Understanding the Impact of New Zealand Milk Seasonality on Dairy Product Quality

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Abstract

In seasonal calving countries like New Zealand, the seasonal variations in milk composition and properties are particularly pronounced due to the significant impact of the stage of lactation on milk characteristics. This study investigated the seasonal variations in the composition and properties of New Zealand milk, as well as the impact of seasonality on product systems including acid milk gel, yoghurt, whipping cream and UHT milk. In addition, this study also demonstrated the effect of processing on the physicochemical properties of milk and the quality of products.

The study of milk composition and properties over two full milking seasons demonstrated that the seasonal variations in the composition and physicochemical properties of New Zealand milk were largely controlled by the stage of lactation. Consistent seasonal patterns found include increases in protein and fat and a decrease in lactose in late-season milk, decreasing proportion of α-Lactalbumin in milk protein during the season and increased glycosylation degree of κ-casein in late season milk.

Both acid milk gels made by the addition of glucono delta-lactone and yoghurts made by bacterial fermentation showed significant decreases in firmness during the late season, despite the standardization of protein and fat content. Standardization was not sufficient in controlling the acid gelation properties of late-season New Zealand milk. The seasonal variation in the glycosylation degree of κ-casein might play an important role in affecting the acid gelation process by altering the electrostatic and hydrophobic interactions. An investigation into the use of ultrafiltration for standardization demonstrated that a higher proportion of ultrafiltration retentate in the milk improved the overall acid gelation properties of milk.

The seasonal variations in the fatty acid composition and the melting behaviour of milk fat broadly followed the lactational trend that the high-melting fatty acids (e.g. C16:0) reached the maximum in the mid-season.

The physicochemical changes during the storage of seasonal UHT milk were monitored. The age gelation of UHT milk was least pronounced in the early season. A new hypothesis for gelation mechanism that involves the interaction and sedimentation of κ-CN-depleted casein micelles was proposed.

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List of publications

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List of common abbreviations

α-LA	α-Lactalbumin
β-LG	β-Lactoglobulin
r	Correlation coefficient
ANOVA	Analysis of variance
Ca^{2+}	Ionic calcium
CCP	Colloidal calcium phosphate
CN	Casein
CV	Coefficient of variation
FA	Fatty acid
GDL	Glucono-δ-lactone
MFGM	Milk fat globule membrane
PAGE	Polyacrylamide gel electrophoresis
RP-HPLC	Reversed phase-high performance liquid chromatography
SCC	Somatic cell count
SDS	Sodium dodecyl sulfate
SFC	Solid fat content
SOL	Stage of lactation
UF	Ultrafiltration or ultrafiltered
UHT	Ultra-high temperature
WP	Whey protein

Chapter 1 - Introduction

The seasonality of bovine milk can be defined as the recurring changes in the composition, characteristics and processing properties of the milk at different times of the year. The seasonal variations in milk properties have a major impact on the processing properties of milk and the quality of dairy products. They offer difficulties for the dairy industry to produce high-quality products consistently throughout the year. A better understanding of how the processing properties of milk vary with season would allow the dairy manufactures to better control product quality and develop strategies to optimize profit.

Although many studies have been carried out on the seasonal variations in milk composition and processing properties, it is important to distinguish the patterns of seasonal variation between different dairy management systems applied in different countries and regions. Unlike many other developed dairy countries, dairy farms in New Zealand implement the seasonal calving system to maximize the utilization of pasture as a low-cost feed source. All the cows calve at the same time around early spring, which results in a lactation-dependent seasonal pattern in milk composition and properties. The most well-known seasonal variation behaviour in milk composition in seasonal calving countries like New Zealand is the drastic increases in milk fat and protein contents during the late season (Autumn). Such variations are not observed in non-seasonal calving countries, where the seasonal variation in milk composition and properties are attributed to the change in feed or climate.

Among different aspects of milk characteristics, milk composition, fatty acid composition and the cheese-making properties have been reported most extensively to

be affected by seasonal variations. However, there are limited reports on the impact of seasonal variations on the quality of a number of other products, including yoghurt, UHT milk and whipping cream.

In the current study, fresh seasonal milk was taken regularly from a seasonal-calving New Zealand farm across three milking seasons. Milk composition and a broad range of physiochemical properties before and after processing (e.g. heating and ultrafiltration) were analysed, along with the properties of acid milk gel, yoghurt, UHT milk and whipping cream made from the seasonal milk. The objectives of this study were to demonstrate and understand the seasonal variations in the characteristics of raw milk, processed milk and the aforementioned product systems. This study provides up-to-date information on the seasonal variations of milk composition and processing properties in New Zealand and contributes to a better understanding of the correlation between milk properties and product properties.

Chapter 4 laid the foundation of this study by investigating the seasonal variations in the composition and characteristics of New Zealand milk, as well as the heat-induced changes in the physicochemical properties of the milk under two typical heat treatments used in the dairy industry. Despite the previous studies on the seasonal variations in milk composition, the physicochemical changes induced by heat treatment have rarely been reported in seasonal studies, which play an important role linking raw milk properties to product properties. The study in Chapter 4 was conducted for two full milking seasons in order to identify the robust seasonal variations in milk characteristics.

Chapter 5 demonstrated the seasonal variations in the acid gelation properties of milk induced by the addition of Glucono-δ-lactone (GDL). Two sample systems were studied for their acid gelation properties and compared: seasonal skim milk (unstandardized) and standardized milk. The efficacy of standardization (of fat and protein content) in stabilizing the acid gelation properties of milk over the seasons was investigated.

Chapter 6 investigated the seasonal variations in the quality of different types of yoghurt, e.g. set yoghurt, stirred yoghurt and Greek-style yoghurt. Greek-style yoghurt was chosen as one of the sample systems of interest for its growing popularity and market share in recent years. The main objective of Chapter 6 was to study whether the different types of yoghurt, due to their different textural characteristics, would vary in their properties differently over the seasons. Comparison between the seasonal variation patterns of the yoghurts and the GDL-induced acid gel was also made to determine whether GDL-induced acid gelation could be used as an indicator for the variation in yoghurt properties during the year.

In the acid gelation study conducted in Chapter 5, the proportion of UF retentate used for the standardization of milk varied during the season, which also correlated with the acid gelation properties of the milk significantly. Therefore, the impact of ultrafiltration (UF) process on the acid gelation properties of milk was studied in Chapter 7. The changes in the structure of milk components and the salt equilibrium induced by UF was hypothesized to influence the acid gelation process.

Chapter 8 demonstrated the seasonal variations in the fatty acid composition and the melting behaviour of milk fat, as well as the properties of whipping cream. Previous

studies demonstrated the variation pattern of fatty acid composition over the seasons or during different stages of lactation. However, the seasonal variation in the whipping properties of unhomogenized pasteurized cream has not been reported in a seasonal calving country like New Zealand. The hypothesis was that the seasonal variations in the melting properties of milk fat, the size of fat globules and serum phase composition would contribute to the variation in whipping cream properties.

Chapter 9 focused on UHT milk and its physicochemical changes during storage, particularly age gelation. UHT skim milk and UHT whole milk made in different seasons were monitored for the occurrence of age gelation. An important aim of this study was to further the understanding of the mechanism of age gelation, specifically in indirectly-heated UHT milk. Therefore the connections between milk composition, the physicochemical changes during storage and the propensity of age gelation were investigated and discussed.

Chapter 2 - Literature review

2.1 Milk characteristics and the effect of processing

2.1.1 Milk composition

Milk contains about 13% total solids, mainly consist of lactose, fat, protein and minerals. Lactose is the primary carbohydrate of milk, which is a disaccharide composed by glucose and galactose. Milk fat is naturally present in globules, coated by the milk fat globule membrane (MFGM). The protein fraction of milk can be divided into two types, namely caseins (CN) and whey proteins (WP). Caseins are defined as the proteins precipitating from milk around pH 4.6, which consist of ~80% of milk proteins. Milk proteins that remain soluble at pH 4.6 are called whey proteins or serum proteins.

2.1.2 Milk protein properties

2.1.2.1 Caseins

In bovine milk, there are four casein fractions (α_{s1}-, α_{s2}-, β- and κ-casein, in approximate ratios of 4: 1: 3.5: 1.5). α-caseins and β-caseins are highly phosphorylated on their serine residues, allowing these caseins the capability of binding calcium ions. κ-Casein displays unique features among caseins. It has a low level of phosphorylation, has a low sensitivity to calcium and is the only one of the caseins to occur in the glycosylated form (Huppertz, 2013).

In milk about 95% of the caseins are present in the form of casein micelles. The casein micelles are complex colloids formed by highly phosphorylated caseins (α- and β-

caseins) interacting and aggregating with calcium phosphate, covered by a hairy layer of κ-casein (Dalgleish, 2011). The N-terminal of κ-casein is associated with the micelle core whereas the C-terminal protrudes into the surrounding serum, reducing surface hydrophobicity of the casein micelles (Walstra, 1990). Casein micelles are highly hydrated, containing ~3 to 4 g water per g of protein. The dry matter of the micelles consists of ~94% protein and ~6% inorganic salt, mainly in the form of calcium phosphate. The structure of casein micelles have been studied and reviewed extensively (Dalgleish, 2011; Garnier & Dumas, 1970; Holt, 1992; Horne, 1998, 2006; Schmidt, 1982; Slattery & Evard, 1973; Walstra, 1990, 1999). A number of models of casein micelle structure have been proposed. Some studies suggested casein micelles are formed by the assembly of substructures called submicelles, which are aggregates of a similar number of casein molecules (Schmidt, 1982; Walstra, 1990, 1999). Other works indicated that the substructures might not be submicelles but calcium phosphate nanoclusters (Dalgleish, 2011; Holt, 1992, 1998). In the "dual-binding" model proposed by Horne (1998), it was suggested that caseins interact with one another in the hydrophobic parts whereas the hydrophilic parts containing phosphoserine are linked to colloidal calcium phosphate (CCP).

The surface layer formed by κ-casein stabilizes casein micelles from aggregation by electrostatic and steric repulsion. Milk curd formation occurs upon destabilizing the κ-casein surface layer, e.g. by rennet addition or acidification, which is the basis of products as cheese and yoghurt. Rennet contains proteolytic enzyme chymosin, which splits off the κ-casein macro-peptides (residue 105-168 of κ-casein) which constitute the polyelectrolyte hairy brush on casein micelles. The cleaving of κ-casein surface

layer leads to the loss of steric stabilization, allowing effective attraction between casein micelles and finally curd formation. Upon acidification of milk, the stability of the casein hairy brush remains until around pH 5. Then over a small pH range, the κ-casein brush collapses allowing aggregation of casein micelles. The isoelectric precipitation of casein micelles enables the production of caseinates, fermented milk products and acid-coagulated cheeses (O'Mahony & Fox, 2013).

2.1.2.2 Whey proteins

Whey proteins in milk are consist of a diverse group, including β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin, immunoglobulins, proteose-peptones and other minor whey proteins (Fox, 2003). Most whey proteins are globular proteins and prone to heat denaturation. Heat-induced denaturation of whey proteins is the initial step of many changes in milk during processing.

β-lactoglobulin is the most prevalent whey protein in milk, comprising more than 50% of total whey proteins. One molecule of β-LG contains five cysteine residues, of which four are involved in disulphide (S-S) bonds, leaving one free thiol group equally distributed between Cys119 and Cys121. The free thiol group will be exposed during heating at temperatures above ~70°C and become reactive, which initiate complex changes in milk during heating.

α-lactalbumin is the second most abundant whey protein. It contains four intramolecular disulphide bonds but no sulfhydryl group. α-LA binds strongly with calcium, which protects it from irreversible denaturation during heating. A biological function of α-LA is the coenzyme in lactose synthesis.

2.1.3 Milk fat and fatty acid composition

The composition, structure and properties of milk lipids have been reviewed extensively (Christie, 1983; Fox & McSweeney, 1998; Jensen, 2002; MacGibbon & Taylor, 2006). Milk lipids are mainly present in globules emulsified in the aqueous phase. The core of milk fat globule contains mainly triacylglycerol, coated by the MFGM containing bipolar materials, e.g. phospholipids, proteins, sterols, etc. The sizes of milk fat globules range from < 0.2 to > 15 μm in diameter. Fat globules with diameters around 4 μm account for most of the mass (Jensen, 2002). The properties of milk fat globules contribute to the colour and creaming of milk and are very important for the processing properties of various dairy products, e.g. cream, ice cream and butter (Huppertz & Kelly, 2006).

Milk fatty acid (FA) composition has been studied extensively for its impact on the quality of dairy products and its potential influence on human health. The approximate composition of major FA in milk is shown in Table 2.1.

Table 2.1: Major fatty acid composition of New Zealand milk fat

Fatty acid		Content % (w/w)
4:0	Butyric	3.9
6:0	Caproic	2.5
8:0	Caprylic	1.5
10:0	Capric	3.2
12:0	Lauric	3.6
14:0	Myristic	11.1
14:1	Myristoleic	0.8
15:0		1.2
16:0	Palmitic	27.9
16:1	Palmitoleic	1.5
18:0	Stearic	12.2
18:1 cis	Oleic	17.2
18:1 trans		3.9
18:2	Linoleic	1.4
18:2 conj	Conjugated Linoleic acid	1.1
18:3	Linolenic	1.0
Minor acids		6.0

From Creamer and MacGibbon (1996)

FA composition has been found to influence the textural and sensory properties of a number of dairy products including cheese, ice cream, butter and yoghurt (Chen et al., 2004). FA composition influences the properties of dairy products owing to its impact on the melting properties of milk fat. The melting point of fat generally increases with FA chain length and decreases with unsaturation degree. Milk fat melts over a wide range from approximately -35°C to 38°C (MacGibbon & Taylor, 2006). Therefore, milk fat contains both liquid and solid fractions under normal consumption and storage

temperatures. A common measure of the melting property of milk fat is the solid fat content (SFC) at a certain temperature.

Milk fatty acids have two main origins: *de novo* synthesis in the mammary glands and plasma (or blood) lipids. The latter includes three pathways: directly from the diet, formation by biohydrogenation by rumen microorganisms and mobilisation of FA from adipose tissue (MacGibbon & Taylor, 2006; Palmquist, Denise Beaulieu, & Barbano, 1993).

Short-chain and medium-chain fatty acids, including 4:0 – 14:0 FA and part of 16:0 FA, are synthesized *de novo.* The rest of 16:0 FA and C18 FA derive from plasma lipids. Approximately 45% of all milk fatty acids are synthesised *de novo* whereas the rest is accounted for by dietary origin (Moore & Christie, 1979).

2.2 Processing effects on milk properties

2.2.1 Heat treatment

Heat treatment is a common practice during milk processing to ensure food safety and improve keeping quality by deactivating microorganisms. In addition, proper heating of milk can improve the quality of dairy products, e.g. yoghurt (Vasbinder & de Kruif, 2003). The properties of dairy products are influenced by the heat treatments to which the milk has been subjected during their preparation (Smits & Brouwershaven, 1980).

Upon heating at temperatures above 70°C, irreversible denaturation of whey proteins occurs as aggregation among whey proteins and between whey proteins and casein micelles take place (Wijayanti, Bansal, & Deeth, 2014). The heat-induced denaturation

of β-LG initiate complex reactions in heated milk. The β-LG monomer contains two disulphide bridges (Cys106-Cys119 and Cys66-Cys160) and one free cysteine (Cys121). The free thiol group (Cys121) is buried at the sheet-helix interface, which will be exposed after heat-induced unfolding and become reactive (Vasbinder, 2002). The reactive thiol group of β-LG can form disulphide bonds with κ-CN at the casein micelle surface via thiol/disulphide (–SH/S–S) interchange reactions (Considine, Patel, Anema, Singh, & Creamer, 2007; Jang & Swaisgood, 1990). The interaction between WP and κ-CN results in casein micelles stabilized by a hairy brush of κ-CN associating with whey proteins (Vasbinder, 2002). In the association between casein micelles and WP, the formation of intermolecular disulphide bonds between whey proteins with κ-CN plays an important role, but hydrophobic bonds are also involved (Smits & Brouwershaven, 1980). Oldfield, Singh, Taylor, and Pearce (1998b) suggested that the hydrophobic groups of β-LG are exposed during the early stage of the denaturation and aggregation reactions, leading to the formation of hydrophobically associated aggregates of β-LG. These hydrophobic aggregates are rapidly converted into S–S-bonded aggregates upon heating to a higher temperature (above 75°C). In the presence of α-LA, denatured β-LG initiates inter-protein reactions with α-LA forming hydrophobically linked β-LG /α-LA aggregates at < 80 °C and S–S-linked β-LG /α- LA aggregates at >80 °C. Nguyen, Anema, Guyomarc'h, Wong, and Havea (2015) found the distribution of κ-CN, β-LG and α-LA between the colloidal and serum phases was not affected even though a significant proportion of the disulphide bonds of κ-CN were reduced after the addition of reducing agent β–mercaptoethanol, suggesting non-covalent bonds may be as important as disulphide interactions in the heat-induced aggregation of the whey proteins with κ-CN.

Besides the interactions between WP and casein micelles, the formation of aggregates occurs in the serum phase of heated milk by interactions of denatured β-LG with cysteine-containing proteins in including κ-CN, α-LA and other β-LG. As a result, heat treatment of milk results in a complex mixture of native whey proteins, soluble whey protein/κ-casein aggregates and whey protein coated casein micelles. The final distribution of whey proteins between serum and casein micelles in heated milk depends on the temperature-time profile and pH of the heat treatment (Law & Leaver, 2000). Vasbinder, Alting, and de Kruif (2003) reported that after 10 min heat treatment at 75°C, 20% of all β-LG in skim milk was denatured and about 17% of all β-LG was associated with casein micelles. As for skim milk heated at 90°C for 10 min, 95% of β-LG was denatured and 65% of β-LG was found bound to casein micelles.

The method of heating was also found to affect the interaction between denatured whey proteins and casein micelles. It is suggested under slow heating conditions, whey proteins tend to form smaller aggregates. In contrast, in milk heated rapidly (e.g. direct steam injection), all the whey proteins denature in a short period, allowing the whey proteins to form larger aggregated species. It was proposed that the smaller aggregates formed under the slow heating conditions may associate with the micelles more efficiently than the larger aggregates formed under rapid heating conditions. As a consequence, a greater level of whey proteins associates with the micelles under slow heating conditions than under rapid heating conditions (Oldfield, Singh, & Taylor, 1998a).

Varying the pH of milk prior to heat treatment can influence the kinetics of whey protein denaturation and association with casein micelles (Anema & Klostermeyer,

1997; Corredig & Dalgleish, 1996; Oldfield, Singh, Taylor, & Pearce, 2000; Singh & Creamer, 1991; Singh & Latham, 1993; Vasbinder & de Kruif, 2003). Anema and Klostermeyer (1997) reported that heat treatment at higher pH results in forming more soluble whey protein aggregates while lower pH during heating leads to more association of whey proteins with casein micelles. This theory is supported by other researchers (Anema, 2007; Vasbinder & de Kruif, 2003), including under ultra-high temperature heating conditions (Singh & Latham, 1993). Vasbinder and de Kruif (2003) suggested the pH during heating not only affect the amount of whey proteins associated with casein micelles but also the homogeneity of the coating. At lower pH, κ-CN on casein micelle surface is more likely to associate with a cluster of whey proteins rather than one, which results in an inhomogeneous coating of whey proteins on casein micelles comparing to that at higher pH value.

Dissociation of κ-CN from casein micelle surface occurs during heating of milk (Anema & Klostermeyer, 1997; Dalgleish & Law, 1989; Donato, Guyomarc'h, Amiot, & Dalgleish, 2007b; Singh & Fox, 1985; Singh & Latham, 1993). It was found that the dissociation of κ-CN increases with both increasing heating temperature and pH (Anema & Klostermeyer, 1997; Singh & Latham, 1993). The dissociation of κ-CN has been reported to have a correlation with the distribution of denatured WP between the colloidal phase and the serum phase after heating but contradicting results have been found. It was reported that a strong relationship between serum phase denatured WP and serum phase κ-CN was found when heating milk at different pH. This relation leads to a conclusion that the distribution of denatured WP between the colloidal phase and the serum phase after heating is controlled by the distribution of κ-casein (Anema,

2007). However, according to Donato et al. (2007b), the addition of κ-casein in milk has little or no effect on the number of soluble complexes formed during heating, indicating the κ-CN in serum phase is somehow less favourable for interaction than κ-CN on casein micelles. It was suggested that the heat-induced aggregates may dissociate into the serum at a later stage of heating (Donato et al., 2007b).

2.2.2 Ultrafiltration

Membrane processing is widely used in the dairy industry for separating different milk components based on their differences in size (Kumar et al., 2013). Ultrafiltration (UF) utilizes membranes with a pore size of around 0.01 μm and a molecular weight cut-off range of 1 to 200 kDa (Rosenberg, 1995). UF is widely used in the dairy industry for various purposes, including concentrating milk proteins, manufacturing whey protein ingredients such as whey protein concentrates and whey protein isolates, and the purification of individual milk proteins (Kumar et al., 2013). The concentration of milk using UF aims at the retention of all milk proteins while allowing the permeation of water, lactose, soluble salts and non-protein nitrogen components (Singh, 2007). With increasing concentration factors (CF), proteins become progressively concentrated in the UF retentate whereas the calcium to protein and lactose to protein ratios decrease. The temperature at which the UF of milk is performed affects the permeation rate, the retention of milk components and the microbial quality of the retentates (Liu, Weeks, Dunstan, & Martin, 2014; McKenna, 2000; St-Gelais, Haché, & Gros-Louis, 1992). At low temperatures (below 10°C), β-CN dissociates from the casein micelles (Atamer et al., 2017; Dalgleish & Law, 1988; Post, Arnold, Weiss, & Hinrichs, 2012), which affect the serum protein composition of milk concentrated by UF (Liu et al., 2014). This

review focuses on the studies on the UF of milk carried out in the range of 40-50°C, which is commonly used in the dairy industry for the concentration of milk.

Diafiltration (DF) is an optional process during UF involves diluting the retentate with water, which improves the separation efficiency and the recovery of the permeable components (Rosenberg, 1995). As a result of the dilution, the proportion of lactose and salts in the UF/DF retentate was lower than UF retentate without DF (equal protein content). An important application of the UF/DF process is the production of milk protein concentrates (MPC), a type of milk protein ingredient with low contents of lactose and salts (Carr & Golding, 2016). The MPC is commonly used in the production of cheese, yoghurt and dairy beverages to enrich protein and enhance their rheological properties (Singh, 2007). This review focuses on the impacts of UF on the physicochemical properties of milk components and its effect on the quality of some fermented dairy products.

2.2.2.1 The effects of UF on the physicochemical properties of milk

Previous studies demonstrated that the UF process could alter the structure and the physicochemical properties of milk components. Three major aspects of milk properties were reported to be affected by UF, i.e. the structure and properties of the casein micelles, the equilibrium of milk calcium and the structure of whey proteins.

2.2.2.1.1 Casein micelle structure and properties

The structure of the casein micelles plays an important role in the processing properties of milk, which were demonstrated to be affected by the UF process (Broyard & Gaucheron, 2015).

There are some contradicting results on how UF affect the size of the casein micelles. The study of Srilaorkul, Ozimek, Ooraikul, Hadziyev, and Wolfe (1991) using electron microscopy showed that the mean casein micelle size decreased from 118 nm in unconcentrated milk to 92 nm in retentate concentrated 3 times by UF (CF3). The size range with the highest proportion of casein micelles shifted from 80–100 nm in milk to 60–80 nm in retentate (CF5). This was in agreement with Erdem (2000) who reported that the casein micelles became most compact and reduced in size after UF. In contrast, in another electron microscopy study, McKenna (2000) reported no apparent change in the casein micelle size during UF (without DF), although a progressive increase in micelle size was found during UF/DF. The micellar dissociation and swelling during DF were suggested to cause an increase in micelle size. Other authors reported that there was no consistent change in the casein micelle size after UF (Green, Scott, Anderson, Griffin, & Griffin, 1984), particularly after diluting the UF retentate with permeate to the original concentration of milk (Ferrer, Alexander, & Corredig, 2011; Liu et al., 2014). Insignificant differences in casein micelle size were also reported between milk and UF/DF retentate (Ferrer, Alexander, & Corredig, 2014; Martin, Williams, & Dunstan, 2010), as well as between milk and the MPC80 powder (Martin et al., 2010).

The behaviour of micellar dissociation during UF has been reported, particularly at high concentration factors (Green et al., 1984) and with the involvement of DF (McKenna, 2000; Singh, 2007). However, Green 1984 suggested that the lower fraction of micellar casein at high CF might be an artefact of determination of caseins in the centrifugation pellet since the higher viscosity of retentate at high CF might retard the

palletisation of caseins. Interestingly, Liu et al. (2014) reported that although the concentration of caseins in the serum of UF retentate increased with CF, the concentration of serum phase casein in original milk during UF at 40°C was generally stable with a slightly decreasing trend, suggesting a net shift of caseins from the serum phase to the micellar phase. Indeed, the removal of water from the serum phase by UF and the concentration of soluble caseins in serum could result in an increase in the concentration of serum phase caseins in UF retentate as observed by McKenna (2000). Ferrer et al. (2011) found no significant effect of concentration by UF on the composition of soluble proteins.

Previous studies also investigated other properties of the casein micelles in milk concentrated by UF. The concentration of UF was suggested to induce structural rearrangement and surface modifications of the casein micelles (Corredig, Nair, Li, Eshpari, & Zhao, 2019; Nair & Corredig, 2015). Erdem (2000) reported that the number of surface hydrophobic sites of casein micelles reduced during UF, which did not recover to the initial level after diluting the retentate with permeate to the concentration of unconcentrated milk. Liu et al. (2014) reported that micelle hydration increased by around 10% at CF3 and CF4. The zeta-potential of casein micelle was found not affected by UF (Ferrer et al., 2014).

2.2.2.1.2 Calcium equilibrium

Since the UF process separates milk components by their size and permeability through the membrane, the retention of milk salts depends on their distribution in the colloidal phase and the serum phase (Green et al., 1984; Premaratne & Cousin, 1991). Calcium is the most important milk salt in dairy processing for its crucial role in stabilizing the

casein micelle structure (Horne, 1998). It greatly affects the heat stability, rennet gelation and the heat-induced protein interactions in milk (Donovan & Mulvihill, 1987; Lewis, 2011). About two-thirds of calcium in milk present in the casein micelles in the form of CCP whereas the rest is soluble calcium (Holt, Dalgleish, & Jenness, 1981). The calcium equilibrium in milk, i.e. the transfer of calcium between the colloidal and the serum phases, is affected by various conditions, including temperature, dilution, concentration, pH, etc. Since the UF/DF process involves concentration and dilution of milk components, its impact on the calcium equilibrium has been investigated in previous studies.

There appears to be disagreement on whether UF (without DF) causes loss of colloidal calcium from the casein micelles. In the review by Corredig et al. (2019), it was suggested that the CCP in the casein micelles remain intact during UF. This agreed with Liu et al. (2014) who found no micellar calcium loss in the retentate during UF (up to CF 4) at 40°C. In contrast, Lin, Grandison, and Lewis (2015) reported a loss of colloidal calcium and magnesium during UF, which suggested a transfer of divalent cations from the casein micelles to the serum phase. The extents of this loss calculated from the data of Lin et al. (2015) were similar at CF 1.5-3 (~12%) and higher at CF 4 (18%). Other authors reported that the micellar calcium loss during UF was apparent only at high CF. Ferrer et al. (2011) reported that at a lower CF of 2, both total calcium and insoluble calcium concentrations of reconstituted retentate (with own permeate to the original concentration of milk) were similar to those of unconcentrated skim milk. However, at a higher CF of 5, the reconstituted retentate contained significantly lower calcium and insoluble calcium than skim milk, even after dialysis again the original

skim milk. This agreed with Li and Corredig (2014) that UF at high CF altered the CCP equilibrium, which was not significant at low CF. A small-angle neutron scattering study of UF (5×) retentate suggested partial dissolution of CCP in the casein micelles (Alexander, Nieh, Ferrer, & Corredig, 2011). The inconsistent reports on this topic might arise from the difference in UF equipment and the different methods for the determination of colloidal calcium in different studies. It seems that the calcium equilibrium can be altered by UF (without DF) to some extent, in which the CF plays a role.

As for UF/DF, its effect on solubilizing colloidal calcium has been reported consistently, the extent of which increases with CF and the amount of water added (Alexander et al., 2011; Corredig et al., 2019; Ferrer et al., 2014; Li & Corredig, 2014; Liu et al., 2019). The dilution of the serum phase surrounding the casein micelles appears to provide the driving force of micellar calcium solubilization.

2.2.2.1.3 Structural modification of whey proteins

A number of studies reported that the structure of globular whey proteins in milk could be affected by UF permeation. Van Audenhaege, Pezennec, and Gesan-Guiziou (2013) reported that α-LA in the UF permeate (dead-end UF, 10 kDa cut-off) had a slightly higher fraction of aggregates and improved heat stability compared with the original α-LA. They suggested that the physical shear stress in the membrane pores upon permeating the membrane altered the tertiary structure of α-LA. Besides, β-LG in the permeate of dead-end UF resulted in increased random coil structures, similar to those induced by thermal or chemical denaturation (Portugal, Crespo, & Lima, 2007; Van Audenhaege et al., 2010). These structural modifications of whey proteins by UF were

only found in the permeate and not in the retentate (Portugal et al., 2007; Van Audenhaege et al., 2013).

2.2.2.2 The impact of UF on fermented dairy products

The UF process is commonly used in the production of fermented dairy products such as cheese and yoghurt (Kumar et al., 2013; Rosenberg, 1995). Numerous studies investigated the effects of UF on the rennet-coagulation properties and cheese-making of milk, which were recently reviewed by (Soodam & Guinee, 2018). In general, compared with unconcentrated milk, milk concentrated with UF has shortened rennet gelation time and a higher rennet gel firmness. Cheese made from UF-standardized milk had lower moisture content, improved fat recovery, higher yield and lower proteolysis rate during ripening.

Concentration of milk with UF is widely used in the dairy industry to increase the protein content of yoghurt (Jørgensen et al., 2019). UF can be used for increasing the solids content of Greek-style yoghurt (GSY) both before and after the fermentation (Paredes Valencia, Doyen, Benoit, Margni, & Pouliot, 2018). Standardization of milk using UF for producing fermented dairy products was suggested to be superior in enhancing product quality than fortifying milk bases with evaporated milk or milk powder (Rosenberg, 1995). Some previous studies examined the effect of UF on the gelation properties of yoghurts, but in milk bases with different protein contents. Biliaderis, Khan, and Blank (1992) reported that compared with yoghurts made from SMP–fortified skim milk, yoghurts made from UF retentate at equal total solids content had higher gel strength, shorter gelation time and higher final loss tangent (LT). However, it should be noted that UF retentate has higher protein to lactose ratio than

the fortified skim milk due to the lactose removal during the UF process. Consequently, the standardization based on total solid content in the study of Biliaderis et al. (1992) resulted in significantly higher protein content in the retentate-standardized milk than in the SMP-standardized milk. Since proteins contribute considerably more than lactose to the structure of yoghurt, the superior yoghurt-making properties of retentate-standardized milk might have resulted solely from its higher protein content. In other studies, UF (with or without DF) was used as a means of lowering lactose content in the milk base, exploring the possibility of developing yoghurt-type products with lower lactose to protein ratios (Alvarez et al., 1998; Kosikowski, 1979). There is no published information on the effect of the UF process on the acid gelation properties or yoghurt-making properties of milk in a protein-standardized sample system.

In summary, UF is a common process used in the dairy industry for the standardization of milk composition for the manufacture of fermented dairy products. UF has been demonstrated in previous studies to affect the physicochemical properties of milk, including the structural characteristics of the casein micelles and whey proteins, as well as the salt equilibrium, which might affect the processing properties of the milk. The extents of these effects by UF depend on the extent of concentration (as indicated by CF). Some previous studies investigated the effect of UF on the quality of fermented dairy products. However, most of these studies used UF to alter the total protein content of the milk base, which might have overshadowed the potential contribution of the altered physicochemical properties of ultrafiltered milk to product quality.

2.3 Seasonal variation of milk composition and processing properties

2.3.1 Milk composition

The composition of raw milk determines, to a large extent, the nutritional value and processing properties of milk. Therefore, the composition of raw milk is of prime importance for the dairy industry, and there is interest in the variations of raw milk composition and physicochemical properties (Chen, Lewis, & Grandison, 2014; Heck, van Valenberg, Dijkstra, & van Hooijdonk, 2009). Bovine milk composition seasonal variations have been recorded extensively in different countries and regions (Auldist, Walsh, & Thomson, 1998; Barłowska, Litwińczuk, & Kowal, 2014; Chen et al., 2014; Heck et al., 2009; Lindmark-Månsson, Fondén, & Pettersson, 2003; Ng-Kwai-Hang, Hayes, Moxley, & Monardes, 1984; O'Brien, Mehra, Connolly, & Harrington, 1999; Phelan, O'keeffe, Keogh, & Kelly, 1982). The composition of milk varies with the stage of lactation, feeding, health status of the cow, milking interval and genetic factors (Fox & McSweeney, 1998). In different countries and regions, the patterns of seasonal variations in milk composition vary considerably, owing to different feeding regimes, calving practices and climates (Grimley, Grandison, & Lewis, 2009; Heck et al., 2009).

Milk production in New Zealand, Ireland and most of Australia has a seasonal pattern different from other developed dairying countries (Auldist, Coats, Rogers, & McDowell, 1995; Grimley et al., 2009; Lucey, 1996; O'Brien et al., 1999). In order to maximize utilization of pasture as the major feed source, seasonal calving is widely adopted in these countries. Dairy cows calve just before spring and are dried off for periods of 8 to 10 weeks during winter. Therefore, all cows are at a similar stage of

lactation (SOL) at any time during the year (Nicholas, Auldist, Molan, Stelwagen, & Prosser, 2002). The synchronized calving pattern leads to a lactation-dependent variation in milk composition (Phelan et al., 1982). This practice has created irregularities in the supply of milk to processors in terms of both quantity and composition, and is accompanied by seasonal variations in the manufacturing properties of the milk (Auldist et al., 1998).

The compositions of major milk components in New Zealand have been found to be correlated closely with the stage of lactation. An example of seasonal variations in milk production, protein content and fat content in New Zealand is shown in Figure 2.1. Data are collected from Massey No.4 dairy farm over five milking seasons. Cows calve around the end of July to early August and start to produce milk. Milk production reaches the maximum in spring around September to October and gradually decreases until the end of lactation in May. Milk fat and protein contents follow similar patterns during the season. Fat and protein contents decrease in early lactation and stabilize during most of the spring, after which they increase gradually as milk production declines and finally increase steeply in autumn till the end of lactation in May - June. The seasonal variation pattern in milk composition is consistent with reports in Australia (Auldist et al., 1995) and Ireland (Phelan et al., 1982), as well as variation pattern during lactation cycle (Barłowska et al., 2014; Ng-Kwai-Hang, Hayes, Moxley, & Monardes, 1982; Walstra & Jenness, 1984).

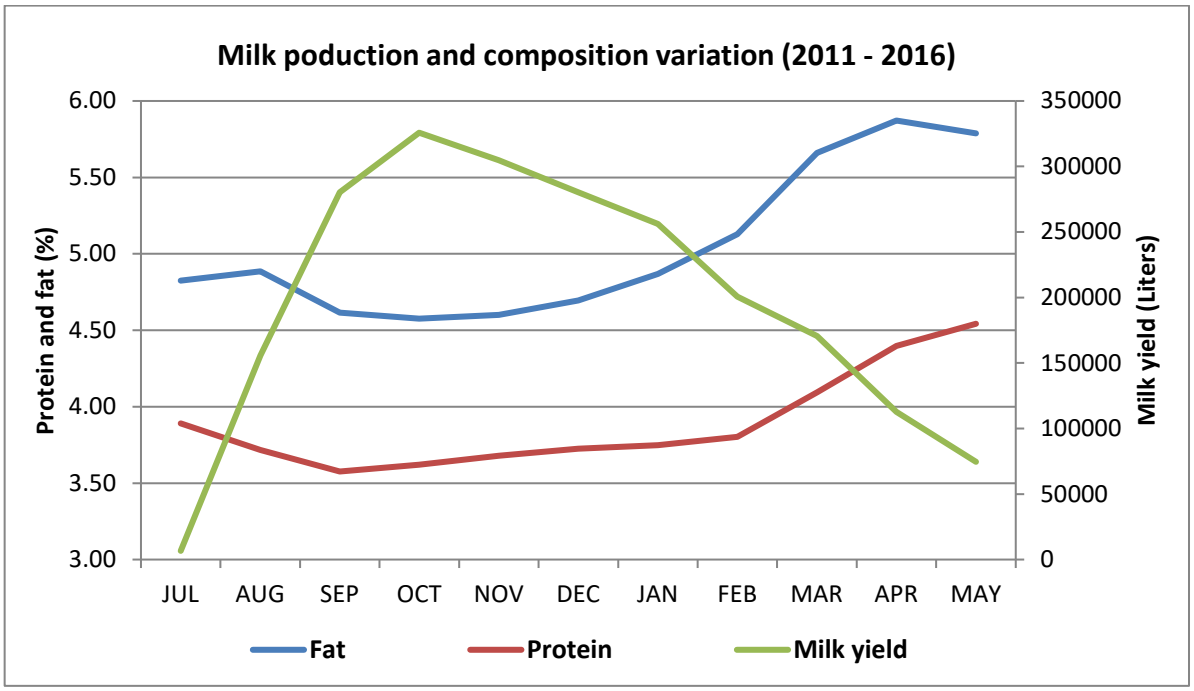

Figure 2.1: Seasonal variations in New Zealand milk production and composition (Massey University dairy Farm No.4, average data 2011 – 2016)

The lactose concentrations in New Zealand milk were found highest in summer and lowest in winter, independent of SOL (Auldist et al., 1998). This trend is in agreement with results reported in other countries where seasonal calving is not applied (Bruhn & Franke, 1977; Heck et al., 2009). The variations in lactose content are smaller than variations in protein and fat (Fox & McSweeney, 1998; Walstra, Wouters, & Geurts, 2005). Chen et al. (2014) reported lactose of British milk did not show significant variation among seasons. During the lactation cycle, milk lactose content has been reported to decrease (Auldist et al., 1995; Auldist et al., 1998; Walstra & Jenness, 1984).

The variations in mineral content have been mainly attributed to the influence of SOL, but feed also plays a role. It has been reported consistently that sodium and calcium

contents are higher in late-lactation comparing to mid-lactation, whereas potassium content remains stable during early- and mid-lactation and decrease in late-lactation (Auldist et al., 1995; Keogh, Kelly, O'keeffe, & Phelan, 1982; Walstra & Jenness, 1984). Independent of SOL, sodium concentration in New Zealand milk was found highest in winter and lowest in summer, while potassium concentration showed the opposite trend (Auldist et al., 1998). The citrate content has been reported to decrease both in late lactation and by consumption of immature pasture comparing to mature pasture or concentrates (Keogh et al., 1982). Change in the citrate concentration of milk would directly influence the Ca^{2+} concentration owing to the strong Ca^{2+} binding capacity of citrate (McSweeney & Fox, 2009).

2.3.2 Somatic cell count and milk protease

The somatic cell count (SCC) and the activity of milk protease are important factors affecting milk quality, whose variations have been mostly attributed to the change during lactation rather than the season.

It is well known that somatic cell count tends to increase in late-lactation milk (Auldist et al., 1995; Auldist et al., 1996a; Barbano, Rasmussen, & Lynch, 1991; Lucey, 1996; Ng-Kwai-Hang et al., 1984). This increase in SCC was suggested to be caused by decreasing milk volume in combination with the degeneration of mammary gland during late lactation (Auldist et al., 1995). A number of studies reported seasonal variations in SCC but the results were not consistent (Chen et al., 2014; Heck et al., 2009; Lindmark-Månsson et al., 2003; Nicholas et al., 2002; Politis, Ng Kwai Hang, & Giroux, 1989b)

The increase of SCC in late lactation milk is associated with enhanced proteolytic activity, specifically plasmin activity (Auldist et al., 1995; Auldist et al., 1996a; Politis, Lachance, Block, & Turner, 1989a). Plasmin is a trypsin-like serine proteinase in milk, especially active on α_{s2}- and β-caseins (Kelly & McSweeney, 2003). Plasmin activity is regulated by a complex system involving its inactive precursor plasminogen, plasminogen activators, and the inhibitors of plasmin and plasminogen activators, as shown in Figure 2.2:

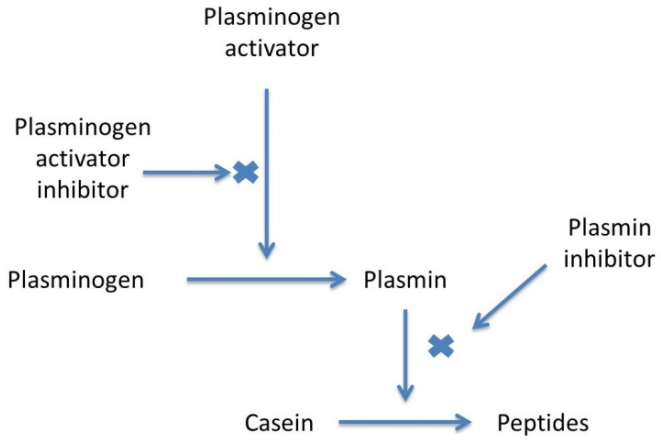

Figure 2.2: Schematic representation of the plasmin system in milk

It has been reported extensively that plasmin activity and plasminogen derived activity increase in late lactation (Bastian, Brown, & Ernstrom, 1991; Donnelly & Barry, 1983; Kelly & McSweeney, 2003; Nicholas et al., 2002; Politis et al., 1989b). Somatic cells contain a type of plasminogen activator that converts plasminogen into the active plasmin in the mammary gland (Gilmore, White, Zavizion, & Politis, 1995; Lucey, 1996). With regard of season, plasminogen derived activity has been reported to be the highest in spring in New Zealand independent of SOL, but no significant effect of SOL or season on plasmin activity was found (Nicholas et al., 2002). Other studies reported

higher plasmin activity in autumn and winter (Bastian et al., 1991; Politis et al., 1989b). It was suggested that the overall effects of SOL of proteolytic activity in New Zealand milk were greater than, and independent of, different time of the year (Nicholas et al., 2002).

2.3.3 Protein composition

The protein composition of milk is of great importance for the yield and quality of dairy products, particularly cheese (Walstra et al., 2005). Auldist et al. (1998) reported that independent of SOL, both casein and whey protein concentrations in New Zealand milk were highest in winter, whereas the casein: whey protein ratio was highest in winter and lowest in summer. It was suggested that the lower availability of pasture in winter may increase the proportions of whey proteins in milk (Gray & Mackenzie, 1987). With regard to SOL, both casein and whey protein contents were higher in late-lactation milk yet no significant variation was found for casein: whey protein ratio (Auldist et al., 1998). This is in agreement with (Coulon, Hurtaud, Remond, & Verite, 1998a; Coulon, Verdier, Pradel, & Almena, 1998b; Walstra & Jenness, 1984). However, a number of studies reported casein: total protein ratio tended to be lower in late-lactation comparing to mid-lactation (Auldist et al., 1995; Auldist et al., 1996a; Ng-Kwai-Hang et al., 1982; Phelan et al., 1982). Although contradicting results have been found regarding impact of SOL on the proportion of casein in protein, somatic cell count, on the other hand, has been related consistently to lower casein: total protein ratio (Auldist et al., 1995; Auldist et al., 1996a; Coulon et al., 1998a; Ng-Kwai-Hang et al., 1982). Two factors related to SCC were suggested to account for the change in casein: protein ratio in late-lactation milk, i.e. enhanced influx of plasma proteins

through ruptured mammary epithelia (Auldist et al., 1996a; Donnelly & Barry, 1983) and higher level of enzymatic breakdown of caseins by milk protease (Auldist et al., 1996a; Donnelly & Barry, 1983; Lucey, 1996). Donnelly and Barry (1983) reported significantly lower concentrations of α_s-casein and β-casein and a higher concentration of γ-casein (product of β-casein hydrolysis) in late lactation milk comparing to mid-lactation milk.

2.3.4 Fatty acid composition

Seasonal variation in fatty acid composition has been attributed mainly to the variation in diet, but also the stage of lactation and the energy status of dairy cows. The diet of cows has a major impact on milk FA composition (Fox & McSweeney, 1998; MacGibbon & Taylor, 2006; Palmquist et al., 1993). In summer, higher levels of plasma-derived FA and lower levels of de novo-synthesized FA have been reported consistently in France (Wolff, Bayard, & Fabien, 1995), The Netherlands (Heck et al., 2009), Germany (Precht & Molkentin, 1999), Switzerland (Collomb et al., 2008) and Sweden (Lindmark-Månsson et al., 2003). This seasonal pattern has been attributed to the similar feeding strategies used in these countries, which change from feeding mainly hay and concentrates in winter to fresh grass in spring and summer. In New Zealand, a different season pattern has been reported. Blood derived 18:1 decrease during spring after milking season starts to minimum values in summer, then increase during autumn and winter. In contrast, C6-C16 fatty acids increase to a maximum in late spring to summer and then slightly decrease during autumn (Auldist et al., 1998; Gray, 1973). Both SOL and feed composition were reported to contribute to this change during the year. At the beginning of lactation, cows are in negative energy balance,

leading to inhibited *de novo* synthesis of most short-chain and medium-chain FA and the release of long-chain FA from adipose tissue (Auldist et al., 1998; Palmquist et al., 1993). Nevertheless, Auldist et al. (1998) reported that the overall effects of the season were greater than the effects of SOL in a study investigating the influence of season and SOL on FA composition independent from each other. This is supported by the works of Gray (1973) and Parodi (1970), which suggested that the change in FA composition were related to the stage of maturity of the ryegrass in the pasture.

2.3.5 Product quality and processing properties of milk

The variations in processing properties of seasonal milk have been reported by many researchers, particularly in seasonal calving countries like New Zealand due to the impact of lactation stages (O'Brien & Guinee, 2011). The effects of milk seasonality on cheese-making properties, heat stability and the hardness of butter have been quite well described.

2.3.5.1 Cheese-making property

The cheese-making properties have been reported most extensively to be affected by SOL and season, particularly in countries where seasonal calving is adopted. Many researchers have demonstrated the inferior properties of late lactation milk for cheese making, including higher cheese moisture (Auldist et al., 1996a; Hickey, Kilcawley, Beresford, Sheehan, & Wilkinson, 2006; Lucey, 1996; O'Keeffe, 1984; O'Keeffe, Phelan, Keogh, & Kelly, 1982), longer rennet coagulation time (Auldist et al., 1996a; Lucey, 1996; O'Keeffe et al., 1982), weaker gel (Halmos, Pollard, Sherkat, & Seuret, 2003; Lucey, 1996; O'Keeffe et al., 1982) and undesirable sensory properties (Coulon

et al., 1998b; Hickey et al., 2006). As discussed in Section 2.2.2, late lactation milk has been correlated to higher SCC and proteolytic activity, which subsequently affect the protein composition of milk. A higher level of SCC in milk was suggested to magnify the inferior cheese-making properties of late lactation milk (Auldist et al., 1996a; Politis & Ng-Kwai-Hang, 1988). In these reports, lowered casein number and elevated proteolytic breakdown of intact caseins in late-lactation milk were suggested to be accountable for the impaired rennet coagulation properties and textural defects of cheese; whereas the inferior sensory properties were attributed to higher free fatty acid content and bitter peptides produced by the action of protease. However, a number of reports also indicated that cows in late lactation can produce milk suitable for cheese manufacture given high-quality feed and improved milking practices (Auldist et al., 1996a; Bastian et al., 1991; Guinee, O'Brien, & Mulholland, 2007; Kefford, Christian, Sutherland, Mayes, & Grainger, 1995; Lucey, 1996; Lucey & Fox, 1992). Kefford et al. (1995) suggested that feed quality had a more significant impact on cheese moisture content rather than SOL. This is reasonable considering the fact that, in spring-calving regions, cows enter late lactation during autumn/winter when high-quality feed is in short supply (O'Brien & Guinee, 2011).

2.3.5.2 Heat stability

The heat stability of milk can be defined as the relative resistance of milk to coagulation upon sterilization. Extensive reviews of milk heat stability have been published over the past 60 years (Fox, 1981; Fox & Morrissey, 1977; Rose, 1963; Singh, 2004; Singh, Creamer, & Newstead, 1995). The coagulation of milk on heating at high temperatures is a consequence of diminishing casein micelle stability. The

colloidal stability of casein micelles is influenced by a number of factors including pH, calcium ions, protein composition and the non-protein nitrogen fraction of milk, which all have been reported to vary with season and/or stage of lactations (Walstra & Jenness, 1984). Lower heat stability of winter milk comparing to summer milk has been reported in Scotland (Holt, Muir, & Sweetsur, 1978; Sweetsur & Muir, 1982). This is in agreement with Burton (1967), who reported that the amount of deposit formed during heating of milk with hot-wire laboratory apparatus was higher in the autumn and winter months and lower in summer months. In contrary, Chen, Grandison, and Lewis (2015a) reported better heat stability of autumn and winter milk comparing to spring and summer in England. This was supported by (Barłowska et al., 2014), who found heat stability of Polish milk is higher in autumn and winter. Gaucher et al. (2008a) reported the heat stability of spring milk subjected to UHT treatment is lower than autumn milk in France. Pouliot and Boulet (1991) found no distinctive instability period of the year of concentrated milk whereas the stabilizing effects of Na_2HPO_4 were more pronounced in summer milk than in winter milk.

2.3.5.3 Butter hardness

Regarding the hardness of butter and the SFC of milk, consistent seasonal patterns have been reported by a number of studies in New Zealand (Auldist et al., 1998; MacGibbon & McLennan, 1987; Meagher, Holroyd, Illingworth, van de Ven, & Lane, 2007; Norris, Gray, & Dolby, 1973) and Australia (Versteeg, Logan, & Müller, 2016; Walker, Wijesundera, Dunshea, & Doyle, 2013). Both hardness of butter and SFC increased sharply from late winter, when the milking season started, to late spring around December; they remained at high levels during summer and finally decreased in

autumn. This trend corresponds with the seasonal pattern of fatty acid composition in seasonal calving countries. The amount of *de novo* synthesized 6:0 – 16:0 fatty acids increase to a maximum in late spring to summer and then slightly decrease during autumn (Auldist et al., 1998; Gray, 1973). Among the FA synthesized *de novo*, the main contributor of the correlation with butter hardness is likely to be palmitic acid (16:0), the most abundant FA in milk. A higher level of 16:0 fatty acids is generally associated with a greater hardness of fat (MacGibbon & Taylor, 2006). In contrary to the seasonal pattern in New Zealand and Australia, Dutch butter made from summer fat was reported to be softer and more spreadable comparing to winter butter (Heck et al., 2009). This is probably due to different seasonal trends in fatty acid composition between two types of dairy management systems, as discussed in Section 2.2.4.

