The impact of once-a-day milking in New Zealand on milk composition and processing

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

Doctor of Philosophy

in

Food Technology

at Massey University, Palmerston North, Manawatū, New Zealand.

Marit Stephanie van der Zeijden

2023

Abstract

Almost 10% of farmers in New Zealand have adopted a once-a-day (OAD) milking system, but the impact of OAD milking beyond the major milk components is not well understood. The aim of this thesis was to study the effect of OAD milking on the proximate, protein and mineral composition of milk and the potential implications for processing. Milk was sampled throughout the lactation from individual cows, of the Holstein-Friesian, Holstein-Friesian×Jersey and Jersey breeds, selected and balanced for parity, breeding worth and calving date. This milk was characterised for proximate and protein composition. For processing purposes, the milk was mixed proportional to the respective yields to firstly study the heat-induced changes to milk proteins and secondly the rennet- and acid-induced gelation properties.

It was found that OAD, compared to TAD, milking impacts the protein composition of the milk. This effect was more pronounced in mixed milk samples. On an individual level, only the κ-casein and α-lactalbumin contents increased and decreased, respectively. The breed effect was explored but the sample size was too small to draw firm conclusions. In the mixed milk samples, the κ-casein content, the κ-casein glycosylation degree, and the α_{s2}-casein content were higher, while the α_{s1}-casein, β-lactoglobulin and α-lactalbumin contents in the protein fraction were lower in OAD than in TAD milk. The calcium, magnesium and phosphorus concentrations and distribution in the milk were unaffected by the milking frequency. In contrast, the sodium, chloride, and copper contents were lower, and the potassium and iodine contents were higher in OAD milk. Casein micelles were smaller in OAD than in TAD milk. The proportions of κ-casein and α_{s2}-casein in the serum phase were higher in raw and pasteurised OAD than in TAD milk. In UHT milk from a OAD milking system, the degree of whey protein denaturation was lower than in TAD milk. The fate of denatured whey proteins, either aggregation in the serum phase or association with the casein micelle, was affected by the type of heat treatment, the type of protein, milking frequency, and the stage of lactation. Despite differences in milk composition and the heat-induced changes to the proteins in the milk, the gelation properties did not differ between OAD and TAD milk. The gelation pH of acid-induced gelation was higher for OAD than for TAD milk.

The results of this study provided valuable insights in the potential implications of OAD milking for processing properties and product quality.

Acknowledgements

Firstly, I would like to express my sincere gratitude towards my supervisors for their support and advice, great discussions, and critical questions. Ashling, thank you for the endless support and kindness. Warren, thank you for sharing your wisdom along the way, for my development as a researcher as well as a person, and for always having time for a chat. Nicole, thank you for your thorough reviews and great eye for detail. Nicolas, thank you for your patience in teaching and for sharing your enthusiasm, I always walked away from our meetings with new energy and fresh motivation. And finally, Siqi, many thanks for your help in the laboratory, the interesting discussions and answering my never-ending questions.

I would also like to thank the Riddet Institute for the friendly and supportive environment. Many thanks to the NZ3M (New Zealand Milks Mean More) project for the scholarship and the NZ3M team for sharing their knowledge and valuable insights. Thank you to the staff at the Riddet Institute for your help in many different ways. Maggie, Jacinda, and Linda for the laboratory support. Natascha, Anneminke and Paloma, thank you for helping me on the farms and in the laboratory and always with a smile (even at five in the morning), which made the long days much more fun. Thank you to the people in the office, Terri, John, Rebekah, Michelle, Alex, and Meg, for the chats and always being there to help, and of course, Ansley, you were such a beautiful person.

I would also like to thank the staff at the Massey University. Firstly, the people at the dairy farms, Fiona, Norton, Jolanda, and Josh. You have made the sampling days and early mornings much easier. Many thanks also go to Garry, Warrick, and Michelle for your help in the Pilot Plant and Massey University labs.

Many thanks to my fellow students, for the fun, the laughs, for sharing the pain at times and the many wins. I very much enjoyed all the chats in the office and lab. And it was always great to meet other members of the Riddet family from different parts of the country at the Riddet Colloquia. You have all made this ride a lot easier and more enjoyable and I hope you see you all again in the future during our next adventures.

Finally, I would like to thank my friends and family, both in New Zealand and in the Netherlands. Covid has definitely made life challenging at times, not being able to visit home when I wanted to. However, you were always there to listen and offer advice. Also, many thanks to Heneriata for trusting Dusty in my care for the past few years. Having him to look after offered a great distraction and I loved taking him to events and for rides on the beach.

Table of contents

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

CHAPTER 9

List of Tables

List of Figures

Abbreviations

Dairy No.1	Massey University Dairy Farm No.1
Dairy No.4	Massey University Dairy Farm No.4
DIM	Days in milk
F	Holstein-Friesian
F×J	Holstein-Friesian×Jersey crossbreed
GDL	Glucono-δ-lactone
HPLC	High-performance liquid chromatography
J	Jersey
MFGM	Milk fat globule membrane
OAD	Once-a-day
SCC	Somatic cell count
SCS	Somatic cell score
TAD	Twice-a-day
TAGs	Triacylglycerides
UHT	Ultra-high temperature
α-LA	α-lactalbumin
α_{s1}-CN	α_{s1}-casein
α_{s2}-CN	α_{s2}-casein
β-CN	β-casein
β-LG	β-lactoglobulin
κ-CN	κ-casein

Chapter 1

General introduction

Most of the milk production systems in New Zealand are pasture-based where cows begin lactating after calving in spring and are dried off in late autumn of the following year. The timing of these events coincides with the pattern of pasture growth so that the seasonal nutrient requirements of the herd align with the pattern of pasture growth (Holmes, 2002). Most farmers milk their cows twice-a-day (TAD), but an increasing number (nearly 10% of farms in New Zealand) have switched to a once-a-day (OAD) milking system (Edwards, 2018). Various motivations can lead to this decision, including changes in lifestyle expectations and the lack of long-term staff. Other reasons could be the design of the farm, for example, when cows are required to walk long distances to the milking shed, increasing the chance of lameness (Bewsell et al., 2008).

To optimise the productivity and breeding worth of the animals, studies have looked at the genetics of the cows and the management of the farm (Hickson et al., 2006; Lembeye, Lopez-Villalobos, et al., 2016b; Stelwagen et al., 2013). Some research has focused on the impact of a reduction in milking frequency on concentrations of protein, fat, and lactose, as well as the yields of these components (Stelwagen et al., 2013). Limited studies have focused on the impact of OAD milking beyond these major components. It raises the question of whether minor components, such as individual proteins, are impacted by the milking frequency.

Milk is used in a range of dairy products, and the importance of milk composition for processing is being highlighted in many studies. For example, milk from different species, such as sheep and goats, has different product properties than products made from cow's milk (Barlowska et al., 2011). Additionally, seasonal variation in the composition and processing properties of milk has been shown (Li et al., 2019; Timlin et al., 2021). The increasing solids content and somatic cell counts in late lactation can have detrimental effects on the quality of the final products and have offered challenges to the industry to produce dairy products satisfactory to the consumer (Timlin et al., 2021).

The aim of this thesis was to explore the changes in bovine milk composition and processing properties of milk from a OAD milking system compared to a TAD milking system. The objectives, hypotheses and thesis structure are discussed at the end of the literature review in Section 2.4.

Chapter 2

Literature review

2.1 Milk composition and structural properties

The main components in milk are fat, protein and lactose, of which the approximate composition is outlined in Table 2.1. In addition to these macronutrients, vitamins, minerals, and enzymes are present in milk. The variation in the composition can be a result of several factors. Across the world, different breeds and farming systems are being used to suit the conditions of a specific country. However, even within one herd of cows, the milk yield and composition may change due to changes in the weather, animal health or feed offered to the animals. Throughout the season, variation in milk composition occurs (Li et al., 2019; Timlin et al., 2021). Variability in milk composition is discussed in more detail in Section 2.1.6. The first few sections of this chapter focus on the different components in milk.

Table 2.1. Proximate composition of bovine milk, displayed as the range of the content (%) of major milk components reported across the literature (Barlowska et al., 2011; Claeys et al., 2014; Li et al., 2019).

	Reported range in bovine milk (%)
Total dry matter	11.8 - 13.0
Protein	2.5 - 4.7
Casein	2.46 - 2.80
Whey protein	0.55 - 0.70
Fat	3.2 - 5.6
Lactose	4.4 - 5.6

2.1.1 Protein

The protein fraction in milk can be divided into two main classes, whey protein, and casein. The casein fraction contains the proteins that precipitate at pH 4.6. The remaining proteins in solution represent the whey protein fraction (Fox et al., 2015). The casein fraction is the main protein fraction, with approximately 80% of the total milk protein

(Fox et al., 2015; Park et al., 2017; Park et al., 2007). Table 2.2 shows the reported range of casein and whey proteins in bovine milk. Each protein can have different genetic variants. This phenomenon is referred to as genetic polymorphism and results in differences in the amino acid sequence (Piredda and Pirisi, 2005). This change affects the physicochemical properties of the protein and, thereby, the structure of the micelle or, for example, protein functionality (Park et al., 2007).

Table 2.2. Reported range of the protein composition (g/L) in bovine milk (Claeys et al., 2014).

	Content in bovine milk (g/L)
Total casein	24.6 – 28.0
α_{s1}-casein	8.0 - 10.7
α_{s2}-casein	2.8 - 3.4
β-casein	8.6 - 9.3
κ-casein	2.3 - 3.3
Total whey protein	5.5 - 7.0
β-lactoglobulin	3.2 - 3.3
α-lactalbumin	1.2 - 1.3
Serum albumin	0.3 - 0.4
Lactoferrin	0.02 - 0.5
Immunoglobulins	0.15 - 1.0

2.1.1.1 Casein

Approximately 95% of the casein is present in the form of casein micelles. Several models of the micelle structure have been proposed. Although research on the casein micelle structure is ongoing, there is a consensus about the basic structure (Dalgleish, 2011). The colloidal particles comprise the major caseins, α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN) and β-casein (β-CN), and nanoclusters of colloidal calcium phosphate, which consists mainly of calcium, magnesium, phosphate, and citrate. κ-casein (κ-CN) forms a hairy layer on the outside of the casein micelles. The casein micelles are voluminous and can hold a high amount of water, approximately four grams per gram of protein. The hydrophobic bonds and cross-links between peptide chains also play a role in the structure and arrangement of the micelles (Dalgleish, 2011; Fox et al., 2015; Walstra et al., 2005). Apart from their crucial function in cheese, casein and caseinates are also used in many other products, e.g., as emulsifiers, gelling agents, stabilisers, or to improve foaming properties (Fox et al., 2015).

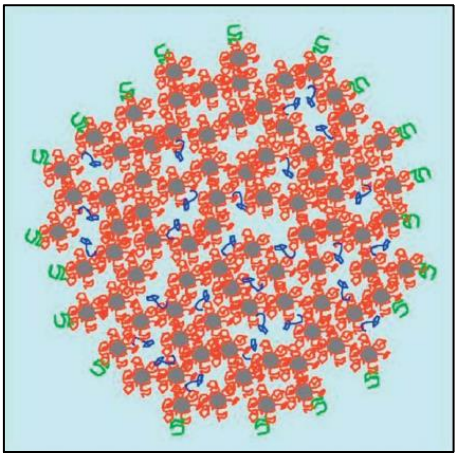


Figure 2.1. A proposed schematic structure of the casein micelle by Dalgleish (2011) incorporates calcium phosphate nanoclusters (grey) with their attached caseins (red) and the surface-located κ-casein (green). The ‘‘hydrophobically bound’’ β-casein is blue within the water channels inside the micelle (the relative sizes of the individual components are not to scale) (Dalgleish, 2011), figure used with permission from a Copyright Clearance Center/Royal Society of Chemistry license.

Caseins are phosphorylated proteins that have little secondary and tertiary structure. As a result, hardly any denaturation occurs when heated. They have a hydrophobic nature, but the high degree of phosphorylation and the low number of sulphur-containing amino acids contribute to their solubilities (Fox et al., 2015). The α_{s1}, α_{s2} and β-CN are calcium-sensitive, while κ-CN is not (Fox et al., 2015; Walstra et al., 2005).

The proportion of each casein in the casein fraction in milk varies across publications (Alichanidis et al., 2016; Ceballos et al., 2009; Ingham et al., 2018; Inglingstad et al., 2010; Ono et al., 1989; Storry et al., 1983). α-CN is the most abundant casein ranging from about 42-50% of the total casein, of which the majority is α_{s1}-CN, with about 36-37% of the total casein, although values as high as 58% have been reported. The α_{s2}-CN is present in much smaller proportions than α_{s1}-CN in bovine milk. The concentration of β-CN is approximately 37%, and the κ-CN content ranges from 11-16% of the total casein (Alichanidis et al., 2016; Ceballos et al., 2009; Ingham et al., 2018; Inglingstad et al., 2010; Ono et al., 1989; Storry et al., 1983). κ-CN is a minor casein in bovine milk that is the smallest of the four, with 169 amino acid residues and a molecular mass of 19 kDa (Walstra et al., 2005). Unlike the other caseins, κ-CN has a low calcium sensitivity and can be glycosylated (McSweeney and Fox, 2013). The glycosylation contributes to the solubility of κ-CN (Fox et al., 2015). The two cysteine residues allow crosslinking through disulphide bonds (Farrell Jr et al., 2004; McSweeney and Fox, 2013). These cysteine residues are important for structural changes when milk is heated. Like κ-CN, α_{s2}-CN has a free thiol group (Farrell Jr et al., 2004).

2.1.1.2 Whey proteins

The non-casein fraction of the protein is referred to as whey (Walstra et al., 2005). The major whey proteins are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA); the remaining fraction comprises serum albumin, lactoferrin, immunoglobulins and other minor proteins. From being a waste product of the cheese-making industry, whey has become a valuable source of protein and protein-derived ingredients with many applications. Whey proteins are added to beverages (e.g., sports drinks or medical nutrition products) to increase their nutritional value. Due to the excellent foaming, emulsifying, gelling and solubility properties, whey proteins are also commonly used as a stabiliser or to improve textural properties (Bansal and Bhandari, 2016).

The globular structure of most of the whey proteins allows them to unfold at the interface (e.g., water-oil), form bonds through disulphide bridges and thereby form a stabilising network (Dickinson and Matsumura, 1991; Lefèvre and Subirade, 2003; McClements et al., 1993). The whey fraction in milk is constituted of approximately 47-59% β-LG, 20% α-LA, 12% immunoglobulins, 6-7% serum albumin and 2-3% lactoferrin (Alichanidis et al., 2016; Heine et al., 1991; Inglingstad et al., 2010; Moatsou et al., 2005; Pintado et al., 1999; Storry et al., 1983).

β-LG is a globular protein of 18.3 kDa and the most abundant whey protein in milk (Farrell Jr et al., 2004). It is composed of 162 amino acids and primarily present as a dimer at neutral pH (Kontopidis et al., 2004). Not all milk, including human milk, contains β-LG and it is one of the allergenic proteins in milk, which can be responsible for bovine milk allergy in infants (McSweeney and O'Mahony, 2016). While the functions of other proteins in milk are well understood, the function of β-LG remains unclear. Various functions have been suggested, including being a ligand or carrier for other components in milk, such as fatty acids, vitamin A and vitamin D (Kontopidis et al., 2004; McSweeney and Fox, 2013). Unlike the function, the structure of β-LG has been studied thoroughly and is well-defined (Kontopidis et al., 2004).

α-LA is a smaller globular protein of 14.2 kDa and made up of 123 amino acids. It is high in tryptophan, the precursor for serotonin, compared to other proteins in milk (Heine et al., 1991) and non-dairy proteins. The composition of α-LA makes it an excellent source of protein for infant nutrition, as tryptophan is one of the limiting amino acids when comparing milk-based infant formula to human milk (Heine et al., 1991; Layman et al., 2018). α-LA participates in the synthesis of lactose, and the mechanism is well understood. The structure of α-LA is compact and similar to the structure of lysozyme (Fox et al., 2015).

2.1.2 Lipid

The primary function of the lipid fraction in milk is to provide energy for the neonate. Additionally, it acts as a carrier for fat-soluble vitamins, essential fatty acids, and bioactive components such as phospholipids. The concentration of lipids in the milk varies largely between different species and to a smaller extent throughout the lactation stage and among breeds. Triacylglycerols (TAGs) make up 97-98% of the lipid fraction (Fox et al., 2015). The remaining comprises free fatty acids, cholesterol, phospholipids, sphingolipids and gangliosides (Walstra et al., 2005).

Fat is present in the form of milk fat globules surrounded by a tri-layer of phospholipids referred to as the milk fat globule membrane (MFGM) (Figure 2.2). The MFGM is composed of primarily phospholipids, cholesterol and (glycated) proteins and allows emulsification of the fat throughout the milk (Walstra et al., 2005). The MFGM is a unique structure with numerous health benefits and has been a topic of interest in dairy science in the past decade (Fong et al., 2007; Gallier et al., 2015). The size of the fat globules in raw milk ranges from less than 0.1 μm up to more than 10 μm, but most globules are between 2.8 and 4.6 μm (Claeys et al., 2014; Lopez, 2011). The milk fat globule size affects the rate of digestion, curd formation and crystallisation/melting behaviour, among other properties (Armand et al., 1999; Lopez et al., 2002; Michalski et al., 2003).

Fatty acids vary mainly in chain length and double bond number, position, and configuration (Jensen, 2002; Walstra et al., 2005). The composition and structure of the lipid fraction of bovine milk have been reviewed elsewhere (Argov et al., 2008; Jensen, 2002; Martini et al., 2016; McPherson and Kitchen, 1983; Singh and Gallier, 2017). The majority of the fatty acids are made up of an even number of carbon atoms, ranging from 4 to 18 (Walstra et al., 2005). The fatty acid composition significantly affects a product's quality such as structural properties, flavour characteristics and stability of milk fat (Chen et al., 2004). For example, a high level of unsaturated fatty acids increases oxidation and the formation of off-flavours and rancidity in milk or milk fat-containing products such as butter and cream. The melting and crystallisation behaviour of milk fat is affected by the fatty acid composition and distribution among TAGs (Walstra et al., 2005). Milk with smaller fat globules has relatively more MFGM, which provides more stability to the fat globules than milk with larger fat globules (Walstra et al., 2005).

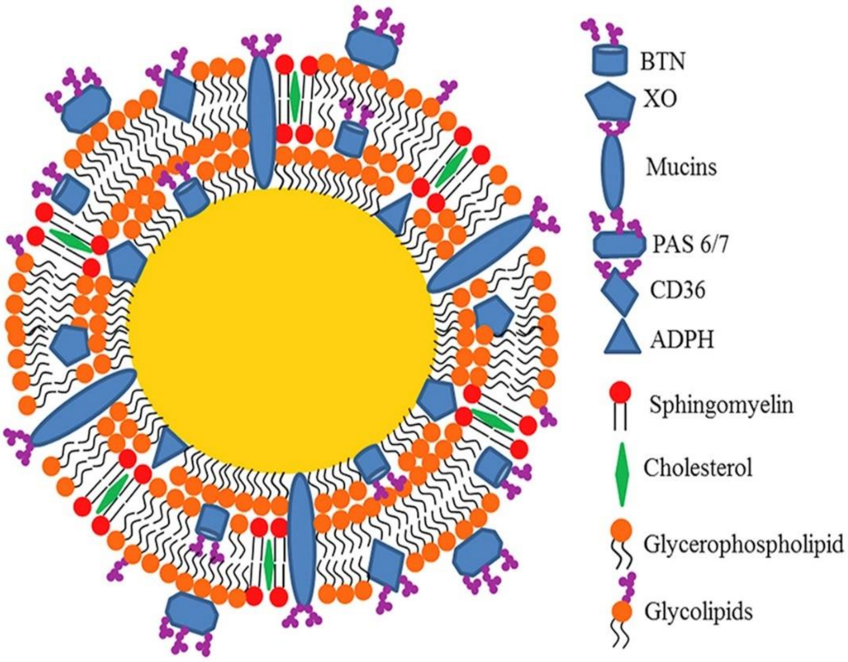


Figure 2.2. Graphical display of a milk fat globule showing the phospholipid tri-layer and the inclusion of various proteins (Singh and Gallier, 2017), figure used with permission from a Rightslink/Elsevier license.

2.1.3 Lactose

Lactose is the major carbohydrate in milk, with a concentration of approximately 4.6%. The primary purpose of lactose in milk is to provide energy to the neonate, and this sugar is unique to milk (Walstra et al., 2005). During milk synthesis, lactose is involved in regulating the osmotic pressure between the blood and the milk (Fox et al., 2015). Lactose is a disaccharide of glucose and galactose. It is a reducing sugar which allows it to participate in the Maillard reaction and thereby contributing to flavour changes in heated-milk products (Colahan-Sederstrom and Peterson, 2005; Walstra et al., 2005). Lactose is the primary source of energy for lactic acid bacteria that convert lactose to lactic acid during the production of yoghurt and cheese and when fouling of raw milk occurs, causing a sour taste (Nguyen et al., 2014; Walstra et al., 2005). In the dairy industry, payment systems for milk are most commonly based on the protein and fat contents and not the lactose content, despite the relatively high lactose concentration (Costa et al., 2019). However, lactose is commonly used in food products and as a filling agent in pharmaceuticals (Walstra et al., 2005).

2.1.4 Minerals

The main minerals in bovine milk are calcium, phosphate, sodium, potassium, chloride, magnesium, and citrate (Fox et al., 2015; Gaucheron, 2013). Other minerals in trace concentrations are also found in milk, including iron, zinc, copper, manganese, iodine, selenium, and fluoride. The mineral concentration and composition vary depending on the breed, stage of lactation and feed or udder health (Gaucheron, 2013; Patiño et al., 2007; Tallamy and Randolph, 1970; Wegner and Stull, 1978). For example, higher protein content is often accompanied by a higher calcium content, as most calcium is protein-bound in the casein micelle (Gaucheron, 2013).

Calcium, phosphate, and magnesium are partly bound to casein and are included in the micelle structure. These minerals, therefore, play an essential role in the structural properties and stability of the milk. There is an equilibrium between the micelle phase and the serum phase, which may shift due to changes in the pH, salt concentration or temperature (Fox et al., 2015; Gonzalez-Jordan et al., 2015; Marchin et al., 2007). For example, calcium and phosphate are released from the micelle when the pH is lowered or when the milk is alkalised, which destabilises the micelle (Ahmad et al., 2009; Gonzalez-Jordan et al., 2015; Marchin et al., 2007; Vaia et al., 2006). The soluble salts in the serum phase may be present in the ionic form or in a complex with other ions (Fox et al., 2015; Neville et al., 1995).

The distribution of calcium, phosphorus and some other minerals in the milk also affects the coagulation properties of the milk (Malacarne et al., 2014). Examples of how changes in the salt concentration impact the functionality of the milk and milk proteins can be found in research on calcium fortification of milk. Calcium fortification is commonly done to improve the nutritional value or to alter the functionality (Deeth and Lewis, 2015; Philippe et al., 2003). Calcium can be added in a different form of which some soluble calcium salts add ionic calcium to the milk, which results in pH shifts, altered gelling properties and, most importantly, decreased heat stability, often undesirable (Deeth and Lewis, 2015; Singh et al., 2007; Vyas and Tong, 2004).

2.1.5 Vitamins and other minor components

Although in small quantities, milk contains some vitamins that are valuable for nutrition and especially the contents of vitamins A, most of the B vitamins contribute significantly to the recommended daily intake of humans with up to 56% in a 250 mL serving. The fat-soluble vitamins D, E, and K and the water-soluble vitamin C are also present in milk but contribute to the recommended daily intake to a smaller extent (less than 10%). Urea makes up a large part of the non-protein nitrogen fraction in milk (up to 50%), in addition to various peptides and other components (Fox et al., 2015; Walstra et al., 2005). Higher heat stability has been found with higher urea content (Crowley et al., 2014; Dalgleish and Pouliot, 1987; Muir and Sweetsur, 1976).

Naturally, enzymes are present in milk, including lipases and proteases. Most of the enzymes do not have a function in the milk, but they can cause significant damage, such as rancidity or bitter flavours in raw milk or improperly processed milk (Walstra et al., 2005). Heat treatment inactivates most of the native enzymes in milk, but plasmin is relatively heat-stable, and high concentrations of this enzyme may cause issues in pasteurised milk (Barbano et al., 2006b; Walstra et al., 2005). Enzymes may originate from the cow via secretion into the milk or from bacteria present in the milk. A high somatic cell count (SCC) has been related to a high concentration of proteolytic or lipolytic enzymes (Barbano et al., 2006b).

2.1.6 Variation in milk composition

2.1.6.1 Season and lactation stage

Throughout the year, milk composition and the functionality of components such as protein and fat change. These differences are more pronounced when seasonal milk production is used (O'Brien and Guinee, 2016). In New Zealand, most dairy farms have seasonal calving and dry off the cows in late autumn until calving starts again in July. In a seasonal milk production system, the lactation stage aligns with the different seasons, with early lactation in late winter-spring and late lactation at the end of autumn or early winter. This way, pasture availability is optimally used by aligning peak milk production with grass growth.

The milk yield increases during the first six weeks post-partum and then gradually decreases towards the end of the lactation and milking season. The fat and protein contents increase during the lactation, while the lactose content is the lowest at the end of the season (Li et al., 2019; O'Brien and Guinee, 2016; Sorensen et al., 2008). In contrast, the yield of fat and protein decreases towards the end of the season (Auldist et al., 1998). The SCC increases towards the end of the season, as does the calcium content (Li et al., 2019; Underwood and Augustin, 1997). Most of the calcium in milk is bound to casein, which explains why increased content correlates with increased protein content during the season. Li et al. (2019) studied the seasonal variation in milk protein composition. They found an increase in the κ-CN and β-LG contents and the κ-CN glycosylation degree and a decrease in the α_{s2}-CN and α-LA contents throughout the lactation. Late-season milk was also found to have smaller fat globules and, therefore, a higher proportion of phospholipids (Li et al., 2019; Liu et al., 2017; Walker et al., 2013).

Issues with processing arise because of changes in the composition of the milk during the milking season and lactation. Examples of issues in the late season include a lower cheese yield, changes in heat stability, and inability to produce certain products (Auldist et al., 1998). Acid gelation properties are also worse in late lactation (Li et al., 2020; Underwood and Augustin, 1997).

2.1.6.2 Feed

In New Zealand, dairy farming is predominantly pasture-based. Grass and pasture are widely available, and utilisation of this feed source reduces the cost of milk production (Dillon et al., 2005). It is well known that feed composition impacts the composition of milk produced by cows and resulting dairy product properties. The effect of feed composition has been studied extensively, including the use of supplementation of certain nutrients to improve the nutritional quality of the milk. Pasture-based nutrition increases the amount of unsaturated fatty acids, especially conjugated linoleic acid, α-linolenic acid and vaccenic acid. The inclusion of supplementary feed such as silage, maize, palm kernel and other types of concentrate increases the saturated and short-chain fatty acids in the milk (Elgersma, 2015; Kalač and Samková, 2010). The protein content of milk is insensitive to changes resulting from specific feed regimes. The total metabolisable energy of the feed is of importance, though, as restriction of this can result in a decreased protein content (Beever et al., 2001; Cowley et al., 2015; Walker et al., 2004).

2.1.6.3 Health

The SCC is an important factor used in the dairy industry to check milk quality. A high SCC (150,000-200,000 cells/mL or higher) can indicate the presence of a bacterial infection in the udder (mastitis) and poor health status of the cow. Correlations between the SCC and compositional and structural factors of the milk have been reported (Auldist, 2011). Some components have been found to increase in concentration (free fatty acids, sodium, chloride, γ-CN, whey protein and most of the minor proteins), while other components (lactose, potassium, casein, α_s-CN, β-CN, α-LA and β-LG) decrease in concentration and yield when the cow has mastitis (Auldist and Hubble, 1998; Tallamy and Randolph, 1970; Wegner and Stull, 1978). A high SCC value can reduce dairy product quality, such as decreased cheese yield, off-flavours and increased enzymatic activity (Auldist et al., 1996; Barbano et al., 2006a; Ma et al., 2000; Murphy et al., 2016).

2.1.6.4 Other factors influencing milk composition

Other factors that can impact the physicochemical properties of milk include genetics, parity, and environmental factors. The main breeds in New Zealand are Holstein-Friesian, Jersey and a cross between the two breeds. Herds are usually composed of a mix of different breeds, of which the ratios vary per dairy farm. Jerseys are known to have a higher fat and protein content but a lower yield of milk solids (Cerbulis and Farrell, 1975;

Lembeye, Lopez-Villalobos, et al., 2016c). On the other hand, Holstein-Friesian cows produce higher volumes of milk that is lower in protein and fat. Holstein-Friesian×Jersey is a popular breed, making up almost half of the national herd in New Zealand in 2021 (LIC and DairyNZ, 2021). Crossbreeding improves the production traits of dairy cows (Lopez-Villalobos et al., 2000) and has been subject to research to understand the heterosis effects on different milk characteristics (Ahlborn and Dempfle, 1992; Back and Lopez-Villalobos, 2007; Lembeye, Lopez-Villalobos, et al., 2016a). The parity of a cow (number of calving) was positively correlated with the milk yield up to the third year in lactation while the fat content decreases (Ikonen et al., 2004; Lembeye, Lopez-Villalobos, et al., 2016c).

2.2 Processing of bovine milk

Many processing technologies are applied to milk and milk-derived ingredients to obtain a range of desirable dairy product characteristics. Most milk is processed before consumption to ensure food safety and quality. The processing conditions of, for example, heating, homogenisation, and acidification are adjusted to achieve structural and chemical properties, as well as functions that are desired product properties.

2.2.1 Heat treatment

One of the most common processes is heat treatment, which, most importantly, ensures the safety of a product and extends its shelf-life. Heat treatment can also be used to modify milk proteins to obtain specific functional properties.

Pasteurisation is one of the heat treatments of which the temperature and time are sufficient to kill pathogenic bacteria and increase the shelf-life up to 21 days. The conditions of pasteurisation may vary but are at least 72 °C for 15 seconds (Moatsou, 2013). Ultra-high temperature (UHT) treatment involves a higher temperature for a few seconds (130-145 °C for 1 to 5 seconds). It aims to obtain a commercially sterile product with all micro-organisms and spores killed and most of the enzymes deactivated. The shelf-life is increased up to a few months, while there are minimal changes to the physical and chemical properties of the milk constituents (Moatsou, 2013; Walstra et al., 2005).

Additional to these two heat treatments, other heat treatments such as thermalisation, high-temperature pasteurisation, sterilisation and extended shelf-life treatment are also applied depending on the desired outcome product (Moatsou, 2013; Walstra et al., 2005). A heat treatment (85-95 °C for several minutes) used in the yoghurt manufacturing process is applied to increase gel strength (Anema et al., 2004). The adjustment of the temperature, time and pH during the heat treatment has an impact on structural properties such as firmness and graininess of the acid gel (Anema et al., 2004; Kücükcetin, 2008; Udabage et al., 2010). The time and temperature used for this type of heat treatment is usually around 90 °C for up to 10 – 30 minutes but can vary depending on the desired properties of the product (Lucey and Singh, 1997; Udabage et al., 2010; Walstra et al., 2005).

When milk is heated, multiple changes occur, affecting different components. Structural modifications such as whey protein denaturation, changes in the casein micelle integrity and shifts in the salt equilibrium are often highlighted in the literature. These changes are described below.

Casein does not have much of a tertiary structure and is relatively heat-stable (Broyard and Gaucheron, 2015). In contrast, whey proteins are sensitive to thermal processing. They are responsible for one of the major consequences of heat treatments, denaturation, which has multiple effects on the chemical, physical and nutritional properties of the milk (Wijayanti et al., 2014). Heating of milk can induce both reversible or irreversible changes to the proteins, depending on the conditions of the heat treatment and compositional factors (Walstra et al., 2005; Wijayanti et al., 2014). β-LG is the most abundant whey protein and is essential in aggregation behaviour involving whey proteins. The denaturation and aggregation pathways have been studied for a long time; the involvement of disulphide bridges in irreversible aggregation is well-established, but there is no consensus on the exact mechanisms involved (Wijayanti et al., 2014).

Whey proteins form aggregates with caseins, especially κ-CN, and other whey proteins and interact with MFGM proteins (Havea et al., 2001; Lee and Sherbon, 2002). Different whey proteins respond differently to heat treatments (Dannenberg and Kessler, 1988). For example, α-LA forms aggregates more readily in the presence of β-LG or bovine serum albumin. The temperature at which different proteins denature and form aggregates also vary (Gezimati et al., 1996; Havea et al., 2000). The heat-induced gelling properties of α-LA are weaker than those of β-LG (Wijayanti et al., 2014).

The association of whey proteins to casein micelles during heat treatment increases the micelle size (Anema and Li, 2003). The extent to which this happens depends on temperature, heating time, pH, ionic strength, and protein concentration (Anema, 2008c). Denatured whey proteins also form aggregates with dissociated κ-CN in the serum phase, but research has suggested that association with micellar κ-CN is preferred (Anema, 2008b; Donato, Guyomarc'h, et al., 2007; Guyomarc'h et al., 2009). During heat treatment, κ-CN dissociates from the micelles into the serum phase. The amount of dissociated casein in the serum phase increases with increasing temperature, although the dissociation of α_s-CN and β-CN decreases between 70 °C and 100 °C before increasing again (Anema, 1998). The dissociation of casein from the micelle during heating is also pH-dependent (Anema, 1998).

Besides thiol-disulphide exchange reactions, other chemical changes occur during the heating of the milk. The Maillard reaction is a well-known series of reactions between a reducing sugar and an amino group resulting in the glycation of proteins. Consequences include the loss of available lysine and the changes in colour and flavour (Burton, 2012; Qi et al., 2015; Walstra et al., 2005). Although this reaction does occur at low temperatures, the rate is minimal and at relatively mild heat treatments such as pasteurisation, almost negligible (Fenaille et al., 2006). Heat treatment also impacts the flavour characteristics of the milk (Fox et al., 2015).

2.2.2 Homogenisation

Like most lipids, milk fat, comprised of 97-98% TAGs, is hydrophobic and needs to be emulsified to remain dispersed in the water phase. In the natural state of milk, the MFGM fulfils this role, but due to the size of the globules, the milk fat in unprocessed milk is unstable against creaming. Homogenisation is a process that decreases the size of the milk fat globules by applying high pressure, thereby preventing destabilising mechanisms such as creaming and consequential partial coalescences (Walstra et al., 2005). Commonly, pressures of between 13-20 MPa are used, and milk is homogenised in two stages; the second stage homogenisation step is at a lower pressure to break up the clumps of milk fat globules formed during the first step. The size of the globules is reduced from 2.8-4.6 μm to smaller than 1 μm (Fox et al., 2015).

