 Rheological characterisation of calcium-added skim milk with high acyl gellan gum

5.3.2.2.1 Heating and holding periods

During the heating period, with the addition of 10mM calcium lactate, the *G'* remained unchanged in the samples with no added high acyl gellan gum and 0.1% high acyl gellan gum (Figure 5.14(a)). With 0.2% and 0.3% added high acyl gellan gum, the *G* started to increase at 65 ºC to reach 2.83 Pa at 75ºC. With 15mM added calcium lactate and 0.1, 0.2 and 0.3% high acyl gellan gum, the *G'* increased rapidly when the heating temperature reached 65 ºC (Figure 5.14(b)).

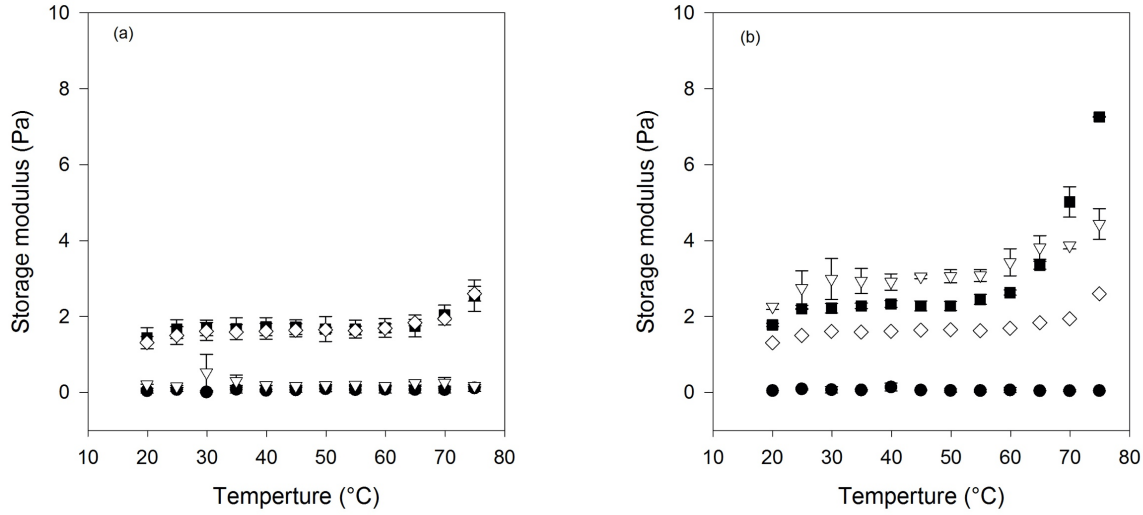

Figure 5.14 Changes in *G'* during heating of the samples with added calcium lactate and high acyl gellan gum heated to 75±0.5 ºC. (a) 10mM added calcium lactate; (b) 15mM added calcium lactate. ● without high acyl gellan gum; ▽ 0.1% high acyl gellan gum; ■ 0.2% high acyl gellan gum; ◇0.3% high acyl gellan gum. Each data point is a mean ± standard deviation (n=2 replicates).

During the holding period, the samples with 10mM added calcium lactate, the *G'* of the samples increased steadily with time in the samples with 0.2% and 0.3% high acyl gellan gum were added. The *G'* of the sample with 0.1% high acyl gellan gum only began to rise gradually after approximately 600 seconds holding (Figure 5.15(a)). The *G'* sample with no added high acyl gellan gum did not increase during the holding period. In samples with 15mM added calcium lactate, the *G'* increased rapidly in the first 800 seconds with 0.1% to 0.3% high acyl gellan gum (Figure 5.15(b)). However, the change of *G'* in the samples with 15mM added calcium lactate and 0.2% high acyl gellan gum only achieved half the *G'* observed with the samples with 0.1% high acyl gellan gum. This sample was replicated four times on the rheometer and similar results were not significantly different

($p<0.05$). The final *G'* did not behave as expected to increase with increasing high acyl gellan gum concentration. This may be due to the interaction or competition between high acyl gellan gum, calcium and milk proteins, which influenced gelling properties. The 15mM calcium lactate sample with no high acyl gellan gum showed an increase in *G'* similar to that observed in Chapter 4.

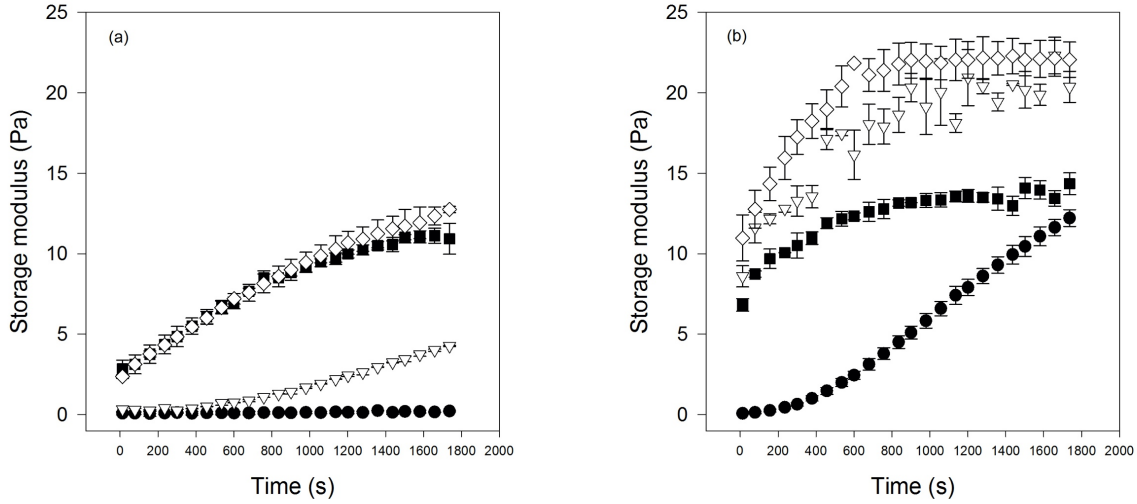

Figure 5.15 Changes in *G'* with holding time of the samples with added calcium lactate and high acyl gellan gum held at 75±0.5 °C for 30 min. (a) 10mM added calcium lactate; (b) 15mM added calcium lactate. ● without high acyl gellan gum; ▽ 0.1% high acyl gellan gum; ■ 0.2% high acyl gellan gum; ◇0.3% high acyl gellan gum. Each data point is a mean ± standard deviation (n=2 replicates).

5.3.2.2.2 The change of *G'* in cooling period

The change of *G'* during the cooling period is shown in Figure 5.16. With the addition of 10mM calcium lactate, the samples with no added high acyl gellan gum and 0.1% high acyl gellan gum, the final *G'* of the two samples were both below 20 Pa at the end of cooling (Figure 5.16(a)). The samples with added high acyl gellan gum and 10mM added calcium lactate, the *G'* of the samples with 0.2% and 0.3% high acyl gellan gum increased rapidly on cooling (Figure 5.16(a)). In the samples with the addition of 15mM calcium lactate and high acyl gellan gum, the *G'*s of all samples increased as temperature decreased to 20 °C (Figure 5.16(b)). The sample with 0.3% high acyl gellan gum had the highest final *G'* of 42.19±1.32 Pa, followed by 0.2% high acyl gellan gum at 37.42±2.03 Pa.

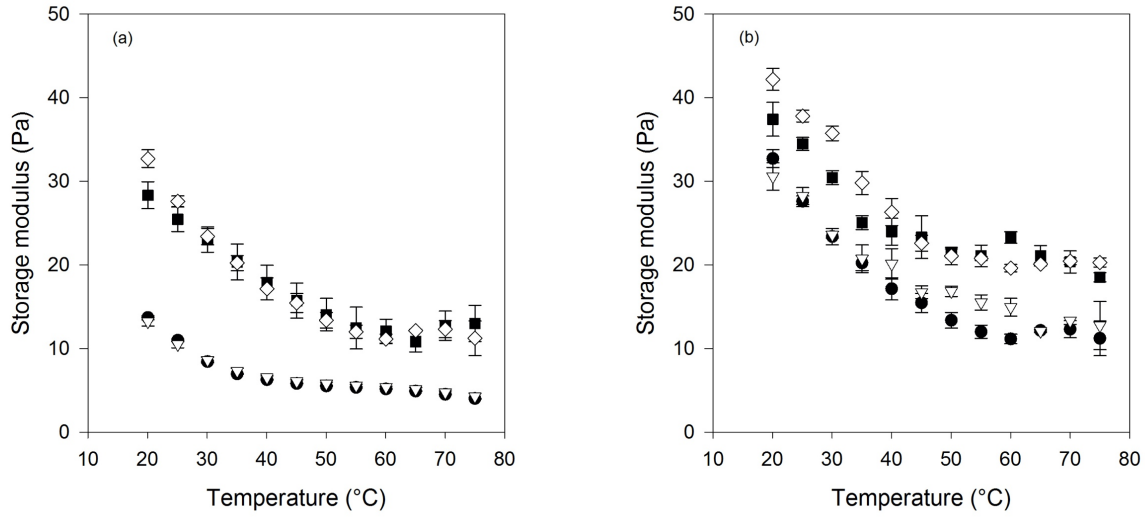

Figure 5.16 Changes in *G'* during cooling of the samples with added calcium lactate and high acyl gellan gum cooled to 20±0.5 ºC. (a) 10mM added calcium lactate; (b) 15mM added calcium lactate. ● without high acyl gellan; ▽ 0.1% high acyl gellan; ■ 0.2% high acyl gellan gum; ◇0.3% high acyl gellan gum. Each data point is a mean ± standard deviation (n=2 replicates).

The change in final *G'* of the samples with 0, 10 and 15mM added calcium lactate and added high acyl gellan gum from 0 to 0.5 wt % is shown in Figure 5.17. The significance of the change in final *G'* at various concentrations of high acyl gellan gum with ANOVA (Appendix 4). The final *G'* of the samples with no added calcium lactate increased significantly ($p<0.05$) with increasing concentration of high acyl gellan gum from 0% to 0.5% (Appendix 4). The final *G'* with the addition of 0.5% high acyl gellan gum and no calcium lactate was 41.01±0.02 Pa. With the addition of 10mM calcium lactate, there was no significant increase in final *G'* when the concentration of high acyl gellan gum increased from 0% to 0.1% ($p>0.05$). There was an increase in the *G'* of approximately 15 Pa when the high acyl gellan gum concentration increased to 0.2%. But from 0.2 to 0.3% added high acyl gellan gum, there was no significant increase ($p>0.05$).

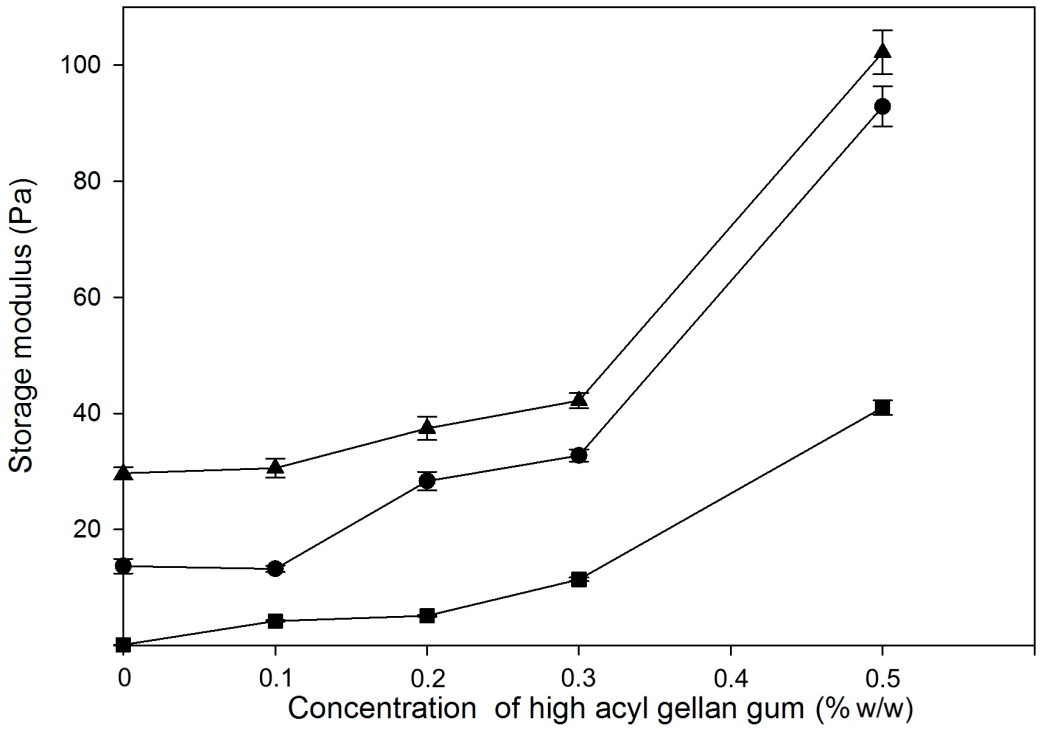

Figure 5.17 The final of *G'* of the samples with various concentrations of high acyl gellan gum. ■ without calcium lactate; ● 10mM added calcium lactate; ▲ 15mM added calcium lactate. Each data point mean ± standard deviation (n=2 replicates).

It was found that as the concentration of added calcium lactate and high acyl gellan gum in the sample increased, a greater final *G'* was achieved (Figure 5.17). The samples with 15mM added calcium lactate showed the highest final *G'*, which was determined to be 92.86±3.48 Pa at 0.5% added high acyl gellan gum. The final *G'* of the samples with 10mM or 15mM added calcium lactate increased significantly when the concentration of high acyl gellan gum increased from 0.3% to 0.5% ($p<0.05$). These results agreed with the visual observation, at which hard gels were formed at all concentrations of high acyl gellan gum to skim milk. Particularly, a firm gel was observed, and the mouthfeel was gritty with 10mM calcium lactate, but it was improved with 15mM calcium lactate.

The addition of high acyl gellan gum increased the gel strength beyond just calcium lactate alone as shown by the significant increase in final *G'* from 0.3% to 0.5% added high acyl gellan gum. High acyl gellan gum is widely used as a thickener and gelling agent in dairy, and it forms gels when hot solutions are cooled (Fallourd & Viscione, 2009). It was reported that high acyl gellan gum were incompatible with casein micelles and it could possibly result in a micro-phase separation. However, physical stability of the gels was increased as the presence of high acyl gellan gum are capable to bind water (Buldo et al., 2016). Carboxylic acid groups, a negatively charged part of gellan gum,

tend to crosslink with the calcium ions present in the milk environment as a result of electrostatic attraction. This promotes the formation of junction zones, where the milk proteins can also interact with the gellan gum (Buldo et al., 2016; Phillips & Williams, 2009). It was also found that high acyl gellan gum molecules were preferential to bind to whey proteins rather than casein micelles and a decrease of casein to whey protein ratio could improve the textural properties (Buldo et al., 2016). The molecules of high acyl gellan gum tend to bind with calcium ions in the system due to electrostatic attraction as high acyl gellan gum is negatively charged. At lower concentration of high acyl gellan gum, the interaction between high acyl gellan gum, calcium ions and proteins may impact an influence on the properties of the gel. However, the mouthfeel of the gels was sandy when the concentration of high acyl gellan gum from 0.1 to 0.3%. But the mouthfeel was improved slightly with increasing concentration of high acyl gellan gum.

5.3.3 The effect of guar gum on calcium-added skim milk

5.3.3.1 Visual observation of milk gels in vials and in petri dishes

Skim milk samples with 10mM and 15mM added calcium lactate with added guar gum at 0.1%, 0.2% and 0.3 wt % , all formed a firm gel after heating to 75 ^{o}C and holding for 30 min (Figures 5.18 and 5.19). When guar gum was added to skim milk with 10mM and 15mM calcium lactate solutions, the observations are reported in Table 5.6. A soft gel was observed with 0.2% guar gum. With the addition of 0.3% guar gum to the skim milk and 10mM or 15mM calcium lactate sample, a semi-liquid gel with small aggregates was observed (Figures 5.19). The mouthfeel of the gels with guar gum was found be gritty and sandy.