2.4 Selected product systems for investigation

In this section, the three main product systems to be investigated in the current research will be introduced, namely yoghurt and acid gel, UHT milk and whipping cream.

2.4.1 Yoghurt and acid milk gels

2.4.1.1 Mechanism of acid-induced milk gelation

Acidification of milk to the isoelectric point of caseins ~4.6 leads to destabilization of casein micelles and subsequently gelation of milk. Early views on the mechanism of acid-induced milk gelation suggested that casein micelles aggregate as a result of charge neutralisation, leading to the formation a three-dimensional network among casein micelles (Davies, Shankar, Brooker, & Hobbs, 1978). Later studies suggested that the gelation process also involves solubilisation of CCP and partial disintegration

of the casein micelles during acidification before aggregation (Donato, Alexander, & Dalgleish, 2007a; Fox & Mulvihill, 1990; Heertje, Visser, & Smits, 1985; Lucey & Singh, 1997; Schorsch, Wilkins, Jones, & Norton, 2001).

2.4.1.2 Yoghurt manufacture

Yoghurt is a very popular dairy product around the world, in which milk is fermented using *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* as the main starter cultures. These lactic acid bacteria convert lactose into lactic acid resulting in a reduction in pH, which facilitates gel formation.

A representative processing scheme of set yoghurt and stirred yoghurt is shown in Figure 2.3. Set yoghurt is incubated in retail containers until the required pH value (normally 4.4–4.7) is reached, leading to an undisturbed gel. Stirred yoghurt is inoculated and incubated in large fermentation vessels, after which the formed gel is then gently stirred to obtain a smooth and viscous, but still pourable, product, and finally packed (Jaros & Rohm, 2003).

In recent years, Greek-style yoghurt or concentrated yoghurt has a drastic growth in popularity and market share (Bong & Moraru, 2014; Desai, Shepard, & Drake, 2013; Dharmasena, Okrent, & Capps Jr, 2014). Traditionally, Greek-style yoghurt is produced by straining yoghurt in cloth bags until the desired level of total solids is achieved (Nsabimana, Jiang, & Kossah, 2005). Modern manufacture process of Greek-style yoghurt can be achieved by increasing solids content, by means of the addition of dry ingredients or concentrating milk base by ultrafiltration.

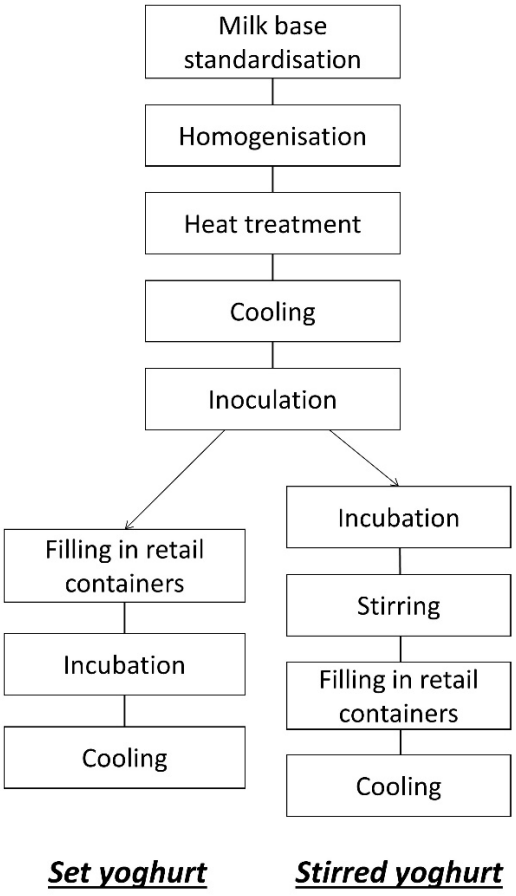

Figure 2.3: Representative processing flowchart of set yoghurt and stirred yoghurt

The textural property of yoghurt is an important aspect of its quality. The firmness of set yoghurts is commonly analysed using texture analyser or rheometer, whereas stirred yoghurt is characterized for its viscosity. The viscosity of yoghurt can be described by the Krieger Dougherty equation (Equation 2.1, Krieger & Dougherty, 1959),

$$\eta = \eta_s \left(1 - \frac{\varphi}{\varphi_{max}}\right)^{-2.5\,\varphi_{max}} \quad \textbf{(2.1)}$$

where η is the viscosity of the dispersion, ηs is the viscosity of the serum phase, φ is the volume fraction of the solid particles and φ_{max} is the maximum volume fraction of the densely packed particles.

2.4.1.3 Acid gelation induced by glucono-δ-lactone

Acidification by glucono-δ-lactone (GDL) has been used extensively for studying acid-induced milk gelation. GDL is hydrolyzed into gluconic acid when mixed with milk, resulting in a gradual reduction in pH (Dalgleish & Law, 1989; de Kruif, 1997; Lucey & Singh, 1997; Lucey, Tamehana, Singh, & Munro, 1998a).

There are differences in the acidification process and gel properties between acidifications with GDL and bacteria cultures. GDL is rapidly hydrolysed to gluconic acid after addition to milk, whereas acidification by starter bacteria has an initial lag phase (Lucey & Singh, 1997). The final pH of GDL-induced gels is a function of the amount initially added to milk, whereas starter bacteria can continue to produce acid until a very low pH (e.g. ~4.0). The different mode of acidification may affect the degree of rearrangement that aggregating particles may undergo at an early stage of the gelation process. Lucey et al. (1998a) found that both rheological and structural properties of acid milk gels were different made by acidification by GDL and bacteria culture and therefore suggested that conclusions from GDL model studies should be verified in experiments using bacterial cultures.

2.4.1.4 Milk acid gel properties and influencing factors

Two major aspects of milk acid gels properties are textural properties (e.g. firmness of set milk gels, the viscosity of stirred yoghurt) and whey separation during incubation (also referred to as syneresis and whey-off). Yoghurt firmness is commonly analysed with penetration or back extrusion methods using a texture analyser (Damin, Alcântara, Nunes, & Oliveira, 2009; Herrero & Requena, 2006; Lauber, Henle, & Klostermeyer, 2000). Alternatively, low amplitude oscillatory rheology has been used to monitor the

change of storage modulus (G′) and loss modulus (G″) during the gelation process, particularly in GDL induced milk gels. Whey separation occuring during milk gelation is a common defect in yoghurts. Spontaneous syneresis is the contraction of a gel without applying any external forces and is related to instability of the gel network (i.e., large scale rearrangements) resulting in the loss of the ability to entrap all the serum phase (Walstra, 1993). Whey separation has been studied by measuring the amount of whey either spontaneously expelled on gel surface after fermentation or under applied force (e.g. centrifugation); the water holding capacity (WHC) under certain centrifugation conditions have been studied commonly for both set and stirred yoghurts (Keogh & O'kennedy, 1998; Lucey, 2001; Lucey et al., 1998a).

The factors affecting yoghurt and acid gel properties have been studied extensively, as reviewed by (Jaros & Rohm, 2003; Sodini, Remeuf, Haddad, & Corrieu, 2004). Enhanced gel firmness and WHC can be achieved by increasing total solids content, increasing protein: total solids ratio, increasing fat content (homogenized), proper casein: whey protein ratio and heat treatment.

The heat treatment of milk is one of the most important processes affecting the texture, microstructure, and the rheological properties of milk acid gels. The intensities of heating encountered in yoghurt manufacture usually range between 75°C for 1–5 min to 95°C for 5–10 min (Sodini et al., 2004). It is well known that heat treatment of milk, which causes denaturation of whey proteins, significantly improves the firmness and viscosity of yoghurts and reduces syneresis compared with yoghurts made from unheated milk (Dannenberg & Kessler, 1988; Lucey & Singh, 1997). Heat treatment of milk results in elevated gelation pH and reduced gelation time during acidification

(Anema, Lee, Lowe, & Klostermeyer, 2004; Donato et al., 2007a; Guyomarc'h, Queguiner, Law, Horne, & Dalgleish, 2003; Lakemond & van Vliet, 2008; Lucey & Singh, 1997; Lucey, Tamehana, Singh, & Munro, 1998b; Schorsch et al., 2001; Vasbinder et al., 2003). The mechanism for the shift in gelation pH is believed to be the coverage of denatured whey proteins on the surface of casein micelles during heating, as whey proteins have high isoelectric points than caseins.

2.4.1.5 Seasonal variation of yoghurt and acid gel properties

There are very limited reports on the seasonal variation of yoghurt and acid gel properties. Underwood and Augustin (1997) reported that the acid gel made with skim milk reconstituted from skim milk powders produced during March to May (late lactation) had lower gel strength and took longer to form a gel comparing to acid gel made from skim milk powders manufactured during early lactation and mid-lactation. Cheng, Clarke, and Augustin (2002) conducted a similar study, using reconstituted concentrated skim milk to make yoghurt by bacterial fermentation. Variations over the year have been found in set yoghurt firmness, stirred yoghurt viscosity and whey separation yet no clear trend for different seasons was reported. The fermentation time to reach pH 4.6 was found not affected by the time of year.

2.4.2 UHT milk

Ultra-high temperature (UHT) process of milk involves heating milk at a very high temperature (~135-145°C) for a short time (1-10s). Milk subjected to UHT treatment followed by aseptic packaging can be kept at room temperature for a shelf-life of several months.

One major change that occurs during the storage of UHT milk is irreversible gel formation, which is commonly known as “age gelation”. The propensity of age gelation decreases as the heating intensity increases (Anema, 2017; Datta & Deeth, 2001; Newstead, Paterson, Anema, Coker, & Wewala, 2006). UHT skim milk is more likely to gel than whole milk (Datta & Deeth, 2001; García-Risco, Ramos, & López-Fandiño, 1999). The optimum temperature for the development of age gelation is 25-30°C (Datta & Deeth, 2001; Kocak & Zadow, 1985; Malmgren et al., 2017).

Despite studies during the past few decades, the mechanism of age gelation is not yet fully understood. The principles of UHT milk processing and age gelation have been reviewed by (Anema, 2019; Datta & Deeth, 2001; Datta & Deeth, 2007). Three mechanisms of age gelation have been proposed, as mainly induced by proteolysis of plasmin, by proteolysis of bacterial proteases or by the κ-CN depleted casein micelles. The most well-known theory of age gelation, proposed by McMahon (1996), is based on a two-stage process, initiated by the proteolysis of caseins by plasmin. In the first stage, proteinases cleave the peptide bonds on αs-CN and β-CN which anchor κ-caseins to the casein micelles, releasing the κ-CN/ β-LG complexes from micelle surface. In the second stage, when a critical concentration of the κ-CN/ β-LG complexes is reached, they would aggregate and connect casein micelles into a three-dimensional matrix. In UHT milk with high residue activity of bacterial proteases, the gelation was suggested to form due to the cleavage of κ-CN and the consequent destabilization of the casein micelles similar to that of rennet-induced gelation (D'Incecco et al., 2019; Zhang, Bijl, & Hettinga, 2018). Anema (2017) proposed a physicochemical mechanism for the age gelation of UHT milk. This type of gelation was suggested to occur in UHT milk with

minimal proteolysis and was caused by the aggregation of casein micelles depleted in κ-CN due to UHT treatment.

Various changes occur in the physicochemical properties of UHT milk during storage but none of them appears to be a good indicator of whether a UHT milk sample will gel (Anema, 2017, 2019). According to the mechanisms of gelation described above, the proteolysis during storage is an important factor in the development of age gelation. However, there was no relation between the extent of proteolysis by plasmin and the propensity of gelation (Auldist et al., 1996b; Kelly & Foley, 1997; Kocak & Zadow, 1985; Manji & Kakuda, 1988; Rauh et al., 2014). More proteolysis occurs at 40°C than at 30 or 20°C but gelation is retarded in milk stored at 40°C (Kocak & Zadow, 1985; Manji, Kakuda, & Arnott, 1986). Rauh et al. (2014) found only low levels of κ-casein and β-LG in the gelled UHT milk and suggested the cleaving and releasing of κ-CN/ β-LG complexes was not the main cause of gelation. Excessive hydrolysis of the caseins may not allow the proteins to associate into a gel network (Auldist et al., 1996b; Kelly & Foley, 1997; Rauh et al., 2014). It was suggested that a low level of proteolysis was necessary for gelation in milk but factors other than proteolysis may be involved (Kelly & Foley, 1997; Manji & Kakuda, 1988). The pH of UHT milk decreases during storage (Anema, 2017; Gaucher et al., 2008a; Manji et al., 1986; Newstead et al., 2006; Rauh et al., 2014; Venkatachalam, McMahon, & Savello, 1993; Zadow & Chituta, 1975), particularly at higher storage temperatures (Gaucher et al., 2008a; Gaucher, Mollé, Gagnaire, & Gaucheron, 2008b; Newstead et al., 2006; Zadow & Chituta, 1975). Proteolysis (Gaucher et al., 2008b; Kelly & Foley, 1997; Rauh et al., 2014) and Maillard reaction (Anema, 2017; Auldist et al., 1996b; Gaucher et al., 2008b; Manji et

al., 1986; Venkatachalam et al., 1993) were suggested to be responsible for this decline in pH of UHT milk. However, studies showed that the extent of pH reduction was not a good indicator of age gelation (Anema, 2017; Auldist et al., 1996b).

Some previous studies examined the seasonal and lactational variations of UHT milk gelation. Auldist et al. (1996b) conducted a seasonal study in Australia using indirect UHT treatment and reported that early-season UHT milk gelled faster than late-season UHT milk. This was in agreement with other studies conducted in Australia (Hardham, 1998; Zadow & Chituta, 1975) that UHT milk produced in early lactation gelled faster than UHT milk produced in late season.

2.4.3 Whipping cream

Whipping cream is a product contains typically 30- 40% fat, primarily designed to be beaten into foam known as whipped cream. Whipped cream is a dispersion of gas bubbles surrounded by partially coalesced fat globules at the air/serum interface, supported by high viscosity in the serum phase (Smith, Goff, & Kakuda, 2000). The whipping cream process and whipping process have been well described in previous studies (Smiddy, Kelly, & Huppertz, 2009; Walstra et al., 2005). It generally involves incorporation and stabilization of air bubbles during the whipping process in earlier stages and partial coalescence of fat globules in later stages. Whipping cream properties are commonly characterized by an increase in volume (normally referred to as overrun), whipping time, whipped cream firmness and subsequent serum leakage (Walstra et al., 2005).

One important variable for standardized whipping cream is the crystallisation state of the lipids. The presence of solid fat crystals at whipping temperature is required to induce partial coalescence among fat globules and prevent destabilisation of foam through spreading of excessive amounts of liquid lipid over the interface (Smiddy et al., 2009). Therefore, the solid fat content at whipping temperature may have a potential impact of the whipping properties, which is subjected to seasonal variation (Section 2.2.5.3).

There are a few reports on the seasonal variation of whipping cream properties. Keogh (1978) reported shorter whipping time and higher foam firmness in December – early March comparing to June and July in Ireland (spring calving), whereas no seasonal trend in overrun was found. This was partly in agreement with Needs, Anderson, and Kirby (1988), who reported creams produced in the summer had shorter whipping time and were less stiff than winter creams in England, but there was no change in overrun. It should be noted that in this research an autumn-calved herd was used. The seasonal sampling covered a change from winter feeding to summer grazing and from early to late lactation (Needs et al., 1988). Chen, Lewis, and Grandison (2015b) reported in England that whipping cream produced in winter and spring had higher overrun and serum stability comparing to summer cream.

2.5 Summary of literature

A considerable number of studies have been undertaken to describe and understand the seasonal variations in milk composition, processing properties and dairy product quality. In particular, the seasonal variations in milk composition, fatty acid

composition and cheese-making properties have been reported extensively. Nevertheless, a clear correlation between seasonal change in milk composition and physicochemical properties and some products has not been revealed or understood. In addition, the seasonal variations in milk and product properties reported in different countries should be distinguished based on different dairy management systems, owing to the prime impacts of feed and stage of lactation on milk composition and properties. It is therefore important to provide up-to-date and comprehensive information on the seasonal variations in milk composition and properties, and their influences on dairy products whose variabilities with the season are less understood. This would allow the dairy industry to better control and manipulate the quality of dairy products made from seasonal milk, particularly in seasonal calving countries.

Chapter 3 - Materials and methods

3.1 Milk supply and initial processing

Raw bovine milk was taken from Massey University No.4 dairy farm (Palmerston North, New Zealand) from August 2016 to Dec 2018. The herd consisted of 614 cows, mostly Friesian–Jersey crossbreed. The herd was offered pasture as the major feed source with low to moderate levels of supplemental feed throughout the seasons. The herd started calving in late July and dried off in May of the following year in each of the three milking seasons covered in this study, i.e. 2016-2017 season, 2017-2018 season and 2018-2019 season. Within each year, the early season, mid-season, and the late season were defined as 8–100 days, 101–220 days, and 221–300 days in milk, corresponding to August-October, November-February, and March-May. "Seasonal variation" or "seasonality" in this thesis was defined as the recurring changes in the characteristics of bovine milk and dairy products during different times of the milking season in a typical New Zealand spring-calving system. Thus, the stage of lactation was treated as an inherent contributor to the seasonality in this thesis, rather than an independent variable as in the study of Auldist et al. (1998).

In three chapters (Chapter 4, 5 and 6), the studies were conducted for two milking seasons (2016-2017 season and 2017-2018 season) and the comparisons between the two milking seasons were made. The 2016-2017 season and 2017-2018 season are referred to as "year 16/17" and "year 17/18" in these chapters, to prevent confusion in the use of the word "season". The purpose of sampling for two years was to identify seasonal variations that were robust in both years.

On sampling days, 30 litres of milk were collected before 9 am. The milk was processed in the Food Pilot of Massey University. Raw milk was pasteurized at 72°C for 15 s within one hour of collection and separated into cream and skim milk using a centrifugal separator (Model 103 AE, Alfa-Laval, Sweden) at 50°C. Batch Ultrafiltration (UF) of part of the skim milk was performed at 50°C using an ultrafiltration unit equipped with an HFK-131 membrane (observed separation range of 10 kDa; Koch Membrane Systems, Wilmington, MA). The pressure during the UF process was adjusted for optimum separation efficiency and was maintained at below 3 bar. The UF process was terminated until the retentate reached a protein content of around 12% (equal to around 20% total soluble solids), monitored using a refractometer PAL-1 (Atago Co., Ltd., Tokyo, Japan). The concentration factor of each UF batch varied with the protein content of seasonal milk (results presented in Chapter 7). Skim milk, cream, UF retentate and UF permeate originating from the same batch of raw milk were used for the standardization of the products. An overview of milk processing in this thesis is shown in the flowcharts in Figure 3.1.

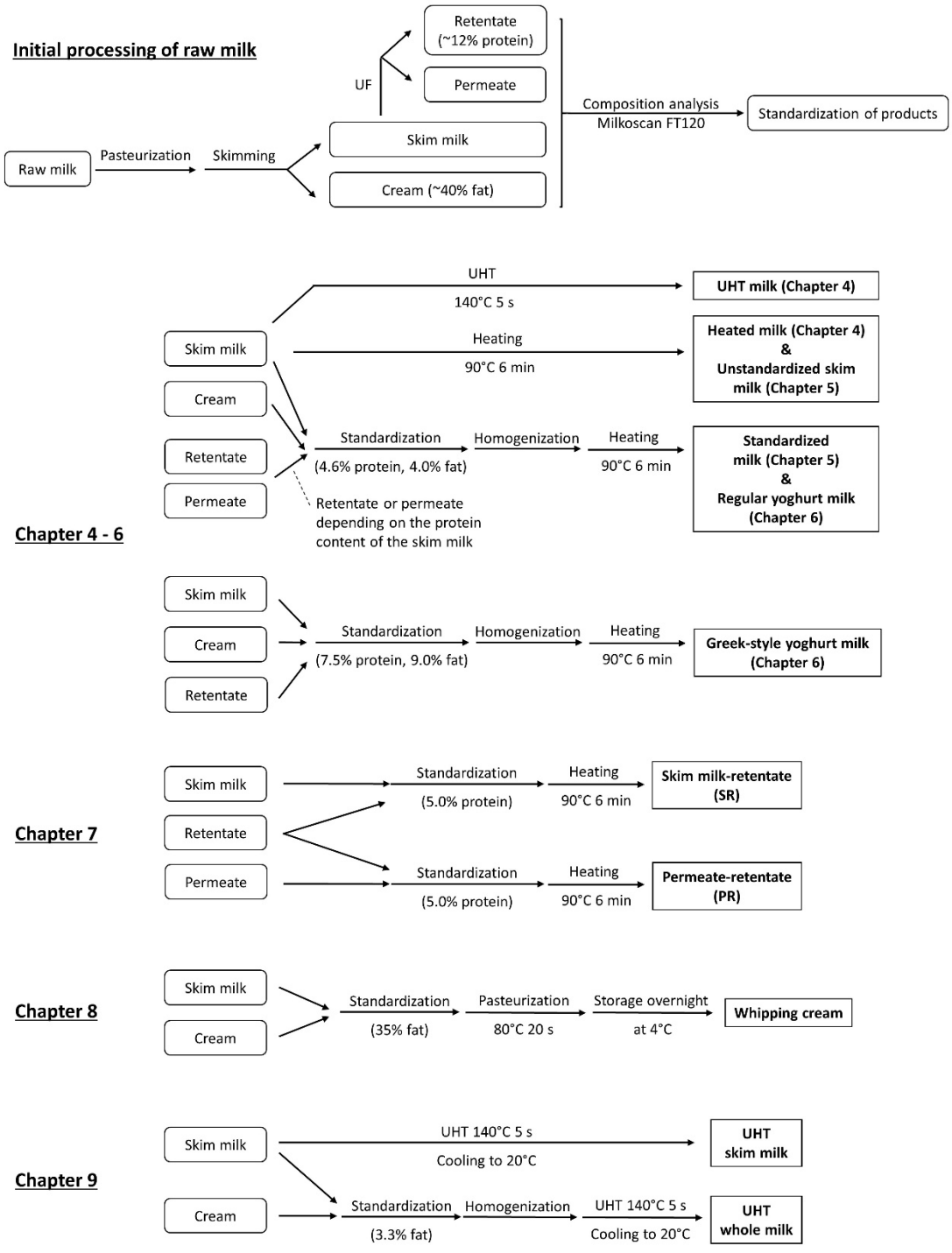

Figure 3.1: Processing flowcharts

3.2 Characterization of physicochemical properties

3.2.1 Milk composition

The contents of fat, protein, lactose, total solids and solid non-fat of milk, cream, UF retentate, permeate and standardized milk mixtures were analysed with a MilkoScan FT120 (FOSS, Denmark). Somatic cell count (SCC) data were kindly provided by the farm.

The total calcium and inorganic phosphorus contents of the milk were analyzed on an RX Daytona Plus analyzer using reagents CA 8309 and PH 8328 (Randox Laboratories, Crumlin, County Antrim, UK).

3.2.2 pH and ionic calcium measurement

The pH values of raw and heated milk were measured using a CyberScan pH 510 pH/mV Meter (Eutech Instruments-Thermo Scientific). The ionic calcium (Ca^{2+}) concentration of raw milk and heated milk were analysed with a calcium electrode (Orion 9720BNWP Thermo Scientific) in combination with a CyberScan pH 510 pH/mV Meter (Eutech Instruments-Thermo Scientific). Calibration was carried out with five standards containing 1.0 – 5.0 mM of $CaCl_2$. The ionic strengths of standards were adjusted to 80 mM with potassium chloride as described by (Geerts, Bekhof, & Scherjon, 1983). All milk samples and calibration solutions were allowed to equilibrate for at least 1 h in the same water bath with tap water before analysis. This practice allowed all calibrations and measurements to be performed at the same temperature (20±1°C) and largely mitigate the variation of room temperature over different time of the year.

3.2.3 Determination of milk fat globule size

The fat globule sizes of raw whole milk, homogenized yoghurt milk and UHT milk were determined using a Malvern MasterSizer 2000 (Malvern Instruments Ltd, Worcestershire, UK). The sample preparation and measurement were carried out according to (Ye, Singh, Taylor, & Anema, 2002). Milk samples were mixed with a solution containing 2% SDS and 50 mM EDTA, pH 6.7, to dissociate casein micelles prior to the measurements. The refractive index of milk fat globules was set as 1.46. The volume-weighted mean diameter was reported as the fat globule size.

3.2.4 Determination of casein micelle size

Dynamic light scattering was used to measure casein micelle sizes. Measurements were carried out with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The Z-average hydrodynamic diameter of casein micelles was determined according to Bijl, de Vries, van Valenberg, Huppertz, and Van Hooijdonk (2014). Skim milk samples were diluted 50 times in milk ultrafiltration permeate made fresh from seasonal skim milk. The diluted milk samples were filtered using a 0.45μm PVDF syringe filter to remove large particles. The viscosity and the refractive index of milk permeate were set according to literature (viscosity of 1.154 cP and refractive index of 1.342 at 20°C, Beliciu & Moraru, 2009). Samples were measured at 20°C using a scattering angle of 173°. Each measurement was at least triplicated.

3.2.5 Buffering capacity and ethanol stability

The buffering capacity of milk was measured in duplicate according to Park (1991). The buffering capacity (dB/dpH) after the addition of 4 mL of 0.5 M HCl was calculated using the equation given by Van Slyke (1922):

$$\frac{\mathrm{dB}}{\mathrm{dpH}} = \frac{\text{Volume of acid or base added} \times \text{Normality of acid or base}}{\text{Volume of sample} \times \text{pH change produced}}$$

The ethanol stability of the pasteurized skim milk was determined as described by Huppertz, Grosman, Fox, and Kelly (2004).

3.2.6 Heat-induced Whey Protein Denaturation Determined by Native-PAGE

Native whey proteins were separated from the casein micelles and the denatured whey proteins using acetic acid precipitation as described by Vasbinder and de Kruif (2003). The supernatant containing the native whey proteins was collected for analysis by native-PAGE using a Bio-Rad Mini-Protean electrophoresis system as described by (Singh & Creamer, 1991). The staining intensities of the protein bands, as an indication of the relative protein concentrations, were quantified on a Gel-Doc XR+ system (Bio-Rad, Hercules, CA). The extent of whey protein (WP) denaturation was calculated as:

$$\text{WP denaturation \%} = \left(1 - \frac{\text{WP staining intensity in the serum of heated milk}}{\text{WP staining intensity in the serum of raw milk}}\right) \times 100\%$$

3.2.7 High-performance liquid chromatography (HPLC)

Reversed-phase HPLC was used to analyze milk and milk serum samples in order to determine the protein composition of the milk, the glycosylation of κ-CN, and the extent of association between the whey proteins and the casein micelles in heated milk.

Milk serum was obtained from raw milk and heated milk by ultracentrifugation at 88,000 x *g* for 60 min at 20°C.

The chromatographic conditions, protein identification, and protein quantification were performed as described by Bobe, Beitz, Freeman, and Lindberg (1998) on a reversed-phase C18 column (Aeris Widepore 3.6µm XB-C18 RP, Phenomenex). The glycosylated κ-CN (G-κ-CN) and nonglycosylated κ-CN (NG-κ-CN) peaks were identified. The glycosylation degree (GD) of κ-CN was defined as the peak area ratio of G-κ-CN in total κ-CN. The extent of whey protein–casein micelle association was calculated as the difference in whey protein concentration between raw and heated milk serum samples. The percentage of whey proteins forming aggregates in the serum phase was calculated as the difference between the extent of whey protein denaturation and whey protein-casein micelle association. Each sample was prepared and analyzed at least in duplicate.

3.2.8 Transmission electron microscopy

The transmission electron microscopy (TEM) was performed at Manawatu Microscopy and Imaging Centre (MMIC). Samples of milk and acid milk gel were injected into agarose tube made from 3% agarose using a pipette. The ends were sealed with agarose to form an enclosed capsule and samples were placed into 3% gluteraldehyde in 0.1M sodium Cacodylate buffer (pH 7.2) for at least 24h. Then the samples were washed in 0.1M sodium cacodylate (pH 7.2) 3 times for 45min each. Following post-fix in 1% Osmium Tetroxide in 0.1M sodium Cacodylate buffer for 1 hour at room temperature, overnight at 4°C and for another hour at room temperature, the samples were washed 3 times again as described above.

Samples were dehydrated through a graded acetone series (25%, 50%, 75%, 95%, 100%, 100%, 100%) for 45min each and then put into 50: 50 resin: acetone and placed on the stirrer overnight. This was replaced by a fresh 100% resin (Procure 812, ProSciTech Australia) for 8 hours on the stirrer. This step was repeated four more times (overnight in 100% resin, 8 hours in 100% resin, overnight in 100% resin, 8 hours in 100% resin). Finally, samples were embedded in moulds with fresh resin and cured in a 60°C oven for 48 hours.

Light microscope sections were cut at 1 micron using a glass knife on the ultramicrotome (Leica EM UC7, Germany) and heat-fixed onto glass slides. These were stained with 0.05% Toluidine Blue for approximately 12 seconds and viewed under the light microscope. The block was then trimmed down to the selected area and cut using a Diamond Knife (Diatome, Switzerland) at 100nm. These were stretched with chloroform and mounted on a grid using a Quick Coat G pen (Daido Sangyo, Japan).

Grids were stained in Saturated Uranyl Acetate in 50% Ethanol for 6.5 min, washed with 50% ethanol and MilliQ water and then stained in Lead Citrate for a further 6.5 minutes. This was followed by a wash in MilliQ water. Samples were imaged with a Tecnai G2 Spirit BioTWIN (FEI company, Czech Republic) paired with a Veleta TEM camera (Olympus SIS Germany).

3.3 Statistical analysis

Statistical analysis was performed using IBM SPSS version 24. A value followed by a mean and "±" is its standard error. Independent sample t-test and one-way analysis of

variance (ANOVA) with Tukey's post-hoc test were used to determine the significant effects of seasonal variation, between-year variation and different heat treatments independent of other variables (e.g. the effect of the season in each year). Two-way ANOVA was used to analyze the interactions between the variables (e.g. season, year and heat treatment). The correlation coefficient (*r*) among the parameters was analyzed. The coefficient of variation (CV, the percentage of the standard deviation in the mean) was used to indicate the extent of variation. For each parameter, statistical analyses were performed with 3–8 results per season per year.

Chapter 4 - Seasonal variations in composition, properties and heat-induced changes in New Zealand milk

4.1 Introduction

The composition and the physicochemical properties of milk are of great importance for the dairy industry. They have marked impacts on the processing properties of the milk and the quality of dairy products (O'Brien & Guinee, 2011). The characteristics of milk are affected by various factors, including breed, season, stage of lactation (SOL), feeding, parity, and the health status of the cow (Fox & McSweeney, 1998).

The patterns in the seasonal variation in the composition and properties of milk vary considerably in different countries, because of the differences in calving system, feeding strategy, and climate (Grimley et al., 2009; Heck et al., 2009). In seasonal calving countries, e.g., New Zealand, Australia, and Ireland, most cows calve in late winter to spring to maximize the utilization of pasture as the major feed source. This synchronized calving practice results in a pattern of seasonal variation in the milk composition that is controlled by the SOL (Auldist et al., 1998; Lucey, 1996). In most European countries, calving is not seasonal and a change in diet has been regarded as being the main reason for the seasonal variation in the milk properties. The cows consume mainly silage and concentrates in winter and fresh grass in the spring and summer months (Grimley et al., 2009; Heck et al., 2009; Lindmark-Månsson et al., 2003). In countries with a warmer climate, the negative effect of heat stress in summer on the composition of the milk has been emphasized (Bernabucci, Lacetera, Ronchi, & Nardone, 2002; Bertocchi et al., 2014).

Previous studies in seasonal calving countries reported that the protein and fat contents of the milk increased towards the end of the milking season, whereas the lactose content decreased (Auldist et al., 1995; Auldist et al., 1998; Phelan et al., 1982). Such seasonal trends were consistent with the pattern of variation in the milk composition during the lactation cycle (Barłowska et al., 2014; Ng-Kwai-Hang et al., 1982; Walstra & Jenness, 1984). However, there were contradictions among different studies in the seasonal and lactational variation patterns of some milk characteristics, e.g., protein composition and pH. The differences in herd management, climate, and the health status of the herd might account for the inconsistent seasonal trends of these parameters (Chen et al., 2014). In particular, the SCC, as an important indicator for udder health and milk quality, differed considerably in different studies (Ng-Kwai-Hang et al., 1982; O'Connell, Kelly, Tobin, Ruegg, & Gleeson, 2017; Ostersen, Foldager, & Hermansen, 1997). Therefore, the impact of a high SCC on the milk characteristics might confound the variations during different seasons or different SOL. In addition, as most seasonal studies were conducted for one year, they could not account for the between-years differences, in climate for example, that affect the quality of milk.

Heat treatment is a common practice during milk processing to improve the keeping quality of the milk by deactivating microorganisms and enzymes. In addition, it can also enhance the textural quality of some dairy products, e.g., yoghurt (Vasbinder & de Kruif, 2003). Upon heating at temperatures above 70°C, there is irreversible denaturation of the whey proteins and their association with the casein micelles (Wijayanti et al., 2014). Anema and Li (2003) demonstrated that the whey protein–casein micelle association resulted in an increase in the casein micelle size. Heating of

milk also induces the dissociation of κ-CN from the casein micelle surface, which influences the whey protein–casein micelle interactions and the heat stability of the milk (Anema & Klostermeyer, 1997; Donato et al., 2007b; Singh & Fox, 1985). The heat-induced protein association and dissociation were reported to affect the acid gelation properties of milk, the age gelation of UHT milk, and the solubility of protein isolates (Donato & Guyomarc'h, 2009; Grufferty & Mulvihill, 1987; McMahon, 1996). The effect of seasonality on the heat-induced changes in milk has rarely been reported.

The aim of the present study was to investigate the seasonal variations in the composition and characteristics of milk in a seasonal calving New Zealand herd, as well as the heat-induced changes in the physicochemical properties of the milk during the season under two typical heat treatments used in dairy processing. The analysis was carried out for two full milking seasons, to identify which quality parameters varied with the season consistently regardless of the between-year effects.

4.2 Materials and methods

4.2.1 Milk supply and heat treatment

Herd information, milk collection and initial processing of the milk are described in Section 3.1. In this study, milk was collected over two complete milking seasons, referred to as year 16/17 (2016–2017 season) and year 17/18 (2017–2018 season). In total, 39 samples were collected in the two milking seasons. The weather information during the two years was obtained from the Palmy Weather website (Palmy Weather, 2019).

The skim milk obtained from initial processing was then heated either at 90°C for 6 min (batch heating) or at the UHT condition of 140°C for 5 s on an indirect tubular UHT plant (Massey University, Palmerston North, New Zealand). The flowrate for UHT processing was 1.6 L/min. Samples were preserved with 0.02% (wt/wt) sodium azide. Aliquots of samples were frozen at –20°C prior to compositional analysis.

4.2.2 Characterizations of milk

The characterizations of raw and heated milk were performed as described in Section 3.2, including milk composition, SCC, calcium and inorganic phosphorus (Section 3.2.1), pH and Ca^{2+} (Section 3.2.2), fat globule size (Section 3.2.3), casein micelle size (Section 3.2.4), protein composition and κ-CN Glycosylation (Section 3.2.7). The buffering capacity and ethanol stability of seasonal pasteurized skim milk were determined only in year 17/18 as described in Section 3.2.5.

Heated milk was analysed for the degree of whey protein denaturation (Section 3.2.6), the extent of κ-CN dissociation and the extent of whey protein-casein micelle association (Section 3.2.7).

4.3 Results and discussion

4.3.1 Milk composition

4.3.1.1 Major components

Table 4.1 shows the composition of the major milk components and minerals. The fat and protein contents in the milk decreased from calving to their lowest levels around September–October in early-season and then gradually increased. In late-season, particularly April–May, the fat and protein contents increased markedly to their highest levels. The seasonal trends for fat and protein were consistent over the 2 years. Milk from year 17/18 contained more protein than milk from year 16/17 ($P < 0.05$). The concentration and dilution effect contributes to the seasonal variation in the fat and protein contents as the milk volume reaches a maximum in mid-lactation and decreases towards late-lactation (Auldist et al., 1998).

Table 4.1: Compositional properties of seasonal milk (*n* = 5–8 for each parameter per season per year)

		Means			Effects		
	Season	16/17	17/18	SEM	Season	Year	Season × year
Fat (%)	Early	4.56[b]	4.74[b]	0.07	L > E, M	NS	NS
	Mid	4.70[b]	4.90[b]				
	Late	5.48[a]	5.55[a]				
Protein (%)	Early	3.44[c]	3.60[b]	0.08	L > M > E	17/18 > 16/17	NS
	Mid	3.74[b]	3.78[b]				
	Late	4.43[a]	4.65[a]				
Lactose (%)	Early	4.95[a]	4.97[a]	0.02	E, M > L	NS	NS
	Mid	4.95[a]	4.92[a]				
	Late	4.78[b]	4.65[b]				
SCC (1,000 cells/mL)	Early	96[ab]	137[b]	9	L > E, M	17/18 > 16/17	NS
	Mid	58[b]	133[b]				
	Late	120[a]	190[a]				
Total calcium (mM)	Early	26.9[b]	33.7[b]	0.5	L > E, M	17/18 > 16/17	*
	Mid	29.0[b]	32.7[b]				
	Late	32.0[a]	36.0[a]				
Calcium to protein ratio (mM/g)	Early	0.76[a]	0.91[a]	0.5	E > M > L	17/18 > 16/17	*
	Mid	0.76[a]	0.84[b]				
	Late	0.70[b]	0.75[c]				
Inorganic phosphorus (mM)	Early	24.2[b]	26.3	0.4	M, L > E	NS	*
	Mid	27.8[a]	27.0				
	Late	29.7[a]	27.6				
Ionic calcium (mM)	Early	2.33[a]	2.46[b]	0.04	E, L > M	17/18 > 16/17	*
	Mid	2.04[b]	2.35[b]				
	Late	2.21[a]	2.71[a]				

[a–c] Means of the same parameter within a column with different superscripts differ ($P < 0.05$).
E, early season; M, mid-season; L, late season.
*$P < 0.05$.

The lactose content was stable until mid-season and dropped significantly during the late season. Lactose synthesis is suppressed in late-lactation to adjust for the increased osmotic pressure caused by the influx of blood constituents, because of the degeneration of the mammary epithelia towards the end of lactation (Fox, 2009).

The seasonal trends for milk fat, protein, and lactose were in agreement with reports in countries with seasonal calving (Auldist et al., 1995; O'Connell et al., 2017; Phelan et al., 1982), as well as with variation patterns during the lactation cycle (Barłowska et al., 2014; Fox & McSweeney, 1998; Ng-Kwai-Hang et al., 1982). These comparisons indicated that the SOL largely controls the seasonal variations in the composition of the major milk components in a seasonal calving system.

4.3.1.2 Somatic Cell Count

The SCC of all milk samples were lower than 200,000 cells/mL, with annual averages of 86,158 and 152,053 cells/mL in year 16/17 and year 17/18, respectively. The SCC was low compared with the European Union requirements of 400,000 cells/mL and with the target annual average SCC of 150,000 cells/mL recommended by DairyNZ (Hamilton, New Zealand). The low overall SCC in this study was an indication of good udder health and milk quality, minimizing the influence of excessively high SCC or mastitic milk on the seasonal variations in the milk properties.

Late-season milk had the highest SCC during both years, consistent with the well-documented trend that the SCC increases in late-lactation (Auldist et al., 1996a; Lucey, 1996; Ng-Kwai-Hang et al., 1984). Auldist et al. (1995) suggested that a joint effect of the decreasing milk volume and the degeneration of the mammary gland resulted in an increase in SCC during late-lactation.

Milk from year 17/18 had a higher SCC than milk from year 16/17. The higher environmental temperature in year 17/18 might have contributed to the higher SCC in the milk. Heat stress has been reported consistently to increase the SCC in milk

(Bernabucci et al., 2002; Bertocchi et al., 2014; Igono, Johnson, Steevens, Hainen, & Shanklin, 1988). The number of days with a daily maximum temperature of 24°C or higher (equivalent to maximum temperature-humidity-index of 74 or higher at 75% mean daily relative humidity) were 35 d in year 16/17 and 80 d in year 17/18 (Palmy Weather, 2019). The daily maximum temperature-humidity-index of 74 was a threshold as reported by Bertocchi et al. (2014), above which the SCC of milk would increase significantly.

4.3.1.3 Milk Minerals

The calcium contents of the milk were highest in the late season in both years, consistent with reports that the calcium content was higher in late-lactation than in mid-lactation (Auldist et al., 1995; Auldist et al., 1996a; O'Keeffe et al., 1982). Auldist et al. (1995) reported that the milk calcium content varied during the year in a similar pattern to the milk casein content because a large proportion of the calcium in milk is present in the casein micelles in the form of colloidal calcium phosphate. This may explain why milk from year 17/18 had higher total calcium and protein contents than milk from year 16/17. The inorganic phosphorus content of the milk was lower in the early season in year 16/17 but did not vary significantly in year 17/18.

The mean Ca^{2+} concentration was lowest in mid-season in both years, although, in year 17/18, the Ca^{2+} concentration was not significantly higher in the early season than in mid-season. The Ca^{2+} concentration had a strong correlation with the total calcium content ($r = 0.642$, $P < 0.01$) and more so with the calcium to inorganic phosphorus ratio ($r = 0.867$, $P < 0.01$). This may have been because the salt balance between

calcium and calcium-binding anions such as phosphates and citrates greatly influences the Ca^{2+} concentration in milk (Lewis, 2011).

4.3.2 Physicochemical properties

Table 4.2 shows the physicochemical properties of milk. The pH displayed different seasonal trends in the two years (16/17, early-season > mid-season; 17/18, early-season < mid-season and late-season, $P < 0.05$). Different SCC in the two years might account for the different patterns of the variation in pH. The pH correlated with the SCC ($P < 0.05$), which has been well documented (Auldist et al., 1995; Auldist et al., 1996b; Kandeel, Megahed, Ebeid, & Constable, 2019). Similar to the current study, Albenzio et al. (2004) found that the pH of high SCC ewe milk increased significantly in mid- and late-lactation, whereas the pH of low SCC ewe milk did not increase with the SOL.

Table 4.2: Raw milk physicochemical properties (*n* = 4–8 for each parameter per season per year)

		Means			Effects[1]		
	Season	16/17	17/18	SEM	Season	Year	Season × year
pH	Early	6.70[a]	6.65[b]	0.01	L > E	17/18 > 16/17	**
	Mid	6.67[b]	6.72[a]				
	Late	6.68[ab]	6.75[a]				
Fat globule size (D_{43}, μm)	Early	5.18[a]	5.39[a]	0.07	E > M, L	NS	NS
	Mid	4.51[b]	4.50[b]				
	Late	4.40[b]	4.29[b]				
Casein micelle size (d.nm)	Early	160	158	1	NS	NS	NS
	Mid	162	159				
	Late	157	160				
Buffering capacity (dB/dpH)	Early		0.027[b]	0.001	L > E, M		
	Mid	ND	0.027[b]				
	Late		0.032[a]				
Ethanol stability (%)	Early		77.9[a]	0.6	E, M > L		
	Mid	ND	76.1[a]				
	Late		72.1[b]				

[a,b] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).
[1]Analyzed using two-way ANOVA
ND, not determined; NS: nonsignificant; E, early season; M, mid-season; L, late season.
**$P < 0.01$.

The milk fat globule sizes were largest in the early season. The patterns of variation were consistent during the two years and were in accordance with previous studies (Fleming et al., 2017; Huppertz & Kelly, 2006; Wiking, Stagsted, Björck, & Nielsen, 2004). Wiking et al. (2004) found that the fat globule size correlated negatively with the diurnal fat yield of cows, suggesting that the lack of MFGM materials relative to the enhanced fat synthesis resulted in the increase in fat globule size. This may explain

the seasonal trend in fat globule size observed in the present study, considering that the fat yield in milk reaches a maximum at about 60 days, i.e., around September–October in the early season.

The casein micelle size did not vary with season or year. This was in agreement with de Kruif and Huppertz (2012), who reported that the casein micelle size of the milk from individual cows was stable during lactation. In a nonseasonal calving system, Chen et al. (2014) also reported that the casein micelle size did not vary significantly with the season in the UK.

The buffering capacity was highest in the late season. It correlated with the quantities of the major buffering constituents in the milk, including protein ($P < 0.01$), calcium ($P < 0.01$), and inorganic phosphorus ($P < 0.05$).

The ethanol stability (determined in year 17/18) was lowest in the late season and correlated negatively with the protein content, the Ca^{2+} concentration, and the calcium to inorganic phosphorus ratio ($P < 0.01$). It is expected that higher concentrations of protein, particularly casein, will lower the ethanol stability by promoting aggregation among the casein micelles. The negative correlation between ethanol stability and Ca^{2+} concentration was in accordance with previous studies (Horne & Parker, 1981; Lin, Lewis, & Grandison, 2006). Ionic calcium could destabilize the micelles by binding to the caseins, thus reducing their negative charges (Horne & Parker, 1981). Both Donnelly and Horne (1986) and Tsioulpas, Lewis, and Grandison (2007) reported that the $\frac{\text{Calcium + Magnesium}}{\text{Phosphate + Citrate}}$ ratio correlated negatively with the ethanol stability, similar to the findings for the calcium to inorganic phosphorus ratio and the ethanol stability in the

present study. Calcium and magnesium were suggested to destabilize the casein micelles whereas phosphate and citrate counteract this destabilizing effect by lowering their activities.

4.3.3 Protein composition

Table 4.3 shows the relative concentrations in the total protein of total casein, total whey protein, and individual proteins, and the GD of κ-CN.

Table 4.3: Protein composition (weight percentage in total protein) and the degree of glycosylation of κ-CN (*n* = 5–8 for each parameter per season per year)

		Means		CV	Effects[1]		
	Season	16/17	17/18	(%)	Season	Year	Season × year
Total CN (%)	Early	85.2	84.5	0.8	NS	16/17 > 17/18	NS
	Mid	85.5	84.2				
	Late	85.4	83.9				
α_{s2}-CN (%)	Early	6.6[c]	7.8	10.5	M, L > E	NS	**
	Mid	7.7[b]	8.3				
	Late	8.8[a]	7.6				
α_{s1}-CN (%)	Early	28.9[a]	31.0[a]	4.8	E > M > L	17/18 > 16/17	**
	Mid	27.8[b]	28.3[b]				
	Late	27.1[b]	27.8[b]				
β-CN (%)	Early	37.4[ab]	33.8	5.1	NS	16/17 > 17/18	**
	Mid	38.2[a]	34.4				
	Late	36.5[b]	35.0				
κ-CN (%)	Early	12.7[a]	12.3[b]	6.2	L > E, M	17/18 > 16/17	**
	Mid	11.9[b]	13.3[a]				
	Late	13.0[a]	13.8[a]				
Glycosylated κ-CN (%)	Early	4.8[b]	4.6[c]	14.9	L > E, M	17/18 > 16/17	**
	Mid	4.6[b]	5.3[b]				
	Late	5.6[a]	6.5[a]				
κ-CN GD (%)	Early	37.9[b]	37.1[b]	9.7	L > E, M	NS	*
	Mid	38.6[b]	39.6[b]				
	Late	42.7[a]	46.9[a]				
Total whey proteins (%)	Early	14.8	15.5	5.0	NS	17/18 > 16/17	NS
	Mid	14.5	15.8				
	Late	14.6	16.1				
α-LA (%)	Early	2.9[a]	3.2[a]	16.3	E > M > L	NS	**
	Mid	2.7[b]	2.7[b]				
	Late	2.2[c]	2.0[c]				
β-LG (%)	Early	11.8	12.2[c]	7.1	L > E, M	17/18 > 16/17	**
	Mid	11.9	13.1[b]				
	Late	12.3	14.1[a]				

[a–c] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).

[1]Analyzed using two-way ANOVA

GD, glycosylation degree; E, early season; M, mid-season; L, late season; NS: nonsignificant.

*$P < 0.05$, **$P < 0.01$.

4.3.3.1 Total casein and whey protein

The proportion of casein in the total protein did not vary with the season but was lower in year 17/18 than in year 16/17. The low SCC (Table 4.1) of the seasonal milk in this study, and thus the good overall health of the cow udders, might be the reason for the stable casein to protein ratio across different seasons. In agreement with the current study, a number of authors found that the proportion of casein did not vary over lactation (Auldist et al., 1998; Coulon et al., 1998a; Walstra & Jenness, 1984), although other authors reported a lower proportion of casein in late-lactation milk (Auldist et al., 1996a; Lucey, 1996; Ng-Kwai-Hang et al., 1982). The differences in the milk SCC in these studies might be the main reason for this discrepancy. Unlike the contradicting reports on the effect of the SOL on the proportion of casein, a higher SCC has been consistently related to a lower percentage of casein in the total protein (Auldist et al., 1995; Coulon et al., 1998a; Ng-Kwai-Hang et al., 1982). Two factors related to the SCC were suggested to account for the change in the casein to total protein ratio in late-lactation milk, i.e., enhanced influx of plasma proteins through ruptured mammary epithelia and higher levels of enzymatic breakdown of the caseins by proteases (Auldist et al., 1996a; Donnelly & Barry, 1983; Lucey, 1996). Coulon et al. (1998a) reported that the casein to protein ratio significantly reduced with the SCC only when the SCC was greater than 200,000 cells/mL. This might explain the nonsignificant seasonal variation in the proportion of total casein in this study, considering that none of the seasonal milk samples had an SCC higher than 200,000 cells/mL.

However, the higher SCC in year 17/18 might have contributed to the higher proportion of whey proteins (particularly β-LG) than in year 16/17, considering that the between-

year difference in SCC was greater than that within each year (Table 4.1). In addition, the higher environmental temperature in year 17/18 might have caused heat stress of the cow and a decline in casein synthesis (Bernabucci et al., 2002; Bertocchi et al., 2014).

4.3.3.2 Individual Proteins and Glycosylation of κ-CN

The percentages of all individual proteins were significantly affected by season x year interactions, suggesting that the seasonal variation patterns were different during the two years. However, the same seasonal trends during the two years were observed for α_{s1}-CN (early-season > mid-season and late-season), α-LA (early-season > mid-season > late-season), and the GD of κ-CN (late-season > early-season and mid-season). Interestingly, the concentrations of G-κ-CN (CV 14.9%) and α-LA (CV 16.3%) also varied the most among all milk proteins across the seasons (Table 4.3). The stable seasonal trends and the large variations in G-κ-CN and α-LA are discussed below.