Upon a decrease in fat globule size while maintaining the volume of the fat, the surface area of the fat globules increases. The MFGM ruptures during the process, and proteins, including casein micelles, casein submicelles, and whey proteins, adsorb to the interface (Lee and Sherbon, 2002; Walstra et al., 2005). The protein concentration of the lipid-serum interface is increased, with predominantly casein adsorbing to the interface (Zahar and Smith, 1996). The composition of the casein fraction at the interface differs from that in serum; β-CN and κ-CN fractions are higher at the interface, and the α-CN fraction is lower at the interface than in the serum (Zahar and Smith, 1996). When the milk is heat-treated after homogenisation, the denatured whey proteins interact with the interfacial casein as well as MFGM proteins (Corredig and Dalgleish, 1996; Dalgleish and Sharma, 1993; Lee and Sherbon, 2002; Michalski and Januel, 2006). These changes in the interface composition impact the gelling properties, depending on the processing conditions and composition of the milk (Lucey and Singh, 1997; Michalski et al., 2002).

2.2.3 Rennet gelation

In their natural state, casein micelles are stabilised against aggregation by the layer of κ-CN on the outside. Upon addition of rennet, the κ-CN is enzymatically hydrolysed, thereby removing the steric and electrostatic stabilisation. Consequently, the casein micelles coagulate and form a curd which is the start of the cheesemaking process (Lucey, 2002). The composition of milk and the protein fraction in milk affect the coagulation properties. A higher total protein and casein-to-protein ratio will result in higher cheese yield. However, varying results have been reported on the correlation between protein content and gel strength (Auldist et al., 2004; Glantz et al., 2010; Ikonen et al., 2004). Higher proportions of κ-CN, α_{s1}-CN and β-CN resulted in better rennet coagulation properties (Wedholm et al., 2006), and a higher κ-CN glycosylation degree resulted in longer coagulation times (Bonfatti et al., 2014). Also, a high SCC has been associated with poor cheese-making properties regarding yield and cheese quality (Auldist et al., 1996).

Calcium is involved in the network formation, forming bridges between destabilised casein micelles (Lucey, 2002). Calcium is even added to cheese milk to improve the cheese yield (Fox et al., 2017). Ionic calcium also screens charges on the micelles improving the aggregation (Dalgleish, 1983), but an excess in calcium (over 10 mM) had an opposite effect and did not further improve gelation properties (Udabage et al., 2001)

Additional to milk composition, the structural properties of the milk also impact gel formation. For example, smaller casein micelles result in higher gel strength (Glantz et al., 2010). Milk fat globules in their native state do not participate in the gel structure. Upon homogenisation, the increased surface area of milk fat globules is covered in protein allowing fat globules to be incorporated into the network (Kelly et al., 2008). However, homogenisation of cheese milk leads to a higher moisture content of the curd which impacts the ripening of the cheese, and due to disruption of the fat globule membrane increased lipolysis can occur without proper heat treatment (Kelly et al., 2008). Although pasteurisation is usually applied in commercial cheese manufacturing, intense heating impairs gel formation (Britten and Giroux, 2022; Dinkov and Dushkova, 2007; Kethireddipalli et al., 2010). The complex formation between whey proteins and κ-CN during higher-intensity heat treatments negatively impacts coagulation (Guyomarc'h, 2006).

2.2.4 Acid gelation

Lowering the pH is another way in which the proteins in milk destabilise and form a gel. Acid-induced gelation is part of the yoghurt manufacturing process. During this process, milk is first heated to improve the gelation properties (increased firmness and lower gelation times). The heat treatment of 80-95 °C for up to 30 minutes is applied to obtain a high level of whey protein denaturation (Lucey and Singh, 1997; Lucey et al., 2022; Walstra et al., 2005). As discussed in Section 2.2.1, denatured whey proteins form complexes with mostly κ-CN. The denatured whey proteins in the serum phase and aggregated with the micellar κ-CN increase the gelation pH due to its higher isoelectric point, lowering the gelation time. Additionally, the distribution of denatured whey proteins between the serum and colloidal phase affects the acid gelation properties. Higher levels of serum whey protein aggregates increase gel firmness (Anema et al., 2004).

Milk composition also impacts the acid gelation properties. Li et al. (2020) studied the seasonal variation in acid gelation properties of bovine milk in New Zealand. Their results showed that late lactation milk had inferior acid gelation properties, which they attributed predominantly to the correlation with a higher κ-CN glycosylation degree. Whey proteins form an integral part of the gel network, but β-LG has better gelling properties than α-LA (Graveland-Bikker and Anema, 2003; Matumoto-Pintro et al., 2011). Also, small changes in the pH of the milk before and after heat treatment affects acid gelation properties (Anema et al., 2004; Del Angel and Dalgleish, 2006; Lakemond and van Vliet, 2008).

2.3 The effect of a once-a-day milking production system on the yield and composition of bovine milk

In New Zealand, dairy farming is pasture-based, and most dairy farms follow a seasonal system where cows calf in late winter and early spring and are dried off in early winter. Typically, in New Zealand, cows are milked twice-a-day (TAD) from the start of the season (90-92% of farms). Towards the end of the season, the milking frequency is sometimes reduced to three times in two days or once-a-day (OAD). The reduction in milking frequency could be applied due to, for example, feed shortage or health reasons of the herd (Bewsell et al., 2008). Less frequent milking decreases the number of times the cows have to walk to the milking shed and, thereby, the incidence of lameness and may lower heat stress during peak lactation in the summer months (Kendall et al., 2008; Stelwagen et al., 2013). Finally, with increasing herd sizes and changing lifestyle expectations, finding long-term quality staff to manage the high workload that TAD dairy farming demands is increasingly difficult. This factor is one of the motivations for farmers to adopt the OAD milking practice all year round (Bewsell et al., 2008). Compared to the more conventional TAD milking routine, it reduces total expenses and increases flexibility (Bewsell et al., 2008; Lazzarini et al., 2018).

Reducing the milking frequency from TAD to OAD is not without consequences. Decreasing the milking frequency reduces the milk yield by 10-30% on average worldwide and approximately 11% in New Zealand (Bewsell et al., 2008; Edwards, 2018; Lacy-Hulbert et al., 1999; Lazzarini et al., 2018; Lembeye, Lopez-Villalobos, et al., 2016c; Murney et al., 2015; O'Brien et al., 2002; Rémond et al., 2004; Stockdale, 2006). Payment in New Zealand is predominantly based on the total milk solids, so a decrease in the milk yield results in a reduction in income. However, the cost of day to day running of OAD milking is lower compared to TAD milking, and therefore the system is financially viable (Edwards, 2019). It should be noted that not all farm systems are suitable for OAD milking, and adjustments may need to be made according to DairyNZ recommendations. Such adjustments are related to the herd, as not all cows are suitable for OAD milking. Udder health and support are important factors as the cow will hold the milk for extended periods, and the udder must hold a higher volume of milk. Additionally, the breed of the cow is also of importance, with Holstein-Friesian and crossbreeds less likely to be selected for OAD milking than Jersey breeds (Rocha et al., 2018).

The composition of the milk is also affected by OAD milking practices. The milk, protein, and fat yields of each of the main New Zealand dairy cattle breeds are lower in OAD milking than in TAD milking (Lembeye, Lopez-Villalobos, et al., 2016c). The protein and fat contents increase by 2-8% (Lembeye, Lopez-Villalobos, et al., 2016c; Rémond et al., 2004). The SCC has been found to increase in some studies by up to 86%, but not in all, and the incidence of mastitis does not increase (Clark et al., 2006; Rémond and Pomiès, 2005; Stelwagen et al., 2013). Some studies reported a decrease in the casein-to-whey ratio (Auldist and Prosser, 1998; Klei et al., 1997; Martin et al., 2009; O'Brien et al., 2002; Pomiès et al., 2007; Sorensen et al., 2008), but others did not (Rémond et al., 2004). Recently, researchers reported an increase in the lactoferrin content from 508 mg/L in TAD milk to 574 mg/L in OAD milk (Gedye et al., 2020) which is high compared to a reported range of 20 to 500 mg/L with an average value of 100 mg/L (Claeys et al., 2014; Fox et al., 2015). The milk in this study (Gedye et al., 2020) was collected during early lactation, one week post-partum, which explains the high values as lactoferrin is higher in colostrum milk and early lactation milk (Fox et al., 2015).

Studies on the effects of milking frequency on milk composition and yield are mainly from an agricultural point of view. Therefore, most of the data focuses on aspects like genetics and milk yield, and the compositional properties are often limited to only the fat and protein contents. Additionally, a lot of the research was conducted in the late 1990s and early 2000s. Only in the last few years has the interest in this topic increased again, possibly due to an increasing number of farms adopting a OAD milking system for the reasons described above. Research on the impact of OAD milking, with a focus on the protein composition, is also limited. One study reports the secretory activity of different proteins but included no compositional analysis of the milk. Their findings suggest a decrease in the α-CN, β-CN, α-LA and β-LG contents and an increase in the bovine serum albumin, lactoferrin, κ-CN and γ-CN contents in the milk (Murney et al., 2015). O’Brien et al. (2001) looked at the effect of milking frequency on the processing quality of milk (O'Brien et al., 2002). They did not find OAD milking to negatively impact processability as analysed by curd rennet coagulation time, rate of curd aggregation and curd firmness. Additionally, they did not observe a difference in casein content and composition (α_{s1}-

CN, α_{s2}-CN, β-CN, κ-CN, and γ-CN) but reported an increased whey protein content and thereby decreased casein-to-whey ratio in OAD milk (O'Brien et al., 2002).

2.3.1 Massey University dairy farms

Massey University has several farms in Palmerston North near the university campus that are used for teaching and research purposes. Two of these farms are Dairy No. 1, which is the farm where the cows are milked OAD and Dairy No. 4, which uses the conventional TAD. Besides the teaching and research objectives, the aim of the set-up of Dairy No. 1 is “to explore environmental and financial aspects of once-a-day milking” while meeting local environmental requirements. Dairy No. 1 is a functional dairy farm delivering milk to Fonterra. At the same time, it allows access to researchers and students from different disciplines at Massey University. This dairy farm switched to OAD milking in 2013, and since then, the herd has been optimised to perform well under the OAD milking routine. Researchers have been working with the farm to select the right cows and implement a low-input (of, e.g., concentrate feeding and soil fertiliser) farm management system.

The herd at Dairy No. 1 is smaller than at Dairy No. 4, with approximately 250 cows compared to 600 cows at Dairy No. 4. While Dairy No. 1 has been relatively consistent over the years, the use of Dairy No. 4 and herd management differs each year depending on the research projects that are working with this farm. Examples of implemented changes are temporary switches to OAD milking and indoor housing during the 2016/2017 season, and the milk obtained from differently managed cows often ends up in the same bulk tank. However, the history and breed composition of individual cows are well-known.

Besides, between year changes in herd management, there are some differences regarding the feed of the cows. Dairy No. 1 is pasture-based and does not use concentrates or supplements. The main crop is ryegrass-white clover, and in addition to that, lucerne, plantain, chicory, red clover, turnips, rape, kale, and maize are grown in minor quantities of which the allocation has differed each year since 2014. Dairy No. 4 is also pasture-based with predominantly perennial ryegrass and white clover as used on Dairy No. 1. Additionally, summer crops like chicory and turnips are fed and a variety of supplementary feed, including silage, bailage, molasses, meal, hay, maize, and straw. The composition of the feed differs per year. Finally, the herd composition in terms of breeds also differs. The herd at Dairy No. 1 is composed of 50% crossbred cows, just over 25% Holstein-Friesian and just under 25% Jersey cows. Dairy No. 4 has approximately 60% crossbred cows, 40% Holstein-Friesian and less than 10% Jersey cows.

The production data from 2015-2019 has been collected as part of this PhD thesis and is displayed below. Table 2.3 shows the number of cows and the average production per cow given as the total milk solids, litres, protein yield and fat yield. This average value per yield factor was calculated by dividing the total yield of either milk solids, litres, protein, or fat by the maximum number of cows milked during the season. The actual values may differ as the total yield values are provided by Fonterra, who collects the milk. Milk may have been taken for research purposes, or certain cows may have been milked separately (e.g., when the cow is suffering from mastitis, this milk is not allowed in the tank), which results in a lower amount in the tank. Nevertheless, these values do give a good indication of the comparison between OAD and TAD milking. As discussed at the beginning of this chapter, a reduction in milking frequency from OAD to TAD results in a decrease in the total milk yield. This is also the case when comparing the Dairy No. 1 farm (OAD) to the Dairy No. 4 farm (TAD). From 2015 until 2018, the difference in milk solids yield is 11-13% but increased to 31% in the 2018-2019 season. In the 2019-2020 milking season, the difference is lower again (23%) but not as low as the seasons before 2018-2019. It is unclear what the reason for this variation is. The lowest milk solids (kg) per cow at Dairy No. 4 (TAD milking) in the 2017-2018 season can be explained by the switch to OAD milking from November 2017 until the end of the season due to a drought which limited the availability of feed and decreased the production of the cows.

Table 2.3. Number of cows per season on Massey University Dairy Farm No. 1 and Dairy No. 4 (Dairy No. 1 and Dairy No. 4, respectively), and yearly yield per cow given as milk solids (in kg), litres, protein (in kg) and fat (in kg) during the seasons 2015-2020, including the difference between the farms (%).

		2015/ 2016	2016/ 2017	2017/ 2018	2018/ 2019	2019/ 2020
Number of cows	Dairy No. 1	264	258	251	242	264
	Dairy No. 4	608	597	578	565	602
Milk solids yield (kg//cow)	Dairy No. 1	351	358	349	320	345
	Dairy No. 4	402	410	390	466	447
	Difference	*-13%*	*-13%*	*-11%*	*-31%*	*-23%*
Total yield (L/cow)	Dairy No. 1	3800	3780	3781	3442	3652
	Dairy No. 4	4574	4810	4513	5550	5281
	Difference	*-17%*	*-21%*	*-16%*	*-38%*	*-31%*
Protein yield (kg/cow)	Dairy No. 1	153	155	152	138	150
	Dairy No. 4	173	180	171	207	200
	Difference	*-12%*	*-14%*	*-11%*	*-33%*	*-25%*
Fat yield (kg/cow)	Dairy No. 1	199	203	197	182	195
	Dairy No. 4	228	231	219	259	247
	Difference	*-13%*	*-12%*	*-10%*	*-30%*	*-21%*

The protein content of milk from Dairy No. 1 and Dairy No. 4 farms throughout different seasons is shown in Figure 2.3. As expected from previous research, the protein content is higher in milk from OAD milked cows than in milk from TAD milked cows in each of the seasons shown. Both milking routines result in a steep increase toward the end of the season.

Figure 2.4 shows the fat content of each of the milk samples throughout the various seasons and was also found to be higher in milk from OAD milked cows than that of TAD milked cows. While the fat content in milk from Dairy No. 1 is similar between different seasons, large variability can be seen between seasons at Dairy No. 4. The latter could be the result of the differences in farm management per year. For example, in the 2016-2017 season, the cows on the Dairy No. 4 farm were split into multiple herds, of which some were housed inside, and some were milked OAD for some time. However, the milk was collected in the same tank, which would have affected the composition of bulk milk. Although there are some differences in the fat content between seasons from 2014-2018, the fat content across the last two seasons was similar.

In the 2017-2018 season, the difference in protein and fat contents in milk from the Dairy No. 1 farm and the Dairy No. 4 farm decreased from December. The average temperature (21.2 °C, Figure 2.6) during this summer was higher than most of the other years, and the average rainfall in November and December 2017 was low compared to other years. These two factors could have caused some degree of heat stress in the cows and a lack of feed, both decreasing milk production. It is unknown whether OAD milked cows were affected differently by this than TAD milked cows, but Holstein-Friesians are known to be more sensitive to heat stress than Jersey (Smith et al., 2013). The higher fraction of Holstein-Friesians in the Dairy No. 4 herd can explain this change in protein and fat contents during the 2017/2018 season. The SCC is expected to be higher in milk from OAD milked cows compared to milk from TAD milked cows. This result was the case for most of the seasons except the 2017/2018 season, where the SCC value in milk from Dairy No. 4 farm seems to be higher compared to the other years (Figure 2.5), which can also be explained by the more extreme weather conditions during this summer.

To summarise the findings on the comparison of Massey University Dairy Farms No. 1 and No. 4, the overall trend aligns with the previously reported impact of OAD milking. The milk and milk solids yield are lower from OAD milked cows than from TAD milked cows, and the protein content, fat content and SCC are higher. There are some between-year differences, which differences in herd composition and meteorological impacts could explain.

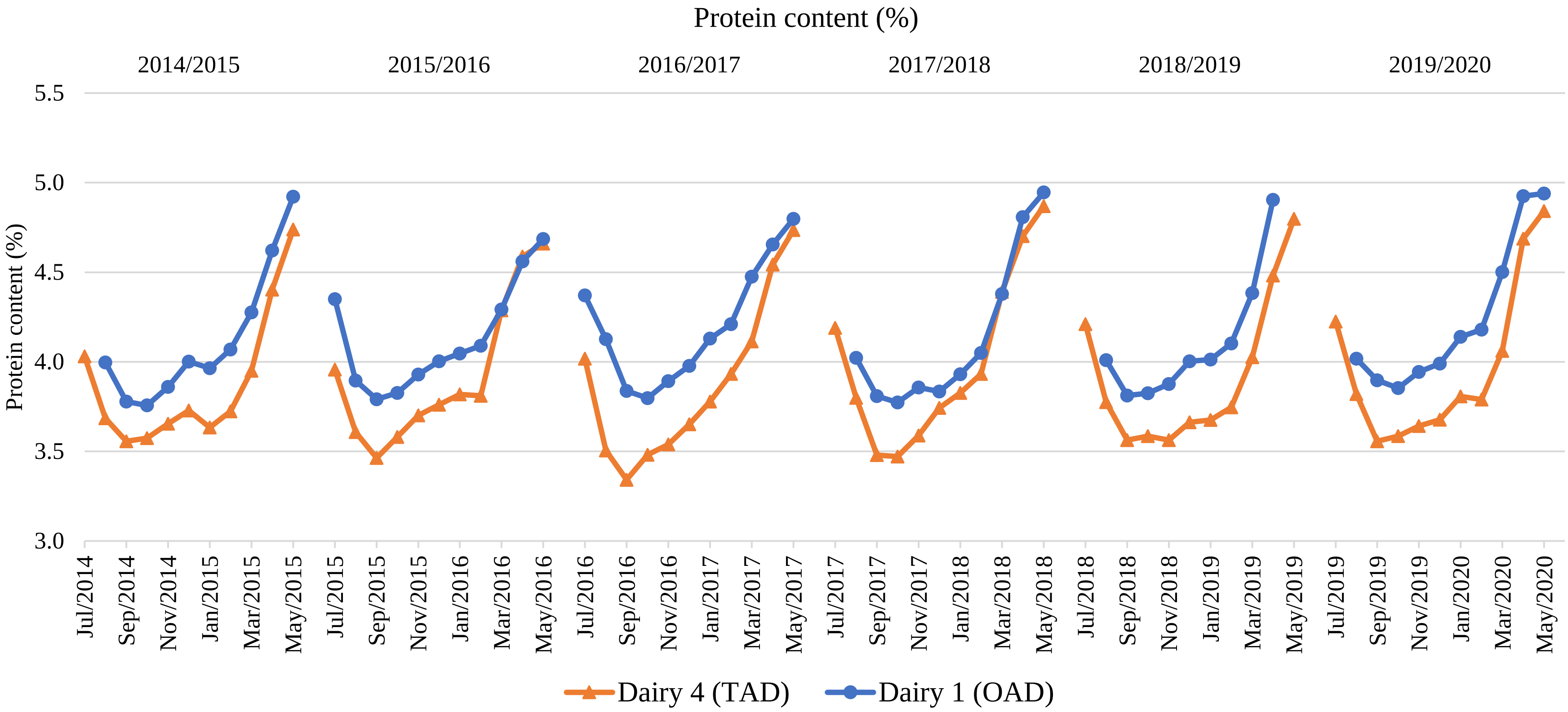


Figure 2.3. The protein content of milk from Massey farms Dairy No. 1 (OAD milking) and Dairy No. 4 (TAD milking) throughout the season from 2014-2020, given as average values per month.

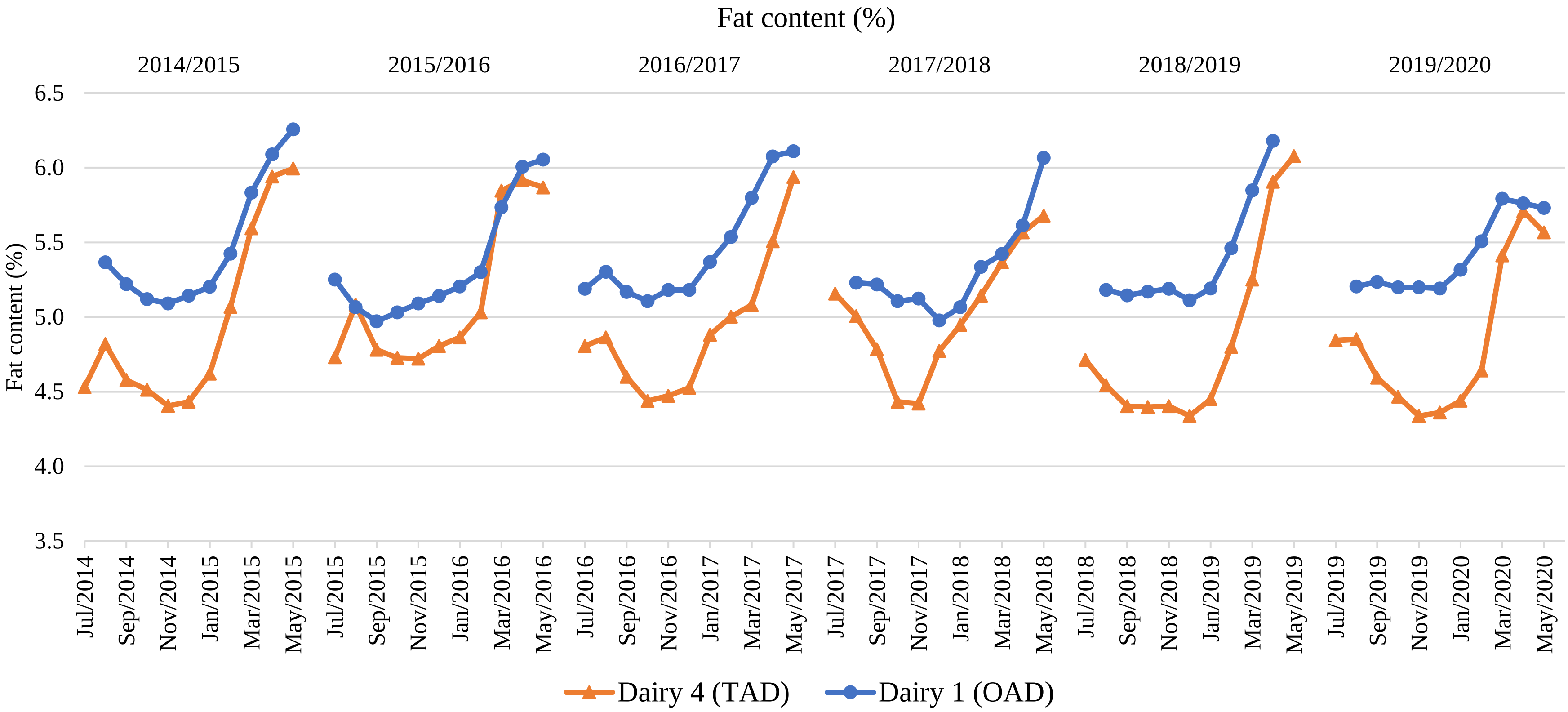


Figure 2.4. The fat content of milk from Massey farms Dairy No. 1 (OAD milking) and Dairy No. 4 (TAD milking) throughout the season from 2014-2020, given as average values per month.

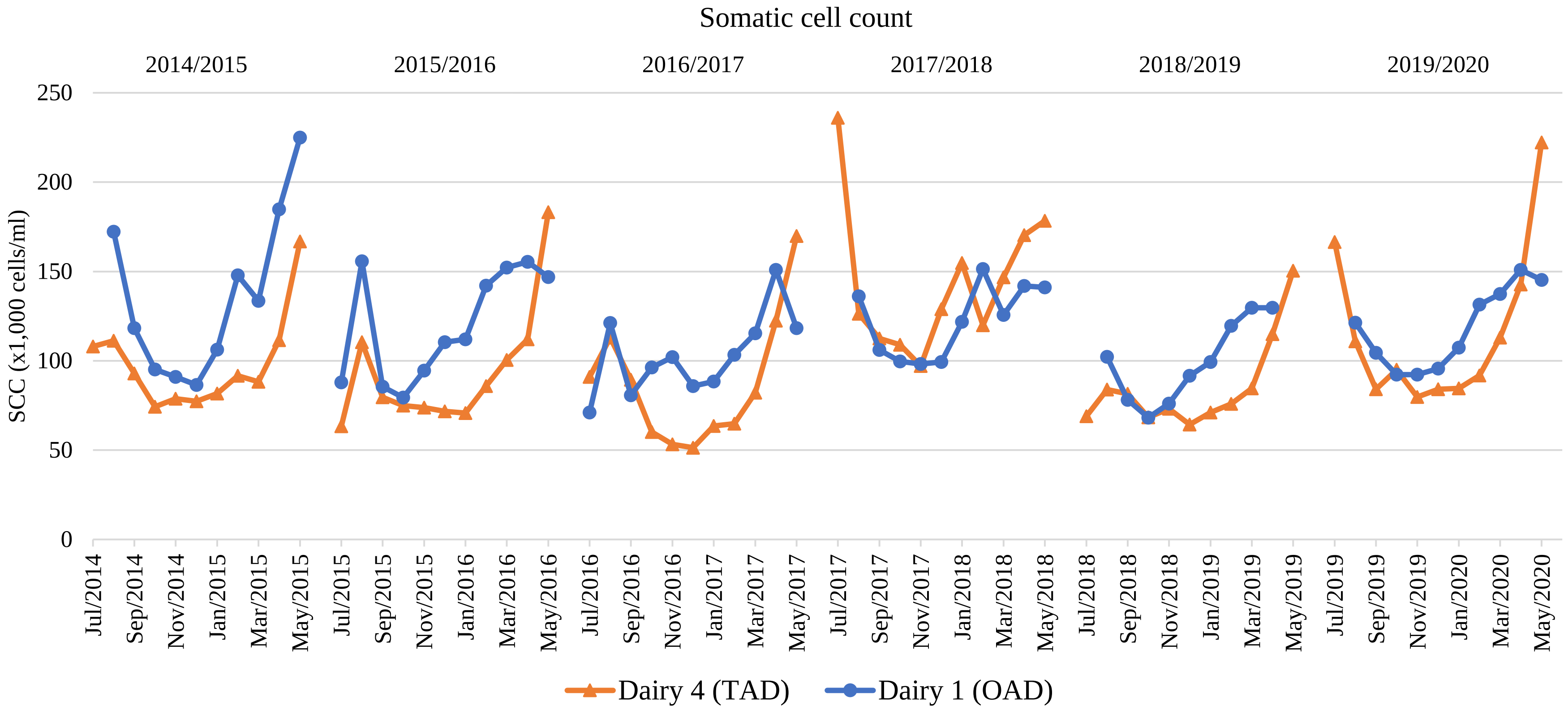


Figure 2.5. The somatic cell count (SCC) of milk from Massey farms Dairy No. 1 (OAD milking) and Dairy No. 4 (TAD milking) throughout the season from 2014-2020, given as average values per month.

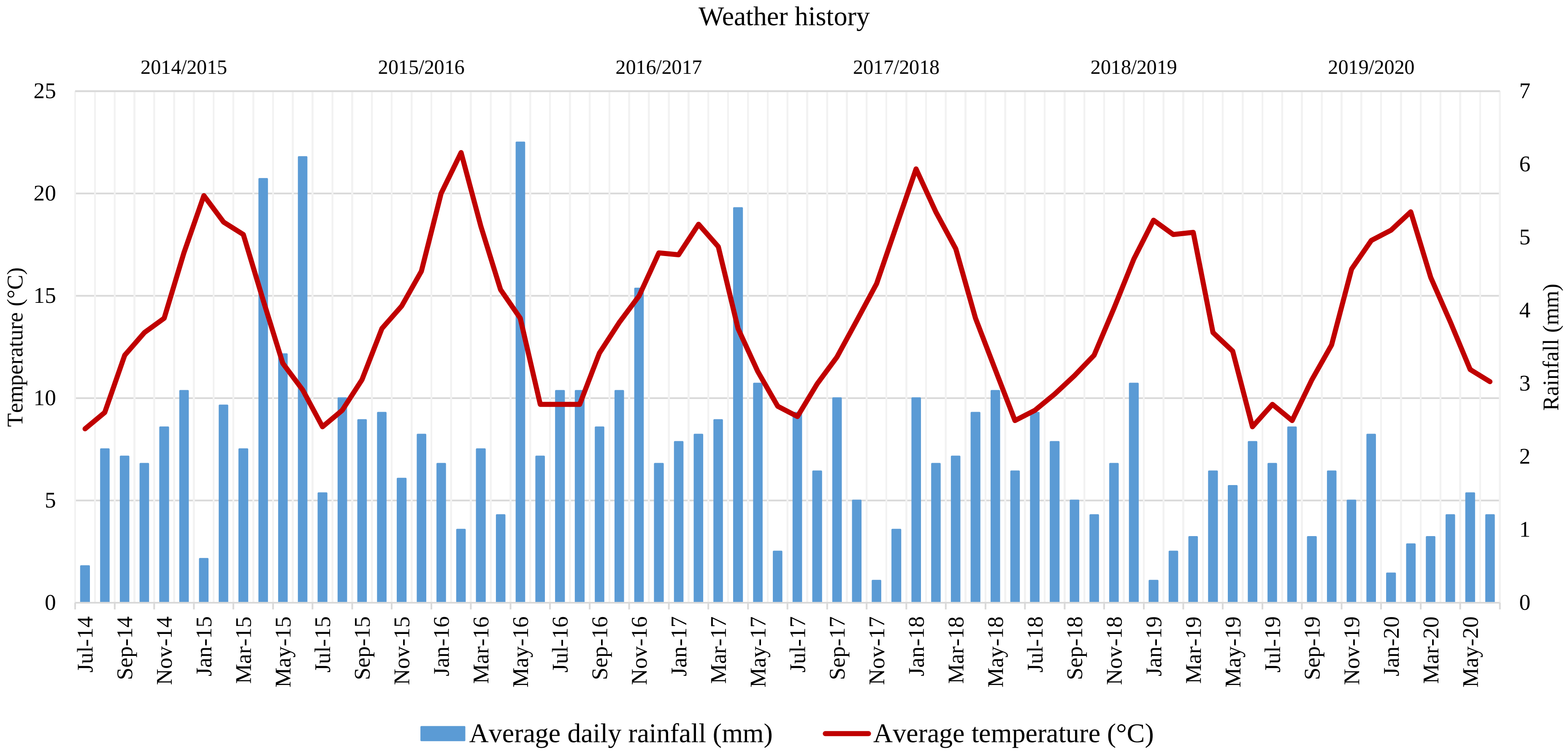


Figure 2.6. Weather history in Palmerston North: the average temperature (°C) and average daily rainfall (mm).

Source: www.palmyweather.co.nz/trendshistoric.php.

2.4 Concluding remarks

Several studies have focused on the impact of OAD milking on milk composition and yield. This past research has shown that a reduction in milking frequency affects the content and yield of protein and fat. However, these studies are either limited to the proximate composition of milk or only reduce the milking frequency temporarily. For processing purposes, other minor components such as individual proteins and minerals, play a crucial role in the product quality. The literature has highlighted the importance of these components and other physicochemical characteristics of milk for heating and gelation properties.

Although the research on the protein and mineral profiles of bovine milk in a farm system where cows are milked OAD all year round is lacking, previous studies indicate that OAD milking not only impacts the major components but also minor components in milk. Potential changes in these components could impact the processing properties of the milk. If the number of farms adopting a OAD milking production systems continues to increase, it is essential to understand the potential compositional changes and the impact these may have on the dairy industry.

The aim of this thesis was to obtain a better understanding of the impact of OAD milking on the compositional properties of bovine milk. It was hypothesised that milk from cows milked OAD, compared to milk from cows milked TAD, has a different protein and mineral composition which will affect the processing properties of the milk. To test this hypothesis the following objectives have been set:

1. To study the effect of OAD, compared to TAD, milking on the proximate and protein composition of milk.

Milk was sampled in early, mid-, and late lactation from individual cows from a farm that milks TAD for the majority of the milking season and from a farm that milks OAD all year round. These cows were selected based on their breed, parity, breeding worth and calving date to limit the effect of confounding factors. The feed of the cows was not controlled in this study since the diet is part of the differences in the farm management systems. This difference in the diet is a confounding factor, however, and therefore, the feed of the cows prior to milk sampling was analysed and its composition presented together with the milk composition results. The milk and milk solids yield, proximate composition and protein composition were determined. Initially, the effect of milking frequency and stage of lactation was studied (Chapter 4). Then the role of breed in milk yield and composition was also studied in each milking system (Chapter 5).

2. To study the effect on the proximate, protein and mineral composition of bulk milk prior to processing.

In reality, milk from multiple cows is collected in one milk tank and mixed according to respective yields. Milk was collected throughout the lactation from individual cows milked either OAD or TAD, and selected based on their breed, parity, breeding worth and calving date, was mixed according to the respective yields. This milk was characterised for the proximate, protein and mineral composition as well as some physicochemical properties (Chapter 6).

3. To study the effect of OAD milking on the processing properties of milk.

Following the previous objective, mixed milk samples were used to firstly study the heat-induced changes in milk (Chapter 7). The following heat treatments were used:

- Pasteurisation at 72 °C for 15 seconds

- High heat treatment: 95 °C for 5 minutes, a heat treatment used during yoghurt production
- UHT treatment at 140 °C for 5 seconds
- Raw milk was used as a control

Secondly, the effect of OAD milking on the rennet and acid gelation properties were studied (Chapter 8). Pasteurised, with and without a homogenisation step, milk was used for rennet-induced gelation. Milk heated at 95 °C for 5 minutes was used for acid-induced gelation. These properties will provide valuable insight into potential implications of OAD milking for cheese and yoghurt productions. Figure 2.7 shows an overview of the structure of the thesis.