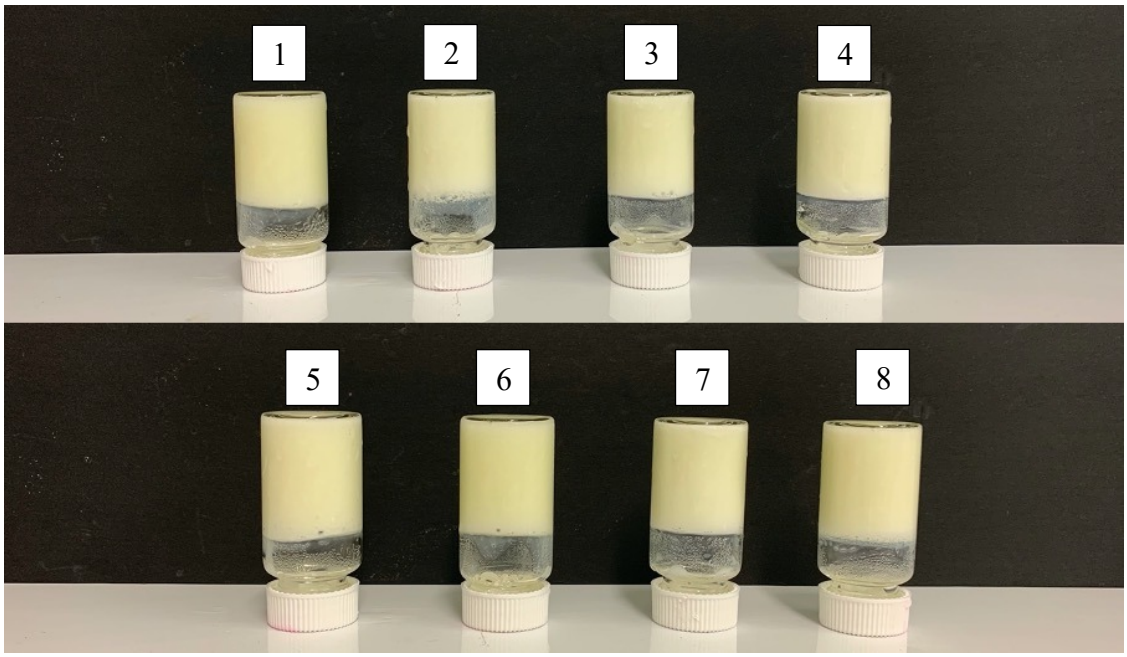

Figure 5.18 Skim milk samples with added calcium lactate and guar gum after heating at 75 ºC for 30 min. 1, 2, 3, 4 10mM calcium lactate; 5, 6, 7, 8 15mM calcium lactate. 1. no added guar gum; 2. 0.1% guar gum; 3. 0.2% guar gum; 4. 0.3% guar gum; 5. no added guar gum; 6. 0.1% guar gum; 7. 0.2% guar gum; 8. 0.3% guar gum.

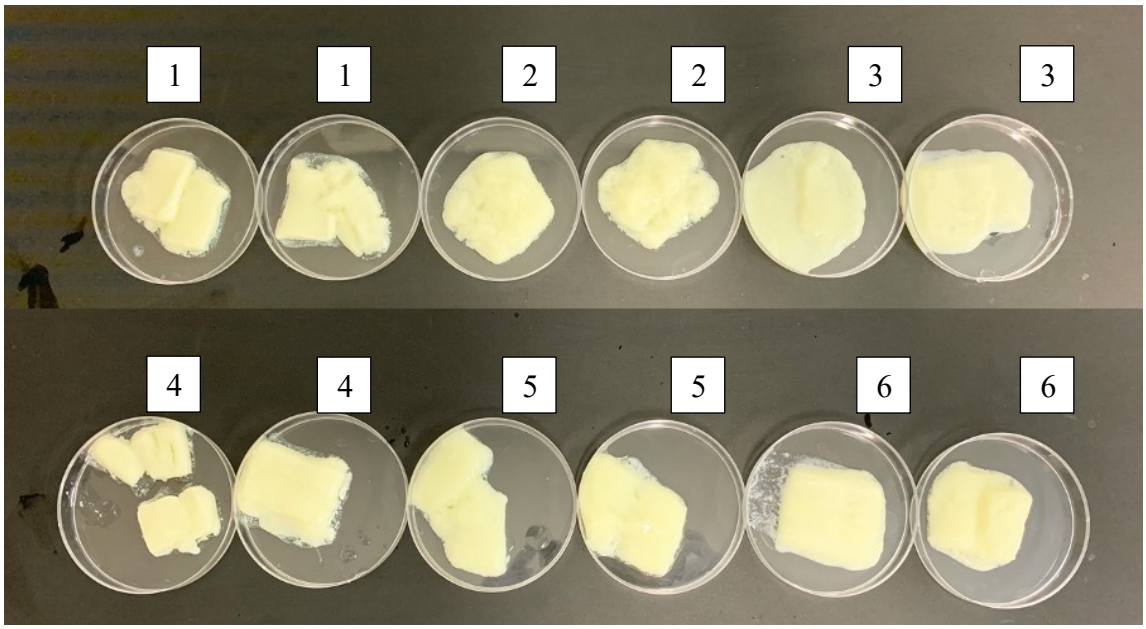

Figure 5.19 Skim milk samples with added guar gum and 10mM calcium lactate on petri dishes after heat treated at 75±2 ºC and held for 30 min and cooled to 20±2 ºC. 1. 10mM calcium lactate and 0.1% guar gum; 2. 10mM calcium lactate and 0.2% guar gum; 3. 10mM calcium lactate and 0.3% guar gum; 4. 15mM calcium lactate and 0.1% guar gum; 5. 15mM calcium lactate and 0.2% guar gum; 6. 15mM calcium lactate and 0.3% guar gum.

Table 5.6 Description of the gels observed with added calcium lactate and guar gum after being heated and held at 75 °C for 30 min and cooled to 20 °C, based on visual observation and evaluation of mouthfeel.

Concentration (wt %)	**10mM calcium lactate**	**15mM calcium lactate**
0	Hard gel	Hard gel
	Gritty and sandy mouthful	Gritty and sandy mouthful
0.1	Hard gel	Hard gel
	Gritty mouthfeel	Gritty and sandy mouthful
0.2	Soft gel	Soft gel
	Gritty and sandy mouthful	Sandy and watery mouthfeel
0.3	Semi- liquid gel	Soft gel
	Sandy mouthfeel	Sandy and watery mouthfeel

5.3.3.2 Rheological characterisation of calcium-added skim milk with guar gum

5.3.3.2.1 Heating period and holding period

The change of *G'* of the samples with added guar gum during the heating period is shown in Figure 5.20. For both 10mM and 15mM added calcium lactate samples, addition of guar gum showed no significant change in the *G'* during the heating period ($p<0.05$).

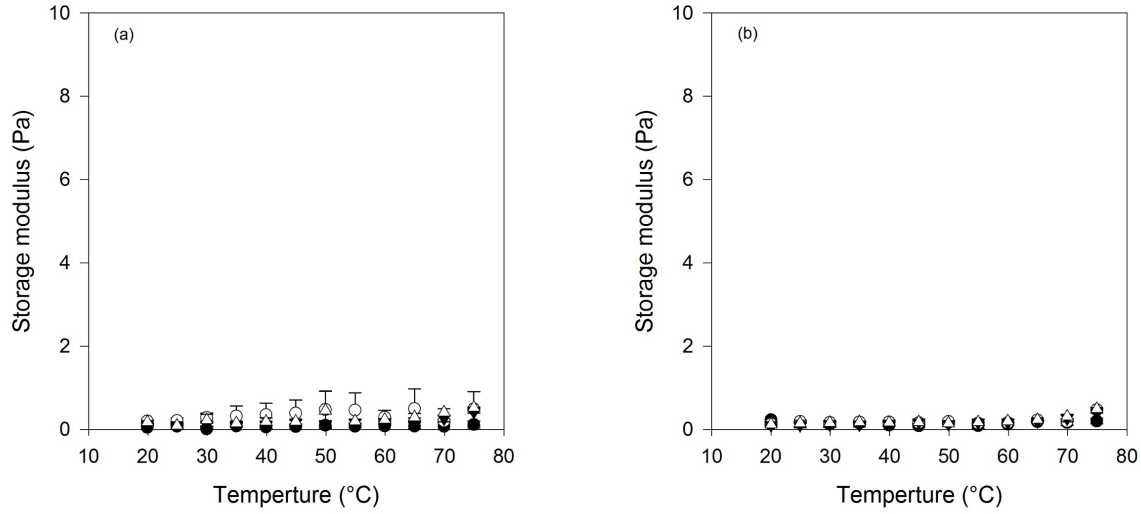

Figure 5.20 Changes in *G'* during heating of the samples with added calcium lactate and guar gum heated to 75±0.5 °C. (a) 10mM added calcium lactate; (b) 15mM added calcium lactate. ● without guar gum; ▽ 0.1% guar gum; ■ 0.2% guar gum; ◇0.3% guar gum. Each data point is a mean ± standard deviation (n=2 replicates).

During the holding period, with the addition of guar gum, the *G'* of the samples with 10mM calcium lactate increased steadily with increasing concentrations of guar gum except for the sample with no added guar gum (Figure 5.21(a)). With the addition of 15mM added calcium lactate, the *G'* also increased over time with increasing

concentration of guar gum (Figure 5.21(b)). But the *G'* values achieved were less than the control with no added guar gum. Hence the *G'* achieved during holding was reduced with increasing additions of guar gum, it appeared to be inhibiting gelation. It was observed that the *G'* of the samples with 0.2% and 0.3% guar gum increased slowly but were less than 5 Pa by the end of the holding period. With the 0.1% added guar gum and 15mM calcium lactate, the *G'* of the sample was greater than 5 Pa, which was higher than the *G'* achieved with higher concentrations of guar gum.

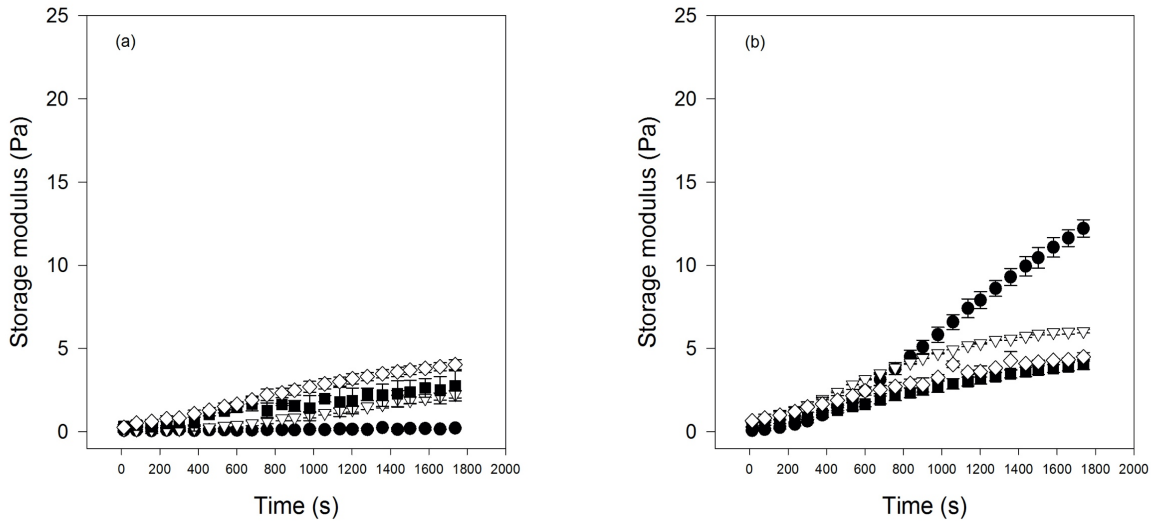

Figure 5.21 Changes in *G'* with holding time of the samples with added calcium lactate and guar gum held at 75±0.5 ºC for 30 min. (a) 10mM added calcium lactate; (b) 15mM added calcium lactate. ● without guar gum; ▽ 0.1% guar gum; ■ 0.2% guar gum; ◇0.3% guar gum. Each data point is a mean ± standard deviation (n=2 replicates).

5.3.3.3.2 The change of G' in cooling period

On cooling, the samples with 10mM calcium lactate and guar gum showed that the *G'*s increased slowly as temperature decreased. The final *G'*s were all below 20 Pa at all concentrations of guar gum (Figure 5.22). With the addition of 15mM calcium lactate, the *G'* of the samples without added guar gum increased rapidly on cooling, also corresponding to the high *G'* achieved after the holding period and confirms results in Chapter 4. It was greater than 30 Pa and reached the highest *G'* among all samples. However, the *G'* of the samples with 0.1%, 0.2% or 0.3% guar gum increased from approximately 4 Pa to 10 Pa, similar to the results observed from the samples with 10mM calcium lactate and guar gum.

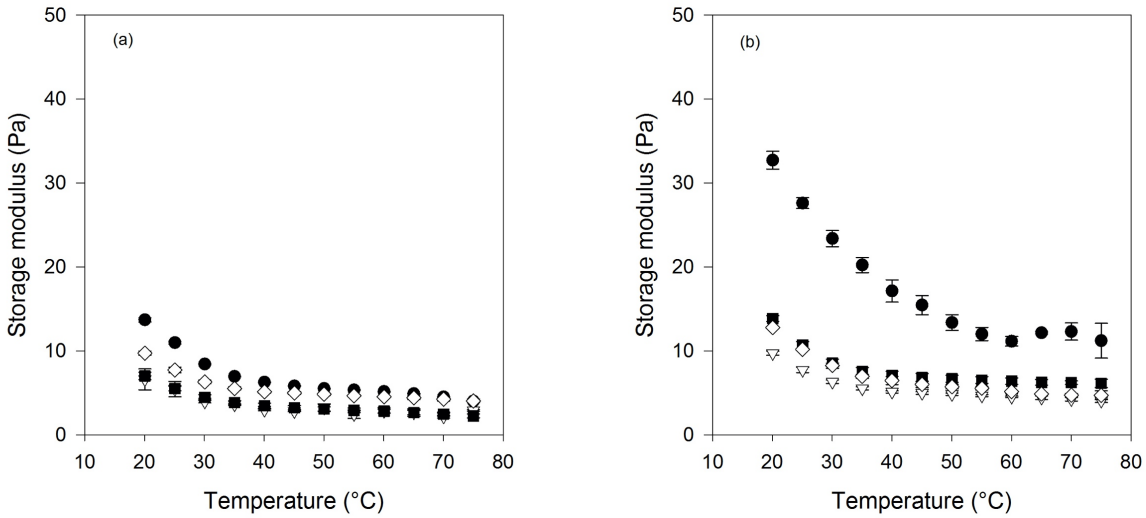

Figure 5.22 Changes in *G'* during cooling of the samples with added calcium lactate and guar gum cooled to 20±0.5 ºC. (a) 10mM added calcium lactate; (b) 15mM added calcium lactate. ● without guar gum; ▽ 0.1% guar gum; ■ 0.2% guar gum; ◇0.3% guar gum. Each data point is a mean ± standard deviation (n=2 replicates).

The change in final *G'* at different concentrations of guar gum is shown in Figure 5.23 and results were analysed with ANOVA (Appendix 5). In the samples with no added calcium lactate, the addition of guar gum from 0% to 0.5% to the samples showed no significant increase in final *G'* ($p>0.05$). With the addition of 10mM and 15mM calcium lactate, the final *G'* values both dropped significantly from 0 to 0.1% added guar gum ($p<0.05$) and then the *G'* increased steadily with increasing concentration of guar gum (0.1 to 0.5 wt %). With the addition of 10mM calcium lactate, the final *G'* of did not show a significant increase or drop when concentration of guar gum increased from 0.1% to 0.3% ($p>0.05$). The highest final *G'* was determined to be 14.98±0.45 Pa at 0.5% guar gum. The highest final *G'* was found to be 29.67±1.06 Pa when 15mM calcium lactate was added in the sample without guar gum With the addition of 15mM calcium lactate, no significant change in final *G'* was observed as the concentration of guar gum increased from 0.1% to 0.3% ($p>0.05$).