The mean G-κ-CN concentrations were highest in the late season in both years. Both the mean concentration of total κ-CN and the GD of κ-CN were highest in the late season. Higher total κ-CN in late-lactation than in mid-lactation was reported by O'Connell et al. (2017), whereas Barry and Donnelly (1980) reported no significant difference in κ-CN during different SOL. The κ-CN GD reached a maximum in the late season in both years, in agreement with reported trends during the lactation cycle (Bonfatti, Chiarot, & Carnier, 2014; Robitaille, Ng-Kwai-Hang, & Monardes, 1991).

The decrease in the relative concentration of α-LA during the season was consistent with the change during lactation (Ostersen et al., 1997; Regester & Smithers, 1991). The α-LA concentration correlated with the lactose content ($P < 0.01$), which was also observed by Heck et al. (2009) in a nonseasonal calving system. The role of α-LA as a co-enzyme in lactose synthesis probably gave rise to this correlation (Brew, 2003).

4.3.4 Heat-induced changes in seasonal milk

4.3.4.1 Ionic Calcium

The trends in the seasonal and between-year variations in the Ca^{2+} concentration of milk heated under both conditions (90°C for 6 min and UHT treatment) were broadly consistent with those in raw milk (Table 4.4). There were strong correlations among the Ca^{2+} concentrations in raw milk, heated (90°C for 6 min) milk, and UHT milk ($P < 0.01$).

Both heating at 90°C for 6 min and UHT treatment resulted in significant decreases in the Ca^{2+} concentration compared with that in raw milk ($P < 0.05$). The extents of heat-induced Ca^{2+} reduction were similar for the two heating conditions ($P > 0.05$). The season x year interactions were significant for the Ca^{2+} decrease under both heat treatments. In year 16/17, the heat-induced decrease in Ca^{2+} was significantly higher in the early season than during the rest of the year. However, in year 17/18, there was no significant seasonal variation. A possible explanation for the between-year difference was that Ca^{2+} was considerably higher in year 17/18 than in year 16/17 (Table 4.1). Consequently, the mean Ca^{2+} reduction during heating was higher in year 17/18 ($P < 0.05$), which made the variation among seasons less apparent.

Table 4.4: Ionic calcium of heated milks (*n* = 4–8 for each parameter per season per year)

			Mean		Effects[1]		
		Season	16/17	17/18	Season	Year	Season × year
Ionic calcium concentration (mM)	Heat	Early	2.01 ± 0.07[ab]	2.18 ± 0.04[ab]	E, L > M	17/18 > 16/17	NS
		Mid	1.86 ± 0.03[b]	2.06 ± 0.07[b]			
		Late	2.10 ± 0.02[a]	2.38 ± 0.04[a]			
	UHT	Early	1.95 ± 0.06[b]	2.16 ± 0.06[b]	L > E, M	17/18 > 16/17	NS
		Mid	1.89 ± 0.01[b]	2.09 ± 0.09[b]			
		Late	2.11 ± 0.02[a]	2.44 ± 0.06[a]			
Heat-induced Ca^{2+} reduction (%)	Heat	Early	12.6 ± 1.4[a]	14.4 ± 0.9	E > M, L	17/18 > 16/17	**
		Mid	9.1 ± 1.1[ab]	11.9 ± 1.5			
		Late	5.0 ± 1.3[b]	14.8 ± 0.9			
	UHT	Early	15.1 ± 1.6[a]	12.2 ± 1.5	E > M, L	NS	**
		Mid	7.3 ± 0.8[b]	11.8 ± 1.8			
		Late	4.4 ± 0.7[b]	9.6 ± 0.9			

[a, b] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).

[1]Analyzed using two-way ANOVA

E, early-season; M, mid-season; L, late-season; Heat, 90°C for 6 min, UHT, 140°C for 5 s.

**$P < 0.01$; NS: nonsignificant.

4.3.4.2 Serum-phase κ-CN and Heat-induced κ-CN Dissociation.

Figure 4.1 depicts the percentages of serum-phase κ-CN in the total κ-CN of raw milk and heated milk. In raw milk, the percentages of serum-phase κ-CN were comparable with those reported by Singh and Latham (1993) and Singh, Roberts, Munro, and Teo (1996). In both years, the proportion of serum-phase κ-CN increased significantly as the milking season progressed.

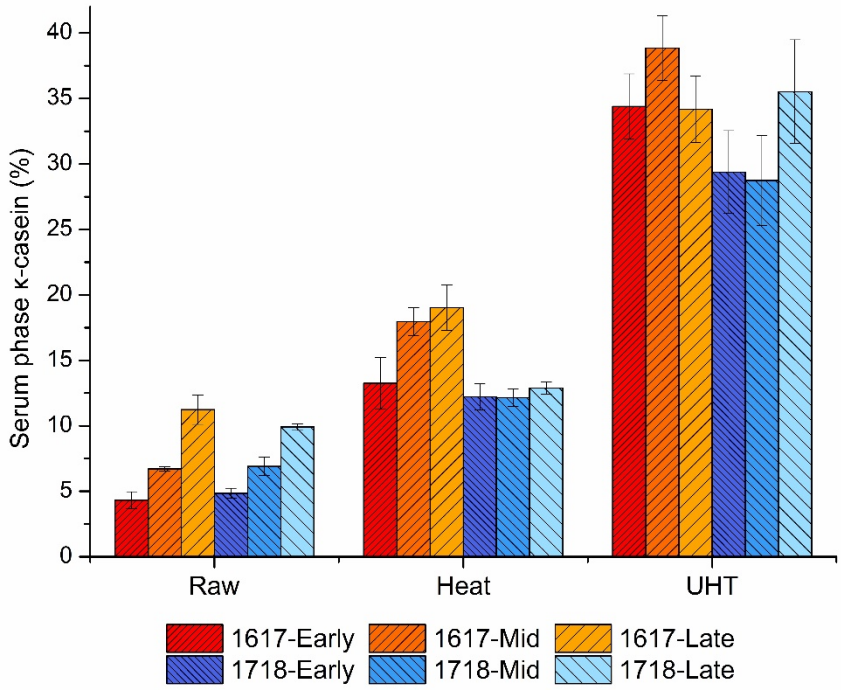

Figure 4.1: Percentage of serum-phase κ-CN in total κ-CN of milk. Heat, 90°C for 6 min; UHT, 140°C for 5 s. (*n* = 3–8 for each parameter per season per year.)

Both heating conditions resulted in significant dissociation of κ-CN ($P < 0.05$). UHT milk had a considerably higher amount of serum-phase κ-CN (33.3 ± 1.3%) than milk heated at 90°C for 6 min (15.1 ± 0.7%). There was no significant seasonal variation in serum-phase κ-CN after either heat treatment. For the between-year variation, heated milk (90°C for 6 min) from year 16/17 had a higher level of serum-phase κ-CN than

that from year 17/18. A possible explanation might be that milk from year 17/18 had higher calcium to inorganic phosphorus ratio (Table 4.1); a higher proportion of calcium relative to phosphate has been reported to reduce the dissociation of κ-CN (Singh & Fox, 1987).

4.3.4.3 Serum-phase κ-CN Glycosylation

To understand how the glycosylation of κ-CN affected its partitioning between the micelles and the serum phase, the serum: milk ratio of κ-CN GD is shown in Figure 4.2. In raw milk, the mean serum: milk κ-CN GD ratio was 0.71 ± 0.03, indicating that a higher proportion of NG-κ-CN than G-κ-CN presented in the serum phase. Early-season milk serum contained a significantly higher proportion of G-κ-CN than serum in the rest of the year, regardless of the fact that the GD of total κ-CN in the milk was highest in the late season and low in the early season (Table 4.3). The GD of κ-CN in the milk correlated with a lower serum: milk GD ratio ($r = –0.762$, $P < 0.01$). The results suggested that the natural presence of G-κ-CN and NG-κ-CN in the milk serum was independent of the κ-CN GD of the milk or even that a higher κ-CN GD in milk might somehow contribute to lowering the proportion of G-κ-CN in the serum phase. The mechanism of this phenomenon is unclear.

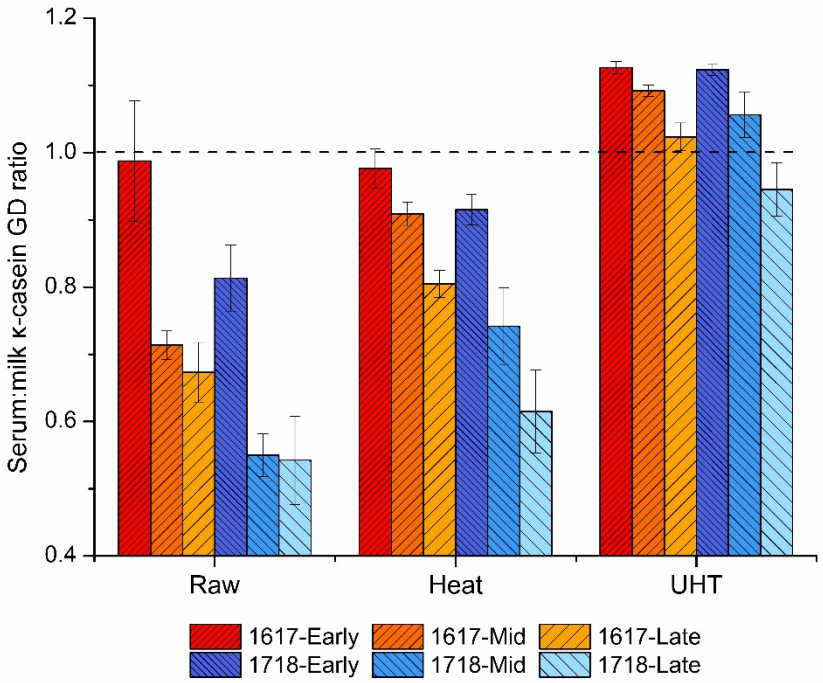

Figure 4.2: Serum: milk ratios of the degree of glycosylation of κ-CN. Heat, 90°C for 6 min; UHT, 140°C for 5 s. (Dashed line indicates a ratio of 1.0; *n* = 3–8 for each parameter per season per year.)

Heat treatments, particularly UHT treatment, resulted in a greater proportion of G-κ-CN in the serum phase. The mean serum: milk GD ratios were 0.83 ± 0.02 in heated milk (90°C for 6 min) and 1.06 ± 0.01 in UHT milk. Under heating at 90°C for 6 min, dissociation of NG-κ-CN was still favoured, as in raw milk, but proportionally more G-κ-CN was present in the serum. However, in UHT milk, the extent of dissociation of G-κ-CN increased greatly. The mean serum: milk κ-CN GD ratio of UHT milk was higher than 1.0, indicating that the dissociation of G-κ-CN was favoured, in contrast to that of raw milk and heated milk (90°C for 6 min). UHT treatment seemed to trigger significant dissociation of G-κ-CN, which could not be attained under heating at 90°C for 6 min. This might also explain the significantly higher extent of κ-CN dissociation in UHT milk (Figure 4.1). These results suggested that the mechanism of heat-induced

κ-CN dissociation might be different under different heating conditions, in which the glycosylation of κ-CN plays a role.

Milk heated under both conditions followed similar seasonal patterns of the serum: milk κ-CN GD ratio to that of raw milk, despite the marked impact of heating on κ-CN dissociation. The proportion of G-κ-CN in the serum phase generally decreased as the milking season progressed in both types of heated milk. Under both heating conditions, late-season milk, with the highest κ-CN GD, still had the lowest proportion of G-κ-CN in the serum phase. Nevertheless, the impact of heating was evident, considering that the extent of variation in the serum: milk κ-CN GD ratio was lowest in UHT milk (CV 7.2%), followed by heated (90°C for 6 min) milk (CV 15.9%), and was highest in raw milk (CV 24.1%). In particular, the serum: milk GD ratio of late-season milk had the largest increase from raw and heated (90°C for 6 min) milks to UHT milk. This could be explained by the hypothesis that UHT conditions triggered the dissociation of G-κ-CN and the fact that late-season milk had the highest κ-CN GD among the different seasons.

When the two years were compared, both raw milk and heated (90°C for 6 min) milk had higher serum: milk κ-CN GD ratios in year 16/17 than in year 17/18, whereas there was no significant difference for UHT milk. The higher proportion of serum phase G-κ-CN in year 16/17 may have been associated with the higher κ-CN GD of the milk in year 17/18 than in year 16/17, although the between-year difference of the latter was not statistically significant ($P = 0.052$).

4.3.4.4 Heat-induced Whey Protein Denaturation and Association with Casein Micelles

As shown in Figure 4.3, heating at 90°C for 6 min and UHT treatment resulted in similar extents of whey protein denaturation. However, the fraction of micelle-bound whey proteins was significantly higher in heated (90°C for 6 min) milk than in UHT milk. The differences in the heating rate and the dissociation of κ-CN might account for the different extents of whey protein–casein micelle association. Oldfield et al. (1998a) suggested that whey protein–casein micelle association was more favoured under slower heating conditions, compared with rapid heating conditions, such as the UHT treatment used in this study. In addition, UHT treatment induced a considerably higher extent of κ-CN dissociation (Figure 4.1), which was reported to correlate with a higher proportion of serum-phase denatured whey proteins in heated milk (Anema & Li, 2003; Singh, 2004). This dissociated κ-CN could form complexes with denatured whey proteins in the serum phase, competing with κ-CN on the micelle surface.

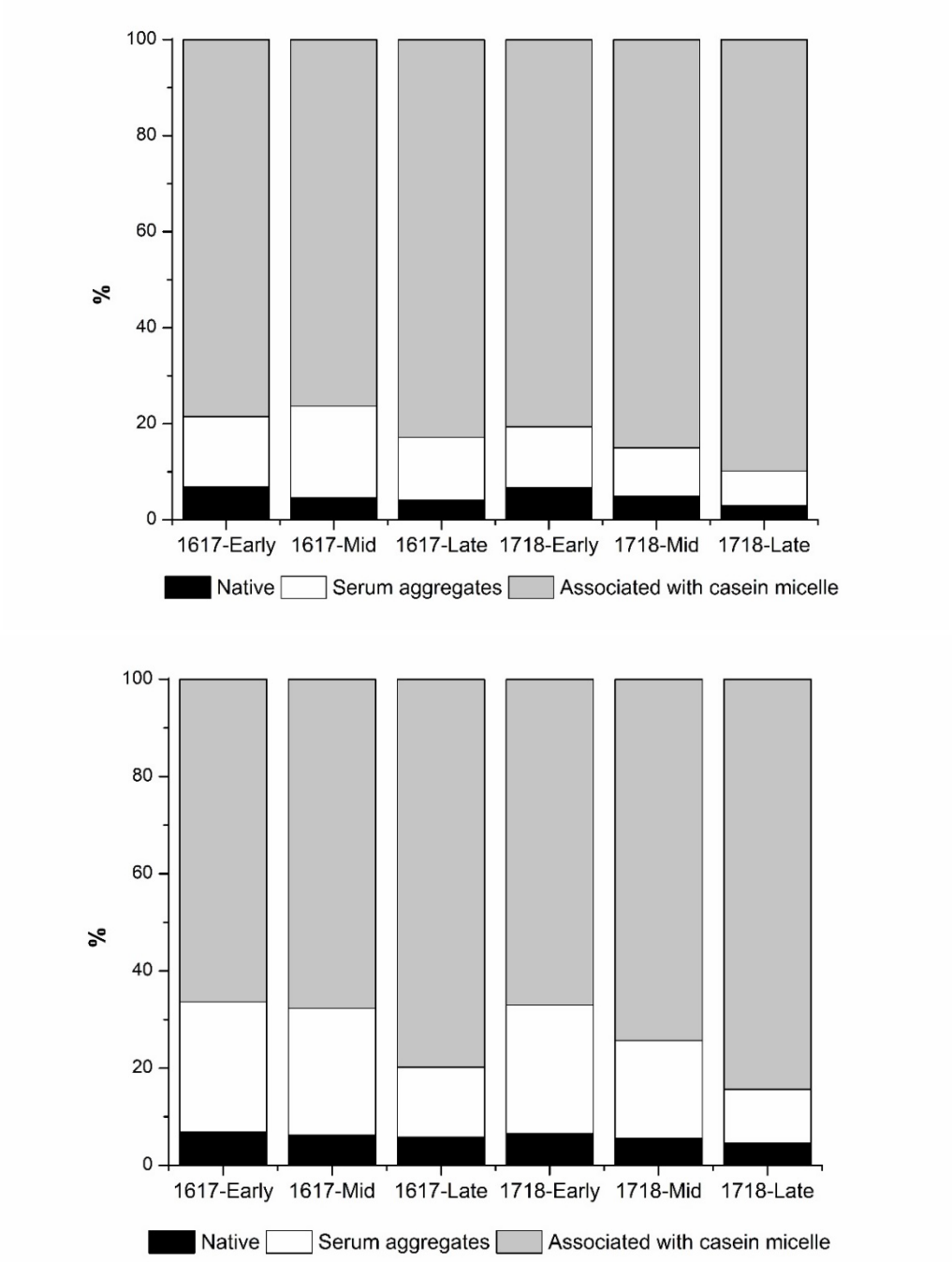

Figure 4.3: Whey protein distribution in milk heated at 90°C for 6 min (top) and UHT milk (bottom). Fractions of whey proteins that remained native, formed aggregates in the serum, and associated with the casein micelles are shown as black, white, and grey bars, respectively. (*n* = 3–8 for each parameter per season per year.)

The extent of whey protein denaturation was lowest in the early season for heated (90°C for 6 min) milk ($P < 0.05$) but did not vary with the season in UHT milk ($P > 0.05$). Nevertheless, the extents of whey protein–casein micelle association were highest in the late season ($P < 0.05$) under both heat treatments. This agreed with Auldist et al. (1996b) and Oldfield (1996), who reported that late-lactation milk contained a higher amount of denatured whey proteins complexed with the casein micelles than milk produced in earlier stages of lactation. When the two years were compared, heated (90°C for 6 min) milk in year 17/18 had a greater extent of whey protein–casein micelle association than that in year 16/17 although there was no significant difference in the whey protein denaturation.

Three factors might have contributed to the seasonal and between-year variations in the denaturation of the whey proteins and their association with the casein micelles, i.e., the proportion of β-LG in the total whey protein, the extent of dissociation of κ-CN, and the Ca^{2+} concentration.

The proportion of β-LG in the total whey proteins increased as the milking season progressed, and was higher in year 17/18 than in year 16/17. It correlated with the denaturation of the whey proteins and their association with the casein micelles under both heating conditions ($P < 0.05$). β-LG not only is the main type of whey protein that is involved in forming complexes with κ-CN, but also catalyzes the irreversible denaturation of α-LA by forming disulfide bonds. Thus, a higher proportion of β-LG in the whey proteins would promote the denaturation of the total whey proteins and their association with the casein micelles. Oldfield (1996) also attributed the high whey

protein denaturation and extent of association with the casein micelles in late-season New Zealand milk to its higher content of β-LG and therefore more free thiol groups.

As discussed previously, serum-phase κ-CN could form complexes with denatured whey proteins, competing with κ-CN on the micelle surface, and could consequently lower the extent of association of the whey proteins with the casein micelles. In heated (90°C for 6 min) milk, the percentage of serum-phase κ-CN correlated negatively with the extent of whey protein–casein micelle association ($P < 0.01$). This may explain the between-year variation, i.e., milk in year 17/18 had less serum-phase κ-CN and a greater extent of association of the whey proteins with the casein micelles ($P < 0.05$).

The Ca^{2+} concentration correlated positively with the whey protein–casein micelle association in the milk under both heating conditions ($P < 0.01$). By binding to milk proteins, Ca^{2+} may reduce their negative charges and promote aggregation among the proteins (Donovan & Mulvihill, 1987). Law and Leaver (2000) reported that the addition of $CaCl_2$ to skim milk resulted in a greater extent of both the denaturation of whey proteins and their association with the casein micelles.

4.3.4.5 Casein Micelle Size

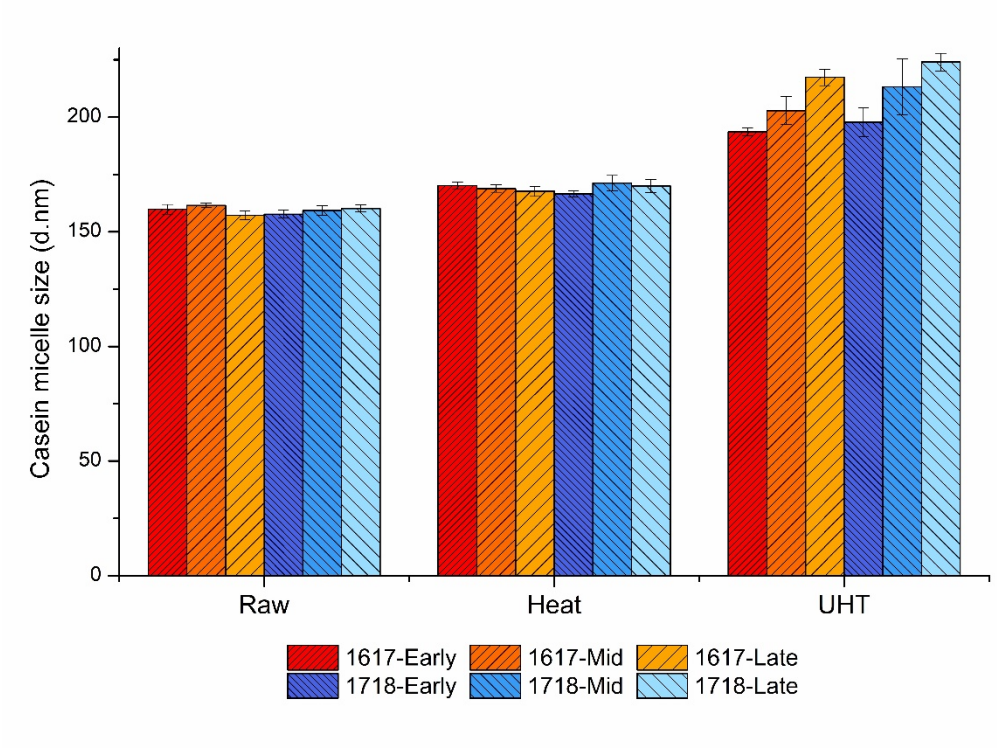

Figure 4.4: Mean hydrodynamic diameter of casein micelles in raw and heated milk. Heat, 90°C for 6 min; UHT, 140°C for 5 s. (*n* = 4–8 for each parameter per season per year.)

Both heating conditions resulted in a significant increase in the casein micelle size (Figure 4.4, $P < 0.05$). UHT milk had much larger micelle size (207 ± 1 nm) than milk heated at 90°C for 6 min (169 ± 1 nm). The association between denatured whey proteins and casein micelles has been well demonstrated to be the major contributor to the heat-induced increase in casein micelle size in milk heated at under 100°C (Anema, 2007; Anema & Li, 2003). This study found that UHT milk had a larger micelle size than heated (90°C for 6 min) milk, despite having a lesser extent of whey protein–casein micelle association. This suggested that, in UHT milk, mechanisms other than whey protein–casein micelle association played an important role in altering the casein

micelle size. Aggregation among the partially κ-CN-depleted micelles might be the main cause for the greater increase in casein micelle size in UHT milk.

As discussed previously, UHT treatment induced a considerably higher level of κ-CN dissociation (33.3 ± 1.3%) compared with heated (90°C for 6 min) milk (15.1 ± 0.7%). Partially κ-CN-depleted casein micelles are prone to aggregation in the presence of Ca^{2+} (Singh, 2004). Gaur, Schalk, and Anema (2018) reported that sediment in UHT milk was mainly composed of κ-CN-depleted casein micelles, whose quantity increased with increasing level of ionic calcium. It is proposed that UHT treatment, because of its stronger κ-CN depletion effect, might have induced aggregation among part of the casein micelles, which resulted in the increase in the average casein micelle size. In contrast, in milk heated at 90°C for 6 min, the aggregation among the micelles was negligible and the increase in the casein micelle size was likely to result from the association with whey proteins.

Seasonality had different impacts on heated (90°C for 6 min) milk and UHT milk. In heated (90°C for 6 min) milk, there was no significant seasonal or between-year variation in casein micelle size. This indicated that the seasonal variations in the extent of whey protein–casein micelle association, although statistically significant, were not sufficient to induce a significant change in the casein micelle size. This was reasonable considering the overall high extent of heating-induced whey protein–casein micelle association of 81.6 ± 1.0%.

In UHT milk, the casein micelle size was significantly larger in the late season than in the early season in both years ($P < 0.05$). Based on the hypothesis above, the extent of aggregation among the partially κ-CN-depleted casein micelles possibly determined the

increase in casein micelle size in the UHT milk. It is proposed that seasonal milk containing greater amounts of protein, serum phase κ-CN, and Ca^{2+} may favour aggregation among the casein micelles. The casein micelle size of the UHT milk correlated strongly with both the protein content and the serum-phase κ-CN content of the raw milk ($P < 0.01$). A higher protein content presumably accelerated the rate of aggregation. Raw milk with a higher proportion of serum-phase κ-CN, thus a lower proportion of κ-CN on the micelle surface, would be more readily reaching the critical level of κ-CN depletion during heating that leads to significant aggregation among the casein micelles. Consequently, in milk with a higher serum-phase κ-CN content, more casein micelles may reach this critical level of κ-CN depletion during heating, and this critical level may be reached earlier, allowing more time for aggregation among the partially κ-CN-depleted micelles. The insignificant positive correlation ($P = 0.055$) between the Ca^{2+} concentration and the casein micelle size might have been confounded by the fact that early-season milk had the lowest protein content and serum-phase κ-CN content but a high Ca^{2+} concentration. If the aggregation of some κ-CN-depleted casein micelles indeed caused the observed increases in the average micelle size induced by UHT treatment, it would be interesting to look at whether the sedimentation in UHT milk reported by Gaur et al. (2018) has a seasonal variation pattern similar to that of the casein micelle size of UHT milk in this study.

4.4 Conclusions

The results of this study indicated that the seasonal variation patterns in the composition and physicochemical properties of milk in a seasonal calving system were largely affected by the SOL. Despite significant between-year effects on some milk

characteristics, the fat, protein, and lactose contents, the fat globule size, and the G-κ-CN and α-LA concentrations had consistent seasonal variation patterns, similar to those during the lactation cycle. For the first time, the variation in κ-CN glycosylation was highlighted in a seasonal milk study. Two typical heat treatments of milk, heating at 90°C for 6 min and UHT treatment, resulted in significantly different extents of κ-CN dissociation (particularly G-κ-CN) and whey protein–casein micelle association, and different increases in casein micelle size. In particular, UHT treatment might have triggered significant dissociation of G-κ-CN and subsequent aggregation among the casein micelles. Seasonality affected the heat-induced protein dissociation and association, possibly because of the differences across the different seasons in the GD of κ-CN, the protein composition, and the Ca^{2+} concentration of the milk. This information will contribute to an understanding of and the control of the processing properties of milk and the quality of dairy products throughout the year.

Chapter 5 - Impact of seasonal variations on the acid gelation of milk

5.1 Introduction

Seasonal variations in the composition and properties of milk result in considerable changes in its processing properties and the quality of dairy products (O'Brien & Guinee, 2011). For instance, the inferior cheese-making properties of late-lactation milk have been demonstrated by previous studies (Hickey et al., 2006; Lucey, 1996; Lucey & Fox, 1992). For mainly 2 reasons, it is difficult to fully understand and control the seasonal variations in dairy product quality. Firstly, different milk characteristics change concurrently during the season, making it difficult to pinpoint the main contributor. Secondly, the seasonal variation patterns might be different in different herds or even in different years of the same herd, because of the influences of herd management, climate, the health status of the cow, etc.

The acid gelation of milk is important for the production and quality of fermented dairy products, e.g., yoghurt. The acidification of milk to the isoelectric point (pH 4.6) of caseins leads to destabilization of the casein micelles and subsequent gelation of the milk. Upon acidification, solubilization of the colloidal calcium phosphate and partial disintegration of the casein micelles occur before aggregation among the milk proteins (Donato et al., 2007a; Heertje et al., 1985; Lucey & Singh, 1997). Typically, milk for yoghurt making is heated at 80–95°C for several minutes prior to acidification, to induce a high level of whey protein denaturation. The denatured whey proteins participate in the acid gel structure, resulting in elevated gelation pH, reduced gelation

time, and a firmer gel structure compared with unheated milk (Lakemond & van Vliet, 2008; Lucey et al., 1998b; Vasbinder et al., 2003).

Standardization is a common practice in the dairy industry, to control its processing properties and the quality of dairy products, e.g., yoghurt. However, standardization does not account for the variations in the physicochemical properties of milk and their potential impact on dairy products.

Limited published information on the seasonal or lactational variations in the acid gelation properties of milk is available. Underwood and Augustin (1997) reported that acid gels made with milk reconstituted from skim milk powders produced during the late season (from March to May) in Australia had the lowest gel strength and the longest gelation time. However, there is no published study on acid milk gels made using fresh seasonal milk with standardization based on protein content.

The aim of this study was to investigate the seasonal variations in the rheological properties of acid milk gels over 2 full milking seasons in a typical seasonal calving New Zealand herd. The acid gelation properties of the seasonal milk samples characterized in Chapter 4 were determined in this study in 2 milk systems, i.e., natural skim milk (unstandardized) and standardized milk (4.6% protein, 4.0% fat). The two sample systems were compared to determine the effectiveness of standardization in maintaining the acid gelation properties of seasonal milk throughout the year. The correlations between acid gelation properties and milk characteristics demonstrated in Chapter 4 were investigated to determine the possible cause of the seasonal variations in the acid gelation of milk.

A better understanding of the acid gelation behaviour would assist in controlling the quality of yoghurt and other fermented dairy products. In addition, the variations in the acid gelation properties may in turn highlight certain differences in seasonal milk properties, as well as provide new perspectives on the acid gelation mechanisms.

5.2 Materials and methods

5.2.1 Milk sampling and processing

Seasonal milk was taken for 2 full milking seasons, referred to as year 16/17 (2016-2017 season) and year 17/18 (2017-2018 season). Milk sampling and initial processing were described in Section 3.1. Skim milk, cream, UF retentate and permeate obtained from the initial processing of the milk were used to prepare the milk for the acid gelation analysis.

Acid gelation analysis was conducted with 2 sample systems, i.e., unstandardized seasonal skim milk and standardized whole milk (4.6% protein, 4.0% fat). A flowchart of milk processing and standardization is shown in Figure 5.1. The unstandardized skim milk was heated at 90°C for 6 min right after skimming, followed by cooling in ice water to 20°C. The standardized milk was made by mixing skim milk, cream, and UF retentate (or permeate) processed from the same batch of raw milk in calculated ratios. Permeate was added instead of retentate only when the protein content of the skim milk was too high to achieve 4.6% protein in the mixture. The standardized milk mixture was then homogenized at 70°C (2,000 psi in the first stage and 500 psi in the second stage) and heated at 90°C for 6 min. After heating, the milk was cooled to 20°C immediately in ice water. The heated milk samples were stored at 4°C and were

analyzed within 2 days. Samples were preserved with 0.02% (wt /wt) sodium azide. Aliquots of samples were frozen at –20°C prior to compositional analysis.

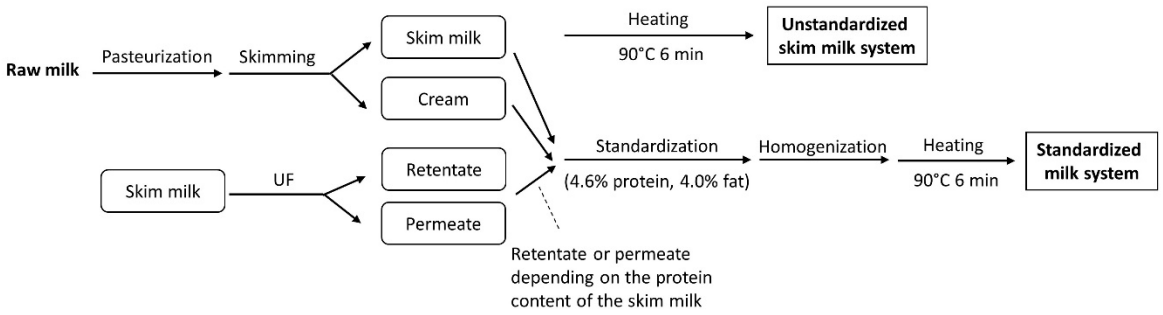

Figure 5.1: Flowchart of milk processing and the preparation of sample systems

5.2.2 Characterization of milk composition and properties

A MilkoScan FT120 (Foss Electric, Hillerød, Denmark) was used to determine the fat and protein contents of the cream, UF retentate, and UF permeate, as well as the composition of the unstandardized seasonal skim milk and the standardized milk including fat, protein, lactose, total solids, and solids-non-fat.

Milk characterizations were performed as described in Section 3.2. The characterizations of raw milk included total calcium, inorganic phosphorus, pH, ionic calcium, ethanol stability, buffering capacity, casein micelle size, protein composition, and the glycosylation of κ-casein (κ-CN). The extents of heat-induced κ-CN dissociation, whey protein denaturation, and whey protein–casein micelle association of heated unstandardized skim milk (90°C, 6 min) were also determined. Among the characterizations, pH, Ca^{2+}, and buffering capacity were also determined for the heated standardized whole milk.

5.2.3 Rheological analysis of acid milk gels

Acid milk gels were made by acidification using glucono-δ-lactone (GDL) and were analyzed on an AR-G2 magnetic bearing rheometer (TA Instruments, Crawley, West Sussex, UK) with standard Peltier concentric cylinder geometries (including a cup and a rotor with radii of 15 and 14 mm, respectively). Low amplitude oscillation tests were performed using a method modified from Anema et al. (2004). Milk was warmed to 30°C, 2.0% (wt/vol) GDL was added, and the mixture was stirred for 2 min prior to analysis. Based on preliminary tests, measurements were taken for 6 h for the unstandardized skim milk and for 8 h for the standardized milk, allowing the storage modulus (G′) to reach a stable level with a final pH of around 4.2. The gelation time was defined as the time from the addition of GDL to the time at which the G′ was greater than 1.0 Pa. The loss tangent (LT) of the final acid gel was determined. In year 17/18, the pH changes during acidification were monitored simultaneously using a TitraLab 856 pH-stat titration workstation (Radiometer Analytical, Lyon, France). The time differences among the addition of GDL, the measurements in the rheometer, and the measurements in the pH-stat titrator were recorded to determine the actual gelation time, gelation pH (the pH at gelation time), and final pH. Acid gelation analysis of each sample was at least duplicated. Acid gelation tests were conducted on 19 unstandardized skim milk and 15 standardized milk samples in year 16/17, and 15 unstandardized skim milk and 15 standardized milk samples in year 17/18 (4–8 samples in each season of each year).

5.3 Results and discussion

5.3.1 Milk composition and properties

Table 5.1 shows the composition, buffering capacity, pH, and Ca^{2+} concentration of the unstandardized skim milk and standardized milk. Unstandardized skim milk from the late season had significantly lower lactose and higher protein, solids-non-fat, and total solids than the milk from early- and mid-season. As expected, the variation pattern of the composition of the skim milk followed the trend of seasonal whole milk demonstrated in Chapter 4.

Table 5.1: Composition and chemical properties of unstandardized skim milk and standardized milk

		Unstandardized skim milk		Standardized milk	
	Season	2016–17	2017–18	2016–17	2017–18
Protein (%)	Early	3.52 ± 0.04[b]	3.71 ± 0.10[b]	4.54 ± 0.10	4.61 ± 0.03
	Mid	3.82 ± 0.05[b]	3.90 ± 0.07[b]	4.53 ± 0.03	4.66 ± 0.04
	Late	4.65 ± 0.13[a]	4.82 ± 0.12[a]	4.60 ± 0.06	4.73 ± 0.06
Fat (%)	Early	0.11 ± 0.02	0.08 ± 0.02	4.11 ± 0.07	4.03 ± 0.04
	Mid	0.13 ± 0.02	0.17 ± 0.09	3.95 ± 0.09	4.08 ± 0.10
	Late	0.09 ± 0.01	0.09 ± 0.01	4.07 ± 0.03	4.05 ± 0.06
Lactose (%)	Early	5.20 ± 0.02[a]	5.22 ± 0.02[a]	5.28 ± 0.07	5.36 ± 0.03[a]
	Mid	5.19 ± 0.03[a]	5.19 ± 0.03[a]	5.22 ± 0.05	5.26 ± 0.05[a]
	Late	5.03 ± 0.05[b]	4.92 ± 0.02[b]	5.10 ± 0.06	5.11 ± 0.02[b]
Total solids (%)	Early	9.38 ± 0.06[c]	9.61 ± 0.09[b]	14.50 ± 0.12	14.68 ± 0.10
	Mid	9.66 ± 0.04[b]	9.82 ± 0.11[b]	14.24 ± 0.09	14.61 ± 0.21
	Late	10.28 ± 0.12[a]	10.38 ± 0.11[a]	14.33 ± 0.07	14.46 ± 0.06
Solids-non-fat (%)	Early	9.24 ± 0.04[c]	9.52 ± 0.10[b]	10.27 ± 0.09	10.51 ± 0.07
	Mid	9.49 ± 0.04[b]	9.59 ± 0.07[b]	10.20 ± 0.06	10.39 ± 0.10
	Late	10.06 ± 0.10[a]	10.18 ± 0.11[a]	10.19 ± 0.05	10.32 ± 0.04
Buffering capacity (dB/dpH)	Early	ND	0.027 ± 0.000[b]	ND	0.032 ± 0.000
	Mid		0.027 ± 0.001[b]		0.031 ± 0.001
	Late		0.032 ± 0.001[a]		0.032 ± 0.000
pH (heated)	Early	6.72 ± 0.01[a]	6.67 ± 0.01[b]	6.68 ± 0.03	6.66 ± 0.01[b]
	Mid	6.67 ± 0.01[b]	6.76 ± 0.01[a]	6.65 ± 0.02	6.74 ± 0.01[a]
	Late	6.68 ± 0.01[ab]	6.75 ± 0.02[a]	6.66 ± 0.02	6.74 ± 0.02[a]
Ca^{2+} (heated) (mM)	Early	2.00 ± 0.07[ab]	2.18 ± 0.04[ab]	1.95 ± 0.09	2.10 ± 0.02
	Mid	1.86 ± 0.03[b]	2.06 ± 0.07[b]	1.81 ± 0.04	2.09 ± 0.09
	Late	2.10 ± 0.02[a]	2.37 ± 0.04[a]	2.03 ± 0.03	2.30 ± 0.05

[a–c] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).

ND: not determined; Ca^{2+}: ionic calcium.

Standardized milk eliminated the variations in milk composition during different seasons and between the 2 years, except for the lactose content in year 17/18, which does not actively contribute to the acid gelation induced by GDL. Standardization erased the elevated buffering capacity in late-season skim milk, presumably because of the strong contribution of proteins to the milk buffering capacity (Table 5.1). The pH and Ca^{2+} contents of heated skim milk and standardized milk were broadly similar, both correlating well with the pH and Ca^{2+} content of raw milk (Chapter 4). In summary, standardization equalized the composition and the buffering capacity of seasonal milk but did not change the pH and Ca^{2+} content significantly. This allowed a fair comparison of the acid gelation properties of seasonal milk.

5.3.2 Acid gelation properties

5.3.2.1 Unstandardized skim milk.

Table 5.2 shows the acid gelation properties of unstandardized skim milk. The seasonal trends in the final G′ were different in the 2 years, as indicated by the significant interactions between seasonal and year effects ($P < 0.01$). In year 16/17, acid gels made from early-season skim milk had significantly lower G′ than those made in the mid- and late season, whereas, in year 17/18, there was no significant seasonal difference in G′. It was interesting to note that, although late-season milk had considerably higher protein contents than early- and mid-season milk in both years (Table 5.1), late-season milk acid gels did not show higher G′ than those in other seasons, except for the early season of year 16/17. When the 2 years were compared, the mean G′ values of acid gels made from mid- and late-season milks in year 17/18 were lower than those in year 16/17 ($P < 0.05$), despite their similar protein contents (Table 5.1).

Table 5.2: Acid gelation properties of unstandardized skim milk

		Means ± SE		Effects[1]		
	Season	2016–17	2017–18	Season	Year	Season × year
Final G′ (Pa)	Early	360.1 ± 24.7^{a}	436.1 ± 30.7	NS	16/17 > 17/18	**
	Mid	456.0 ± 15.5^{bA}	362.4 ± 14.8^{B}			
	Late	502.0 ± 22.3^{bA}	358.2 ± 29.8^{B}			
Gelation time (min)	Early	39.1 ± 1.2^{aB}	43.9 ± 1.39^{aA}	L > E, M	17/18 > 16/17	*
	Mid	40.4 ± 0.4^{aB}	48.6 ± 2.62^{aA}			
	Late	58.3 ± 5.5^{bB}	81.9 ± 7.59^{bA}			
Gelation pH	Early	ND	5.22 ± 0.29^{a}	E, M > L		
	Mid		5.20 ± 0.34^{a}			
	Late		5.05 ± 0.37^{b}			
Final LT	Early	0.254 ± 0.001	0.254 ± 0.019^{a}	L > E, M	17/18 > 16/17	NS
	Mid	0.253 ± 0.001	0.256 ± 0.023^{a}			
	Late	0.257 ± 0.002	0.262 ± 0.044^{b}			

$^{a, b}$ Means of the same parameter within a column with different lowercase superscripts differ ($P < 0.05$, one-way ANOVA). $^{A, B}$ Means within a row with different uppercase superscripts differ ($P < 0.05$, independent sample t-test).
[1]Analyzed using two-way ANOVA
G′: storage modulus; LT: loss tangent; E: early season; M: mid-season; L: late season; ND: not determined; NS: nonsignificant; SE, standard error.
*$P < 0.05$, **$P < 0.01$.

The gelation time was significantly longer in the late season than in the rest of the year, particularly in year 17/18, in which the mean gelation time of late-season milk was almost double that of early-season milk. The gelation time was longer in year 17/18 than in year 16/17 in all 3 seasons. The gelation pH results (year 17/18) echoed the seasonal variation in gelation time, as late-season milk gelled at a significantly lower pH of 5.05 compared with those of early- and mid-season milks (around pH 5.20). It is expected that milk with a lower gelation pH would take longer to form a gel during acidification. The extents of variation in the final LT of the acid gels were not as large as those in the other gelation properties. Nevertheless, the final LT was significantly

higher, indicating less elastic or "solid-like" properties of the acid gels, in the late season and in year 17/18.

The results for the unstandardized skim milk acid gels suggested that the acid gelation properties of the milk were poor in the late season, among the seasons, and in year 17/18, between the 2 years. However, because of the difference in protein content, it is difficult to interpret the variation, e.g., the higher protein content of the late-season milk presumably increased the gelation time because of their higher buffering capacity (Table 5.1). Therefore, the variation in the acid gelation properties of seasonal milk can be better understood by studying the standardized milk system.

5.3.2.2 Standardized Milk

Figure 5.2 and Table 5.3 show the acid gelation properties of standardized milk. The final G′ was significantly lower in the late season than in early- and mid-season, which was consistent in both years. There was no significant between-year difference in the G′ of acid gels made in either the early season or mid-season. However, the acid gels made in the late season of year 17/18 had significantly lower G′ than those made in the late season of year 16/17. The seasonal and between-year variation patterns in gelation pH, gelation time, and final LT were consistent with those of unstandardized skim milk.

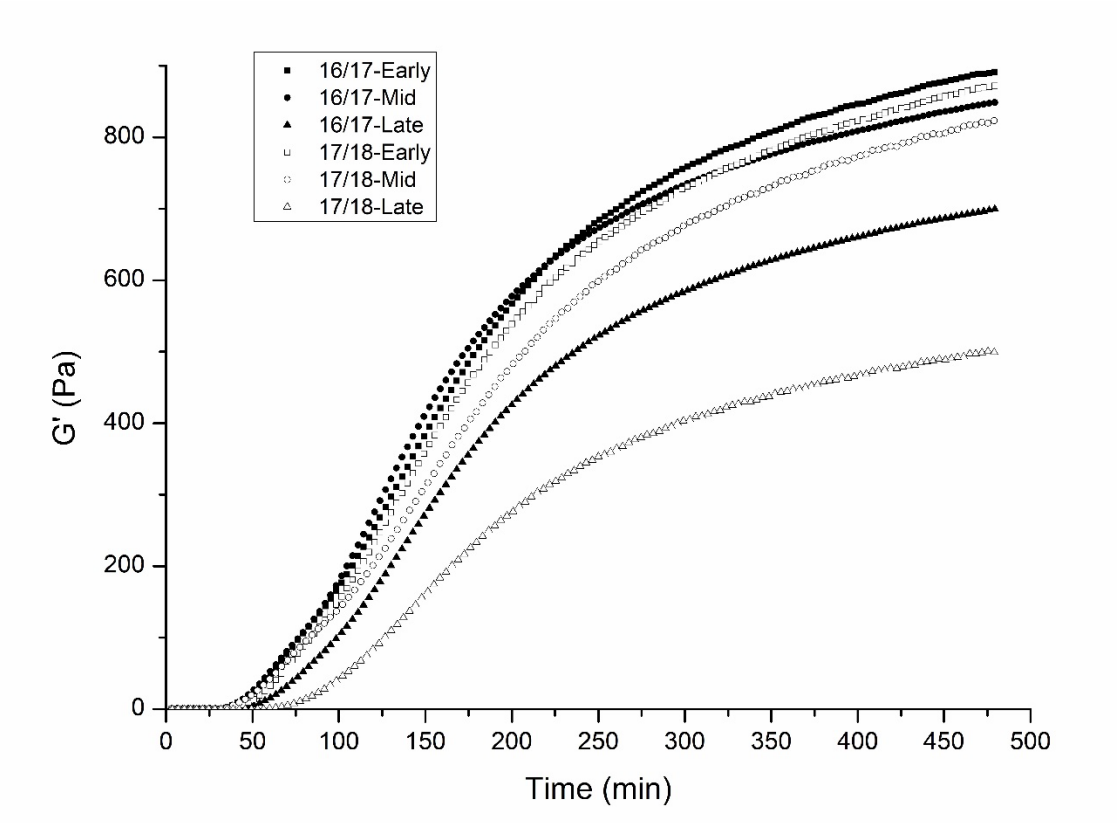

Figure 5.2: Change of storage modulus (G′) with acidification time for acid gels made from standardized milk from: early season of year 16/17 (■), mid-season of year 16/17 (●), late season of year 16/17 (▲), early season of year 17/18 (□), mid-season of year 17/18 (○), and late season of year 17/18 (△).

Table 5.3: Acid gelation properties of standardized milk

		Means ± SE		Effects[1]		
	Season	2016–17	2017–18	Season	Year	Season × year
Final G′ (Pa)	Early	862.6 ± 8.1^{a}	891.2 ± 21.8^{a}	E > M > L	16/17 > 17/18	*
	Mid	819.7 ± 37.8^{a}	764.4 ± 31.2^{a}			
	Late	672.2 ± 36.6^{bA}	513.4 ± 58.0^{bB}			
Gelation time (min)	Early	38.7 ± 1.0^{aB}	43.5 ± 0.8^{aA}	L > E, M	17/18 > 16/17	*
	Mid	38.0 ± 0.3^{aB}	43.6 ± 2.3^{aA}			
	Late	49.7 ± 2.3^{bB}	64.8 ± 4.4^{bA}			
Gelation pH	Early	ND	5.29 ± 0.04^{a}	E, M > L		
	Mid		5.26 ± 0.04^{a}			
	Late		5.07 ± 0.04^{b}			
Final LT	Early	0.225 ± 0.001^{B}	0.230 ± 0.001^{aA}	L > E	17/18 > 16/17	NS
	Mid	0.226 ± 0.002^{B}	0.234 ± 0.001^{bA}			
	Late	0.229 ± 0.001^{B}	0.233 ± 0.000^{bA}			

$^{a, b}$ Means of the same parameter within a column with different lowercase superscripts differ ($P < 0.05$, one-way ANOVA). $^{A, B}$ Means within a row with different uppercase superscripts differ ($P < 0.05$, independent sample t-test).

[1]Analyzed using two-way ANOVA

G′: storage modulus; LT: loss tangent; E: early season; M: mid-season; L: late season; ND: not determined; NS: nonsignificant; SE, standard error.

*$P < 0.05$.

Comparing the 2 sample systems, standardized milk gave rise to higher overall G′ and final LT values, probably resulting from the involvement of homogenized milk fat in the mixture and the higher protein content of 4.6% compared with most seasonal skim milk samples. In early- and mid-season, standardization was effective in stabilizing the G′ both across seasons and between the 2 years. However, the late-season standardized milk acid gel had the lowest G′ across the 3 seasons consistently in both years, which

was confounded in the unstandardized skim milk system by the highest protein contents of the late-season milk.

Standardization did not alter the gelation pH significantly ($P > 0.1$ in all seasons). This indicated that it was not affected by the protein concentration of the system or the involvement of fat, but by the differences in the physicochemical properties of the seasonal milk components.

By comparing the gelation times of the 2 milk systems over the seasons, it is found that standardization alleviated the increase in gelation time in the late season. However, the gelation time of late-season standardized milk was still significantly higher than that of the milk from the other seasons. The alleviation of the prolonged gelation time in the late season by standardization probably resulted from the standardized protein content, and thus the standardized buffering capacity. The buffering capacity of unstandardized skim milk was highest in the late season, whereas that of standardized milk did not vary with the season (Table 5.1, $P = 2.27$).

Analysis of the acid gelation property in a standardized milk system highlighted the overall inferior acid gelation properties of late-season milk, including the lowest G′, the longest gelation time, and the lowest gelation pH. The findings of the present study agreed with Underwood and Augustin (1997), who reported that acid gels made from late-season reconstituted milk in Australia had the lowest gel strength, the longest gelation time, and the highest final LT, i.e., the least solid-like properties. It is also worth noting that these authors reconstituted the milk based on the total solids content and that the late-season milk powders had the highest protein contents during the season. Consequently, the late-season reconstituted milk had the highest protein content

but the worst gelation properties, which was consistent with the results in the present study, particularly in year 17/18 (Tables 5.2 and 5.3). In agreement with Nguyen, Afsar, and Day (2018), the differences in the acid gelation properties were greatly affected by the physicochemical properties of the milk components, independent of the differences in protein content. Standardization was not sufficient in stabilizing the acid gelation properties of milk during the year in a seasonal calving system.