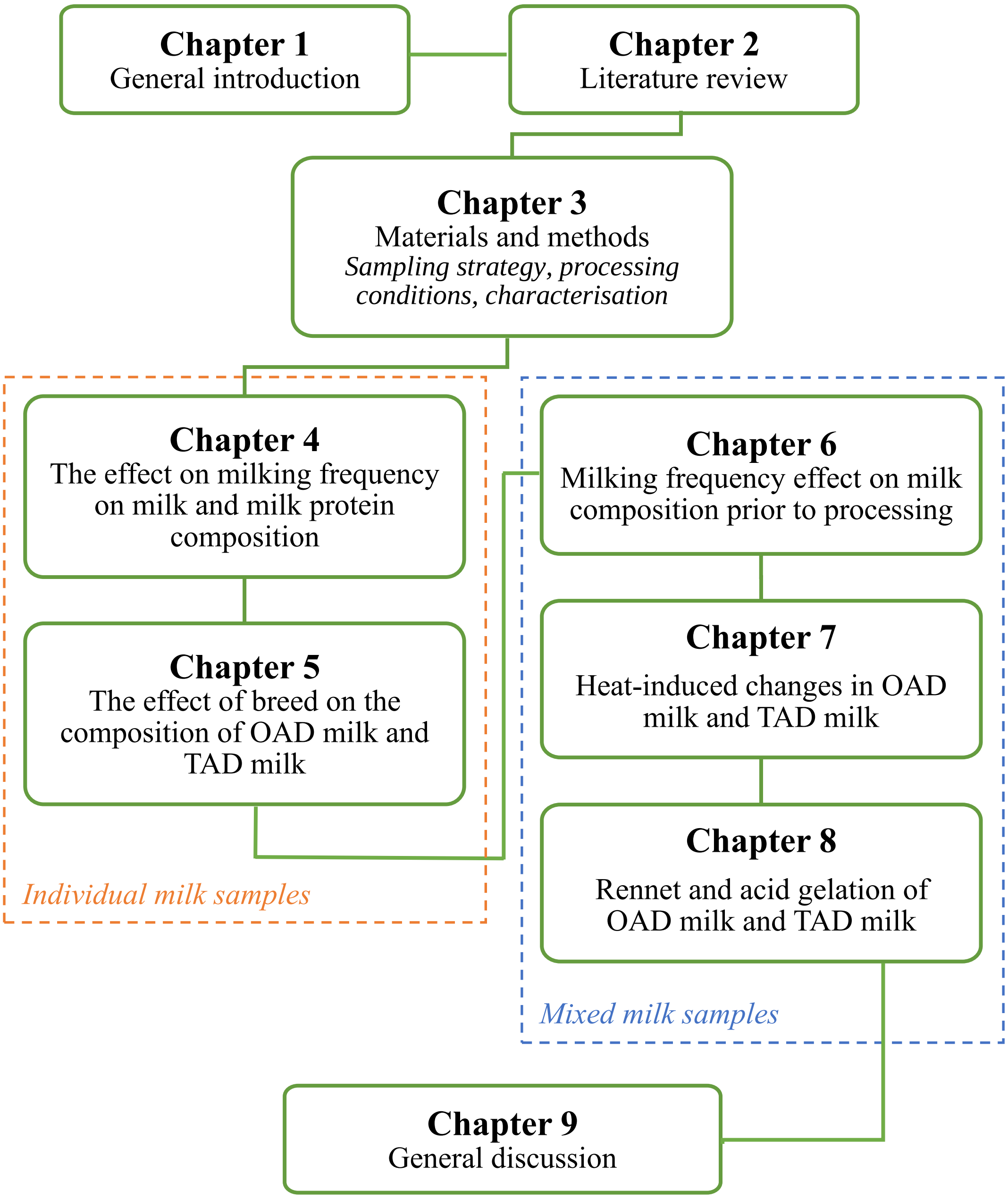


Figure 2.7. Thesis structure.

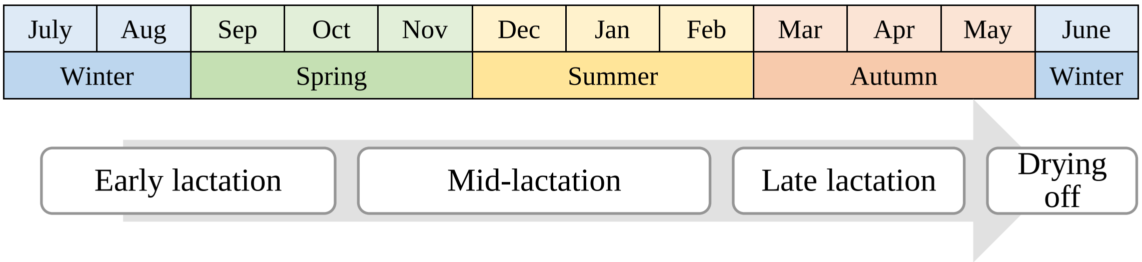
Chapter 3

Materials and methods

3.1 Milk sampling

The milk used for this study was obtained from the Massey University Dairy Farm No. 1 (OAD milking) and Massey University Dairy Farm No. 4 (TAD milking). These farms are used for research and teaching purposes as well as supplying milk to a dairy company. Milk was collected in the 2020-2021 production season. Samples were taken from individual cows that were selected according to the criteria outlined in Section 3.1.1.

For each timepoint (early, mid-, and late lactation), three replicates were included meaning that milk was collected three times, each one week apart. In New Zealand, the stages of lactation align with the meteorological season (Figure 3.1). Calving on each of the farms was scheduled from late July until the end of September, with some cows expected to calve in October. To ensure there were enough cows available that would meet the selection criteria, the first early lactation sampling was scheduled in the middle of September. Mid-lactation sampling was scheduled during peak production in late spring to early summer, from the last week of November until the middle of December. Late lactation sampling was scheduled from the end of March until the middle of April. Eventually, the days in milk (DIM, number of days since calving on the sampling day) for each of the cows were known and used to determine the lactation stage of each cow at the time of sampling. The different stages of lactation were defined as early (DIM ≤ 90 days), mid (DIM > 90 day and ≤ 180 days) and late (DIM > 180 days).



July	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	June
Winter		Spring			Summer			Autumn			Winter

Figure 3.1. A graphic display of how the milking season and meteorological seasons align with the calendar months on most farms in New Zealand.

3.1.1 Selection criteria

The cows were selected based on breed, parity, calving date, and breeding worth. The breed of the cow was determined as previously described by Handcock et al. (2019). The breed composition of each cow is expressed as a fraction of 16. Any cows that were of a partial breed that was either unknown or other than Holstein-Friesian or Jersey were excluded. The classification criteria are outlined in Table 3.1.

Table 3.1. Classification of breeds and their breed composition used in the study.

Breed	Abbreviation	Breed composition
Holstein-Friesian	F	F ≥ 14/16
Holstein-Friesian×Jersey crossbreed	F×J	F < 10/16 and J < 10/16
Jersey	J	J ≥ 14/16

After excluding cows that did not meet the criteria described above, cows with a parity lower than three or higher than four were excluded. Cows in third and fourth parities, the peak milk production years, were selected. Unfortunately, there were not enough cows of the Jersey breed at Massey University Dairy Farm No. 4 to meet this parity criterium. As a result, the TAD milked Jersey cows were of the second and sixth parities. Calving was spread out over multiple months, but the aim was to limit this spread in the cows that were selected for the study. Initially, cows that calved in August were selected. However, throughout the year some cows were replaced due to health issues (e.g., mastitis or treatment for lameness). Some of these cows had a calving date in September. Finally, where possible, cows with similar breeding worth values were selected. The breeding worth is the index that is used to rank cows and bulls according to their ability to effectively convert feed into profit (DairyNZ, 2021). From each farm, three Holstein-Friesian, three Jersey and three Holstein-Friesian×Jersey crossbreed cows that met the criteria as discussed above were selected.

3.1.2 Sampling procedure

From each cow, a full milking was collected in a test bucket. The amount of milk was recorded by weighing the bucket. After collection, the milk was immediately taken to the laboratory for further analysis. Milk from Dairy No. 4 (TAD milking system) was collected in the afternoon and on the following morning. Afternoon milk was stored at 4 °C overnight and mixed with the morning milk in the same ratio as the total afternoon: morning milk yield. This 24-hour milk sample was then used for further analysis and to make up the mixed milk sample.

Mixed milk samples were prepared to mimic tank milk for analyses in Chapters 6 to 8. Milk from Holstein-Friesian and Holstein-Friesian×Jersey breeds were mixed in the same ratio as the total milk yields. This mixed milk sample was processed in the Massey University Pilot Plant. This mixing step was a major difference with the individual samples used for the analyses reported in Chapters 4 and 5, since milk from higher yielding cows have a larger effect on the milk composition of tank milk than milk from lower yielding cows. A sample of this mixed milk sample was also sent to MilkTestNZ for somatic cell count analysis. Upon arrival in the laboratory, 0.02% sodium azide was added as a preservative.

3.1.3 Feed analysis

The diet of the cows from both farms mainly consisted of pasture, which contains a mix of perennial ryegrass and clover. In the summer months, chicory (both Dairy No. 1 and Dairy No. 4) and turnips (Dairy No. 1) were also fed. The latter was not fed when milk was collected. Fresh pasture and chicory samples were taken no more than 48 hours before the cows would start grazing the paddock. A “W” walking pattern was followed through the paddock and a random sample was taken every 10-15 meters. Additional concentrates (such as maize silage, bailage, and soy) that were fed prior to milk sampling were also sampled, but only once from each batch.

All feed samples were analysed by the Massey University Nutrition Lab. For soybean meal, dry matter (AOAC 925.10, 930.16), neutral detergent fibre and acid detergent fibre (Fibertec, Foss Analytics, Hillerød, Denmark), organic matter digestibility (Roughan and Holland, 1977) and crude protein (Dumas, AOAC 986.06) were determined separately. The chemical composition of molasses and turnips was taken from DairyNZ (DairyNZ, 2022). All other feed samples were analysed using Near Infrared spectroscopy analysis (Bruker MPA, Ettlingen, Germany).

3.2 Processing of milk samples

The mixed milk samples processed in the Massey University Pilot Plant. The raw milk was first pasteurised at 72 °C for 15 seconds. Consequently, the milk was either skimmed using a centrifugal separator (Model 103 AE, Alfa-Laval, Lund, Sweden) at 50°C or homogenised at 200/50 bar before further heat treatment. Figure 3.2 displays the processing steps the milk underwent for Chapters 7 and 8.

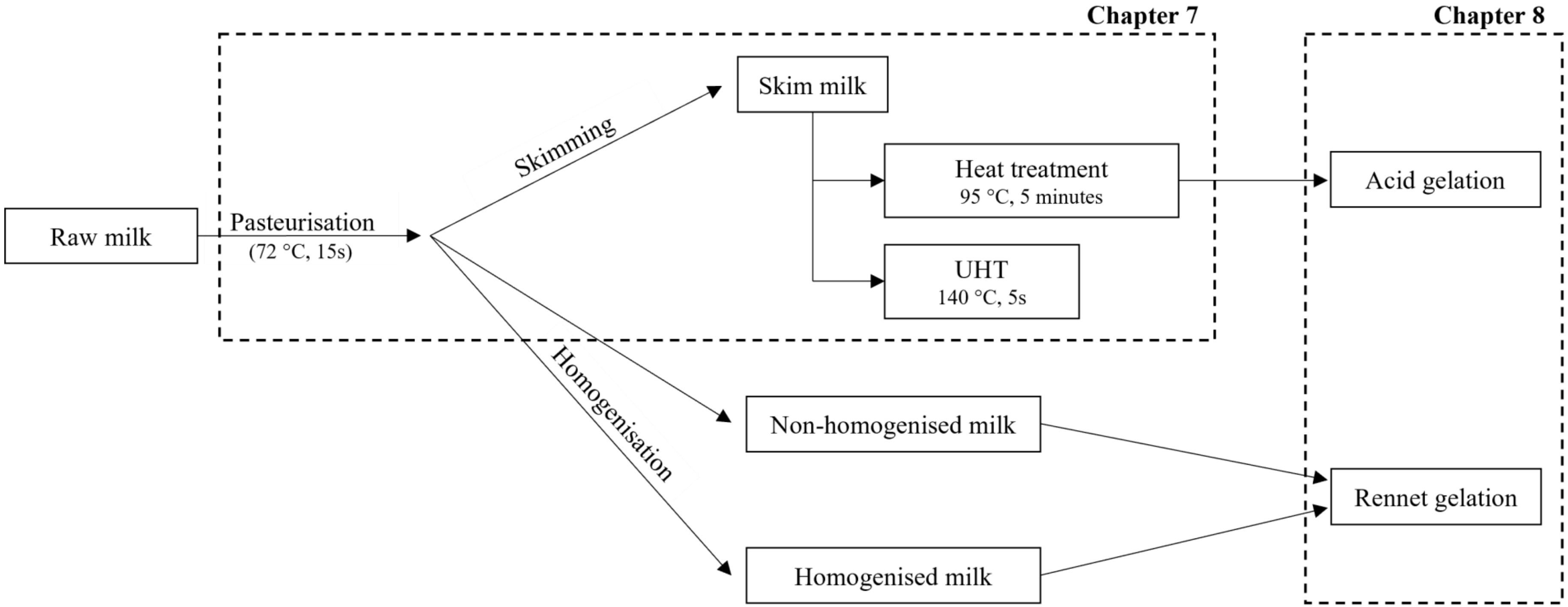


Figure 3.2. Flowchart for processing of mixed milk sample.

3.3 Characterisation

3.3.1 Yield

The milk of each collection per cow was weighed initially. This weight was used to mix the milk to create 24-hour samples for the TAD milk and the bulk sample. The density was given from the MilkoScan measurement, which, together with the protein and fat contents, was used to calculate the protein and fat yield.

3.3.2 Proximate composition

The proximate composition, including the protein, fat, and lactose contents, was analysed using the MilkoScan FT1 (Foss Analytics, Hillerød, Denmark).

3.3.3 Protein composition

The protein composition was analysed using high-performance liquid chromatography (HPLC) with a method modified from the method by Bobe et al. (1998). Samples were prepared as follows. 0.5 mL of sample was mixed with 0.5 mL of Solution A (21 mg/mL Bis-Tris, 573 mg/mL guanidine HCl, 1.57 mg/mL trisodium citrate and 3 mg/mL dithiothreitol in MilliQ water, pH 7.0). After 1 hour at room temperature, the samples were centrifuged for 5 minutes at 14,100g, 300 μL was taken and mixed with 900 μL of Solution B (430 mg/mL guanidine HCl in 10% acetonitrile in MilliQ water with 0.1% trifluoroacetic acid, pH 2.0). Finally, the samples were filtered through a 0.2 μm RC syringe filter before analysis. An Aeris Widepore 3.6 μm XB-C18 RP column (Phenomenex, Torrance, CA, USA) was used. The total run time was 45 minutes at a flow rate of 0.6 mL/min. The separation gradient was started with solvent B (water: acetonitrile, 1:9; 0.1% trifluoroacetic acid) set at 27%, followed by an increase to 32% in 2 minutes, then to 45.6% in 29 minutes, then to 50.2% in 1 minute followed by a hold at 50.2% for 2 minutes, and was then returned to 27% in 2 minutes and held for 9 min. The UV wavelength was set at 220 nm for protein detection. The peaks of each protein were identified using a standard solution of α-casein (4 mg/mL, containing α_{s1}-casein and α_{s2}-casein), β-casein (3 mg/mL), κ-casein (κ-CN; 1.5 mg/mL), β-lactoglobulin (β-LG; 1 mg/mL), and α-lactalbumin (α-LA; 0.5 mg/mL) (all from Sigma-Aldrich, St. Louis, MO, USA), and the areas under the peaks were used to quantify each protein in the milk samples. The glycosylated κ-CN peaks were also identified and divided by the total peak area for κ-CN to obtain the glycosylation degree.

3.3.4 Mineral composition and ionic calcium

The mineral composition of raw skim milk and serum was determined externally by Hill Laboratories (Hamilton, New Zealand). Calcium, phosphorus, magnesium, potassium, and sodium were analysed by ICP-OES. Copper, iodine, selenium, and zinc were analysed by ICP-MS. Chloride was analysed by potentiometric titration. The ionic calcium was determined using a calcium-sensitive electrode (Orion 9720BNWP Thermo Scientific, Singapore) together with a CyberScan pH 510 pH/mV Meter (Eutech Instruments-Thermo Scientific, Singapore). The electrode was calibrated using 1-10 mM of $CaCl_2$, of which the ionic strengths were adjusted to 80 mM with potassium chloride, as described by Li et al. (2019).

3.3.5 Heat-induced protein denaturation and aggregation

The levels of protein denaturation and aggregation were determined by analysing the protein composition in the skim milk, in the serum and native whey fraction of the milk. The methods used for separating these fractions are described below. The results from the raw milk samples and the heat-treated samples were compared to study changes in the protein distribution and denaturation degrees.

The milk was fractionated as follows. Serum was isolated from skim milk samples by ultracentrifugation at 63,000g for 1 hour. Serum was carefully pipetted from the middle of the tube while aiming to limit the stirring and mixing in of the top fat layer. Aliquots of 0.5 mL were taken and frozen at -20 °C until analysis for protein composition. The native whey protein fraction in the milk was isolated according to a method described by Vasbinder and de Kruif (2003). Milk (400 μL) was transferred to an Eppendorf tube and mixed with MilliQ water (800 μL) at 40 °C and 40 μL of 10% acetic acid. This mixture was left for 10 minutes before adding 40 μL of 1M sodium acetate and 720 μL of MilliQ water at 40 °C followed by mixing using a vortex. After 1 hour at room temperature, the mixture was centrifuged for 5 minutes at 3000g. The supernatant was frozen in aliquots of 0.5 mL until analysis for protein composition.

The spectra of the protein composition of raw and heated skim milk, serum phase and native whey isolate samples were used to determine the degree of denaturation of whey protein, proportion of individual caseins in the serum, proportion of whey proteins associated with the casein micelles and the proportion of whey protein forming aggregates in the serum as described below.

The proportion of individual casein in the serum was calculated by dividing the peak area of a specific protein in the serum by the peak area of the same protein in the skim milk sample. The degree of denaturation was determined using the peak area of α-LA and β-LG in the native whey isolates from heated milk samples and compared to the native whey isolate sample from raw milk.

$$\text{Degree of denaturation} = 1 - \frac{(\text{peak area of whey protein in native whey isolate in heated milk})}{(\text{peak area of whey protein in native whey isolate in raw milk})} \times 100\%$$

The proportion of whey protein associated with the casein micelles was calculated by taking the difference in the peak area of a protein in the milk serum samples between the raw and heated samples divided by the peak area of that protein in the raw milk serum sample, multiplied by 100% to obtain a percentage. The proportion of whey protein forming aggregates in the serum was calculated by subtracting the proportion of whey proteins associated with casein micelles from the degree of denaturation.

3.3.6 Casein micelle size

The casein micelle size was determined using the Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK), according to a method adapted from Bijl et al. (2014). Skim milk samples (raw and heated) were diluted 50x in an imidazole buffer (20 mM imidazole, 5 mM $CaCl_2$, 30 mM NaCl, pH 7.0) and left for 10 minutes. The diluted sample was then filtered through a 0.45 μm PVDF (polyvinylidene difluoride) syringe filter before analysis. The measurement was performed at 25 °C with a scattering angle of 173 degrees. Each measurement was performed in triplicate, each consisting of 13 sub-measurements.

3.3.7 Fat globule size

The fat globule size of the raw and heat-treated samples was determined using the Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK). Each milk sample was mixed 1:1 with a 2% SDS and 50 mM EDTA solution (pH 6.7) prior to analysis to break weak protein bonds and dissociate the casein micelles.

3.3.8 Rennet gelation

Rennet-induced gelation properties were analysed according to the method described by Glantz et al. (2010). Both homogenised and non-homogenised pasteurised milk samples were analysed. A microbial rennet (HANNILASE® XP 1050 NB, Christian Hansen A/S, Hørsholm, Denmark) was used for rennet gelation. Milk was heated to 36-37 °C, inoculated at 38 international milk clotting units per litre and mixed for approximately 1 minute before being transferred into the rheometer cup. Gelation analyses were carried out at 32 °C for 40 minutes using 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 a radius of 15 mm and 14 mm, respectively). The gelation time was defined as the time from the addition of the rennet until the time when the storage modulus (G′) had reached 1.0 Pa.

3.3.9 Acid gelation

The acid gelation properties were analysed according to the method described by Li et al. (2020). Milk heated at 95 °C for 5 minutes was used. This type of heat treatment results in the level of whey protein denaturation and consequent aggregation with casein micelles required for yoghurt productions. 2 g of glucono-δ-lactone (GDL) was added to 100 mL of milk at 36-37 °C followed by stirring for 2 minutes. 20 mL of sample was transferred to the rheometer cup. The gelation properties were measured for 8 hours at 30 °C using 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 a radius of 15 mm and 14 mm, respectively). The remaining of the sample was used to track the pH in a jacketed beaker connected to a waterbath at 30 °C. pH measurements were taken at 1-minute intervals using an Edge Blu pH meter (Hanna Instruments, Woonsocket, RI, USA), alongside the rheology analysis. The gelation time and gelation pH were defined as the time from the addition of the GDL and the pH at that time, at which the G′ was 1.0 Pa. The final gel strength was determined at 480 minutes.

3.4 Statistical analysis

The statistical analysis was performed using the MIXED procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The appropriate model for each research aim is described in the corresponding chapter.

Chapter 4

The effect of milking frequency on milk composition and yield

To be submitted

van der Zeijden, M., Ellis, A., Lopez-Villalobos, N., Li, S., Roy, N., McNabb, W. The protein composition of bovine milk from once-a-day and twice-a-day milking production systems in New Zealand. *Dairy*

4.1 Abstract

The majority of the farms in New Zealand utilise a twice-a-day (TAD) milking production system. However, an increasing number (10% of all farms) of dairy farmers have adopted once-a-day (OAD) milking production system. The objective of this chapter was to evaluate the effect of OAD milking on the protein composition in milk from individual cows. Milk was sampled in early, mid-, and late lactation from cows kept at Massey University farms Dairy No. 1 (OAD milking) and Dairy No. 4 (TAD milking) in the Manawatu region, New Zealand. The yields of total milk and milk solids, the proximate and protein composition were determined. OAD milking yielded less milk and milk solids than TAD milking. However, the protein, fat and lactose contents were similar between OAD and TAD milk. While the proportions of total casein, total whey proteins, α_{s1}-casein, β-casein and β-lactoglobulin were not affected by the milking frequency, milk from a OAD milking system contained higher proportions of α_{s2}-casein and κ-casein, and lower proportions of α-lactalbumin. These proteins followed a different trend throughout the milking season in a OAD and a TAD milking system. Differences in yield affect the payout for the farmer, and changes in the composition could have implications for processing properties and product quality.

4.2 Introduction

In New Zealand, most farms follow a twice-a-day (TAD) milking production system. Once-a-day (OAD) milking is increasing in popularity because of a lack of good quality long-term staff and a change in lifestyle expectations, among other reasons (Bewsell et al., 2008; Kendall et al., 2008; Stelwagen et al., 2013). In the 2018-2019 milking season, almost 10% of dairy farms in New Zealand milked their cows using a OAD milking system (DairyNZ, 2020). The total milk yield, as well as the protein and fat yields, decreased when the milking frequency was reduced (Bewsell et al., 2008; Edwards, 2018; Lacy-Hulbert et al., 1999; Lazzarini et al., 2018; Lembeye, Lopez-Villalobos, et al., 2016c; Murney et al., 2015; O'Brien et al., 2002; Rémond et al., 2004; Stockdale, 2006). This variation in composition is an important consideration since the value of milk to the farmer in New Zealand is based on the yield of milk solids, including fat and protein. Previous research has also shown higher milk protein and fat contents from cows milked OAD compared to cows milked TAD (Lembeye, Lopez-Villalobos, et al., 2016c; Rémond et al., 2004). Another study looked at gene expression for various milk proteins and found a change for all major proteins in milk (Murney et al., 2015), suggesting a difference in the protein composition of the milk, but this has not been confirmed or measured in previous studies.

Additional to the milking frequency, other factors also impact milk composition. One of these factors is the stage of lactation. In New Zealand, most farms work with a seasonal calving system where calving happens from late winter to spring. As a result, the meteorological seasons are aligned with the milking season and the stage of lactation. From calving, the protein and fat contents slightly decrease until peak lactation around 6-8 weeks postpartum before slowly increasing again. Closer to drying off, the proportion of protein and fat increases more significantly, alongside a decrease in milk yield (Li et al., 2019; O'Brien and Guinee, 2016; Sorensen et al., 2008).

The milk and milk protein compositions are essential contributors to the processing properties of the milk. However, as far as the author is aware, no studies have analysed the milk protein profile of cows milked in a OAD compared to a TAD milking production system. An increasing number of farms adopting a OAD milking system increases the need to understand the impact of this change in milking frequency on the composition of milk.

Therefore, the aim of this chapter was to understand the consequences of a OAD production system on the protein composition of individual cows. In this study, the milk from cows from Massey University Dairy Farm No. 1 (OAD milking) was compared to the milk from cows from Massey University Dairy Farm No. 4 (TAD milking), both farms operating in the Manawatu region, New Zealand. Firstly, the overall impact of OAD milking on the milk and milk solids yield and the proximate composition across the milking season was investigated. Secondly, the average protein composition across the lactation was studied. Finally, the effect of stage of lactation on milk yield and the proximate and protein composition of milk from OAD and TAD milking systems were analysed.

4.3 Methods

The sampling strategy and analytical methods are described in detail in Chapter 3. A summary is given below.

4.3.1 Sampling strategy

Cows from two Massey University research farms, one using a OAD (Dairy No. 1) and one using a TAD (Dairy No. 4) milking production system, were selected based on their genetics, breeding worth and expected calving date. Nine cows from each farm were sampled, and each group had an even distribution of breeds (Holstein-Friesian (n=3), Holstein-Friesian×Jersey crossbred (n=3) and Jersey (n=3)). At Dairy No. 4, the same cows were used for each sampling. At Dairy No. 1, some cows were excluded during the season due to either mastitis or treatment for other health issues (e.g., lameness). These cows were replaced with cows that matched the criteria for the study. Milk was sampled nine times throughout the milking season; three were scheduled at the beginning, three in the middle and three towards the end of the season. The milk samples were categorised as early, mid-, and late lactation based on the days in milk (DIM, number of days since calving on the sampling day) of the cow on the day of sampling. To create a 24-hour milk sample for the TAD milked cows, the milk from the afternoon and the following morning was mixed in the laboratory proportional to the respective yields.

4.3.2 Sample analysis

The milk yield of each cow was determined by recording the weight on the farm immediately after the milking. The proximate composition of the milk was analysed using the MilkoScan (Foss Analytics, Hillerød, Denmark). The obtained values for protein, fat and lactose content and the total milk yield were used to calculate the yield of each component in kilograms. The protein composition was analysed using a high-performance liquid chromatography (HPLC) method modified from the method by Bobe et al. (1998). The method is detailed in Chapter 3.

4.3.3 Statistical analysis

The data was analysed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A mixed model (using the MIXED procedure) was used to obtain the least square means and standard errors of the parameters analysed in this study. The following model was used:

$$Y_{ijkl} = \mu + M_i + B_j(M_i) + S_k + M_iS_k + B_j(M_i)S_k + \beta_1 p_{ijkl} + \beta_2 p^2_{ijkl} + \beta_3 d_l + C_l + e_{ijkl}$$

Y_{ijkl} is the observation for the trait for milking frequency i, breed j, lactation stage k, and cow l

μ is the population mean

M_i is the fixed effect of milking frequency i (i = OAD and TAD)

$B_j(M_i)$ is the fixed effect of breed j nested in milking frequency i (j = F, FxJ and J)

S_k is the fixed effect of the stage of lactation k (k = early, mid, and late)

β_1 and β_2 are the regression coefficients of the linear and quadratic effects of parity p (years) of cow l

β_3 is the regression coefficient of the linear effect of deviation (days) from herd median calving date d of cow l

C_l is the random effect of cow l

e_{ijkl} is the residual random error assumed with mean zero and variance s_e^2

Marginal means and standard errors for each level of the fixed effects were obtained and used for multiple mean comparisons using Fisher's least significant difference test. Significant differences were declared at $P < 0.05$.

4.4 Results

4.4.1 **Descriptive statistics**

The average parameters of the cows used in the study are set out in Table 4.1. The median calving date was taken from the entire herd on each farm: for Dairy No. 1, this was 7 August 2020, and for Dairy No. 4, the median calving date was 27 July 2020. Table 4.1 also shows the mean parity, breed proportions and the breeding worth at the start of the season. All cows, except three cows from the TAD milking system (second and sixth parity), were in their third or fourth parity. For each trait analysed in this study, the order of the effect of parity was studied. A linear and a quadratic effect were tested, and the closest fit was used in the model described in Section 4.3.3. A quadratic effect was the best fit for all traits except the lactose and α-lactalbumin (α-LA) content. Therefore, only the linear effect was included in the model, and no *F*-value of the quadratic effect for these two characteristics is included in Table 4.3.

Table 4.2 shows the descriptive statistics of the daily milk and milk solids yield, proximate milk composition and protein composition of all samples. Table 4.3 shows the *F*-values and significance levels of the fixed effects in the statistical model used in this study for the different traits analysed. Overall, a larger *F*-value indicates a larger contribution of a certain parameter from the model. For example, the stage of lactation generally affected all traits more than the deviation from the median calving date did, and milking frequency had a greater effect on the protein yield than on the fat yield.

Table 4.1. Number of records and descriptive statistics of cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

	Milking frequency	
	OAD	TAD
Number of observations	81	81
Deviation from the median calving date (days)	17 ± 13	15 ± 10
Parity (years)	3.4 ± 0.5	3.8 ± 1.3
Proportion of Friesian	0.481	0.563
Proportion of Jersey	0.519	0.438
Breeding worth at the start of the season	139 ± 32	148 ± 33

Table 4.2. Descriptive statistics of daily milk yield, proximate composition, and protein composition of milk from cows once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

Trait	N	Mean	SD	CV	Min	Max
Milk yield (kg/cow/day)	162	22.1	7.4	33.7%	9.3	42.0
Protein yield (kg/cow/day)	162	0.80	0.21	26.1%	0.39	1.28
Fat yield (kg/cow/day)	162	1.04	0.32	30.3%	0.44	2.22
Lactose yield (kg/cow/day)	162	0.99	0.35	35.2%	0.39	1.95
Protein content (%)	162	3.8	0.5	11.9%	2.8	5.0
Fat content (%)	162	5.0	0.9	18.7%	2.6	9.0
Lactose content (%)	162	4.6	0.2	3.3%	4.2	5.0
Casein (%)	162	80.0	3.8	4.7%	73.1	87.7
α_{s1}-casein (%)	162	22.6	1.6	7.2%	19.1	27.3
α_{s2}-casein (%)	162	5.7	1.1	19.1%	3.6	8.9
β-casein (%)	162	37.4	2.4	6.5%	31.4	43.8
κ-casein (%)	162	14.3	1.5	10.7%	9.9	17.1
Gly-κ-CN[1] (% of κ-casein)	162	43.6	5.3	12.1%	29.8	57.3
Whey protein (%)	162	20.0	3.8	18.9%	12.3	26.9
β-lactoglobulin (%)	162	16.9	3.9	23.2%	8.7	23.9
α-lactalbumin (%)	162	3.1	0.4	14.4%	1.9	4.0

[1] Gly-κ-CN = glycosylated κ-casein

Table 4.3. *F*-values and significance levels for factors affecting the composition of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

	Factor[1]							
Trait	M	B	S	M×S	B×S(M)	p	p^2	d
Milk yield	15.6***	4.9**	457.3***	2.9	6.8***	2.4	2.4	0.1
Protein yield	20.7***	5.1**	213.4***	0.3	2.4*	2.1	2.0	1.4
Fat yield	6.2*	0.4	140.6***	4.4*	2.5*	0.8	0.7	0.0
Lactose yield	15.6***	4.5**	405.2***	1.7	5.6***	2.4	2.4	0.3
Protein content	0.2	0.7	202.5***	6.2**	2.2*	0.4	0.4	0.4
Fat content	0.5	3.2*	8.3***	1.5	0.7	0.1	0.2	0.0
Lactose content	0.2	2.6*	62.8***	6.5**	4.0***	7.0**		2.3
Casein	0.2	1.8	40.7***	1.0	0.3	0.1	0.1	0.3
α_{s1}-casein	1.6	0.8	51.5***	0.5	0.5	1.3	1.3	1.7
α_{s2}-casein	15.9***	2.4	36.0***	3.7*	0.8	0.9	1.2	0.1
β-casein	1.0	2.0	28.4***	2.8	1.2	1.7	1.7	0.0
κ-casein	10.7**	8.7***	8.3***	4.0*	3.5**	6.4*	6.6*	0.0
Glycosylated κ-casein	3.8	2.2	90.7***	3.3*	2.5*	1.2	1.2	0.3
Whey protein	0.2	1.8	40.7***	1.0	0.3	0.1	0.1	0.3
β-lactoglobulin	0.1	2.0	60.3***	0.5	0.4	0.1	0.1	0.4
α-lactalbumin	6.4*	4.2**	58.3***	3.4*	0.9	7.7**		0.1

[1] M = milking frequency (OAD and TAD), B = breed (Holstein Friesian, Friesian-Jersey crossbreed, and Jersey), S = stage of lactation (early, mid and late), p = parity (2-6), d = deviation from the median calving date of the herd for each farm. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.5$.

4.4.2 Feed composition

Like most farms in New Zealand, both Massey University dairy farms have a pasture-based feeding system. The diet composition and chemical composition of the diet offered to the cows prior to sampling are presented in Table 4.4. The data is expressed as the mean per stage of lactation of the rations offered on the day before each sampling day. Overall, the chemical composition of the diets fed on each farm was similar. However, the total dry matter (DM) allowance was higher for Dairy No. 4 (TAD) than for Dairy No.1 (OAD), with approximately 16 to 17 kg DM per cow per day, whereas Dairy No.4 offered 18 to 24 kg DM per cow per day. In addition, the crude protein per kg dry matter (DM) was higher in early and mid-lactation in the OAD feed compared to the TAD feed. In contrast, neutral detergent fibre content was lower in mid- and late than in early lactation and soluble sugar and starch content was lower in each stage of lactation in a OAD milking system than in a TAD milking system.