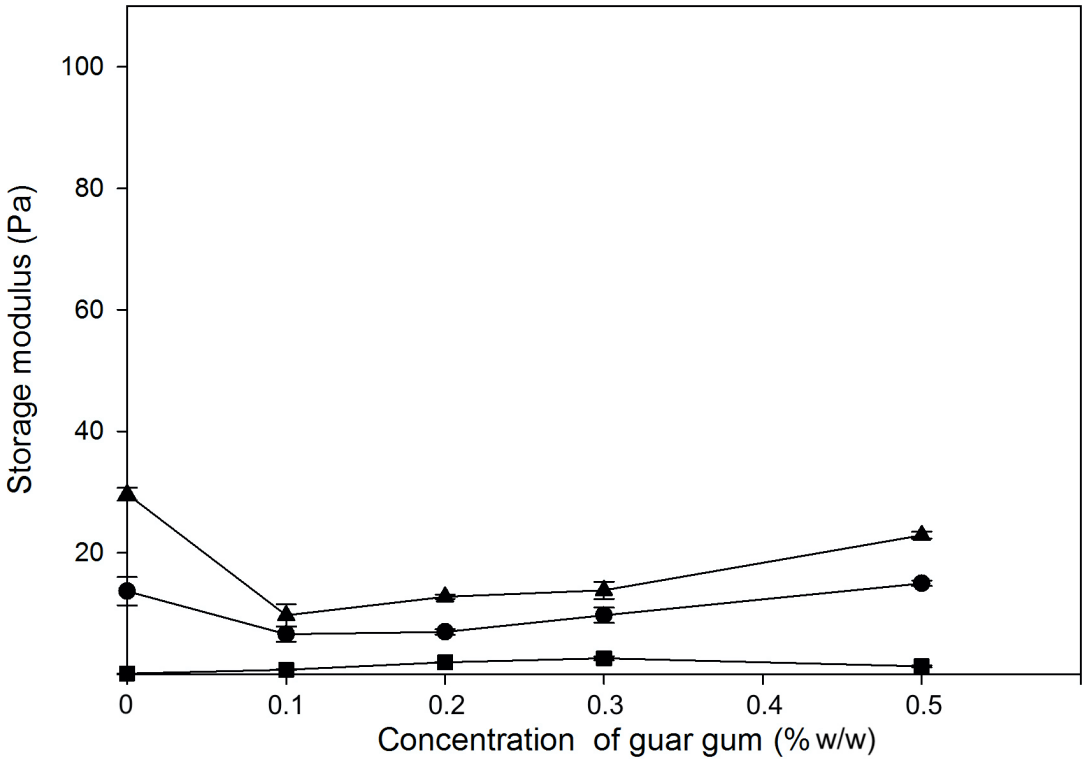

Figure 5.23 The final of *G'* of the samples with various concentrations of guar gum. ■ without calcium lactate; ● 10mM added calcium lactate; ▲ 15mM added calcium lactate. Each data point mean ± standard deviation (n=2 replicates).

Guar gum is neutral and the interaction with calcium ions and milk proteins was minimal (Bourriot et al., 1999; Mudgil et al., 2014). It was reported that the dissolution and viscosity of a guar gum solution increased with decreasing pH and increasing temperature, guar gum can hydrate rapidly in cold water and develop viscous solutions (Mudgil et al., 2014). The addition of guar gum can reduce syneresis and improve texture (Brennan & Tudorica, 2008; Gupta & Variyar, 2018). However, guar gum has been reported to be thermodynamically incompatible with milk proteins, which often causes phase separation (Gupta & Variyar, 2018). This was observed visually on the milk gels, where water separated from the gels particularly at higher concentration of guar gum. It was recommended that guar gum was suitable for products which undergo high-temperature-short-time processing to minimise the issues with the proteins and phase separation (Mudgil et al., 2014). It was also suggested guar gum can be added along with other polysaccharides to minimise micro-phase separation and stabilise the matrix (George et al., 2019). Guar gum was not found to be suitable to improve the texture of skim milk and calcium lactate gels.

5.3.4 Microscopic observation of milk gels

Table 5.7 lists the samples that were investigated by SEM. As it discussed in Sections 5.3.1, 5.3.2 and 5.3.3, the addition of xanthan gum had a different impact on the final *G'* of the samples compared with the samples with high acyl gellan gum or guar gum. Hence, the structure of gels formed with three concentrations of xanthan gum were investigated with 15mM calcium lactate. The samples with 15mM calcium lactate and 0.3% high acyl gellan gum or guar gum were only assessed. The samples were scanned and imaged under two magnifications (300× and 2400×) and the images are shown in Figures 5.24, 5.25 and 5.26.

Table 5.7 The concentration of calcium lactate and polysaccharide in samples evaluated by SEM.

Sample	1	2	3	4	5	6	7
Concentration of calcium lactate (mM)	10	15	15	15	15	15	15
Concentration of polysaccharide	N/A	N/A	0.1% xanthan gum	0.3% xanthan gum	0.5% xanthan gum	0.3% high acyl gellan gum	0.3% guar gum

The microstructure of the milk gels with only calcium lactate addition at 10mM and 15mM under 300× magnification are shown in Figure 5.24 A and B, respectively. The microstructure of the milk gels with 15mM added calcium lactate appeared to be denser and more compact, whereas the sample with 10mM added calcium lactate had a more open porous network as more air pockets were shown in its microstructure as it can be seen in Figure 5.24 C and D under 2400× magnification. This could indicate that there was a stronger interaction occurred between calcium ions and milk proteins in the with 15mM added calcium lactate sample. This was in agreement with the visual observations of prepared gels in Section 5.3.1.1 and the final *G'* in Section 5.3.1.2.2 which showed that the skim milk solution with high concentration of calcium lactate form a hard gel.

The microstructure of the milk gels with 15mM added calcium lactate and added xanthan gum (0.1, 0.3, 0.5%) are shown in Figure 5.25. The SEM images showed different microstructures in the samples with different concentrations of xanthan gum. The gel with 15mM calcium lactate and 0.1% xanthan gum showed a loose and porous structure with long spherical shaped structures (Figures 5.25 A and 5.25 D). The microstructure of the sample with 0.3% xanthan gum was distinct from the one with 0.1% xanthan gum. The

SEM images show that the gel structure had a very different shape, there were strands of interlinking fibres which could be xanthan chains providing the appearance of crosslinking in the matrix (Figures 5.25 B and 5.25 E). The network showed air spaces and voids.

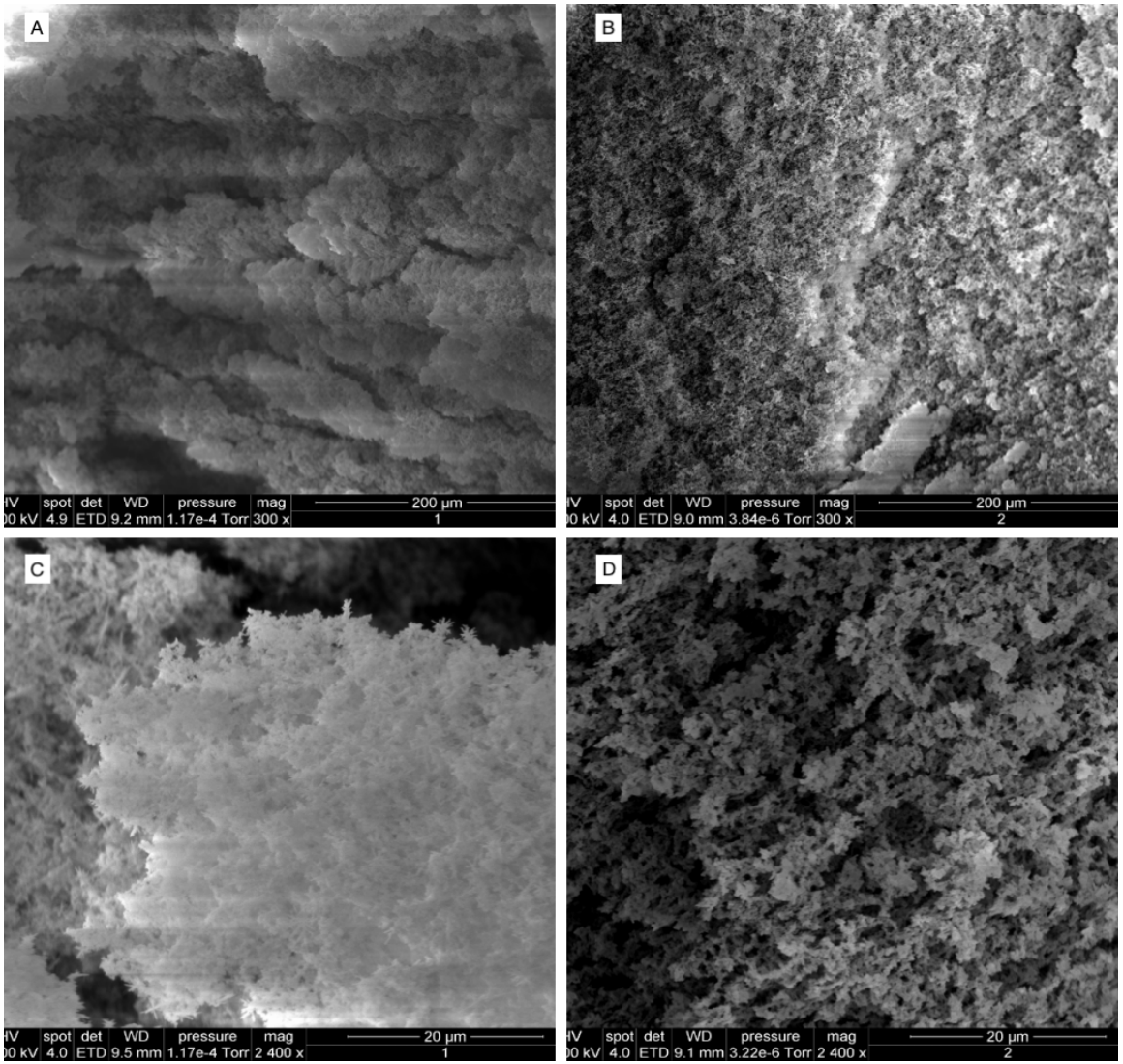

Figure 5.24 SEM images of skim milk gels after being heated and held at 75ºC for 30 min and cooled to 20ºC. A 10mM added calcium lactate 300× magnification; B 15mM added calcium lactate 300× magnification; C 10mM added calcium lactate 2400× magnification; D 15mM added calcium lactate 2400× magnification.

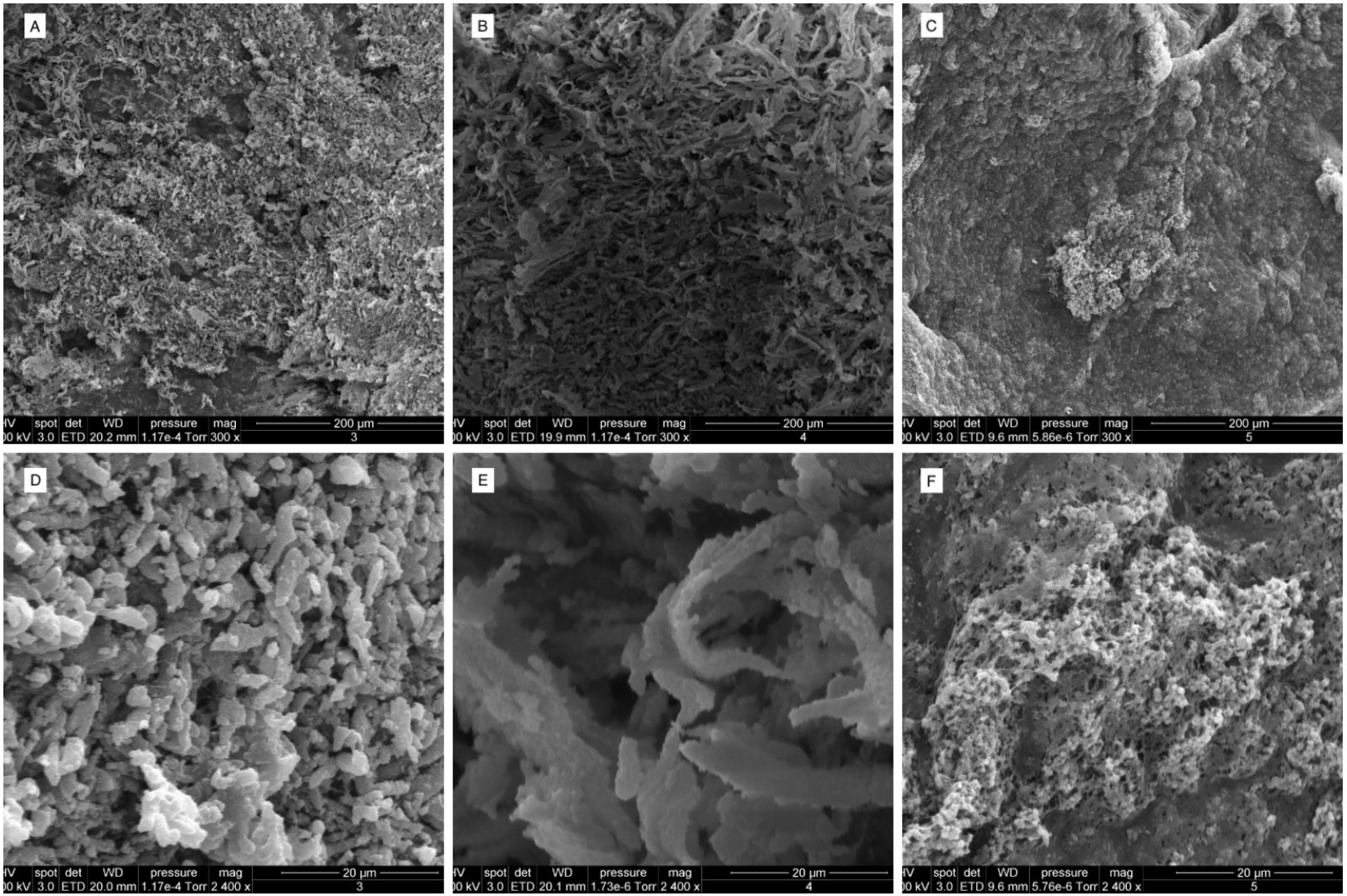

Figure 5.25 SEM images of skim milk gels with added xanthan gum after being heated and held at 75ºC for 30 min and cooled to 20ºC. A 15mM added calcium lactate and 0.1% xanthan gum 300× magnification; B 15mM added calcium lactate and 0.3% xanthan gum 300× magnification; C 15mM added calcium lactate and 0.5% xanthan gum 300× magnification; D 15mM added calcium lactate and 0.1% xanthan gum 2400× magnification; E 15mM added calcium lactate and 0.3% xanthan gum 2400× magnification; F 15mM added calcium lactate and 0.5% xanthan gum 2400× magnification.

The gel with 0.5% xanthan gum shows a more compact and denser structure, in which a well-ordered and crosslinked network can be seen (Figures 5.25 C and F). It also showed small fine filaments in the network with more crosslinking, in which a more ordered structure was formed. From the visual observation, viscous liquid was observed at 0.3% and 0.5% xanthan gum and this may be caused by the thickening properties of xanthan and the network was more crosslinked in the presence of xanthan in the system.

A number of researchers have studied the changes in microstructure of heat-induced egg white gels and whey protein isolate gels with added xanthan gum, and skim milk gels with added xanthan and locust bean gum mixture using SEM (Nayebzadeh et al., 2006; Sanchez et al., 2000; Zhang et al., 2019). It was observed in egg white gels, the number of pores in the gel with 0.12 wt % added xanthan gum decreased with a denser and more smooth structure, which possessed better mouthfeel and texture (Nayebzadeh et al., 2006). It was reported that this was mainly caused by the electrostatic attraction between egg white proteins and xanthan, leading to tight binding between molecules (Nayebzadeh et al., 2006). It was observed by Zhang et al. (2019) that the surface of the whey isolate gels with added xanthan gum were dense and smooth with less void spaces under SEM. These findings agreed with the results presented in this research from the visual observation and SEM, in which a more viscous liquid was formed, and the mouthfeel was smoother as the concentration of xanthan gum increased.