5.3.3 Correlation and Interpretation

Table 5.4 shows the significant correlations between the acid gelation properties of standardized milk and the milk characteristics (Chapter 4). Desirable acid gelation properties, i.e., higher gel strength, shorter gelation time, and higher gelation pH, were correlated with higher α-LA content, lower total calcium, Ca^{2+}, κ-CN, G-κ-CN, and β-lactoglobulin (β-LG) contents, as well as lower levels of heat-induced whey protein denaturation and whey protein–casein micelle association. When interpreting these correlations, it should be noted that the seasonal variations in the acid gelation properties resulted from the combined effects of various milk characteristics that changed concurrently during the season. As some of the milk quality parameters are interrelated with each other, they might not have affected the gelation properties *per se* to the extents that the correlations would suggest. Therefore, it is important to interpret these correlations with theoretical underpinnings. Four aspects of seasonal milk properties and their potential impacts on the acid gelation properties of seasonal milk are discussed below, i.e., the glycosylation of κ-CN, the heat-induced whey protein denaturation and association with the casein micelles, the α-LA: β-LG ratio, and the contents of calcium and Ca^{2+}. The interrelations among these parameters are also discussed.

Table 5.4: Pearson correlation coefficients between the seasonal milk characteristics and the acid gelation properties of standardized milk

	G′	Gelation pH	Gelation time	Final LT
κ-CN	–0.759***	–0.567*	0.762***	0.582**
Glycosylated κ-CN	–0.920***	–0.792***	0.897***	0.506**
Non-glycosylated κ-CN	0.579*	0.664**	–0.709**	NS
κ-CN GD	–0.927***	–0.834***	0.883***	0.429*
Serum:milk κ-CN GD ratio of heated milk	0.768***	0.541*	–0.705***	–0.515**
α-LA	0.890***	0.759**	–0.722***	NS
β-LG	–0.609***	–0.646**	0.598**	0.533**
WP denaturation	–0.609***	–0.716**	0.516**	NS
WP–CN micelle association	–0.651***	–0.733**	0.671***	0.453*
Total calcium	–0.593**	–0.770***	0.752***	0.685***
Ca^{2+} – Raw milk	–0.463*	–0.859***	0.728***	0.518**
Ca^{2+} – Heated standardized milk	–0.552**	–0.786***	0.733***	0.473**

GD: glycosylation degree; WP: whey protein; CN: casein; Ca^{2+}: ionic calcium; G′: storage modulus; LT: loss tangent; NS: nonsignificant.
Significance levels: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

5.3.3.1 Glycosylation of κ-CN

The content of G-κ-CN, which was highest in late-season milk in both years, had the highest correlation coefficients with lower G′ and longer gelation time of the standardized milk acid gels among all parameters determined (Table 5.4, $P < 0.001$). In

contrast, non-glycosylated κ-CN correlated with higher G′, high gelation pH, and shorter gelation time ($P < 0.05$). This agreed with Cases, Vidal, and Cuq (2003), who demonstrated that the deglycosylation of κ-CN with neuraminidase resulted in higher acid gel firmness and shorter gelation time. They attributed the enhanced acid gelation properties to the higher surface hydrophobicity of the deglycosylated casein micelles. In a recent study, Oka, Ono, Ohara, Noguchi, and Takano (2018) reported that κ-CN-depleted casein micelles had higher acid gelation pH than native casein micelles because of their lower contents of sialic acid, higher surface hydrophobicity, and lower negative charge.

κ-CN forms the surface layer of casein micelles, stabilizing the micelles by providing electrostatic and steric repulsion. Because of their importance in the stabilization of casein micelles, even minor changes in the properties of κ-CN could have a significant impact on casein micelle interactions. G-κ-CN carries hydrophilic sugar moieties that both increase the negative charge and reduce the hydrophobicity of κ-CN (Holland & Boland, 2014; Vreeman, Both, & Brinkhuis, 1977). Upon the acidification of milk, the added negative charge on the surface of the micelles by glycosylation might provide stronger resistance to charge neutralization. As such, a higher proportion of G-κ-CN might lower the pH required for micelle aggregation and might delay the gelation. Subsequently, a longer gelation time would shorten the time available for the development of the gel structure, resulting in a lower G′ of the final gel. In addition, a higher concentration of κ-CN, particularly G-κ-CN (both highest in the late season), may reduce the hydrophobicity of the protein matrix, weakening the hydrophobic interactions in the acid gel network. The importance of hydrophobic interactions in the

development of acid milk gel structure has been well established (Donato & Guyomarc'h, 2009; Jean, Renan, Famelart, & Guyomarc'h, 2006; Morand, Dekkari, Guyomarc'h, & Famelart, 2012).

In addition to the variations in the GD of κ-CN of milk, the serum: milk ratio of the GD of κ-CN in heated (90°C for 6 min) milk correlated with the positive acid gelation properties. A lower serum: milk κ-CN GD ratio, as in late-season milk, indicates that a higher proportion of G-κ-CN remained on the micelle surface compared with that in the serum phase. As such, the GD of micelle-bound κ-CN was even higher than the GD of total κ-CN in late-season milk, which may further promote the negative effects of κ-CN glycosylation on the acid gelation properties of milk. Further investigation is needed to test this hypothesis.

In summary, the amount of G-κ-CN might play an important role in affecting the acid gelation properties of seasonal milk based on the consistent and strong correlations supported by previous work and theoretical understanding. The variation in the distribution of G-κ-CN between the micelle and the serum phase might further amplify the impact of κ-CN glycosylation on the acid gelation properties of milk.

5.3.3.2 Whey Protein Denaturation and Association with Casein Micelles

The extent of heat-induced whey protein denaturation and the extent of whey protein–casein micelle association, both highest in late-season milk, correlated with lower G′ and longer gelation time (Table 5.4). The negative correlations between the extent of denaturation of the whey proteins and the desirable acid gelation properties were expected, given the high extent of whey protein denaturation and its small amount of

variation in the heated milk in this study (94.9 ± 0.3%). In most samples, β-LG was completely denatured and the variation in total whey protein denaturation arose mostly from the variation in the extent of denaturation of α-LA. Compared with β-LG, denatured α-LA does not contribute to the acid gel structure in the same way because of its lack of a free thiol group (Graveland-Bikker & Anema, 2003), lower isoelectric point (Paulsson, Hegg, & Castberg, 1986), and lower hydrophobicity (Morand, Guyomarc'h, & Famelart, 2011; Mottar, Bassier, Joniau, & Baert, 1989). As a result, the variation in the extent of whey protein denaturation, although statistically significant, might not affect the acid gelation of milk greatly.

Similar to the whey protein denaturation, the higher extent of whey protein–casein micelle association, as in late-season milk, correlated with inferior gelation properties. This agreed with the results of a number of other researchers, i.e., the soluble whey protein aggregates in heated milk contributed more to the acid gel structure than did the micelle-bound whey proteins (Anema, 2018; Anema et al., 2004; Lakemond & van Vliet, 2008; Vasbinder, van de Velde, & de Kruif, 2004). Anema (2008) emphasized the role of soluble whey protein aggregates in interconnecting colloidal casein particles with disulfide bonds, suggesting that the inter-particle disulfide bonds would give rise to a higher gel rigidity compared with the disulfide bonds present on the individual micelles.

5.3.3.3 α-LA:β-LG Ratio

A higher percentage of α-LA and a lower percentage of β-LG in the milk proteins correlated with better acid gelation properties (Table 5.4). These correlations contradicted that reported by Graveland-Bikker and Anema (2003), who demonstrated

that the addition of β-LG to whey-protein-depleted milk resulted in significantly higher G′, higher gelation pH, and shorter gelation time of acid gels compared with the addition of an α-LA/β-LG mixture (1:2 ratio) or α-LA alone at the same concentration. This discrepancy was understandable considering that α-LA made up only 2.6% of the total protein and 17.3% of the whey proteins in this study, compared with the study of Graveland-Bikker and Anema (2003), in which α-LA made up at least 33.3% of the added whey proteins. β-LG had a rather small extent of variation (CV 7.1%) and did not vary significantly with the season in year 16/17 (Chapter 4). Despite this insignificant seasonal variation in the β-LG content in year 16/17, late-season milk from year 16/17 still had significantly worse acid gelation properties than the milk from the rest of the year. It was likely that the variation in the α-LA:β-LG ratio *per se* did not contribute greatly to the variation in the acid gelation properties of the seasonal milk in this study.

5.3.3.4 Milk Calcium

The concentrations of both calcium and Ca^{2+} correlated with lower G′, longer gelation time, and higher final LT of the acid gels (Table 5.4). Underwood and Augustin (1997) also reported that late-season reconstituted milk had both the highest Ca^{2+} content and the worst acid gelation properties. Some studies showed that moderate calcium removal or its chelation by trisodium citrate improved the acid gel or yoghurt properties (Meletharayil, Patel, & Huppertz, 2015; Ozcan-Yilsay, Lee, Horne, & Lucey, 2007). Ozcan-Yilsay et al. (2007) reported that partial removal of the colloidal calcium phosphate by trisodium citrate prior to acidification gave rise to a higher level of protein rearrangement during the gelation process, resulting in a stronger gel network.

However, there have been contradicting reports on the effect of calcium on the acid gelation of milk. Ozcan, Lucey, and Horne (2008) did not find a positive effect of another calcium-chelating agent, tetrasodium pyrophosphate, on yoghurt firmness. Ramasubramanian, Restuccia, and Deeth (2008) reported only a minor effect of calcium addition or removal prior to heating on yoghurt structural properties. Morand et al. (2011) suggested that Ca^{2+} binding did not affect the acid gel structure. In addition, the extents of natural variation in calcium and Ca^{2+} in the present study were markedly smaller than the adjusted levels in previous studies. The effect of the natural variation in milk calcium on the rheological properties of acid milk gels is inconclusive until further, detailed investigations are made.

5.3.3.5 Interrelations Among Milk Quality Parameters

The GD of κ-CN correlated ($P < 0.001$) with all parameters discussed above that had significant correlations with the acid gelation properties, i.e., lower α-LA, higher β-LG, higher total calcium and Ca^{2+}, and higher extents of heat-induced whey protein denaturation and association with the casein micelles. In addition, a higher proportion of β-LG in the total whey proteins and a higher Ca^{2+} content have been demonstrated to enhance the extent of whey protein denaturation and the extent of whey protein–casein micelle association (Law & Leaver, 2000; Oldfield, 1996). Considering these interrelations, it is concluded that the extent of heat-induced whey protein–casein micelle association might play a part in affecting the acid gelation properties of seasonal milk but its impact is confounded, to some extent, by its correlation with the GD of κ-CN. The variations in the denaturation of the whey proteins, the α-LA: β-LG ratio and the contents of calcium and Ca^{2+} might not have great direct impacts on the

acid gelation of seasonal milk. Their correlations with the acid gelation properties were probably caused by their correlations with the GD of κ-CN and with the distribution of the denatured whey proteins between the micelles and the serum phase.

5.4 Conclusions

The acid gelation properties of seasonal milk deteriorated consistently in the late season of both years. The comparison between unstandardized skim milk and standardized milk demonstrated that the inferior acid gelation properties of late-season milk resulted from its unique physicochemical properties, independent of the varied protein content. Standardization alleviated the elevated gelation time in the late season but this was still significantly longer than that in early- and mid-season. In addition, standardized milk from the late season had the lowest acid gelation pH and the lowest G′ during the year. Standardization of the protein and fat contents was not sufficient in stabilizing the acid gelation properties throughout the year. Inferior gelation properties correlated significantly with the GD of κ-CN, the heat-induced whey protein denaturation and whey protein–casein micelle association, the α-LA: β-LG ratio, and the contents of calcium and Ca^{2+}. Based on the strong correlations and consistent literature reports, it is proposed that the glycosylation of κ-CN plays an important role in milk acid gelation by altering the hydrophobic and electrostatic interactions. From the present study, it is difficult to draw a firm conclusion on how other parameters affect acid gelation because of their interrelations and the lack of consistent supporting evidence in the literature.

Chapter 6 - Effects of seasonal variations in the quality of set yoghurt, stirred yoghurt and Greek-style yoghurt.

6.1 Introduction

Yoghurt is a popular dairy product around the world not only for its unique texture and flavour but also for its health benefits (Macori & Cotter, 2018). A better understanding of the seasonal impacts on yoghurt properties would allow better control of the product quality, as well as assist in the development of new types of fermented dairy products to meet the ever-changing consumer preferences.

The manufacture of Yoghurt involves the acid gelation of milk. The effects of some important processing parameters on yoghurt, e.g. heating, were displayed in acid gelation studies using GDL (Guyomarc'h et al., 2003; Lucey & Singh, 1997; Lucey, Teo, Munro, & Singh, 1997). However, Lucey et al. (1998a) suggested that the observations from GDL-induced gelation tests should be validated in yoghurt made with bacterial cultures, because of rheological and structural differences between GDL-induced acid gels and bacterial-fermented yoghurt gels. The GDL-induced acid gelation of milk and yoghurt fermentation were typically carried out at different temperatures, to achieve similar incubation times to the desired pH (around 4.6). Temperature affects some physicochemical properties of milk (e.g. salt equilibrium) as well as the reactions during the gelation process (e.g. hydrophobic interactions). In addition, the acidification rate was demonstrated to be different between GDL and yoghurt culture, which would affect the structural development and rearrangement of the acid gel structure (Lucey et al., 1998a). For these reasons, the seasonal variations in the quality

of yoghurts were studies in this chapter in addition to the acid gelation study in Chapter 5.

There is very limited published information on the seasonal variations in yoghurt properties. Cheng et al. (2002) studied the properties of yoghurts made from reconstituted concentrated skim milk produced at different times of the year in Australia. They reported that there were seasonal variations in the firmness of set yoghurt, the viscosity of stirred yoghurt and the extent of syneresis. However, no clear trend during the year was reported by Cheng et al. (2002).

As reviewed in Section 2.4.1, different types of yoghurt e.g. set yoghurt, stirred yoghurt and Greek-style yoghurt (GSY), are made by varying the process and milk composition. These types of yoghurt have different structural and textural characteristics. Therefore, to provide a more comprehensive understanding of the impact of seasonality on the quality of yoghurts, it is worthwhile to investigate different types of yoghurts.

The objective of this chapter was to evaluate the seasonal variations in the quality of three types of yoghurt, namely regular set yoghurt, regular stirred yoghurt and Greek-style yoghurt. The firmness of set yoghurt and Greek-style yoghurt and the viscosity of stirred yoghurt were determined. Besides, the syneresis and the water holding capacity of different yoghurts were also studied. The same standardized milk base (4.6% protein, 4% fat) was used for GDL-induced acid gel (Chapter 5), set yoghurt and stirred yoghurt study, which allowed the direct comparisons between GDL-induced acid gel and yoghurts, as well as between different types of yoghurt products. It was of interest

to explore the usefulness of GDL-induced acid gel was as an indicator of the variations in yoghurt quality.

6.2 Materials and methods

6.2.1 Yoghurt milk preparation and fermentation

The process of yoghurt milk base preparation and fermentation is described below and presented in Figure 6.1 and Figure 6.2.

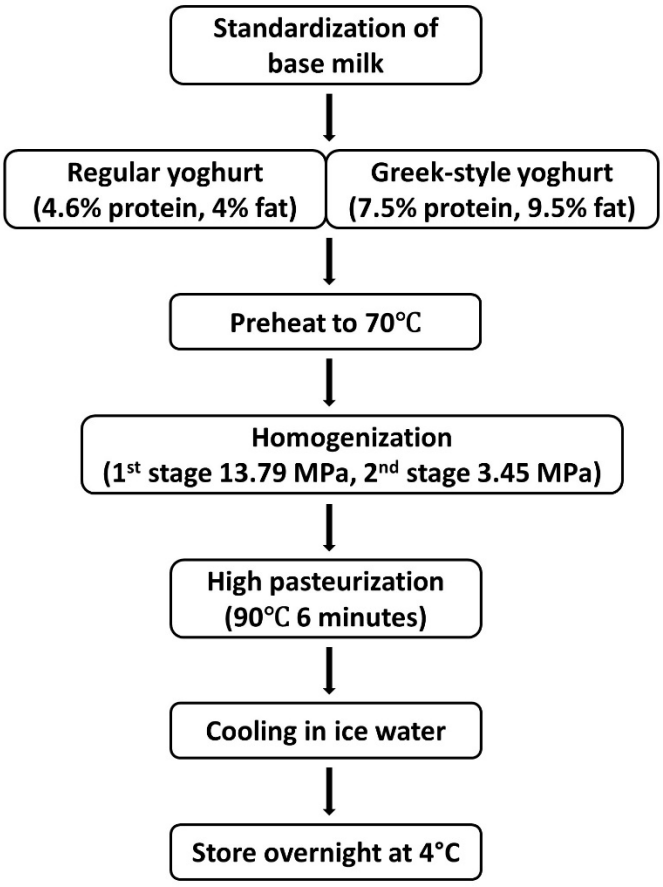

Figure 6.1: Flowchart of yoghurt milk base preparation

Regular yoghurt milk base (4.6% protein, 4% fat) and Greek-style yoghurt milk base (7.5% protein, 9.0% fat) were standardized by mixing skim milk, cream and UF retentate (or permeate) obtained from the initial process of raw milk as described in Section 3.1. Both milk mixtures were preheated to 70°C and homogenized in a two-stage homogenizer under pressure of 13.79 MPa (2000 psi) in the first stage and 3.45 MPa (500 psi) in the second stage. After homogenization, the milk mixture was heated at 90 ± 0.5°C for 6 minutes. The heated yoghurt milk was chilled immediately in ice water and stored at 4°C overnight before fermentation.

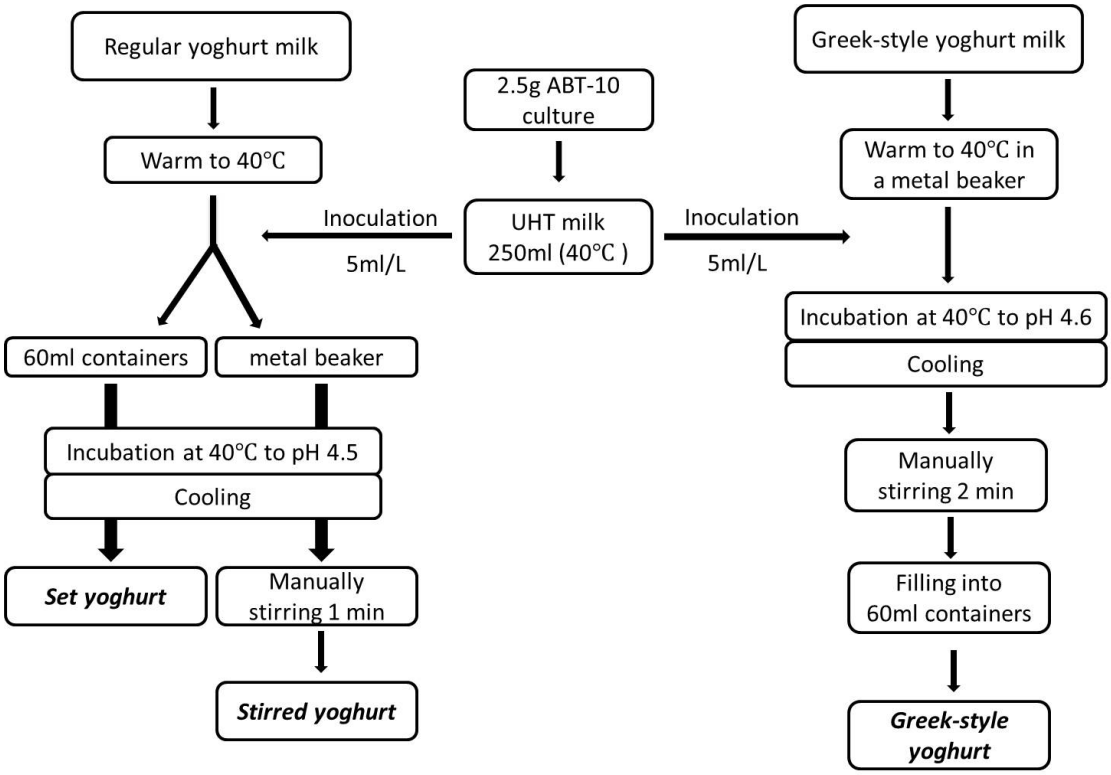

Figure 6.2: Flowchart of yoghurt making

Yoghurts were fermented using an exopolysaccharide-producing ABT-10 culture (Chr. Hansen, Hoersholm, Denmark). Prior to inoculation, milk bases were warmed to 40°C in a temperature-controlled water bath. 2.5 g of culture was dissolved in 250 ml of

commercial UHT milk and stirred for 2 min. This cultured milk was used to inoculate yoghurt milk bases at a dosage of 5 ml/L. Fermentation was carried out at 40°C in an incubator.

The fermentation of regular yoghurts was performed in three types of containers. Cylinder-shaped 60 ml containers (43 mm diameter) were used to ferment set yoghurts for texture analysis and the determination of spontaneous syneresis. The inoculated regular yoghurt milk was filled in the containers to the marked line of 50 ml (35 mm height). The second part of regular yoghurt (around 45 g) was fermented in 50 ml Falcon centrifuge tubes to determine the water-holding-capacity (WHC) of set yoghurts. The rest of regular yoghurt mixture was fermented in a cylinder metal beaker (diameter 15.5 cm, height 20.5 cm). The termination pH for regular yoghurt was 4.5. After fermentation, the yoghurts were chilled immediately at 4°C. The regular yoghurt was stirred manually in the metal containers with a spatula (4.7 cm x 5.5 cm) in a circular motion for 1 min. The stirring time was determined in preliminary tests to make the reference yoghurt visually smooth. All measurements of stirred yoghurts were performed after overnight storage at 4°C.

The fermentation of Greek-style yoghurt was also performed in three types of containers as described above. Greek-style yoghurt was fermented until the termination pH of 4.6. Greek-style yoghurt was stirred in the same way described above for 2 min. After the stirring process, Greek-style yoghurt was filled into the 60 ml containers described above to the marked line of 50 ml. During the filling process, the containers were gently tapped on the bench to ensure no air bubbles were trapped in the sample.

This stirred sample was used to determine the firmness of Greek-style yoghurt. All samples were stored at 4°C until further analysis.

6.2.2 Texture analysis of set yoghurt

The firmness of set yoghurt was analysed with a back extrusion programme using a texture analyser TA.XT Plus (Stable Micro System, Surrey, UK). A geometry with a round flat surface (40 mm diameter) was used for back extrusion test, allowing 1.5 mm space between the geometry and the sample container (43 mm diameter). The analysis was performed by lowering the geometry at a speed of 1mm/s into the yoghurt sample for 30 s. The measurement was triggered automatically when a force of 0.05 N is reached. The measured force increased during the first 5 s and then stabilized. The firmness (N.s) of yoghurt is defined as the area under the time - force curve from 5 to 30 seconds. Set yoghurt firmness was measured after 24 h and 7 d storage. All measurements were made in triplicate.

6.2.3 Apparent viscosity measurement of stirred yoghurt

The apparent viscosity of stirred yoghurt was determined by an AR-G2 Magnetic Bearing Rheometer (TA Instruments, Crawley, West Sussex, UK) in combination with a cone and plate geometry (Steel cone of 40 mm diameter, 4° cone angel). A constant shear rate of 50 s^{-1} was applied to simulate oral processing (Wood, 1968). Apparent viscosity readings (Pa.s) were taken every 10 seconds for 3 minutes at 4°C. Initial viscosity (V_0) and the median viscosity during 3 minutes (V_{median}) were determined. The V_{median}/V_0 ratio was also analysed to indicate the resistance of stirred yoghurt structure against shearing.

6.2.4 Spontaneous syneresis and water holding capacity

Spontaneous syneresis of yoghurt was defined as the whey separation on the surface of yoghurts during fermentation. The extent of spontaneous syneresis was determined as the weight percentage of free whey in the total yoghurt weight, immediately after fermentation. Spontaneous syneresis measurements were made in triplicate.

The water holding capacity (WHC) of both set and stirred yoghurts was defined as the weight percentage of concentrated yoghurt under centrifugation at 1,000 g for 10 min. About 45.0 g of yoghurt was centrifuged in 50 ml Falcon centrifuge tubes for the WHC test. Regular set yoghurt and GSY were fermented in centrifuge tubes whereas stirred yoghurt was filled into the tubes after stirring and overnight storage at 4°C. WHC tests were duplicated.

6.3 Results and discussion

Table 6.1: Fermentation time and firmness of set yoghurt and Greek-style yoghurt

		Means ± SE		Effects[1]		
	Season	16/17	17/18	Season	Year	Season × year
Fermentation time – regular yoghurt (min)	Early Mid Late	362 ± 16 356 ± 18 359 ± 35	347 ± 2 329 ± 15 335 ± 25	NS	16/17 > 17/18	NS
Fermentation time – Greek-style yoghurt (min)	Early Mid Late	409 ± 3 419 ± 7 416 ± 13	407 ± 9 373 ± 14 415 ± 15	NS	NS	NS
Set yoghurt firmness – average (N.s)	Early Mid Late	235 ± 13[a] 202 ± 7[b] 193 ± 11[b]	240 ± 1[a] 191 ± 9[b] 179 ± 5[b]	E > M, L	NS	NS
Greek-style yoghurt firmness - average (N.s)	Early Mid Late	518 ± 49.8[a] 387 ± 41.1[b] 344 ± 7.6[b]	449 ± 19[a] 335 ± 17[b] 343 ± 38[b]	E > M, L	NS	NS
Greek-style yoghurt firmness - overnight (N.s)	Early Mid Late	537 ± 49[a] 348 ± 32[b] 274 ± 15[b]	411 ± 23[a] 297 ± 15[b] 289 ± 37[b]	E > M, L	16/17 > 17/18	NS

[a–b] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).
[1]Analyzed using two-way ANOVA
E: early-season; M: mid-season; L: late-season; NS: nonsignificant; SE, standard error.

6.3.1 Fermentation time

The fermentation time of regular yoghurt was not affected by seasons in either year, but the overall fermentation time was longer in year 16/17 than in year 17/18 (Table 6.1). The consistent fermentation time during different seasons agreed with the finding that standardization eliminated the variation in buffering capacity of seasonal milk, as well as the final acid gelation pH induced by GDL (Chapter 4 and Chapter 5). Cheng et al. (2002) also reported the fermentation time of yoghurt made from reconstituted milk

was not affected by seasons in which the milk powders were produced in Australia. The longer fermentation time in year 16/17 might be a result of a higher casein content, hence a stronger buffering capacity, of the milk from year 16/17. Longer fermentation time correlated with higher proportion of caseins in total milk proteins (r = 0.384, P = 0.04). Similar to regular yoghurt, the fermentation time of Greek-style yoghurt also did not vary with season. Milk from year 16/17 also had a longer fermentation time than milk from year 17/18, although the difference was not statistically significant (P = 0.054).

6.3.2 Firmness of set yoghurt and Greek-style yoghurt

6.3.2.1 Set yoghurt

There was no significant effect of storage for 7 d on the firmness of set yoghurt. For this reason, the average firmness of yoghurts stored for 24 h and 7 d are used for further discussion. Yoghurts made in the early seasons of both years had the highest firmness comparing to the rest of the year. Late-season yoghurts had the lowest mean firmness in both years, although it was not significantly lower than that of yoghurts made in mid-seasons. The firmness of set yoghurt correlated with the G′ of GDL-induced acid gel made from the same standardized milk (P < 0.01, Chapter 5). The results suggested that despite the difference in acidification rate and structural rearrangements (Lucey et al., 1998a; Ozcan, Horne, & Lucey, 2015), GDL-induced acid gel could represent the seasonal variation in the firmness of yoghurt in this study. Nevertheless, the extent of variation, represented by coefficients of variance (CV), was higher for GDL-induced acid gels (CV 18.5%) than for set yoghurts (CV 14.7%). Exopolysaccharide produced

by the starter culture could contribute to the yoghurt structure, diminishing the variation arising from physiochemical properties of seasonal milk.

6.3.2.2 Greek-style yoghurt

The average firmness of Greek-style yoghurt measured after storage of 1 d, 7 d, 14 d and 28 d were 363.7 N.s, 411.6 N.s, 411.5 N.s and 415.1 N.s, respectively. The firmness increased in the first 7d of storage and remained stable until 28 d. This increase of the firmness by about 10% of set-yoghurts during storage agreed with previous studies (Matumoto-Pintro, Rabiey, Robitaille, & Britten, 2011; Sodini et al., 2004). In the late season, the increase in the firmness during storage was largest and the most significant ($P = 0.016$) among seasons (Table 6.1). The firmness results during storage indicated that GSY became firmer during the first 7d of storage, presumably owing to the protein network fusion and "rebodying" after breakage of the original gel structure by mixing (Lee & Lucey, 2010). In particular, in the late season where the initial firmness of GSY was the lowest, the increase in firmness during storage was the most pronounced.

The firmness of Greek-style yoghurt followed the same seasonal trend as the set yoghurt in that the early-season samples had the highest firmness (Table 6.1). The average firmness of GSY correlated with regular set yoghurt firmness and the G′ of GDL-induced acid gel ($P < 0.01$). The results indicated that the seasonal variation in the textural properties of a high protein yoghurt was similar to, and can be modelled by, GDL-induced acid gel or regular set yoghurt. However, comparing the extent of variation in firmness, Greek-style yoghurt had a larger extent of variation in firmness

(CV 22.3%) than regular set yoghurt (CV 14.7%), suggesting the impact of seasonal milk properties on yoghurt structure was greater in a high protein system.

The lower firmness of yoghurts in the late season, particularly GSY, might be a potential advantage in designing high protein yoghurt products. Jørgensen et al. (2015) reported that high protein yoghurts with lower firmness had better sensory properties (i.e. smoother texture with less graininess). Attempts have been made to alleviate the undesirable sensory properties of high protein yoghurts, such as post-fermentation processing (Jørgensen et al., 2019). A high-protein low-viscosity yoghurt product might be appealing to certain groups of consumers. Late-season milk that is both naturally rich in protein and able to produce yoghurts with lower firmness might have the potential for the development of specialized high protein yoghurt products with certain textural characteristics.

Table 6.2 shows the Pearson correlation coefficients between the yoghurt firmness and the characteristics of milk. The firmness of both set yoghurt and Greek-style yoghurt had similar correlations with milk characteristics as the G′ of GDL-induced acid gels. Higher firmness of both types of yoghurts significantly correlated with higher non-glycosylated κ-CN ($P < 0.05$), lower glycosylated κ-CN ($P < 0.01$), thus lower glycosylation degree (GD) of κ-CN ($P < 0.01$, Table 6.2) of the milk. In addition, yoghurt firmness correlated with proportions of α-LA and β-LG, the extents of whey protein denaturation and whey protein-casein micelle association, similar to the G′ of GDL-induced acid gels (Table 6.2). These correlations suggested that the physicochemical implications of milk characteristics on GDL-induced acid gelation, as discussed in Chapter 5, also largely contributed to affecting the firmness of yoghurt.

For this reason, the same correlations between yoghurt firmness and milk characteristics are not discussed further in this chapter.

Table 6.2: Pearson correlation coefficients between the seasonal milk characteristics and the acid gelation properties of standardized milk

	Average firmness – set yoghurt (N.s)	Average firmness – GSY (N.s)
κ-CN	–0.436*	NS
Glycosylated κ-CN	–0.620***	–0.503**
Non-glycosylated κ-CN	0.472**	0.464*
κ-CN GD	–0.671***	–0.566**
α-LA	0.703***	0.572**
β-LG	–0.393*	-0.359 (P = 0.056)
WP denaturation	–0.614***	–0.607***
WP–CN micelle association	–0.443*	–0.535**
Total calcium	NS	–0.410*
Ca^{2+} (Raw milk)	NS	NS

GD: glycosylation degree; NS: nonsignificant; WP: whey protein; CN: casein; Ca^{2+}: ionic calcium; GSY, Greek-style yoghurt.
Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001.

Nevertheless, the extents of correlations between yoghurt firmness and milk characteristics were smaller than between the G′ of acid gels and milk characteristics. Unlike acid gels made by acidification using GDL, yoghurt firmness did not correlate significantly with ionic calcium in milk. These differences might arise from the

difference in the acidification rate between GDL and yoghurt culture (Lucey et al., 1998a). Besides, spontaneous syneresis occurred during the yoghurt fermentation but not during the acidification process using GDL, which might have played a part in affecting the seasonal trends in yoghurt firmness. These results and correlation analysis of GDL-induced acid gel and yoghurt highlighted the important roles of the GD of κ-CN and to a lesser extent the whey protein-casein micelle association, on the acid gelation process during yoghurt making. As discussed in Chapter 5, factors such as calcium and whey protein composition might not contribute to the acid gelation properties of seasonal milk. Their correlations with the acid gel strength and yoghurt firmness were likely to be resulting from the interrelations with other parameters, such as the GD of κ-CN.

6.3.3 Syneresis and water holding capacity of yoghurts

Table 6.3 shows the extent of spontaneous syneresis and the water holding capacity of different types of yoghurts. Mid-season set yoghurts had the highest level of spontaneous syneresis and the lowest WHC in both years. Similarly, Greek-style yoghurt made in mid-season also had the highest level of spontaneous syneresis and the lowest WHC, although the variation in spontaneous syneresis was not statistically significant ($P = 0.059$). In contrast, the WHC of stirred yoghurt was not affected by seasonal or between-year variation.

Table 6.3: Syneresis and water holding capacity of yoghurts

		Means ± SE		Effects[1]		
	Season	16/17	17/18	Season	Year	Season × year
Spontaneous syneresis - Set yoghurt (%)	Early	2.1 ± 0.3^{ab}	2.3 ± 0.2^{ab}	M > E, L	NS	NS
	Mid	3.0 ± 0.5^{a}	3.6 ± 0.6^{a}			
	Late	1.1 ± 0.1^{b}	1.8 ± 0.2^{b}			
Spontaneous syneresis - Greek -style yoghurt (%)	Early	1.0 ± 0.2	3.0 ± 0.3	NS	17/18 > 16/17	NS
	Mid	2.0 ± 0.6^{a}	2.9 ± 0.5			
	Late	0.5 ± 0.1^{b}	2.1 ± 0.6			
WHC - Set yoghurt (%)	Early	91.4 ± 1.9	94.8 ± 0.7^{a}	E > M	NS	NS
	Mid	87.8 ± 2.2	88.0 ± 1.3^{b}			
	Late	91.0 ± 2.1	92.8 ± 1.9^{a}			
WHC - Stirred yoghurt (%)	Early	92.8 ± 1.3	92.9 ± 0.9	NS	NS	NS
	Mid	91.8 ± 1.7	92.8 ± 0.5			
	Late	94.2 ± 0.5	93.6 ± 0.7			
WHC - Greek - style yoghurt (%)	Early	94.9 ± 0.2^{ab}	96.1 ± 0.6^{ab}	E, L > M	17/18 > 16/17	NS
	Mid	91.8 ± 1.8^{b}	94.7 ± 0.6^{b}			
	Late	95.8 ± 0.4^{a}	97.8 ± 0.7^{a}			

[a–b] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).
[1]Analyzed using two-way ANOVA
E: early-season; M: mid-season; L: late-season; WHC: Water holding capacity; NS: nonsignificant; SE, standard error.

The WHC under centrifugation followed the order of Greek-style yoghurt > stirred yoghurt > set yoghurt ($P < 0.001$). The level of spontaneous syneresis was also lower in Greek-style yoghurt (1.94%) than in regular set yoghurt (2.32%), although the difference was not statistically significant ($P = 0.142$). The higher WHC of Greek-style yoghurt compared with regular yoghurt was presumably due to a more effective water-binding and trapping owing to higher solids content (Sodini et al., 2004). This was probably the reason for the lack of significance in WHC among different seasons in Greek-style yoghurt. The WHC of stirred yoghurts also did not vary significantly with the season, which might be resulted from the effects of stirring and storage. For set

yoghurts, the structural arrangement of the yoghurt during fermentation greatly affects their WHC, whereas for stirred yoghurts the structure of the yoghurt gel was broken and allowed to rearrange during the overnight storage. The effect of structural disruption and rebodying probably played a more important role in determining the WHC of stirred yoghurts than the variation in the structure of the original set yoghurt. The slightly higher WHC of stirred yoghurts compared with set yoghurts suggested that the rebodying process effectively entrapped the whey separated both during fermentation and after the stirring process.

6.3.4 Stirred yoghurt viscosity

The seasonal variation in the viscosities of stirred yoghurts and their resistance to thinning under shearing displayed different seasonal trends with the firmness of set yoghurts (Figure 6.3 and Table 6.4). The initial viscosity (V_0) of stirred yoghurt was significantly affected by season-year interactions, suggesting different seasonal trends in the two years. In year 17/18, early-season yoghurts had significantly higher V_0 than those from mid- and late-season, whereas in year 16/17 there was no significant seasonal variation. Although not statistically significant, mid-season yoghurts in year 16/17 had the lowest mean V_0 of 2.55 Pa.s during the year while early and late-season yoghurts had similar viscosity of 2.76 Pa.s and 2.73 Pa.s respectively. The median viscosity during 3 min of constant shearing (V_{median}) was the lowest in the mid-season of both years. The V_{median}/V_0 ratio, as an indicator of the resistance of stirred yoghurt viscosity against shear-induced thinning, was consistently higher in the late season than in the early season and mid-season.

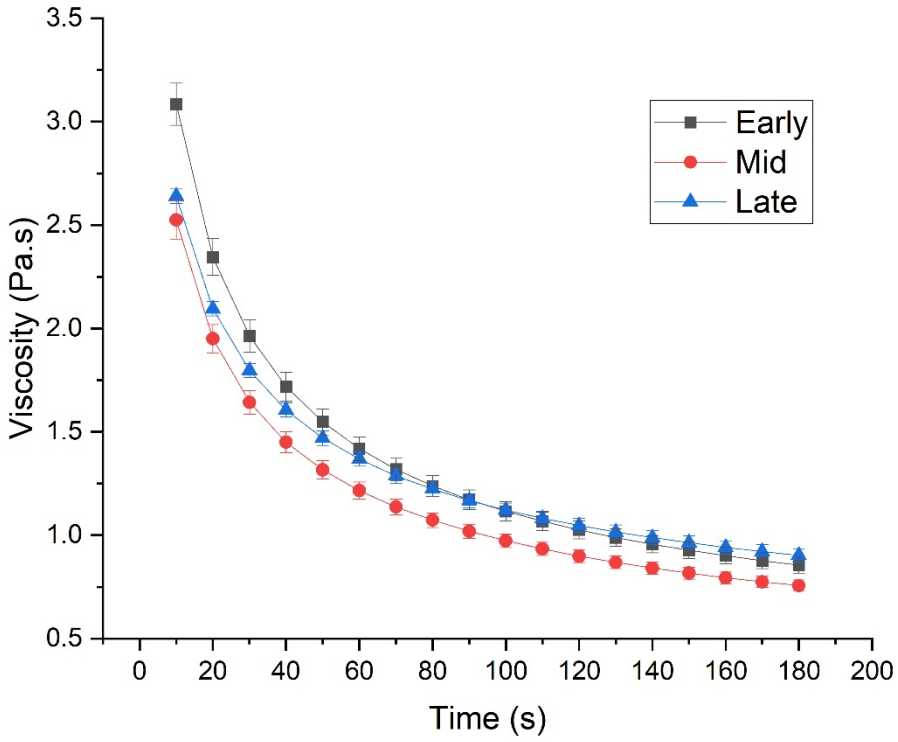

Figure 6.3: Viscosity reduction of stirred yoghurts over time under a constant shear rate of 50 s^{-1}.

Table 6.4: Viscosity of stirred yoghurts

		Means ± SE		Effects[1]		
	Season	16/17	17/18	Season	Year	Season × year
V_0 (Pa.s)	Early	2.76 ± 0.08	3.08 ± 0.10[a]	E > M, L	NS	*
	Mid	2.55 ± 0.04	2.52 ± 0.09[b]			
	Late	2.73± 0.10	2.64 ± 0.04[b]			
V_{median} (Pa.s)	Early	1.08 ± 0.03[ab]	1.14 ± 0.05	E, L > M	NS	NS
	Mid	0.99 ± 0.01[b]	1.00 ± 0.03			
	Late	1.18 ± 0.04[a]	1.14 ± 0.03			
Ratio V_{median} / V_0	Early	0.39 ± 0.01[b]	0.37 ± 0.00[c]	L > E, M	NS	NS
	Mid	0.39 ± 0.01[b]	0.39 ± 0.01[b]			
	Late	0.43 ± 0.00[a]	0.43 ± 0.01[a]			

[a–b] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).
[1]Analyzed using two-way ANOVA
E: early-season; M: mid-season; L: late-season; NS: nonsignificant; SE, standard error; V_0: Initial viscosity of stirred yoghurt, V_{median} : The median viscosity of stirred yoghurt during 180 s of shearing at 50 s^{-1}.

Despite being made from set yoghurt by stirring, the viscosity of stirred yoghurt did not follow the same seasonal trends of the firmness of set yoghurt. The results suggested that the viscosity of stirred yoghurt was affected by properties other than the firmness of the yoghurt before stirring, which varied with season. The V_0 followed similar seasonal trends with set yoghurt firmness, with a strong correlation ($r = 0.64$, $P < 0.001$,). Nevertheless, late-season stirred yoghurt in year 16/17 had comparable V_0 with early-season stirred yoghurt, regardless of the significantly lower set yoghurt firmness in the late season (Table 6.1). In contrast to V_0, V_{median} did not correlate with the initial firmness of set yoghurts. Stirred yoghurts made in the late seasons of both years had similar V_{median} to the yoghurts made in the early seasons, higher than those from mid-

seasons. The viscosity test under constant shearing can be seen as a continuation of the stirring process. The highest V_{median} /V_0 ratios in the late season indicated that late-season yoghurts had the strongest resistance against shear thinning. The results suggested that the inferior acid gelation and yoghurt making properties of late-season milk demonstrated in this study did not affect the structural quality of late-season stirred yoghurts.

The higher viscosity of late-season yoghurts relative to their lower initial firmness might be related to their more viscous properties of the yoghurt gels and the higher WHC than those of mid-season yoghurts. The loss tangent at pH 4.6 of GDL-induced acid gels made from standardized yoghurt milk was the highest in the late season (0.31) and the lowest in the early season (0.28). The highest loss tangent at pH 4.6 indicated that the late-season milk acid gels and yoghurts would have the most viscous properties. As a result, relatively more shear stress might dissipate through late-season yoghurt structure rather than causing a structural breakdown. As such, the decrease in viscosity under shearing would be lower in yoghurts from the late season than those from the rest of the year. Moreover, the WHC of set yoghurts correlated with both V_0 ($r = 0.544$, $P < 0.01$) and V_{median} ($r = 0.494$, $P < 0.05$). The lowest WHC of mid-season yoghurts might have contributed to the expelling of more liquid during stirring and shearing. According to the Krieger Dougherty equation (Equation 2.1, Section 2.4.1.2), the loss of entrapped water would lower the apparent viscosity of stirred yoghurts by reducing the effective volume fraction of the protein network. As a result, the viscosity of stirred yoghurts in the mid-season might be reduced to the greatest extent across different seasons by their highest extent of whey separation. This hypothesis was in

agreement with Matumoto-Pintro et al. (2011), who also reported relationships between the extent of syneresis and both the viscosity of stirred yoghurts and the firmness of set-style yoghurts.

The V_{median} /V_0 ratio significantly correlated with the GD of κ-CN (r = 0.808, P < 0.001). Since the glycan groups on glycosylated κ-CN are hydrophilic, milk with higher GD of κ-CN would have casein micelles with more hydrophilic surfaces thus greater water-binding capacities. Consequently, the higher GD of κ-CN in late-season milk might have contributed to lower syneresis of late-season yoghurts by increasing the amount of water bound to the proteins. This hypothesis was supported by the significant negative correlation between the GD of κ-CN and the extent of spontaneous syneresis (r = -0.489, P < 0.01). Besides, higher hydration of the proteins resulting from higher κ-CN GD might also promote the viscous properties of acid gels from the late season.

6.4 Conclusions

The fermentation time to reach certain pH did not vary with the season for either regular yoghurt or GSY. The firmness of set yoghurt and GSY was the highest in the early season, correlating significantly with the G′ of GDL-induced acid gel. GDL-induced acid gelation could represent the seasonal variations in the structural properties of set yoghurt and GSY. This suggested that the physicochemical properties that correlated with the rheological properties of acid gels discussed in Chapter 5, such as the glycosylation of κ-CN, might also play an important part in affecting the structure development of yoghurt. Mid-season yoghurts had the highest level of syneresis and the

lowest WHC. The viscosity of stirred yoghurts displayed different seasonal trends to the firmness of set yoghurts. Late-season stirred yoghurts had the strongest resistance to the shear-induced thinning behaviour among different seasons. This might have resulted from the unique viscoelastic property of late-season yoghurt, as suggested by the rheological test of GDL-induced acid gels. The lowest WHC and thus most amount of whey separation of mid-season yoghurts might also play a role in lowering its viscosity after stirring.

Chapter 7 - The effect of ultrafiltration on the acid gelation properties of milk

7.1 Introduction

The use of ultrafiltration (UF) for the standardization of fermented dairy products, particularly the high-protein types, is a common practice in the dairy industry (Jørgensen et al., 2019; Kumar et al., 2013). It is used as a tool to control the variable processing properties and the composition of milk during the season. Different ways of milk standardization involving UF include concentrating the milk to the desired protein content, the addition of milk protein concentrate (MPC), or the addition of fresh UF retentate as in the current study.

It was found from the acid gelation study in year 16/17 (Chapter 5) that the proportion of proteins from UF retentate in the standardized yoghurt milk was higher in early- and mid-seasons than in the late season, resulting from the seasonal variation in the protein content of milk (Figure 7.1). Besides, the concentration factors (CF) of UF in order to reach a certain protein content in the retentate (12%) also differed in different seasons, which might affect the physicochemical properties of milk components differently (Ferrer et al., 2011; Li & Corredig, 2014; Liu et al., 2014).

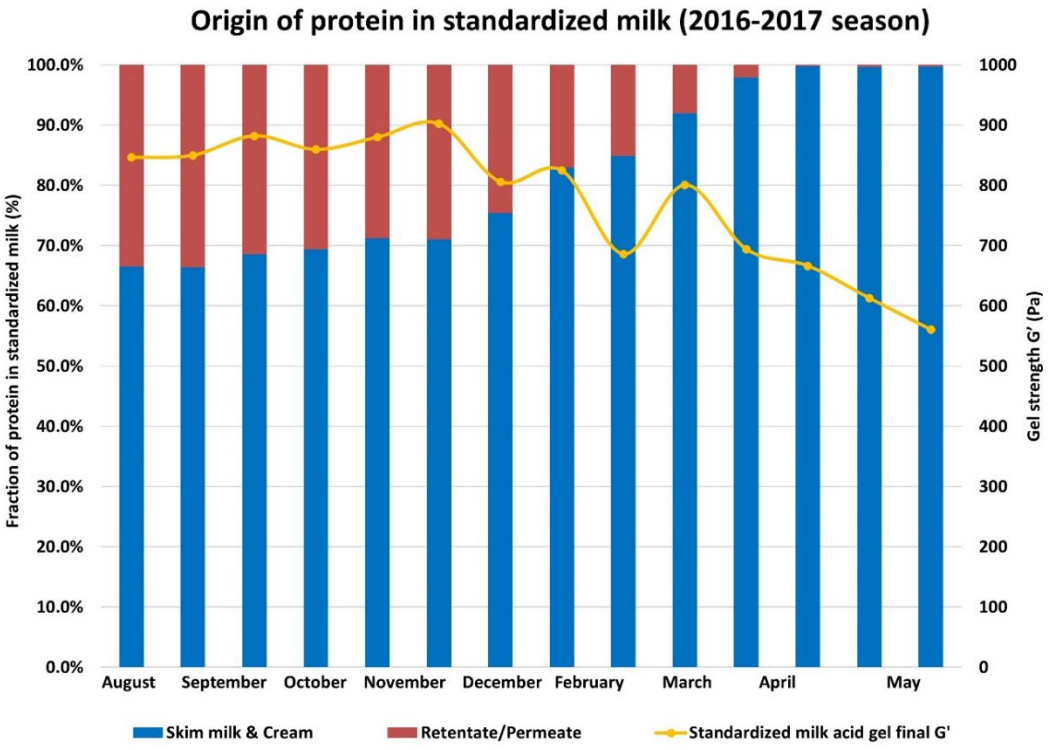

Figure 7.1: Protein sources of standardized milk and acid gel strength (2016-2017 season).

There was a significant positive correlation between the proportion of retentate in the milk mixture and the final G′ of the acid gels ($r = 0.89$, $P < 0.001$). Although this correlation could solely arise from the interrelations between the natural protein content of milk and other milk characteristics that affect the acid gelation properties as discussed in Chapter 5, it would be interesting to explore whether the UF process played a role as well. If the UF process could affect the acid gelation properties in a positive way as the seasonal correlation suggested, it could be used as a tool for tuning the quality of fermented dairy products.

Numerous studies have investigated the effect of UF on the rennet-coagulation and cheese-making properties of milk (Benfeldt, 2006; da Cunha, Viotto, & Viotto, 2006; Soodam & Guinee, 2018). However, there is limited information available on the effect of UF on the acid gelation properties of milk. Biliaderis et al. (1992) reported that yoghurts made from UF retentate had higher gel strength, shorter gelation time and higher final loss tangent (LT) than yoghurts made from skim milk fortified with skim milk powder at the same total solids content. However, since UF retentate had higher protein to lactose ratio than the fortified skim milk, standardization based on solids content resulted in significantly lower protein content for the skim-milk-standardized samples than the retentate-standardized milk. Since proteins contribute much more than lactose to the structure of yoghurt gels, the superior yoghurt-making properties of retentate-standardized milk in the study of Biliaderis et al. (1992) might have resulted solely from its higher protein content. There is no published information on the effect of the UF process on the acid gelation properties of milk in a protein-standardized sample system.

As reviewed in Section 2.2.2, the UF process has been reported to affect the physicochemical properties of milk, including the structure of casein micelles, salt equilibrium, etc (Donato & Guyomarc'h, 2009; Erdem, 2000; Ferrer et al., 2011; Green et al., 1984; McKenna, 2000; Srilaorkul et al., 1991). The acid gelation properties of milk were found to be affected by these characteristics of milk. UF could also affect protein hydrophobicity (Morand et al., 2012) and the amount of micellar calcium (Ozcan-Yilsay et al., 2007).

The main aim of this study was to determine whether the UF process contributes to the seasonal variation in the acid gelation properties of milk by affecting the physicochemical properties of milk components. The hypothesis was that ultrafiltered skim milk had better acid gelation properties than regular skim milk in a protein-standardized system. In addition, the seasonal variations in the acid gelation properties of milk described in Chapter 5 could be verified in this chapter in fat-free milk systems with higher protein content.

7.2 Materials and methods

7.2.1 Standardization and heat treatment of milk mixtures

Pasteurized skim milk, UF retentate and permeate were obtained from the processing of raw milk as described in Section 3.1. The concentration factor (CF) of the UF process for each sample to obtain a retentate with 12% protein was calculated based on the protein content of the skim milk and controlled by volume reduction.