Table 4.4. Dietary and chemical composition of the feed offered per cow daily at Massey University Dairy Farms No.1 (OAD) and No. 4 (TAD) prior to sampling days in early, mid- and late lactation during the 2020-2021 production season.

	OAD			TAD		
	Early	Mid	Late	Early	Mid	Late
Chemical composition						
ME[1] (MJ/kg DM)	11.4	11.2	10.6	11.4	11.1	10.9
CP[2] (g/100g DM)	22.2	21.4	19.7	20.9	19.7	19.6
NDF[3] (g/100g DM)	37.6	40.9	41.6	37.5	44.1	44.7
ADF[4] (g/100g DM)	19.1	21.4	25.6	17.3	21.2	25.7
SSS[5] (g/100g DM)	16.0	11.7	11.4	18.7	16.3	13.9
Lipid (g/100g DM)	4.4	4.6	4.3	3.6	4.2	4.6
Diet composition (kg DM/cow/day)						
Pasture	11.7	11.3	7.3	17.0	17.0	7.5
Chicory	1.3	2.7	1.0	-	-	-
Maize silage	1.0	-	0.3	4.3	5.0	2.7
DDG[6]	1.8	-	-	-	1.0	0.5
Tapioca	0.8	-	1.3	-	-	-
Molasses	-	-	-	1.0	-	-
Concentrates[7]	-	-	2.0	1.0	-	0.7
Dry roughage	-	-	-	0.1	-	0.1
Bailage	-	2.0	4.0	0.7	1.0	6.2

[1]ME = metabolisable energy, [2]CP = crude protein, [3]NDF = neutral detergent fibre, [4]ADF = acid detergent fibre, [5]SSS = soluble sugars and starch, [6]DDG = distillers dried grain, [7]Either soy- or corn-based concentrate.

4.4.3 Effect of milking frequency on milk yield, proximate and protein composition

The estimated marginal means and standard errors for the different milk characteristics from cows milked OAD and TAD are presented in Table 4.5. The milk yield shows the total milk volume per cow per day in litres. In a OAD milking system, cows yielded 17.0 litres daily on average compared to a significantly higher volume of 25.2 litres in a TAD milking system. The total protein, fat, and lactose yields from the OAD cows were also significantly lower than those from the TAD cows, 29%, 26%, and 33%, respectively. The mean protein content of the milk was 3.9% in OAD milk and 3.8% in TAD milk, but no significant effect of the milking frequency was observed. The mean fat content (OAD, 5.2%; TAD, 4.8%) and lactose content (OAD, 4.6%; TAD, 4.6%) in the milk did not differ across the two milking systems.

The average protein composition across the milking season in milk from OAD and TAD milked cows is shown in Table 4.5 as a percentage of the sum of all proteins measured by HPLC. The total whey protein percentage given is the sum of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), and the total casein percentage is the sum of α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN), and κ-casein (κ-CN). No significant effect of milking frequency was found on the total casein and the total whey protein percentage in the milk, nor the α_{s1}-CN, β-CN, and β-LG contents. The proportion of α_{s2}-CN in OAD milk was 43% higher than in TAD milk, 6.7% and 4.7%, respectively. The κ-CN percentage was also higher (P = 0.001) in OAD milk (15.5%) than in TAD milk (13.3%) by 18%. There was a trend of a higher degree of glycosylation of κ-CN in OAD milk than the TAD milk (P = 0.054). The difference in the proportion of α-LA was significant (P = 0.013) and approximately 9% lower in OAD milk (2.9%) than in TAD milk (3.2%).

Table 4.5. Estimated marginal means and standard errors of daily milk yield, proximate composition, and protein composition of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

	Milking frequency		M[1] effect
Trait	OAD	TAD	*(P-value)*
Milk yield (L)	17.0 ± 1.13	25.2 ± 1.19	< 0.001
Protein yield (kg)	0.65 ± 0.032	0.92 ± 0.034	< 0.001
Fat yield (kg)	0.87 ± 0.067	1.18 ± 0.070	0.014
Lactose yield (kg)	0.78 ± 0.053	1.16 ± 0.055	< 0.001
Protein content (%)	3.9 ± 0.14	3.8 ± 0.15	0.657
Fat content (%)	5.2 ± 0.25	4.8 ± 0.26	0.464
Lactose content (%)	4.6 ± 0.03	4.6 ± 0.03	0.693
Casein (%)	80.5 ± 1.64	79.2 ± 1.81	0.680
α_{s1}-casein (%)	21.8 ± 0.62	23.3 ± 0.66	0.216
α_{s2}-casein (%)	6.7 ± 0.27	4.7 ± 0.29	< 0.001
β-casein (%)	36.5 ± 0.93	38.2 ± 0.99	0.312
κ-casein (%)	15.5 ± 0.39	13.1 ± 0.42	0.001
Gly-κ-CN[2] (% of κ-CN)	46.8 ± 1.63	41.0 ± 1.73	0.054
Whey protein (%)	19.5 ± 1.64	20.8 ± 1.76	0.680
β-lactoglobulin (%)	16.5 ± 1.68	17.6 ± 1.81	0.729
α-lactalbumin (%)	2.9 ± 0.07	3.2 ± 0.07	0.013

[1]M = milking frequency (OAD and TAD), [2]Gly-κ-CN = glycosylated κ-casein.

4.4.4 The effect of stage of lactation on the milk from OAD and TAD milked cows

The previous section focused on the season average of different characteristics of milk from OAD and TAD milking production systems. In this section, the effect of the stage of lactation on milk (solids) yield, the proximate composition and protein composition in each milking system were investigated. Stage of lactation influenced each of the traits analysed in the study, as seen in Table 4.3. Table 4.6 shows the milk and milk solids yield per cow per day, the proximate composition, and the protein composition in early, mid-, and late lactation for OAD and TAD milk. The last column also shows the interaction effect between milking frequency and the stage of lactation.

4.4.4.1 Milk and milk solids yield

Overall, the yield, both in litres and in terms of the different major components, decreased throughout the season and was higher in early lactation for both milking systems (OAD, 21.8 L, TAD, 31.0 L) and lower in late lactation (OAD, 11.5 L, TAD, 19.6 L). The total milk yield from OAD cows compared to TAD cows was 30% and 29% lower in early and mid-lactation, respectively. The largest difference in milk yield was observed in late lactation, where the total milk yield was 41% lower in a OAD compared to a TAD milking system. The difference in protein yield between OAD and TAD milking at different stages of lactation was smaller than the difference in the total milk yield. In early and mid-lactation, the protein yield was 26% and 28% lower ($P < 0.001$) in OAD milking than in TAD milking. In late lactation, this difference was 35%. The lactose yield followed a similar trend as the total milk volume yield. In early and mid-lactation, the lactose yield was 30% and 31% lower in OAD milking, respectively. This difference was 43% in late lactation. The total lactose yield decreased more between mid- and late lactation in a OAD milking system than in a TAD milking system. No significant interaction effect was found for this trait, nor total milk or protein yield.

As shown in Table 4.6, an interaction effect between milking frequency and stage of lactation for the total fat yield indicates a different stage of lactation effect for OAD and TAD systems. In the OAD milking production system, the fat yield decreased more evenly between the different stages of lactation by 0.21 kg and 0.27 kg per cow per day between early and mid-lactation and between mid- and late lactation, respectively. On the contrary, in the TAD milking production system, the fat yield decreased much more between early and mid-lactation with a 0.40 kg reduction, while between mid- and late lactation, the total fat yield per cow per day only decreased by 0.11 kg. Overall, the total fat yield fell more over the whole lactation in OAD milking than in TAD milking. The difference in fat yield between OAD and TAD milked cows was 26%, 18% and 35% in early, mid-, and late lactation, respectively.

4.4.4.2 Proximate milk composition

In both production systems, the highest protein content was observed in late lactation (OAD, 4.3%, TAD, 4.1%), and the stage of lactation effect was significant. Milk from the TAD milking system had the lowest protein content in early lactation (3.5%), which was significantly different ($P < 0.001$) from the protein content at mid-lactation (3.7%). In OAD milk, however, the protein content in early and mid-lactation was similar, both 3.7%. No significant difference was found between the protein content of OAD and TAD milk at each stage of lactation.

In OAD milk, the effect of stage of lactation on the fat content was less evident than in TAD milk. The fat content in OAD milk at different stages of lactation did not significantly differ from each other while they did in TAD milk. The fat content of the

TAD milk decreased significantly from early lactation to mid-lactation and then increased significantly in the late lactation. The fat content was not significantly different between OAD and TAD milk at any stage of the lactation.. For both production systems, the highest fat content was observed in late lactation (OAD, 5.3%, TAD, 5.2%) and the lowest in mid-lactation (OAD, 5.1%, TAD, 4.5%).

The highest lactose content was in early lactation both in the OAD milking system (4.7%) and the TAD milking system (4.7%). In OAD milk, the lactose content decreased throughout the milking season, with the lowest value observed in late lactation (4.5%). In TAD milk, the lactose content in early lactation was different from that in mid- (4.6%) and late lactation (4.5%), but the lactose content at mid- and late lactation was not significantly different from each other. There was an interaction effect between milking frequency and stage of lactation for the lactose content. The lactose content in OAD milk decreased more throughout the lactation than in TAD milk.

4.4.4.3 Protein composition

The estimated marginal means of the proportion of each of the major milk proteins and the casein and whey protein contents at different stages of lactation are presented in Table 4.6. There was a significant stage of lactation effect on each of the individual proteins analysed in the study. Milk in late lactation contained the lowest proportion of casein (OAD, 79.3%, TAD, 78.1%) and the highest proportion of whey proteins (OAD, 20.7%, TAD, 21.9%) in both OAD and TAD milking systems. In OAD milk, the casein content in early and mid-lactation was similar, while in TAD milk, the casein content was higher in early than in mid-lactation. However, there was no significant interaction effect between milking frequency and stage of lactation.

The α_{s1}-CN content was the highest in early lactation (OAD, 22.7%, TAD, 21.0%) and the lowest in late lactation (OAD, 21.0%, TAD, 22.4%). There was no significant difference between the OAD and TAD milk at any stage of lactation. An interaction effect between milking frequency and stage of lactation was found for α_{s2}-CN, however. In Section 4.4.3, the results showed a significant increase in the proportion of α_{s2}-CN in OAD compared to TAD milk. Although the lowest α_{s2}-CN content was in early lactation for both milking systems, the content increased significantly in mid-lactation in OAD milk from 6.2% to 6.9% but remained similar in TAD milk with 4.5% and 4.6%, respectively. A significant increase was observed in late compared to mid-lactation in TAD milk (5.1%) but not in OAD milk (7.1%). The proportion of β-CN in OAD milk was significantly different in early, mid-, and late lactation, with the highest value in mid- (37.3%) and the lowest value in late lactation (35.5%). The β-CN content in TAD milk was also lowest in late lactation (37.7%) but did not differ significantly between early (38.4%) and late (38.6%) lactation.

As with the α_{s2}-CN content, an interaction effect was found for the proportion and the degree of glycosylation of κ-CN. In a OAD milking system, the κ-CN concentration was lowest in mid-lactation (15.2%) and was similar in early (15.5%) and late (15.6%) lactation. In comparison, in TAD milk, the proportion of κ-CN was highest in early lactation (13.3%) and similar in mid- (12.9%) and late lactation (13.0%). At each stage of lactation, the proportion of κ-CN was higher in OAD than in TAD milk. The glycosylation degree of κ-CN was most strongly affected by the stage of lactation of all individual proteins, as indicated by the higher *F*-value shown in Table 4.3. In both OAD and TAD milking systems, the seasonal trend for κ-CN glycosylation degree was similar. The highest value was found in late lactation (OAD, 51.0%, TAD 43.8%), and the lowest was found in early lactation (OAD, 43.1%, TAD 38.5%). However, when comparing

OAD and TAD milk at each stage of lactation, the difference was only significant in late lactation.

The proportion of β-LG followed a similar trend throughout the season as that of total whey proteins. The highest β-LG concentration was found in late lactation for both milking frequencies (OAD, 18.0%, TAD, 19.0%). While the difference between early (15.7%) and mid- (15.9%) lactation in OAD milk was not significant, in TAD milk, the proportion of β-LG was lower in early (16.6%) than in mid- (17.3%) lactation. An interaction effect was found between milking frequency and stage of lactation for the proportion of α-LA. In a OAD milking system, the α-LA content decreased significantly between each stage of lactation from 3.2% to 2.7% in early- to late-lactation, respectively. In a TAD milking system, only the α-LA content in late lactation (2.9%) was significantly lower than in early and mid-lactation (both 3.3%). Additionally, the proportion of α-LA was lower in OAD than in TAD milk in mid- and late lactation but not in early lactation.

Table 4.6. Estimated marginal means and standard errors of daily milk yield, proximate composition, and protein composition of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

	OAD			TAD			*P-value*	
	Early	Mid	Late	Early	Mid	Late	S	M×S^{1}
Milk yield (L)	21.8 ± 1.14^{bc}	17.6 ± 1.22^{d}	11.5 ± 1.16^{e}	31.0 ± 1.23^{a}	24.9 ± 1.23^{b}	19.6 ± 1.23^{cd}	< 0.001	0.061
Protein yield (kg)	0.80 ± 0.033^{bc}	0.65 ± 0.037^{d}	0.51 ± 0.034^{e}	1.08 ± 0.036^{a}	0.90 ± 0.036^{b}	0.78 ± 0.036^{c}	< 0.001	0.728
Fat yield (kg)	1.10 ± 0.069^{bc}	0.89 ± 0.077^{de}	0.62 ± 0.072^{f}	1.48 ± 0.074^{a}	1.08 ± 0.074^{bd}	0.97 ± 0.074^{ce}	< 0.001	0.015
Lactose yield (kg)	1.02 ± 0.053^{bc}	0.79 ± 0.058^{d}	0.51 ± 0.055^{e}	1.45 ± 0.058^{a}	1.14 ± 0.058^{b}	0.90 ± 0.058^{cd}	< 0.001	0.185
Protein content (%)	3.7 ± 0.14^{bcd}	3.7 ± 0.15^{bcd}	4.3 ± 0.15^{a}	3.5 ± 0.16^{d}	3.7 ± 0.16^{c}	4.1 ± 0.16^{ab}	< 0.001	0.003
Fat content (%)	5.1 ± 0.26^{ab}	5.1 ± 0.28^{ab}	5.3 ± 0.26^{ab}	4.9 ± 0.27^{a}	4.5 ± 0.27^{b}	5.2 ± 0.27^{a}	< 0.001	0.228
Lactose content (%)	4.7 ± 0.03^{a}	4.5 ± 0.03^{b}	4.5 ± 0.03^{c}	4.7 ± 0.03^{a}	4.6 ± 0.03^{b}	4.5 ± 0.03^{bc}	< 0.001	0.002
Casein (%)	81.1 ± 1.64^{abc}	81.1 ± 1.67^{abc}	79.3 ± 1.65^{def}	80.1 ± 1.77^{ad}	79.5 ± 1.77^{be}	78.1 ± 1.77^{cf}	< 0.001	0.359
α_{s1}-casein (%)	22.7 ± 0.63^{ace}	21.8 ± 0.66^{bdf}	21.0 ± 0.64^{g}	24.0 ± 0.68^{ab}	23.4 ± 0.68^{cd}	22.4 ± 0.68^{efg}	< 0.001	0.618
α_{s2}-casein (%)	6.2 ± 0.28^{b}	6.9 ± 0.30^{a}	7.1 ± 0.28^{a}	4.5 ± 0.30^{d}	4.6 ± 0.30^{d}	5.1 ± 0.30^{c}	< 0.001	0.027
β-casein (%)	36.6 ± 0.93^{be}	37.3 ± 0.95^{ad}	35.5 ± 0.94^{cf}	38.4 ± 1.00^{abc}	38.6 ± 1.00^{abc}	37.7 ± 1.00^{def}	< 0.001	0.066
κ-casein (%)	15.5 ± 0.39^{a}	15.2 ± 0.40^{b}	15.6 ± 0.40^{a}	13.3 ± 0.42^{c}	12.9 ± 0.42^{d}	13.0 ± 0.42^{d}	< 0.001	0.020
Gly-κ-CN^{2} *(% of κ-CN)*	43.1 ± 1.65^{cdf}	46.4 ± 1.76^{be}	51.0 ± 1.69^{a}	38.5 ± 1.78^{f}	40.7 ± 1.78^{de}	43.8 ± 1.78^{bc}	< 0.001	0.039
Whey protein (%)	18.9 ± 1.64^{abc}	18.9 ± 1.67^{abc}	20.7 ± 1.65^{def}	19.9 ± 1.77^{ad}	20.5 ± 1.77^{be}	21.9 ± 1.77^{cf}	< 0.001	0.359
β-lactoglobulin (%)	15.7 ± 1.68^{adf}	15.9 ± 1.71^{adf}	18.0 ± 1.69^{bce}	16.6 ± 1.81^{ef}	17.3 ± 1.81^{cd}	19.0 ± 1.81^{ab}	< 0.001	0.623
α-lactalbumin (%)	3.2 ± 0.07^{a}	2.9 ± 0.09^{b}	2.7 ± 0.08^{c}	3.3 ± 0.08^{a}	3.3 ± 0.08^{a}	2.9 ± 0.08^{b}	< 0.001	0.036

^{1}M = milking frequency (OAD and TAD), S = stage of lactation (early, mid, and late), ^{2}Gly-κ-CN = glycosylated κ-casein, ^{a,b,c,d,e,f,g} values with different superscripts within one row differ significantly ($P < 0.05$).

4.5 Discussion

4.5.1 Effect of milking frequency on milk yield

The decrease in yield, which was 33% lower in a OAD milking system than in a TAD milking system, found in this study agrees with the literature. However, yield loss from previous studies ranges from 8% (Gedye et al., 2020) up to 50 % (Davis et al., 1999) that accompanies a reduced milking frequency. Most research, including some performed in New Zealand, shows a decrease of approximately 20-30% (Correa-Luna et al., 2021; Edwards, 2018; Lembeye, Lopez-Villalobos, et al., 2016c; O'Brien et al., 2002; Rémond et al., 2004). Edwards (2018) compared OAD and TAD milking systems four years before and four years after dairy farms switched from TAD to OAD. In the first year of OAD milking, the milk yield decreased by 11% compared to the previous years and was 22% lower than the TAD farms. Compared to these results, the decrease in milk yield in the present study (33%) is higher than previously recorded in New Zealand (Stelwagen et al., 2013). Breed, between-year variation and stage of lactation could explain this difference. Some of the studies referenced above are full lactation studies, but some were conducted at a certain stage of lactation. The breed effect is further discussed in the next chapter.

As with the milk volume yield, protein, fat, and lactose losses were on the higher end of the reported range. Since lactose is highly correlated with milk yield, the loss in lactose yield is expected to be similar to the total milk volume yield. With an expected increase in protein and fat contents, the decrease in their yields will not be as large as the total milk yield. In some studies, the decrease in fat yield was also lower than the decrease in protein yield (Lembeye, Lopez-Villalobos, et al., 2016c; O'Brien et al., 2002; Rémond et al., 2004) while in other studies, the opposite was observed (Clark et al., 2006; Lacy-Hulbert et al., 1999). In New Zealand, the payout is based on the protein and fat yield. Therefore, decreasing the total protein and fat yields per cow will result in a lower payout for the farmer but also the overall cost (Edwards, 2019).

OAD milking is sometimes implemented when feed availability is low or to improve the body condition score of the cows (Bewsell et al., 2008) and is often accompanied by lower supplement use and stocking rate (Stelwagen et al., 2013). However, there is little evidence to show that OAD-milked cows have lower DM requirements (Stelwagen et al., 2013). The mean ration at each stage of lactation displayed in Table 4.4 is the amount the farmers offered to the cows whereas the actual intake per animal probably differs. Therefore, there would have been variation in feed and energy intake between the animals on the same farm despite the same feeding regime.

4.5.2 The effect of OAD milking on the concentration of major components

The protein and fat contents in the milk were expected to be higher in OAD than in TAD milk. However, no significant effect of the milking frequency on either of these components was found. Previous studies have found an increase in the protein content ranging from 3% to 8% (Clark et al., 2006; Gedye et al., 2020; Martin et al., 2009; O'Brien et al., 2002; Rémond et al., 2004). On the other hand, the increase in fat content showed more variation in previous studies with a range of 1-10% (Clark et al., 2006; Gedye et al., 2020; Martin et al., 2009; O'Brien et al., 2002; Rémond et al., 2004). This larger variation in the increase in fat content was also seen in the present study, where the coefficient of variation was higher for the fat content than for the protein content, as shown in Table 4.2.

In contrast to the protein and fat contents, the lactose content was previously found to decrease when the milking frequency is reduced from TAD to OAD (Clark et al., 2006; Gedye et al., 2020; O'Brien et al., 2002; Rémond et al., 2004). But this difference was also not always significant (Rémond et al., 2004), which agrees with the results in the present study.

4.5.3 The effect of OAD milking on the protein composition

The present study found no significant effect on the proportion of casein and whey proteins. In contrast, most studies (Auldist and Prosser, 1998; Klei et al., 1997; Martin et al., 2009; O'Brien et al., 2002; Pomiès et al., 2007), but not all (Rémond et al., 2004), reported a decrease in casein-to-whey ratio in the milk with less frequent milking. Both the casein and whey protein fractions have been found to increase in previous research with a lower milking frequency, likely caused by an increase in protein content. A larger increase in the whey protein content contributed to a change in the ratio between the two protein groups. This increase in whey protein content has been attributed to increased permeability of the mammary tissue due to the loss of tight junctions leading to an increased influx of serum proteins when milking frequency is reduced (Davis et al., 1999).

No studies have looked at the protein composition of milk from a OAD milking system and of milk from a TAD milking system. Some authors reported an increase in the expression of the gene for α-LA in the mammary tissue with increasing milking frequency (Alex et al., 2015; Murney et al., 2015). In the present study, the α-LA content was lower in OAD than in TAD milk. α-LA is involved in lactose production, and lactose is involved in regulating milk volume by creating osmotic pressure between the blood and alveoli (Strucken et al., 2015; Zhao and Keating, 2007). Therefore, the downregulation of milk production when the milking frequency is reduced could be positively correlated with the α-LA concentration in the milk and explain this result. However, an increase in the α-LA concentration after 7 and 15 days of decreased milking frequency was also found (Kelly et al., 1998). Murney et al. (2015) also found an increase in mRNA abundances for α_{s1}-CN, β-CN, and β-LG in the udder half that was milked four times daily compared to the other half that was milked once daily. Most of these studies, however, focused on the effect of a temporary change in milking frequency. Therefore, the exact mechanism behind changes in the protein profile remains unclear. Genetic polymorphism of proteins is also known to affect the concentration of individual proteins (Huang et al., 2012), but this was not measured in this study.

4.5.4 The effect of stage of lactation on the milk from OAD and TAD milked cows

4.5.4.1 Milk and milk solids yield

The difference in milk yield between OAD and TAD milk was within the expected range of 20-30% in early and mid-lactation. However, in late lactation, the difference was much higher than found in other studies (Correa-Luna et al., 2021; Edwards, 2018; Lembeye et al., 2016c; O'Brien et al., 2002; Rémond et al., 2004). While the milk yield in the TAD milking system decreased by approximately 20% between each stage of lactation, in the OAD milking system, the milk yield decreased by 19% between early and mid-lactation and by 35% between mid- and late lactation. There was an interaction effect (P = 0.061) between milking frequency and stage of lactation for total milk yield. Previous studies have reported larger production losses in early and mid-lactation than in late lactation when the milking frequency was temporarily reduced (Carruthers et al., 1993; Stelwagen and Knight, 1997; Stelwagen et al., 2013). The cows in the present study were milked OAD throughout the entire lactation and have been so for previous milking seasons. Few studies have reported the impact of lactation persistency in cows milked OAD compared to cows milked TAD (Hickson et al., 2006; Lembeye et al., 2016). Hickson et al. (2006) found that TAD milked cows tend to have better persistency than OAD milked cows, while Lembeye, Lopez-Villalobos, Burke, Davis, et al. (2016) found opposite results.

The loss in fat yield as a result of a lower milking frequency was larger in late than in early and mid-lactation. This finding contrasts with previous studies that showed higher fat yield loss in early and mid-lactation compared to late lactation (Carruthers et al., 1993; Stelwagen and Knight, 1997). The results from the present study also suggest a higher persistency in OAD milked cows than in TAD milked cows. Both higher (Lembeye, Lopez-Villalobos, et al., 2016c) and lower (Hickson et al., 2006) persistency of milk fat yield in OAD compared to TAD milking was found in earlier studies. The fat content decreased significantly in TAD milk between early and mid-lactation, while this remained the same in OAD milk. Since the trend in milk yield throughout the lactation was similar in OAD and TAD milk, this change in the fat content was likely to drive the difference in the lactation curve for the fat yield.

4.5.4.2 Proximate milk composition

In late lactation, milk from both TAD and OAD milking production systems had the highest protein and fat content. The protein and fat contents increase throughout lactation until drying off (Auldist et al., 1998; Li et al., 2019; O'Brien and Guinee, 2016; Sorensen et al., 2008). TAD milk followed this trend, but OAD milk remained similar in protein content in the first few months of lactation. Li et al. (2019) also found no significant difference between the protein content in early and mid-lactation in one of the two milking seasons studied. Previous studies have found that the lactose content decreases towards the end of lactation (Auldist et al., 1998; Li et al., 2019; O'Brien and Guinee, 2016; Sorensen et al., 2008). It has been suggested that a reduction in milking frequency leads to an enhanced involution process (Bernier-Dodier et al., 2010; Knight and Dewhurst, 1994; Lembeye et al., 2016), decreasing the number of epithelial cells and thus decreasing lactose and total milk production. Whether this is the case when cows are milked OAD throughout the entire season is unclear.

4.5.4.3 Protein composition

In line with the lactational trend for the total casein content observed in the present study, previous research has also shown a decrease in the casein fraction in milk in late lactation compared to earlier stages (Ng-Kwai-Hang et al., 1982). This decrease has been attributed to the involution process, weakening the tight junctions, and increasing the influx of serum protein (Auldist and Prosser, 1998; Davis et al., 1999). However, some studies did not find a difference in the casein content at different stages of lactation (Auldist et al., 1998; Coulon et al., 1998; Li et al., 2019).

Although similar lactation trends were previously observed (Li et al., 2019) for the α_{s1}-CN and α_{s2}-CN contents in the milk, the stage of lactation effect on the κ-CN content in bovine milk is inconsistent across the literature. Li et al. (2019) studied the seasonal trend of the main proteins in milk from TAD milked cows in New Zealand over two consecutive milking seasons. While the seasonal trend observed in OAD milk in the present study was in line with the results Li et al. (2019) reported for the first year, the trend in TAD milk here was in contrast with their findings from either of the two milk seasons that were studied. O'Connell et al. (2017) found an increase in the κ-CN content between mid- and late lactation. They also studied a herd managed in a seasonal calving system that was mostly grass fed during mid-lactation and the start of late lactation (O'Connell et al., 2017). In another study, no effect of the stage of lactation on the κ-CN content was found (Barry and Donnelly, 1980). Ostersen et al. (1997) found a decrease in the κ-CN content throughout lactation. The increase in the degree of glycosylation of κ-CN throughout lactation is consistent across the literature (Bonfatti et al., 2014; Li et al., 2019; Robitaille et al., 1991). The mechanism behind the increasing glycosylation degree remains to be investigated, but there is evidence that genetics contribute to the variation (Bonfatti et al., 2014).

Finally, the increase in β-LG content towards the end of lactation was reported in some studies but not all. Li et al. (2019) found an increase in β-LG throughout the lactation of one year but not the other. O'Connell et al. (2017) found an increase between mid- and late lactation for β-LG variant B but not for variant A. In agreement with the present study, Li et al. (2019) also reported a decrease in the α-LA content throughout lactation. The involvement of α-LA in the regulation of lactose production could explain the decrease in α-LA content in late lactation with a decline in milk volume (Brew, 2003; Li et al., 2019; Strucken et al., 2015)

4.6 Conclusion

The results of this study confirmed that cows in a OAD milking system have lower yields of milk and milk solids than cows in a TAD milking system. In contrast to previous studies, the difference in the protein and fat content between the OAD and TAD milking production systems was not significant, possibly due to the small sample size. This study is the first to compare the protein profile of milk from a OAD milking system with that from a TAD milking system. An effect of milking frequency on the proportion of κ-CN, α_{s2}-CN and α-LA was found, and the effect of the glycosylation degree approached significance. Additionally, these proteins followed a different lactation curve in OAD than in TAD milk. α_{s2}-CN and α-LA are both minor proteins, so the implications for processing may be minimal. However, the proportion and the degree of glycosylation of κ-CN could influence the heating and gelation properties of the milk. These processing characteristics are further investigated in Chapters 7 and 8.

Chapter 5

The effect of breed on the protein composition of milk from dairy cows milked once- or twice-a-day

To be submitted:

van der Zeijden, M., Ellis, A., Lopez-Villalobos, N., Li, S., Roy, N., McNabb, W. The effect of breed on the protein composition of milk from dairy cows milked once- or twice-a-day. *New Zealand Journal of Animal Science and Production.*

5.1 Abstract

Breed plays an essential role in determining milk composition and yield. In New Zealand, Holstein-Friesian (F), Jersey (J) and their crossbreed (F×J) are the most common breeds. With an increasing number of farmers switching from a twice-a-day (TAD) milking system to a once-a-day (OAD) milking system, there is a need to understand better the consequences of this change in milking frequency for milk quality. The objective of this chapter was to investigate the effect of breed on the protein composition of milk from cows in a OAD and in a TAD milking system. Milk was sampled in early, mid-, and late lactation from cows kept at Massey University Dairy Farms No. 1 (OAD milking) and No. 4 (TAD milking) located in Palmerston North, New Zealand. The total milk and milk solid yield, the proximate composition and the protein composition were determined. The total milk, protein, and lactose yield (F > F×J, J), and fat (F > F×J > J), lactose (J > F, F×J), κ-casein (F×J > F, J) and α-lactalbumin (F×J > F in OAD milk) contents were significantly affected by breed. Additionally, in the OAD milking system, the proportion of total casein in the milk was significantly higher, and the β-LG lower in F×J than in F and J cows. Overall, breed seemed to affect milk composition more in a OAD milking system than in a TAD milking system.

5.2 Introduction

In the 2020-2021 milking season, the national herd in New Zealand consisted of 32.4% Holstein-Friesian (F), 8.2% Jersey (J) cows, 49.6% Holstein-Friesian×Jersey (F×J) crossbreed and the remaining 9.7% of various other breeds (LIC and DairyNZ, 2021). The herd composition also varies per farm.

The genetic composition of a cow is a major contributor to milk yields and composition. For example, Jersey cows have a higher fat and protein content but a lower yield of milk solids (Cerbulis and Farrell, 1975; Lembeye, Lopez-Villalobos, et al., 2016c). In contrast, Holstein-Friesian cows yield milk lower in protein and fat but yield higher total volumes. Crossbreeding has been subject to various studies aiming to understand the heterosis effect on various milk components (Ahlborn and Dempfle, 1992; Back and Lopez-Villalobos, 2007; Lembeye, et al., 2016a). Not only are composition and yield important for the pay-out to the farmer, but they are also crucial for the industry undertaking milk processing. For example, milk with a high proportion of casein has improved cheese yield and quality (Auldist et al., 2004).

In New Zealand, most farms milk twice-a-day (TAD), but once-a-day (OAD) milking is increasing in popularity. From previous studies, it is known that a reduction in milking frequency results in an increase in protein and fat contents but a decrease in yield (Bewsell et al., 2008; Edwards, 2018; Lacy-Hulbert et al., 1999; Lazzarini et al., 2018; Lembeye et al., 2016; Murney et al., 2015; O'Brien et al., 2002; Rémond et al., 2004; Stockdale, 2006). The consequence of this change in the milking production system for protein composition is not well understood.

The previous chapter examined the impact of OAD milking compared to TAD milking on milk (solids) yield, the proximate milk composition and protein composition at different stages of lactation. To our knowledge, no research has looked at the effect of breed on protein composition in a OAD milking system and in a TAD milking system.

Thus, this chapter aims to study the role of breed (F, F×J, and J) on the impact of milking frequency on milk yield and composition throughout lactation. In this study, the milk from cows kept at Massey University Dairy Farm No. 1 (OAD milking) was compared to the milk from cows kept at Massey University Dairy Farm No. 4 (TAD milking). The results were separated into different breed-milking frequency groups.

5.3 Methods

The sampling strategy and analysis methods are described in detail in Chapter 3. A summary is given below.

5.3.1 Sampling strategy

Cows from Massey University Dairy Farm No.1 (Dairy No. 1; OAD milking) and Massey University Dairy Farm No. 4 (Dairy No. 4; TAD milking) were selected based on their genetics, breeding worth and expected calving date. The median calving date was taken from the entire herd on each farm: for Dairy No. 1, this was 7 August 2020, and for Dairy No. 4, the median calving date was 27 July 2020.

From the OAD farm, milk was sampled from three animals, each of the F, F×J and J breeds. From the TAD milking farm, milk was sampled from three animals of the F and F×J breeds. Cows with a breed composition of F ≥ 14/16 were classified as F, cows with a breed composition of J ≥ 14/16 as J, and cows with both F < 10/16 and J < 10/16 were classified as F×J. Cows containing any breed other than F or J were excluded from the study.

Milk was sampled nine times throughout the milking season, of which three were scheduled at the beginning, three in the middle, and three towards the end of the season. The milk samples were categorised as early, mid-, and late lactation based on the days in milk (DIM, number of days since calving) of the cow on the day of sampling. The milk from the afternoon and the following morning was mixed proportional to the PM, and AM milk yield per cow to create a 24h milk sample for the TAD milked cows.

5.3.2 Sample analysis

The yield of each cow was determined by recording the weight on the farm immediately after the milking. The proximate composition of the milk was analysed using the MilkoScan (Foss Analytics, Hillerød, Denmark). The obtained values for protein, fat and lactose contents were used together with the total milk yield to calculate the yield of each component in kilograms. The protein composition was analysed using a high-performance liquid chromatography (HPLC) method modified from the method by Bobe et al. (1998). The method is detailed in Chapter 3.