The microstructure of the gel with 15mM added calcium lactate and 0.3% high acyl gellan gum is shown in Figures 5.26 B and E. It was observed the gel has a fibrous or strands-like network. Yang et al. (2019) reported a similar finding of hydrogels with 0.1% high acyl gellan gum, the gel exhibited fibres or a strand-like network. Coronato et al. (2012) reported that the addition of 0.125% gellan gum to milk formed a gel which showed large pores under SEM and the pores may be responsible for the firmness and the strength of gels, the firmness of the gel decreased with larger pores. Mao et al. (2001) also observed similar rounded pores in the 1% gellan gels containing various concentrations of calcium ions (4, 6, 20 and 60mM) under SEM. Large pores with a fibrous network was observed in the samples with 0.3% high acyl gellan gels in this study in Figures 5.26 B and E.

The microstructure of the milk gel with 0.3% added guar gum showed individually elongated swollen clusters (Figures 5.26 C and F). The formation of the elongated swollen clusters may contribute to the increasing final *G'* of the gels as concentration of guar gum increased. It was

reported in a study of yoghurt with hydrolysed guar gum using SEM that the guar gum at higher concentration (greater than 1%) embedded in the network of the milk proteins and formed a layer supporting the casein structure (Hussain et al., 2017). However, it also reported the phase separation occurred in casein and guar gum association because of thermodynamic incompatibility with the casein network (Bourriot et al., 1999; Hussain et al., 2017; Tuinier et al., 2000). The addition of guar gum at low concentration formed a weak gel structure as the gel network was not well developed (Green, 1980). This agreed with the finding in this study, the phase separation in the samples occurred and lost its initial texture and hardness after 12 hours storage (Figure 5.19).

The results from the scanned images by SEM showed the microstructure of the milk gels with different polysaccharides. With increasing concentration of xanthan gum, the microstructure of the samples showed a more compact network with more crosslinks. This may explain the results found previously that the gel was a firm gel with a smooth mouthfeel but at higher xanthan gum concentrations, the solutions became more viscous.

The microstructural network of the milk gels with high acyl gellan gum and guar gum were different in comparison to the samples with added xanthan gum. The microstructure of the milk gel with added high acyl gellan gum showed fibrous network with strands, whereas the one with guar gum showed individually elongated fibres. Results shown in Section 5.3.2.1 showed firm gels were formed with the addition of high acyl gellan gum at all concentrations and the mouthfeel of the gels improved at 0.3% high acyl gellan gum. The final *G'* of the samples with added high acyl gellan gum increased as its concentration increased from 0% to 0.3%, and it increased rapidly as its concentration increased from 0.3% to 0.5%. The formation of a fibrous network with crosslinks observed under SEM may explain the mouthfeel and increase in final *G'*. It was reported Coronato et al. (2012) and Mao et al. (2001) that the firmness and strength of the gel was enhanced with added gellan gum and calcium ions.

For the gels with added guar gum, the microstructure showed a less ordered network, the final *G'* increased slowly with increasing guar gum concentration and did not achieve a high final *G'* at all concentrations compared with the samples with added xanthan or high acyl gellan gum. This may explain although hard gels with 0.1% added guar gum was formed and soft gels were formed at 0.2% and 0.3% added guar gum. And yet a sandy and gritty mouthfeel was perceived for all gels with added guar gum.

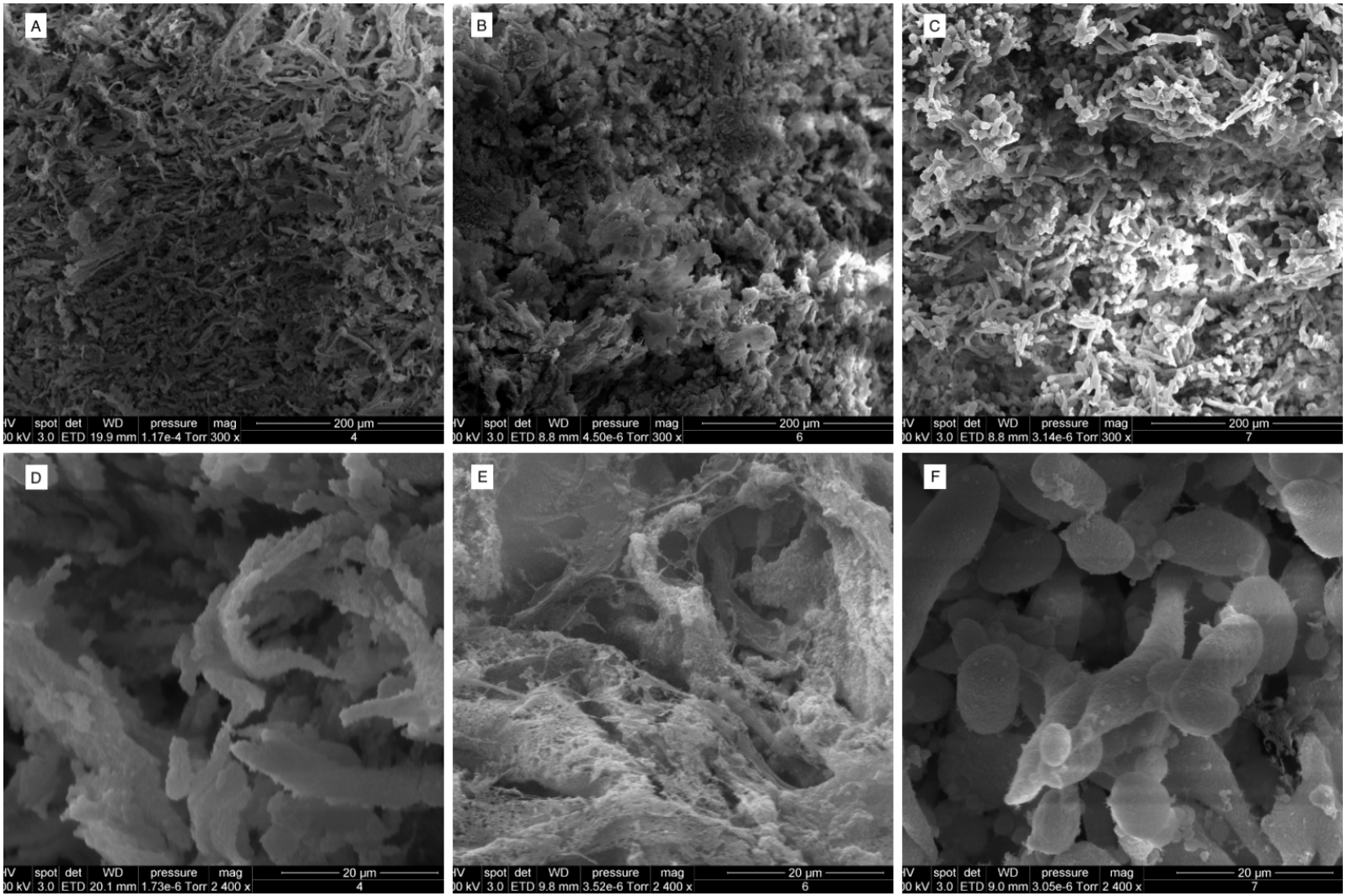

Figure 5.26 SEM images of skim milk gels with added three polysaccharides after being heated and held at 75ºC for 30 min and cooled to 20ºC. A 15mM added calcium lactate and 0.3% xanthan gum 300× magnification; B 15mM added calcium lactate and 0.3% high acyl gellan gum 300× magnification; C 15mM added calcium lactate and 0.3% guar gum 300× magnification; D 15mM added calcium lactate and 0.3% xanthan gum 2400× magnification; E 15mM added calcium lactate and 0.3% high acyl gellan gum 2400× magnification; F 15mM added calcium lactate and 0.3% guar gum 2400× magnification.

5.4 Conclusions

The addition of calcium lactate and xanthan gum with various concentrations resulted in a distinct change in final *G'*, at which final *G'* of the samples with added calcium lactate reached its lowest *G'* with 0.1% xanthan gum and highest *G'* with 0.15% xanthan gum. The SEM images showed a compact microstructure with crosslinks at higher concentration of xanthan gum (greater than 0.3%) of xanthan gum. As the xanthan concentration was above 0.3%, liquid gels were formed and the samples became more viscous, while the final *G'* decreased steadily at 0.3-0.5% xanthan gum. The results showed a change of the interactions in the samples with xanthan gum at various concentrations. With the addition of high acyl gellan gum, the final *G'* increased as the concentration of high acyl gellan gum increased. It agreed with the visual observations, where a firmer and more rigid gel was formed with increasing concentration of high acyl gellan gum. With the addition of guar gum, the final *G'* increased insignificantly with increasing concentration of guar gum and soft gels were formed with gritty mouthfeel.

Chapter 6 Overall Discussion

The objectives of this study were to investigate the effect of the addition of a calcium salt to skim milk at increasing concentrations with different heat treatments, and to determine the effect of the addition of three stabilisers (xanthan gum, high acyl gellan gum or guar gum) on calcium-added skim milk. To carry out these objectives, the formation of calcium-added skim milk gels with and without added stabilisers was investigated by studying the physical, rheological properties and microstructure of the skim milk samples.

With the addition of various concentrations of calcium lactate, the skim milk samples without added stabilisers were given different heat treatments, the changes on their physical and rheological properties were investigated and the mechanisms of the development of skim milk gels without added stabilisers was studied in Chapter 4.

The skim milk samples with added stabilisers were heated and held at 75 ^{o}C for 30 min, followed by cooling to 20 ^{o}C. The physical, rheological properties and microstructure were studied to evaluate possible interactions between milk protein, added calcium ions and stabilisers on the development of a milk gel. In Chapter 5 of this study, various concentrations of calcium lactate and each of three stabilisers were added in the skim milk samples to investigate the effect on the apparent physical and rheological properties, microstructures and mechanisms involved in the skim milk gel development.

6.1 Calcium ion-protein interaction in calcium-added skim milk without added polysaccharides under various heat treatments

The addition of calcium ions to skim milk can affect the stability of milk proteins through electrostatic interaction and/or changes in the ionic strength (Damodaran & Parkin, 2017; Tsioulpas et al., 2007). The severity and degree of heat treatment also had an impact on the interaction between calcium ions and milk proteins (Ju & Kilara, 1998b; Ramasubramanian et al., 2012). In Chapter 4, results showed the physical and rheological properties of the skim milk gels after various heat treatments. At all heat treatments evaluated, firm milk gels were formed when 15mM or 20mM calcium lactate was added. The rheology results showed that the final *G'* after cooling increased with increasing concentration of added calcium lactate and was influenced by heating temperature and

holding time. The results could be attributed to a number of possible mechanisms. Increasing the concentration of calcium lactate results in a greater number of calcium ions and higher calcium activity in the system. The free calcium ions may act as calcium bridges between two adjacent proteins and this promotes a shifting of the mechanism towards the formation of calcium phosphate as there are more calcium ions available in the system (Bryant & McClements, 1998; Dalgleish, 1998). An increase in calcium phosphate can potentially be transferred to the colloidal phase, which could lead to interaction with the casein micelle and promote crosslinking of casein molecules (Philippe et al., 2003). In addition, more hydrogen ions are released from dihydrogen phosphate ions as a result of the shifting of the equilibrium towards the formation of calcium phosphate (Section 4.4 in Chapter 4). As more hydrogen ions are present in the system, the pH of the milk samples decreased and electrostatic repulsion reduces, which leads to the destabilisation of the milk proteins with the effects of heat (Croguennec et al., 2016; Lewis et al., 2011). Upon heating of the skim milk, followed by cooling with the effect of calcium ions, the denaturation and aggregation of the milk proteins will occur if the sample is heated to 70 ºC, this will result in a higher final *G'* and the formation of firm milk gels. Lin et al. (2018) reported similar findings that the addition of calcium salts with higher calcium activity could induce higher final *G'* of milk gels and form stronger gels.

In Chapter 4, the skim milk samples with added calcium lactate at different heat treatments showed high final *G'* values (>10 Pa) only after 15mM to 20mM calcium lactate was added. A higher heating temperature and/or a longer holding time resulted in a significant increase in the final G' with increasing calcium salt concentration. This can be explained by the thermodynamics and kinetic theory, at which the molecules move at a more rapid rate as a result of temperature rise (Chalmers, 2009). Upon protein unfolding, the enthalpy change increases significantly at high temperature, which is driven by a faster rate of hydrophobic interaction (Chalmers, 2009). The hydrophobic interaction leads to the crosslinking and bond formation of the protein molecules and thereby the aggregation of the proteins (Baldwin, 1986). Overall, the addition of calcium lactate and

a higher degree of heat treatment tended to induce and promote the interaction of the milk proteins and calcium ions, which leads to the gelation of the skim milk.

6.2 Calcium-protein-polysaccharides interaction in calcium-added skim milk with added polysaccharides

This study aimed to provide insights and pave a pathway to the potential development and formulation of thickened skim milk drinks for dysphagic patients. Previous studies reported that the addition of calcium salts to skim milk could lead to the formation of milk gels and there was lack of information on stability of milk gels and the information on the mouthfeel of milk gels (Hongsprabhas & Barbut, 1996; Ju & Kilara, 1998a; Lin et al., 2018; Ramasubramanian et al., 2014). Without heating, the milk gels were not be able to form with added calcium salts. The formation of milk gels with added calcium salts required external factors such as heat to induce gelation. The addition of polysaccharide to skim milk with added calcium lactate aimed to stabilise the milk system upon heating and provide desirable mouthfeel. In the literature review, the functionalities of each gum and they were all used in different dairy products although under different heat treatments (Section 2.4, Table 2.5). The polysaccharide was added to the skim milk samples before the heat treatment and milk gels with higher concentration of calcium lactate (>15mM) were formed after heating. The polysaccharides were in powder form and it was difficult to add powdered polysaccharides to solid milk gels and expect them to perform their functions. It was likely to break the milk gel matrix and lose its structure if external forces were applied to mix the polysaccharide with the milk gels. However, the addition of polysaccharides can be considered for those milk gels which remained in a liquid form after heating.

The addition of calcium lactate and polysaccharides may exert a synergistic or antagonist effect on the apparent physical, rheological properties and microstructure depending on the concentration of calcium lactate and polysaccharides added in the skim milk samples. In this study (Chapter 5), xanthan gum, high acyl gellan gum and guar gum were respectively added in the skim milk samples with added calcium lactate. All the skim milk samples were heated and held at 75 ^{o}C for 30 min, followed by cooling to 20 ^{o}C.

Sections 6.2.1, 6.2.2 and 6.2.3 discuss the possible interactions between calcium ions and milk proteins with each stabiliser.

6.2.1 Xanthan gum

The rheology results showed a variation in final *G'* of the skim milk samples with various concentrations of calcium lactate and added xanthan gum. Several possible mechanisms may occur simultaneously, which was mainly driven by the interaction between calcium ions, milk proteins and xanthan gum molecules. When calcium lactate was added in the skim milk samples, a calcium lactate molecule was present as one positively calcium ion and two negatively lactate anions. This results in increasing concentration of calcium ions in the skim milk. On the other hand, xanthan gum molecules are negatively charged due to the presence of pyruvate unit and guluronic acid (Butler, 2016). When the concentration of calcium ions increased in the skim milk systems, they inevitably move towards and come into contact with the negatively charged xanthan gum molecules as the principle electrostatic interaction is formed by the pyruvate and guluronic acids (Groves & Chaw, 2015; Phillips & Williams, 2009). The calcium ions tend to bind with the outer end position of the xanthan gum molecules side chains where the pyruvate and guluronic units reside, which confer small steric hindrance (Groves & Chaw, 2015). In other words, the pyruvate units bind with the calcium ions via electrostatic interaction. This could result in calcium ion bridging with xanthan gum and the proteins (Belitz et al., 2009; Mikac et al., 2010). However, the rheological properties and microstructure of the skim milk samples in Chapter 5 varied with different concentrations of added calcium lactate and xanthan gum. It was found that the final *G'* of the skim milk samples with 10mM or 15mM added calcium lactate dropped when the concentration (wt %) of xanthan gum increased from 0% to 0.1%.