Two milk systems were standardized to 5.0% protein for further analysis. One system consisted of skim milk and retentate (SR), in which up to about 40% of the protein came from retentate and the rest from skim milk. The other system consisted of UF permeate and retentate (PR), where almost all of proteins came from UF retentate. After standardization, both milk mixtures were heated at 90 ± 0.5°C for 6 minutes and chilled immediately in ice water. Samples were stored at 4°C until further analysis. In total, 10 samples across two milking seasons were analysed, i.e. 3 samples from the early season (year 18/19), 4 samples from the mid-season (2 samples from year 17/18, 2 samples from year 18/19) and 3 samples from the late season (year 17/18).

7.2.2 Characterizations of milk mixtures

Milk composition, total calcium, pH, ionic calcium, buffering capacity, casein micelle size, protein composition, heat-induced changes of milk (i.e. whey protein denaturation, κ-CN dissociation, whey protein-casein micelle association and the formation of serum-phase whey protein aggregates) were determined as described in Section 3.2.

In addition to the total calcium content of milk, the soluble calcium content of milk mixtures was determined by measuring the calcium content of the ultracentrifugation supernatant of the milk mixtures (88,000 g, 60 min at 20°C). The soluble fraction of calcium was calculated as the percentage of soluble calcium in total calcium of the milk mixtures.

7.2.3 Acid gelation analysis

The rheological analysis of the acid gelation process was carried out as described in Section 5.2.3. The acid gelation process was monitored for 8 h. The water holding capacity (WHC) of acid gels was determined as described in Section 6.2.4.

7.2.4 Transmission electron microscopy of acid milk gels

Acid milk gels made from milk mixtures in different seasons were imaged using transmission electron microscopy (TEM) as described in Section 3.2.8.

7.3 Results and discussion

7.3.1 Milk composition and processing parameters

Table 7.1 shows the milk characteristics, concentration factors (CF) of UF and the origins of proteins in the SR mixtures. The mean protein content and SCC of milk used to make milk mixtures followed the same seasonal trends reported in Chapter 4 that late-season milk had the highest content of protein and the highest SCC. Correlating well with the natural protein content of the skim milk, the proportions of proteins originating from skim milk in SR mixtures were similar in early- and mid-season but increased drastically in the late season. Besides, the mean concentration factors were 3.35 ± 0.00, 3.21 ± 0.03 and 2.48 ± 0.05 in early-, mid- and late-season, respectively, to obtain the retentate protein content of 12%.

Table 7.1: Seasonal milk properties and processing parameters

	Season	Means ± SE
Protein content of skim milk (%)	Early	3.59 ± 0.01^{b}
	Mid	3.75 ± 0.04^{b}
	Late	4.84 ± 0.12^{a}
SCC (1,000 cells/mL)	Early	72 ± 26^{b}
	Mid	96 ± 29^{ab}
	Late	188 ± 8^{a}
Protein from skim milk in SR mixtures (%)	Early	57.7 ± 0.7^{b}
	Mid	60.8 ± 2.6^{b}
	Late	94.3 ± 3.2^{a}
Concentration factor	Early	3.35 ± 0.00
	Mid	3.21 ± 0.03
	Late	2.48 ± 0.05

[a, b] Means of the same parameter within a column with different lowercase superscripts differ ($P < 0.05$, one-way ANOVA).
SCC, somatic cell count; SR, skim milk-retentate mixture; SE, standard error.

The composition of milk mixtures is shown in Table 7.2. As a result of standardization, the protein and fat contents of the two milk systems (SR and PR) were neither different from each other nor vary with season. However, in mid- and late-season, the protein contents of PR mixtures were slightly higher than SR mixtures (Table 7.2). For this reason, the composition of milk mixtures corrected to 5.0% protein basis was also shown in Table 7.2. After this correction based on protein content, the variations between SR and PR in lactose, total solids and solids non-fat were eliminated. The seasonal variation in the content of lactose was also no longer significant after correction, but the mean lactose contents of both mixtures still decreased as the season progressed, as reported in Chapter 4.

Table 7.2: Variations in the composition of milk mixtures

		Means ± SE		Effects[1]		
	Season	SR	PR	Season	SR/PR	Interaction
Protein (%)	Early	4.97 ± 0.01	5.01 ± 0.01	NS	NS	NS
	Mid	5.04 ± 0.03	4.93 ± 0.14			
	Late	5.06 ± 0.03	4.87 ± 0.10			
Fat (%)	Early	0.08 ± 0.00	0.08 ± 0.00	NS	NS	NS
	Mid	0.14 ± 0.01	0.14 ± 0.02			
	Late	0.08 ± 0.03	0.10 ± 0.03			
Lactose (%)	Early	5.06 ± 0.01	5.01 ± 0.01[a]	E, M > L	SR > PR	NS
	Mid	5.06 ± 0.05	4.88 ± 0.03[b]			
	Late	4.90 ± 0.02	4.77 ± 0.04 [b]			
Total solids (%)	Early	11.09 ± 0.05[a]	11.04 ± 0.07[a]	E, M > L	SR > PR	NS
	Mid	10.98 ± 0.03[a]	10.63 ± 0.15[ab]			
	Late	10.68 ± 0.01[b]	10.33 ± 0.07[b]			
Solids-non-fat (%)	Early	10.84 ± 0.00[a]	10.78 ± 0.02[a]	E > M, L	SR > PR	NS
	Mid	10.71 ± 0.06[a]	10.36 ± 0.14[ab]			
	Late	10.44 ± 0.01[b]	10.12 ± 0.08[b]			
Lactose - corrected for 5% protein (%)	Early	5.10 ± 0.02	5.00 ± 0.01[a]	NS	NS	NS
	Mid	5.03 ± 0.06	4.96 ± 0.16[b]			
	Late	4.84 ± 0.04	4.90 ± 0.11[b]			
Total solids - corrected for 5% protein (%)	Early	11.16 ± 0.07[a]	11.02 ± 0.06	E > L	NS	NS
	Mid	10.89 ± 0.05[b]	10.80 ± 0.17			
	Late	10.55 ± 0.05[c]	10.62 ± 0.16			
Solids non-fat - corrected for 5% protein (%)	Early	10.92 ± 0.03[a]	10.76 ± 0.02	E > L	NS	NS
	Mid	10.63 ± 0.08[b]	10.53 ± 0.22			
	Late	10.31 ± 0.05[c]	10.39 ± 0.14			
Total calcium (mM)	Early	40.7 ± 1.1	41.2 ± 1.7	NS	NS	NS
	Mid	40.5 ± 0.7	39.3 ± 1.4			
	Late	39.0 ± 1.5	38.2 ± 1.5			
Total calcium corrected for 5% protein (mM)	Early	41.0 ± 1.1	41.1 ± 1.7	NS	NS	NS
	Mid	40.4 ± 0.5	40.4 ± 0.3			
	Late	38.5 ± 1.4	39.2 ± 0.7			

[a–c] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).

[1]Analyzed using two-way ANOVA

E: early-season; M: mid-season; L: late-season; SR, skim milk-retentate mixture; PR, permeate-retentate mixture; NS: nonsignificant; SE, standard error.

The reduction in the extent of seasonal variation in lactose content in the milk mixtures compared with raw milk is likely to result from the use of retentate for standardization. Retentate has lower lactose to protein ratio because part of the lactose transfers into permeate during the UF process. Therefore, adjusting the protein content by adding retentate reduces the lactose to protein ratio of the milk mixtures. During early- and mid-seasons, natural milk samples had lower protein contents, thus larger proportion of retentate was added to milk mixtures than in the late season. Consequently, the lactose to protein ratio of milk mixtures from early- and mid-season was reduced more than those from the late season, which might have diminished the difference in the lactose content of the milk mixtures.

The total calcium contents of SR and PR mixtures did not differ significantly, and these differences were even smaller when adjusted by protein content (Table 7.2), indicating that there was no alteration of the total calcium content by the UF and the standardization processes. This was expected considering that diafiltration was not used during the UF process. It was reported that during UF some calcium precipitates on and causes the fouling of polyethersulfone (PES) membranes (Ferrer et al., 2011; Rabiller-Baudry, Le Maux, Chaufer, & Begoin, 2002). There was no significant difference in total or soluble calcium contents of 2x retentate diluted to original concentration with UF permeate (Ferrer et al., 2011), although the total calcium of diluted 5x retentate was significantly lower. Calcium contents of milk mixtures did not vary significantly with the season, although there was a slight trend of decreasing calcium as the season progressed. This was consistent with the trend observed in Chapter 4 that the calcium to protein ratio decreased with the progressing season (Table 4.1).

7.3.2 Protein composition of milk mixtures

Table 7.3 shows the protein composition of the SR and PR mixtures. No significant difference in the content of any milk protein was observed between SR and PR. The process of UF and standardization did not affect the protein composition of the two samples.

Table 7.3: Protein composition (g/100 g of total protein) and the degree of glycosylation of κ-CN of milk mixtures

	Season	Means ± SE		CV (%)	Seasonal variation[1]
		SR	PR		
Total CN	Early	87.6 ± 0.2	87.8 ± 0.1	1.2	NS
	Mid	86.9 ± 0.7	87.0 ± 0.7		
	Late	85.9 ± 0.2	86.1 ± 0.3		
α_{s2}-CN	Early	8.1 ± 0.5	8.5 ± 0.4	7.2	NS
	Mid	8.3 ± 0.3	8.4 ± 0.2		
	Late	7.6 ± 0.2	7.6 ± 0.1		
α_{s1}-CN	Early	32.0 ± 0.1^{a}	31.7 ± 0.2^{a}	3.4	E > L
	Mid	30.5 ± 0.5^{ab}	30.7 ± 0.6^{ab}		
	Late	29.9 ± 0.1^{b}	29.9 ± 0.0^{b}		
β-CN	Early	34.4 ± 0.1	34.4 ± 0.4	1.7	NS
	Mid	35.3 ± 0.2	35.4 ± 0.2		
	Late	35.3 ± 0.2	35.5 ± 0.2		
κ-CN	Early	13.0 ± 0.3	13.2 ± 0.2	3.6	NS
	Mid	12.7 ± 0.3	12.6 ± 0.3		
	Late	13.1 ± 0.1	13.1 ± 0.1		
Glycosylated κ-CN	Early	5.0 ± 0.3^{b}	4.9 ± 0.2^{b}	15.8	L > E, M
	Mid	4.7 ± 0.3^{b}	4.6 ± 0.3^{b}		
	Late	6.3 ± 0.2^{a}	6.3 ± 0.2^{a}		
κ-CN GD (%)	Early	38.7 ± 1.2^{b}	37.4 ± 0.6^{b}	13.8	L > E, M
	Mid	36.9 ± 1.6^{b}	36.7 ± 1.7^{b}		
	Late	48.3 ± 1.4^{a}	48.0 ± 1.3^{a}		
Total whey proteins	Early	12.4 ± 0.2	12.2 ± 0.1	7.9	NS
	Mid	13.1 ± 0.7	13.0 ± 0.7		
	Late	14.1 ± 0.2	13.9 ± 0.3		
α-LA	Early	2.6 ± 0.0^{a}	2.6 ± 0.0^{a}	14.4	E, M > L
	Mid	2.5 ± 0.1^{a}	2.5 ± 0.1^{a}		
	Late	1.8 ± 0.1^{b}	1.9 ± 0.0^{b}		
β-LG	Early	9.9 ± 0.1^{b}	9.6 ± 0.1^{b}	11.2	L > E
	Mid	10.6 ± 0.6^{ab}	10.5 ± 0.6^{ab}		
	Late	12.3 ± 0.2^{a}	12.1 ± 0.2^{a}		

$^{a–b}$ Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).

[1]Analyzed using two-way ANOVA

SR, skim milk-retentate mixture; PR, permeate-retentate mixture; GD, glycosylation degree; CV, coefficient of variation; NS: nonsignificant; SE, standard error.

The patterns of seasonal variation in protein composition were identical for SR and PR, both correlating well with the seasonal variation trends discussed in Chapter 4. Similar to the observation across two full milking seasons (Table 4.3), the most variable proteins across the seasons determined in the SR and PR mixtures were Glycosylated κ-CN (CV 15.8%) and α-LA (CV 14.4%). The three consistent seasonal trends reported in Chapter 4, i.e. the decreasing contents of α_{s1}-CN and α-LA with the season and the elevated content of G-κ-CN in the late season, were observed in the SR and PR mixtures. However, there were some differences in the seasonal variation patterns between these results and those shown in Chapter 4. In Chapter 4, significant seasonal variations in the contents of α_{s2}-CN, β-CN and β-LG were found only in one of the two years, whereas the significant seasonal trends in the content of κ-CN were different in the two years (Table 4.3). Table 7.3 shows that in SR and PR mixtures, there was no significant seasonal variation in the content of α_{s2}-CN, β-CN and κ-CN. In the case of β-LG, SR and PR mixtures from the late season had significantly higher β-LG than those from the early season, consistent with the finding in year 17/18 (Table 4.3, Chapter 4) when the late-season milk for SR and PR was sampled. Besides, the content of α-LA in early-season milk was higher than that in mid-season milk consistently in both years (Table 4.3, Chapter 4), whereas in the SR and PR mixtures, there was no significant difference between the content of α-LA of early-season or mid-season samples (Table 7.3).

The comparisons between the protein composition results in Chapter 4 and this chapter further supported the findings reported in Chapter 4 that α_{s1}-CN, α-LA and G-κ-CN have the most significant seasonal variations. On the contrary, the contents of α_{s2}-CN,

β-CN, κ-CN and β-LG do not vary with season consistently. The slight difference in the variation patterns of α-LA might have resulted from the fact that the SR and PR study had less sampling points (3 or 4 in each season) across two milking seasons.

7.3.3 Physicochemical properties of milk mixtures

Table 7.4 shows the physicochemical properties of milk mixtures before and after heating. The pH of milk was similar for SR and PR, both before and after heating, and did not vary with season significantly. A slight increase was observed in late-season samples, in agreement with the trend shown in Chapter 4. Heating resulted in a slight drop in pH of about 0.02 units. The pH reduction upon heating is a well-known phenomenon because of the transfer of soluble calcium into the casein micelles (Walstra & Jenness, 1984) and the simultaneous release of hydrogen ions as indicated roughly by the reaction below (Equation 7.1):

$$Ca^{2+} + H_2PO_4^- \rightarrow Ca_3(PO_4)_2 + H^+ \quad \textbf{(7.1)}$$

The contents of soluble calcium and ionic calcium in relation to total calcium were in agreement with previous reports (Ferrer et al., 2011; Lewis, 2011; Pyne, 1962). Neither the percentage of soluble calcium in total calcium nor the ionic calcium concentration differed between SR and PR mixtures. However, despite the lack of difference in Ca^{2+} between SR and PR, the Ca^{2+} concentration prior to heating was likely to be higher in SR than in PR. By analysing 17 samples from year 17/18, it was found that the retentates diluted to the original protein contents (CF of 1) with its own UF permeate for 1-2 hours had 6.8% lower Ca^{2+} than the original skim milk ($P = 0.014$). UF permeates had an average Ca^{2+} of 1.49 mM, similar to that reported by Lin et al. (2015). There seemed to be a re-equilibration process of Ca^{2+} after dilution of retentate with permeate, as reported by (Brule & Fauquant, 1981). Since the Ca^{2+} of milk mixtures were determined after being stored overnight, the re-equilibration of Ca^{2+} likely erased the difference in Ca^{2+} concentrations between SR and PR (Table 7.4).

Table 7.4: Physicochemical properties of milk mixtures before and after heating

		Means ± SE			
	Season	SR	PR	SRH	PRH
pH	Early	6.73 ± 0.03	6.74 ± 0.02	6.71 ± 0.02	6.71 ± 0.02
	Mid	6.72 ± 0.01	6.73 ± 0.01	6.70 ± 0.01	6.71 ± 0.01
	Late	6.76 ± 0.00	6.76 ± 0.00	6.75 ± 0.01	6.74 ± 0.01
Soluble calcium in total calcium (%)	Early	29.0 ± 1.4	29.9 ± 1.7	27.8 ± 1.3	27.5 ± 1.4
	Mid	26.2 ± 0.2	26.3 ± 1.8	25.7 ± 0.9	24.8 ± 0.7
	Late	27.2 ± 0.9	26.5 ± 0.5	24.9 ± 0.7	25.4 ± 0.7
Ionic calcium (m*M*)	Early	2.36 ± 0.04[b]	2.37 ± 0.04[a]	2.21 ± 0.02[b]	2.21 ± 0.03[b]
	Mid	2.14 ± 0.04[c]	2.13 ± 0.05[b]	2.02 ± 0.03[c]	2.01 ± 0.03[c]
	Late	2.56 ± 0.01[a]	2.54 ± 0.02[a]	2.41 ± 0.03[a]	2.42 ± 0.02[a]
Ionic calcium in soluble calcium (%)	Early	20.1 ± 1.7	19.5 ± 1.7	18.3 ± 1.0	18.4 ± 1.4
	Mid	19.9 ± 0.6	20.4 ± 0.9	18.6 ± 0.8	18.9 ± 0.4
	Late	24.3 ± 1.2	25.2 ± 1.3	23.7 ± 1.4	24.2 ± 1.8
Heat-induced ionic calcium decrease (%)	Early			6.3 ± 1.7	7.0 ± 0.4
	Mid			5.5 ± 0.6	5.3 ± 0.7
	Late			5.7 ± 0.9	4.4 ± 0.7
Casein micelle diameter (nm)	Early	159.7 ± 2.6	164.1 ± 4.3	166.5 ± 1.3	168.1 ± 3.0
	Mid	168.9 ± 2.4	165.5 ± 2.3	174.7 ± 2.5	175.3 ± 2.6
	Late	161.0 ± 2.7	161.1 ± 1.9	170.6 ± 2.7	169.8 ± 1.7
Buffering capacity (dB/dpH)	Early			0.036 ± 0.001	0.036 ± 0.001
	Mid			0.035 ± 0.000	0.035 ± 0.000
	Late			0.035 ± 0.000	0.034 ± 0.000

[a–c] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).

SR, skim milk-retentate mixture; PR, permeate-retentate mixture; SRH, skim milk-retentate mixture heated at 90°C for 6 min; PRH, permeate-retentate mixture heated at 90°C for 6 min; SE, standard error.

Heat treatment reduced the concentrations of both soluble calcium and ionic calcium, to a similar extent in both SR and PR. This could be explained by Equation 7.1 as discussed above.

Interestingly, seasonality had different impacts on soluble calcium and ionic calcium concentrations. Early-season samples had the highest proportion of soluble calcium both before heating ($P = 0.026$) and after heating ($P = 0.025$), whereas mid- and late-season samples had similar contents of soluble calcium. The ionic calcium concentrations were highest in the late season and the lowest in mid-season. This indicated that late-season milk had the highest proportion of ionic calcium in soluble calcium (Table 7.4), which was similar for early- and mid-season samples, either before or after heat treatment. The heat-induced reduction in ionic calcium was higher in the early season, but the difference was not statistically significant ($P = 0.20$).

The casein micelle size did not vary between SR and PR mixtures. Similar to the findings reported in Chapter 4, there was no significant seasonal variation in the casein micelle size. Milk mixtures heated at 90° for 6 min had increased casein micelles size (SRH, 170.6 ± 1.5 nm; PRH, 171.1 ± 1.6 nm) compared with unheated milk mixtures (SR, 163.2 ± 1.8 nm; PR, 163.6 ± 1.6 nm). These results indicated that the UF process did not significantly affect the hydrodynamic size of casein micelles, in agreement with some previous reports also using dynamic light scattering methods (Ferrer et al., 2011; McKenna, 2000). Liu et al. (2014) reported that alterations of the hydrodynamic size of casein micelles by UF process were observable only in the concentrated form of the retentates.

The buffering capacity of heated SR and PR mixtures did not differ from each other. This was expectable considering their similar contents of protein and total calcium.

7.3.4 Physicochemical changes induced by UF and heat treatment

7.3.4.1 Serum phase κ-CN in unheated milk

Table 7.5 shows the amount of serum phase κ-CN, the degree of whey protein denaturation and the extent of whey protein-casein micelle association in heated milk. In unheated milk, the overall content of serum phase κ-CN in total κ-CN was comparable to that in raw milk (7.3 ± 0.5%) as reported in Chapter 4. Besides, there was also a seasonal variation trend of increasing serum phase κ-CN as the season progressed (Figure 4.1, Chapter 4). However, SR and PR mixtures from early- and mid-seasons contained more serum phase κ-CN than in raw milk (early-season 4.6%, mid-season 6.8%), whereas late-season milk mixtures had similar fractions of serum phase κ-CN to late-season raw milk (10.7%). There was no significant difference in the contents of total serum phase caseins or serum phase κ-CN between SR and PR, in agreement with previous reports of UF (without DF) studies performed at 40°C (Ferrer et al., 2011; Liu et al., 2014). However, interestingly, PR mixtures on average had 0.4% and 0.2% more serum phase κ-CN than SR mixtures in early- and mid-season, but 0.1% less serum phase κ-CN than SR in the late season (Table 7.5). The results echoed the seasonal differences between the milk mixtures (both SR and PR) and raw milk. This suggested that the UF process might have resulted in some dissociation of κ-CN, the extent of which was affected by different seasons. The difference in the concentration factors of the UF process among different seasons (Table 7.1) might have resulted in the slightly different levels of κ-CN dissociation.

Table 7.5: Heat-induced changes in milk proteins. Results are presented as the percentages of in the total amount of specified proteins in milk

		Means ± SE		Effects[1]		
	Season	SR	PR	Season	SR/PR	Interactions
Serum phase κ-CN - unheated milk (%)	Early	7.7 ± 0.5[b]	8.1 ± 0.3[b]	L > E, M	NS	NS
	Mid	8.6 ± 0.4	8.8 ± 0.1[b]			
	Late	10.2 ± 0.4[a]	10.1 ± 0.3[a]			
Heat-induced κ-CN dissociation (%)	Early	4.7 ± 0.6	5.3 ± 0.8	NS	NS	NS
	Mid	4.3 ± 0.7	4.9 ± 1.4			
	Late	3.1 ± 0.1	3.2 ± 0.2			
WP denaturation (%)	Early	94.6 ± 0.4[a]	91.5 ± 0.6	L > M	SR > PR	NS
	Mid	93.7 ± 0.6[b]	89.9 ± 0.7			
	Late	96.4 ± 0.5[a]	91.2 ± 0.5			
α-LA denaturation (%)	Early	75.0 ± 2.3	60.2 ± 3.2[a]	E> M	SR > PR	0.039
	Mid	68.8 ± 2.5	46.8 ± 4.3[ab]			
	Late	78.1 ± 4.5	43.1 ± 2.2[b]			
Denatured α-LA in denatured WP (%)	Early	16.5 ± 0.3	14.0 ± 0.5[c]	E > M > L	SR > PR	NS
	Mid	14.0 ± 0.7	10.0 ± 1.0[b]			
	Late	10.5 ± 0.6	6.3 ± 0.2[a]			
WP serum aggregates (%)	Early	12.5 ± 0.9[a]	12.7 ± 2.4	E, M > L	NS	NS
	Mid	10.6 ± 1.0[a]	10.9 ± 2.1			
	Late	6.8 ± 0.2[b]	6.9 ± 0.6			
WP micelle association (%)	Early	82.2 ± 0.8[b]	78.7 ± 2.1[b]	L > E, M	SR > PR	NS
	Mid	83.0 ± 1.6[b]	78.7 ± 3.0[b]			
	Late	89.6 ± 0.6[a]	84.3 ± 0.1[a]			
α-LA micelle association (%)	Early	59.7 ± 0.9[b]	46.9 ± 1.8	L > M	SR > PR	0.003
	Mid	55.2 ± 1.8[b]	40.7 ± 3.6			
	Late	71.3 ± 2.1[a]	41.9 ± 1.0			
β-LG micelle association (%)	Early	87.3 ± 0.7	86.5 ± 2.1	L > E	NS	NS
	Mid	88.8 ± 2.2	87.4 ± 2.7			
	Late	92.4 ± 0.5	91.0 ± 0.3			

[a–c] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).

[1]Analyzed using two-way ANOVA

SR, skim milk-retentate mixture; PR, permeate-retentate mixture; E: early-season; M: mid-season; L: late-season; NS: nonsignificant; SE, standard error.

Micellar dissociation during the UF process has been reported, whose extent increased significantly with extensive diafiltration (Ferrer et al., 2014; McKenna, 2000; Singh, 2007). DF effectively reduces the calcium and phosphate contents in the serum phase, stimulating the solubilization of CCP to maintain the salt equilibrium between the micellar phase and serum phase. As a result of the CCP removal, solubilization of some caseins from the micelles occurs (Dalgleish & Law, 1989). Although this behaviour seemed less prominent during the UF process without DF, McKenna (2000) still observed non-micellar material in the TEM images of UF retentates, the amount of which increased greatly in the UF-DF retentates. Lin et al. (2015) reported a loss of colloidal calcium and magnesium during the UF process (without DF) at 50°C, the extent of which increased from CF 2 to CF 4. These reports indicate that micellar dissociation could occur during the UF process because of colloidal calcium removal, but its extent is considerably lower than that during a combined UF-DF process.

7.3.4.2 Heat-induced κ-CN dissociation

After heating at 90°C for 6 min, the concentration of serum-phase κ-CN was not significantly different between SR and PR or among different seasons, in agreement with the two-year results from Chapter 4 that the serum-phase κ-CN in heated milk (90°C 6 min) was not significantly affected by seasonality.

7.3.4.3 Denaturation of whey proteins

The extent of heat-induced denaturation of whey proteins was significantly higher in SR than in PR ($P < 0.001$). For both SR and PR, mid-season milk mixtures had the lowest level of WP denaturation, although for PR the variation was not statistically significant. For SR, the extent of whey protein denaturation correlated with the

concentration of ionic calcium ($r = 0.813$, $P < 0.01$). As discussed in Section 4.3.4.4, ionic calcium promotes the denaturation of whey proteins (Donovan & Mulvihill, 1987; Law & Leaver, 2000).

The seasonal trend in the denaturation of whey proteins was different from the results of heated natural skim milk from Chapter 4, where early-season skim milk had the lowest extent of whey protein denaturation. The between-year variations as a result of sampling early-season milk in year 18-19 and late-season milk in year 17-18 might have played a part in shifting the seasonal trend in the extent of whey protein denaturation. However, the difference between the natural skim milk with varied protein contents (Chapter 4) and the protein-standardized milk mixtures in this chapter might have contributed to the different seasonal trends in the denaturation of whey proteins. Natural early-season milk had low protein content and high ionic calcium concentration (Table 4.1). Despite the effect of ionic calcium on enhancing whey protein denaturation, the lower protein content of early-season milk might limit the denaturation rate, considering the aggregations between proteins (β-LG/β-LG, β-LG/κ-CN and β-LG/α-LA) play an important part in the irreversible denaturation of whey proteins. However, in the case of protein-standardized milk mixtures, the differences in protein contents across seasons were minimized and the seasonal variation pattern in whey protein denaturation was similar to that of ionic calcium (Early- and late-season > mid-season).

Similarly, the difference between SR and PR in the extent of whey protein denaturation could be explained by the difference in ionic calcium. As discussed in Section 7.3.3, PR mixtures might have lower ionic calcium concentrations than SR mixtures during heat

treatment. Because of this effect, the seasonal variations in the ionic calcium of PR prior to heating might be smaller than SR, which might be the reason for the smaller extent of seasonal variation in the extent of whey protein denaturation of PR mixtures.

Compared with β-LG which was fully denatured in most samples, the extent of variation in the denaturation of α-LA was significantly greater. The variation in the denaturation of total WP was mostly contributed by the variable extent of the α-LA denaturation. Compared with α-LA in SR mixtures, α-LA in PR mixtures was less likely to denature under the same heating condition ($P < 0.001$). Considering that the extent of α-LA denaturation in SR was comparable to previous reports in milk (above 75%, Anema, 2001), the effect of UF and/or the standardization process of PR on the denaturation of α-LA was considerable. Considering that denatured whey proteins have a much greater contribution to the acid gelation of milk than native whey proteins (Lucey et al., 1998b), the composition of denatured whey proteins in the milk mixtures would be of greater importance for acid milk gelation compared with total whey protein composition. The proportions of denatured α-LA in total denatured whey proteins are shown in Table 7.5. They decreased significantly in both SR and PR as milking season progressed ($P < 0.001$). Besides, SR contained a higher proportion of denatured α-LA in total denatured whey proteins than PR ($P < 0.001$), the extent of which was consistent across different seasons.

It was unclear how the denaturation of α-LA was suppressed in PR. Both the UF process and the standardization process of PR mixtures might have an impact on the denaturation of α-LA.

Among all major milk proteins, α-LA has unique behaviour during UF. A number of previous studies reported that α-LA was the major component of the foulant on PES UF membranes (Ng, Dunstan, & Martin, 2018; Tong, Barbano, & Jordan, 1989; Tong, Barbano, & Rudan, 1988). The smaller size of α-LA was suggested to enable access to more pores on the UF membrane (Ng, Haribabu, Harvie, Dunstan, & Martin, 2017). For the small amount of proteins that could pass through the membrane, Barbano, Sciancalepore, and Rudan (1988) reported that 90% of the proteins in UF permeate (10 kDa cut-off) consisted of α-LA. The small size and high mobility of α-LA during the UF process might make it more prone to process-induced structural and functional alterations than other milk proteins. Van Audenhaege et al. (2013) reported that α-LA in the permeate obtained by dead-end UF with a PES membrane of 10 kDa cut-off had a slightly higher fraction of aggregates and elevated denaturation temperature. They attributed these changes to the tertiary structural modifications caused by the physical shear stress in the membrane pores. On the other hand, dead-end UF of β-LG resulted in the presence of more random coil structures, similar to that induced by thermal or chemical unfolding (Portugal et al., 2007; Van Audenhaege et al., 2010). These different reported effects of UF on permeated α-LA (enhanced heat stability) and β-LG (initiated unfolding of structure) might contribute to the reduced denaturation of α-LA in PR mixtures, considering the UF permeate contained roughly 0.2% of proteins (similar to that reported by Liu et al. (2014).

As discussed in Section 7.3.3, the standardization of PR mixtures resulted in an initial decrease in ionic calcium. A lower Ca^{2+} concentration of was reported to correlate with a lower denaturation rate of whey proteins (Deeth & Lewis, 2015; Law & Leaver,

2000; Tolkach, Steinle, & Kulozik, 2005). Considering a) the denaturation of β-LG happens at a higher rate than the denaturation of α-LA at 90°C (Law & Leaver, 2000; Oldfield et al., 1998b), and b) the rate of irreversible denaturation of α-LA in milk relies on the denaturation of β-LG (de la Fuente, Singh, & Hemar, 2002; Wijayanti et al., 2014), the lower Ca^{2+} in PR possibly reduced the extent of denaturation of α-LA more than that of β-LG.

7.3.4.4 Distribution of denatured whey proteins between micelle and serum phase

Denatured whey proteins in heated milk either associate with the casein micelles or form aggregates (with themselves or κ-CN) in the serum phase. Looking at the effect of seasonality, the proportion of whey proteins that formed serum phase aggregates was lower in the late season than in early- and mid-season ($P < 0.01$). Correspondingly, late-season milk mixtures had a significantly higher extent of whey protein-casein micelle association than milks from early- or mid-season ($P < 0.01$). These results were consistent with the heating study of seasonal skim milk discussed in Chapter 4; the whey proteins in late-season milk were more prone to associate with the casein micelles under heat treatment.

The extent of heat-induced κ-CN dissociation had a strong positive correlation with the amount of whey proteins forming serum phase aggregates ($r = 0.908$, $P < 0.001$) and a strong negative correlation with whey protein-casein micelle association ($r = -0.857$, $P < 0.001$). This is in agreement with the well-known phenomenon that κ-CN dissociation contributes to the formation of serum phase protein aggregates (Anema & Li, 2003; Singh, 2004), as well as the results from Chapter 4. It was interesting, however, that the total amount of serum phase κ-CN in heated milk did not vary

significantly with season nor it correlated with the distribution of denatured whey proteins between the serum and micelle phase. This suggests that the κ-CN present in the serum prior to heat treatment may not contribute to the formation of κ-CN/β-LG complexes in the serum phase as much as the κ-CN dissociated from the micelles during heating. This phenomenon could be explained by assuming that the serum-phase whey protein-κ-CN aggregates were formed initially on the micelle surface, which then dissociated into the serum phase in the later phase of heating (Donato & Guyomarc'h, 2009; Donato et al., 2007b). This hypothesis was supported by the study of Anema and Li (2000), which demonstrated that the addition of thiol-blocking agent to milk, which prevented the S-S bond formation between κ-CN and β-LG, reduced the extent of κ-CN dissociation. Donato et al. (2007b) reported that the purified κ-CN did not react with whey proteins in the presence of casein micelles and attributed it to a different structure of the purified κ-CN, which made the hydrophobic sites and thiol groups less accessible than κ-CN on the micelle surface. In the current study, the serum phase κ-CN present in unheated milk, although not purified as in the study of Donato et al. (2007b), might also have a structure that protected their hydrophobic sites and thiol groups. Consequently, the distribution of denatured whey proteins between micelle and serum phase was affected more by the κ-CN dissociated during heating rather than the total serum phase κ-CN in heated milk.

In SR and PR mixtures, the proportions of whey proteins forming aggregates in the serum phase were almost the same across all seasons, despite the different extent of whey protein denaturation (Table 7.5). The extent of whey protein-casein micelle association of PR was significantly lower than that of SR ($P < 0.01$). This difference

between SR and PR in the extent of micelle association of total whey proteins resulted mostly from the difference in the amount of α-LA associating with casein micelles. For β-LG, the extent of association with the casein micelles was similar for SR and PR across different seasons. This was consistent with the difference between SR and PR in whey protein denaturation that the denaturation of α-LA differed significantly but β-LG were mostly denatured in both SR and PR mixtures (Table 7.5). In SR, the seasonal variations trend of α-LA-casein micelle association followed that of β-LG and total WP. In PR, however, the extent of α-LA-casein micelle association did not vary with season significantly.

7.3.5 Acid gelation properties of milk mixtures

7.3.5.1 Transmission electron microscopy images of acid gels

Figure 7.2 shows the microstructure of acid milk gels made from SR and PR mixtures in different seasons examined using TEM. The acid gel structures resembled those in previous studies using TEM (Kalab, Emmons, & Sargant, 1976; Sanchez, Zuniga-Lopez, Schmitt, Despond, & Hardy, 2000). Casein micelles formed into chains and clusters in acid milk gels. Surrounded by the protein chains were pores with sizes ranging from 1 to 5 μm, consistent with the observations of Sanchez et al. (2000).
No apparent difference in the microstructure of acid gels was observed between SR (Figure 7.2-A, C) and PR (Figure 7.2-B, D) or between early season (Figure 7.2-A, B) and late season (Figure 7.2-C, D).

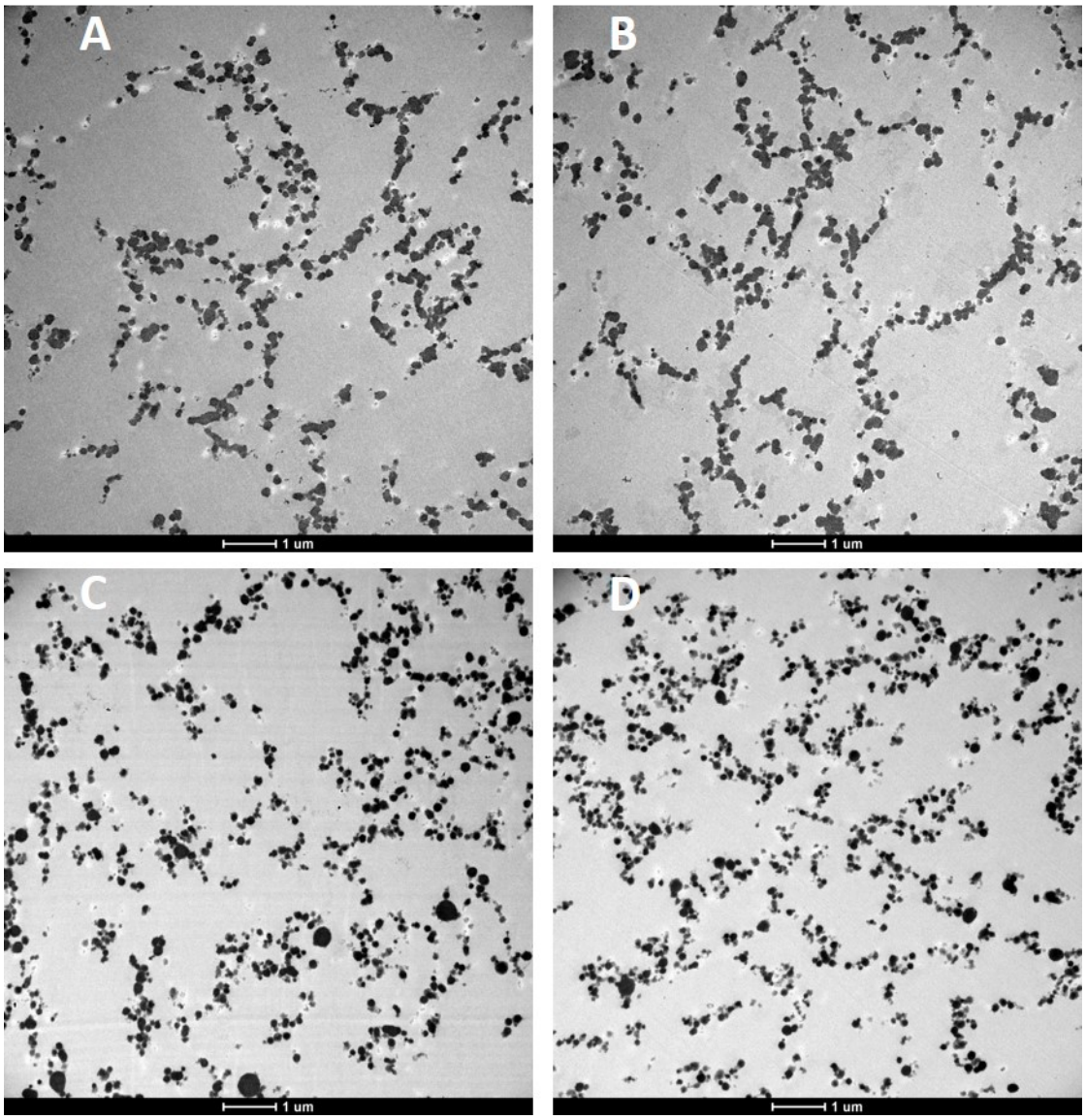

Figure 7.2: Transmission electron microscopy (TEM) images of acid gels made from A) SR in the early season, B) PR in the early season, C) SR in the late season, D) PR in the late season.

7.3.5.2 Comparisons of acid gelation properties

The acid gelation properties of milk mixtures are shown in Figure 7.3 and Table 7.6. To account for the minor error in standardization (mostly in mid- and late-season), the

final G′ corrected to 5.0% protein (assuming a linear relationship between G′ and protein content) is also shown in Table 7.6.

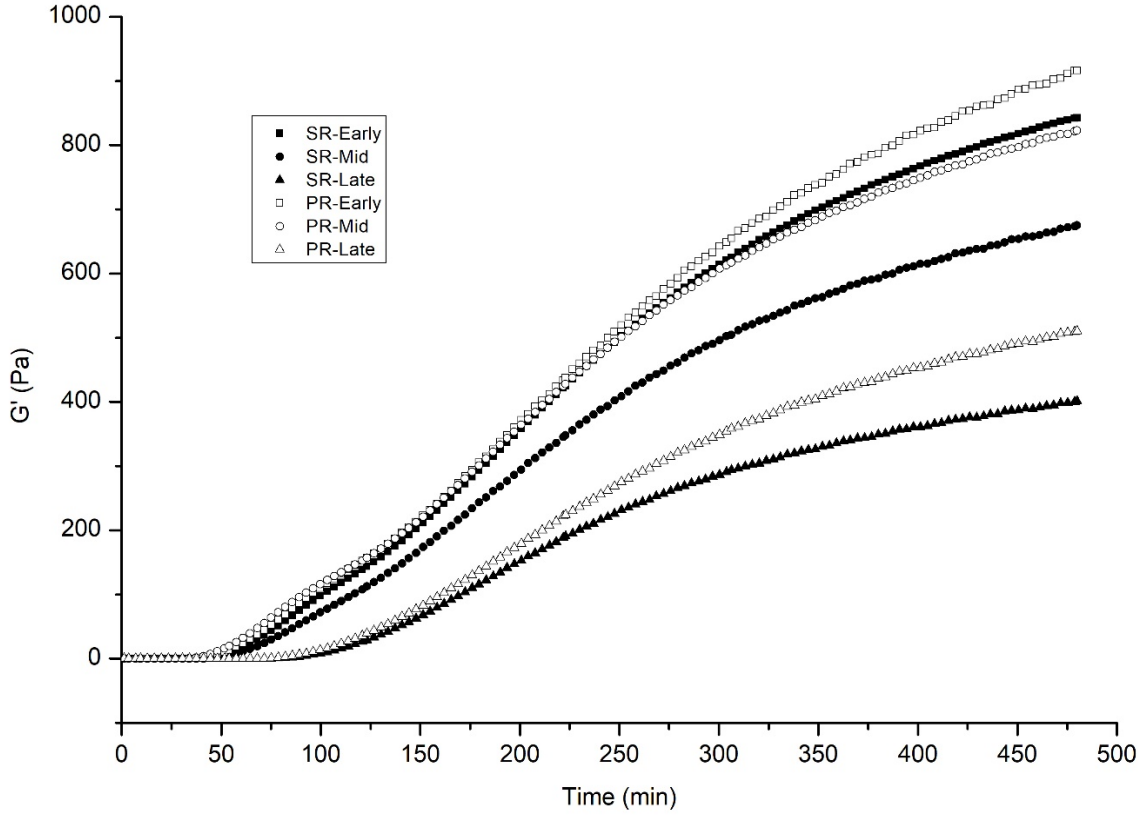

Figure 7.3: Development of storage modulus (G′) with time of acid gels made from SR in early season (■), SR in mid-season (●), SR in late season (▲), PR in early season (□), PR in mid-season (○) and PR in late season (△). SR, skim milk-retentate mixture (5% protein); PR, permeate-retentate mixture (5% protein).

Table 7.6: Acid gelation properties of milk mixtures

		Means ± SE		PR/SR difference	Effects[1]		
	Season	SR	PR		Season	SR/PR	Interactions
G′ (Pa)	Early	847.7 ± 14.5[a]	900.7 ± 12.9[a]	+6.3%	E > M > L	PR > SR	NS
	Mid	717.3 ± 43.4[a]	820.9 ± 31.5[a]	+14.4%			
	Late	445.3 ± 28.1[b]	529.7 ± 11.1[b]	+19.0%			
G′ per 5% protein (Pa)	Early	853.9 ± 15.4[a]	898.3 ± 12.5[a]	+5.2%	E > M > L	PR > SR	NS
	Mid	712.2 ± 43.6[a]	834.0 ± 35.0[a]	+17.1%			
	Late	439.8 ± 28.6[b]	544.7 ± 21.9[b]	+23.8%			
Gelation time (min)	Early	48.1 ± 0.9[b]	46.0 ± 1.0[b]	-4.4%	L > M, E	SR > PR	NS
	Mid	48.6 ± 2.1[b]	43.2 ± 1.0[b]	-11.1%			
	Late	80.5 ± 4.4[a]	70.5 ± 5.9[a]	-12.4%			
Gelation pH	Early	5.21 ± 0.02[a]	5.25 ± 0.01[ab]	+0.8%	E, M > L	NS	NS
	Mid	5.27 ± 0.03[a]	5.32 ± 0.05[a]	+0.9%			
	Late	5.06 ± 0.04[b]	5.11 ± 0.04[b]	+1.1%			
WHC (%)	Early	94.3 ± 1.8	95.3 ± 1.1	+1.1%	L > M	NS	NS
	Mid	92.5 ± 1.8	96.0 ± 0.9	+3.8%			
	Late	97.6 ± 0.3	97.8 ± 0.3	+0.2%			

[a–c] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).
[1]Analyzed using two-way ANOVA
SR, skim milk-retentate mixture; PR, permeate-retentate mixture; E: early-season; M: mid-season; L: late-season; WHC, water holding capacity; LT, loss tangent; NS: nonsignificant; SE, standard error.

For both SR and PR mixtures, the seasonal variation patterns of major acid gelation properties were consistent with acid gels made from standardized milk (Chapter 5). Late-season milk mixtures had the lowest final G′, longest gelation time and the lowest gelation pH. These results confirmed the seasonal variation patterns observed in Chapter 5 in fat-free milk systems with higher protein content. The consistency in the seasonal variation pattern also indicated that the milk characteristics contributed to the seasonal changes in the acid gelation properties of milk were not affected by the UF

process. Considering both SR and PR mixtures, the WHC of acid gels was significantly higher in the late season than in mid-season ($P = 0.043$). However, compared with PR, the WHC of SR had a more similar seasonal variation pattern to the WHC of yoghurts in Chapter 6 that was lowest in mid-season.

Compared with acid gels made from SR, acid gels made from PR had higher G′ ($P < 0.01$), shorter gelation time ($P < 0.05$) and higher final LT ($P < 0.01$). In addition, acid gels made from PR had slightly higher gelation pH and higher WHC than SR, although the differences were not statistically significant. The difference by percentage in acid gelation properties between SR with PR was calculated as (Mean $_{PR}$ – Mean $_{SR}$) / Mean $_{SR}$ (Table 7.6). The improvement from SR to PR in the G′ of acid gels was highest in the late season (19.0%), followed by the mid-season (14.4%) and the early season (6.3%). When corrected by protein content, the enhancement in the final G′ of acid gels by replacing SR with PR was even higher in the mid-season (17.1%) and the late season (23.8%). The mean gelation times of acid gels made from PR were 2.1 min, 5.4 min and 10.0 min (corresponding to 4.4%, 11.1% and 12.4%) shorter than acid gels made from SR in early-, mid- and late-season, respectively. The mean gelation pH of PR was 0.04 to 0.05 higher than SR across the seasons. Comparing SR with PR, the WHC of acid gels was most improved in mid-season (3.8%), where the WHC of SR acid gels was the lowest.

In summary, PR displayed better overall acid gelation properties than SR, without altering the patterns of seasonal variation. The results indicated that the UF process might modify the physicochemical properties of milk components in ways that improved their acid gelation properties. It is difficult, however, to pinpoint what and

how modifications contributed to the acid gelation process. The increase in G′ and the reduction in gelation time of PR over SR were the most pronounced in the late season when the milk had the worst gelation properties during the year.

7.3.5.3 Possible factors contributing to the variations

To better understand the superior acid gelation properties of PR compared with SR, the correlations between the acid gelation properties and milk characteristics were investigated. However, both the seasonal variations and the differences between SR and PR contributed to the correlations between milk characteristics and the acid gelation properties. Therefore, the differences in the physio-chemical properties between SR and PR that contributed to their different acid gelation properties cannot be distinguished from the effect of seasonality by solely looking at the correlations. The characteristics of milk that might contribute to the different acid gelation properties between SR and PR are discussed in detail below.

7.3.5.3.1 Whey protein denaturation and association with casein micelles

Among the milk characteristics determined in this study, the most pronounced difference between SR and PR was the extent of denaturation and subsequent micelle-association of whey proteins, particularly α-LA. It was counterintuitive that PR with significantly lower extent of α-LA denaturation than SR had better acid gelation properties, considering that the protein composition and the extent of β-LG denaturation of SR and PR were almost the same. This correlation suggested the possibility that limiting the involvement of α-LA in the acid gel network by suppressing its heat-induced denaturation might somehow improve the acid gelation properties of heated milk.

As for the distribution of denatured whey proteins, desirable acid gelation properties (higher G′, shorter gelation time and higher gelation pH) correlated with lower extents of whey protein-casein micelle association and higher proportions of whey proteins forming serum phase aggregates in heated milk ($P < 0.001$), in agreement with the correlations observed in Chapter 5. Numerous previous studies also demonstrated that the serum phase whey protein aggregates improved the acid gelation properties of milk more than the micelle-bound whey proteins (Anema, 2018; Anema et al., 2004; Lakemond & van Vliet, 2008; Vasbinder et al., 2004). However, this might not explain the superior acid gelation properties of PR in the current study. Despite having a lower proportion of micelle-bound denatured whey proteins compared with SR, PR had a similar amount of denatured whey proteins forming serum aggregates to that of SR (Table 7.5). An extra amount of denatured whey proteins associating with the casein micelles in SR compared with that in PR was not likely to weaken its acid gelation properties. Therefore, it appeared that the denatured whey proteins in SR and PR were not equally effective in improving the gelation properties, which might be resulted from the different composition of denatured whey proteins, i.e. the proportion of denatured α-LA and denatured β-LG.

7.3.5.3.2 Proportion of α-LA and β-LG in denatured whey proteins

Analysis of the denaturation and association of whey proteins in heated milk mixtures in the previous section suggested that the different proportions of denatured α-LA and β-LG might have played a role in the acid gelation of milk mixtures. PR contained significantly lower proportions of denatured α-LA in denatured whey proteins than SR (Table 7.5). The extent of this difference between PR and SR (calculated as a

percentage in SR) increased as the season progressed (early-season 15.2%, mid-season 28.6%, and late-season 40.0%). It correlated significantly with the increment (by percentage) in the corrected G′ of the acid gels made from PR instead of SR as shown in Table 7.6 (r = 0.731, P = 0.016). This correlation further suggested that the lower extent of denaturation of α-LA in PR, thus the lower proportion of denatured α-LA in denatured whey proteins, might have contributed to its improved acid gelation properties.

As discussed in Chapter 5, α-LA contributes less to the acid gelation of milk than β-LG. This is probably due to lack of free thiol group (Graveland-Bikker & Anema, 2003), lower pI (Paulsson et al., 1986), and lower hydrophobicity of α-LA (Morand et al., 2011). Involvement of more denatured α-LA in whey protein aggregates was suggested to reduce their surface hydrophobicity (Morand et al., 2011; Mottar et al., 1989). Besides, α-LA limited the size of the heat-induced whey protein aggregates (de la Fuente et al., 2002) and was stabilized partially by hydrophobic interactions within the aggregates, in contrast to β-LG which was bonded mostly via the stronger S-S bonds (Havea, Singh, Creamer, & Campanella, 1998; Lakemond & van Vliet, 2008). Consequently, the protein complexes containing more α-LA might be less effective in promoting the acid gelation process than those containing less or no α-LA. This was supported by the reports that higher proportion of α-LA in whey protein mixtures significantly reduced gel strength of both acid-induced whey protein gels (Rabiey & Britten, 2009) and yoghurt fortified with whey protein ingredients (Matumoto-Pintro et al., 2011).