5.3.3 Statistical analysis

The data was analysed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A mixed model (using the MIXED procedure) was used to obtain the least square means and standard errors of the parameters analysed in this study. The following model was used:

$$Y_{ijkl} = \mu + M_i + B_j(M_i) + S_k + MS_{ik} + BS_{jk}(M_i) + \beta_1 p_{ijkl} + \beta_2 d_l + C_l + e_{ijkl}$$

Y_{ijkl} is the observation for the trait for milking frequency i, breed j, stage of lactation k, and cow l

μ is the population mean

M_i is the fixed effect of milking frequency i (i = OAD and TAD)

$B_j(M_i)$ is the fixed effect of breed j nested in milking frequency i (j = F, F×J and J)

S_k is the fixed effect of the stage of lactation k (k = early, mid, and late)

MS_{ik} is the effect of interaction between milking frequency i and stage of lactation k

$BS_{jk}(M_i)$ is the effect of the interaction of breed j and stage of lactation k nested in milking frequency i

β_1 is the regression coefficient of the linear effect of parity p (years) of cow l

β_2 is the regression coefficient of the linear effect of deviation (days) from herd median calving date d of cow l

C_l is the random effect of cow l assumed with mean zero and variance σ_c^2

e_{ijkl} is the residual random error assumed with mean zero and variance σ_e^2

Marginal means and standard errors for each level of the fixed effects were obtained and used for multiple mean comparisons using Fisher's least significant difference test. Significant differences were declared at $P < 0.05$.

5.4 Results

5.4.1 Descriptive statistics

Descriptive statistics are presented in Table 5.1, outlining the mean, standard deviation, coefficient of variance and minimum and maximum values for each of the analysed traits. *F*-values and significance levels for each of the factors included in the statistical model are shown in Table 5.2. The stage of lactation had a significant effect on all traits analysed in this study. Breed effects were significant for total milk, protein and lactose yields, the fat, lactose, κ-casein and α-lactalbumin contents in the milk.

Table 5.1. Descriptive statistics of daily milk yield, proximate composition, and protein composition of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

Trait	N	Mean	SD	CV	Min	Max
Milk yield (kg/cow/day)	135	22.3	7.5	33.5%	9.0	41.2
Protein yield (kg/cow/day)	135	0.81	0.22	26.9%	0.39	1.28
Fat yield (kg/cow/day)	135	1.05	0.33	31.1%	0.44	2.22
Lactose yield (kg/cow/day)	135	1.03	0.36	34.6%	0.39	1.95
Protein content (%)	135	3.7	0.4	11.2%	2.84	4.93
Fat content (%)	135	4.8	0.8	17.4%	2.64	7.63
Lactose content (%)	135	4.6	0.1	3.1%	4.33	5.02
Casein (%)	135	80.5	3.6	4.5%	73.1	87.7
α_{s1}-casein (%)	135	22.8	1.6	6.8%	19.6	27.3
α_{s2}-casein (%)	135	5.7	1.1	18.3%	3.7	8.9
β-casein (%)	135	37.8	2.3	6.1%	31.4	43.8
κ-casein (%)	135	14.2	1.6	11.1%	9.9	17.1
Gly-κ-CN[1] (% of κ-CN)	135	43.9	5.3	12.1%	29.8	57.3
Whey protein (%)	135	19.5	3.6	18.5%	12.3	26.9
β-lactoglobulin (%)	135	16.4	3.7	22.8%	8.7	23.6
α-lactalbumin (%)	135	3.1	0.4	14.1%	1.9	4.0

[1] Gly-κ-CN = glycosylated κ-casein

Table 5.2. *F*-values and significant levels for factors affecting the composition of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

Trait	Factor[1]						
	M	B(M)	S	M×S	B×S(M)	p	d
Milk yield	53.3***	5.8**	430.1***	8.9***	5.1***	2.0	0.0
Protein yield	64.6***	5.8**	188.7***	1.2	2.6*	1.7	1.2
Fat yield	18.8***	0.5	125.8***	8.3***	1.6	1.7	0.1
Lactose yield	54.9***	5.4**	371.5***	6.2**	3.8**	1.6	0.3
Protein content	1.6	0.8	169.2***	6.4**	2.6*	0.2	0.4
Fat content	4.0*	3.9*	11.3***	3.3*	0.3	0.3	0.0
Lactose content	0.8	4.5**	46.3***	6.9**	4.5***	1.7	4.6*
Casein	0.3	2.3	126.7***	3.8*	0.8	0.5	0.6
α_{s1}-casein	2.1	1.3	61.6***	0.2	0.7	1.2	2.8
α_{s2}-casein	30.8***	2.1	30.7***	2.8	0.3	0.1	0.2
β-casein	1.3	2.5	25.0***	3.2*	1.3	1.5	0.0
κ-casein	9.9**	8.7***	13.2***	9.9	1.1	3.5	0.0
Glycosylated κ-casein	2.1	2.4	59.0***	7.0***	1.5	0.6	0.3
Whey protein	0.3	2.3	126.7***	3.8**	0.8	0.5	0.6
β-lactoglobulin	0.1	2.5	260.6***	2.7*	1.2	0.5	0.6
α-lactalbumin	15.6***	3.1*	70.0***	3.1	0.5	1.7	0.5

[1] M = milking frequency (OAD and TAD), B(M)= breed (Holstein-Friesian, Holstein-Friesian×Jersey crossbreed and Jersey) nested within M, S = stage of lactation (early, mid, and late), p = parity (3-4) included as a covariate, d = deviation from the median calving date of the herd for each farm included as a covariate. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

5.4.2 Yield

Marginal means of the milk yield and the proximate and protein composition of milk from F, F×J and J cows in the OAD and the TAD milking systems are presented in Table 5.3. In the OAD milking system, F cows produced more milk per day than F×J and J cows. This breed effect was also observed for daily protein and lactose yield but not for fat yield, which did not differ significantly between breeds. In contrast, in the TAD milking system, protein and fat yields per cow per day were both similar in F and F×J cows. The daily lactose yield from TAD-milked F cows was higher than that from F×J cows, which aligned with the daily milk yield.

The daily yield of milk, protein, fat, and lactose all decreased significantly from early to late lactation for each breed-milking frequency combination (Figure 5.1). The daily milk and lactose yields followed similar patterns throughout the lactation in both milking systems. Daily yields of milk and lactose of OAD milked F×J and J cows decreased less from early to mid-lactation than those of OAD milked F cows. The lactational curves of milk, protein, fat, and lactose yields were similar for F and F×J cows milked TAD. In contrast, the protein and fat yields of F×J and J cows decreased less between early and mid-lactation than from OAD milked F cows.

5.4.3 Proximate composition

Breed effects within milking frequency were significant ($P < 0.05$) on fat and lactose contents. In OAD, the fat content in milk from J cows was higher ($P < 0.05$) than in milk from F cows, with an intermediate mean fat content in milk from F×J cows between the fat content in F and J milk but did not significantly differ from either of these breeds. In the TAD milking system, compared with F cows, F×J cows produced milk with significantly higher ($P < 0.05$) fat content. The lactose content did not differ between F and F×J in both milking systems. Milk from OAD milked J cows had a higher (*P* < 0.05) lactose content than F and F×J cows milked OAD.

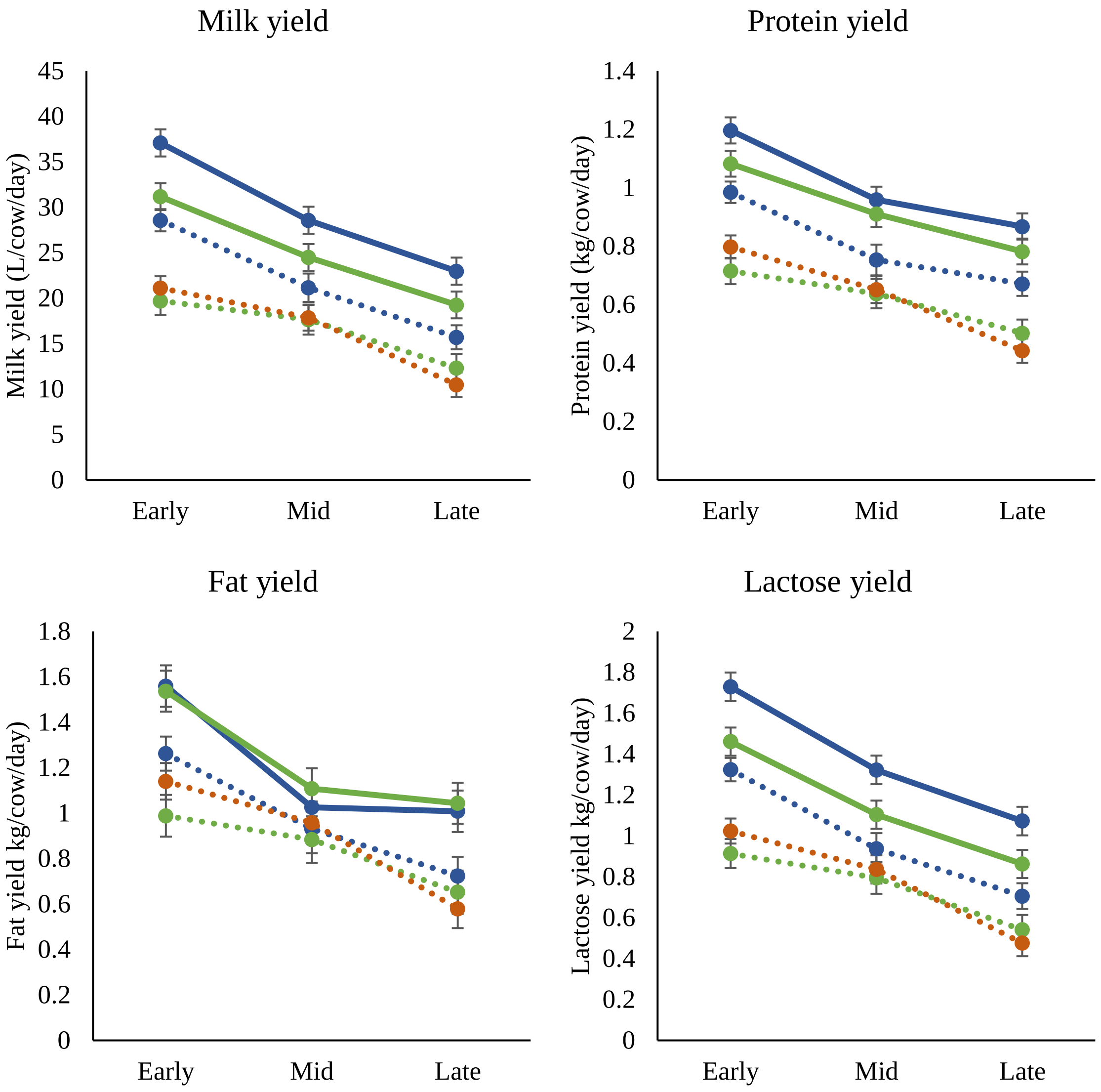


Figure 5.1. The daily yield of milk, protein, fat, and lactose from OAD milked Holstein-Friesian (•••••, blue), Holstein-Friesian×Jersey (•••••, green) and Jersey (•••••, orange) cows, and TAD milked Holstein-Friesian (——, blue) and Holstein-Friesian×Jersey (——, green) cows in early, mid-, and late lactation at Massey Dairy farms No. 1 and No.4 in production season 2020-2021.

Table 5.3. Marginal means of milk yield, proximate composition, and protein composition of milk from Holstein-Friesian (F), Friesian-Jersey crossbreed (F×J) and Jersey (J) cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in the production season 2020-2021.

	OAD			TAD	
	F	F×J	J	F	F×J
Milk yield (L)	21.8 ± 1.22^{b}	16.6 ± 1.48^{c}	16.5 ± 1.23^{c}	29.5 ± 1.40^{a}	25.0 ± 1.38^{b}
Protein yield (kg)	0.80 ± 0.036^{b}	0.62 ± 0.042^{c}	0.63 ± 0.036^{c}	1.01 ± 0.040^{a}	0.93 ± 0.039^{a}
Fat yield (kg)	0.97 ± 0.074^{b}	0.84 ± 0.086^{b}	0.89 ± 0.073^{b}	1.20 ± 0.081^{a}	1.23 ± 0.080^{a}
Lactose yield (kg)	0.99 ± 0.057^{b}	0.75 ± 0.068^{c}	0.78 ± 0.057^{c}	1.37 ± 0.065^{a}	1.14 ± 0.064^{b}
Protein content (%)	3.7 ± 0.15	3.8 ± 0.19	3.9 ± 0.16	3.5 ± 0.18	3.8 ± 0.18
Fat content (%)	4.5 ± 0.27^{bc}	5.2 ± 0.32^{ab}	5.4 ± 0.27^{a}	4.0 ± 0.31^{c}	4.9 ± 0.30^{ab}
Lactose content (%)	4.5 ± 0.04^{c}	4.5 ± 0.04^{c}	4.7 ± 0.04^{a}	4.7 ± 0.04^{ab}	4.6 ± 0.04^{bc}
Casein (%)	78.3 ± 1.57^{b}	84.7 ± 2.03^{a}	79.1 ± 1.66^{b}	79.0 ± 1.94^{b}	80.7 ± 1.91^{ab}
α_{s1}-casein (%)	21.7 ± 0.58^{b}	22.9 ± 0.72^{ab}	22.3 ± 0.59^{ab}	22.5 ± 0.68^{ab}	23.9 ± 0.67^{a}
α_{s2}-casein (%)	6.2 ± 0.27^{ab}	7.0 ± 0.33^{a}	6.0 ± 0.28^{b}	4.7 ± 0.31^{c}	5.1 ± 0.31^{c}
β-casein (%)	37.2 ± 0.96^{ab}	39.3 ± 1.21^{a}	35.6 ± 0.99^{b}	39.7 ± 1.16^{a}	37.3 ± 1.14^{ab}
κ-casein (%)	13.3 ± 0.40^{bc}	15.5 ± 0.51^{a}	15.1 ± 0.42^{a}	12.1 ± 0.49^{c}	14.5 ± 0.48^{ab}
Gly-κ-casein[1] (% of κ-CN)	48.6 ± 1.76^{a}	44.5 ± 2.14^{ab}	42.8 ± 1.77^{b}	44.3 ± 2.03^{ab}	41.2 ± 2.00^{b}
Whey (%)	21.7 ± 1.57^{b}	15.3 ± 2.03^{a}	20.9 ± 1.66^{b}	21.1 ± 1.94^{b}	19.3 ± 1.91^{ab}
β-lactoglobulin (%)	19.0 ± 1.59^{b}	12.2 ± 2.06^{a}	18.1 ± 1.68^{b}	17.9 ± 1.96^{b}	15.8 ± 1.94^{ab}
α-lactalbumin (%)	2.7 ± 0.11^{c}	3.1 ± 0.12^{b}	2.9 ± 0.10^{bc}	3.1 ± 0.12^{ab}	3.4 ± 0.11^{a}

[1] Gly-κ-CN = glycosylated κ-casein, $^{a, b, c, d, e, f}$ Means with different superscripts within one row are significantly different ($P < 0.05$).

5.4.4 Protein composition

In the OAD milking system, the proportion of casein in the milk was higher ($P < 0.05$) for F×J (84.7%) cows than for F (78.3%) and J (79.1%) cows. In the TAD milking system, the proportion of casein did not differ significantly between F (79.0%) and F×J (80.7%) cows. In both milking systems, the proportions of α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN, and the glycosylation degree of κ-CN were similar across F and F×J cows. The proportions of α_{s1}-CN, α_{s2}-CN, β-CN, and glycosylated κ-CN were similar in F and F×J. The κ-CN content was higher ($P < 0.05$) in milk from F×J cows than in milk from F cows in both milking systems. OAD milked J cows produced milk with a similar α_{s1}-CN content as OAD milked F and F×J cows. The contents of α_{s2}-CN and β-CN, as percentages of protein, in J milk were like those of milk from OAD milked F cows but were significantly ($P < 0.05$) lower than those in F×J milk. The κ-CN content and degree of κ-CN glycosylation in OAD J milk were similar to the milk from OAD F×J cows but higher and lower ($P < 0.05$), respectively, than in milk from F cows. In OAD milked cows, the proportion of β-LG in the milk of F×J cows was significantly ($P < 0.05$) lower than that of F and J cows. In the OAD milking system, the α-LA content in milk from F×J cows was significantly ($P < 0.05$) higher than in milk from F cows, but the α-LA content in J milk did not differ significantly from the other breeds. In contrast, in the TAD milking system, the proportions of β-LG and α-LA did not differ significantly between F and F×J cows.

The proportion of casein in milk protein from each of the different breeds milked OAD decreased significantly ($P < 0.05$) in late lactation (Figure 5.2). The TAD-milked F and F×J cows both produced milk with a casein content decreasing throughout the lactation. Similar patterns in milk from different breeds within each milking system were found for all individual caseins (α_{s1}-CN, α_{s2}-CN, κ-CN, and β-CN; Figure 5.2 and Figure 5.3). The degree of κ-CN glycosylation increased as the lactation progressed for each breed-milking frequency combination (Figure 5.3). In the OAD milking system, there was a difference of approximately 9% between early and late lactation for F and F×J cows, but a smaller increase of about 5% in milk from J cows. In the TAD milking system, the glycosylation degree of κ-CN increased by 5% in milk from F but only by 2.5% in F×J milk. The changes in the proportions of β-LG and α-LA in the milk throughout lactation were consistent across different breeds within each milking system (Figure 5.3).

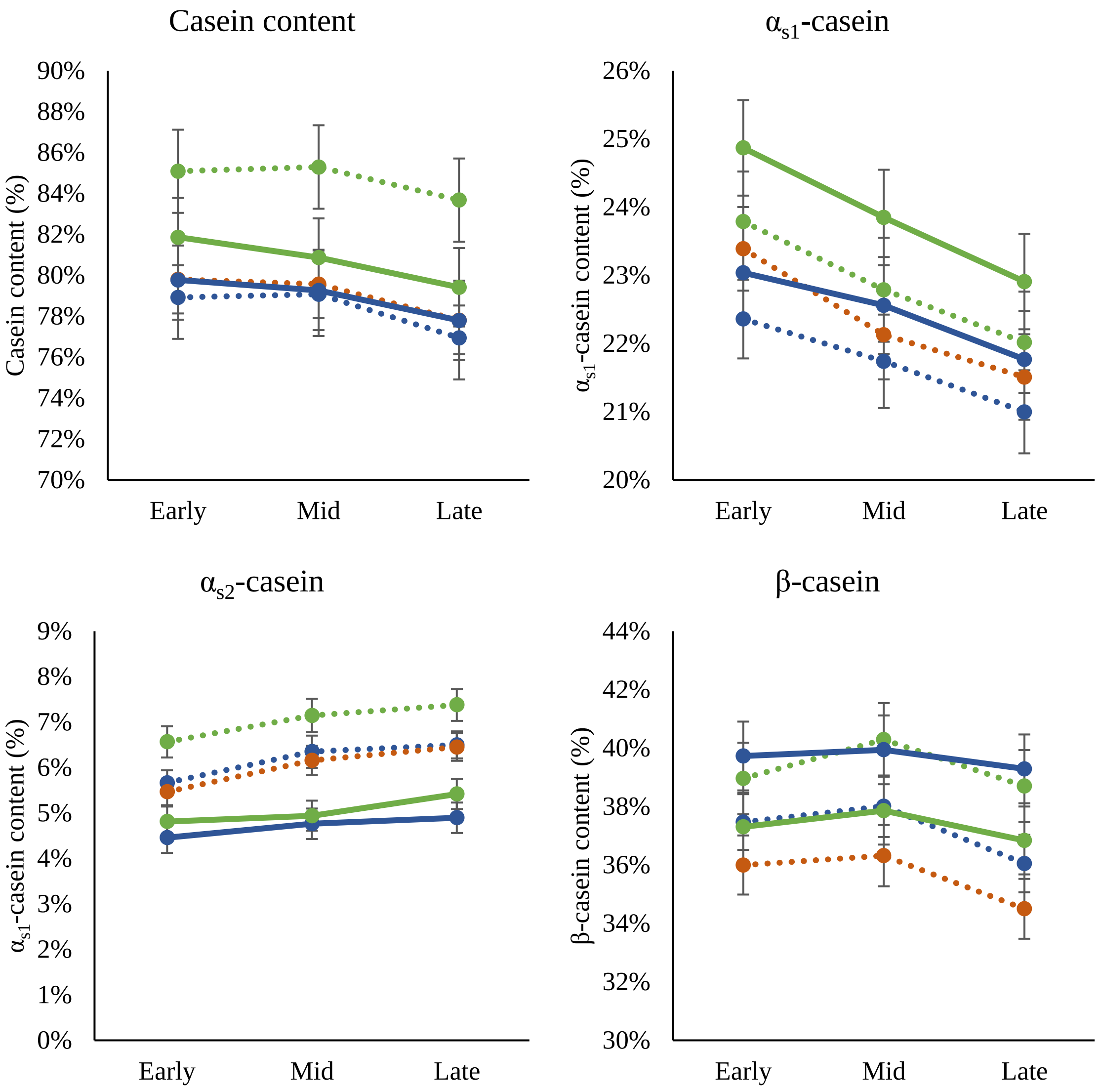


Figure 5.2. The proportions of casein, α_{s1}-casein, α_{s2}-casein and β-casein, in milk from OAD milked Holstein-Friesian (••••, blue), Holstein-Friesian×Jersey (••••, green) and Jersey (••••, orange) cows, and TAD milked Holstein-Friesian (—, blue) and Holstein-Friesian×Jersey (—, green) cows in early, mid-, and late lactation at Massey Dairy farms No. 1 and No.4 in production season 2020-2021.

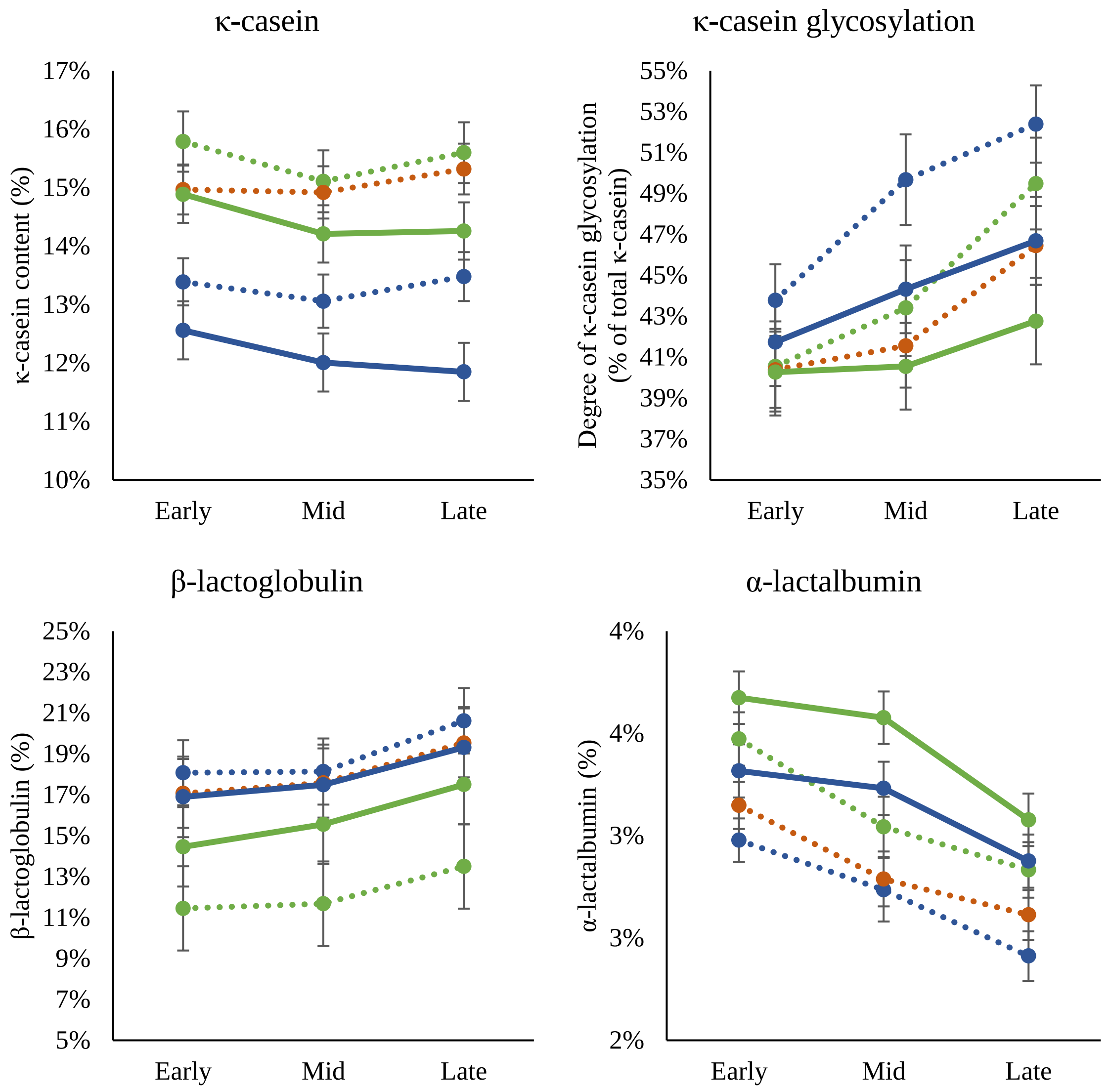


Figure 5.3. The proportions of κ-casein, β-lactoglobulin and α-lactalbumin, and degree of κ-casein glycosylation in milk from OAD milked Holstein-Friesian (·····, blue), Holstein-Friesian×Jersey (·····, green) and Jersey (·····, orange) cows, and TAD milked Holstein-Friesian (—, blue) and Holstein-Friesian×Jersey (—, green) cows in early, mid-, and late lactation at Massey Dairy farms No. 1 and No.4 in production season 2020-2021.

5.5 Discussion

5.5.1 Yield

The breed effects on daily milk, fat and lactose yields were similar in the OAD and TAD milking systems, with F cows producing larger amounts than F×J cows. However, in the OAD milking system, the total daily milk yield from F was 31% higher than F×J, while this was only 18% in the TAD milking system. A decrease in milk and milk solids yield with a decreasing breed proportion of F aligns with previous research (Back and Lopez-Villalobos, 2007; Prendiville et al., 2011). However, other studies have also shown a larger heterosis effect on milk yield in a OAD than in a TAD milking system (Lembeye, et al., 2016a). Crossbreeding has been adopted widely in the New Zealand dairy industry to improve the production traits of dairy cows and has been found to improve profitability (Lopez-Villalobos et al., 2000). The results from the present study suggest that crossbreed F×J cows could do better in a TAD milking system than in a OAD milking system. The protein yield did not differ between F and F×J in the TAD milking system, while in the OAD milking system, F×J cows yielded less protein than F cows.

Jersey cows in the OAD milking system yielded similar amounts of milk, protein, fat, and lactose as F×J crossbreed cows but less than F cows. Higher yields for F cows compared to J cows have been reported in other studies (Back and Lopez-Villalobos, 2007; Prendiville et al., 2009), while some did not (Kedzierska-Matysek et al., 2011). Generally, as shown in previous research, F×J cows tend to have yields that are intermediate between F and J cows, whether that is closer to F (positive heterosis) or not (Back and Lopez-Villalobos, 2007; Lembeye, et al., 2016a; Penasa et al., 2010; Prendiville et al., 2011). Some studies also found yields from F and F×J cows to be similar (Ormston et al., 2022). In the present study, in the OAD milking system, either J cows produced exceptionally well, or the F×J crossbreed cows produced less than expected.

Overall, the yields of milk, protein, fat, and lactose in the OAD milking system were lower than the yields in the TAD milking system. Since the pay-out to the farmer in New Zealand is based on the protein and fat yields from the milk, such a decrease is potentially unfavourable for the farmer. However, costs are lower with the OAD milking system, and research has shown that this system is financially viable (Edwards, 2019).

5.5.2 Milk composition

Fat content was not different between F and F×J and between F×J and J cows in the OAD milking system. The difference in fat content between F and F×J cows was larger in the TAD milking system. A similar pattern was found for the protein content in the present study, but no significant differences were found. Previous research consistently shows increasing fat and protein contents with an increasing proportion of J cows (Bland et al., 2015; Clark et al., 2006; Ormston et al., 2022; Prendiville et al., 2009). The small number of cows and the variation between individual cows could be why there was no effect of breed in the present study.

In the OAD milking system, milk from F×J cows had a higher content of casein in the protein fraction than F cows. This result was mostly driven by the higher contents of β-CN and κ-CN and lower contents of β-LG in the milk. Ormston et al. (2022) also found lower total casein levels in milk from F compared to F×J cows. Higher contents of casein have been associated with improved coagulation properties (Auldist et al., 2004) and cheese yield (Pretto et al., 2013). The proportion of total casein decreased in late lactation in all breed-milking frequency combinations, which aligns with previous research (Li et al., 2019). A decrease in the casein-to-whey ratio has been attributed to an increased influx of serum protein as a result of increased permeability of the mammary tissue due to the loss of tight junctions during the involution process near the end of a milking season (Davis et al., 1999).

In the present study, the κ-CN content in the protein was higher in F×J than in F cows in both milking systems. K-CN plays an important part in heat-induced changes to the proteins in the milk and gelation properties (Donato and Guyomarc'h, 2009). Higher proportions of κ-CN have also been associated with smaller aggregate formation during heat treatment which may be beneficial for the heat stability of milk (Guyomarc'h et al., 2009). In the OAD milking system, the degree of κ-CN glycosylation was lower in J than in F cows. The κ-CN glycosylation degree in milk from F×J cows lay between these two breeds but did not differ from either. In the TAD milking system, the κ-CN glycosylation degree was also lower in milk from F×J than from F cows, but this difference was not significant.

Additionally, an increase in the κ-CN glycosylation degree throughout lactation was found, which aligns with previous research (Li et al., 2019). The underlying mechanism causing variation in the glycosylation degree is unknown, but there is evidence that genetics contribute to the variation (Bonfatti et al., 2014). This evidence also supports the difference between breeds, where an increasing proportion of J in the cow genetics seems to negatively correlate with the κ-CN glycosylation degree. Lower gel strength and longer gelation times have been associated with the degree of κ-CN glycosylation (Li et al., 2020). This finding would suggest that milk from J or F×J crossbred cows is more suitable for yoghurt production than milk from F cows.

A considerably lower (36%) β-LG content was found in milk from F×J cows compared to F cows in the OAD milking system. As β-LG is the most abundant whey protein in milk, this likely contributed strongly to the lower whey protein and, thereby, higher casein content of the F×J milk. Therefore, a lower β-LG content in the milk indirectly contributes to improved yield associated with higher casein contents. Additionally, β-LG content has been negatively correlated with cheese processing properties (Cipolat-Gotet et al., 2018).

The α-LA content in milk from OAD milked F cows was lower than F×J cows, while there was no difference between the two breeds in the TAD milking system. A-LA is involved in lactose production, which drives the milk volume (Strucken et al., 2015).

5.5.3 Future recommendations

The present study did not include J cows in the TAD milking system. The number of J cows at the Massey University Dairy Farm No.4 (TAD milking) was limited, resulting in this group not meeting the parity criteria (third and fourth) for the study. It was considered to include TAD-milked J cows in the analysis. However, preliminary analysis of the data resulted in the following. As a result of large standard errors induced by a large deviation from the mean parity (second and sixth compared to third and fourth in all other cows), this group of J cows was not a good representation of J cows. Therefore, TAD-milked J cows were excluded from the dataset, and results are not reported in this study.

For future research on the effect of breed on the protein composition, it is recommended firstly to select the animals more strictly for parity and secondly to increase the sample size. Since parity was included in the statistical model as a covariate, the deviation from the parity of other cows in the dataset caused increased errors in the estimated marginal means for TAD-milked J cows. Genetics also affect protein composition, especially κ-CN and β-LG, which differ between breeds. In the present study, cows were not selected based on the genetic variants of some of the main proteins, such as κ-CN and β-LG, in the milk. Including the polymorphism of milk proteins in the selection criteria or the statistical model would be a better approach to studying the effect of breed on milk composition in different milking frequency systems.

The small sample size likely contributed to the nonsignificant difference in protein and fat content between F and F×J cows, especially in the OAD milking system. A power analysis was done to determine the sample size required to study the breed effect on protein composition in a seasonal calving system. Results from another study (Back and Lopez-Villalobos, 2007) were used to calculate the relative composition of the main proteins in the milk in peak lactation. The coefficient of variance was assumed at 10%, the probability for a type 1 error was set at 0.05, and the power was set at 80%. To examine the breed effect on κ-CN and β-LG, which are two of the most important proteins determining the heat-induced changes and processing properties, a sample size of at least 8 (for κ-CN) or 30 (for β-LG) animals per group is required.

5.6 Conclusion

Breed affected total milk, protein and lactose yields, the fat and lactose contents and κ-CN and α-LA contents. F cows produced more milk, protein, and lactose than F×J and J cows in the OAD milking system. Similar trends were observed in the TAD milking system, but differences between F and F×J were smaller and protein yield did not differ between breeds. Protein and fat contents showed an increasing trend with an increasing proportion of J in the cow genetics. Again, differences were smaller in the TAD milking system than in the OAD milking system. The lactose content was similar in the milk of F and F×J cows but was higher in the milk of J cows in the OAD milking system. In the OAD system, milk from F×J cows had a considerably higher casein content and a lower β-LG content than F and J cows in the same milking system. The α-LA content was lower in F than in F×J milk from OAD-milked cows but similar between these breeds in the TAD milking system. The contents of α_{s2}-CN and β-CN in the milk protein from OAD-milked J cows were lower than in milk protein from OAD milked F×J cows. In both milking systems, the κ-CN content was higher in F×J than in F milk protein. The glycosylation degree of κ-CN decreased with an increasing proportion of J, potentially improving the gelation properties of the milk.

Chapter 6

The protein and mineral composition of bovine milk: comparing once- and twice-a-day milking of pasture-fed cows at different stages of lactation

Manuscript in preparation.