The drop in final *G'* was possibly caused by the interaction between xanthan gum molecules and calcium ions. When the concentration of xanthan gum was less than 0.15%, positively charged calcium ions tended to bind with the pyruvate and guluronic acids of the xanthan gum molecules chains. And yet there was a very small number of xanthan gum molecules at this low concentration available to bind to the available calcium ions. Hence, calcium ions will be in excess, leaving free calcium ions in the system. The

interaction between xanthan and calcium ions reduced the number of free calcium ions in the system. Subsequently, this may result in less interactions between free calcium ions and caseins during the heat treatment, which resulted in a reduction in final *G'* as the degree of protein aggregation and gelation was reduced. However, it was likely that the calcium ions still dominated the properties of the skim milk gels at this stage, where the skim milk gels were formed with the addition of calcium lactate. In contrast, the final *G'* the skim milk samples without added xanthan gum increased gradually with increasing concentration of calcium lactate. The more free calcium ions present in the system, the higher degree of association and interaction between the milk proteins and calcium ions was likely to occur and this would have led to a higher final *G'* of the skim milk samples and firmer gels.

In addition, the final *G'* of the skim milk samples with 10mM or 15mM added calcium lactate increased rapidly with the addition of 0.1% to 0.15% xanthan gum in the samples. As the concentration of xanthan gum increased from 0.1% to 0.15%, a rise in final *G'* may be caused by the formation of ion bridging between calcium-xanthan gum molecules and milk proteins (Groves & Chaw, 2015; Mohammed et al., 2007). During this period, xanthan gum molecules possibly bond to most of available free calcium ions and formed calcium-xanthan bridging, which promoted the development of crosslinking with milk proteins and formed molecule complexes. As less free calcium ions were present in the system, the predominating force of the gelation could possibly be the formation of molecule complexes which were resulted from the crosslinking and ion bridging with calcium ions, xanthan gum molecules and milk proteins.

For the skim milk samples with 15mM added calcium lactate, the final *G'* reached its peak at concentration of 0.15% xanthan gum. For the skim milk samples with 10mM added calcium lactate, the final *G'* reached its peak at concentration of 0.2% xanthan gum. This increase in the final *G'* was possibly caused by the interaction between calcium ions, milk proteins and xanthan gum molecules (Groves & Chaw, 2015; Mohammed et al., 2007). When the calcium ions bind to the side chains of xanthan gum molecules, it elongates the molecule chains via electrostatic interaction, and this leads to an decrease

in viscosity. This was observed visually and corresponded with the semi-liquid gels, where the gels were small aggregates and the solution was less viscous than when the concentration of xanthan gum ranged from 0.1% to 0.2%.

However, the final *G'* of 10mM and 15mM added calcium lactate skim milk samples began to drop at 0.2% and 0.15% added xanthan gum concentration, respectively. This was likely caused by the functional properties of xanthan gum. Xanthan gum is used as a thickener which results in more viscous fluids when it is added (Phillips & Williams, 2009). The viscosity of the fluid generally increases with increasing concentration of added xanthan gum (Butler, 2016; Phillips & Williams, 2009). This also agreed with their microstructure changes, where the molecules became more crosslinked and exhibited a more uniformed three-dimensional network as it was shown in Section 5.3.4. This formation of ordered network results from the addition of increasing concentrations of xanthan gum in the skim milk samples. This may indicate an improvement in the mouthfeel of the skim milk samples. Binding of the excess calcium with xanthan molecules may have reduced the gritty mouthfeel in the calcium milk gels.

The rheology results also showed with the addition of 10mM or 15mM calcium lactate, the final *G'* decreased constantly from 0.3% to 0.5% added xanthan gum. At this stage, the function of xanthan gum may dominate the properties of the skim milk gel as the more viscous the skim milk sample becomes, then calcium ions will not come into contact this xanthan or protein molecules leading to a calcium-induced gel. The results of visual observation showed the skim milk sample with added xanthan gum thickened when the addition of xanthan gum was from 0.2% - 0.5%. The viscosity of the samples with xanthan gum increased when the concentration of xanthan gum was above 0.3%. It was reported that the viscosity of the xanthan dominated the viscosity of the mixture when the concentration of xanthan gum was less than 1 wt % (Hemar et al., 2001). This finding in some extent agreed with the observation and postulated mechanism of xanthan-protein interactions when the concentration of xanthan gum was between 0.2% - 0.5%. In addition, a more ordered and structured network was observed in their microstructures when the concentration of xanthan gum increased from 0.2% to 0.5% with the addition

of 10mM or 15mM calcium lactate. The interaction between calcium ions and milk proteins may still occur and yet the properties of xanthan gum may compete with the impact of the interaction. It was possibly the dominance of functional properties of xanthan gum determined the physical properties of the skim milk gels. However, the final *G'* may not the best parameter to identify the changes as no solid gels were formed when the concentration of xanthan gum was between 0.2% to 0.5%.

Xanthan gum is thermally stable and relatively independent of heating temperature (Schmidt & Smith, 1992). However, the denaturation of proteins particularly whey occurs could occur at a faster rate and this causes unfolding of the whey proteins and may expose additional binding sites for xanthan molecules (Schmidt & Smith, 1992). With addition of xanthan gum, the calcium equilibrium was likely to gradually shift from the caseins to the xanthan molecules. However, the calcium equilibrium change was likely influenced by the concentration of xanthan gum and calcium lactate. Without adding calcium lactate, it was reported the addition of xanthan to skim milk resulted in the formation of protein-rich phase and xanthan-rich phase, which leads to phase separation (Hemar et al., 2001; Rohart & Michon, 2014). The phase separation was likely caused by the depletion flocculation of casein micelles due to the presence of xanthan macromolecules.

6.2.2 High acyl gellan gum

The rheology results of the skim milk sample with added high acyl gellan gum showed a continuous and steady increase in the final *G'* when the concentration of added high acyl gellan gum increased from 0% to 0.3%. The final *G'* increased when the concentration of high acyl gellan gum increased from 0.3% to 0.5%. The addition of high acyl gellan gum to skim milk with added calcium lactate resulted in significantly higher final *G'* only at concentration of 0.3% and 0.5% high acyl gellan gum ($p<0.05$). The high acyl gellan gum is a negatively charged polysaccharide and its molecules tend to bind with calcium ions due to electrostatic attraction. This interaction occurred as the calcium ions could directly crosslink adjacent gellan gum molecules and form calcium-gellan molecule complexes, which resulted in a stronger gel strength and gelation properties (Chandrasekaran, 1990, 1991). Sworn (2009) also proposed a mechanism of milk gelation with added gellan gum, where the disordered polymer chains along with other molecules formed junction zones

and this resulted in ordered coil-helix network. The addition of cations promoted aggregation of the gellan double helices and transition of a more ordered structure after the cooling of a hot solution (Sworn, 2009). However, the aggregation of the gellan gum with acyl substituents such as high acyl gellan gum is restricted by its acetyl group. The presence of acetyl groups inhibit the intermolecular association via the steric hindrance, which leads to a gel with less continuity and homogeneity (Sworn, 2009). This agreed with the visual observations and evaluation of mouthfeel, where firm gels were formed with increasing concentration of high acyl gellan gum. It was also found that the final *G'* almost tripled when the concentration of high acyl gellan gum increased from 0.3% to 0.5%. This was possibly caused by the excessive concentration of high acyl gellan gum in the system, which resulted in the properties of high acyl gellan gum dominating over the properties of the gel. It was reported that high acyl gellan gum is capable of forming self-supporting gels when the concentration was above 0.2% (Sworn, 2009). However, the setting temperature may be affected by the concentration of calcium ions. As the calcium concentration increased from 2 to 80mM, the setting temperature for high acyl gellan gum increased from 71 ºC to 80 ºC (Huang et al., 2004). Overall, the interaction between calcium ions, high acyl gellan gum molecules and milk proteins may be favoured at lower concentrations of high acyl gellan gum up to 0.3%. As the concentration of the gum increased above 0.3%, the driving force for gelation may be due to the functional properties of high acyl gellan gum along, which results in a high final *G'* and firm gels.

6.2.3 Guar gum

The addition of guar gum in the skim milk samples did not show a significant change in its final *G'* and microstructure. Guar gum molecules are neutral unlike high acyl gellan gum and xanthan gum. Calcium-induced skim milk gels were observed without stabilisers, but the addition of guar gum inhibited the gel formation. The final *G'* of the skim milk samples decreased as the concentration of guar gum increased from 0% and 0.1%. As the concentration of guar gum was 0.1%, the formation of solid gels with 10mM or 15mM calcium lactate was observed. Any interactions between calcium ions and milk proteins may dominate the properties of the gel and hence hard gels were formed. However, the less crosslinking in the network and phase separation observed may be caused by the depletion interaction between the casein micelles and guar gum (Bourriot et al., 1999;

Tuinier et al., 2000). Gupta and Variyar (2018) reported that guar gum suits ice cream using high-temperature short-time processing and it is often added with other hydrocolloids such as κ-carrageenan, to prevent phase separation (Mudgil et al., 2014). The mixture of guar gum and other polysaccharides could be considered for future experiments.

6.3 Experimental improvements and potential development for thickened drinks

This work has provided a better understanding of the rheological properties and microstructure of thickened skim milk or milk gels with the addition of calcium lactate and three polysaccharides. The key outcomes of this project were the characterisation of the *G'* during the heat treatment with the effect of added calcium lactate and various polysaccharides, the identification of the microstructure of various milk gels and the visual observation of the thickened skim milk and milk gels. However, there are still more improvements to be done on the rheology, sensory evaluation and microstructure imaging for future development of potential products.

The preparation of the samples for SEM imaging was difficult, it was observed that some of the milk gels were fragile and difficult to prepare the gels with fixative. The fragile gels were likely to lose their structure and have phase separation. The milk gels were embedded with droplets of agarose to minimise the structural change caused by phase separation. But this was suboptimal due to the penetration issues with the fixative and the heat of the agarose affecting the gels. The results obtained from the SEM illustrate some of the samples were quite prone to flaring which causes the uneven illumination, even after multiple conductive coatings. Therefore, this type of gel is recommended to use cryo-SEM to observe the microstructure of the milk gels, at which the gels were treated under extreme cold conditions and structural changes were minimised.

In addition, the glass vials and the plastic moulds used to induce milk gelation were made from different materials and they are different in size and shape. Different materials may have different heat conductivity and they may transfer heat to the sample at different rates. This could lead to different gelation times, which may show different physical properties in visual observations. Also, some of gels were not intact when they were removed from

the plastic moulds, some of them stuck on the wall of the moulds and broke easily. A layer of non-stick paper or foil is recommended to lay on the wall of the moulds before transferring samples in.

The mouthfeel and texture of some of current products is improved by using thickeners and/or stabilisers (Mills, 2008; Nicholson et al., 2008). Different types and the concentration of the thickeners and stabiliser can affect the thickness and consistency of the fluids (Garcia et al., 2008). Hadde (2017) studied the rheological properties of some commercial thickened fluids with different types of thickeners or a mixture of thickeners, and it was found there were significant variabilities in rheological properties of the products with different thickeners and dispersing media (Hadde, 2017). The major findings in this study may be used as a part of guideline to develop thickened milk drinks for people who suffer from swallowing difficulties. This study investigated the effect of three polysaccharides on calcium-added skim milk and there are several suggestions for the potential development of thickened milk drinks. Firstly, with the addition of 10mM or 15mM calcium lactate, the skim milk samples became thickened and more viscous when the concentration of xanthan gum was greater than 0.15%. The mouthfeel was improved when the concentration of xanthan gum was greater than 0.15%. With 10Mm and 15mM calcium lactate, a smooth mouthfeel was perceived when 0.5% xanthan gum added to the skim milk with 10 wt % total solids. Secondly, the addition of high acyl gellan gum in the skim milk promoted the formation of hard and firm milk gels. Firmer gels were formed with increasing concentration of high acyl gellan gum. Thirdly, mouthfeel of the milk gels or thickened milk with xanthan gum was smoother compared with the addition of high acyl gellan or guar gum. However, the addition of guar gum to skim milk was not recommended as mouthfeel was undesirable and phase separation was observed.

Chapter 7 Conclusions & Recommendations

7.1 Conclusions

This project sets out to investigate and study the properties of thickened milk or milk gels with added calcium lactate and polysaccharides. The findings of this work are concluded below:

The increasing concentration of calcium lactate promoted the gelation of milk along with higher temperatures for heat treatment and longer holding times. Both calcium lactate concentration and heat treatment conditions impact on the gelation of skim milk. Hard and firm gels were formed with the concentration of added calcium lactate greater than 10mM, and yet skim milk samples remained in a liquid state when less than 10mM calcium lactate was added despite high heating temperatures and long holding times. The highest final *G'* was achieved with the addition of 20mM calcium lactate when the sample was heated at 80 ^{o}C for 60 min.

Different polysaccharides showed different interactions when they were added to skim milk with calcium lactate. When xanthan gum was added to skim milk with 10mM and 15mM calcium lactate, soft gels were formed at low concentration of xanthan gum (less than 0.1%), due to xanthan gum, calcium ions and milk protein interactions. When the concentration of xanthan was greater than 0.2%, liquid gels were formed and gels became more viscous with increasing concentration of xanthan, which was a result of xanthan controlling the viscosity of the solution reducing other interactions. Firm, soft or semi-liquid gels can be formed by the addition of calcium lactate and xanthan to skim milk after the sample was heated at 75 ^{o}C and held for 30 min. Gels with smooth mouthfeel were formed at 10mM and 15mM calcium lactate when 0.5% xanthan gum was added.

The addition of high acyl gellan gum to the skim milk with 10mM and 15mM added calcium lactate produced firm gels at all concentrations (0.1-0.5%). The final *G'* increased with increasing concentration of high acyl gellan gum and the highest final *G'* was achieved at 0.5% high acyl gellan gum. A fibrous network was observed under SEM

when 0.3% high acyl gellan gum was added to the skim milk samples. The addition of high acyl gellan gum may promote the formation of firmer and stronger gels as the *G'* increased with increasing concentration of high acyl gellan gum.

For the samples with added guar gum, hard gels were formed at 10mM and 15mM calcium lactate when 0.1% guar gum was added. The addition of 0.2 and 0.3% guar gum to calcium-added skim milk produced soft gels with small aggregates and phase separation was observed when the concentration of guar gum was at 0.5%. With the addition of 0.3% guar gum, a less compact network was observed under SEM. The final *G'* did not increase significantly with increasing concentration of guar gum. The addition of guar gum may inhibit the interactions between calcium ions, milk proteins and guar gum molecules.

7.2 Recommendations for future work

This work has provided some insights to the physical, rheological and microscopic properties of calcium-added skim milk with different polysaccharides. However, there is still more work to be done for the development of thickened milk drinks in the future. The effect of three polysaccharides on the skim milk was studied and it had indications of the concentration and the type of gums could be potentially used and adjusted in thickened skim milk or milk gels as it discussed in Section 6.3. The addition of hybrids of gums and starch or multi-gums in the skim milk may have advantageous impacts on the properties of skim milk and provide a desirable mouthfeel and texture. This may also be an option for future development of thickened skim milk products. The processing condition also plays an important role in characterising the properties of thickened skim milk or milk gels as results showed in Chapter 4 where the final *G'* rose rapidly with a higher heating temperature and/or a longer holding time condition. The processing parameters and conditions ought to be considered accordingly with the product. The appropriate processing condition should be considered and complied with other factors to acquire an optimal and desirable properties of the product. Measurements of the rheological parameters should comply with the processing parameters. A suitable sensory evaluation is recommended to be designed and conducted on thickened skim milk/milk gels and potential prototypes.

Chapter 8 References

Adams, M., & Smith, B. S. (2012). *Dysphagia : risk factors, diagnosis, and treatment*. Nova Science Publishers.

Akkerman, M., Rauh, V. M., Christensen, M., Johansen, L. B., Hammershøj, M., & Larsen, L. B. (2016). Effect of heating strategies on whey protein denaturation—Revisited by liquid chromatography quadrupole time-of-flight mass spectrometry. *Journal of Dairy Science, 99*(1), 152-166.

Ala'A, A. J., Katzka, D. A., & Castell, D. O. (2015). Approach to the patient with dysphagia. *The American journal of medicine, 128*(10), 1138. e1117-1138. e1123.

Alting, A. C., Hamer, R. J., de Kruif, C. G., & Visschers, R. W. (2003). Cold-set globular protein gels: Interactions, structure and rheology as a function of protein concentration. *Journal of Agricultural and Food Chemistry, 51*(10), 3150-3156.