The additional denatured α-LA in heated milk, as in the SR mixtures in the current study, might compete for the available thiol groups to form protein complexes that are inferior in enhancing the acid gelation properties of milk. The counterbalance between the positive effect of additional proteins and the negative effect of a higher proportion of α-LA in denatured whey proteins on acid gelation properties might have resulted in the weaker acid gel structure of SR compared with that of PR. This hypothesis is supported by the work of Graveland-Bikker & Anema (2003), who investigated the acid gelation properties of whey-protein-depleted (WPD) milk with added α-LA and β-LG in different amounts and ratios. They found that the addition of β-LG to WPD milk significantly reduced the gelation time and increased the G′ of acid gel, with similar effects either with or without additional α-LA. Furthermore, in some cases, additions of α-LA to WPD milk containing β-LG even slightly impaired their acid gelation properties. For instance, the addition of 0.15% α-LA to WPD milk containing 0.3% β-LG reduced the acid gelation pH and prolonged the gelation time by 3 to 6 minutes. The addition of 0.45% α-LA to WPD milk containing 0.9% of β-LG A and the addition of 0.6% α-LA to WPD milk containing 1.2% β-LG A resulted in lower final G′ values of the acid gels made from WPD milks containing only 0.9% and 1.2% of β-LG A, respectively.

7.3.5.3.3 Changes in the structure and functionality of proteins

Reported effects of UF on milk proteins include lowering the surface hydrophobicity of casein micelles (Erdem, 2000), structural modification of whey proteins (Portugal et al., 2007; Van Audenhaege et al., 2013), altering casein micelle size (Erdem, 2000;

Srilaorkul et al., 1991) and inducing micellar dissociation (Lin et al., 2015; McKenna, 2000).

The UF process was reported to lower the surface hydrophobicity of casein micelles, the effect of which was not fully recovered after dilution to the original concentration with UF permeate (Erdem, 2000). Lower surface hydrophobicity of the casein micelles prior to heating is not likely to affect the acid gelation process directly, because the association with whey proteins during heating significantly increases the surface hydrophobicity of the casein micelles (Donato & Guyomarc'h, 2009; Guyomarc'h, Renan, Chatriot, Gamerre, & Famelart, 2007). However, the lowering of micelle surface hydrophobicity during UF might affect the interactions between whey proteins and the casein micelles during heat treatment. Hydrophobic interactions play an important part in the initial stage of β-LG /κ-CN complex formation (Haque & Kinsella, 1988). Lower surface hydrophobicity of the casein micelles would retard the whey protein-casein micelle association, thus promoting the formation of more protein complexes in the serum phase. Consequently, a shift of the distribution of the denatured whey proteins towards the serum phase might contribute to the enhanced acid gelation properties of the ultrafiltered milk. Various previous results, including those in Chapter 5, indicated that the serum phase whey protein aggregates were more effective in promoting the acid gelation properties of milk than the whey proteins bound to casein micelles (Anema, 2008; Anema, 2018; Vasbinder et al., 2004). As a result, the reported effect of UF on the surface hydrophobicity of the casein micelles might contribute to the superior acid gelation properties of the PR mixtures, which contained proteins exclusively originating from the UF retentate. Interestingly, this hypothesis might also

help to explain the seasonal variations in the extent of heat-induced casein micelle-whey protein associations (Table 7.5) of the SR mixtures. Late-season SR mixtures contained significantly lower proportions of UF retentate than the SR mixtures from early- and mid-season (Table 7.1), which correlated well with the highest extent of whey protein-casein micelle association of SR from the late season (r = 0.753, P=0.012). This possible effect of UF was not likely to be the main reason for the seasonal variation in whey protein-casein micelle association, considering that the PR mixtures had the same seasonal trend. Nevertheless, the increase in the amount of whey proteins associating with the casein micelles from early- and mid-seasons to the late season was higher in SR (7%) than in PR (5.6%).

The effect of UF on modifying the molecular structure of whey protein might alter the denaturation kinetics of α-LA and β-LG, as discussed in Section 7.3.4.3. As a result of this modification, a lowered ratio of denatured α-LA: β-LG in the PR mixtures might contribute to its better acid gelation properties than the SR mixtures as discussed in Section 7.3.5.3.2. The casein micelle size measured by DLS in the current study was found not to be affected by UF (Table 7.4), in agreement with other previous results (Ferrer et al., 2011; Lin et al., 2015; McKenna, 2000). Similarly, the micellar dissociation reported previously was not significantly different between SR and PR (Table 7.5).

7.3.5.3.4 Milk calcium equilibrium

The equilibrium of calcium during two processes might affect the acid gelation properties of milk, i.e. (1) calcium dissolution from the casein micelles during UF and (2) lowered ionic calcium in fresh PR mixtures.

(1) The proportions of soluble calcium were not different for SR and PR in this study (Table 7.4), in agreement with some previous reports (Brule & Fauquant, 1981; Corredig et al., 2019; Liu et al., 2014) that there was no micellar calcium loss during UF. Even if there was partial solubilization of CCP during UF (Lin et al., 2015), its impact on the acid gelation properties of milk was inconsistent (Ozcan-Yilsay et al., 2007; Ozcan et al., 2008; Ramasubramanian et al., 2008), as discussed in Chapter 5.

(2) The lowered ionic calcium in fresh PR mixtures prior to heating might affect the denaturation kinetics of α-LA and β-LG as discussed in Section 7.3.4.3. Similar to the effect of the possible structural modifications by UF on the whey proteins, the lower ionic calcium in fresh PR might have contributed to its improved acid gelation properties by reducing the α-LA: β-LG ratio in denatured whey proteins.

7.3.5.3.5 General discussion and future suggestions

The lower ratio of α-LA: β-LG in the denatured whey proteins in PR compared with SR, possibly arising from shear-induced structural modifications of α-LA and β-LG in the permeate and/or lower ionic calcium in fresh PR mixtures before heating, might be an important factor contributing to the superior acid gelation properties of PR, despite its lower extent of denaturation of α-LA. This hypothesis was proposed based on the consistent evidence that the aggregates formed by denatured whey proteins containing a higher proportion of α-LA were inferior in promoting acid gelation, and supported by the reports that addition of α-LA to milk containing β-LG did not improve, even in some cases reduced, their acid gelation properties.

Besides, the reported effect of UF in reducing the surface hydrophobicity of the casein micelles might also play a part in the better the acid gelation properties of PR than SR by promoting the formation of more serum-phase whey protein aggregates, which was reported to be better at promoting the acid gelation of milk than the micelle-bound whey proteins.

Based on the discussion above, the superior acid gelation properties of PR compared with SR might be resulted from both the effect of the UF process on protein functionality and the standardization process by adding UF permeate. The former would be helpful in tuning the properties of fermented milk and other dairy products using UF retentate, whereas the latter would provide a better understanding of the processing properties of the dairy products standardized using UF permeate. It is difficult, however, to distinguish between the two possible effects. The milk mixtures used in this study were designed to standardize the contents of both milk proteins and salts, where the addition of UF permeate is required. Future studies could be performed using stored PR mixture (to restore the salt equilibrium) or a UF system with a smaller pore size (e.g. 6 kDa) to strictly control the permeation of proteins.

7.4 Conclusions

The SR and PR milk mixtures investigated in this study were not significantly different in their compositions, including the contents of protein and calcium, and the composition of individual proteins. In addition, the physicochemical properties determined, including pH, ionic calcium, buffering capacity and casein micelle size, were also not significantly different between SR and PR. However, a re-equilibration process of ionic calcium after mixing UF permeate and retentate (as in PR mixture) was

observed that the Ca^{2+} concentrations of PR mixtures prior to heating were likely to be lower than the SR mixtures. The extent of heat-induced denaturation of α-LA was significantly lower in PR than in SR, whereas β-LG were almost completely denatured in both SR and PR. This might be caused by the reported modification effect of UF permeation on the structure of α-LA and the reduced ionic calcium concentrations of fresh PR mixtures. As a result of suppressed α-LA denaturation in PR, the proportion of denatured α-LA in denatured whey proteins was considerably higher in SR than in PR. The proportion of whey proteins forming serum phase aggregates were similar for SR and PR. Whereas the extent of whey protein-casein micelle association was lower in PR than in SR. The seasonal variations in most compositional and physicochemical properties of milk reported in Chapter 4 were verified in the milk mixtures.

The overall acid gelation properties of PR were greater than SR. Compared with acid gels made from SR, those made from PR had significantly higher final G′ and shorter gelation time, as well as slightly elevated gelation pH and WHC. The advantages of PR over SR in increasing final G′ and reducing gelation time were most pronounced in the late season when the acid gelation properties of milk were the most inferior during the year. Similarly, PR improved the WHC of acid gels the most over SR in mid-season, when the WHC of yoghurts were the lowest across the seasons as demonstrated in Chapter 6.

The possible connections between the composition and properties of milk mixtures and their acid gelation properties were discussed. Although PR had a lower extent of denaturation of α-LA than SR, its lower proportion of α-LA in the denatured whey proteins might have played an important part in its superior acid gelation properties.

Besides, the reported effect of UF in reducing the surface hydrophobicity of the casein micelles might have contributed to the superior acid gelation properties of PR by promoting the formation of more serum-phase whey protein aggregates.

This study demonstrated the effect of the UF process in enhancing the acid gelation properties of milk. UF might have contributed to the seasonal variation in the acid gelation properties of milk standardized by adding UF retentate (e.g. the SR mixture in this chapter and the standardized milk in Chapter 5) since the CF of UF and the proportion of retentate varied with season. However, the extent of improvement by replacing SR with PR was not sufficient to overcome the seasonal variations. Besides, this study also contributed to expanding the understanding of the functionalities of UF retentate and permeate, allowing the dairy industry to optimize the use of UF in processing and formulation.

Chapter 8 - Seasonal variations in milk fat and whipping properties of cream

8.1 Introduction

In milk, 98% of the fat is present in the form of triglycerides (esters of fatty acids and glycerol), located in the milk fat globules (FG) that are covered by the milk fat globule membrane (MFGM). Milk fat plays an important role in the nutritional value of the milk and the quality of various dairy products relating to the fatty acid (FA) composition. Some fatty acids in milk fat were demonstrated to be beneficial to human health, as reviewed previously (Parodi, 1997; Pereira, 2014). In particular, conjugated linoleic acid (CLA) in milk received considerable research interest for its anticancerous effects and other health benefits (Kelly, Kolver, Bauman, Van Amburgh, & Muller, 1998; Lock & Bauman, 2004; MacDonald, 2000; Parodi, 1997; Pereira, 2014). Among different isomers of CLA, the *cis* 9, *trans* 11 isomer makes up 75-90% of total CLA in milk (Lindmark Månsson, 2008). Moreover, the fatty acid composition of bulk milk fat plays an important role in the quality of fat-rich products like butter, cheese and ice cream (Chen et al., 2004).

The fatty acid composition of milk affects product properties because of its influence on the melting behaviour of milk fat. Milk fat melts over a wide temperature range from approximately -35°C to 38°C (MacGibbon & Taylor, 2006). The proportion of milk fat in the solid state at a certain temperature is defined as the solid fat content (SFC). Previous studies demonstrated that the hardness of butter had strong correlations with the SFC of milk fat (Couvreur, Hurtaud, Lopez, Delaby, & Peyraud, 2006;

MacGibbon & McLennan, 1987). Among the fatty acids, C16:0 is the greatest contributor to a higher SFC whereas C18:1 is associated with a lower SFC (Couvreur et al., 2006; O'Callaghan et al., 2016).

Variations in the properties of milk fat arise from different aspects including the fat globule size and the fatty acid composition. As discussed in Chapter 4, fat globule size decreases as the lactation progresses (Fleming et al., 2017; Wiking et al., 2004). The fat globule size of milk was found to affect the quality of cheese and whipping cream (Edén, Dejmek, Löfgren, Paulsson, & Glantz, 2016; Michalski et al., 2004a). The variations in fatty acid composition and the melting behaviour of milk fat were studied over the seasons in seasonal calving countries (Auldist et al., 1998; Versteeg et al., 2016; Walker et al., 2013) and during the lactational cycle (Auldist et al., 1998; Garnsworthy, Masson, Lock, & Mottram, 2006; Mele et al., 2009; Soyeurt, Dehareng, Mayeres, Bertozzi, & Gengler, 2008; Stoop, Bovenhuis, Heck, & Van Arendonk, 2009). Consistent variations were found during the first 2-3 months of lactation, as the proportion of *de-novo* synthesized fatty acids increased at the expense of long-chain fatty acids. However, from mid- to late lactation, some studies reported reverse trends to those during the early lactation (Auldist et al., 1998; Mele et al., 2009; Soyeurt et al., 2008), while others reported no significant variations (Garnsworthy et al., 2006; Lynch, Barbano, Bauman, Hartnell, & Nemeth, 1992; Stoop et al., 2009).

Whipping cream is a product typically contains 30-40% of milk fat, mainly to be consumed after being whipped into a stiff foam known as whipped cream. The whipping process of cream was described in Section 2.4.3. In brief, it involves the incorporation and stabilization of air bubbles during the initial stages of whipping,

followed by the partial coalescence of fat globules (both shear-induced and surface-induced) in the later stages. Whipping cream properties are affected by the fat droplet size (Edén et al., 2016), the composition of the serum phase (Börjesson, Dejmek, Löfgren, Paulsson, & Glantz, 2015; Needs & Huitson, 1991), and the crystalline state of milk fat (Drelon et al., 2006; Ihara et al., 2010), all of which could vary over the seasons in fresh cream. A few previous studies investigated the seasonal variations in the whipping properties of homogenized UHT cream (Keogh, 1978) and pasteurized cream (Chen et al., 2015b; Needs et al., 1988). However, no study on the seasonal variation in the whipping properties of unhomogenized pasteurized cream has been done in a seasonal calving country like New Zealand.

The aim of this study was to investigate the seasonal variations in the properties of bulk milk fat, including the FA composition, FG structure, thermal properties of milk fat and whipping properties of the pasteurized unhomogenized cream. Unlike most previous studies that investigated one or two of these aspects, this study would contribute to a more comprehensive understanding of the interactions among the parameters. In addition, it could also help to identify the major contributors to the seasonal variations in the whipping properties of the cream among all the factors that change concurrently during the year.

8.2 Materials and methods

8.2.1 Whipping cream manufacture

Whipping cream samples were produced from fresh milk in the 2017-2018 season. Cream (~40% fat) and skim milk were obtained from pasteurized whole milk using a centrifugal separator as described in Section 3.1.

Whipping cream was standardized to fat content of 35% by mixing cream and skim milk, and pasteurized at 80°C for 20 s before being cooled in an ice-water bath. The whipping cream samples were stored overnight at 4°C before analysis. In total, 16 whipping cream samples were produced (6 in the early season, 5 in the mid-season and 5 in the late season).

8.2.2 Characterization of milk and cream

The composition of milk and cream, fat globule size, calcium, ionic calcium were determined as described in Section 3.2.

8.2.3 Fat extraction

Fat extraction started with churning chilled cream (35-40% fat) into butter granules using a Kenwood Major Titanium Mixer (Kenwood, Havant, UK). Then, milk fat was extracted from the butter granules using a physical method as described in ISO 14156:2001 (ISO, 2001).

8.2.4 Fatty acid composition

The fatty acid methyl esters (FAME) were prepared from extracted milk fat according to ISO 15884:2002 (ISO, 2002). The composition of the FAME was analyzed on an Agilent 7890A gas chromatograph with a flame ionization detector. The column was a Supelcowax-10 (Supelco Analytical, Bellefonte, PA) fused silica capillary column (30 m × 0.32 mm id, 0.5 μm film thickness). The separation started at an oven temperature of 60°C, which was maintained for 3 min before increasing to 180°C at a rate of 8°C per min. After holding at 180°C for 1 min, the temperature was increased to 210°C at a rate of 2°C per min. For quantification, nonadecanoic acid (C19:0) was used as an internal standard. The theoretical response correction factors given by Christie (1989) were used to correct the peak area of different FAME. In this study, CLA refers to the *cis* 9, *trans* 11 isomer unless specified otherwise. 5-7 samples were analysed for FA composition in each of the early, mid- and late season. Each milk fat sample was prepared and analysed in triplicate.

Fatty acid unsaturation indices for C10, C14, C16, C18 and CLA were calculated as the ratio between unsaturated product fatty acid and the sum of both the precursor and the product fatty acid as described by Schennink et al. (2008).

8.2.5 Thermal analysis of milk fat

The thermal behaviour of extracted milk fat was determined by differential scanning calorimetry (DSC). The melting profile of the milk fat was analyzed using a DSC Q 2000 (TA Instruments, New Castle, DE). Milk fat (8.5-9.0 mg) was transferred into a hermetic aluminium pan and sealed. An empty sealed aluminium pan was used as a

reference. The samples were heated to 65°C and equilibrated isothermally for 15 min to melt all fat crystals and nuclei. Then the samples were cooled to −40°C at 10°C/min and held for 10 min. Finally, the temperature was increased to 60°C at 10°C/min. To determine the solid fat content (SFC), a baseline was drawn on the melting thermogram between the start of melting (around -35°C) to the end of melting (around 40°C) and the area between the baseline and the melting curve was integrated using the Universal Analysis 2000 software (TA Instruments, New Castle, DE). The SFC at temperature T was calculated as the percentage of the area between the start of melting to temperature T in the total area between the baseline and the melting curve. The fractions of milk fat melt below 5°C, between 5 to 20°C and above 20°C are defined as the low melting fraction (LMF), the medium melting fraction (MMF) and the high melting fraction (HMF), respectively. From the DSC results, the proportion of milk fat in each melting fraction was calculated as a percentage of total milk fat. In each season, 4 samples were analysed with DSC and the analysis of each sample was at least duplicated.

8.2.6 Determination of whipping properties

Whipping tests of cream samples were performed in a Major Titanium mixer (Kenwood, Havant, UK) at speed setting 5. The samples, the whipping bowl and the whisk were chilled at 4°C until the experiment. The cream was whipped until stiff peaks were formed. Whipping test of each sample was at least duplicated. The whipping time was recorded and the overrun of whipped cream was calculated as described by Smiddy et al. (2009) from the weights of cream and whipped cream with equal volume.

The firmness of whipped cream was analysed using a texture analyser TA.XT Plus (Stable Micro System, Surrey, UK). To account for the impact of temperature differences in the whipped cream samples caused by the varying whipping time, the whipped cream was filled into containers (60 mm diameter, 45 mm height) and chilled at 4°C for about 2 hours before texture analysis. A geometry with a round flat surface (40 mm diameter) was lowered into the whipped cream samples to a maximum depth of 30 mm at a speed of 1mm/s. The firmness of the whipped cream is defined as the maximum force recorded during the test. The measurement was triggered automatically when a force of 0.05 N is reached. Firmness measurements of each whipped cream sample were triplicated.

8.3 Results and discussion

8.3.1 Composition and properties of seasonal milk and whipping cream

The seasonal patterns of the composition and characteristics of the milk used for preparing whipping cream, as shown in Table 8.1, were consistent with those across two full milking seasons reported in Chapter 4. The amount of fat in milk was highest in the late season. The protein content of cream correlated with that of the milk (r = 0.91, $P < 0.001$), both increased to the highest levels in the late season (Table 8.1). The concentrations of calcium and ionic calcium were both highest in late-season milk. The mean fat globule sizes were largest in the early season and decreased as the season progressed (Table 8.1).

Table 8.1: Seasonal variations in the composition and properties of milk and whipping cream

	Season	Means ± SE	Seasonal variation[1]	*P* value
Fat – milk (%)	Early	4.74 ± 0.07	L > E, M	< 0.001
	Mid	4.97 ± 0.11		
	Late	5.59 ± 0.08		
Protein – milk (%)	Early	3.60 ± 0.10	L > E, M	< 0.001
	Mid	3.83 ± 0.08		
	Late	4.65 ± 0.12		
Protein – cream (%)	Early	2.38 ± 0.05	L > E	0.007
	Mid	2.51 ± 0.07		
	Late	2.69 ± 0.06		
Milk calcium (mM)	Early	33.7 ± 0.6	L > M	0.01
	Mid	32.7 ± 0.3		
	Late	35.9 ± 0.8		
Milk ionic calcium (mM)	Early	2.46 ± 0.03	L > E, M	0.001
	Mid	2.36 ± 0.07		
	Late	2.71 ± 0.04		
Milk pH	Early	6.68 ± 0.01	M, L > E	0.002
	Mid	6.76 ± 0.01		
	Late	6.75 ± 0.02		
Fat globule size (D_{43}, μm)	Early	5.39 ± 0.13	E > M > L	<0.001
	Mid	4.52 ± 0.07		
	Late	4.30 ± 0.04		

[1]Analyzed using one-way ANOVA

D_{43}, volume-weighted mean diameter. E: early season; M: mid-season; L: late season; SE, standard error.

8.3.2 Fatty acid composition

Table 8.2 shows the variation in the fatty acid composition of the milk produced in early, mid- and late seasons. The detailed variation patterns of C4:0, C16:0, C18:1 *cis* and CLA are shown in Figure 8.1.

Table 8.2: Seasonal variation in the fatty acid composition (g /100g of fatty acids) of milk

	Season	Means ± SE	CV (%)	Seasonal variation[1]	*P* value
C4:0	Early	3.7 ± 0.1	9.6	E > M, L	< 0.001
	Mid	3.2 ± 0.0			
	Late	3.0 ± 0.0			
C6:0	Early	2.2 ± 0.0	8.2	E, M > L	0.004
	Mid	2.1 ± 0.1			
	Late	1.9 ± 0.0			
C8:0	Early	1.3 ± 0.0	8.1	NS	0.127
	Mid	1.3 ± 0.0			
	Late	1.2 ± 0.0			
C10:0	Early	2.9 ± 0.2	15.4	NS	0.182
	Mid	3.2 ± 0.2			
	Late	2.7 ± 0.1			
C12:0	Early	3.1 ± 0.3	17.6	NS	0.09
	Mid	3.8 ± 0.3			
	Late	3.3 ± 0.1			
C14:0	Early	10.0 ± 0.5	11.2	M > E	0.014
	Mid	12.0 ± 0.4			
	Late	10.8 ± 0.2			
C14:1	Early	0.6 ± 0.1	26.5	M, L > E	< 0.001
	Mid	1.0 ± 0.1			
	Late	1.1 ± 0.0			
C15:0	Early	0.8 ± 0.0	17.7	M, L > E	< 0.001
	Mid	1.2 ± 0.0			
	Late	1.2 ± 0.0			
C16:0	Early	27.0 ± 0.8	10.2	M > E, L	0.001
	Mid	32.4 ± 0.8			
	Late	28.4 ± 0.9			
C16:1	Early	1.5 ± 0.0	10.1	L > E, M	0.003
	Mid	1.5 ± 0.1			
	Late	1.8 ± 0.0			
C17:0	Early	0.6 ± 0.0	8.6	E, M > L	0.001
	Mid	0.6 ± 0.0			
	Late	0.5 ± 0.0			
C18:0	Early	12.8 ± 0.8	15.7	E > M	0.014
	Mid	10.1 ± 0.6			
	Late	10.7 ± 0.3			

Table 8.2 (continued)

C18:1 *cis*	Early Mid Late	21.0 ± 1.0 16.1 ± 0.8 19.5 ± 0.3	15.1	E, L > M	0.001
C18:1 *trans*	Early Mid Late	3.5 ± 0.1 2.4 ± 0.1 3.1 ± 0.2	19.6	E, L > M	< 0.001
C18:2	Early Mid Late	2.0 ± 0.1 1.7 ± 0.1 1.8 ± 0.1	11.2	E > M	0.022
CLA	Early Mid Late	0.7 ± 0.0 0.8 ± 0.0 1.2 ± 0.1	25.2	L > E, M	< 0.001
C18:3	Early Mid Late	0.8 ± 0.0 0.8 ± 0.1 0.8 ± 0.1	17.4	NS	0.993
Minor fatty acids	Early Mid Late	5.3 ± 0.1 5.7 ± 0.1 6.4 ± 0.3	10.0	L > E, M	0.002
Saturated fatty acids	Early Mid Late	63.7 ± 1.0 69.3 ± 1.0 63.1 ± 0.9	5.6	M > E, L	0.001

CLA, C18:2 *cis* 9, *trans* 11; SE: Standard error; CV: coefficient of variation; E: early season; M: mid-season; L: late season; NS, not significant.

[1]Analyzed using one-way ANOVA

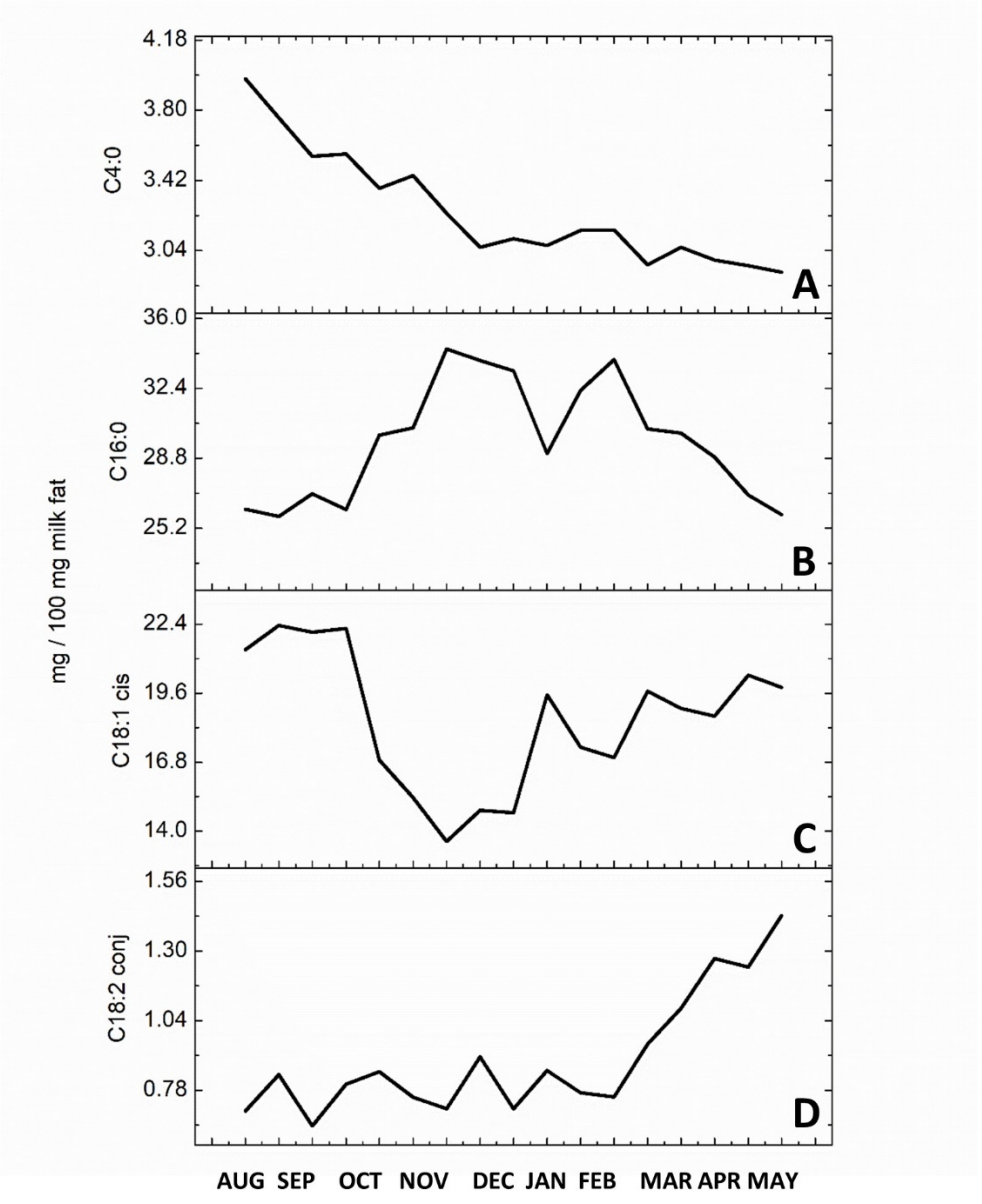

Figure 8.1: Detailed variation patterns of the concentrations of butyric acid (A), palmitic acid (B), oleic acid (C) and C18:2 *cis* 9, *trans* 11 CLA (D) in milk.

8.3.2.1 Overall seasonal patterns

The proportions of the identified fatty acids in the milk fat were in good agreement with previous studies on New Zealand milk fat (Auldist et al., 1998; Creamer & MacGibbon, 1996; Gray, 1973). Total saturated fatty acids (SFA) accounted for 65.8 ± 0.9% of fatty acids in the milk. This level is lower than those reported in the US (69%, Jensen, 2002), Sweden (70%, Lindmark-Månsson et al., 2003), Denmark (69%, Larsen, Andersen, Kaufmann, & Wiking, 2014), the Netherlands (70-71%, Heck et al., 2009; Stoop et al., 2009), and closer to the reports in New Zealand (67%, Creamer & MacGibbon, 1996; Gray, 1973) and Australia (66%, Walker et al., 2013). The differences between the countries might arise from the pasture-based feeding systems used in seasonal calving countries like New Zealand and Australia. It is well established that feeding fresh grass that is rich in LCUSFA results in a lower proportion of SFA in milk fat compared with feeding diets based on silage and concentrates (Hanuš, Samková, Křížová, Hasoňová, & Kala, 2018; Heck et al., 2009; Lindmark Månsson, 2008).

Three major seasonal variation patterns were observed that were likely related to the progressing stages of lactation.

1) The proportion of C4:0 in milk fat was highest after calving and decreased gradually as the season progressed (Figure 8.1-A).

2) C6-16 fatty acids increased to maximum levels in 3 to 4 months and maintained at high levels in the mid-season (Table 8.2).

3) The proportions of most C18 fatty acids, including C18:0, C18:1 *cis* and C18:1 *trans*, were highest in the early season and decreased drastically into the mid-season (Table 8.2, Figure 8.1-C).

All of the three seasonal patterns were consistent with previous reports during the season in New Zealand (Auldist et al., 1998; Gray, 1973), Australia (Walker et al., 2013) and during the lactation cycle (Auldist et al., 1998; Garnsworthy et al., 2006; Hanuš et al., 2018; Lynch et al., 1992; Mele et al., 2009; Palmquist et al., 1993; Soyeurt et al., 2008).

The mechanism for the changes in fatty acids profiles during early to mid-lactation has been well demonstrated in previous studies. At the beginning of the lactation, the cows are in negative energy balance (NEB). As a result, long-chain fatty acids (mainly C18:0 and C18:1 *cis*) in the adipose tissue are mobilized and transferred into milk fat (Auldist et al., 1998; Gross, van Dorland, Bruckmaier, & Schwarz, 2011; Palmquist et al., 1993; Stoop et al., 2009). The greater uptake of these long-chain fatty acids suppresses the *de-novo* synthesis of C6-16 fatty acids (Garnsworthy et al., 2006; Glasser et al., 2008; Palmquist et al., 1993) by inhibiting acetyl-CoA carboxylase (Bauman & Davis, 2013; Beaulieu & Palmquist, 1995; Palmquist et al., 1993). Acetyl-CoA carboxylase catalyzes the *de novo* synthesis of fatty acids from acetate via the malonyl-CoA pathway (Bauman & Davis, 2013). Unlike other *de-novo* synthesized fatty acids, the proportion of C4:0 in milk fat was highest at the beginning of the season because of its unique synthesis pathways that are independent of the action of acetyl-CoA carboxylase. Firstly, C4:0 can be formed directly from the 4 carbon unit β-hydroxybutyrate (McCarthy & Smith, 1972; Moore & Christie, 1981; Palmquist et al.,

1993), which is particularly abundant at the beginning of lactation (Garnsworthy et al., 2006; Lynch et al., 1992). Secondly, C4:0 could also be synthesized by a β-reduction pathway (Moore & Christie, 1979; Palmquist et al., 1993).

8.3.2.2 Early to mid-season

Despite the overall seasonal trend of *de-novo* synthesized fatty acids, different patterns were found for individual fatty acids that were probably linked to their chain length. C6:0 (CV 8.2%) and C8:0 (CV 8.1%) had the smallest extent of variation among the individual fatty acids during the season (Table 8.2). The concentrations of C8:0, C10:0 and C12:0 did not vary significantly in different seasons. With increasing chain length, the mean concentrations of C6-C16 fatty acids in mid-season milk fat became progressively higher than milk fat from the rest of the year (Table 8.2). The maximum levels were observed in late October for C6:0 and C8:0 and in late November for C12:0, C14:0 and C16:0. C10: 0 had the two of the highest levels in both late October (3.66 mg/ 100 mg fat) and late November (3.69 mg/ 100 mg fat).

As discussed above, adipose tissue mobilization due to NEB in early lactation inhibits the *de-novo* synthesis of fatty acids. The extent of inhibition increases as the chain lengths of the fatty acids increases (Beaulieu & Palmquist, 1995; Palmquist et al., 1993) because the chain elongation process uses solely acetate (Garnsworthy et al., 2006). This might be the reason for the less significant increase from early season to mid-season in the amounts of C6-10 compared with C12-C16, and the fact that the maximum levels of these fatty acids with longer chain length were reached later in the season. This hypothesis could also explain the results of Garnsworthy et al. (2006), who reported that the proportions of C6:0 and C8:0 in milk fat did not vary

significantly with lactation, whereas those of C10-14 were significantly lower in early lactation than mid- and late lactation.

8.3.2.3 Mid- to late season

In late-season milk fat, the proportion of C16 significantly increased and the proportion of C18:1 *cis* decreased significantly compared with mid-season milk fat (Table 8.2). To lesser extents, *de-novo* synthesized C10-14 fatty acids also decreased into the late season (Table 8.2). These trends were consistent with reports in New Zealand (Auldist et al., 1998; Gray, 1973) and over the lactation (Mele et al., 2009; Soyeurt et al., 2008). However, in some other studies during the lactation cycle, changes from mid-lactation to late lactation in these fatty acids, if any, were far less significant (Garnsworthy et al., 2006; Lynch et al., 1992; Stoop et al., 2009). The disagreements might arise from the differences in feeding systems. The cows were fed total mixed ration in the works of Garnsworthy et al. (2006) and Lynch et al. (1992), and the milk was sampled only in the winter housing period in the study of Stoop et al. (2009). In contrast, studies in New Zealand followed the pasture-based feeding throughout the milking season (Auldist et al., 1998; Gray, 1973). In New Zealand, the pasture is less mature in spring (early season) and autumn (late season) than in summer (mid-season). Immature pasture contains higher polyunsaturated fatty acids (Heck et al., 2009), which might contribute to higher levels of long-chain fatty acids (e.g. C18:1 *cis*) in the milk fat and suppress the *de novo* synthesis of C10-16 fatty acids (Hawke, 1963). Due to the synchronized calving practice in New Zealand, the seasonal variation in the maturity of the pasture probably contributed to the significant shift in the proportions of these fatty acids from mid-lactation to late lactation in the present study and other previous reports in New

Zealand (Auldist et al., 1998; Gray, 1973). Nevertheless, it is interesting that these variation patterns in New Zealand were also found in the lactational studies conducted in Europe (Mele et al., 2009; Soyeurt et al., 2008). In these two studies conducted in Europe, the milk was sampled over 6 months in Italy (Mele et al., 2009). and over 21 months in Belgium (Soyeurt et al., 2008). In European countries, calving spreads across the year but the main feed of the cows switch from fresh grass during spring and summer to silage and concentrates in the winter (Grimley et al., 2009; Heck et al., 2009). Therefore, in the studies of Mele et al. (2009) and Soyeurt et al. (2008), cows in their late lactations could access fresh grass in spring and summer months. In contrary, in the studies that found less significant changes in the contents of C10-16 and C18:1 *cis* from mid- to late lactation (Garnsworthy et al., 2006; Lynch et al., 1992; Stoop et al., 2009), the cows had no access to fresh grass during the whole sampling period. This suggested that the consumption of fresh grass might alter the fatty acid composition in two ways. 1) By direct uptake and subsequent inhibition of the *de novo* synthesis of fatty acids in relation to pasture maturity, as over the seasons in New Zealand. 2) By interacting with the SOL in a way that results in a similar shift in the fatty acid profile, i.e. increased C18:1 and decreased C10-16 from mid-lactation to late lactation, regardless of the maturity of the grass. The second hypothesis was supported by the work of Auldist et al. (1998), who reported that in New Zealand, late-lactation milk fat had a lower proportion of C16:0 and a higher proportion of C18:1 *cis* compared with mid-lactation milk fat within spring, summer and autumn, but not in winter when the access to pasture was limited.

8.3.2.4 Odd-chain fatty acids and polyunsaturated fatty acids

The proportion of C15:0 was lowest in the early season and the proportion of C17:0 was lowest in the late season (Table 8.2). The variation patterns were likely caused by the adipose mobilization during early lactation since the C17:0/C15:0 ratio in the adipose tissue is about 4 times as much as that in milk (Fievez, Colman, Castro-Montoya, Stefanov, & Vlaeminck, 2012).

Polyunsaturated fatty acids C18:2 and C18:3 did not vary greatly across different seasons compared with other fatty acids (Table 8.2). However, early-season milk fat was significantly richer in C18:2 than those from the rest of the year. Previous studies also reported that the content of C18:2 in milk fat is highest in early lactation (Boatman, Hotchkiss, & Hammond, 1965; Lynch et al., 1992; Walker et al., 2013). Consistent with the present study, Kay et al. (2005) reported no significant change in the proportion of C18:3 until 16 weeks into the lactation. Walker et al. (2013) observed the highest levels of C18:3 from July to September in Australia for both spring-calved and autumn-calved herds. It appeared that the SOL does not contribute greatly to the variation in C18:3 in bovine milk fat.

8.3.2.5 CLA and unsaturation indices

The amount of CLA in milk fat ranged from 0.6% in September to 1.4% in May. The CLA concentration in milk was stable during early and mid-season and then increased drastically in the late season (Figure 8.1-D). This seasonal trend in CLA agreed with those reported during the lactation cycle (Auldist et al., 1998; Mele et al., 2009; Soyeurt et al., 2008; Stoop et al., 2009; Wang et al., 2013), and an earlier study carried out in Palmerston North, New Zealand (Schwendel et al., 2015). In contrast, Kelsey,

Corl, Collier, and Bauman (2003) found no effect of SOL on the CLA content in milk fat. MacGibbon, Van der Does, Fong, Robinson, and Thomson (2001) reported that the concentration of CLA in New Zealand milk was higher in the early and late seasons than in the mid-season, although some of the lowest values were observed in the milk from August (beginning of the season). Auldist et al. (1998) examined the effect of lactation and time of year on milk FA composition separately in New Zealand and reported that CLA content in milk fat increased with proceeding lactation, from 0.79% in early lactation to 0.97% in late lactation. When examining milk produced across all stages of lactation in different seasons, the contents of CLA in milk fat were highest in spring and were lowest in summer (Auldist et al., 1998). There might be a lactation × season interaction effect on the content of CLA in the milk fat that is related to the variation in pasture maturity during the year in New Zealand (as discussed in Section 8.3.2.3). Enhanced uptake of polyunsaturated fatty acids via consumption of immature pasture in the spring might consequently lead to elevated levels of CLA in the milk fat (Chilliard, Ferlay, & Doreau, 2001).

As shown in Table 8.3, all individual fatty acid unsaturation indices determined were highest in the late season. The total unsaturation index was lowest in mid-season and similar in early and late season, agreeing with previous reports during the lactation circle (Mele et al., 2009; Stoop et al., 2009). The variation patterns of all individual unsaturation indices were consistent with those reported by Mele et al. (2009) during the lactation circle and broadly agreed with Soyeurt et al. (2008) who reported that the C14 and C16 indices increased gradually over lactation. In contrast, Garnsworthy et al. (2006) reported no effect of SOL on the C14 index hence the activity of Δ^9-desaturase.

Interestingly, this might explain the difference that the contents of C14:1, C18:1 *cis*-9 and CLA were similar in mid-lactation and late-lactation milk fat in the study of Garnsworthy et al. (2006), but were significantly higher in the late season than in the mid-season in the present study and the work of Mele et al. (2009). The seasonal and lactational variation in the activity of Δ^9-desaturase appeared to contribute to the variations in the contents of desaturation products in milk fat.

Table 8.3: Seasonal variation in the fatty acid unsaturation indices

	Season	Means ± SE	CV (%)	Seasonal variation[1]	*P* value
C10 index	Early	9.0 ± 0.4	9.8	M, L > E	0.005
	Mid	10.4 ± 0.3			
	Late	10.7 ± 0.1			
C14 index	Early	5.7 ± 0.3	20.0	L > M > E	<0.001
	Mid	7.6 ± 0.3			
	Late	9.2 ± 0.1			
C16 index	Early	5.4 ± 0.2	13.6	E, L > M	<0.001
	Mid	4.4 ± 0.1			
	Late	5.8 ± 0.1			
C18 index	Early	62.3 ± 0.5	2.9	L > E, M	0.006
	Mid	61.5 ± 0.6			
	Late	64.5 ± 0.3			
CLA index	Early	19.8 ± 0.9	14.8	L > M > E	<0.001
	Mid	24.7 ± 0.7			
	Late	27.9 ± 0.5			
Total unsaturation index	Early	43.1 ± 2.2	16.2	E, L > M	0.001
	Mid	32.9 ± 1.6			
	Late	42.8 ± 0.9			

[1]Analyzed using two-way ANOVA

CLA, C18:2 *cis* 9, *trans* 11; SE: Standard error; CV: coefficient of variation; E: early season; M: mid-season; L: late season.

The concentration of CLA correlated with the C14 index, the C16 index, the C18 index and the CLA index ($P < 0.01$ in all cases). Previous studies demonstrated endogenous synthesis by the action of Δ^9-desaturase largely accounts for the CLA in milk fat

(Collomb, Schmid, Sieber, Wechsler, & Ryhänen, 2006; Corl et al., 2001; Griinari et al., 2000). The results of the present study suggested that the activity of Δ^9-desaturase, as indicated by the unsaturation indices, increased to the highest levels in the late season, which might contribute to the highest content of CLA in late-season milk fat (Table 8.2).

8.3.2.6 Correlation between fatty acid composition and fat globule size

Table 8.4: Pearson correlation coefficients between the fatty acid composition and the fat globule size

Fatty acid	D (3, 2)	D (4, 3)
4:0	0.93***	0.94***
6:0	0.63*	0.59*
14:0	-0.52*	-0.61*
14:1	-0.88***	-0.94***
15:0	-0.83**	-0.90**
16:0	-0.38	-0.53*
16:1	-0.56*	-0.51
17:0	0.84***	0.77***
18:0	0.75**	0.80***
18:1 *cis*	0.48	0.58*
18:1 *trans*	0.39	0.57*
18:2	0.46	0.60*
CLA	-0.75**	-0.64**

CLA, C18:2 *cis* 9, *trans* 11; D (3, 2), surface-weighted mean diameter; D (4, 3), volume-weighted mean diameter; CLA, C18:2 *cis* 9, *trans* 11.
Significance levels: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Table 8.4 shows the significant correlations between the contents of individual fatty acids and the fat globule size. Larger fat globule size correlated with higher proportions of short-chain fatty acids C4:0 and C6:0 and preformed long-chain fatty acids (≥17C), and lower proportions of *de-novo* synthesized C14-16 fatty acids and CLA (Table 8.4). These results agreed with previous studies that larger fat globules contain higher C18:0 (Briard, Leconte, Michel, & Michalski, 2003; Fauquant, Briard, Leconte, & Michalski, 2005; Lopez et al., 2011; Lu et al., 2016; Timmen & Patton, 1988; Wiking et al., 2004) and lower C12-16 fatty acids (Briard et al., 2003; Fauquant et al., 2005; Lopez et al.,

2011). The reports on the relationship between fat globule size and other fatty acids showed mixed results. For instance, some previous reports demonstrated that the content of C18:1 *cis* was higher in the larger fat globules (Briard et al., 2003; Lopez et al., 2011; Wiking et al., 2004). However, some reports suggested that the smaller fat globules were richer in C18:1 *cis* (Lu et al., 2016; Timmen & Patton, 1988). Agreeing with the present study, some previous studies indicated that larger fat globules contained lower amounts of CLA than the smaller fat globules (Lopez et al., 2011; Lu et al., 2016; Michalski, Briard, & Juaneda, 2005) whereas other studies reported the opposite correlation (Timmen & Patton, 1988) or no significant difference (Fauquant et al., 2005). It should be noted that, in the present study, the fatty acid composition and fat globule size were both analysed in bulk milk fat rather than fractionated fat globules as in the previous studies. The disagreements among different studies might arise from the differences in 1) the preparation of the fat globule fractions and 2) the milk sampling, which might induce other factors that affect the correlations between fat globule size and the fatty acid composition. It was demonstrated that the fatty acid composition of fat globules separated in different size groups varied with different stages of lactation (Mesilati-Stahy & Argov-Argaman, 2014) and different seasons (Michalski, Ollivon, Briard, Leconte, & Lopez, 2004b).

8.3.3 Melting behaviour and solid fat content

Figure 8.2 shows the representative melting curves of milk fat from different seasons obtained by DSC. Table 8.5 shows the percentages of milk fat in each of the low melting fraction (LMF), the medium melting fraction (MMF) and the high melting fraction (HMF), and the SFC at 5°C and at 10°C.

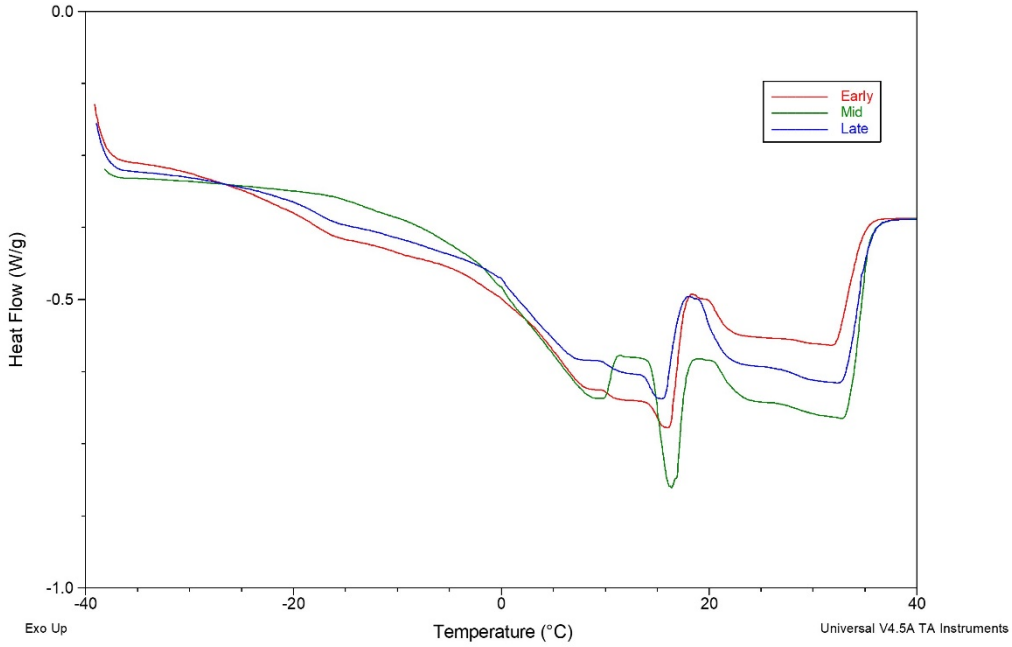

Figure 8.2: Melting thermograms of milk fat from early season, mid-season and late season.

Table 8.5: Variation in the melting fraction and the solid fat content of milk fat

	Season	Means ± SE	Seasonal variation	*P* value
Low melting fraction (%, < 5°C)	Early	33.0 ± 0.8	E > L > M	<0.001
	Mid	21.1 ± 1.0		
	Late	28.1 ± 1.1		
Medium melting fraction (%, 5-20°C)	Early	42.2 ± 0.9	E, M > L	0.001
	Mid	40.2 ± 0.6		
	Late	36.8 ± 0.4		
High melting fraction (%, > 20°C)	Early	24.8 ± 1.4	M, L > E	<0.001
	Mid	38.8 ± 1.1		
	Late	35.1 ± 0.8		
SFC_{10} (%)	Early	54.4 ± 0.2	M > L > E	<0.001
	Mid	65.3 ± 1.1		
	Late	59.2 ± 1.6		
SFC_5 (%)	Early	67.7 ± 0.4	M > L > E	<0.001
	Mid	78.9 ± 1.0		
	Late	72.0 ± 1.1		

[1]Analyzed using one-way ANOVA

$SFC_{t:}$ solid fat content at t°C; E: early season; M: mid-season; L: late season; SE, standard error.

The proportion of milk fat in the LMF decreased in the order of early-season > late-season > mid-season, whereas the opposite order was found in the high melting fraction (Table 8.5). The proportion of fat in the MMF (CV 6.6%) was less variable than those in LMF (CV 19.7%) and HMF (CV 19.8%). Nevertheless, it was still significantly lower in the late season (Table 8.5). The results in the seasonal variation of the melting behaviour of milk fat were consistent with previous studies in New Zealand (Norris et al., 1973), as well as during the lactation cycle (Lynch et al., 1992). The SFC_{10} was highest in the mid-season (65.3%), followed by that in the late season (59.2%) and was lowest in the early season (54.4%, $P < 0.001$). The SFC_5 results showed the same trend over the season. The seasonal patterns in SFC agreed with previous studies during the season in New Zealand (Auldist et al., 1998; MacGibbon & McLennan, 1987; Norris et al., 1973) and Australia (Versteeg et al., 2016; Walker et al., 2013).

The vast shift in the proportions of milk fat in the LMF and HMF due to the change in fatty acid composition over the season probably played a major role in affecting the SFC levels. Both the proportion of HMF and SFC_{10} correlated positively with saturated fatty acids C14:0, C15:0 and C16:0 and correlated negatively with C18:0, C18:1 *cis* and C18:1 *trans* ($P < 0.05$). As two of the most abundant fatty acids in bovine milk, the contents of C16:0 and C18:1 are good indicators of the SFC of the milk fat (Couvreur et al., 2006). The high-melting C18:0 correlated with lower SFC presumably due to its similar seasonal variation pattern with C18:1 *cis* (Larsen et al., 2014). As the cows recover from NEB during the early lactation, the proportion of c18:1 *cis* decreases and the proportion of C16:0 increases (Figure 8.1-B, C), resulting in a concurrent decrease in the LMF and an increase in the HMF (Table 8.5, Lynch et al., 1992). Interestingly, besides the effect of SOL, the change in the pasture maturity in New Zealand from spring to autumn could also result in the same changes in the melting behaviour of milk fat from early lactation to late lactation. Auldist et al. (1998) demonstrated that cows at the same stage of their lactations produced fat with higher SFC_{10} in summer than in spring and autumn, probably due to the variation in the ryegrass maturity as discussed in Section 8.3.2.3. The effect of this shift in diet composition corresponding to different times of the year on the SFC of milk fat was suggested to be greater than the effect of SOL in New Zealand (Auldist et al., 1998).