Van der Zeijden, M., Ellis, A., Lopez-Villalobos, N., Li, S., Roy, N., McNabb, W. The protein and mineral composition of bovine milk: comparing once- and twice-a-day milking of pasture-fed cows at different stages of lactation. *Dairy*

6.1 Abstract

There is limited knowledge of the effect of once-a-day (OAD) milking on milk composition beyond the protein, fat, and lactose content. The increasing number of farms implementing a OAD milking system raises the question of whether such a change in milking frequency affects other compositional and structural properties related to processing characteristics. The research presented in this chapter aimed to determine the proximate, protein and mineral composition as well as fat globule and casein micelle size in milk from OAD and twice-a-day (TAD) milking production systems. Milk from Holstein-Friesian and Holstein-Friesian×Jersey crossbred cows was sampled in early, mid-, and late lactation and mixed proportionally to the yields of each cow to mimic tank milk. The protein and fat contents, casein percentage, and somatic cell counts were higher in OAD than in TAD milk. The concentrations of all major proteins, except β-casein, were affected by the milking frequency. The κ-casein content, the κ-casein glycosylation degree and the α_{s2}-casein content were higher, while the contents of α_{s1}-casein, β-lactoglobulin and α-lactalbumin in the protein fraction were lower in OAD than in TAD milk. All traits above were affected by the stage of lactation. The calcium, magnesium and phosphorus concentrations and distribution in the milk were unaffected by the milking frequency. In contrast, the sodium and chloride contents were lower, and the potassium content was higher in OAD milk. These findings provide insights into the underlying physiological mechanism in milk composition changes due to less frequent milking. In addition, copper (OAD < TAD), iodine (OAD > TAD) and selenium (OAD < TAD) concentrations were also found to be affected by milking frequency, which a difference in diet composition could explain. Casein micelles were smaller in OAD than in TAD milk, but their size changed similarly throughout the lactation. These findings lay the foundation for understanding the potential implications for processing properties of

milk from a OAD milking production system compared to a TAD milking production system.

6.2 Introduction

The proximate, mineral and protein compositions of bovine milk are important factors for processing and product properties (Auldist, 2011; Donato and Guyomarc'h, 2009). For example, seasonal changes in the milk have been linked to altered product quality (O'Brien and Guinee, 2016). Proteins play a crucial role in the structure and stability of products such as cheese and yoghurt, but minor components such as minerals are not to be ignored. Minerals add important nutritional value to milk and are crucial for protein stability (Gaucheron, 2005).

Changes in farm management, including milking frequency, are known to impact milk composition. A lower milking frequency yields lower milk volumes and milk solids while the protein and fat contents of the milk (Clark et al., 2006; Gedye et al., 2020; Martin et al., 2009; O'Brien et al., 2002; Rémond et al., 2004). From an agricultural point of view, this offers a good insight into optimising a OAD milking system in terms of yield and cow reproduction. However, few studies have investigated changes in the concentration of individual proteins or minerals in milk due to a reduced milking frequency. For processing purposes, more knowledge is required to understand further implications of OAD milking across the lactation.

This chapter aimed to investigate the consequences of a OAD, compared to a TAD, milking system on the proximate, protein and mineral composition and physicochemical properties of milk mimicking bulk tank milk. This approach offers a more accurate insight into the impact OAD milking has on processing in an industry context compared to the approach used in Chapter 4, where milk from individual cows managed in a OAD or a

TAD milking production system were compared. Here, milk samples from Holstein-Friesian and Holstein-Friesian×Jersey crossbred cows were mixed to study the composition and structural differences between OAD and TAD milk. These raw milk samples were then analysed for the proximate protein and mineral composition, fat globule size, casein micelle size and pH. Milk was also sampled and analysed at different stages of lactation to understand the lactational effect in each milking production system.

6.3 Methods

6.3.1 Preparation of mixed milk samples

Milk was sampled three times in early, mid-, and late lactation from individual cows that met the study criteria described in Chapters 3 and 4. Only milk from Holstein-Friesian (F) and Holstein-Friesian×Jersey crossbred (F×J) cows were used as the Jersey cows from one of the farms did not strictly meet the parity criteria for the study. Three cows from each breed on each farm were selected, so six cows from the OAD milking system and six cows from the TAD milking system were used. The yield of milk from each cow was recorded on the farm by weight. Pooled milk was used for further characterisation; this mixed milk sample was created as follows. In the laboratory, the milk was mixed proportionally to the yields of each cow. The average proportion of milk from F cows was 59.9% and 54.1% in OAD and TAD milk, respectively. For F×J cows, the average proportion of milk from this breed in the mixed milk sample was 40.1% and 45.9% in OAD and TAD milk, respectively.

6.3.2 Characterisation

A detailed description of the analytical methodologies is given in Chapter 3. Below, a summary of each method is provided.

6.3.2.1 *Milk composition*

The proximate composition, including protein, fat, and lactose content, was analysed using the MilkoScan FT1 (Foss Analytics, Hillerød, Denmark). The pH was measured using an Edge Blu pH meter (Hanna Instruments, Woonsocket, RI, USA).

6.3.2.2 *Somatic cell count*

The somatic cell count (SCC) of each mixed milk sample was determined externally by MilkTestNZ (Hamilton, New Zealand) using the CombiFoss (Foss Analytics, Hillerød, Denmark).

6.3.2.3 *Protein composition*

Skim milk was used for the determination of the protein composition. The milk was skimmed by centrifugation for 15 minutes at 3000g at 4 °C. The protein composition of the skim milk was analysed using a high-performance liquid chromatography (HPLC) method modified from the method by Bobe et al. (1998). The method is detailed in Chapter 3.

6.3.2.4 *Mineral composition and ionic calcium*

The mineral composition of raw skim milk and serum was determined externally by Hill Laboratories (Hamilton, New Zealand). The ionic calcium was determined using a calcium electrode (Orion 9720BNWP Thermo Scientific, Singapore) together with a CyberScan pH 510 pH/mV Meter (Eutech Instruments-Thermo Scientific, Singapore). The electrode was calibrated using 1-10 mM of $CaCl_2$, of which the ionic strengths were adjusted to 80 mM with potassium chloride, as described by Li et al. (2019).

6.3.2.5 *Casein micelle size*

The casein micelle size was determined using the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Skim milk samples were diluted 50x in an imidazole buffer and filtered before analysis. The measurement was performed at 25 °C, each in triplicate, each consisting of 13 sub-measurements.

6.3.2.6 Fat globule size

The fat globule size of the raw and heat-treated samples was determined using the Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK). Each milk sample was mixed 1:1 with a 2% SDS and 50 mM EDTA solution (pH 6.7) prior to analysis.

6.3.3 Statistical analysis

Marginal means of the dependent variables for each level of milking frequency (OAD and TAD), stage of lactation (early, mid, and late) and combinations of milking frequency and stage of lactation were obtained using the MIXED procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) with the following linear model:

$$y_{ijk} = \mu + M_i + S_j + MS_{ij} + e_{ijk}$$

Y_{ik} is the observation for the trait for milking frequency i and lactation stage j

μ is the population mean

M_i is the fixed effect of milking frequency i (i = OAD and TAD)

S_j is the fixed effect of stage of lactation j (j = early, mid, and late)

MS_{ij} if the fixed effect of interaction between milking frequency i and stage of lactation j

e_{ijk} is the residual random error associated with observation y_{ijk}.

The residuals were modelled with heterogeneous variance across the combinations of milking frequency and stage of lactation. Significant differences were declared at $P < 0.05$.

6.4 Results

6.4.1 Major components

The proximate composition is given in Table 6.1 and Table 6.2. The protein content ranged from 3.5% in mid-lactation up to 4.1% in late lactation for OAD milking and from 3.3% in early lactation up to 3.9% in late lactation in TAD milk. Only in early lactation was the protein content significantly higher in OAD milk than in TAD milk, by almost 8%. However, overall, the milking frequency effect was significant ($P = 0.025$). In the OAD milking system, the protein content increased only in late lactation compared to early and mid-lactation, during which the protein content did not change significantly. In contrast, in the TAD milking system, the protein content increased throughout the entire lactation, and the protein content was lower in early than in mid- and late lactation. The interaction effect between milking frequency and stage of lactation was significant ($P < 0.05$).

The fat content of OAD milk ranged from 4.7% in mid-lactation up to 5.1% in late lactation. In TAD milk, the fat content ranged from 4.0% in mid-lactation to 4.8% in late lactation. The fat content of OAD and TAD milk differed significantly only in mid-lactation. In both systems, the fat content increased significantly from mid- to late lactation. The effects of stage of lactation and milking frequency on the fat content were both significant ($P < 0.05$).

In early and mid-lactation, the lactose contents of OAD and TAD milk were similar, while OAD milk had a significantly lower lactose content (4.5 ± 0.02%) than TAD milk (4.6 ± 0.02%) in late lactation. A significant effect of stage of lactation was observed, with the lactose content decreasing throughout the lactation.

Table 6.1. Estimated marginal means and standard errors of proximate composition, protein composition and structural components of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

	OAD	TAD	M[1] effect (*P-value*)
Protein (%)	3.7 ± 0.03	3.6 ± 0.05	0.025
Fat (%)	4.8 ± 0.09	4.5 ± 0.13	0.050
Lactose (%)	4.5 ± 0.02	4.6 ± 0.02	0.137
SCS[2]	6.6 ± 0.13	4.1 ± 0.08	< 0.001
SCC[3] (x 1000 cells/mL)	109 (87-130)	17 (15-20)	< 0.001
pH	6.70 ± 0.012	6.69 ± 0.010	0.437
Casein micelle size (nm)	153 ± 0.5	159 ± 1.6	0.008
Fat globule size (μm)	4.8 ± 0.07	4.8 ± 0.04	0.785
Total casein (%)	82.1 ± 0.24	80.1 ± 0.16	< 0.001
α_{s1}-casein (%)	22.5 ± 0.14	23.1 ± 0.11	0.012
α_{s2}-casein (%)	6.7 ± 0.15	4.9 ± 0.06	< 0.001
β-casein (%)	38.4 ± 0.21	38.6 ± 0.15	0.497
κ-casein (%)	14.5 ± 0.05	13.5 ± 0.10	< 0.001
Gly-κ-CN[3] (% of κ-casein)	46.2 ± 0.32	42.5 ± 0.38	< 0.001
Total whey protein (%)	17.9 ± 0.24	19.9 ± 0.16	< 0.001
β-lactoglobulin (%)	15.1 ± 0.19	16.7 ± 0.16	<.0001
α-lactalbumin (%)	2.8 ± 0.07	3.2 ± 0.04	< 0.001

[1]M = milking frequency, [2]SCS = somatic cell score calculated as SCS = $\log_2$ (SCC/1000), [3]SCC = somatic cell count (means with the confidence interval between brackets), [3]Gly-κ-CN = glycosylated κ-casein.

6.4.1.1 Somatic cell count

The SCC was measured in the mixed milk samples to confirm that they were suitable for processing and to analyse the impact of OAD milking on this trait. The SCC was transformed to somatic cell score SCS = Log_2(SCC/1000) for statistical analysis. There was a significant milking frequency effect on the SCS. In OAD milk, the SCC ranged from 68,000 cells/mL in early lactation to 159,000 cells/mL in late lactation, while TAD milk contained 14,000 cells/mL in mid-lactation up to 21,000 cells/mL in late lactation. While the SCC in TAD milk did not change throughout lactation, the SCC increased in late lactation in OAD milk.

Table 6.2. Estimated marginal means and standard errors of proximate composition, protein composition and structural components of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021, at different stages of lactation (SOL).

	OAD			TAD			*P-value*[1]	
	Early	Mid	Late	Early	Mid	Late	S	M×S
Protein (%)	3.6 ± 0.08^{bc}	3.5 ± 0.03^{cd}	4.1 ± 0.02^{a}	3.3 ± 0.05^{d}	3.5 ± 0.01^{c}	3.9 ± 0.14^{ab}	< 0.001	0.016
Fat (%)	4.8 ± 0.27^{abc}	4.7 ± 0.07^{b}	5.1 ± 0.06^{a}	4.7 ± 0.20^{abc}	4.0 ± 0.29^{c}	4.8 ± 0.16^{ab}	0.013	0.391
Lactose (%)	4.7 ± 0.05^{a}	4.5 ± 0.02^{b}	4.4 ± 0.01^{c}	4.6 ± 0.03^{a}	4.6 ± 0.04^{ab}	4.6 ± 0.02^{ab}	0.001	0.056
SCS[2]	6.1 ± 0.15^{b}	6.6 ± 0.31^{ab}	7.3 ± 0.17^{a}	4.1 ± 0.03^{c}	3.8 ± 0.11^{d}	4.3 ± 0.21^{c}	0.001	0.017
SCC[3] (x1000 cells/mL)	68 (52-83)	99 (50-148)	159 (121-197)	17 (16-17)	14 (12-16)	21 (14-28)	0.001	0.002
pH	6.66 ± 0.024^{bc}	6.71 ± 0.023^{abc}	6.74 ± 0.009^{a}	6.67 ± 0.015^{c}	6.68 ± 0.015^{bc}	6.73 ± 0.020^{ab}	0.007	0.697
Casein micelle size (nm)	153 ± 1.2^{bc}	152 ± 0.9^{c}	154 ± 0.6^{b}	153 ± 1.7^{bc}	159 ± 3.7^{abc}	164 ± 2.8^{a}	0.011	0.052
Fat globule size (μm)	5.3 ± 0.19^{a}	4.8 ± 0.08^{b}	4.3 ± 0.07^{d}	5.2 ± 0.05^{a}	4.7 ± 0.11^{bc}	4.5 ± 0.03^{c}	< 0.001	0.288
Total casein (%)	82.6 ± 0.32^{a}	83.0 ± 0.11^{a}	80.8 ± 0.62^{bc}	81.6 ± 0.09^{b}	80.4 ± 0.32^{c}	78.3 ± 0.33^{d}	< 0.001	0.014
α_{s1}-casein (%)	23.2 ± 0.17^{b}	22.8 ± 0.13^{b}	21.6 ± 0.38^{c}	24.1 ± 0.22^{a}	23.2 ± 0.09^{b}	21.9 ± 0.21^{c}	< 0.001	0.258
α_{s2}-casein (%)	6.2 ± 0.10^{b}	6.8 ± 0.14^{a}	7.2 ± 0.42^{a}	4.7 ± 0.04^{d}	4.8 ± 0.11^{cd}	5.1 ± 0.13^{c}	0.004	0.059
β-casein (%)	38.7 ± 0.25^{abc}	39.2 ± 0.13^{a}	37.4 ± 0.58^{c}	38.9 ± 0.34^{ab}	39.0 ± 0.16^{a}	38.0 ± 0.25^{b}	0.004	0.355
κ-casein (%)	14.6 ± 0.11^{a}	14.2 ± 0.09^{b}	14.7 ± 0.07^{a}	13.9 ± 0.17^{bc}	13.5 ± 0.15^{cd}	13.2 ± 0.21^{d}	0.029	0.043
Gly-κ-CN[4] (% of κ-CN)	40.6 ± 0.50^{d}	46.4 ± 0.61^{b}	51.5 ± 0.56^{a}	39.9 ± 0.51^{d}	42.2 ± 0.50^{c}	45.4 ± 0.89^{b}	< 0.001	0.002

Total whey (%)	17.4 ± 0.32^{d}	17.0 ± 0.11^{d}	19.2 ± 0.62^{bc}	18.4 ± 0.09^{c}	19.6 ± 0.32^{b}	21.8 ± 0.33^{a}	< 0.001	0.014
β-lactoglobulin (%)	14.3 ± 0.37^{cd}	14.3 ± 0.11^{d}	16.6 ± 0.43^{b}	15.1 ± 0.15^{c}	16.3 ± 0.31^{b}	18.8 ± 0.33^{a}	< 0.001	0.086
α-lactalbumin (%)	3.2 ± 0.05^{b}	2.7 ± 0.04^{d}	2.6 ± 0.19^{d}	3.3 ± 0.12^{ab}	3.3 ± 0.02^{a}	3.0 ± 0.01^{c}	0.006	0.020

[1]M = milking frequency (OAD and TAD), S = stage of lactation (early, mid, and late), [2]SCS = somatic cell score, [3]SCC = somatic cell count (means with confidence intervals between brackets), [4]Gly-κ-CN = glycosylated κ-casein, $^{a, b, c, d, e}$ Means with different superscripts within one row are significantly different ($P < 0.05$).

6.4.2 Protein composition

Table 6.1 and Table 6.2 show the results of the protein composition measured in the mixed milk samples. The total casein and whey protein in the milk were calculated as a percentage of the total protein as measured by HPLC. All proteins except β-casein (β-CN) were affected by milking frequency. The details of this comparison are outlined below.

The percentage of caseins in OAD milk proteins ranged from 80.8% in late lactation to 83.0% in mid-lactation. The casein percentage in TAD milk ranged from 78.3% in late lactation to 81.6% in early lactation. At each stage of lactation, OAD milk had a higher casein content than TAD milk. At both milking frequencies, the casein content dropped in late lactation. The casein fraction did not change with OAD milking during early and mid-lactation. In contrast, with TAD milking, the casein fraction of the milk was found to decrease over the lactation gradually. The effects of both stage of lactation and milking frequency were significant.

κ-casein (κ-CN) content in OAD milk was higher than in TAD milk, with the largest difference in late lactation. In OAD milk, the proportion of κ-CN ranged from 14.2% in mid-lactation up to 14.7% in late lactation, whereas, in TAD milk, the content ranged from 13.2% in late lactation up to 13.9% in early lactation. Also, while the κ-CN content in TAD milk decreased gradually throughout the lactation, the κ-CN content in OAD milk decreased during the early to mid-lactation, followed by a significant increase towards the end of the lactation. The significant interaction effect between milking frequency and stage of lactation confirms this difference in the lactational pattern in the present study.

The degree of κ-CN glycosylation was calculated as the percentage of glycosylated κ-CN in total κ-CN, as shown in Table 6.2. A significant difference between OAD and TAD milk was found in mid- and late lactation but not early lactation. In OAD milk, the glycosylation degree of κ-CN ranged from 40.6% in early lactation to 51.5% in late lactation. In TAD milk, these values ranged from 39.9% in early lactation up to 45.4% in late lactation. Although the glycosylation degree increases between early and late lactation in both milking systems, a more notable change was observed in OAD milk, with an increase of almost 11% from early to late lactation. This increase was only 5.5% in TAD milk.

There was a significant milking frequency effect on the proportion of α_{s1}-CN in the milk such that OAD milk generally contains less α_{s1}-casein (α_{s1}-CN) than TAD milk, although this difference was only significant in early lactation. There was a significant stage of lactation effect on the proportion of α_{s1}-CN, with the concentration decreasing throughout the lactation for both OAD and TAD milk. The α_{s2}-casein (α_{s2}-CN) content was also found to be significantly higher in OAD milk compared to TAD milk at each stage of lactation. The proportion of α_{s2}-CN in OAD milk increased from 6.2% in early to 7.2% in late lactation and in TAD milk from 4.7% in early to 5.1% in late lactation. There was no significant interaction effect between milking frequency and stage of lactation for the α_{s2}-CN content.

Both stage of lactation and milking frequency significantly affected the β-lactoglobulin (β-LG) content. OAD milk contained less β-LG than TAD milk, with values ranging from 14.3% to 16.6% and 15.1% to 18.8%, respectively. At both milking frequencies, the highest proportion was found in late lactation, and the largest increase was found between mid- and late lactation. In OAD milk, the β-LG content remains the same in early and mid-lactation. In TAD milk, however, there was a significant increase between these two stages of lactation. The interaction effect between milking frequency and stage of lactation was not significant.

The α-lactalbumin (α-LA) content was significantly (*P* < 0.001) lower in OAD (2.8%) than in TAD milk (3.2%). In contrast to β-LG, the proportion of α-LA decreased throughout the lactation in both milking systems. However, in the present study, the decrease mainly occurred between early and mid-lactation in OAD milk, while in TAD milk, the content remained similar in early and mid-lactation and decreased in late lactation.

6.4.3 Mineral composition

The total mineral profile of the milk, and the concentration of calcium, phosphorus, and magnesium in the serum phase, are presented in Table 6.3 and Table 6.4. This data allows for determining differences in the distribution of minerals and, thus, their involvement in the casein micelle structure. For calcium, the ionic fraction was also measured. The percentage of bound calcium, calcium bound to the casein micelle, was calculated by subtracting the percentage of serum calcium from the total calcium.

Table 6.3. Estimated marginal means and standard errors of the mineral composition of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

	OAD	TAD	M effect[1] (*P-value*)
Calcium (mg/100g)	126 ± 1.1	126 ± 1.4	1.000
Bound calcium (%)	56.2 ± 1.66	55.9 ± 1.04	0.888
Serum calcium (%)	43.8 ± 1.66	44.1 ± 1.04	0.888
Ionic calcium (%)	8.3 ± 0.45	8.1 ± 0.44	0.772
Phosphorus (mg/100g)	101 ± 1.2	102 ± 1.2	0.611
Serum Phosphorus (%)	56.4 ± 1.77	55.8 ± 1.29	0.808
Magnesium (mg/100g)	11.8 ± 0.09	11.5 ± 0.11	0.068
Serum Magnesium (%)	76.3 ± 0.67	76.8 ± 0.79	0.680
Potassium (mg/100g)	150 ± 1.5	166 ± 1.0	< 0.001
Sodium (mg/100g)	40 ± 0.3	31 ± 0.6	< 0.001
Chloride (mg/100g)	101 ± 1.0	97 ± 1.1	0.012
Copper (µg/100g)	3.5 ± 0.23	4.3 ± 0.27	0.035
Iodine (µg/100g)	8.5 ± 0.41	1.8 ± 0.15	< 0.001
Selenium (µg/100g)	1.6 ± 0.14	2.2 ± 0.14	0.021
Zinc (mg/100g)	0.39 ± 0.009	0.36 ± 0.012	0.126

[1] M = milking frequency

Both the total calcium and calcium distribution in milk were unaffected by the milking frequency. Stage of lactation affected the total calcium content and the proportion of calcium bound in the casein micelle. The total calcium content started at 125 mg/100g of milk in early lactation, then decreased to 122 mg/100g and 123 mg/100g before increasing again to 132 mg/100g and 131 mg/100g milk in the late lactation, in OAD and TAD milk, respectively. In both OAD and TAD milk, the highest proportion of bound calcium was found in mid-lactation (OAD: 57.4%, TAD: 57.5%, respectively). The lowest values were found in early lactation (OAD: 55.6%, TAD: 53.1%). The phosphorus content in the milk was affected by stage of lactation but not by milking frequency. In OAD and TAD milk, the lowest phosphorus concentration was found in mid-lactation. In OAD milk, the highest value was found in early lactation, while the highest value of phosphorus in TAD milk was found in late lactation. The percentage of the total phosphorus concentration in the serum was not significantly affected by either milking frequency or stage of lactation. The concentration of magnesium in the milk was also only affected by stage of lactation and not by milking frequency. In both milking systems, the total magnesium content in early and mid-lactation was 11.0-11.3 mg/100g. In late lactation, this value increased to 12.9 and 12.4 mg/100g in OAD and TAD milk, respectively. The majority of the magnesium in the milk was present in the serum, with 75.2-78.5% of total magnesium, which was not affected by milking frequency but decreased throughout lactation.

Both stage of lactation and milking frequency significantly affected the potassium concentration in the milk. OAD milk contained less potassium than TAD milk at each stage of lactation. In both milking systems, the potassium content decreased significantly in late lactation to 140 mg/100g in OAD milk and 157 mg/100g in TAD milk. The opposite was observed for the sodium content in the milk. Higher concentrations were found in OAD milk than in TAD milk, and the content increased in late lactation to 44.3 mg/100g and 34.3 mg/100g in OAD and TAD milk, respectively. Finally, OAD milk contained less chloride than TAD milk, and in both milking systems, the highest concentration was found in late lactation and the lowest in early lactation.

The iodine content in the milk was significantly affected by milking frequency but not by stage of lactation. OAD milk contained almost five times the amount of iodine as TAD milk. In TAD milk, the iodine concentration ranged from 1.4-2.2 μg/100g, while in OAD milk, the range was 7.8-9.6 μg/100g. Overall, the selenium content in the milk was lower in OAD than in TAD milk. Additionally, the selenium content in the milk increased significantly in late lactation in both milking systems, to 2.7 and 3.9 µg/100g in OAD and TAD milk, respectively. In OAD milk, the concentration of selenium decreased significantly in mid-lactation (0.7 µg/100g) compared to early lactation (1.5 µg/100g). In TAD milk, this concentration was similar in early (1.3 µg/100g) and mid-lactation (1.2 µg/100g). The zinc content in the milk did not differ between the milking frequency. However, there was a significant stage of lactation effect. Although the zinc concentration in OAD milk was significantly higher in late (0.45 mg/100g) than in early (0.38 mg/100g) and mid-lactation (0.33 mg/100g), there was no significant difference between the different stages of lactation in the TAD milking system. Finally, significant milking frequency ($P = 0.035$) and stage of lactation ($P < 0.001$) effects on the copper content were found. In OAD milk, the copper concentration decreased from 5.9 µg/100g in early to 2.1 µg/100g in late lactation. In TAD milk, these values ranged from 7.0 µg/100g in early to 2.5 µg/100g in late lactation.

Table 6.4. Estimated marginal means and standard errors of the mineral composition of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021 at different stages of lactation.

	OAD			TAD			*P-value*^{1}	
	Early	Mid	Late	Early	Mid	Late	S	M×S
Calcium (mg/100g)	125 ± 0.9^{b}	122 ± 2.4^{b}	132 ± 2.1^{a}	125 ± 1.2^{b}	123 ± 0.9^{b}	131 ± 3.8^{ab}	0.019	0.900
Bound calcium (%)	55.6 ± 1.82	57.4 ± 4.61	55.6 ± 0.42	53.1 ± 2.35	57.5 ± 1.73	57.1 ± 1.08	0.425	0.462
Serum calcium (%)	44.4 ± 1.82	42.6 ± 4.61	44.4 ± 0.42	46.9 ± 2.35	42.5 ± 1.73	42.9 ± 1.08	0.425	0.462
Ionic calcium (%)	8.9 ± 0.54	7.0 ± 1.21	8.9 ± 0.30	8.9 ± 0.40	7.1 ± 1.15	8.3 ± 0.48	0.156	0.815
Phosphorus (mg/100g)	106 ± 2.5^{a}	96 ± 1.5^{c}	102 ± 2.3^{abc}	101 ± 1.8^{ab}	100 ± 1.2^{bc}	106 ± 2.7^{ab}	0.008	0.100
Serum Phosphorus (%)	58.7 ± 2.18	53.1 ± 4.39	57.3 ± 1.99	58.8 ± 1.83	54.2 ± 1.83	54.4 ± 2.89	0.179	0.744
Magnesium (mg/100g)	11.3 ± 0.15^{b}	11.2 ± 0.07^{b}	12.9 ± 0.21^{a}	11.0 ± 0.10^{b}	11.1 ± 0.24^{b}	12.4 ± 0.22^{a}	< 0.001	0.644
Serum Magnesium (%)	77.6 ± 0.48^{ab}	76.3 ± 1.52^{ab}	75.2 ± 1.23^{b}	78.5 ± 0.53^{a}	76.0 ± 2.15^{ab}	75.9 ± 0.82^{b}	0.023	0.905
Potassium (mg/100g)	153 ± 3.0^{c}	157 ± 1.8^{c}	140 ± 2.7^{d}	168 ± 1.5^{b}	173 ± 0.9^{a}	157 ± 2.3^{c}	< 0.001	0.960
Sodium (mg/100g)	39 ± 0.3^{b}	37 ± 0.9^{c}	44 ± 0.3^{a}	30 ± 0.3^{d}	30 ± 0.9^{d}	34 ± 1.5^{c}	< 0.001	0.150
Chloride (mg/100g)	94 ± 2.4^{cd}	101 ± 1.7^{bc}	108 ± 1.0^{a}	92 ± 0.3^{d}	96 ± 2.1^{bd}	102 ± 2.6^{abc}	< 0.001	0.654
Copper (µg/100g)	5.9 ± 0.60^{a}	2.6 ± 0.19^{c}	2.1 ± 0.29^{c}	7.0 ± 0.80^{a}	3.5 ± 0.07^{b}	2.5 ± 0.10^{c}	< 0.001	0.359
Iodine (µg/100g)	7.8 ± 0.38^{a}	8.0 ± 0.31^{a}	9.6 ± 1.13^{a}	1.9 ± 0.18^{b}	2.2 ± 0.40^{bc}	1.4 ± 0.09^{c}	0.547	0.172
Selenium (µg/100g)	1.5 ± 0.25^{c}	0.7 ± 0.25^{d}	2.7 ± 0.25^{b}	1.3 ± 0.25^{cd}	1.2 ± 0.25^{cd}	3.9 ± 0.25^{a}	< 0.001	0.037
Zinc (mg/100g)	0.38 ± 0.007^{b}	0.33 ± 0.009^{c}	0.45 ± 0.023^{a}	0.37 ± 0.015^{bc}	0.34 ± 0.009^{c}	0.38 ± 0.032^{abc}	0.001	0.245

[1] M = milking frequency (OAD and TAD), S = stage of lactation (early, mid, and late), $^{a, b, c, d}$Means with different superscripts within one row are significantly different. ($P < 0.05$).

6.4.4 Physicochemical properties

The pH, fat globule size and casein micelle size are shown in Table 6.1 and Table 6.2. The pH is a good measure to check the quality of the milk, and a low pH can indicate fouling of the milk making it less suitable for processing. In the present study, the pH values of the mixed milk samples were all within the expected range of approximately 6.7. There was no milking frequency effect, but the pH increased throughout the lactation in both milking systems. The effects of the milking frequency and stage of lactation on the casein micelle size were significant. On average, across the lactation, the casein micelles were 5 nm smaller in OAD than in TAD milk. The average casein micelle size in raw milk ranged from 152 nm in mid-lactation to 154 nm in late lactation in OAD milk and from 153 nm in early lactation up to 164 nm in late lactation in TAD milk. The fat globule size decreased throughout the lactation, from 5.3 µm and 5.2 µm to 4.3 µm and 4.5 µm in OAD and TAD milk, respectively. There was no significant effect of milking frequency on the fat globule size.

6.5 Discussion

6.5.1 Major components

Less frequent milking increased protein and fat contents in agreement with previous research (Clark et al., 2006; Gedye et al., 2020; Martin et al., 2009; O'Brien et al., 2002; Rémond et al., 2004). The overall variation in the fat content (9.6%) was higher than for the protein content (8.0%), as shown by the relatively larger standard error of the means in Table 6.1, Chapter 4, and previous studies (Clark et al., 2006; Gedye et al., 2020; Martin et al., 2009). The milking frequency did not affect the lactose content in the milk. Some studies have found a decrease in the lactose content with decreased milking frequency (Clark et al., 2006; Gedye et al., 2020; O'Brien et al., 2002; Rémond et al., 2004), but not all (Rémond et al., 2004).

In late lactation, both protein and fat contents in the milk increased. Other studies also found the protein and fat contents to increase in late lactation (Auldist et al., 1998; Li et al., 2019; O'Brien and Guinee, 2016; Sorensen et al., 2008). The stage of lactation effect on the lactose content differed in each milking system, the lactose content decreased in OAD milk throughout the lactation but not in TAD milk despite a slight decrease (0.09%) from early to mid-lactation. Other researchers also found a decreasing lactational pattern for lactose (Li et al., 2019; O'Brien and Guinee, 2016; Sorensen et al., 2008).

6.5.1.1 Somatic cell count

The SCC was higher in OAD than in TAD milk. According to European and New Zealand regulations, any values below 400,000 cells/mL are acceptable for processing. High SCC values can indicate the presence of an infection in the udder, such as mastitis (Auldist, 2011). In this study, the SCC levels of TAD milk were exceptionally low (17,000 cells/mL), while the higher SCC levels of the OAD milk (109,000 cells/mL) are still considered good quality. It is also a determinant of milk quality since high values correlate with poor product quality after processing (Auldist, 2011).

Previous studies have reported increased SCC levels due to a lower milking frequency (Clark et al., 2006; Holmes et al., 1992; Lembeye, et al., 2016c; Stelwagen and Lacy-Hulbert, 1996), likely due to a concentrating effect of a lowered milk yield (Kelly et al., 1998) or damage to the secretory cells (Kitchen et al., 1980; Sorensen et al., 2008). Some studies did not report any changes in SCC levels due to a lower milking frequency (Lacy-Hulbert et al., 1999; O'Brien et al., 2002).

Others reported an increasing trend in the SCC levels throughout the lactation (Li et al., 2019; Sorensen et al., 2008). The stage of lactation effect was more evident in the OAD than in the TAD milking system.

6.5.2 Protein composition

The total casein content was higher, and the proportion of all major proteins (except for β-casein) differed in OAD milk compared to TAD milk. These results contrast with Chapter 4, where only κ-CN and α-LA were affected by milking frequency, and with previous studies that found decreased casein-to-whey ratios (Auldist and Prosser, 1998; Klei et al., 1997; Martin et al., 2009; O'Brien et al., 2002; Pomiès et al., 2007). Another study found no difference in the casein percentage of total protein between OAD and TAD milking (Rémond et al., 2004). The previously observed increase in the proportion of whey proteins has been attributed to an increased influx of serum proteins due to the weakening of tight junctions between the epithelial cells in the mammary glands (Auldist and Prosser, 1998; Davis et al., 1999). The same mechanism causes the decrease in the proportion of casein in the milk during the involution process in late lactation.

It is unclear why an increase in the casein-to-whey ratio was observed here. One potential reason is that the cows from the OAD milking production system (Massey University Dairy Farm No.1) have been selected over the years for this milking frequency based on their production and breeding potential. Previous studies often investigated short-term or part-season (e.g., in late lactation when milk production drops) milking frequency changes (Auldist and Prosser, 1998; Martin et al., 2009; O'Brien et al., 2002; Pomiès et al., 2007). It could be argued that, since the OAD milked cows have been milked at that frequency over the entire lactation in addition to being selected in the breeding process, their bodies have adapted to the OAD milking, and no severe involution occurred, which could increase the percentage of whey proteins. This finding is supported by the low SCC levels in OAD milk (Table 6.1).

The κ-CN content, the degree of κ-CN glycosylation and the α_{s2}-CN content were higher in OAD milk than in TAD milk. In contrast, proportions of α_{s1}-CN, β-LG and α-LA in the protein fraction were lower in OAD than in TAD milk. This is the first study looking at the effect of milking frequency on the protein composition in bovine milk. Other researchers have looked at the mammary expression genes (Alex et al., 2015; Murney et al., 2015), but it is unclear how these results translate to the protein profile of the milk.