Anema, S., Creamer, L., & Singh, H. (1993). The relative importance of protein and mineral concentrations on the dissociation of kappa-casein from the micelles in heated reconstituted skim milk. Protein and fat globule modifications by heat treatment, homogenization and other technological means for high quality dairy products. IDF Seminar, Munich, Germany, 25-28 Aug 1992,

Anema, S. G. (2009). Effect of milk solids concentration on the pH, soluble calcium and soluble phosphate levels of milk during heating. *Dairy Science and Technology, 89*(5), 501-510.

Antonovcan, Y. A., Zhuravskaya, N., & Tolstoguzov, V. (1985). Solubility of protein fibers obtained from casein solutions and liquid two-phase water-casein-sodium alginate systems. *Food/Nahrung, 29*(1), 39-47.

Aoki, T., Umeda, T., & Kako, Y. (1990). Cleavage of the linkage between colloidal calcium phosphate and casein on heating milk at high temperature. *Journal of Dairy Research, 57*(3), 349-354.

Atherton, M., Bellis-Smith, N., Cichero, J. A. Y., & Suter, M. (2007). Texture-modified foods and thickened fluids as used for individuals with dysphagia: Australian standardised labels and definitions. *Nutrition & dietetics, 64*, S53-S76.

Azim, Z., Alexander, M., Koxholt, M., & Corredig, M. (2010). Influence of cross-linked waxy maize starch on the aggregation behavior of casein micelles during acid-induced gelation. *Food Biophysics, 5*(3), 227-237.

Baldwin, R. L. (1986). Temperature dependence of the hydrophobic interaction in protein folding. *Proceedings of the National Academy of Sciences, 83*(21), 8069-8072.

Barak, S., & Mudgil, D. (2014). Locust bean gum: Processing, properties and food applications—A review. *International Journal of Biological Macromolecules, 66*, 74-80.

Barbosa-Cánovas, G. V., Ortega-Rivas, E., Juliano, P., & Yan, H. (2005). *Food powders*.

Baumgartner, S., Pavli, M., & Kristl, J. (2008). Effect of calcium ions on the gelling and drug release characteristics of xanthan matrix tablets. *European Journal of Pharmaceutics and Biopharmaceutics, 69*(2), 698-707.

Belitz, H. D., Grosch, W., & Schieberle, P. (2009). *Food chemistry*. Springer.

Belloque, J., López-Fandiño, R., & Smith, G. M. (2000). A 1H-NMR study on the effect of high pressures on β-lactoglobulin. *Journal of Agricultural and Food Chemistry, 48*(9), 3906-3912.

Bemiller, J. N. (2018). *Carbohydrate chemistry for food scientists*. Elsevier.

Benichou, A., Aserin, A., & Garti, N. (2002). Protein-polysaccharide interactions for stabilization of food emulsions. *Journal of Dispersion Science and Technology, 23*(1-3), 93-123.

Bergmann, D., Furth, G., & Mayer, C. (2008). Binding of bivalent cations by xanthan in aqueous solution. *International Journal of Biological Macromolecules, 43*(3), 245-251.

Bernal, V., Smajda, C., Smith, J., & Stanley, D. (1987). Interactions in protein/polysaccharide/calcium gels. *Journal of Food Science, 52*(5), 1121-1125.

Bolder, S. G., Hendrickx, H., Sagis, L. M., & Van der Linden, E. (2006). Ca2+-induced cold-set gelation of whey protein isolate fibrils. *Applied Rheology, 16*(5), 258-264.

Bourriot, S., Garnier, C., & Doublier, J.-L. (1999). Phase separation, rheology and microstructure of micellar casein–guar gum mixtures. *Food Hydrocolloids, 13*(1), 43-49.

Brennan, C. S., & Tudorica, C. M. (2008). Carbohydrate - based fat replacers in the modification of the rheological, textural and sensory quality of yoghurt: comparative study of the utilisation of barley beta - glucan, guar gum and inulin. *International journal of food science & technology, 43*(5), 824-833.

Bryant, C., & McClements, D. (2000). Influence of xanthan gum on physical characteristics of heat-denatured whey protein solutions and gels. *Food Hydrocolloids, 14*(4), 383-390.

Bryant, C. M., & McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science & Technology, 9*(4), 143-151.

Buldo, P., Benfeldt, C., Carey, J. P., Folkenberg, D. M., Jensen, H. B., Sieuwerts, S., Vlachvei, K., & Ipsen, R. (2016). Interactions of milk proteins with low and high acyl gellan: Effect on microstructure and textural properties of acidified milk. *Food Hydrocolloids, 60*, 225-231.

Butler, M. (2016). *Xanthan Gum : Applications and Research Studies*. Nova Science Publishers.

Cardona, J. A. R., Iriart, C. H., & Herrera, M. L. (2013). Applications of confocal laser scanning microscopy (CLSM) in foods. In *Confocal Laser Microscopy Principles and Applications in Medicine. Biology, and Food Sciences.* (pp. 203-234).

Caroli, A., Poli, A., Ricotta, D., Banfi, G., & Cocchi, D. (2011). Invited review: dairy intake and bone health: a viewpoint from the state of the art. *Journal of Dairy Science, 94*(11), 5249-5262.

Carucci, L. R., & Turner, M. A. (2015). Dysphagia revisited: common and unusual causes. *Radiographics, 35*(1), 105-122.

Castillo, M., Payne, F., Hicks, C., & Lopez, M. (2000). Predicting cutting and clotting time of coagulating goat's milk using diffuse reflectance: effect of pH, temperature and enzyme concentration. *International Dairy Journal, 10*(8), 551-562.

Chalmers, A. (2009). Thermodynamics and the Kinetic Theory. In *The Scientist's Atom and the Philosopher's Stone* (pp. 215-231).

Chandrasekaran, R. (1990). Three-dimensional structures of gel-forming polysaccharides. *Frontiers in Carbohydrate Research 1: Food Applications*.

Chandrasekaran, R. (1991). Interactions of ordered water and cations in the gel-forming polysaccharide gellan gum. In *Water Relationships in Foods* (pp. 773-784).

Cho, H.-M., Yoo, W., & Yoo, B. (2012). Steady and dynamic rheological properties of thickened beverages used for dysphagia diets. *Food Science and Biotechnology, 21*(6), 1775-1779.

Cichero, J. A., Jackson, O., Halley, P. J., & Murdoch, B. E. (2000). How thick is thick? Multicenter study of the rheological and material property characteristics of mealtime fluids and videofluoroscopy fluids. *Dysphagia, 15*(4), 188-200.

Cichero, J. A., Steele, C., Duivestein, J., Clavé, P., Chen, J., Kayashita, J., Dantas, R., Lecko, C., Speyer, R., & Lam, P. (2013). The need for international terminology and definitions for texture-modified foods and thickened liquids used in dysphagia management: foundations of a global initiative. *Current Physical Medicine and Rehabilitation Reports, 1*(4), 280-291.

Coronato, R., Biasutti, E. A., Carvalho, C. W., & Grossmann, M. V. (2012). Gellan gum/cassava starch mixtures in water systems and in milk systems. *Starch-Stärke, 64*(5), 359-366.

Corredig, M., & Dalgleish, D. (1996). Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. *Food Research International, 29*(1), 49-55.

Corredig, M., Sharafbafi, N., & Kristo, E. (2011). Polysaccharide–protein interactions in dairy matrices, control and design of structures. *Food Hydrocolloids, 25*(8), 1833-1841.

Cox, W., & Merz, E. (1958). Correlation of dynamic and steady flow viscosities. *Journal of Polymer Science, 28*(118), 619-622.

Croguennec, T., Jeantet, R., & Schuck, P. (2016). From milk to dairy products. *Handbook of Food Science and Technology 3: Food Biochemistry and Technology*, 1-63.

Dalgleish, D. (1993). The enzymatic coagulation of milk. In *Cheese: Chemistry, physics and microbiology* (pp. 69-100).

Dalgleish, D. (1998). Casein micelles as colloids: surface structures and stabilities. *Journal of Dairy Science, 81*(11), 3013-3018.

Dalgleish, D., van Mourik, L., & Corredig, M. (1997). Heat-induced interactions of whey proteins and casein micelles with different concentrations of α-lactalbumin and β-lactoglobulin. *Journal of Agricultural and Food Chemistry, 45*(12), 4806-4813.

Dalgleish, D. G., & Parker, T. G. (1980). Binding of calcium ions to bovine α sl-casein and precipitability of the protein–calcium ion complexes. *Journal of Dairy Research, 47*(1), 113-122.

Daly, R. M., Petrass, N., Bass, S., & Nowson, C. A. (2008). The skeletal benefits of calcium-and vitamin D3–fortified milk are sustained in older men after withdrawal of supplementation: an 18-mo follow-up study. *The American journal of clinical nutrition, 87*(3), 771-777.

Damodaran, S., & Parkin, K. L. (2017). *Fennema's food chemistry*. CRC press.

Dannenberg, F., & Kessler, H.-G. (1988). Effect of denaturation of beta-lactoglobulin on texture properties of set-style nonfat yoghurt. Firmness and flow properties. *Milchwissenschaft*.

Datta, N., & Deeth, H. C. (2001). Age Gelation of UHT Milk—A Review. *Food and Bioproducts Processing, 79*(4), 197-210.

De Kruif, C., & Holt, C. (2003). Casein micelle structure, functions and interactions. In *Advanced dairy chemistry—1 proteins* (pp. 233-276).

De Wit, J. (1990). Thermal stability and functionality of whey proteins. *Journal of Dairy Science, 73*(12), 3602-3612.

Demirci, U., & Khademhosseini, A. (2016). *Gels handbook : fundamentals, properties and applications*. New Jersey : World Scientific.

Dickinson, E. (2005). *Food colloids : interactions, microstructure and processing*. Cambridge : Royal Society of Chemistry.

Dissanayake, M., Ramchandran, L., Donkor, O., & Vasiljevic, T. (2013). Denaturation of whey proteins as a function of heat, pH and protein concentration. *International Dairy Journal, 31*(2), 93-99.

Djabourov, M., Leblond, J., & Papon, P. (1988). Gelation of aqueous gelatin solutions. Structural investigation. *Journal de Physique, 49*(2), 319-332.

Donato, L., Guyomarc'h, F., Amiot, S., & Dalgleish, D. G. (2007). Formation of whey protein/κ-casein complexes in heated milk: Preferential reaction of whey protein with κ-casein in the casein micelles. *International Dairy Journal, 17*(10), 1161-1167.

Doublier, J. L., Garnier, C., Renard, D., & Sanchez, C. (2000). Protein–polysaccharide interactions. *Current Opinion in Colloid & Interface Science, 5*(3), 202-214.

Du, B., Li, J., Zhang, H., Chen, P., Huang, L., & Zhou, J. (2007). The stabilization mechanism of acidified milk drinks induced by carboxymethylcellulose. *Lait, 87*(4-5), 287-300.

Dumpler, J., Wohlschläger, H., & Kulozik, U. (2017). Dissociation and coagulation of caseins and whey proteins in concentrated skim milk heated by direct steam injection. *Dairy Science & Technology, 96*(6), 807-826.

Edwards, P., Creamer, L., & Jameson, G. (2009). Structure and stability of whey proteins. *Milk Proteins*, 201-242.

Ekberg, O. (2019). *Dysphagia : diagnosis and treatment*. Cham, Switzerland : Springer.

Fallourd, M., & Viscione, L. (2009). Ingredient selection for stabilisation and texture optimisation of functional beverages and the inclusion of dietary fibre. In *Functional and Speciality Beverage Technology* (pp. 3-38). Elsevier.

Ferreira, T. G. (2011). Optimization of Coagulation and Syneresis Processes in Cheesemaking Using a Light Backscatter Sensor Technology.

Fitzsimons, S. M., Mulvihill, D. M., & Morris, E. R. (2007). Denaturation and aggregation processes in thermal gelation of whey proteins resolved by differential scanning calorimetry. *Food Hydrocolloids, 21*(4), 638-644.

Fox, P. F., Uniacke-Lowe, T., McSweeney, P. L. H., & O'Mahony, J. A. (2015). *Dairy chemistry and biochemistry*. Springer.

Gaby, K. (2010). Bioavailability and solubility of different calcium-salts as a basis for calcium enrichment of beverages. *Food and Nutrition Sciences*.

Garcia, J. M., Chambers, E., Matta, Z., & Clark, M. (2005). Viscosity measurements of nectar-and honey-thick liquids: product, liquid, and time comparisons. *Dysphagia, 20*(4), 325-335.

Garcia, J. M., Chambers, E., Matta, Z., & Clark, M. (2008). Serving temperature viscosity measurements of nectar-and honey-thick liquids. *Dysphagia, 23*(1), 65-75.

Gastaldi, E., Pellegrini, O., Lagaude, A., & de la Fuente, B. T. (1994). Functions of added calcium in acid milk coagulation. *Journal of Food Science, 59*(2), 310-312.

Gaucheron, F. (2005). The minerals of milk. *Reproduction Nutrition Development, 45*(4), 473-483.

George, A., Shah, P. A., & Shrivastav, P. S. (2019). Guar gum: Versatile natural polymer for drug delivery applications. *European Polymer Journal, 112*, 722-735.

Germain, I., Dufresne, T., & Ramaswamy, H. (2006). Rheological characterization of thickened beverages used in the treatment of dysphagia. *Journal of Food Engineering, 73*(1), 64-74.

Green, M. (1980). The formation and structure of milk protein gels. *Food Chemistry, 6*(1), 41-49.

Grinberg, V. Y., & Tolstoguzov, V. B. (1997). Thermodynamic incompatibility of proteins and polysaccharides in solutions. *Food Hydrocolloids, 11*(2), 145-158.

Groher, M. E. (1997). *Dysphagia : diagnosis and management*. Boston : Butterworth-Heinemann.

Groves, E., & Chaw, C. S. (2015). Incorporation of calcium salts into xanthan gum matrices: hydration, erosion and drug release characteristics. *Drug Development and Industrial Pharmacy, 41*(10), 1608-1616.

Guéguen, L., & Pointillart, A. (2000). The bioavailability of dietary calcium. *Journal of the American College of Nutrition, 19*(2), 119S-136S.

Gupta, S., & Variyar, P. S. (2018). Guar gum: a versatile polymer for the food industry. In *Biopolymers for Food Design* (pp. 383-407). Elsevier.

Hadde, E. (2017). Understanding the Rheological Parameters of Thickened Fluids for Dysphagia Sufferers.

Hanson, B. (2016). A review of diet standardization and bolus rheology in the management of dysphagia. *Current opinion in otolaryngology & head and neck surgery, 24*(3), 183-190.

Hashizume, K., & Sato, T. (1988). Gel-forming characteristics of milk proteins. 1. Effect of heat treatment. *Journal of Dairy Science, 71*(6), 1439-1446.

Heaney, R. P., Gallagher, J., Johnston, C., Neer, R., Parfitt, A. M., & Whedon, G. D. (1982). Calcium nutrition and bone health in the elderly. *The American journal of clinical nutrition, 36*(5), 986-1013.

Heertje, I., Visser, J., & Smits, P. (1985). Structure formation in acid milk gels. *Food Structure, 4*(2), 10.

Hemar, Y., Tamehana, M., Munro, P., & Singh, H. (2001). Viscosity, microstructure and phase behavior of aqueous mixtures of commercial milk protein products and xanthan gum. *Food Hydrocolloids, 15*(4-6), 565-574.

Hind, J., Divyak, E., Zielinski, J., Taylor, A., Hartman, M., Gangnon, R., & Robbins, J. (2012). Comparison of standardized bariums with varying rheological parameters on swallowing kinematics in males. *Journal of Rehabilitation Research & Development, 49*(9), 1399-1405.

Hongsprabhas, P., & Barbut, S. (1996). Ca2+ -induced gelation of whey protein isolate: effects of pre-heating. *Food Research International, 29*(2), 135-139.

Horne, D., & Lucey, J. (2014). Revisiting the temperature dependence of the coagulation of renneted bovine casein micelles. *Food Hydrocolloids, 42*, 75-80.

Horne, D. S. (1998). Casein interactions: casting light on the black boxes, the structure in dairy products. *International Dairy Journal, 8*(3), 171-177.

Horne, D. S. (2006). Casein micelle structure: models and muddles. *Current Opinion in Colloid & Interface Science, 11*(2-3), 148-153.