8.3.4 Whipping cream properties and relating factors

All whipped cream samples maintained their structural integrity during the storage periods monitored in this study. When stored on laminated sheets, the rosettes of whipped cream showed no visible collapse or serum leakage either after storage at 20°C for 4 h or at 4°C for 24 h.

Table 8.6: Whipping properties of cream

	Season	Means ± SE	CV (%)	Seasonal variation[1]	*P* value
Whipping time (s)	Early	70 ± 5	18.0	M, L > E	0.002
	Mid	80 ± 4			
	Late	97 ± 4			
Overrun (%)	Early	136 ± 2	6.9	E > L	0.033
	Mid	135 ± 5			
	Late	123 ± 2			
Whipped cream firmness (N)	Early	2.02 ± 0.13	16.6	E > L	0.042
	Mid	1.76 ± 0.11			
	Late	1.59 ± 0.06			

[1]Analyzed using one-way ANOVA
E: early season; M: mid-season; L: late season; SE, standard error; CV: coefficient of variation

Table 8.6 shows the seasonal variations in the whipping time and the properties of whipped cream. Whipping time had the most pronounced seasonal variation among all the parameters determined, being significantly lower in the early season than the rest of the year ($P = 0.002$). The overrun of all whipped cream samples was in an ideal range of 115-150% and did not vary greatly over the seasons (CV 6.9%). Nevertheless, the overrun of whipped cream was significantly higher in the early season than in the late

season (Table 8.6). The firmness of early-season whipped cream was significantly higher than the whipped cream from the late season ($P < 0.05$).

Table 8.7: Significant correlations between whipping properties and milk characteristics

	Whipping time (s)	Overrun (%)	Firmness (N)
Whipping time (s)	1	-0.56*	NS
Overrun (%)	-0.56*	1	NS
Firmness (N)	NS	NS	1
Fat globule size (μm)	-0.78***	NS	0.62**
Cream protein (%)	0.77***	-0.62*	NS
Milk protein (%)	0.76**	-0.72**	NS
Milk fat (%)	0.66**	-0.72**	NS
Milk pH	0.56*	NS	-0.72**
Milk ionic calcium (mM)	NS	-0.55*	NS

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 8.7 shows the correlations among the whipping properties and between the whipping properties and the characteristics of milk and cream. Among the whipping properties, overrun had a moderate negative correlation with the whipping time (r = -0.56, $P < 0.05$). The firmness of whipped cream did not correlate with whipping time or overrun.

8.3.4.1 Fat globule size

The most pronounced correlation observed was that larger fat globule size significantly correlated with shorter whipping time (Table 8.7, r = -0.78, $P < 0.001$). Edén et al.

(2016) also reported that the whipping time decreased with increasing fat globule size. This correlation was likely resulted from the higher propensity of partial coalescence of larger fat globules (Fredrick, Walstra, & Dewettinck, 2010). The development of partial coalescence during the whipping process involves close contact among the fat globules and the penetration of solid fat crystals through the globule membranes. Consequently, partial coalescence is accelerated by 1) enhanced close contact among the fat globules (by both frequency and extent of the contact) and 2) more effective capture upon contact due to the penetration by crystals, both of which are favoured in larger fat globule. Firstly, larger fat globules have a larger contact area upon collision (McClements, 2015). They are also more easily deformed under the same shear flow than the smaller globules, further increasing the contact area and the chance of partial coalescence given a crystal is present (Fredrick et al., 2010). Secondly, larger fat crystals are more likely to form in larger fat globules than in smaller fat globules (Fredrick et al., 2010; Lopez et al., 2002; Thivilliers, Laurichesse, Saadaoui, Leal-Calderon, & Schmitt, 2008). Larger crystals have longer protrusion distance and thus are more effective in penetrating through the fat globule membranes upon close contact than the smaller globules (Fredrick et al., 2010). Finally, the crystallization temperatures were found to be lower for smaller fat globules than the larger ones, as a deeper supercooling was required for the crystallization inside a smaller globule (Lopez et al., 2002; Michalski et al., 2004b; Truong, Palmer, Bansal, & Bhandari, 2016). Truong et al. (2016) attributed this to the lower ratio of catalytic impurities relative to the number of fat globules as the globule size decreases. Impurities in fat globules catalyse heterogeneous nucleation, which initiates the fat crystallization in milk fat globules (Walstra & Jenness, 1984). At least one impurity is required in a fat globule

for the crystallization to occur but it is not present in all fat globules under a given condition (Huppertz & Kelly, 2006). Consequently, at equal fat content, more fat globules would be present in the cream with a smaller mean globule size and more impurities would be required to achieve the same level of fat crystallization. The fact that the number of impurities increases as the temperature decreases could explain the lower crystallization temperature of smaller fat globules (Lopez et al., 2002). To summarize, the decreasing fat globule size during the season probably played an important part in prolonging the whipping time as the season progressed by facilitating partial coalescence. Although larger globules would have a lower collision frequency under the flow than smaller globules (McClements, 2015; Walstra & Jenness, 1984), other factors promoting partial coalescence appeared to be more pronounced, resulting in the shorter whipping time for the larger fat globules.

The FG size correlated significantly with higher firmness of the whipped cream (Table 8.7). This might arise from the different crystallization state (Drelon et al., 2006) in fat globules of different sizes, as discussed above. Overrun had a positive but insignificant correlation ($r = 0.33$, $P = 0.20$) with FG size. In contrast, (Edén et al., 2016) reported that the cream with larger FG had significantly higher overrun. The reason for the disagreement might be that the overrun levels were higher in the present study (115-150%) than the levels of 75-100% in the study of Edén et al. (2016), where the cream was whipped to maximum firmness.

8.3.4.2 Protein content

The protein content of the cream significantly correlated with a longer whipping time ($r = 0.77$, $P < 0.001$) and to a lower extent, a lower overrun ($P < 0.05$, Table 8.7). It

should be noted that the negative correlation between protein content and fat globule size ($P < 0.05$) might have exaggerated its correlation with whipping time to some extent. Nevertheless, the positive correlation between the protein content of the cream and the whipping time agreed with previous studies (Börjesson et al., 2015; Hotrum, Stuart, Vliet, Avino, & van Aken, 2005; Needs & Huitson, 1991; Smiddy et al., 2009). Hotrum et al. (2005) also observed a slight decrease in overrun of a whey protein-stabilized emulsion with increasing protein concentration whereas other reports suggested no significant effect of protein content on overrun (Needs & Huitson, 1991; Smiddy et al., 2009). Despite the variation in whipping time, the firmness of whipped cream had no significant correlation with protein content, agreeing with that reported by Needs and Huitson (1991).

Less than 5% (w/w) of the protein in whipping cream is necessary for stabilizing the air bubbles during the initial stage of whipping (Smiddy et al., 2009). Excess proteins might retard partial coalescence and the whipping process in a few ways. Needs and Huitson (1991) suggested that proteins might strengthen the air-water interfaces, protecting it against the fat-air and fat-fat interactions during the early stage of whipping. Besides, higher proteins in the cream might reduce the shear flow during whipping by increasing the viscosity of the serum phase, resulting in delayed shear-induced partial coalescence (Smiddy et al., 2009). Further, Hotrum et al. (2005) demonstrated that the protein layer absorbed onto the air-water interface effectively retarded the entering and spreading of the fat droplets, inhibiting the surface-induced partial coalescence.

8.3.4.3 Milk pH and ionic calcium

Higher pH of milk correlated with longer whipping time ($P < 0.05$) and higher firmness of the whipped cream ($P < 0.01$, Table 8.7). Ionic calcium inversely correlated with the overrun ($P < 0.05$, Table 8.7). Factors promoting the repulsive interactions between the globules play a role in inhibiting partial coalescence by hindering the close contact among fat globules (McClements, 2015). Kiokias, Reiffers-Magnani, and Bot (2004) reported that acidification to a pH of 4.5 enhanced the propensity of partial coalescence of protein-stabilized emulsions. Higher ionic calcium in milk was suggested to reduce the electrostatic repulsion by increasing the ionic strength and binding to caseins, resulting in a shorter whipping time (Börjesson et al., 2015). It should be noted that the extents of variations in pH and Ca^{2+} in the present study (Table 8.1) were considerably lower than those in the studies where acids or salts were added (Börjesson et al., 2015; Kiokias et al., 2004). Therefore, it is difficult to conclude on the effects of pH and Ca^{2+} *per se* on the seasonal variations in the whipping properties. Their significant correlations with whipping properties probably arise from their interrelations with other parameters. For instance, pH had a significant negative correlation with FG size ($P < 0.001$), which could account for its correlations with whipping time and firmness that are opposite to those of FG size.

8.3.4.4 Solid fat content

The SFC at either 10°C or 5°C did not correlate significantly with the whipping properties (Table 8.7, $P > 0.1$). Previous studies suggested that the efficiency of partial coalescence reached maximum at an SFC of around 25% (relative to that at 20-25°C in the present study), which decreased as the SFC increased further due to the limitation

of liquid fat for wetting of the fat crystals (Fredrick et al., 2010; Hinrichs & Kessler, 1997; McClements, 2015). Probably arising from this effect, the whipping time of cream was reported to decrease as the whipping temperature increased from 5°C to 15°C (Ihara et al., 2010). The lack of significant correlation between SFC and whipping time in the present study could be attributed to the high overall SFC level (74.2% at 5°C, 61.3% at 10°C). At SFC of 60% and greater, the decrease in the propensity of partial coalescence with increasing SFC was considerably less pronounced than that at a lower SFC range (McClements, 2015). Besides, the significant impacts of other factors (e.g. fat globule size) probably overwhelmed the influence of SFC on the whipping properties.

8.4 Conclusions

This study investigated seasonal variations in various aspects of New Zealand milk fat. The variation patterns of de-novo synthesized fatty acids and long-chain fatty acids from early to mid-season were consistent with previous studies during the lactation cycle and those in seasonal calving countries, which can be explained by the recovery from the negative energy balance and the adipose mobilization after calving. Odd-chain fatty acids (C15:0 and C17:0) varied during the season in ways that could also be explained by adipose mobilization in the early lactation. However, minor differences in the variation patterns between the current study and previous works were found, particularly from mid- to late season. The consumption of fresh grass might play a part in affecting the shift in the fatty acid composition of milk during the lactation cycle. The CLA (C18:2 *cis* 9, *trans* 11) content in milk fat increased significantly in the late season, which correlated with the unsaturations indices. This indicated that the activity

of Δ^9-desaturase also elevated in the late season, contributing to a higher concentration of CLA in milk fat. Fat globule size decreased as the season progressed, correlating with the contents of some individual fatty acids. These correlations were largely consistent with previous reports on the fatty acid composition of fat globules fractionated by their sizes.

The melting behaviour of milk fat correlated with fatty acid composition, particularly C16:0 and C18:1 *cis*. The solid fat contents at 5°C and 10°C was highest in the mid-season, arising from the shift from low melting fraction to high melting fraction from early season to mid-season. The observed seasonal variations were consistent with previous reports in seasonal calving countries.

Early-season whipping cream had the best properties including the shortest whipping time, the highest overrun and the highest whipped cream firmness, whereas whipping cream produced from late-season milk had the least desirable properties. Among the whipping properties, whipping time of unhomogenized whipping cream (35% fat) varied the most over the seasons. The effects of fat globule size, protein content, pH, ionic calcium and solid fat content on whipping properties were discussed, mostly relating to their roles in the partial coalescence process during whipping. In particular, fat globule size had the most pronounced effect on whipping time. Larger fat globules probably promote partial coalescence by both enhancing close contact among the fat globules and facilitating the formation of more and larger fat crystals.

Chapter 9 - Age gelation of UHT milk: seasonal variations and possible mechanisms

9.1 Introduction

Ultra-high temperature (UHT) milk has a significant share in the global dairy market due to its long shelf life, typically several months at ambient temperatures. The stability of UHT milk allows transportation of the product across countries, thus expanding the market. One major problem with UHT milk products during storage is the irreversible gel formation, commonly referred to as “age gelation”.

Efforts have been made over the past few decades to understand and control the gelation of UHT milk. Several mechanisms of age gelation have been proposed as caused by proteolysis by plasmin, proteolysis by bacterial protease or a physicochemical process. These mechanisms are reviewed in Section 2.4.2. Despite extensive studies on this topic, the understanding of age gelation is far from clear. The development of gelation and the physicochemical changes during storage vary in different studies, due to a number of factors, including milk properties, heating method, preheating and storage conditions. Further studies are still needed to unravel the complex process of age gelation.

Understanding the gelation behaviour of UHT milk is of particular importance for the New Zealand dairy industry. Furthermore, the significant impact of seasonality on milk properties in seasonal calving countries (New Zealand, Australia and Ireland) adds to the complexity of elucidating the seasonal variations in age gelation. A few studies

conducted in Australia indicated that early-season UHT milk was more prone to age gelation than late-season UHT milk (Auldist 1996 Hardham 1998 Zadow & Chituta, 1975). However, Anema 2019 suggested that this seasonal pattern is not expected because early-season milk is low in protein and calcium, which would reduce the propensity of gelation.

The aim of this study was to investigate the changes in the physicochemical properties and the occurrence of age gelation in indirect UHT milk manufactured in different seasons in New Zealand. The gelation behaviour of both UHT skim milk and UHT whole milk were monitored. In addition, the possible mechanism of age gelation was discussed.

9.2 Materials and methods

9.2.1 UHT milk processing

Seasonal raw milk was sampled in the 2016-2017 season for the manufacture of UHT milk. Raw milk pasteurization and skimming were performed as described in Section 3.1. UHT skim milk samples were produced using pasteurized skim milk. UHT whole milk was standardized to 3.3% fat by mixing pasteurized skim milk and cream (~40% fat) and homogenized in a two-stage homogenizer at 70°C. The homogenization pressure was 15 MPa in the first stage and 5 MPa in the second stage. The UHT processing was carried out in a pilot-scale indirect UHT plant described in Section 4.2.1. Prior to UHT milk processing, the UHT system was sterilized at 120°C for 20 min. Milk was preheated to 75°C and then heated at 140°C for 5 s, after which the milk was cooled immediately to 20°C and filled aseptically into sterilised PET bottles (250

ml) in a laminar flow cabinet, sealed with screw caps. The number of UHT skim milk and UHT whole milk samples produced are shown in Table 9.1. These samples were produced in 3 batches in the early season, 5 batches in mid-season and 5 batches in late season. Whole milk samples were only studied for their age gelation behaviour and not further characterized as for UHT skim milk. Unless otherwise specified, "UHT milk" is referring to UHT skim milk in the results and discussion section.

Table 9.1: Number of bottles of UHT milk samples produced in each season

	Early season	Mid-season	Late season	Total
Skim milk	31	53	61	145
Whole milk	29	33	42	104

9.2.2 Storage test

UHT milk was stored in a temperature-controlled room at 30°C. The storage temperature of 30°C was chosen to accelerate gelation based on previous reports that the optimum temperature for age gelation of UHT milk was 25-30°C. Storage temperatures equal to or greater than 35°C were consistently found to prevent gelation (Datta & Deeth, 2001; Malmgren et al., 2017; Manji et al., 1986). The formation of sediment and the onset of gelation was monitored every two weeks. After storage for 2, 4, 6, 8 months, UHT skim milk samples from each batch were taken to be analysed.

9.2.3 Characterization of physicochemical properties

The composition of seasonal raw milk and the skim milk used for UHT milk production, including protein, fat, lactose, total solids, solids-non-fat, total calcium and inorganic phosphorus, was determined as described in Section 3.2.1.

The pH, ionic calcium (Ca^{2+}), casein micelle size of raw milk and UHT skim milk (fresh and stored) were analysed as described in Section 3.2.2 and Section 3.2.4.

9.2.4 Protein composition

The changes in proteins during storage of UHT milk were analysed indicatively by reducing SDS-PAGE and quantitatively by RP-HPLC.

Reducing SDS-PAGE as described by Oldfield (1996) was used to visualize the changes in milk proteins. UHT milk samples produced in the same batch stored for different durations were diluted in SDS-PAGE sample buffer (1: 40, v: v), kept at -20°C until analysed on the same gel.

RP-HPLC described in Section 3.2.7 was used to quantify the protein composition of UHT milk and milk serums stored for 0, 2, 4, 6 and 8 months on a percentage basis. Milk serum was obtained by ultracentrifugation at 88,000g for 60 min at 20°C and taking the supernatant phase. Milk and serum samples were stored at -20°C until analysis. UHT milk samples from the same batch stored for different durations were analysed together by HPLC. The relative protein contents indicated by peak areas in stored UHT milk were compared with those in fresh UHT milk.

9.2.5 Centrifugal sedimentation of UHT milk

A previously described centrifugal sedimentation method was used for the determination of heat stability (Chen, Grandison, & Lewis, 2012). UHT milk samples stored overnight were well mixed before 45.0 g was accurately weighed into a 50 ml centrifuge tube. The samples were then centrifuged at 2,760 g for 30 min. The supernatant was removed and the sediment was oven-dried at 105°C to constant weight. The amount of sediment was expressed as percentages in 45.0 g of milk.

9.2.6 Transmission electron microscopy (TEM)

The structure of casein micelles in raw, fresh UHT milk and stored UHT milk was imaged using TEM as described in Section 3.2.8.

9.3 Results and discussion

9.3.1 Milk composition

Table 9.2 shows the composition of major components in the pasteurized skim milk used for the production of UHT milk samples. The contents of protein, total solids and solid-non-fat were significantly higher in the late season, whereas the lactose content of late-season milk was the lowest during the year. The patterns of seasonal variation of these components were consistent with those reported in Chapter 4 across two complete milking seasons. This implies that the milk sampled for the study is largely representative of the overall seasonal variations in milk composition. The remaining fat content of the skim milk was around 0.1% across different seasons, thus it would not be a factor contributing to the variation in UHT milk properties among seasons. Table 9.2

also shows the concentrations of calcium and inorganic phosphorus in raw milk prior to skimming. They were both highest in the late season, consistent with that discussed in Chapter 4.

Table 9.2: Composition of source milk

	Season	Means ± SE
Protein - skim milk (%)	Early	3.58 ± 0.04^{b}
	Mid	3.82 ± 0.08^{b}
	Late	4.49 ± 0.13^{a}
Fat - skim milk (%)	Early	0.12 ± 0.03
	Mid	0.13 ± 0.02
	Late	0.09 ± 0.01
Lactose - skim milk (%)	Early	5.22 ± 0.03
	Mid	5.21 ± 0.03
	Late	5.08 ± 0.05
Total solids - skim milk (%)	Early	9.46 ± 0.06^{b}
	Mid	9.67 ± 0.07^{b}
	Late	10.22 ± 0.12^{a}
Solids-non-fat - skim milk (%)	Early	9.28 ± 0.06^{b}
	Mid	9.50 ± 0.06^{b}
	Late	10.01 ± 0.11^{a}
Calcium*(mM)	Early	27.0 ± 0.3^{b}
	Mid	29.1 ± 0.7^{b}
	Late	31.6 ± 0.6^{a}
Inorganic phosphorus* (mM)	Early	25.5 ± 0.4^{b}
	Mid	27.9 ± 0.7^{ab}
	Late	29.5 ± 0.5^{a}

[a, b] Means of the same parameter within a column with different lowercase superscripts differ ($P < 0.05$, one-way ANOVA). SE, standard error.
*The contents of calcium and inorganic phosphorus were determined in raw milk prior to skimming.

9.3.2 Physicochemical properties of raw milk and UHT milk

Table 9.3 shows some characteristics of raw milk and fresh UHT skim milk. Compared with raw milk, UHT milk had lower Ca^{2+} concentration ($P < 0.01$) and to a lesser extent, lower pH ($P = 0.07$). These changes induced by UHT presumably arose from the transfer of calcium from the serum phase to the micellar phase during heating and the simultaneous release of hydrogen ion (as discussed in Chapter 4 and Chapter 7). There was no significant seasonal variation in the pH of milk either before or after UHT treatment. As for the Ca^{2+} in milk, UHT heating appeared to shift the seasonal variation trend. Early-season and late-season raw milk had significantly higher Ca^{2+} than raw milk from mid-season. Whereas in UHT milk, the Ca^{2+} concentrations of milk were similar in early season and mid-season, significantly lower than that in the late season. Similar to the observation in Chapter 4 across the whole 2016-2017 season, the extent of heat-induced Ca^{2+} reduction was highest in the early season, followed by mid-season and was lowest in late season.

Table 9.3: Physicochemical characteristics of raw milk and UHT skim milk

	Season	Raw	UHT
pH	Early	6.71 ± 0.02	6.68 ± 0.02
	Mid	6.66 ± 0.01	6.65 ± 0.01
	Late	6.68 ± 0.01	6.65 ± 0.02
Ionic calcium (mM)	Early	2.22 ± 0.07[a]	1.85 ± 0.07[b]
	Mid	2.04 ± 0.01[b]	1.89 ± 0.01[b]
	Late	2.19 ± 0.03[a]	2.10 ± 0.02[a]
Casein micelle diameter (nm)	Early	157 ± 3	195 ± 3[b]
	Mid	162 ± 1	203 ± 6[ab]
	Late	159 ± 1	219 ± 4[a]
Serum phase κ-casein in total κ-casein (%)	Early	4.3 ± 0.9[b]	34.4 ± 2.5
	Mid	6.6 ± 0.2[b]	38.9 ± 2.4
	Late	11.0 ± 1.4[a]	35.0 ± 2.9
Whey proteins denaturation (%)	Early		92.9 ± 2.3
	Mid		93.8 ± 0.3
	Late		93.6 ± 1.2
Micelle-associating whey proteins (%)	Early		66.4 ± 2.4[b]
	Mid		67.7 ± 2.6[b]
	Late		78.6 ± 2.6[a]
Sediment (wt %)	Early		0.056 ± 0.008
	Mid		0.067 ± 0.005
	Late		0.075 ± 0.005

[a, b] Means of the same parameter within a column with different superscripts differ ($P < 0.05$, one-way ANOVA).

The casein micelle size results (Table 9.3) agreed with the observations across the two full milking seasons in Chapter 4. In agreement with previous reports, UHT treatment induced a significant increase in the hydrodynamic diameter of the casein micelles (Chen et al., 2015a; Gaucher et al., 2008a). As discussed in Chapter 4, such an increase might be caused by some initial aggregation among the partially-κ-CN-depleted casein micelles. There was no significant variation in the casein micelle size of raw milk.

However, in UHT milk, the casein micelle size increased significantly as the season progressed.

The fraction of κ-CN presenting in the serum phase of raw milk samples was significantly higher in the late season than in early season or mid-season, consistent with that observed in Chapter 4. UHT treatment induced significant dissociation of κ-CN from the casein micelles (Anema & Klostermeyer, 1997; Donato et al., 2007b; Singh & Fox, 1985). The amount of serum-phase κ-CN in UHT milk was not significantly different across the seasons, similar to that found in Chapter 4.
The extent of whey protein denaturation did not vary significantly during the season (Table 9.3). However, late-season milk had significantly higher proportions of whey proteins associating with the casein micelles, agreeing with the results of Chapters 4 and other previous reports (Auldist et al., 1996b; Oldfield, 1996). Some studies suggested that higher extent of whey protein denaturation and whey protein-casein micelle association might protect the micelles against gelation (Auldist et al., 1996b; García-Risco et al., 1999). In contrast, Anema (2017) reported that the extents of whey protein denaturation were similar in both gelled UHT milk and non-gelled UHT milk. Therefore, the denaturation of whey proteins is not likely to be a major factor in age gelation.

The average amount of dry sediment in UHT milk was 0.067%. This was consistent with the extent of sedimentation in UHT skim milk reported by Grewal, Chandrapala, Donkor, Apostolopoulos, and Vasiljevic (2017b) using the same centrifugal force, but significantly lower than that reported by Chen et al. (2015a) in UHT whole milk. The involvement of milk fat, the differences in milk properties and the heating equipment

between the studies might have contributed to this disagreement. The mean amount of sediment in UHT milk increased with the proceeding seasons, although the variation was not statistically significant (Table 9.3, $P = 0.15$). Nevertheless, the amount of dry sediment correlated with the casein micelle size in UHT milk ($r = 0.615$, $P = 0.025$). It is proposed in Chapter 4 that the aggregation among some κ-CN-depleted casein micelles could be the main contributor to the heat-induced increase in the average casein micelle size of UHT milk. This hypothesis along with the correlation between the casein micelle size and the amount of sediment agreed with Gaur et al. (2018), who reported that the sediment in UHT milk (both direct and indirect heating methods) consisted of mainly κ-CN-depleted casein micelles. One difference between the increase in casein micelle size and sedimentation is the size of the aggregates, which is determined by the extent of aggregation. Aggregates formed by 2 or 3 micelles might contribute to an increase in apparent casein micelle size but these aggregates may not be sedimentable under the centrifugation conditions. The large aggregates that sediment under centrifugation would be removed by filtering prior to size determination. Furthermore, the sedimentation and aggregation of κ-CN-depleted casein micelles under proper physicochemical environments (e.g. pH and Ca^{2+}) was recently proposed to be a crucial step in the development of age gelation at the bottom of the container (Anema, 2017, 2019). The potential correlations between κ-CN depletion, increase in the apparent casein micelle size, sediment formation and age gelation will be discussed later in Section 9.3.7.

9.3.3 Incidence of age gelation

Table 9.4: The occurrence of age gelation in UHT skim milk and UHT whole milk

	Total	2 month	4 month	6 month	8 month
Skim milk	145	[1]10 (6.9%)	19 (13.1%)	22 (15.2%)	23 (15.9%)
Whole milk	104	1 (1.0%)	3 (2.9%)	7 (6.7%)	9 (8.7%)

[1]Numbers indicate the accumulated number of samples gelled during storage. The percentages in parentheses imply the corresponding percentage of the number in the total number of samples

Table 9.4 shows the number and percentage of UHT milk samples gelled during storage. In all samples, a gel-like sedimentation layer developed at the bottom of the container and grew in height gradually. In most gelled samples, the volume of the gel occupied 30-70% of the container, in agreement with the gelation behaviour of direct-UHT milk with severe preheating conditions reported by Anema (2017). The volume of the gels in late-season samples, being greater than 50% of the milk volume in most cases, tended to be larger than those in early- and mid-season samples. This difference might arise from the higher protein and solids contents of late-season milk (Table 9.2). A correlation between the gel volume in UHT milk and milk concentration was also observed by Anema (2017).

Compared with UHT skim milk, UHT whole milk had a significantly lower occurrence rate of age gelation. In addition, the gelation in UHT skim milk developed faster, with 19 samples gelled in the first 4 months and 4 more samples gelled during 4 to 8 months of storage. In contrast, for UHT whole milk, the gelation occurred more evenly across the storage duration of 8 months. This was consistent with previous reports showing

that age gelation was less likely to occur in UHT whole milk than in UHT skim milk (Datta & Deeth, 2001; García-Risco et al., 1999).

Previous studies proposed a few explanations for the lower incidence of age gelation in UHT whole milk compared with skim milk. First, it was suggested that the lower extent of proteolysis in UHT whole milk compared with UHT skim milk (Deeth, Khusniati, Datta, & Wallace, 2002; Kelly & Foley, 1997; López-Fandiño, Olano, Corzo, & Ramos, 1993) might play a part in retarding the age gelation of UHT whole milk (Datta & Deeth, 2001; García-Risco et al., 1999). Second, the higher extent of whey protein-casein micelle association in whole milk compared with skim milk was suggested to contribute to its lower incidence of gelation (Datta & Deeth, 2001; García-Risco et al., 1999) and slower development of sedimentation (Grewal et al., 2017a; Grewal et al., 2017b) during storage. It was suggested that a greater level of whey protein association in UHT whole milk better protected the casein micelles against both the attack of proteases (Grewal et al., 2017a) and the micelle-micelle aggregation (Auldist et al., 1996b; García-Risco et al., 1999). Besides, serum β-LG in UHT milk was suggested to be more active than the β-LG associating with casein micelles in promoting protein-protein interactions, which might be the reason for the more pronounced development of sedimentation in UHT skim milk than whole milk (Grewal et al., 2017a; Grewal et al., 2017b).

Finally, the adsorption of casein micelles onto the fat droplets after homogenization might hinder the physicochemical gelation of UHT milk as proposed by Anema (2017), in which the slow sedimentation of κ-CN-depleted casein micelles during storage was suggested to initiate the gelation process. Casein micelles adsorbed onto the fat droplets

would have a lower apparent density than the micelles in skim milk, resulting in a lower sedimentation rate. Furthermore, as the protein to fat ratio decreases, the fat-associating casein micelles remain suspending or even ascend with the fat droplets to the top of the sample. Similarly, Grewal et al. (2017b) attributed the slower development of sedimentation in UHT whole milk than in UHT skim milk during storage to the potential hindrance from the fat droplets that were low in density. This hypothesis could explain the lower occurrence of gelation and the slower gelation development of UHT whole milk compared with skim milk in the present study.

Table 9.5 shows the occurrence of gelation accumulated over the storage period of UHT milk produced in different seasons. Among the 31 skim milk samples and 29 whole milk samples produced in the early season, only one skim milk sample gelled during the storage period. As for UHT milk produced in mid-season and late season, they had similar occurrence rates of gelation during storage that were significantly higher than that of early-season UHT milk. By the end of the 8 months storage period, around 19% of skim milk samples and 12% of whole milk samples produced in the mid-season and the late season gelled.

Table 9.5: Seasonal variation in the occurrence of age gelation during the storage of UHT milk

		Total	2 months	4 months	6 months	8 months
Skim milk	Early	31	[1]0	0	1 (3.2%)	1 (3.2%)
	Mid	53	4 (7.5%)	10 (18.9%)	10 (18.9%)	10 (18.9%)
	Late	61	6 (9.8%)	9 (14.8%)	11 (18.0%)	12 (19.7%)
Whole milk	Early	29	0	0	0	0
	Mid	33	0	1 (3.0%)	3 (9.1%)	4 (12.1%)
	Late	42	1 (2.4%)	2 (4.8%)	4 (9.5%)	5 (11.9%)

[1]Numbers indicate the accumulated number of samples gelled during storage. The percentages in parentheses imply the corresponding percentage of the number in the total number of samples

These findings contradicted previous reports that early-season UHT milk produced in Australia was more likely to gel during storage than UHT milk produced in the late season (Auldist et al., 1996b; Hardham, 1998; Zadow & Chituta, 1975). Different gelation mechanisms arising from the differences in processing condition and milk property might have caused the disagreements between the present study and the previous reports. Zadow and Chituta (1975) used direct steam injection as the UHT treatment method, which was demonstrated to be less effective in inactivating the plasmin system in milk compared with indirect UHT treatment as used in the present study (Anema, 2019; Datta & Deeth, 2001; Manji et al., 1986). The combination of a less intense heating method and the higher plasmin and plasminogen-derived activity of

late-season milk (Auldist et al., 1996b; Nicholas et al., 2002) could inhibit the development of gelation of late-season UHT milk in the work of Zadow and Chituta (1975) by excessive proteolysis (Auldist et al., 1996b). Besides, both Auldist et al. (1996b) and Hardham (1998) observed unusual gelation behaviour of the UHT milks that the gel layers formed at the surface of the samples instead of from the bottom. This observation differed from the present study and other previous reports (Anema, 2017; Kelly & Foley, 1997; Newstead et al., 2006). It appeared that different mechanisms might be responsible for the different age gelation behaviours and the different patterns of seasonal variations. As raised by Anema (2019), early-season milk with lower contents of protein and calcium would be expected to have lower tendencies for gelation.

9.3.4 Physicochemical changes during storage of UHT milk

9.3.4.1 pH

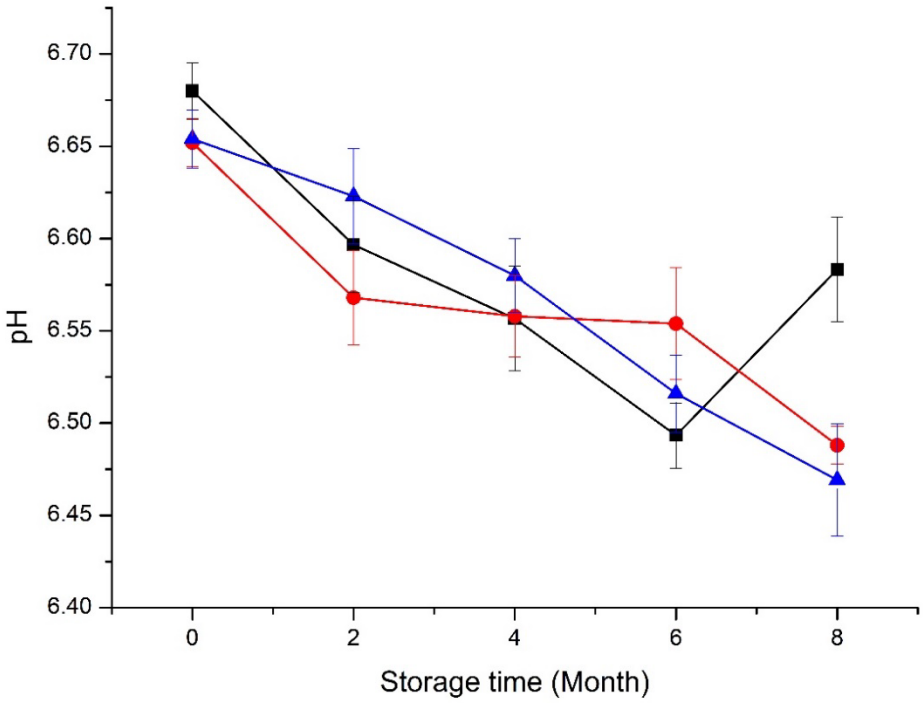

Figure 9.1: The pH of stored UHT milk made in early season (■), mid-season (●) and late season (▲).

Figure 9.1 shows the changes in pH of UHT skim milk during storage at 30°C for up to 8 months. Overall, the pH of UHT milk decreased during storage, consistent with numerous previous reports (Anema, 2017; Gaucher et al., 2008a; Grewal et al., 2017b; Manji et al., 1986; Newstead et al., 2006; Rauh et al., 2014; Venkatachalam et al., 1993; Zadow & Chituta, 1975). The Maillard reaction (Anema, 2017; Auldist et al., 1996b; Gaucher et al., 2008b; Grewal et al., 2017b; Venkatachalam et al., 1993) and proteolysis (Gaucher et al., 2008b; Kelly & Foley, 1997; Rauh et al., 2014) were suggested to be responsible for the reduction in the pH of UHT milk during storage, the rates of both increase at higher storage temperature. Until 6 months of storage, no clear

difference was found in the patterns of pH reduction between UHT milk produced in different seasons. However, an unexpected rise in pH from 6 months to 8 months of early-season UHT milk was observed (Figure 9.1). All of the 3 batches of early-season UHT milk had higher pH at 8 months of storage than that at 6 months of storage. The reason for the rise in pH is unknown. Similarly, Anema (2017) observed unexpected fluctuations in the pH during the storage of UHT milk and suggested that these might be artefacts of variations in temperature and the calibration of the electrodes across the wide time span of the study.

9.3.4.2 Ionic calcium

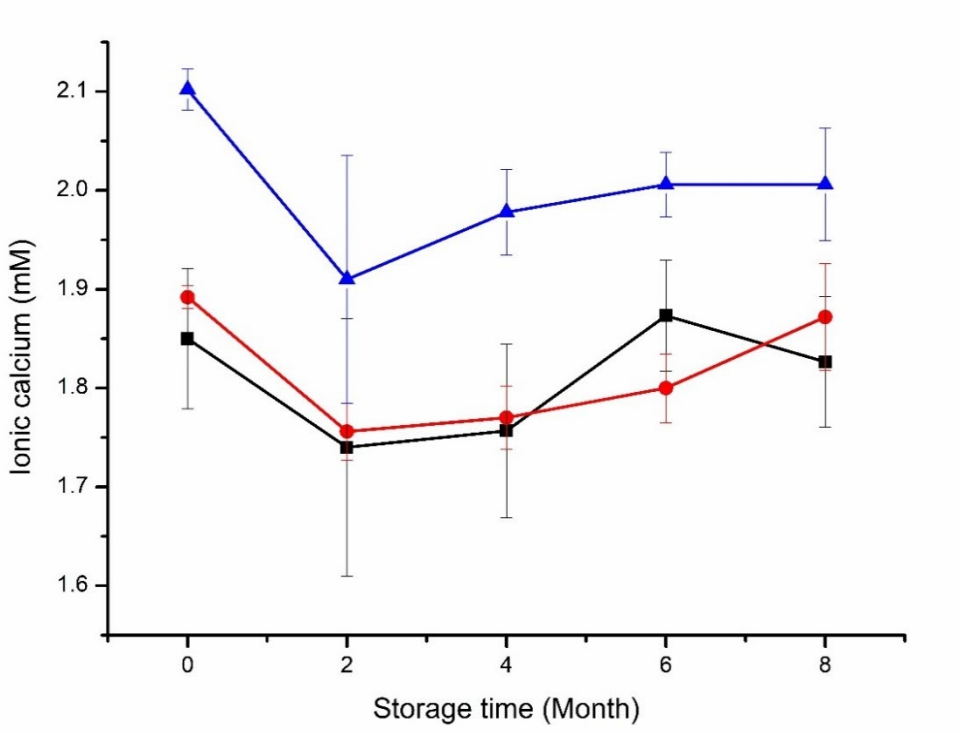

Figure 9.2: The ionic calcium concentration of stored UHT milk made in early season (■), mid-season (●) and late season (▲).

Figure 9.2 shows the storage effects on the ionic calcium (Ca^{2+}) concentration of UHT milk produced in different seasons. The Ca^{2+} concentration in late-season UHT milk remained to be the highest among different seasons during the storage period of 8 months. During the first 2 months of storage, the Ca^{2+} of UHT milk produced in all seasons decreased by 0.1 to 0.2 mM (Figure 9.2). Late-season UHT milk had the highest extent of Ca^{2+} reduction during the seasons, both by concentration (0.19 mM) and by percentage (9.2%). From 2 months to 8 months of storage, the Ca^{2+} concentration in UHT milk slowly increased, except for that the early-season milk stored for 6 months had higher Ca^{2+} than those stored for 8 months.

Considering the Ca^{2+} - pH equilibrium in milk (see Equation 7.1), a drop in the pH of milk during storage is expected to promote solubilization of calcium in milk, thus an increase in the Ca^{2+} concentration (Broyard and Gaucheron 2015). This might explain the changes in the pH and Ca^{2+} in UHT milk from 2 months to 8 months of storage. In addition, the unexpected increase in pH of early-season samples stored for 8 months compared with those stored for 6 months (Figure 9.1) coincided with a drop in Ca^{2+} from 6 months to 8 months. This suggested that the gradual decrease in pH of UHT milk might play a role in the moderate increase in Ca^{2+} during prolonged storage. In contrast, the decrease in Ca^{2+} in UHT milk during the first 2 months of storage could not be explained by the change in pH. Some other mechanism appeared to be responsible for this change in Ca^{2+}. One possibility was that the storage of UHT milk at a higher-than-ambient temperature of 30°C affected the calcium equilibrium that some ionic calcium transferred into the casein micelles (Broyard & Gaucheron, 2015; Koutina, Knudsen, Andersen, & Skibsted, 2014; Lewis, 2011). Although the Ca^{2+} in milk was determined at the same temperature of 20 ± 2°C in the present study, a shift in the calcium equilibrium due to prolonged storage at 30°C might have not recovered prior to the Ca^{2+} measurement. A few previous studies investigated the changes in the ionic calcium or soluble calcium fractions during the storage of UHT milk, but the results reported were not consistent (Anema, 2017; Gaucher et al., 2008b; Grewal et al., 2017b).

9.3.4.3 Casein micelle size

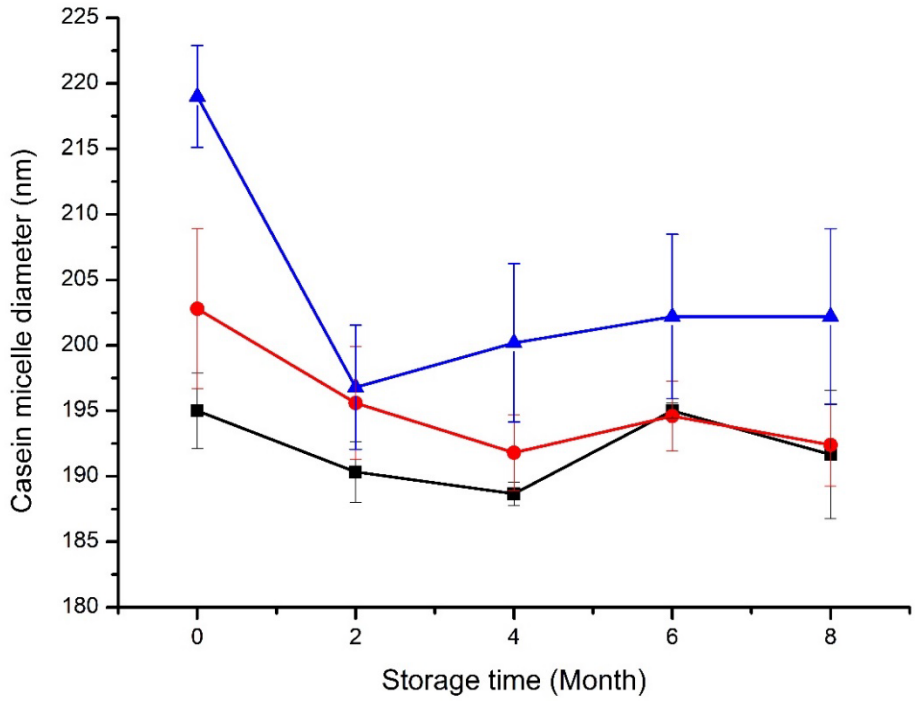

Figure 9.3: The mean hydrodynamic diameters of casein micelles in stored UHT milk made in early season (■), mid-season (●) and late season (▲).

Figure 9.3 shows the change in the casein micelle size of UHT milk stored at 30°C. Decreases in micelle size during the initial 2 to 4 months of storage were observed in UHT milk samples produced in all seasons. During prolonged storage of 4 to 8 months, the casein micelle size of UHT milk was relatively stable, regardless of the season. Due to the larger decrease in casein micelle size of late-season milk, the variation among seasons in the casein micelle size of fresh UHT milk (Table 9.3) was no longer significant after storage of 2 to 8 months.

The initial decrease in casein micelle size during the first 2 months of storage coincided with the decrease in Ca^{2+} (Figure 9.2). An increase in the amount of CCP and stronger hydrophobic interactions at 30°C might result in a more compact micellar structure,

which might be the reason for the decrease in casein micelle size in the UHT milk stored for 2 months. This hypothesis was supported by the fact that late-season UHT milk had the most significant decrease in casein micelle size during the first 2 months of storage, corresponding to its largest extent of Ca^{2+} reduction in the same period (Figure 9.2). Beliciu and Moraru (2009) attributed the reduced casein micelle size of milk at 50°C compared with the micelle size at 20°C to the shrinking effects caused by the greater hydrophobic interactions and the lower solubility of calcium phosphate at a higher temperature. Similarly, Anema (2019) suggested that the storage of UHT milk at a higher temperature would result in a lower level of ionic calcium and a decrease in casein micelle size.

9.3.5 Changes in protein profiles of UHT milk during storage

9.3.5.1 SDS-PAGE

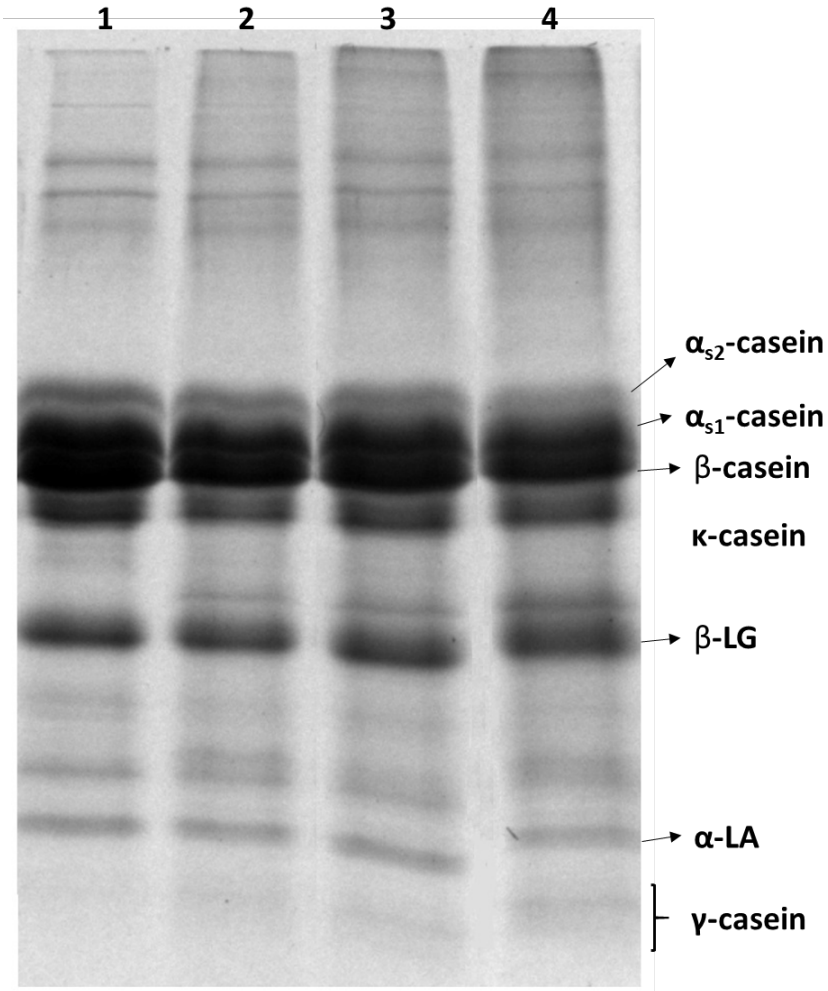

Figure 9.4: Indicative reducing SDS-PAGE patterns of UHT milk stored for 0 months (Lane 1), 2 months (Lane 2), 4 months (Lane 3) and 8 months (Lane 4).

Figure 9.4 shows the indicative patterns of a UHT milk sample in reducing-SDS-PAGE during storage. There appeared to be a subtle decrease in the intensity of bands after 8 months of storage (Figure 9.4), the extent of which was far lower compared with other studies using direct UHT treatments with mild preheating conditions (Anema, 2017; García-Risco et al., 1999; Newstead et al., 2006). This indicated that the indirect UHT treatment used in the current study effectively suppressed the activities of the proteases

in milk, in agreement with the previous reports that indirect heating better inactivates the proteases, particularly plasmin, than direct heating methods (Anema, 2019; Datta & Deeth, 2001; Kelly & Foley, 1997). Nevertheless, bands of γ-CN showed up and slowly intensified during storage (Figure 9.4), implying some β-CN hydrolysis by plasmin. This was consistent with previous reports that the moderate proteolysis resulted from plasmin or plasminogen-derived activity occurred in indirectly heated UHT milk (Auldist et al., 1996b; Kelly & Foley, 1997; Manji et al., 1986). Manji et al. (1986) reported that indirect UHT treatment deactivated 100% of plasmin and 81% of plasminogen. The remaining plasminogen transformed into plasmin during storage and caused proteolysis in the milk.

Besides proteolysis, other possible reasons for the reduction in the staining intensities of intact proteins are protein modifications and covalent protein interactions, e.g. lactosylation and Maillard reaction. Some higher molecular weight materials occurred above the caseins in reducing SDS-PAGE gels whose intensities increased during storage (Figure 9.4), indicating the development of non-disulphide covalent crosslinking among proteins. This observation agreed with previous reports on stored UHT milk, which was attributed to the covalent bonding via Maillard reaction (Andrews, 1975; Anema, 2019; Grewal et al., 2017b) or by lysinoalanine crosslinking (Holland, Gupta, Deeth, & Alewood, 2011). A band above β-LG emerged and developed gradually during the storage at 30°C (Figure 9.4) that might represent the lactosylated β-LG as indicated in previous studies (Dave, Loveday, Anema, Jameson, & Singh, 2014; Holland et al., 2011; Jongberg, Rasmussen, Skibsted, & Olsen, 2013). Lactosylation of whey proteins during the storage of UHT milk was demonstrated in

numerous previous reports (Anema, 2019; Czerwenka, Maier, Pittner, & Lindner, 2006; Holland et al., 2011; Malmgren et al., 2017; Rauh et al., 2015; Siciliano, Rega, Amoresano, & Pucci, 2000).

It is difficult to distinguish the contributions of proteolysis and covalent interactions to the reduction in the concentration of intact proteins during storage. For instance, the α_{s2}-CN displayed the highest reduction in peak intensity during storage among individual milk proteins (Figure 9.4). It was demonstrated that α_{s2}-CN both participated in the formation of the covalently-linked large protein aggregates (above caseins in Figure 9.4, Holland et al., 2011) and prone to the hydrolysis by plasmin (Datta & Deeth, 2001). Overall, the signs of protein modification and crosslinking appeared to be more evident than proteolysis (Figure 9.4). This topic will be discussed further with the qualitative protein composition results obtained by RP-HPLC.