In both milking systems, the casein percentage was the lowest in late lactation. In OAD milk, the casein percentage remained the same in early and mid-lactation, while in TAD milk, the casein content decreased throughout the entire lactation. The κ-CN content also changed differently throughout the lactation in each milking system. While in OAD milk, the κ-CN content was lower in mid- than in early and late lactation, the κ-CN content in TAD milk decreases throughout the lactation. Some studies also found a decrease in the proportion of casein (Ng-Kwai-Hang et al., 1982), increased or decreased κ-CN content (Li et al., 2019; O'Connell et al., 2017; Ostersen et al., 1997) in the milk, while others did not find a stage of lactation effect on these variables (Auldist et al., 1998; Barry and Donnelly, 1980; Coulon et al., 1998; Li et al., 2019). The degree of κ-CN glycosylation increased throughout the lactation in both milking systems, but the increase was greater in OAD than in TAD milk. This stage of lactation effect was also found by other researchers (Bonfatti et al., 2014; Li et al., 2019; Robitaille et al., 1991). The mechanism behind this stage of lactation effect on the κ-CN glycosylation is unknown.

Decreasing α-LA content with stage of lactation in both milking systems might be ascribed to the role of α-LA in lactose production. Lactose regulates milk volume by creating osmotic pressure between the blood and alveoli (Strucken et al., 2015; Zhao and Keating, 2007). Therefore, the downregulation of milk production when milking frequency is reduced might explain this result. However, an increase in α-LA after 7 and 15 days of decreased milking frequency was also found (Kelly et al., 1998).

6.5.3 Casein micelle size

The casein micelle size in OAD milk was smaller than in TAD milk. No other studies have examined the effect of milking frequency on the casein micelle size. The smaller casein micelle size could be a result of the higher κ-CN content (Akkerman et al., 2021; Devold et al., 2000; Donnelly et al., 1984) since κ-casein is present on the outside of the casein micelle.

In the present study, the casein micelle size increased with stage of lactation by approximately 2 nm (OAD) and 11 nm (TAD). A few studies reported no change in casein micelle size across different stages of lactation (Bijl et al., 2014; De Kruif and Huppertz, 2012; Li et al., 2019), while Fleming et al. (2019) showed a downward slope in the micelle size in late lactation. The size of casein micelles has previously been shown to impact gel strength (Glantz et al., 2010; Jørgensen et al., 2017), cheese production properties (Visentin et al., 2017) and storage stability of UHT-treated milk (Akkerman et al., 2021).

6.5.4 Mineral composition

6.5.4.1 Calcium, magnesium, and phosphorus

The calcium, phosphorus and magnesium contents did not differ between OAD and TAD milk, nor did their proportions in serum. Martin et al. (2009) found no change in the calcium content upon temporarily reducing the milking frequency from TAD to OAD. They found a higher phosphorus content in OAD compared to TAD milk. Calcium, phosphorus, and magnesium are partially associated with the casein micelles and are crucial for casein micelle integrity (Gaucheron, 2005). Calcium is the most abundant mineral in milk and contributes to the nutritional value of dairy. The calcium content and distribution are also crucial for protein stability and interactions during processing. Environmental factors such as pH and temperature impact the distribution of calcium, phosphorus, and magnesium between the colloidal and serum phases (Gaucheron, 2005).

Calcium, phosphorus, and magnesium contents were all affected by stage of lactation, similarly in the OAD and TAD milking systems. In late lactation, the calcium content, like the protein content, was highest in both milking systems, which aligns with results from previous studies (Fleming et al., 2019; Gaucheron, 2005; van Hulzen et al., 2009). The lower phosphorus content in mid- than in early lactation in OAD milk, but not in TAD milk, may be due to the lower protein content in OAD milk in mid-lactation. Magnesium content did not change in early and mid-lactation but increased in late lactation. This observation was also found by Auldist et al. (1996), but Gaucheron (2005) and White and Davies (1958) found the highest magnesium content in early lactation.

6.5.4.2 Sodium, potassium, and chloride

The OAD milk contained higher proportions of sodium and chloride than TAD milk, while the opposite was observed for potassium, in agreement with other studies (Davis et al., 1999; De Villiers, 1976). Such changes might be due to weakened tight junctions between epithelial cells of the mammary glands resulting in impaired regulation of their concentrations (Linzell and Peaker, 1971; Stelwagen, Davis, Farr, Eichler, et al., 1994; Stelwagen, Davis, Farr, Prosser, et al., 1994). As discussed earlier, it has been suggested that with a reduced milking frequency, the involution process may be enhanced, reducing the number and activity of the epithelial cells in milk synthesis.

Additionally, the potassium content increased, and sodium and chloride contents decreased in late lactation, which has also been found by other studies (Auldist et al., 1995). These changes can be explained by the same involution phenomenon as described above.

6.5.4.3 Trace minerals

Besides zinc, milking frequency affected all trace minerals (copper, iodine, and selenium) analysed in this study. Copper and selenium contents were lower in OAD than in TAD milk, while the iodine content was higher in OAD than in TAD milk. Selenium and iodine concentrations strongly depend on dietary intake and can be increased by dietary supplementation with these components (Gaucheron, 2013).

The selenium content in milk varies depending on diet, season, and location of the farm (Mehdi and Dufrasne, 2016; Tinggi, 2001). Location and between-farm differences are also important factors affecting the iodine concentration in the milk (Flachowsky et al., 2014). Iodine concentrations are also affected by using iodine-containing cleaning products and teat sprays (French et al., 2016; Gaucheron, 2013). At Dairy No.1 (OAD milking), iodine containing teat spray was used after milking. The diet has little or no effect on the zinc and copper concentrations in the milk (Ianni et al., 2019).

In the present study, copper concentration was higher in early lactation than in mid- and late lactation for both milking systems, which agrees with the results of another study (De Maria and Angelucci, 1978). In OAD milk, zinc concentration decreased between early and mid-lactation, aligning with findings from a previous study (De Maria and

Angelucci, 1978), and increased in late lactation. However, the concentration did not change significantly in TAD milk between stage of lactation .

Stage of lactation did not affect the iodine concentration in the milk. The effect of the stage of lactation on the iodine content in the milk is inconsistent across the literature (Flachowsky et al., 2014; Franke et al., 1983). Cressey (2003) looked at the iodine content of different dairy products in New Zealand. Standard and trim milk sampled in winter contained higher iodine levels than in spring. Such seasonal variation was attributed to, firstly, higher amounts of supplementary feeding and, secondly, to a concentrating effect of decreased milk yield. In the present study, the OAD milking system supplied relatively less concentrates to the cow, but the decreased milk yield could partly explain the increased iodine levels in OAD milk compared to TAD milk. Finally, although the farms in the present study are near each other, differences in location and diets in each farm system differ, which may have impacted the trace mineral concentrations in the milk.

6.5.5 Fat globule size

Milking frequency did not affect, but stage of lactation tended to decrease the fat globule size. Li et al. (2019) also found this stage of lactation effect in a study in New Zealand with cows milked TAD. Wiking et al. (2006) also reported smaller fat globule sizes in milk from udder halve that were milked two times compared to four times daily. Other studies reported no effects of milking frequency on milk fat globule size (Abeni et al., 2005; Komara et al., 2009). Fat globule size has been positively correlated with fat yield (Fleming et al., 2017; Wiking et al., 2004). With increased fat synthesis, relatively less membrane material is available to cover the fat globules (Wiking et al., 2004). The milk fat globule size impacts gel structure during rennet gelation and thus cheese quality (Logan et al., 2015; Michalski et al., 2004).

6.6 Conclusion

This study showed the implications of OAD milking on the proximate protein and mineral composition of milk pooled in the ratio of the relative yields from each cow which offers more realistic insights into the consequences of OAD milking for dairy processing.

Overall, milking frequency increased the protein and fat contents and SCC levels of milk in OAD compared to TAD milk. The total casein content, κ-CN content, the degree of κ-CN glycosylation and the α_{s2}-CN content were higher, while the contents of α_{s1}-CN, β-LG and α-LA in the protein fraction were lower in OAD than in TAD milk. The calcium, magnesium and phosphorus contents and their distribution in milk were unaffected by milking frequency. In contrast, the sodium and chloride contents were lower, and the potassium content was higher in OAD milk. No milking frequency effect was observed for the fat globule size, but the casein micelle size was smaller in OAD than in TAD milk.

The fat and protein content, SCC, κ-CN glycosylation degree, and contents of calcium, magnesium, potassium, and copper increased throughout the lactation, while the casein percentage, α-LA, sodium, and chloride contents decreased in late lactation, in both milking frequencies. The lactose, κ-CN and phosphorus contents were all affected by stage of lactation differently in a OAD milking system than in a TAD milking system.

The reported changes in the protein profile and the casein micelle size can impact heat stability and gelation properties of OAD milking important for dairy processing. This chapter lays the foundation for the following two chapters that compare the heating and gelation properties of OAD and TAD milk.

Chapter 7

Heat-induced changes in milk from a once-a-day and a twice-a-day milking system

Manuscript in preparation.

van der Zeijden, M., Ellis, A., Lopez-Villalobos, N., Li, S., Roy, N., McNabb, W. Heat-induced changes, rennet, and acid gelation properties of milk from a once-a-day and a twice-a-day milking system. *Dairy.*

7.1 Abstract

The composition of milk and the protein fraction in milk are important factors in heat-induced changes to proteins. Heating is applied during dairy processing to ensure safety for consumption and extend shelf-life, but also to modify the characteristics of the final product. With an increasing number of farmers in New Zealand changing from a twice-a-day (TAD) milking system to a once-a-day (OAD) milking system, a need to understand the implications of this change for processing arises. This chapter investigated heat-induced changes to proteins in milk from a OAD milking system compared to milk from a TAD milking system. Milk was sampled from individual cows and mixed to create a mixed milk sample similar to tank milk. This milk was pasteurised at 72 °C for 15 seconds, heated at 95 °C for 5 minutes, or ultra-high temperature (UHT) treated at 140 °C for 5 seconds. The percentages of serum phase caseins (α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, and glycosylated κ-casein), the degree of whey protein denaturation and interaction between whey proteins and casein micelles were analysed. In raw and pasteurised OAD milk, higher proportions of κ-casein and α_{s2}-casein were present in the serum phase compared to TAD milk, but no milking frequency effect was found after heat treatment at higher temperatures. A significant amount of κ-casein dissociated from the casein micelle during these higher temperature treatments, especially in UHT milk. Additionally, in UHT milk from a OAD milking system, the degree of whey protein denaturation was lower than in TAD milk. No difference between milking frequencies was found with other heat treatments. The difference in the degree of denaturation was driven by a lower degree of α-lactalbumin denaturation. The interaction between whey and casein proteins was affected by the type of heat treatment, the type of protein (α-lactalbumin or β-lactoglobulin), the milking frequency and the stage of lactation. In milk heated at 95 °C, a higher proportion of whey proteins had associated with the casein

micelle than in UHT milk. In milk from a OAD milking system, a higher proportion of whey proteins had formed aggregated in the serum phase, likely due to the higher percentage of serum κ-casein. Finally, the casein micelle size was smaller in OAD than in TAD milk overall and became larger with increasing heating temperature.

7.2 Introduction

Milk is heated to assure food safety firstly, but also to increase shelf life and modify product properties. Heat treatments vary greatly in time and temperature, depending on the purpose of the treatment. Pasteurisation is one of the most common processing steps and is aimed at killing pathogens, thereby extending the shelf-life of milk up to 21 days when refrigerated (Moatsou, 2013). Ultra-high temperature (UHT) treatments are used to make dairy products stable for storage at ambient temperatures (Moatsou, 2013). Another heating step commonly used in the industry involves heating at 80-95 °C for up to 30 minutes to improve acid gelation properties in yoghurt production (Lucey and Singh, 1997; Udabage et al., 2010; Walstra et al., 2005).

When milk is heated to temperatures above 70 °C, whey proteins denature, and irreversible aggregation with other whey proteins and caseins occurs (Walstra et al., 2005; Wijayanti et al., 2014). β-lactoglobulin (β-LG) and κ-casein (κ-CN) are most involved in the formation of these complexes (Anema, 2008c). To a minor extent, α-lactalbumin (α-LA) and α_{s2}-casein (α_{s2}-CN) are also included in aggregates (Donato and Guyomarc'h, 2009). However, the ratio between these proteins, as well as other compositional factors, impact the extent and location of this complex formation (Oldfield et al., 2005). Stage of lactation was previously found to not only affect milk and protein composition, but also the whey protein-casein micelle interaction and casein micelle size after heat treatment (Li et al., 2019).

In New Zealand, approximately 90% of farmers use a twice-a-day (TAD) milking system for at least part of the year. An increasing number of farmers have adopted a once-a-day (OAD) milking system for various reasons, including but not limited to, the lack of quality long term staff and the changing lifestyle expectations (Bewsell et al., 2008; Kendall et al., 2008; Stelwagen et al., 2013). To date, most studies on the OAD milking system have focused on changes in milk yield and the protein and fat contents. Some research has looked at the concentration of minor components in the milk. No studies have examined the consequences of OAD milking for heat-induced changes in the milk, which are crucial for shelf-life stability and product properties (Anema, 2008c; Gaur et al., 2018; Lucey et al., 1998).

Chapter 6 looked at the proximate, protein and mineral composition in milk from a OAD milking system and milk from a TAD milking system. These are all important factors in determining the course of heat-induced changes in the milk. OAD milk had higher protein, fat, and casein contents than TAD milk. Additionally, all proteins, except β-CN, were affected by the milking frequency. Higher proportions of κ-CN and α_{s2}-CN, and higher degree of κ-CN glycosylation were found in OAD milk, along with a decrease in the proportions of α_{s1}-CN, β-LG, and α-LA. This variation in the protein composition potentially affects the processability of the milk into dairy products. Heat treatment is one of the most common processes used in the dairy industry and the stability of the milk during heating is impacted by protein and mineral composition.

The aim of this chapter was to explore the impact of OAD milking on the heating properties of bovine milk by studying the casein dissociation, and denaturation and aggregation behaviour of whey proteins in the milk.

7.3 Methods

A detailed description of the analysis methodologies is given in Chapter 3. Below a summary of each method is provided.

7.3.1 Preparation of mixed milk samples

Milk was sampled three times in early, mid-, and late lactation from individual cows that met the study criteria described in Chapters 3 and 4. Only milk from Holstein-Friesian (F) and Holstein-Friesian×Jersey crossbred (F×J) cows were used as the Jersey cows from one of the farms did not strictly meet the parity criteria for the study. Three cows from each breed on each farm were selected. The yield of milk from each animal was recorded on farm by weight. Pooled milk was used for further characterisation; this mixed milk sample was created as follows.

In the laboratory, the milk was mixed proportionally to the yields of each cow. The average proportion of milk from F cows was 59.9% and 54.1% in OAD and TAD milk, respectively. For F×J cows, the average proportion of milk from this breed in the mixed milk sample was 40.1% and 45.9% in OAD and TAD milk, respectively. The milk was mixed within hours of the collection and stored at 4 °C until used for processing. The heat treatments were performed in the FoodPilot (Massey University, Palmerston North, New Zealand) within 36 hours of collection.

7.3.2 Heat treatment and sample preparation

All milk was pasteurised (72 °C, 15 seconds) and skimmed using a centrifugal separator (Model 103 AE, Alfa-Laval, Lund, Sweden) at 50 °C before further heat treatments. Part of the skim milk was used for analysis of pasteurised milk whereas the rest was further heated at either 95 °C for 5 minutes or UHT treated at 140 °C for 5 seconds.

Raw milk was skimmed in the laboratory by centrifugation at 4,000g for 15 minutes at 4 °C followed by careful removal of the fat layer. The skim milk was used for the analysis of protein composition and distribution, and casein micelle size.

7.3.3 Milk fractionation

7.3.3.1 Serum fractionation

Milk serum from raw and heated milks was obtained by ultracentrifugation of skim milk at 63,000g for 1 hour at 20 °C. The serum phase was removed carefully by pipetting and stored frozen at -20 °C until protein composition analysis.

7.3.3.2 Acid precipitation of native whey

The native whey protein fraction in the milk was isolated using acid precipitation according to a method described by Vasbinder and de Kruif (2003).

7.3.4 Characterisation

7.3.4.1 Milk composition

The proximate composition, including protein, fat, lactose, and casein content, was analysed using the MilkoScan FT1 (Foss Analytics, Hillerød, Denmark). The pH was measured using an Edge Blu pH meter (Hanna Instruments, Woonsocket, RI, USA).

7.3.4.2 Somatic cell count

The somatic cell count of each mixed milk sample was determined by MilkTestNZ (Hamilton, New Zealand) using the CombiFoss (Foss Analytics, Hillerød, Denmark).

7.3.4.3 Protein composition

The protein composition of skim milk samples, serum samples and native whey isolates was analysed using a high-performance liquid chromatography (HPLC) method modified from the method by Bobe et al. (1998). The method is detailed in Chapter 3.

7.3.4.4 Mineral composition and ionic calcium

The mineral composition of raw skim milk and serum was determined by Hill Laboratories (Hamilton, New Zealand). The ionic calcium was determined using a calcium electrode (Orion 9720BNWP Thermo Scientific, Singapore) together with a CyberScan pH 510 pH/mV Meter (Eutech Instruments-Thermo Scientific, Singapore). The electrode was calibrated using 1-10 mM of $CaCl_2$, of which the ionic strengths were adjusted to 80 mM with potassium chloride, as described by (Li et al., 2019).

7.3.4.5 Casein micelle size

The casein micelle size was determined using the Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). Skim milk samples were diluted 50x in an imidazole buffer and filtered before analysis. The measurement was performed at 25 °C, each measurement was performed in triplicate, each consisting of 13 sub-measurements.

7.3.4.6 Heat-induced protein denaturation and aggregation

The protein composition results of raw and heated skim milk, serum phase and native whey isolate samples were used to determine the degree of denaturation of whey protein, proportion of individual caseins in the serum, proportion of whey proteins associated with the casein micelles and the proportion of whey protein forming aggregates in the serum as described below.

The proportion of individual casein in the serum was calculated by dividing the peak area of a specific protein in the serum by the peak area of the same protein in the skim milk sample. The degree of denaturation was determined using the peak area of α-LA and β-LG in the native whey isolates from heated milk samples and compared to the native whey isolate sample from raw milk.

$$\text{Degree of denaturation} = 1 - \frac{(\text{peak area of whey protein in native whey isolate in lheated milk})}{(\text{peak area of whey protein in native whey isolate in raw milk})} \times 100\%$$

The proportion of whey protein associated with the casein micelles was calculated by taking the difference in the peak area of a protein in the milk serum samples between the raw and heated samples divided by the peak area of that protein in the raw milk serum sample, multiplied by 100% to obtain a percentage. The proportion of whey protein forming aggregates in the serum was calculated by subtracting the proportion of whey proteins associated with casein micelles from the degree of denaturation.

7.3.5 Statistical analysis

Marginal means of the dependent variables for each level of milking frequency (OAD and TAD), stage of lactation (early, mid, and late) and combinations of milking frequency and stage of lactation were obtained using the MIXED procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) with the following linear model:

$$y_{ijk} = \mu + M_i + S_j + H + MSH_{ijk} + e_{ijkl}$$

Y_{ijk} is the observation for the trait for milking frequency i and lactation stage j

μ is the population mean

M_i is the fixed effect of milking frequency i (i = OAD and TAD)

S_j is the fixed effect of stage of lactation j (j = early, mid, and late)

H_k is the fixed effect of heat treatment k (k = pasteurisation, 95 °C heat treatment and UHT)

MSH_{ijk} if the fixed effect of interaction between milking frequency i, stage of lactation j and heat treatment k

e_{ijkl} is the residual random error associated with observation y_{ijkl}.

The residuals were modelled with heterogeneous variance across the combinations of milking frequency and stage of lactation. Significant differences were declared at $P < 0.05$ and trends at $P < 0.10$.

7.4 Results

7.4.1 Milk composition

The proximate, protein and mineral composition, pH and casein micelle size are shown in Table 7.1. These results are discussed in more detail in Chapter 6. In summary, the protein and fat contents were both higher in OAD than in TAD milk. All major proteins, except for β-CN, were significantly affected by the milking frequency. The calcium, magnesium and phosphorus concentrations and distribution were similar in OAD and in TAD milk. Finally, the casein micelle size was smaller in OAD than in TAD milk.

Table 7.1. Estimated marginal means and standard errors of proximate, protein and mineral composition and casein micelle size of milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

	OAD	TAD	M[1] effect (*P-value*)
Protein (%)	3.7 ± 0.03	3.6 ± 0.05	0.025
Fat (%)	4.8 ± 0.09	4.5 ± 0.13	0.050
Lactose (%)	4.5 ± 0.02	4.6 ± 0.02	0.137
pH	6.70 ± 0.012	6.69 ± 0.010	0.437
Casein micelle size (nm)	153 ± 0.5	159 ± 1.6	0.008
Total casein (%)	82.1 ± 0.24	80.1 ± 0.16	< 0.001
α_{s1}-casein (%)	22.5 ± 0.14	23.1 ± 0.11	< 0.001
α_{s2}-casein (%)	6.7 ± 0.15	4.9 ± 0.06	0.012
β-casein (%)	38.4 ± 0.21	38.6 ± 0.15	< 0.001
κ-casein (%)	14.5 ± 0.05	13.5 ± 0.10	0.497
Gly-κ-casein[2] (% of κ-casein)	46.2 ± 0.32	42.5 ± 0.38	< 0.001
Total whey (%)	17.9 ± 0.24	19.9 ± 0.16	< 0.001
β-lactoglobulin (%)	15.1 ± 0.19	16.7 ± 0.16	< 0.001
α-lactalbumin (%)	2.8 ± 0.07	3.2 ± 0.04	< 0.001
Calcium (mg/100g)	56.2 ± 1.66	55.9 ± 1.04	1.000
Bound calcium (%)	43.8 ± 1.66	44.1 ± 1.04	0.888
Serum calcium (%)	8.3 ± 0.45	8.1 ± 0.44	0.888
Ionic calcium (%)	101 ± 1.2	102 ± 1.2	0.772
Phosphorus (mg/100g)	56.4 ± 1.77	55.8 ± 1.29	0.611
Serum phosphorus (%)	11.8 ± 0.09	11.5 ± 0.11	0.808
Magnesium (mg/100g)	76.3 ± 0.67	76.8 ± 0.79	0.068
Serum magnesium (%)	56.2 ± 1.66	55.9 ± 1.04	0.680

[1]M = milking frequency, [2]Gly-κ-CN = Glycosylated κ-casein.

7.4.2 Serum phase caseins

The majority of the caseins are present in the casein micelle and the remaining caseins are present in the serum phase. The proportions of κ-CN, and α_{s2}-CN in the serum phase were significantly affected by the milking frequency. The distribution of these proteins, as well as of β-CN and the glycosylation degree of κ-CN, were also affected by the heat treatment applied to the milk.

7.4.2.1 κ-casein

The proportion of κ-CN in the serum is shown in Figure 7.1. There was a significant effect ($P = 0.009$) of milking frequency on the proportion of κ-CN in the serum phase. In raw OAD milk, relatively more κ-CN (5.8 – 8.9% of total κ-CN) was present in the serum phase than in raw TAD milk (3.2 – 4.5%), in each stage of lactation. At both milking frequencies, there was an increasing trend of serum κ-CN throughout the lactation. In pasteurised milk, the proportion of κ-CN in the serum phase did not change significantly compared to raw milk. A similar difference between OAD and TAD milk was observed (OAD > TAD) across all stages of lactation. Heat treatment at 95 °C for 5 minutes caused a significant dissociation of κ-CN into the serum. After this treatment, OAD and TAD milk did not differ significantly anymore. At both milking frequencies, the proportion of κ-CN in the serum phase of milk heated at 95 °C was lower in early (OAD, 10.9%; TAD, 8.9%) than in mid- (OAD, 13.8%; TAD, 15.4%) and late (OAD, 14.7%; TAD, 12.3%) lactation. The most pronounced κ-CN dissociation occurred during the UHT treatment, reaching 29-41% of κ-CN in the serum. Only in late lactation, the difference between OAD and TAD was significant ($P = 0.045$, OAD > TAD). In contrast with other heat treatments and raw milk, the serum κ-CN in UHT milk followed a decreasing trend throughout the lactation. Less κ-CN had dissociated from the casein micelles in late lactation during UHT treatment than in early lactation.

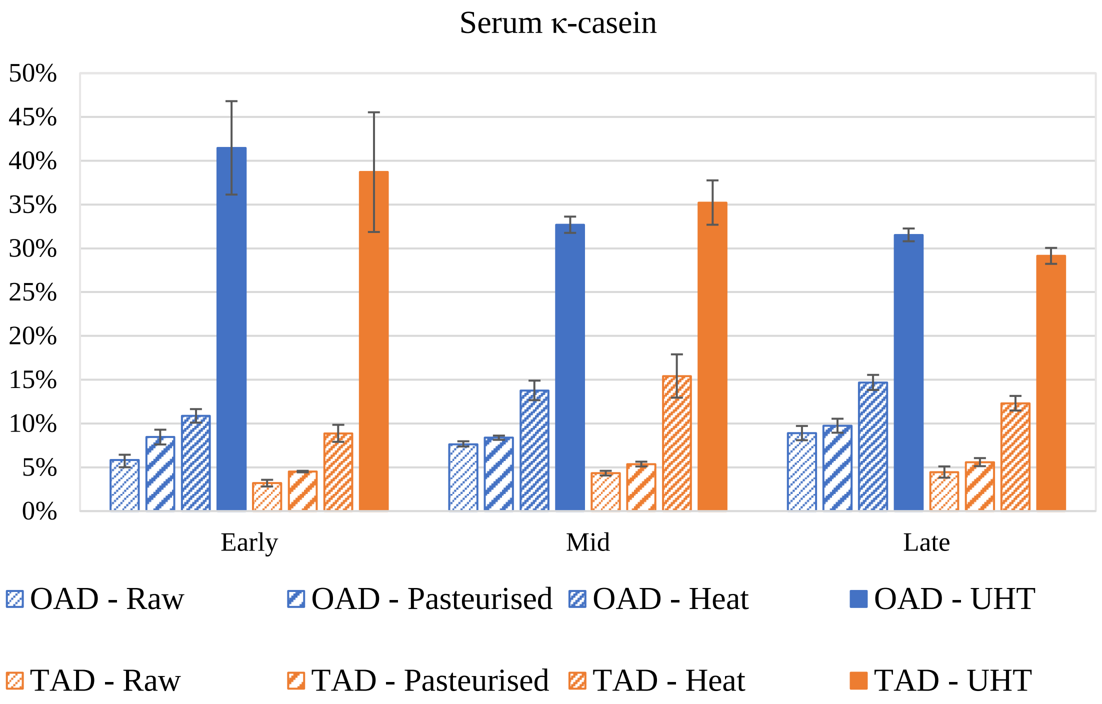


Figure 7.1. The proportion of κ-casein in the serum phase in raw, pasteurised (72 °C, 15 seconds), heated (95 °C, 5 minutes) and UHT (140 °C, 5 seconds) treated milk from cows milked once-a-day (OAD) and twice-a-day (TAD) in early, mid-, and late lactation, collected during the 2020-2021 milking season.

Figure 7.2 shows the proportion of glycosylated κ-CN in the serum phase in milk from a OAD milking system and a TAD milking system before and after various heat treatments at different stages of lactation. A trend, similar to that of serum κ-CN, throughout lactation and during processing was observed. In raw and pasteurised milk, the proportion of glycosylated κ-CN was below 5% and practically zero from mid-lactation onwards. Milking frequency did not significantly affect the distribution, but the stage of lactation did, depending on the type of process. In raw and pasteurised milk, the distribution did not significantly change throughout the lactation. In TAD milk heated at 95 °C, the proportion of serum glycosylated κ-CN increased significantly from early to mid-lactation. A similar trend was observed in OAD milk, but no significant differences were found here. As with serum κ-CN, a significant amount dissociated during UHT treatment, which decreased slowly throughout the lactation. The largest proportion of glycosylated κ-CN in the serum was found in UHT milk in early lactation, 48.5% and 44.5% in OAD and TAD milk respectively.

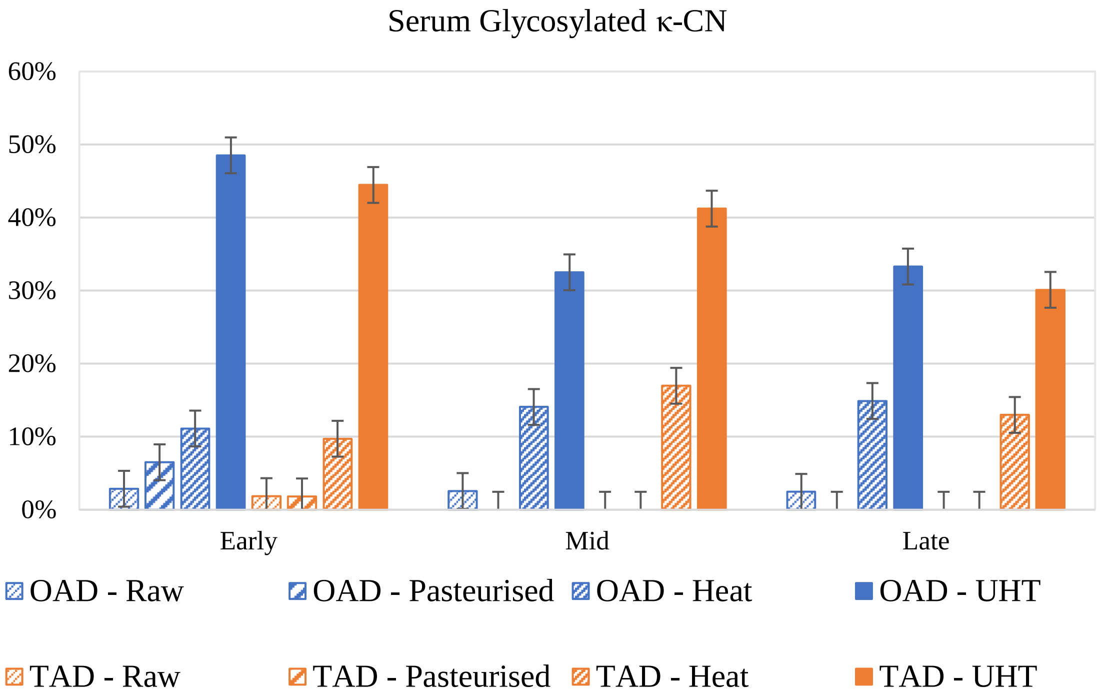


Figure 7.2. The proportion of glycosylated κ-casein in the serum phase in raw, pasteurised (72 °C, 15 seconds), heated (95 °C, 5 minutes) and UHT (140 °C, 5 seconds) treated milk from cows milked once-a-day (OAD) and twice-a-day (TAD) in early, mid-, and late lactation, during the 2020-2021 milking season.

7.4.2.2 α_{s2}-casein

Serum α_{s2}-CN in raw and heated milk throughout the season are shown in Figure 7.3. Similar to κ-CN, of α_{s2}-CN, there was a significantly higher proportion present in the serum phase in OAD than in TAD milk. In raw OAD milk, the proportion of serum α_{s2}-CN ranged from 34.7% in early to 44.7% in late lactation. In raw TAD milk, these values increased from 16.4% in early to 23.1% in late lactation, which is almost half of what was found in OAD milk. Both OAD and TAD milk, however, follow the same upward trend throughout the lactation which is consistent after heat treatments. After each heat treatment, OAD remained to contain a higher proportion of α_{s2}-CN in the serum than TAD milk. In OAD milk, serum α_{s2}-CN decreased after heat treatment. In TAD milk, there only is a small (1%) but significant difference between raw and pasteurised milk in mid-lactation.

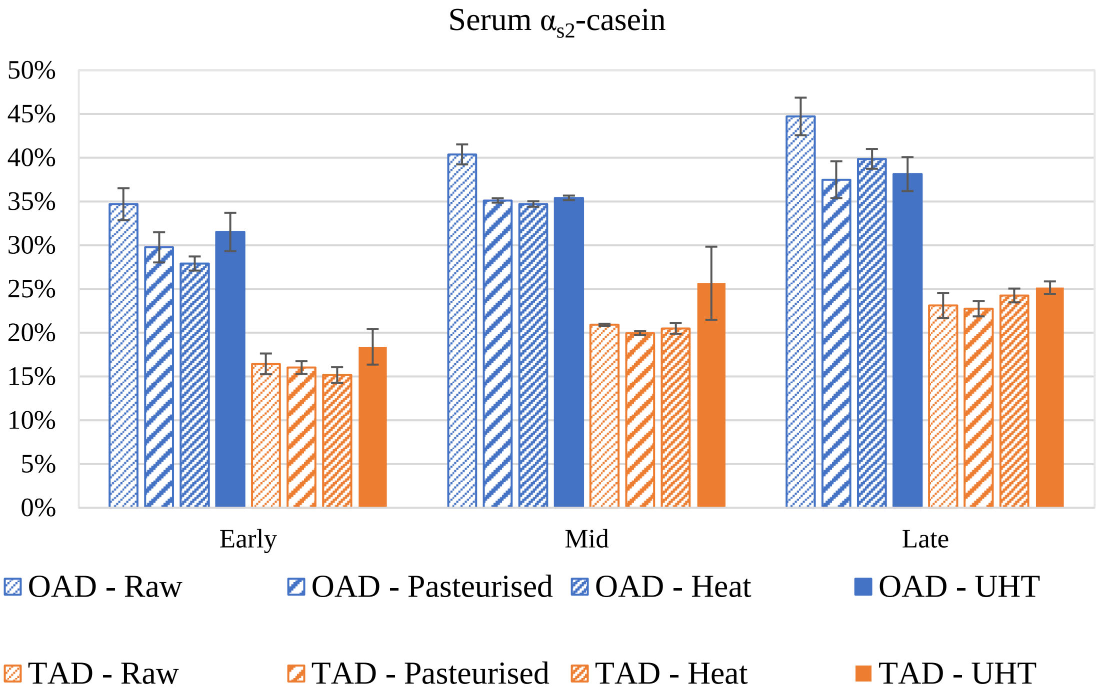


Figure 7.3. The proportion of α_{s2}-casein in the serum phase in raw, pasteurised (72 °C, 15 seconds), heated (95 °C, 5 minutes) and UHT (140 °C, 5 seconds) treated milk from cows milked once-a-day (OAD) and twice-a-day (TAD) in early, mid-, and late lactation, collected during the 2020-2021 milking season .