Huang, Y., Singh, P. P., Tang, J., & Swanson, B. G. (2004). Gelling temperatures of high acyl gellan as affected by monovalent and divalent cations with dynamic rheological analysis. *Carbohydrate Polymers, 56*(1), 27-33.

Hussain, M., Bakalis, S., Gouseti, O., Akhtar, S., Hameed, A., & Ismail, A. (2017). Microstructural and dynamic oscillatory aspects of yogurt as influenced by hydrolysed guar gum. *International journal of food science & technology, 52*(10), 2210-2216.

Hyslop, D. (2003). Enzymatic coagulation of milk. In *Advanced Dairy Chemistry—1 Proteins* (pp. 839-878). Springer.

International Dysphagia Diet Standardisation Initiative. (2017). *IDDSI Flow Test*.

Jang, H. D., & Swaisgood, H. E. (1990). Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *Journal of Dairy Science, 73*(4), 900-904.

Jenkins, P. J., & Donald, A. M. (1998). Gelatinisation of starch: a combined SAXS/WAXS/DSC and SANS study. *Carbohydrate Research, 308*(1-2), 133-147.

Jenness, R. (1974). Chapter one - The Composition of Milk. In *Nutrition and Biochemistry of Milk/maintenance* (pp. 3-107). Academic Press.

Jennifer, C. D., & Mikoto, B. (2000). Evaluation and treatment of swallowing impairments. *Am Fam Physician, 61*(8), 2453-2462.

Jeurnink, T. J., & De Kruif, K. G. (1995). Calcium concentration in milk in relation to heat stability and fouling. *Netherlands Milk and Dairy Journal, 49*, 151-151.

Ju, Z., & Kilara, A. (1998a). Aggregation induced by calcium chloride and subsequent thermal gelation of whey protein isolate. *Journal of Dairy Science, 81*(4), 925-931.

Ju, Z., & Kilara, A. (1998b). Properties of gels induced by heat, protease, calcium salt, and acidulant from calcium ion-aggregated whey protein isolate. *Journal of Dairy Science, 81*(5), 1236-1243.

Karlsson, A. O., Ipsen, R., & Ardö, Y. (2007). Observations of casein micelles in skim milk concentrate by transmission electron microscopy. *LWT-Food Science and Technology, 40*(6), 1102-1107.

Karlsson, M. A., Lundh, Å., Innings, F., Höjer, A., Wikström, M., & Langton, M. (2019). The Effect of Calcium, Citrate, and Urea on the Stability of Ultra-High Temperature Treated Milk: A Full Factorial Designed Study. *Foods, 8*(9), 418.

Kim, Y. H. (2007). *Inconsistency in the line spread test as an objective measurement of thickened liquids.* Seoul, Korea. https://soar.wichita.edu/bitstream/handle/10057/1142/t07025.pdf?sequence=3

Kinsella, J., Rector, D., & Phillips, L. (1994). Physicochemical properties of proteins: Texturization via gelation, glass and film formation. In *Protein structure-function relationships in foods* (pp. 1-21). Springer.

Kosikowski, F., & Mistry, V. V. (1977). *Cheese and fermented milk foods* (Vol. 586). Edwards Bros.

Koutina, G. (2016). Calcium induced skim-milk gelation during heating as affected by pH. *Dairy science & technology, 96*(1), 79-93.

Koutina, G., Christensen, M., Bakman, M., Andersen, U., & Skibsted, L. H. (2016). Calcium induced skim-milk gelation during heating as affected by pH. *Dairy science & technology, 96*(1), 79-93.

Koutina, G., Christensen, M., Bakman, M., & Anderson, U. (2015). Calcium induced skim milk gelation during heating as affected by pH. *Dairy Science & Technology, 96*, 79-93.

Koutina, G., Knudsen, J., & Skibsted, L. (2015). The effect of pH on calcium and phosphorus distribution between micellar and serum phase after enrichment of skim milk with calcium D-lactobionate. *Dairy Science & Technology, 95*(1), 63-74.

Kruif, C. (1996). Skim milk acidification at low temperature: a model for the stability of casein micelles. *Netherlands Milk and Dairy Journal, 50*, 113-120.

Kucharska, E. (2017). Osteoporosis: a social problem in the elderly population. *Horyzonty Wychowania, 16*(40), 37-57.

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.

Le, X. T., & Turgeon, S. L. (2013). Rheological and structural study of electrostatic cross-linked xanthan gum hydrogels induced by β-lactoglobulin. *Soft Matter, 9*(11), 3063-3073.

Lewis, M. (2011). The measurement and significance of ionic calcium in milk–a review. *International Journal of Dairy Technology, 64*(1), 1-13.

Lewis, M., Grandison, A., Lin, M., & Tsioulpas, A. (2011). Ionic calcium and pH as predictors of stability of milk to UHT processing. *Milchwissenschaft, 66*(2), 197-200.

Lin, L., Wong, M., Deeth, H., & Oh, H. (2020). Calcium-induced skim milk gels: Impact of holding temperature and ionic strength. *International Dairy Journal, 104*, 104657.

Lin, L., Wong, M., Deeth, H. C., & Oh, H. E. (2018). Calcium-induced skim milk gels using different calcium salts. *Food Chemistry, 245*, 97-103.

Lin, M., Lewis, M., & Grandison, A. (2006). Measurement of ionic calcium in milk. *International Journal of Dairy Technology, 59*(3), 192-199.

Liu, J., Nakamura, A., & Corredig, M. (2006). Addition of pectin and soy soluble polysaccharide affects the particle size distribution of casein suspensions prepared from acidified skim milk. *Journal of Agricultural and Food Chemistry, 54*(17), 6241-6246.

Loren, N., Langton, M., & Hermansson, A.-M. (2007). Confocal fluorescence microscopy (CLSM) for food structure characterisation. *Understanding and controlling the microstructure of complex foods*, 232-260.

Lu, M., & Wang, N. S. (2017). Chapter 7 - Spoilage of Milk and Dairy Products. In A. Bevilacqua, M. R. Corbo, & M. Sinigaglia (Eds.), *The Microbiological Quality of Food* (pp. 151-178). Woodhead Publishing.

Lucey, J. (2011). Cheese | Rennet-Induced Coagulation of Milk. In *Encyclopedia of Dairy Sciences (Second Edition)* (2 ed., pp. 579-584). Academic Press.

Lucey, J. (2014). Chapter 17 - Milk Protein Gels. In H. Singh, M. Boland, & A. Thompson (Eds.), *Milk Proteins (Second Edition)* (pp. 493-523). Academic Press.

Lucey, J., Munro, P., & Singh, H. (1999). Effects of heat treatment and whey protein addition on the rheological properties and structure of acid skim milk gels. *International Dairy Journal, 9*(3-6), 275-279.

Lucey, J., & Singh, H. (1997). Formation and physical properties of acid milk gels: a review. *Food Research International, 30*(7), 529-542.

Lucey, J., Tamehana, M., Singh, H., & Munro, P. (1998). Effect of interactions between denatured whey proteins and casein micelles on the formation and rheological properties of acid skim milk gels. *Journal of Dairy Research, 65*(4), 555-567.

Lund, M., Heyda, J., & Jungwirth, P. (2010). Ion Binding to Biomolecules.

Mackley, M., Tock, C., Anthony, R., Butler, S., Chapman, G., & Vadillo, D. (2013). The rheology and processing behavior of starch and gum-based dysphagia thickeners. *Journal of Rheology, 57*(6), 1533-1553.

Macosko, C. W., & Larson, R. G. (1994). Rheology: principles, measurements, and applications.

Mahmoudi, N., Mehalebi, S., Nicolai, T., Durand, D., & Riaublanc, A. (2007). Light-scattering study of the structure of aggregates and gels formed by heat-denatured whey protein isolate and β-lactoglobulin at neutral pH. *Journal of Agricultural and Food Chemistry, 55*(8), 3104-3111.

Mao, R., Tang, J., & Swanson, B. (2001). Water holding capacity and microstructure of gellan gels. *Carbohydrate Polymers, 46*(4), 365-371.

Maroziene, A., & De Kruif, C. (2000). Interaction of pectin and casein micelles. *Food Hydrocolloids, 14*(4), 391-394.

Mazzeo, F. A. (2008). Importance of OscillatoryTIme sweep in rheology. *TA Instruments*, 1-4.

McClements, D. J., & Keogh, M. K. (1995). Physical properties of cold-setting gels formed from heat-denatured whey protein isolate. *Journal of the Science of Food and Agriculture, 69*(1), 7-14.

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

Merkus, H. G. (2009). *Particle size measurements: fundamentals, practice, quality* (Vol. 17). Springer Science & Business Media.

Mikac, U., Sepe, A., Kristl, J., & Baumgartner, S. (2010). A new approach combining different MRI methods to provide detailed view on swelling dynamics of xanthan tablets influencing drug release at different pH and ionic strength. *Journal of controlled release, 145*(3), 247-256.

Mills, R. H. (2008). Dysphagia management: using thickened liquids. *The ASHA Leader, 13*(14), 12-13.

Minton, A. P. (2007). Static light scattering from concentrated protein solutions, I: general theory for protein mixtures and application to self-associating proteins. *Biophysical journal, 93*(4), 1321-1328.

Mohammed, Z., Haque, A., Richardson, R., & Morris, E. (2007). Promotion and inhibition of xanthan 'weak-gel'rheology by calcium ions. *Carbohydrate Polymers, 70*(1), 38-45.

Morison, K., Phelan, J., & Bloore, C. (2013). Viscosity and Non-Newtonian Behaviour of Concentrated Milk and Cream. *International Journal of Food Properties, 16*(4), 882-894. https://doi.org/10.1080/10942912.2011.573113

Moritaka, H., Nishinari, K., Taki, M., & Fukuba, H. (1995). Effects of pH, potassium chloride, and sodium chloride on the thermal and rheological properties of gellan gum gels. *Journal of Agricultural and Food Chemistry, 43*(6), 1685-1689.

Mudgil, D., Barak, S., & Khatkar, B. S. (2014). Guar gum: processing, properties and food applications-a review. *Journal of Food Science and Technology, 51(3)*, 409-418.

Mulvihill, D., & Grufferty, M. (1995). Effect of thermal processing on the coagulability of milk by acid.

Muresan, V. (2018). Particle Size Analysis by wet Laser Diffraction for anhydrous food suspensions: Application to sunflower tahini. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca: Food Science and Technology, 75*(1), 43-52.

Nayak, N., & Singh, H. (2019). Milk Protein–Polysaccharide Interactions in Food Systems.

Nayebzadeh, K., Chen, J., Dickinson, E., & Moschakis, T. (2006). Surface structure smoothing effect of polysaccharide on a heat-set protein particle gel. *Langmuir, 22*(21), 8873-8880.

Neutrium. (2012). *Non-Newtonian Fluids*. Neutrium. https://neutrium.net/fluid_flow/viscosity/

Nicholson, T., Torley, P., & Cichero, J. (2008). The measurement of thickened liquids used for the management of dysphagia. AIP Conference Proceedings, Australia.

Nicosia, M. A., & Robbins, J. (2007). The usefulness of the line spread test as a measure of liquid consistency. *Dysphagia, 22*(4), 306-311.

O'Gara, J. A. (1990). Dietary adjustments and nutritional therapy during treatment for oral-pharyngeal dysphagia. *Dysphagia, 4*(4), 209-212.

O'Connell, J., & Fox, P. (2011). Heat Treatment of Milk: Heat Stability of Milk. *Encyclopedia of Dairy Sciences* 744-799. (2)

O'Kennedy, B. (2011). Caseins. In *Handbook of food proteins* (pp. 13-29). Elsevier.

O'Mahony, J. A., & Fox, P. F. (2014). Chapter 2 - Milk: An Overview. In *Milk Proteins* (2 ed., pp. 19-73). Academic Press.

Omoarukhe, E., On-Nom, N., Grandison, A., & Lewis, M. (2010). Effects of different calcium salts on properties of milk related to heat stability. *International Journal of Dairy Technology, 63*(4), 504-511.

Owen, S. R., Tung, M. A., & Paulson, A. T. (1992). Thermorheological studies of food polymer dispersions. *Journal of Food Engineering, 16*(1), 39-53.

Papiz, M., Sawyer, L., Eliopoulos, E., North, A., Findlay, J., Sivaprasadarao, R., Jones, T., Newcomer, M., & Kraulis, P. (1986). The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature, 324*(6095), 383-385.

Pappas, C. P., & Rothwell, J. (1991). The effects of heating, alone or in the presence of calcium or lactose, on calcium binding to milk proteins. *Food Chemistry, 42*(2), 183-201.

Parris, N., Purcell, J. M., & Ptashkin, S. M. (1991). Thermal denaturation of whey proteins in skim milk. *Journal of Agricultural and Food Chemistry, 39*(12), 2167-2170.

Parry, R. (1974). Milk coagulation and protein denaturation. *Fundamentals of Dairy Chemistry, 603*.

Patton, J., & Reeder, W. (1956). New indicator for titration of calcium with (ethylenedinitrilo) tetraacetate. *Analytical Chemistry, 28*(6), 1026-1028.

Pawley, J. (2010). *Handbook of biological confocal microscopy*. Springer Science & Business Media.

Pearce, K. (1977). The complexometric determination of calcium in dairy products. *New Zealand Journal of Dairy Science and Technology*.

Pereira, L. (2016). *Carrageenans : Sources and Extraction Methods, Molecular Structure, Bioactive Properties and Health Effects*. Nova Science Publishers.

Pesic, M. B., Barac, M. B., Stanojevic, S. P., Ristic, N. M., Macej, O. D., & Vrvic, M. M. (2012). Heat induced casein–whey protein interactions at natural pH of milk: A comparison between caprine and bovine milk. *Small Ruminant Research, 108*(1), 77-86.

Phadungath, C. (2005). The mechanism and properties of acid-coagulated milk gels. *Songklanakarin J. Sci. Technol, 27*(2), 433-448.

Philippe, M., Gaucheron, F., Le Graet, Y., Michel, F., & Garem, A. (2003). Physicochemical characterization of calcium-supplemented skim milk. *Le lait, 83*(1), 45-59.

Phillips, G. O., & Williams, P. A. (2009). *Handbook of hydrocolloids*. CRC Press ; Cambridge : Woodhead Pub.

Phillips, G. O., & Williams, P. A. (2011). *Handbook of food proteins*. Elsevier.

Qian, F., Sun, J., Cao, D., Tuo, Y., Jiang, S., & Mu, G. (2017). Experimental and modelling study of the denaturation of milk protein by heat treatment. *Korean Journal for Food science of Animal Resources, 37*(1), 44.

Raikos, V. (2010). Effect of heat treatment on milk protein functionality at emulsion interfaces. A review. *Food Hydrocolloids, 24*(4), 259-265.

Ramasubramanian, L., D'Arcy, B., & Deeth, H. C. (2012). Heat-induced coagulation of whole milk by high levels of calcium chloride. *International Journal of Dairy Technology, 65*(2), 183-190.

Ramasubramanian, L., D'Arcy, B. R., Deeth, H. C., & Oh, H. E. (2014). The rheological properties of calcium-induced milk gels. *Journal of Food Engineering, 130*, 45-51.

Rao, M., & Cooley, H. (1993). Dynamic rheological measurement of structure development in high-methoxyl pectin/fructose gels. *Journal of Food Science, 58*(4), 876-879.

Rao, M. A., & Lopes da Silva, J. A. (2007). *Rheology of fluid and semisolid foods : principles and applications*. New York : Springer.

Reimer, L. (2013). *Scanning electron microscopy: physics of image formation and microanalysis* (Vol. 45). Springer.

Reimers-Neils, L., Logemann, J., & Larson, C. (1994). Viscosity effects on EMG activity in normal swallow. *Dysphagia, 9*(2), 101-106.

Richardson, R. K., & Ross-Murphy, S. B. (1981). Mechanical properties of globular protein gels: II concentration, pH concentration, pH and ionic strength dependence. *British Polymer Journal, 13*(1), 11-16.

Rohart, A., & Michon, C. (2014). Designing microstructure into xanthan gum-enriched acid milk gels. *Innovative Food Science & Emerging Technologies, 25*, 53-57.

Saha, D., & Bhattacharya, S. (2010). Hydrocolloids as thickening and gelling agents in food: a critical review. *Journal of Food Science and Technology, 47*(6), 587-597.