9.3.5.2 HPLC profile

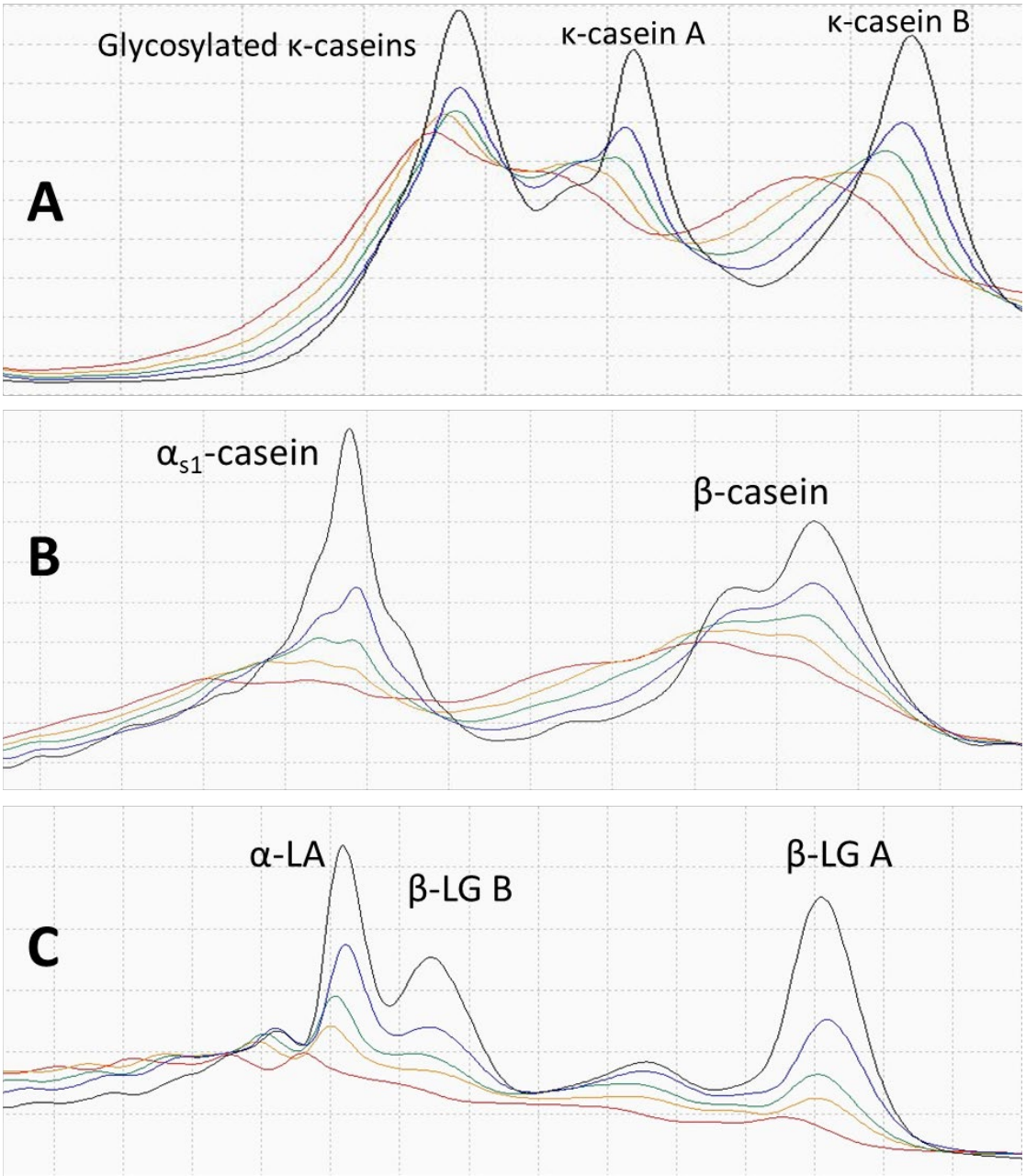

Figure 9.5: Representative HPLC profiles of (A) κ-CN, (B) α_{s1}-CN and β-CN and (C) α-LA and β-LG in UHT milk during storage. Fresh UHT milk (black), UHT milks stored for 2 months (blue), 4 months (green), 6 months (yellow) and 8 months (red).

Figure 9.5 shows the changes in the HPLC profiles of individual proteins during the storage of UHT milk. The peaks of all milk proteins in HPLC reduced in heights, broadened and shifted to the more hydrophilic side, i.e. eluted earlier, with increasing storage time (Figure 9.5). These changes in HPLC profiles, consistent with the SDS-PAGE results discussed in Section 9.3.5.1, indicated lactosylation of proteins during storage at 30° (Anema, 2019; Malmgren et al., 2017; Rauh et al., 2015; Siciliano et al., 2000). Changes in the protein profiles of stored UHT milk due to modifications were reported in numerous studies using HPLC (Czerwenka et al., 2006; Gaucher et al., 2008b; Rauh et al., 2014; Siciliano et al., 2000), capillary electrophoresis (García-Risco et al., 1999; Malmgren et al., 2017) and gel electrophoresis (Anema, 2017; García-Risco et al., 1999; Holland et al., 2011).

The extent of peak shifting in HPLC profiles appeared to be greatest for whey proteins (particularly β-LG), followed by α_s-CN and β-CN, and was least pronounced for κ-CN. This order agreed with previous reports on the rate of lactosylation of different milk proteins (Cardoso, Wierenga, Gruppen, & Schols, 2018; Czerwenka et al., 2006; Rauh et al., 2015; Scaloni et al., 2002). The identification and quantification of peaks might be affected by these modifications. The implications of such effects will be discussed below with the quantification results.

9.3.5.3 Changes in HPLC peak area of UHT milk during storage

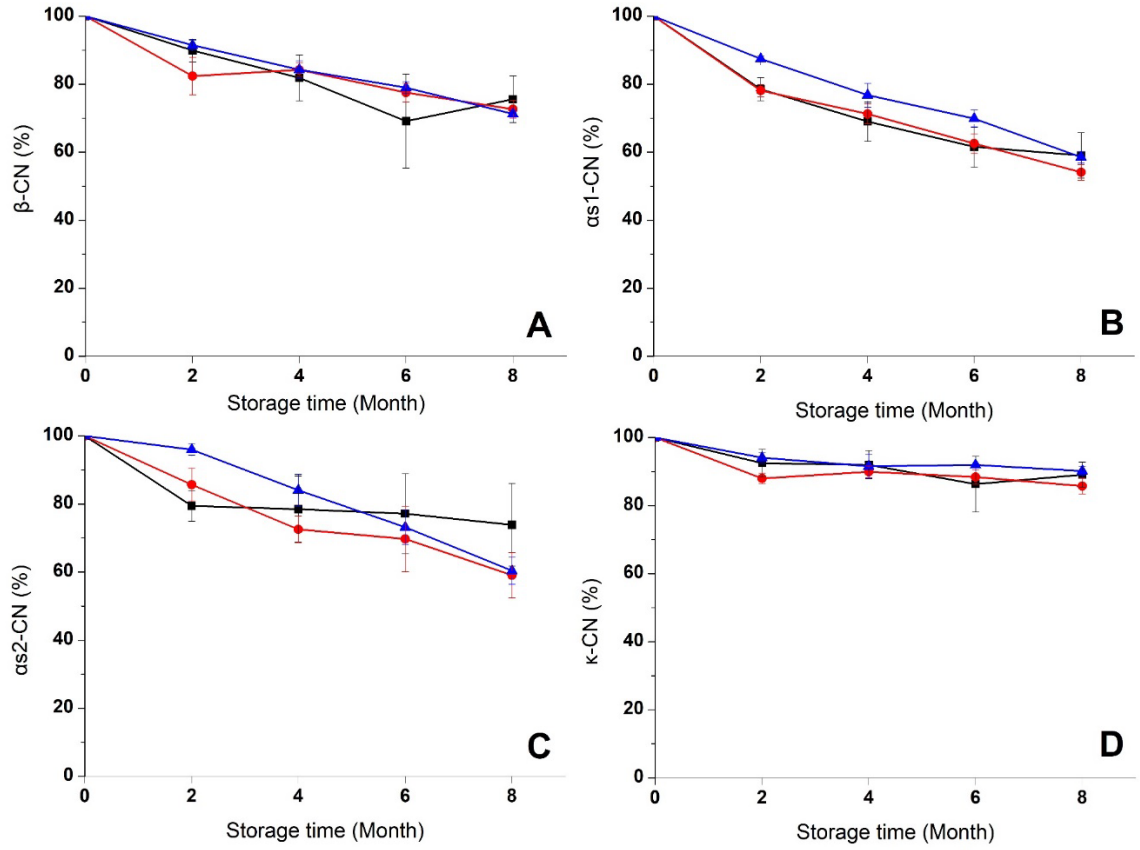

Figure 9.6: Changes in the relative peak area in HPLC of β-CN (A), α_{s1}-CN (B), α_{s2}-CN (C) and κ-CN (D) during the storage of UHT milk made in early season (■), mid-season (●) and late season (▲). The peak areas in fresh UHT milk sample (0 months) is defined as 100%.

Figure 9.6 shows peak areas of caseins in RP-HPLC during storage relative to the peak areas in fresh UHT milk. In UHT milk stored for 8 months, the peak areas of α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN reduced to 57.0%, 63.0% %, 72.8% and 88.2%, respectively, of those in fresh UHT milk. β-CN, as the primary substrate of plasmin, was not hydrolysed extensively during storage (Figure 9.6-A). The peak area of κ-CN was least affected during storage among caseins (Figure 9.6-D), ruling out the possibility of extensive proteolysis by bacterial proteases (Datta & Deeth, 2001; López-Fandiño et

al., 1993; Zhang et al., 2018). Rauh et al. (2014) also observed a slight decrease in the peak area of κ-CN in HPLC during storage of UHT milk and suggested that it was brought about by protein modifications rather than proteolysis. The HPLC results, consistent with the SDS-PAGE patterns (Figure 9.4), indicated that the proteolysis of caseins occurred during storage was limited, presumably due to the effective inactivation of plasmin by the indirect heating method applied. Besides, the modifications caused by Maillard reactions (e.g. lactosylation) might have affected the quantification results of the proteins. The higher reduction rates in peak area of the α_{s2}-CN and α_{s1}-CN than β-CN (Figure 9.6) suggested that the Maillard reactions might play a larger part than the proteolysis by plasmin, considering the reported higher lactosylation rates of α_s-CN compared with β-CN (Rauh et al., 2015) and the highest susceptibility of β-CN and α_{s2}-CN against plasmin among caseins (Datta & Deeth, 2001).

The changes in the peak area of caseins were similar among seasons, except for the slightly slower reduction of α_{s1}-CN ($P < 0.01$) and α_{s2}-CN ($P = 0.07$) during the first 2 months of storage in late-season UHT milk (Figure 9.6-B, C). The lower lactose to protein ratio of late-season milk might play a part in affecting the rate of lactosylation (Table 9.2).

When looking at the HPLC profiles of different fractions of κ-CN (Figure 9.7), it was observed that the peak area of the A variant of non-glycosylated κ-CN (NG-κ-CN) decreased significantly during storage (Figure 9.7-A), whereas the changes in the peak area of glycosylated κ-CN and NG-κ-CN B were minimal (Figure 9.7-B and C).

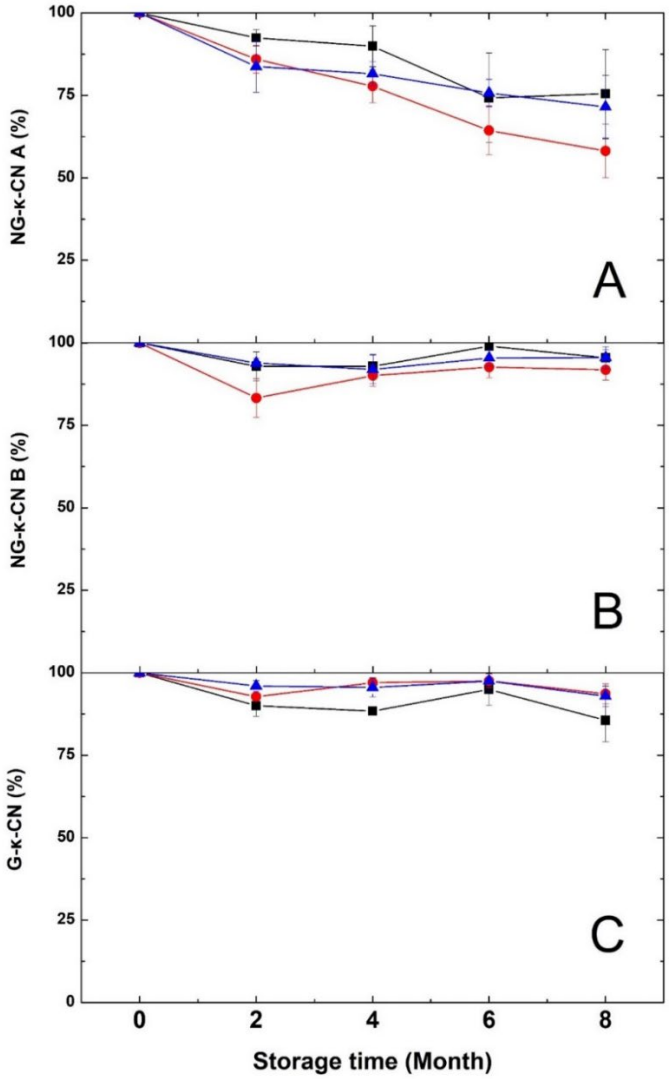

Figure 9.7: Changes in the relative peak area in HPLC of non-glycosylated κ-CN A (A), non-glycosylated κ-CN B (B) and Glycosylated κ-CN (C) during the storage of UHT milk made in early season (■), mid-season (●) and late season (▲). The peak areas in fresh UHT milk sample (0 months) is defined as 100%.

This might be an artefact in HPLC peak identification caused by lactosylation. The signs of modifications, indicated by the peak reduction and shifting in the HPLC profiles, appeared to be similar for κ-CN A and κ-CN B (Figure 9.5-A). However, part of the modified κ-CN A might have coeluted with, and identified as glycosylated κ-CN. Whereas the identification of modified κ-CN B did not seem to be affected, possibly

because of the relatively large difference in elution time between κ-CN B and the earlier peak, κ-CN A.

Significant reductions in the peak area of whey proteins, particularly β-LG, were found during the storage of UHT milk (Figure 9.8). After the storage of 8 months, the reduction in peak area compared with fresh UHT milk was 17.3 ± 3.3% for α-LA (Figure 9.8-A) and 58.9 ± 2.2% for β-LG (Figure 9.8-B). Numerous studies reported the broadening, shifting and reduction of the whey proteins peaks in HPLC in stored UHT milk (Czerwenka et al., 2006; Elliott, Datta, Amenu, & Deeth, 2005; Rauh et al., 2014; Siciliano et al., 2000) and attributed them to the lactosylation of the proteins rather than proteolysis. The shift in the HPLC elution patterns due to lactosylation might have contributed to the difference in the peak size reduction between α-LA and β-LG. Part of the lactosylated β-LG might have coeluted with α-LA in the HPLC (Figure 9.5-C). Furthermore, the higher rate of lactosylation of β-LG than α-LA, due to its higher number of lysine groups (Czerwenka et al., 2006; Rauh et al., 2015), could further contribute the greater extent of peak area reduction of β-LG than α-LA during storage.

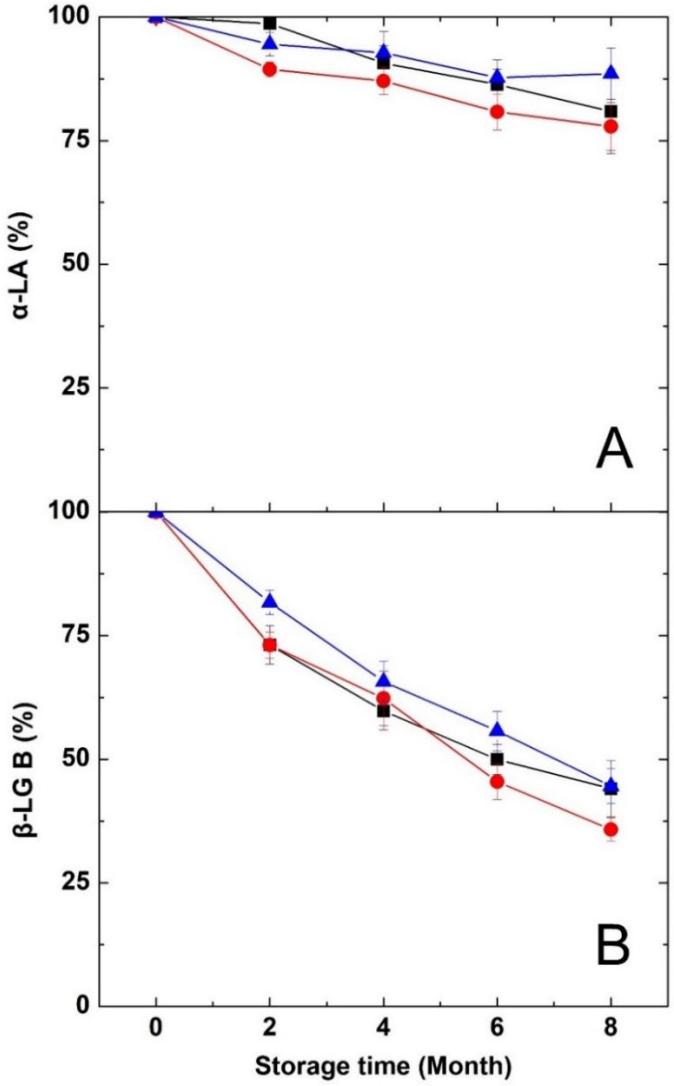

Figure 9.8: Changes in the relative peak area in HPLC of α-LA (A) and β-LG (B) during the storage of UHT milk made in early season (■), mid-season (●) and late season (▲). The peak areas in fresh UHT milk sample (0 months) is defined as 100%.

The patterns of peak area reduction during storage were similar across different seasons. The most significant seasonal difference was the slower reduction of β-LG in late-season milk during the first two months (P = 0.08, Figure 9.8-B), coinciding with the same pattern for α_{s1}-CN and α_{s2}-CN discussed above (Figure 9.6-B and C),

suggesting a slower initial lactoyslation rate of late-season milk, possibly due to their lower lactose to protein ratios (Table 9.2).

9.3.5.4 Changes in HPLC peak area of milk serum during storage

The protein composition of the serums of fresh and stored UHT milk was analysed using HPLC. It is important to note that a change in the serum protein composition of stored UHT milk could be caused by two mechanisms, i.e., 1) proteolysis or protein modifications, which could occur at different rates between the micellar phase and the serum phase 2) the transfer of proteins between the serum and the micelles.

To distinguish the two effects during storage, the results of serum protein composition are expressed in two ways. First, the “amount” of a protein in the serum phase of stored UHT milk is indicated by its peak area as a percentage of the protein peak area in fresh UHT milk (Figure 9.9-A, C, E, G, I). Second, the “proportion” of a protein in the serum phase in stored UHT milk is indicated by the percentage of the serum protein peak area in the peak area of the protein in the corresponding stored milk (Figure 9.9-B, D, F, H, J).

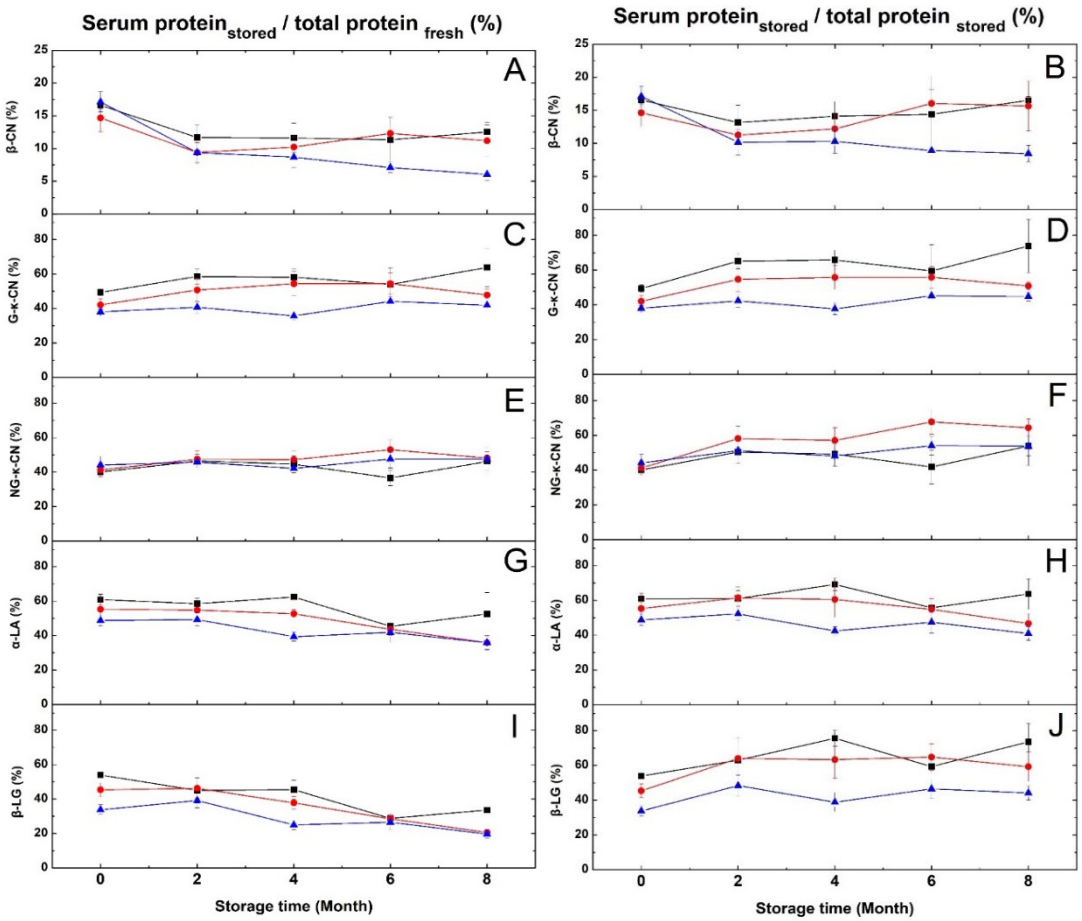

Figure 9.9: Changes in serum-phase proteins in UHT milk stored for up to 8 months, expressed as percentages of the corresponding proteins in fresh UHT milk (A, C, E, G, I) or in stored UHT milk (B, D, F, H, J). A and B: β-CN, C and D: glycosylated κ-CN; E and F: non-glycosylated κ-CN; G and H: α-LA; I and J: β-LG. Different legends indicate the mean values of samples from early season (■), mid-season (●) and late season (▲).

The results at 0 months shown in Figure 9.9 indicate the amounts of serum-phase proteins in fresh UHT milk. The proportions of β-CN in the serum of UHT milk did not vary with different seasons (Figure 9.9-A). Figure 9.9-C, E show that the proportion of G-κ-CN and NG-κ-CN in the milk serum had the opposite trends during the seasons (G-κ-CN, early-season > mid-season > late-season; NG-κ-CN, late-season > mid-season > early-season). Despite the insignificant variations of either G-κ-CN or NG-κ-

CN (G-κ-CN, $P = 0.07$, NG-κ-CN, $P = 0.79$), the serum: milk ratio of the glycosylation degree (GD) was significantly higher in early season than in late season ($P = 0.02$), consistent with the findings in Chapter 4 across the two milking seasons. The portions of both serum β-LG and serum α-LA followed the trend of early-season > mid-season > late-season (Figure 9.9-G, I). This agreed with the findings from Chapter 4 and previous reports that as the season progressed, the extent of heat-induced whey protein-casein micelle association increased and the part of denatured whey proteins remaining in the serum phase decreased (Auldist et al., 1996b; Oldfield, 1996).

The fraction of serum-phase β-CN became smaller during the first 2 months of storage in the UHT milk produce in all seasons (Figure 9.9-A). This might arise from the transfer of β-CN to the colloidal phase due to the storage at a higher-than-ambient temperature of 30°C (Broyard & Gaucheron, 2015; Liu, Weeks, Dunstan, & Martin, 2013). The drop in soluble β-CN coincided with the decreases in casein micelle size and Ca^{2+} during the first two months of storage (Figure 9.2 and Figure 9.3). All of the three changes were suggested to occur in milk stored at a higher temperature (Anema, 2019). During the storage from 2 to 8 months, the amount of soluble β-CN remained relatively stable for UHT milk produced in early season and mid-season, suggesting limited proteolysis in the serum phase. In contrast, the serum β-CN content dropped gradually during storage in late-season UHT milk (Figure 9.9-A). The proportion of serum β-CN (Figure 9.9-B) increased from 2 to 8 months in UHT milk from early and mid-season despite the rather stable content in the serum (Figure 9.9-A). Whereas in late-season milk, the proportion of serum β-CN still decreased during prolonged storage (Figure 9.9-B) but became less pronounced than the decreasing trend in the

amount of serum β-CN as the storage time increased (Figure 9.9-A). These differences indicated a progressively greater loss of β-CN from the micellar phase than in the serum phase. This was likely to be caused by the proteolysis of β-CN which occurred mostly in the micellar phase in milk, since plasmin, plasminogen, and the plasminogen activators are associated with the casein micelles, whereas the inhibitors of plasmin and plasminogen activators are mostly present in the serum phase (Ismail & Nielsen, 2010; Korycha-Dahl, Dumas, Chene, & Martal, 1983). It is unclear why the amount of serum β-CN decreased in late-season UHT milk. It appeared that part of the serum β-CN in late-season UHT milk might have transferred into the colloidal phase during prolonged storage.

For both G-κ-CN and NG-κ-CN in the serum phase, neither the amount (Figure 9.9-C, E) nor the proportion (Figure 9.9-D, F) varied significantly. No consistent changes in serum κ-CN were found during storage, besides the slight increases during the first 2 months in the amount of serum κ-CN (Figure 9.9-C, E), which were more evident by proportion (Figure 9.9-D, F). These changes indicate that the dissociation of micellar κ-CN into the serum phase might have occurred during the first two months of storage. Unlike serum β-CN, whose proportions in stored UHT milk (Figure 9.9-B) became progressively higher than their amounts (Figure 9.9-A), the differences between the proportions (Figure 9.9-D, F) and the amounts (Figure 9.9-C, E) of serum κ-CN were stable during storage. This implies that the changes occur preferentially in either the micellar or the serum phase, such as the hydrolysis by plasmin, did not play a major role in shifting the serum κ-CN composition. This agreed with previous reports that κ-

CN was the least susceptible casein again plasmin (Anema, 2019; Datta & Deeth, 2001).

The amount of α-LA and β-LG in the serum phase did not change greatly during the first 2 months, then gradually decreased from 2 to 8 months of storage (Figure 9.9-G, I). The reduction rate was higher for serum β-LG than for serum α-LA (Figure 9.9-G, I), similar to the trends of β-LG and α-LA in milk during storage (Figure 9.8-A and B). These changes in serum whey proteins might arise from the transfer between the serum and the micellar phases or protein modifications. The proportions of serum-phase α-LA and β-LG in UHT milk (Figure 9.9-H, J) did not vary significantly during storage, except for the increases in the proportion of serum-phase β-LG during the first 2 months of storage (Figure 9.9-J). This initial increase in the proportion of β-LG in the serum phase, coinciding with the increase in serum κ-CN during the first two months (Figure 9.9-D, F), suggested that the dissociation of κ-CN/β-LG complexes from the casein micelles might have taken place. However, these signs of the dissociation of κ-CN/β-LG complexes ceased from 2 to 8 months of storage (Figure 9.9-D, F, J). It is unclear whether the changes in serum β-LG and serum κ-CN during the first 2 months of storage-related with the concurrent decrease in Ca^{2+} concentration, shrinking in micelle size and reduction in serum β-CN contents that were possibly caused by the storage at a higher-than-ambient temperature of 30°C. Anema 2017 reported no significant change in serum phase proteins during the storage of UHT milk at 20°C. It is possible that the micellar rearrangements caused by the increase in storage temperature, e.g. shrinking and transfer of β-CN, could lead to the destabilization and dissociation of the κ-CN/β-LG complexes on the micelle surface. For instance,

shrinkage in micelle size might increase the steric and electrostatic repulsions among the protein complexes associating at the micelle surface and result in their dissociation. In summary, the serum phase protein composition of UHT milk did not change significantly during storage. This observation agreed with Anema (2017) but contradicted to Grewal et al. (2017b) who reported significant dissociation of caseins during storage. Grewal et al. (2017b) attributed the micellar dissociation to the simultaneous solubilization of CCP indicated by the considerable increase in serum calcium and ionic calcium during storage, which was also not observed in the current study or other previous reports on the soluble and ionic calcium of UHT milk during storage (Anema, 2017; Gaucher et al., 2008b).

The storage temperature of 30°C rather than the ambient temperature of around 20°C might have contributed to the initial changes in serum protein composition occurred during the first two months of storage. A slight decrease in serum β-CN and increases in serum κ-CN and β-LG during the first two months of storage suggesting some micellar rearrangements and the dissociation of κ-CN/β-LG complexes from the micelle surface. However, no further sign of the dissociation of β-LG/κ-CN complexes was found during prolonged storage. The results did not fully support the theory of age gelation proposed by McMahon (1996) that the gradual dissociation of κ-CN/β-LG complexes into the serum phase initiates the gelation process.

The proportion of serum β-CN in stored UHT milk (Figure 9.9-B) became progressively higher during storage than that in fresh UHT milk (Figure 9.9-A), indicating the effect of proteolysis by plasmin that takes place primarily in the micellar phase. This pattern was not observed for the κ-CN, α-LA or β-LG in milk serum, all of

which are much less susceptible to plasmin (Datta & Deeth, 2001). Interestingly, the patterns of κ-CN, α-LA and β-LG in the serum of early-season UHT milk all exhibited unusual fluctuation at 6 months (Figure 9.9-D, F, H, J), which was not found for β-CN (Figure 9.9-B). This difference further supported the hypothesis that the proteolysis by plasmin played an important part in the changes in the proportion of serum β-CN but not in the changes of serum κ-CN or the whey proteins. The fluctuations at 6 months in the serum of early-season UHT milk coincided with the fluctuations in milk pH (Figure 9.1) and Ca^{2+} (Figure 9.2), suggesting these differences occurred due to some physicochemical causes that were interrelated.

9.3.6 Transmission electron microscopy

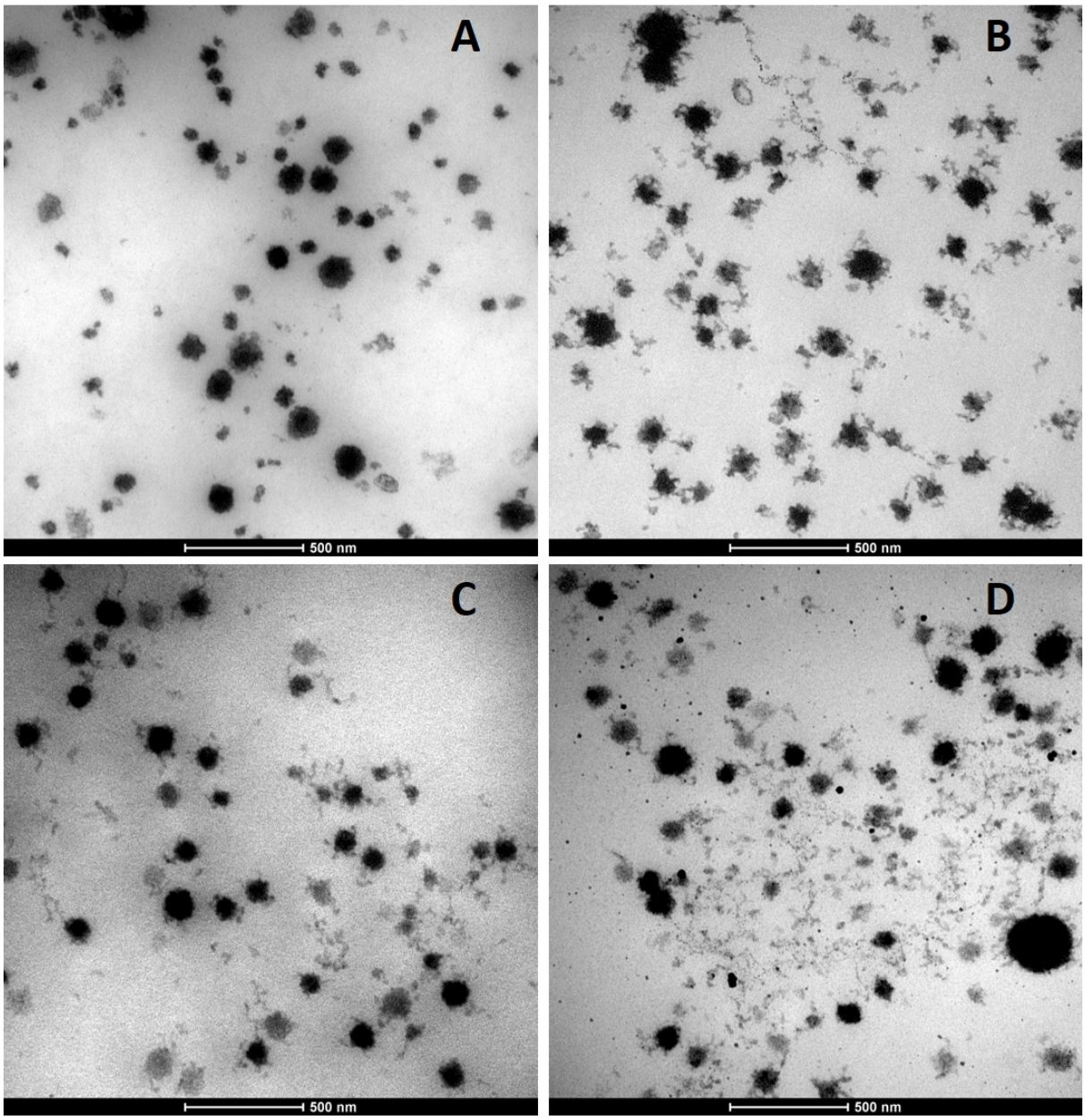

Figure 9.10: Transmission electron microscopy images of raw skim milk (A), fresh UHT milk (B), UHT milk stored for 6 months (C), and UHT milk stored for 8 months (D).

Figure 9.10 shows the TEM images of raw skim milk and UHT milks stored for 0, 6 and 8 months. In raw milk (Figure 9.10-A), the casein micelles have smooth surfaces. UHT treatment of the milk resulted in casein micelles covered with whey protein/κ-CN complexes that are protruding from the micelle surface (Figure 9.10-B). Protein complexes were found in the serum phase of UHT milk, because of the heat-induced whey protein denaturation, and protein-protein association. In addition, aggregates of two or three micelles were found in fresh UHT milk (Figure 9.10-B), which supported the hypothesis proposed in Chapter 4 that the initial aggregation between κ-CN-depleted micelles might be the reason for the increase in the average hydrodynamic size of casein micelles. Figure 9.10-C shows that, in UHT milk stored for 6 months, part of the casein micelles surfaces become smoother without the protruding appendages compared with the micelles in fresh UHT milk (Figure 9.10-B). In the serum phase, more tendrils appeared (Figure 9.10-C) compared with the serum phase of fresh UHT milk (Figure 9.10-B). In one of the two UHT milk samples stored for 8 months that were imaged with TEM (Figure 9.10-D), networks formed by proteins tendrils connecting casein micelles were found. Besides, fewer appendages were observed on the surface of casein micelles in UHT milk stored for 8 months than in fresh UHT milk (Figure 9.10-B), similar to that in UHT milk stored for 6 months (Figure 9.10-C). The implications of these changes observed during storage by TEM are discussed in the following section.

9.3.7 Discussion of gelation mechanisms

In the present study, signs supporting both gelation mechanisms proposed by McMahon (1996) and Anema (2017) were found. Both mechanisms are discussed with the observations in this section to illuminate how gelation was developed in this study.

The development of tendrils and protein network formation were observed using TEM in UHT milk stored for 6 and 8 months. The number of appendages covering the micelle surface after UHT treatment appeared to decrease after storage periods of 6 and 8 months (Figure 9.10). The concurrent increase in the number of tendrils, and the disappearance of the appendages on the micelle surface during storage, suggests that part of the whey protein complexed with the casein micelle surface during UHT treatment might have dissociated into the serum phase. Some previous studies reported the presence of 'strands', 'tendrils' or 'linear fibres' in stored UHT milk, heated both directly (Malmgren et al., 2017; McMahon, 1996) and indirectly (Raynes et al., 2018). Using immunogold labelling, Malmgren et al. (2017) evidenced that the tendrils were composed of β-LG and κ-CN. These findings appear to support the mechanism of age gelation that the κ-CN/β-LG complexes dissociate from the micelles during storage, which gradually forms a network that links the casein micelles (Datta & Deeth, 2001; Holt, Carver, Ecroyd, & Thorn, 2013; McMahon, 1996). However, the serum protein analysis indicates that the dissociation of κ-CN/β-LG complexes might have only occurred during the first two months of storage, resulting from a micellar rearrangement process due to the storage at 30°C (Section 9.3.5.4). Considering the reports that the serum β-LG in UHT milk was likely to be more active in promoting protein-protein interactions (Grewal et al., 2017a; Grewal et al., 2017b), the tendrils

and the protein network observed could also be formed by the κ-CN/β-LG complexes formed during UHT treatment. This hypothesis is supported by the fact that UHT treatments do not cause significant whey protein-casein micelle association compared with other heating methods at a comparable extent of whey protein denaturation (Chapter 4). Particularly, direct UHT with gentle preheating only results in limited extents of whey protein denaturation (Anema, 2017; Rauh et al., 2014), which further reduce the amount of whey proteins bound to the casein micelles. The observations made after 6 and 8 months of storage using TEM (Figure 9.10-C and D) could be due the initial dissociation of κ-CN/β-LG complexes (in the first 2 months) and the development of tendrils along with the κ-CN/β-LG complexes presenting in the serum phase after UHT treatment.

Despite the evidence that tendrils formed by β-LG and κ-CN could develop in UHT milk, their origin and role in age gelation are not fully understood. Venkatachalam et al. (1993) observed 'tendrilar or hairy appendages' protruding from the micelle surface and connecting casein micelles into a network. However, these appendages might have been whey protein-κ-CN complexes that associated with the casein micelles after UHT treatment. Studies on protein composition demonstrated that gelled UHT milk consisted mainly of α_s-CN and β-CN, with similar or lower proportions of κ-CN and β-LG than those in bulk milk (Anema, 2017; Rauh et al., 2014). This composition resembles that of the sediments in stored UHT milk (Gaur et al., 2018). Based on these findings, it was suggested that the κ-CN/β-LG complexes did not actively participate in the gelation process (Anema, 2017; Rauh et al., 2014). The involvement of κ-CN/β-LG complexes in the gel formation can be further investigated by comparing the amount of β-LG in

the colloidal phase of gelled UHT milk to that in the colloidal phase of fresh UHT milk. This would help to understand whether the whey proteins present in the milk gels were associating with the micelles due to the UHT process or during the storage.

The mechanisms of tendril formation and age gelation are likely to be different in UHT milk with extensive or limited proteolysis. Hydrolysis of α_s-CN and β-CN by plasmin was suggested to assist in the dissociation of the κ-CN/β-LG complexes by disrupting their anchor points in the micelles (Datta & Deeth, 2001), thus facilitating the formation of tendrils and the subsequent gelation. In addition, peptides resulting from proteolysis could also participate in the formation of fibrils and tendrils (Holt et al., 2013). Besides, gelled UHT milk that had extensive proteolysis by plasmin was rich in peptides (García-Risco et al., 1999; Malmgren et al., 2017; Rauh et al., 2014). Malmgren et al. (2017) reported that β-CN was fully hydrolysed into γ-CN in UHT milks gelled at 22°C and 30°C. As suggested by Anema (2017), the gelation mechanism of UHT milk that has had severe proteolysis would be different from the gelation of UHT milk with limited proteolysis. In the present study, despite subtle signs of γ-CN formation (Figure 9.4), around 60%–70% of α_s-CN and β-CN remained intact after 8 months' storage. Besides, gelation was developed from the bottom of the container and occupied 30%–70% of the volume of the milk samples, consistent with that of intensely preheated direct UHT milk (Anema, 2017). This suggestes that the indirect UHT treatment used in this study rather effectively inactivated the proteases in milk and that the proteolysis was not the main cause of gelation. Previous studies reported that extensive hydrolysis of α_s-CN and β-CN (>90%) took place prior to gelation in direct-UHT milk with mild preheat treatment (Anema, 2017; Malmgren et

al., 2017; Newstead et al., 2006; Rauh et al., 2014) and UHT milk with additional plasmin, plasminogen or plasminogen activator (Kelly & Foley, 1997; Kohlmann, Nielsen, & Ladisch, 1991; Zhang et al., 2018).

Based on the composition of gelled UHT milk, Anema (2017) proposed a mechanism for age gelation, particularly for UHT milk with limited proteolysis, which is caused by the κ-CN-depleted casein micelles. The gelation process is suggested to be initiated by the gradual settling of the κ-CN-depleted casein micelles. This results in a higher concentration of the micelles at the bottom of the container, which could develop into a gel from the bottom under proper physicochemical conditions (e.g. Ca^{2+} and pH) during prolonged storage (Anema, 2017, 2019). This theory is supported by the hypothesis in the present study that κ-CN-depleted casein micelles might aggregate during the UHT treatment, resulting in the increased apparent casein micelle size and the formation of sediments. The Ca^{2+} concentration, which promotes the aggregation of the micelles (Gaur et al., 2018; Singh, 2004), correlated with both the casein micelle size ($P < 0.01$) and the amount of sediments ($P = 0.03$) in UHT milk. As discussed in Section 9.3.2, subtle aggregation between 2 or 3 κ-CN-depleted casein micelles, as observed in the TEM (Figure 9.10-B), might result in an increase in the apparent casein micelle size (Table 9.3). Further aggregation of the micelles during heat treatment might result in the formation of large aggregates that are sedimentable under a certain centrifugal force in fresh UHT milk (Boumpa, Tsioulpas, Grandison, & Lewis, 2008; Chen et al., 2015a; Grewal et al., 2017b). The aggregation of κ-CN-depleted micelles during UHT treatment, whether or not sedimentable in fresh UHT milk under centrifugation, would promote sedimentation of the micelles during storage due to the increase in density,

which might facilitate the gelation of milk under suitable conditions, based on the theory proposed by Anema (2017). However, in milk with certain environmental conditions (e.g. high Ca^{2+} and low pH), extensive sedimentation would occur prior to gelation (Gaur et al., 2018). It appeared that a certain level of micelle aggregation, both during UHT treatment and during storage, facilitates the gelation process by increasing the concentration of the micelles at the bottom of the pack. Extensive aggregation of the micelles during processing or the early stage of storage might result in the formation of very large particles that precipitate quickly and form a dense sediment layer (Anema, 2019). This process would also deplete the micelles in the milk, retarding gel formation during further storage.

In summary, the age gelation of UHT skim milk appear to be controlled by a delicate and complex balance involving interplaying processes including sedimentation, proteolysis, protein-protein interaction and lactosylation. All of these processes also interact with the physicochemical environment (e.g. temperature, Ca^{2+} and pH). It adds to the complexity that both proteolysis and sedimentation appear to either promote or hinder the gelation of milk, depending on their respective extents and other involving factors.

UHT milk produced in mid- and late season were more likely to gel than those produced in the early season. None of the physicochemical properties determined in fresh and stored UHT milk clearly correlate with this seasonal pattern of age gelation, agreeing with Anema (2019) that there was no known physicochemical indicator of age gelation. Based on the hypothesis extended from the mechanism proposed by Anema (2017), some signs appeared to link the aggregation among κ-CN-depleted micelles

during UHT treatment to increased casein micelle size, the formation of sediments and the likelihood of gelation. Both the micelle size and the amount of sediments in UHT milk increased in the order of early-season < mid-season < late-season (Table 9.3), which might associate with the lowest incidence of gelation in early-season UHT milk. The casein micelle size and the extent of sedimentation in UHT milk correlated with Ca^{2+} in UHT milk ($P < 0.05$). Besides, the pH of UHT milks from the mid- and late season was 6.65, slightly lower than that of early-season UHT milk (pH 6.68, Table 9.3). A pH of 6.65 was suggested to be a threshold, below which significant sedimentation would occur in UHT milk (Gaur et al., 2018; Lewis, Grandison, Lin, & Tsioulpas, 2011). In addition, the lowest protein content of early-season milk (Table 9.2) might also play a part in reducing the micelle-micelle aggregation, and gelation during storage.

9.4 Conclusion

In fresh UHT milk, the mean pH did not vary significantly in the UHT milk produced in different seasons. The Ca^{2+} concentration was highest in the late season. Both the casein micelle size and the amount of sediments increased with proceeding season. UHT treatment caused significant κ-CN dissociation, the extent of which was similar across different seasons. The level of whey protein-casein micelle association was highest in late-season UHT milk.

Changes in the physicochemical properties of UHT milk during storage were broadly similar in different seasons. The pH of UHT milk decreased during the storage period of 8 months. The Ca^{2+} and the casein micelle size decreased during the first two months

of storage. Further changes in Ca^{2+} and micelle size during 2-8 months of storage were insignificant.

Protein analysis of stored UHT milk using SDS-PAGE and HPLC indicated signs of moderate proteolysis by plasmin and noticeable lactosylation of all milk proteins. Investigation of the serum protein profile suggested that proteolysis of β-CN by plasmin mainly occurred in the casein micelles rather than the serum phase. The proportion of serum β-CN decreased while the proportions of serum κ-CN and serum β-LG increased during the initial two months of storage. These changes, along with those in Ca^{2+} and casein micelle size occurred during the first two months of storage, might be interrelated, all resulting from the physicochemical and structural changes caused by the storage at a higher-than-ambient temperature of 30°C. The results indicate that accelerated storage test different temperatures might result in different changes in the physical and chemical characteristics of UHT milk.

Formation of protein tendrils and networks in stored UHT milk was demonstrated using TEM. Besides, the surface of micelles covered with long appendages in fresh UHT milk became smoother with fewer appendages attached. This observation, along with the increase in serum κ-CN and serum β-LG, suggested that the dissociation of β-LG/κ-CN complexes from the casein micelles might have occurred during the first two months of storage.

The incidence of age gelation was considerably higher in UHT milk produced in the mid-season and in the late season than in UHT milk from the early season. The mechanisms of age gelation were discussed. A hypothesis extending the gelation mechanism proposed by Anema (2017) that involves the interaction and sedimentation

of κ-CN-depleted casein micelles was proposed. The seasonal variation in the incidence of gelation might associate with the UHT-induced increase in mean casein micelle size and sediment formation, all of which involve the aggregation of κ-CN-depleted casein micelles.

Chapter 10 - Overall discussion and future recommendations

Dairy plays an important role in the food cultures across the globe and is among the most important source of nutrients to feed the growing world population. Milk is arguably the most versatile food owing to its unique physicochemical and biological characteristics. The understanding of these characteristics and their natural variations lays the foundation for successful applications of dairy processing and product development. This research provided a comprehensive and up-to-date analysis of the seasonal variations in the composition and properties of New Zealand milk. It also investigated the impact of seasonality on several product systems, i.e. acid gel, yoghurt, UHT milk and whipping cream. In addition, this study demonstrated how processing (e.g. heating and UF) affect the physicochemical properties of milk differently across different seasons and the subsequent influences on product properties.

The fact that different factors, e.g. feed, climate and SOL, change simultaneously during the year makes it difficult to interpret seasonal studies on milk properties. One important consideration in the present study was to focus on the robust seasonal variations in milk characteristics. To account for the between-year variation and identify the consistent seasonal patterns, the studies on milk characteristics and heat-induced changes (Chapter 4), milk acid gelation (Chapter 5) and yoghurt properties (Chapter 6) were conducted for two complete milking seasons. In addition, the impact of somatic cell count on milk characteristics was discussed in Chapter 4. It is recommended to take the difference in somatic cell count into consideration when interpreting the data from different studies and when conducting future studies.

The study of milk characteristics over two full milking seasons (2016-2017 and 2017-2018, Chapter 4) indicated that the stage of lactation largely controls the seasonal variations in the composition and physicochemical properties of seasonal-calving New Zealand milk. The most consistent seasonal variation patterns observed, including the change in late-season milk composition, decreasing proportion of α-LA in milk protein, elevated glycosylation degree of κ-CN in the late season, decreasing fat globule size, were reported to occur during the lactation cycle. Besides, the seasonal variations in the fatty acid composition and the melting behaviour of milk fat (Chapter 8) also broadly followed the lactational trend. However, the access to fresh grass and the changing maturity of the pasture during the season also seemed to play a part in affecting the fatty acid composition of milk.

The seasonal variation in the glycosylation degree of κ-CN and its potential impacts on different processing properties of milk were highlighted. Glycosylated κ-CN was found more stable against heat-induced dissociation (Chapter 4) and appeared to play a role in the textural development of acid milk gel and yoghurt (Chapter 5 and 6). The impact of κ-CN glycosylation on the hydrophobic and electrostatic interactions among milk proteins appeared to further affect the stability and interactions of casein micelles. The degree of κ-CN glycosylation was reported to affect the rennet coagulation of milk (Bonfatti et al., 2014). Future studies could explore the effects of κ-CN glycosylation on other processing properties of milk. Besides lactation, the glycosylation degree of κ-CN is also influenced by the breed, parity and the genetic variants of κ-CN (Poulsen, Jensen, & Larsen, 2016; Robitaille et al., 1991). Future research could attempt at

controlling the processing properties of milk by manipulating the glycosylation degree of κ-CN via breeding and genetic selection.

Three chapters of this study were dedicated to the acid gelation of milk and yoghurt (Chapter 5, 6, and 7). Significant correlations were found between the firmness of acid milk gel (standardized), regular set yoghurt and Greek-style yoghurt across the seasons, all of which decreased drastically in the late season. This finding suggested that the seasonal trend found in GDL-induced acid milk gel could represent the variation in the texture of both the regular yoghurt and Greek-style yoghurt. In contrast, the viscosity of stirred yoghurts behaved differently that late-season stirred yoghurt displayed the strongest resistance to shear-induced thinning. This suggested a shift in the viscoelastic properties of the yoghurt during the season. Besides, standardization using UF retentate appeared to play a part in affecting the acid gelation properties of milk, but not enough to the standardize the gel strength over the seasons. Future studies could explore the use of UF and UF-DF to manipulate the processibility of milk. This could not only help to control the processing properties of seasonal milk, but also enable the development of new dairy products.

The study on milk fat in Chapter 8 showed broadly consistent results with previous studies in terms of fatty acid composition and melting behaviour. The stage of lactation played an important role while the change in the diet, particularly the access of fresh grass, appeared to influence the shift in fatty acid composition during lactation in New Zealand. The varying fat globule size was likely to be a major factor affecting the whipping properties of fresh cream. Future studies could look into the whipping properties of UHT whipping cream (homogenized) and ice cream over the seasons.

The age gelation of UHT milk was least prominent in the early season in this study (Chapter 9). The physicochemical mechanism of age gelation proposed by Anema (2017) was extended as related to the heat-induced aggregation between the casein micelles, which was promoted by higher concentrations of protein and Ca^{2+} and lower pH. To test this hypothesis, future studies could investigate the aggregation behaviour of casein micelles in UHT milk and their sedimentation and interactions during storage. Centrifugation and filtration techniques could be utilized to fractionate the aggregated casein micelles for characterization.

In summary, this study provided a comprehensive and up-to-date characterization of seasonal milk in New Zealand and demonstrated the seasonal variations in the quality of a range of dairy products. It also deepened the understanding of the impact of processing (heating and UF) on milk properties and the mechanism involved in determining the quality of different dairy products. This study could not only help the dairy industry to manufacture products with consistent quality during the dairying season, but also assist in the improvement of product quality (overall or in certain seasons). In addition, based on the naturally altering milk composition and characteristics over the seasons., new dairy products with special properties and functionalities could be developed in a certain period of the year.

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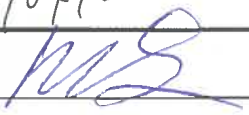
Primary Supervisor's Signature:	
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