Sanchez, C., Zuniga-Lopez, R., Schmitt, C., Despond, S., & Hardy, J. (2000). Microstructure of acid–induced skim milk–locust bean gum–xanthan gels. *International Dairy Journal, 10*(3), 199-212.

Sawyer, L. (2003). β-Lactoglobulin. In *Advanced Dairy Chemistry 1: Proteins* (Vol. 1, pp. 319-386). Kluwer Academic/Plenum Publishers, New York.

Schmidt, K., & Smith, D. E. (1992). Milk reactivity of gum and milk protein solutions. *Journal of Dairy Science, 75*(12), 3290-3295.

Schokker, E., Singh, H., & Creamer, L. (2000). Heat-induced aggregation of β-lactoglobulin A and B with α-lactalbumin. *International Dairy Journal, 10*(12), 843-853.

Schorsch, C., Wilkins, D. D., Jones, M. G., & Norton, I. T. (2001). Gelation of casein-whey mixtures: effects of heating whey proteins alone or in the presence of casein micelles. *Journal of Dairy Research, 68*(3), 471-481.

Siamand, R., Deeth, H. C., & Al-Saadi, J. M. S. (2014). Textural and sensory properties of a calcium-induced milk gel. *Journal of Food Engineering, 139*, 10-12.

Sievanen, K., Huppertz, T., Kelly, A. L., & Fox, P. F. (2008). Influence of added calcium chloride on the heat stability of unconcentrated and concentrated bovine milk. *International Journal of Dairy Technology, 61*(2), 151-155.

Singh, G., Arora, S., Sharma, G., Sindhu, J., Kansal, V., & Sangwan, R. (2007). Heat stability and calcium bioavailability of calcium-fortified milk. *LWT-Food Science and Technology, 40*(4), 625-631.

Skibsted, L. H. (2016). Mineral nutrient interaction: Improving bioavailability of calcium and iron. *Food Science and Biotechnology, 25*(5), 1233-1241.

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

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.

Snoeren, T. H., Both, P., & Schmidt, D. (1976). An electron-microscopic study of carrageenan and its interaction with kappa-casein. *Netherlands Milk and Dairy Journal*.

Sopade, P., Halley, P., Cichero, J., Ward, L., Hui, L., & Teo, K. (2008). Rheological characterisation of food thickeners marketed in Australia in various media for the management of dysphagia. II. Milk as a dispersing medium. *Journal of Food Engineering, 84*(4), 553-562.

Stacey, K. A. (1956). Light-scattering in physical chemistry.

Stading, M., & Hermansson, A.-M. (1990). Viscoelastic behaviour of β-lactoglobulin gel structures. *Food Hydrocolloids, 4*(2), 121-135.

Stading, M., & Hermansson, A.-M. (1993). Rheological behaviour of mixed gels of κ-carrageenan-locust bean gum. *Carbohydrate Polymers, 22*(1), 49-56.

Steele, C. M., James, D. F., Hori, S., Polacco, R. C., & Yee, C. (2014). Oral perceptual discrimination of viscosity differences for non-Newtonian liquids in the nectar- and honey-thick ranges. *Dysphagia, 29*(3), 355-364.

Stephen, A. M., Phillips, G. O., & Williams, P. A. (2006). *Food polysaccharides and their applications* (2 ed.). CRC.

Stetefeld, J., McKenna, S. A., & Patel, T. R. (2016). Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophysical Reviews, 8*(4), 409-427.

Sura, L., Madhavan, A., Carnaby, G., & Crary, M. (2012). Dysphagia in the elderly: management and nutritional considerations. *Clinical interventions in aging, 7*, 287.

Sutherland, I. W. (2001). Microbial polysaccharides from Gram-negative bacteria. *International Dairy Journal, 11*(9), 663-674.

Swaisgood, H. (2003). Chemistry of the caseins. In *Advanced dairy chemistry—1 Proteins* (pp. 139-201). Springer.

Sworn, G. (2009). Gellan gum. In *Handbook of hydrocolloids* (pp. 204-227). Elsevier.

Syrbe, A., Bauer, W., & Klostermeyer, H. (1998). Polymer science concepts in dairy systems—an overview of milk protein and food hydrocolloid interaction. *International Dairy Journal, 8*(3), 179-193.

Thaiudom, S., & Goff, H. (2003). Effect of κ-carrageenan on milk protein polysaccharide mixtures. *International Dairy Journal, 13*(9), 763-771.

Thakur, B. R., Singh, R. K., Handa, A. K., & Rao, M. (1997). Chemistry and uses of pectin—a review. *Critical Reviews in Food Science & Nutrition, 37*(1), 47-73.

Totosaus, A., Montejano, J. G., Salazar, J. A., & Guerrero, I. (2002). A review of physical and chemical protein - gel induction. *International journal of food science & technology, 37*(6), 589-601.

Tromp, R. H., de Kruif, C. G., van Eijk, M., & Rolin, C. (2004). On the mechanism of stabilisation of acidified milk drinks by pectin. *Food Hydrocolloids, 18*(4), 565-572.

Tsioulpas, A., Lewis, M., & Grandison, A. (2007). Effect of minerals on casein micelle stability of cows' milk. *Journal of Dairy Research, 74*(2), 167-173.

Tuinier, R., Rieger, J., & de Kruif, C. G. (2003). Depletion-induced phase separation in colloid–polymer mixtures. *Advances in Colloid and Interface Science, 103*(1), 1-31.

Tuinier, R., Ten Grotenhuis, E., & De Kruif, C. (2000). The effect of depolymerised guar gum on the stability of skim milk. *Food Hydrocolloids, 14*(1), 1-7.

Turgeon, S. L., Schmitt, C., & Sanchez, C. (2007). Protein–polysaccharide complexes and coacervates. *Current Opinion in Colloid & Interface Science, 12*(4), 166-178.

Udabage, P., Mckinnon, I. R., & Augustin, M.-A. (2000). Mineral and casein equilibria in milk: effects of added salts and calcium-chelating agents. *Journal of Dairy Research, 67*(3), 361-370.

Ueda, Y., & Taira, Z. (2013). Effect of anions or foods on absolute bioavailability of calcium from calcium salts in mice by pharmacokinetics. *Journal of experimental pharmacology, 5*, 65.

Van Vliet, T., & Keetels, C. (1995). Effect of preheating of milk on the structure of acidified milk gels. *Netherlands Milk and Dairy Journal, 49*, 27-35.

Vasbinder, A. J., & De Kruif, C. G. (2003). Casein–whey protein interactions in heated milk: the influence of pH. *International Dairy Journal, 13*(8), 669-677.

Vasbinder, A. J., Rollema, H. S., Bot, A., & de Kruif, C. G. (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.

Vavrusova, M., Liang, R., & Skibsted, L. H. (2014). Thermodynamics of dissolution of calcium hydroxycarboxylates in water. *Journal of Agricultural and Food Chemistry, 62*(24), 5675-5681.

Vernon-Parry, K. (2000). Scanning electron microscopy: an introduction. *III-Vs Review, 13*(4), 40-44.

Vivanti, A. P., Campbell, K., Suter, M., Hannan‐Jones, M., & Hulcombe, J. (2009). Contribution of thickened drinks, food and enteral and parenteral fluids to fluid intake in hospitalised patients with dysphagia. *Journal of Human Nutrition and Dietetics, 22*(2), 148-155.

Waito, A. A., Valenzano, T. J., Peladeau-Pigeon, M., & Steele, C. M. (2017). Trends in research literature describing dysphagia in motor neuron diseases (MND): a scoping review. *Dysphagia, 32*(6), 734-747.

Walstra, P. (1999). *Dairy technology: principles of milk properties and processes*. CRC Press.

Walstra, P., & Jenness, R. (1984). *Dairy chemistry & physics*. John Wiley & Sons.

Wang, S., Li, C., Copeland, L., Niu, Q., & Wang, S. (2015). Starch retrogradation: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety, 14*(5), 568-585.

Wang, W., Zhang, L., Li, Y., & Feng, Z. (2012). Heat‐induced protein aggregates and difference in the textural properties of whole milk gel. *Journal of Food Quality, 35*(4), 247-254.

Weaver, C. M. (1998). Calcium in food fortification strategies. *International Dairy Journal, 8*(5-6), 443-449.

Webb, J., & Holgate, J. H. (2003). Microscopy: Scanning Electron Microscopy. In B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition* (2 ed., pp. 3922-3928). Academic Press.

Wehbi, Z., Pérez, M.-D., Sánchez, L., Pocoví, C., Barbana, C., & Calvo, M. (2005). Effect of heat treatment on denaturation of bovine α-lactalbumin: determination of kinetic and thermodynamic parameters. *Journal of Agricultural and Food Chemistry, 53*(25), 9730-9736.

Whelan, K. (2001). Inadequate fluid intakes in dysphagic acute stroke. *Clinical Nutrition, 20*(5), 423-428.

Wijayanti, H. B., Bansal, N., & Deeth, H. C. (2014). Stability of whey proteins during thermal processing: A review. *Comprehensive Reviews in Food Science and Food Safety, 13*(6), 1235-1251.

Williams, P., & Phillips, G. (2009). Introduction to food hydrocolloids. In *Handbook of hydrocolloids* (pp. 1-22). Elsevier.

Winter, H. H. (2009). Three views of viscoelasticity for Cox–Merz materials. *Rheologica acta, 48*(3), 241-243.

Yang, X., Hou, Y., Gong, T., Sun, L., Xue, J., & Guo, Y. (2019). Concentration-dependent rheological behavior and gelation mechanism of high acyl gellan aqueous solutions. *International Journal of Biological Macromolecules, 131*, 959-970.

Zhang, H., Yang, L., Tu, Y., Wu, N., Jiang, Y., & Xu, M. (2019). Changes in texture and molecular forces of heated‐induced egg white gel with adding xanthan gum. *Journal of Food Process Engineering, 42*(4), e13071.

Zhong, L., Hadde, E. K., Zhou, Z., Xia, Y., & Chen, J. (2018). Sensory discrimination of the viscosity of thickened liquids for dysphagia management. *Journal of Sensory Studies, 33*(6), 12464.

Zhong, L., Oostrom, M., Truex, M., Vermeul, V., & Szecsody, J. (2013). Rheological behavior of xanthan gum solution related to shear thinning fluid delivery for subsurface remediation. *Journal of Hazardous Materials, 244*, 160-170.

Chapter 9 Appendices

Appendix 1 Skim milk product specification

Fonterra™
Dairy for life

Certificate of Analysis

COA ID: 2004-500-2

COA Number: 84920024-10-1

Product Description: Skimmilk Powder - Low Heat

Factory: 1575 - Fonterra Clandeboye Powder 1

Batch: 22973382

Customer: FONTERRA NEW ZEALAND

Manufacture Date: 29 January 2019

Buyer Order: 7400097343

Best Before Date: 27 January 2021

Parameter	Units of Measure	Mean Result	Standard Deviation
Protein (6.38 x N) as is	%m/m	33.65	0.16
Fat	%m/m	0.91	0.03
Moisture	%m/m	3.78	0.07
Protein in Milk Solids Non-Fat	%m/m	35.31	0.16
Titratable Acidity	%m/v	0.09	0.00
WPNI	mg/g	6.8	0.1
Insolubility Index	ml	0.1	0.0
Scorched Particles	/25g	A	
Flavour		Typical	
Aerobic Plate Count	cfu/g	240	167
Coliforms	/g	Not Detected	
Escherichia coli	/g	Not Detected	
Yeasts & Moulds	cfu/g	<1	0
Coag Positive Staphylococci	/g	Not Detected	
Salmonella	/750g	Not Detected	

Appendix 2 The final *G'* of the samples with various concentrations of calcium lactate from different heat treatments.

Concentration of calcium lactate (mM)	Final *G'* (Pa) from various heat treatments				
	65 °C for 60 min	70 °C for 60 min	75 °C for 30 min	75 °C for 60 min	80 °C for 30 min
0	0.06 ± 0.01^{cB}	0.10 ± 0.01^{dAB}	0.07 ± 0.01^{cAB}	0.10 ± 0.01^{cAB}	0.16 ± 0.02^{dA}
5	0.21 ± 0.01^{cD}	0.22 ± 0.01^{dD}	0.12 ± 0.01^{cC}	0.11 ± 0.00^{cB}	0.18 ± 0.00^{dA}
7.5	0.16 ± 0.09^{cB}	0.23 ± 0.01^{dB}	0.02 ± 0.00^{cB}	0.25 ± 0.02^{cB}	0.57 ± 0.05^{dA}
10	0.21 ± 0.01^{cD}	1.35 ± 0.02^{cC}	4.63 ± 0.08^{cB}	5.23 ± 0.03^{cB}	29.18 ± 0.36^{cA}
15	10.31 ± 0.75^{bD}	21.29 ± 0.38^{bC}	36.03 ± 3.03^{bB}	41.17 ± 2.93^{bB}	61.41 ± 2.47^{bA}
20	19.91 ± 0.90^{aE}	35.78 ± 0.49^{aD}	44.33 ± 0.71^{aC}	63.21 ± 3.00^{aB}	73.15 ± 0.93^{aA}

Average of mean values ± standard deviation (n = 2 replicates)
Means in the same column followed by different letters are significantly different ($p<0.05$)
Means in the same row followed by different capital letters are significantly different ($p<0.05$)

Appendix 3 The final *G'* of the samples with added xanthan gum and calcium lactate.

Concentration of xanthan gum	Concentration of calcium lactate		
	0mM	10mM	15mM
0%	0.15 ± 0.02^{a}	13.69 ± 2.25^{cd}	29.67 ± 1.06^{b}
0.10%	0.37 ± 0.12^{a}	2.64 ± 1.25^{d}	7.03 ± 1.32^{d}
0.15%	N/A	16.13 ± 0.52^{bc}	54.46 ± 1.42^{a}
0.20%	N/A	36.41 ± 4.68^{a}	53.60 ± 3.74^{a}
0.25%	N/A	27.22 ± 3.69^{ab}	47.20 ± 1.27^{a}
0.30%	1.00 ± 0.09^{a}	18.96 ± 1.85^{ab}	22.91 ± 1.42^{bc}
0.40%	N/A	11.02 ± 0.44^{cd}	16.08 ± 1.30^{cd}
0.50%	8.62 ± 0.26^{b}	8.96 ± 0.60^{cd}	14.01 ± 2.29^{cd}

Average of mean values ± standard deviation (n = 2 replicates)
Means in the same column followed by different letters are significantly different ($p < 0.05$)
N/A: The final *G'* of the sample was not measured.

Appendix 4 The final *G'* of the samples with added high acyl gellan gum and calcium lactate.

Concentration of high acyl gellan gum	Concentration of calcium lactate		
	0mM	10mM	15mM
0%	0.15 ± 0.02^{d}	13.69 ± 1.25^{c}	29.67 ± 1.06^{c}
0.1%	4.24 ± 0.13^{c}	13.22 ± 0.55^{c}	30.58 ± 1.65^{bc}
0.2%	5.14 ± 0.15^{c}	28.34 ± 1.61^{b}	37.42 ± 2.03^{bc}
0.3%	11.39 ± 0.31^{b}	32.73 ± 1.06^{b}	42.19 ± 1.32^{b}
0.5%	41.01 ± 1.25^{a}	92.86 ± 3.48^{a}	102.19 ± 3.77^{a}

Average of mean values ± standard deviation (n = 2 replicates)
Means in the same column followed by different letters are significantly different ($p<0.05$)

Appendix 5 The final *G'* of the samples with added guar gum and calcium lactate.

Concentration of guar gum	Concentration of calcium lactate		
	0mM	**10mM**	**15mM**
0%	0.15±0.02^{c}	13.69±2.31^{ab}	29.67±1.06^{a}
0.1%	0.78±0.05^{a}	6.60±1.25^{b}	9.72±1.83^{c}
0.2%	2.01±0.06^{b}	7.00±0.44^{b}	12.78±0.34^{c}
0.3%	2.65±0.34^{b}	9.72±1.25^{ab}	13.85±1.42^{c}
0.5%	1.33±0.16^{ab}	14.98±0.45^{a}	22.88±0.58^{b}

Average of mean values ± standard deviation (n = 2 replicates)
Means in the same column followed by different letters are significantly different ($p<0.05$)