7.4.2.3 *β-casein*

The proportion of serum β-CN (Figure 7.4) in the milk did not change during pasteurisation or heat treatment at 95 °C for 5 min, but increased significantly in UHT milk in both milking frequencies reaching up to almost five times the amount in raw milk in early lactation. There was no milking frequency effect on the serum β-CN, nor was there a stage of lactation effect.

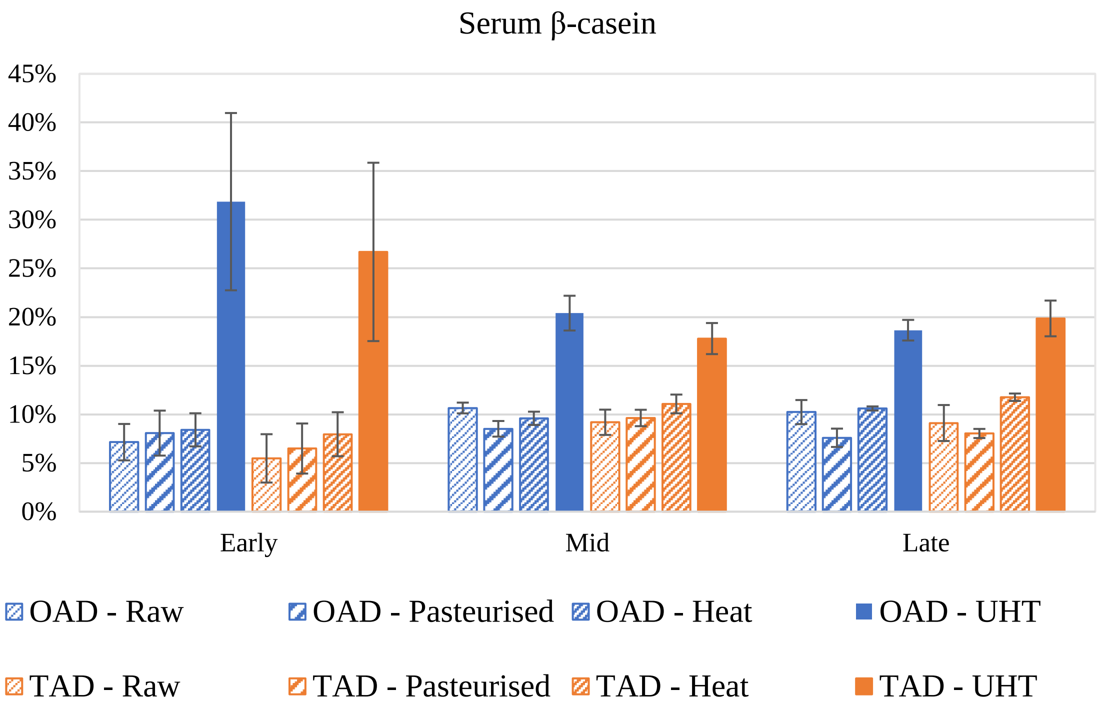


Figure 7.4. The proportion of β-casein in the serum phase in raw, pasteurised (72 °C, 15 seconds), heated (95 °C, 5 minutes) and UHT (140 °C, 5 seconds) treated milk from cows milked once-a-day (OAD) and twice-a-day (TAD) in early, mid-, and late lactation, collected during the 2020-2021 milking season.

7.4.3 Whey protein denaturation

The denaturation degree was calculated by dividing the native whey peak areas in heated milk by the peak areas of native whey in raw milk and subtracting the obtained value from 100%. The degree of denaturation in the pasteurised, heat (95 °C, 5 minutes) and UHT treated milks is shown in Figure 7.5. The lowest level of denaturation was found in pasteurised milk as this is the least intense heat treatment of the three. In pasteurised milk the denaturation degree did not differ significantly between OAD and TAD milk. There was a stage of lactation effect with the lowest degree of denaturation in early lactation at 16.5% and 10.9% in OAD and TAD milk, respectively. The highest degree of denaturation was found in mid-lactation with 35.1% and 32.5%, in OAD and TAD milk, respectively. The denaturation degree increases to at least 95% in milk heated at 95 °C. No effect of milking frequency or stage of lactation was found at this heat treatment. In contrast, in UHT milk a significant difference ($P < 0.01$) between OAD and TAD milk was found. The degree of denaturation following UHT treatment in OAD milk was lower than in TAD milk throughout the whole lactation. The denaturation degree increased from 93.5% in early lactation to 94.2% in late lactation in OAD milk. In TAD milk, these values ranged from 96.2% in early and mid-lactation to 96.5% in late lactation.

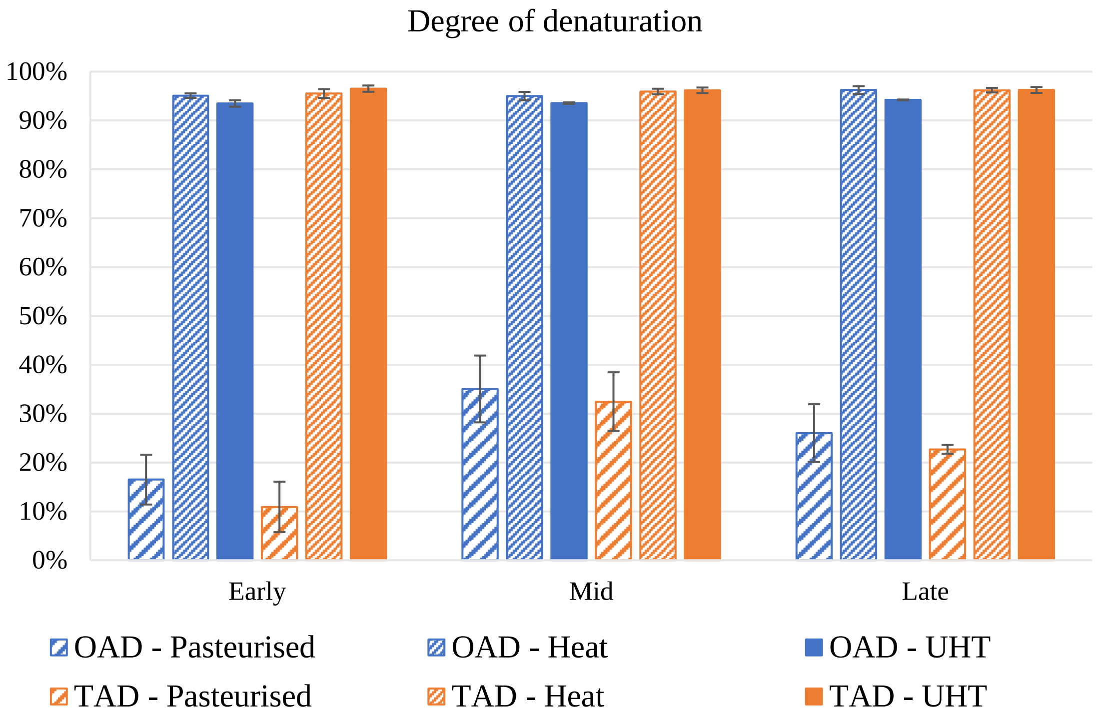


Figure 7.5. Degree of denaturation of whey proteins in pasteurised, heat (95 °C, 5 minutes) and UHT (140 °C, 5 seconds) treated milks from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021 in early, mid-, and late lactation.

7.4.4 Whey protein-casein micelle association

Denatured whey proteins either form aggregates in the serum with other whey proteins or caseins, or they associate with the casein micelles. Figure 7.6 Shows the whey protein distribution in milk heated at 95 °C (5 minutes) and in UHT milk, respectively. There was no significant milking frequency effect, but there were significant changes throughout the season and between heat treatments. In heated milk (95 °C, 5 minutes), 77.4% to 87.2% of whey proteins were associated with the casein micelle after heating. At both milking frequencies, the seasonal effect was similar with the highest amount of whey-casein micelle association observed in late lactation. Casein micelle association was less favoured during UHT treatment. In UHT milk, 63% to 81% of whey proteins were associated with the casein micelle. This proportion was highest in late lactation and lowest in early lactation at both milking frequencies.

Opposite trends were observed for the formation of serum aggregates of whey protein. Again, no significant milking frequency effect was found. In heated milk (95 °C, 5 minutes), the proportion of whey proteins forming aggregates in the serum phase ranged from 9.0% to 18.5%. In both the OAD and TAD milking systems, the highest values were observed in mid-lactation and the lowest values in late lactation. In UHT milk, 15.4% to 30.1% of whey proteins formed aggregates in the serum. A decreasing seasonal trend was found with the lowest values in late lactation in both milking systems.

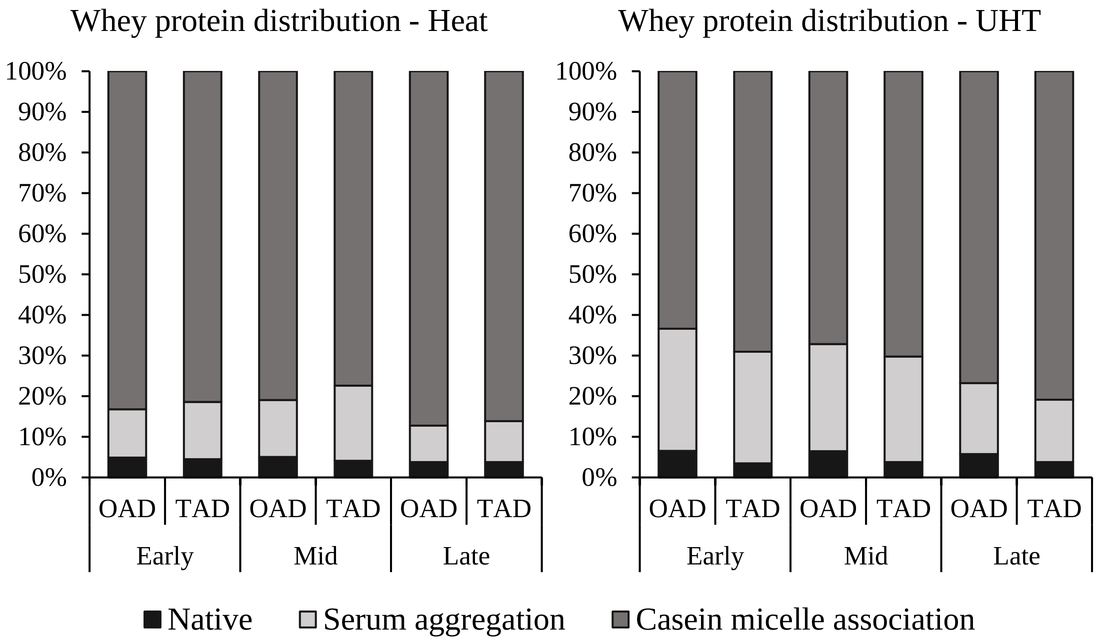


Figure 7.6. Whey protein distribution in heated milk (95 °C, 5 minutes; left) and UHT treated milk (140 °C, 5 seconds; right) from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021 in early, mid-, and late lactation.

7.4.5 Heat-induced changes to α-lactalbumin and β-lactoglobulin

Figure 7.7 shows the distribution of α-LA and β-LG in OAD and TAD milk after heat treatment at 95 °C for 5 minutes. In the 95 °C heat-treated milk, a larger proportion of the α-LA remains in its native state than β-LG for both OAD and TAD systems. Only in mid-lactation, the degree of denaturation for α-LA was higher in TAD than in OAD milk. In addition to the higher degree of denaturation of β-LG compared to α-LA, the aggregation behaviour of β-LG and α-LA were different. The proportion of protein that associated with the casein micelle was lower for α-LA than for β-LG. Both for α-LA and β-LG, a similar stage of lactation effect was observed in OAD and in TAD milk for both the casein micelle association and serum aggregation. In late lactation, the proportion of whey protein associated with the casein micelle increased and the proportion of whey protein forming aggregates in the serum decreased.

Figure 7.7 shows the distribution of α-LA and β-LG in OAD and TAD milk after a UHT treatment at different stage of lactation. While in OAD milk, the degree of denaturation of α-LA was lower in UHT than in heated (95 °C, 5 minutes), the opposite was observed in TAD milk. In OAD UHT milk, the degree of α-LA denaturation ranged from 81.3% to 83.2%; in TAD milk, 89.7% to 92.9% of α-LA was denatured after UHT treatment. Additionally, in OAD milk the proportion of α-LA associating with the casein micelles was also lower in UHT than in milk heated at 95 °C for 5 minutes. The opposite was found for TAD milk. In contrast, in UHT milk, casein micelle association of β-LG was consistently lower than in milk heated at 95 °C for 5 minutes. There was no difference in the level of β-LG casein micelle association between OAD and TAD milk. Seasonal trends were similar in OAD and TAD milk. The degree of denaturation of both whey proteins remained similar throughout the lactation in both OAD and TAD milk. For both α-LA and β-LG, the highest proportions of protein associated with the casein micelles was found in late lactation, while the lowest proportion of serum aggregation occurred in this stage of lactation.

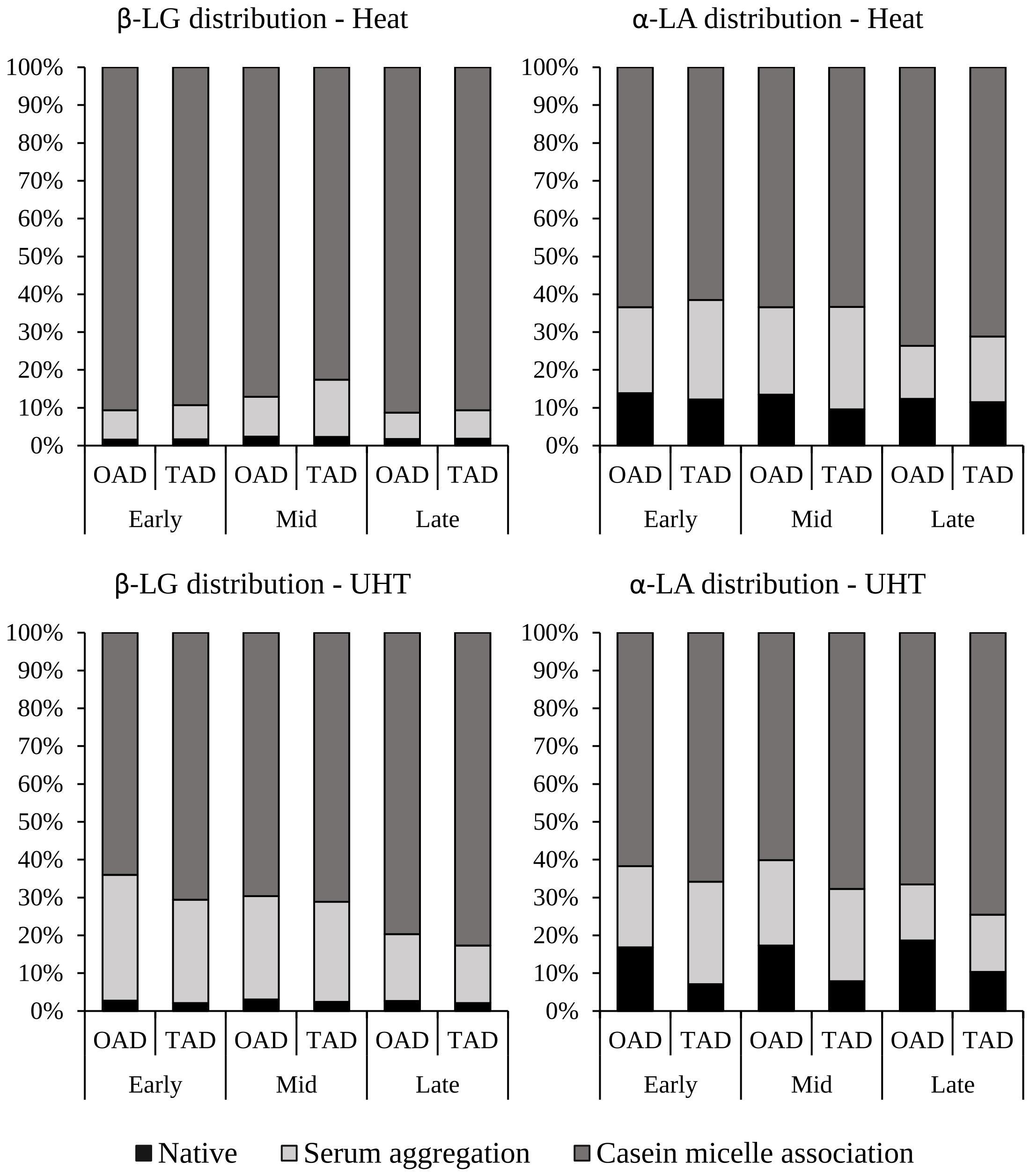


Figure 7.7. The distribution of β-LG (left) and α-LA (right) in heated (95 °C, 5 minutes; top) and UHT (140 °C, 5 seconds; bottom) milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021 in early, mid-, and late lactation.

7.4.6 Casein micelle size

Figure 7.8 shows the casein micelle size in raw milk and in the milk after the different heat treatments. There was a significant milking frequency effect (*P* = 0.034) on the casein micelle size. In raw OAD milk, the micelle size ranged from 152 nm up to 154 nm, while in TAD milk the micelle size was larger at 153 nm in early to 164 nm in late lactation. Additionally, stage of lactation had a significant effect (*P* < 0.001) on the casein micelle size, which increased throughout the lactation. During pasteurisation, the casein micelle size increased in both OAD (by 10 nm) and TAD (by 11 nm) milk in early lactation but not in other stages of lactation. Across the lactation, the casein micelle size ranged from 153 nm to 162 nm in OAD milk, and from 154 nm to 165 nm in TAD milk. At both milking frequencies, the casein micelle size in pasteurised milk was larger in early than in mid- and late lactation, which is in contrast with the seasonal trend in raw milk.

Upon heating at 95 °C for 5 minutes, the casein micelle size increased towards 159 nm to 163 nm in OAD milk, and to 161 nm to 167 nm in TAD milk. Again, a similar increasing trend throughout the lactation was observed. The largest increase in casein micelle size compared to raw milk was observed in UHT milk. The casein micelle size increased up to 229 ± 14 nm in OAD milk and 220 ± 11 nm in TAD milk, both in late lactation, which was significantly larger than in UHT milk in early and mid-lactation in both milking systems. While in TAD milk, the casein micelle size was similar in early and mid-lactation (192 nm), in OAD milk, the casein micelle size was lowest in early lactation (171 nm). This size was also significantly lower than any of the other sizes measured in UHT milk.

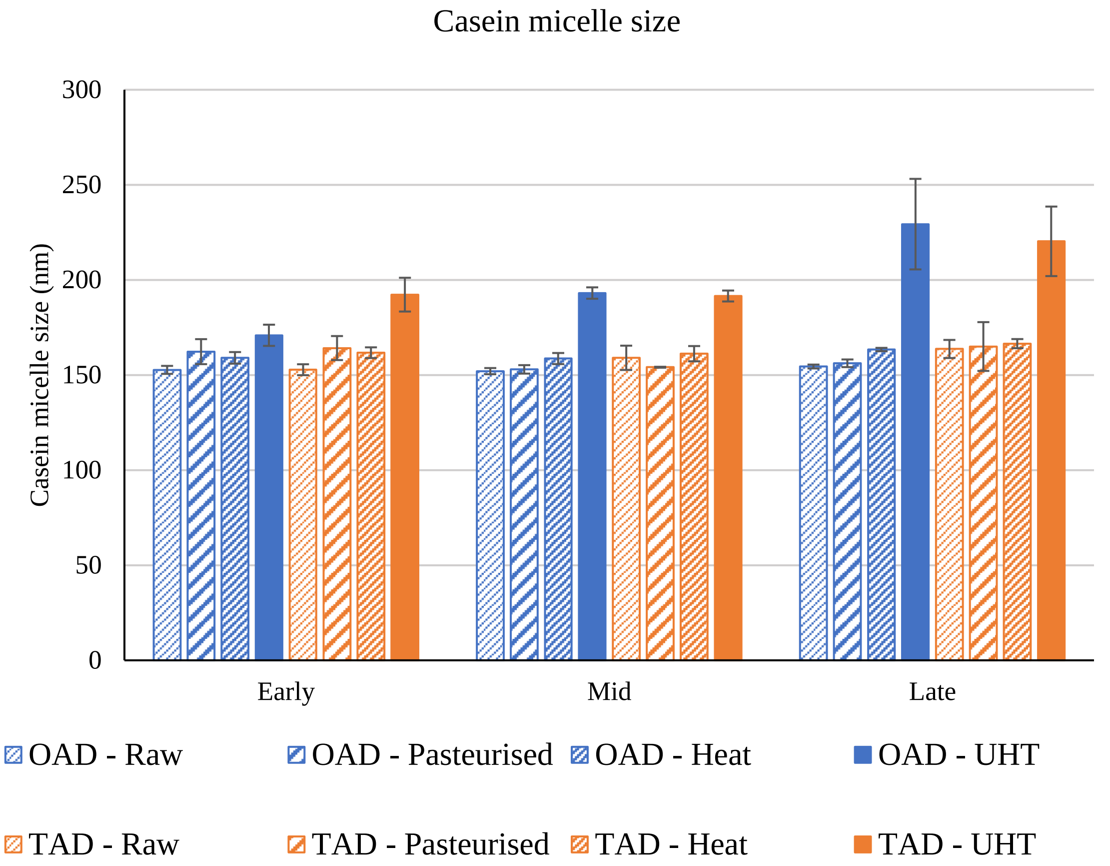


Figure 7.8. Casein micelle size in raw, pasteurised (72 °C, 15 seconds), heated (95 °C, 5 minutes) and UHT-treated (140 °C, 5 seconds) milk from cows milked once-a-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021 in early, mid-, and late lactation.

7.5 Discussion

7.5.1 Milk composition

The protein and fat contents were both higher in OAD than in TAD milk, which is in line with previous research (Clark et al., 2006; Gedye et al., 2020; Martin et al., 2009; O'Brien et al., 2002; Rémond et al., 2004). Additionally, all major proteins, except for β-CN, were affected by the milking frequency. The total casein content was higher in OAD than in TAD milk, as well of the proportions of κ-CN, α_{s2}-CN, and the degree of κ-CN glycosylation. On the other hand, contents of α_{s1}-CN, β-LG and α-LA were lower in OAD than in TAD milk. As a result, the ratio of whey protein to κ-CN was also higher in TAD than in OAD milk ($P < 0.001$). These findings can have implications for the protein interactions during heat treatments and consequently product properties and stability (Elfagm and Wheelock, 1978; Oldfield et al., 2005).

7.5.2 Serum phase caseins

The proportions of κ-CN and α_{s2}-CN in the serum phase of the milk were both higher in OAD than in TAD milk. Milk from OAD milked cows also contained more κ-CN and α_{s2}-CN. The amount and distribution (bound, serum or ionic) of calcium in the milk as well as the pH were similar across the milking systems. Since these factors impact micelle integrity and composition, the combination of the findings above suggests that the excess amount of κ-CN and α_{s2}-CN in OAD milk, compared to TAD milk, is mostly present in the serum phase of the milk. In raw and pasteurised milk, the proportion of serum κ-CN increased throughout the lactation, more in OAD (from 5.8 to 8.9%) than in TAD (from 3.2 to 4.5%) milk. Li et al. (2019) also found this lactational effect, although in their study the difference between early (4-5%) and late lactation (10-12%) was larger.

Upon heat treatments at higher temperatures (95 °C for 5 minutes and 140 °C for 5 seconds), significant dissociation of κ-CN occurred, especially in UHT treated milk. After these heat treatments, no difference between milking frequencies was found anymore. The heat-induced κ-CN dissociation and its extent is consistent with the literature (Li et al., 2019). The seasonal trend in heated milk was similar in OAD and in TAD milk. In milk heated at 95 °C for 5 minutes, the amount of serum κ-CN increased between early and mid-lactation but did not change between mid- and late lactation. In UHT milk, a downward trend was observed. Li et al. (2019) did not find such a trend in their results. Finally, although there was a difference between OAD and TAD milk prior to heating, dissociation during heat treatment led to a similar proportion of serum κ-CN after heat treatments at 95 °C and 140 °C. The dissociation of glycosylated κ-CN followed a similar trend as non-glycosylated κ-CN. However, the difference in serum glycosylated κ-CN between raw and heated (95 °C, 5 minutes) and between raw and UHT milk was larger than for total κ-CN. This finding suggests that the dissociation of glycosylated κ-CN during heat treatment is preferred over the dissociation of non-glycosylated κ-CN. This result is partly in agreement with the findings of Li et al. (2019), who also suggested that dissociation of glycosylated κ-CN during heating was favoured over the dissociation of glycosylated κ-CN.

Finally, an increase in serum β-CN was found after UHT treatment, in OAD and in TAD milk. The proportion of β-CN in the serum doubled in mid- and late lactation. In early lactation serum β-CN in UHT milk was almost five times that in raw milk. However, the stage of lactation effect was not significant, due to the large standard error in early lactation UHT milk. The dissociation behaviour of β-CN is often not highlighted since it tends to be minimal compared to κ-CN dissociation, but some heat-induced β-CN dissociation has been previously reported (Dumpler et al., 2017). The increased levels of β-CN in the serum after UHT treatment could be caused by increased exposure after the dissociation of κ-CN and consequent dissociation of β-CN.

7.5.3 Whey protein denaturation and aggregation

When milk is heated at temperatures above 70 °C, irreversible denaturation of whey protein occurs (Anema, 2008c). These denatured whey proteins then associate with the casein micelle or form aggregates in the serum with other denatured whey proteins or caseins. Although α_s-CN and β-CN can be involved in these aggregates, it is mostly κ-CN that forms complexes with these denatured whey proteins (Donato and Guyomarc'h, 2009).

Overall, the degree of whey protein denaturation did not differ between OAD and TAD when milk was heated at 95 °C for 5 minutes. However, in UHT treated milk, the degree of whey protein denaturation was lower in OAD than in TAD milk which was driven by the lower denaturation degree of α-LA. Additionally, the denaturation degree was similar in heated (95 °C, 5 minutes) and in UHT treated milk from the TAD milking system but not from the OAD milking system. Li et al. (2019), who studied heat-induced changes in milk from a TAD seasonal milking system, also found no difference in the degree of denaturation in milk heated at 90 °C for 6 minutes and UHT treated milk. Stage of lactation had a small but significant effect on the degree of denaturation, with an overall increase across lactation for heat (95 °C, 5 minutes) and UHT treated milk. The protein contents in late lactation could explain this effect since higher protein concentrations have been associated with higher degrees of denaturation (Anema et al., 2006; Law and Leaver, 1997).

In the present study, the denaturation degree of β-LG was similar in both heat treatments, in both milking systems. However, in a OAD milking system, the degree of α-LA denaturation is lower in UHT than in heated (95 °C, 5 minutes) milk, while this was not the case in TAD milk. This difference in α-LA denaturation drives the difference in the overall degree of whey protein denaturation. The higher proportion of serum κ-CN in raw OAD milk could have contributed to this difference. With a higher abundance of κ-CN and α_{s2}-CN for β-LG to associate with in UHT treated milk, less β-LG is available for α-LA to form irreversible aggregates with. α-LA aggregates more readily in the presence of β-LG (Gezimati et al., 1996). Additionally, α-LA has shown the highest level of renaturation of all whey proteins (Rüegg et al., 1977). When less proteins with free thiol groups (β-LG, κ-CN, and α_{s2}-CN) are available to react with, α-LA tends to fold back to its natural structure (Anema, 2022).

The proportions of whey protein, both β-LG and α-LA, associating with the casein micelle or forming serum aggregates did not differ between milking frequencies. The aggregation behaviour differed between the different heat treatments, and between β-LG and α-LA. In UHT treated milk, more whey proteins had formed serum aggregates than in milk heated at 95 °C for 5 minutes. This finding is in line with previous research (Li et al., 2019; Oldfield et al., 1998). While the proportion of α-LA in serum aggregates was similar in both heat treatments, β-LG had formed more serum aggregates in UHT than in heat (95 °C, 5 minutes) treated milk. Additionally, a significantly higher proportion of κ-CN was present in the serum after UHT treatment than after heating at 95 °C. Predominantly β-LG and κ-CN form complexes during heat treatments. Whether the κ-CN-β-LG complexes are formed in the serum after κ-CN dissociation or whether β-LG interacts with micellar casein first followed by dissociation of these newly formed complexed, remains to be investigated (Donato and Guyomarc'h, 2009).

The proportion of whey protein forming serum aggregates was lowest in late lactation. This finding aligns with decreasing κ-CN concentrations and whey protein:κ-CN ratios throughout the lactation. With less κ-CN available for complex formation, casein micelle association tends to be preferred (Anema, 2007). Li et al. (2019) also found a negative correlation between the proportion of serum κ-CN and casein micelle association.

7.5.4 Casein micelle size

Casein micelles in OAD milk were smaller than in TAD milk and increased in size throughout the lactation. Although previous research has been inconsistent regarding the correlation between κ-CN and the casein micelle size (Akkerman et al., 2021; De Kruif and Huppertz, 2012; Devold et al., 2000; Donnelly et al., 1984), the higher κ-CN levels in OAD milk could account for the smaller casein micelle size. The casein micelle size increased during heat treatment at 95 °C for 5 minutes. Denatured whey proteins that are associated with κ-CN on the casein micelle surface have previously been found to increase the micelle size up to 40 nm (Anema and Li, 2003). A greater increase in casein micelle size compared to raw milk, occurred during UHT treatment. In this case, the association of denatured whey protein to micellar κ-CN is not the only mechanism behind the increase in size. UHT heat treatment resulted in a significant amount of κ-CN dissociation from the casein micelle into the serum. κ-CN depletion of casein micelle thus results in destabilisation against aggregation. These larger casein micelles found in UHT milk are larger aggregates of κ-CN depleted casein micelles. Previous research has shown that sediment in UHT milk mostly consists of these κ-CN depleted micelles (Gaur et al., 2018).

7.6 Conclusion

This study was the first to investigate heat-induced changes in milk from OAD and TAD milking production systems. In raw and pasteurised OAD milk, higher proportions of κ-CN and α_{s2}-CN were present in the serum phase. Upon heating at 95 °C for 5 minutes and 140 °C for 5 seconds (UHT), a significant amount of κ-CN dissociated from the casein micelle, especially during the UHT treatment. The degree of denaturation did not differ between OAD and TAD milk after heat treatment at 95 °C. In UHT milk, the denaturation degree of whey proteins was lower in OAD than in TAD milk and increased in late lactation in OAD but not in TAD milk. The lower denaturation degree of whey proteins was driven by a difference in α-LA denaturation. The fate of denatured whey proteins was affected by the heat treatment, type of protein (α-LA or β-LG), milking frequency and stage of lactation. Overall, more casein micelle association occurred in milk treated at 95 °C than in UHT treated milk. In UHT milk from a TAD milking system, higher proportions of whey protein were associated with the casein micelle than in OAD milk, due to higher proportions of κ-CN in the serum of OAD milk. The casein micelle size was smaller in OAD than in TAD milk overall and increased upon heating and in late lactation. The most marked increase in size occurred during UHT treatment, as a result of the destabilisation of κ-CN depleted micelles. These findings contribute to the understanding of the implications of OAD milking on the processing properties of bovine milk.

Chapter 8

Gelation properties of milk from once-a-day and twice-a-day milked cows

Manuscript in preparation.

van der Zeijden, M., Ellis, A., Lopez-Villalobos, N., Li, S., Roy, N., McNabb, W. Heat-induced changes, rennet, and acid gelation properties of milk from a once-a-day and a twice-a-day milking system. *Dairy*.

8.1 Abstract

The rennet and acid gelation properties of milk from cows from a once-a-day (OAD) and cows from a twice-a-day (TAD) milking system were investigated. Cows were selected to balance for breed, parity, and breeding worth from a OAD milking farm and a TAD milking farm, both Massey University farms in Palmerston North, New Zealand. Milk was sampled at different stages of lactation from individual cows, including Holstein-Friesian and Holstein-Friesian×Jersey breeds, which were mixed proportionally to the yields to mimic tank milk. Pasteurised homogenised and pasteurised non-homogenised milk were used for rennet gelation. Homogenised milk heated at 95 °C for 5 minutes was used to study the acid gelation properties. Milking frequency did not significantly affect the rennet gelation time, storage modulus, or loss tangent. Stage of lactation affected the rennet gelation time and storage modulus. The rennet gelation time was highest, and the storage modulus was lowest in mid-lactation. The acid gelation pH of OAD milk was higher than that of TAD milk and was lowest in late lactation at both milking frequencies. The acid gelation time was also affected by the stage of lactation, with the longest gelation times observed in late lactation.

8.2 Introduction

Acid and rennet-induced gelation are processes involved in common dairy food products such as yoghurt and cheese. Rennet gelation is part of the cheese-making process and involves the enzymatic cleavage of κ-casein (CN). κ-CN forms a hairy layer on the outside of the casein micelles, providing both steric and electrostatic stabilisation against coagulation. Upon removal of this protective layer during the renneting process, the casein micelles aggregate and form a curd (Lucey, 2002). Acid gelation of milk occurs during the production process of yoghurt. Heat treatment is usually applied prior to gelation to improve the gel properties. The heating conditions vary but are usually between 80-95 °C for up to 30 minutes (Lucey and Singh, 1997; Lucey et al., 2022; Walstra et al., 2005). Denatured whey proteins, both in the form of soluble aggregates and attached to the casein micelle, increase the gelation pH and gel strength, and decrease the gelation time, which are considered favourable gelation properties (Del Angel and Dalgleish, 2006; Donato, Alexander, et al., 2007; Lucey, 2002).

In New Zealand, cows are conventionally milked twice a day (TAD), but once-a-day (OAD) milking is becoming more appealing to farmers for various reasons, including but not limited to changes in lifestyle expectations and labour challenges (Bewsell et al., 2008; Kendall et al., 2008; Stelwagen et al., 2013). There is limited knowledge of the processing properties of milk from a OAD milking system compared to a TAD milking system. One previous study has shown no effect of OAD milking on the rennet gelation properties of bovine milk (O'Brien et al., 2002). However, another study suggested a change in protein composition due to a reduction in milking frequency (Murney et al., 2015). Research has shown that, among other factors, protein composition affects gelation properties (Amalfitano et al., 2019; Glantz et al., 2010; Li et al., 2020). Chapter 6 showed that the composition, both proximate and that of the protein fraction, is different in OAD compared to TAD milk.

In this chapter, the same mixed milk samples used in Chapters 6 and 7 are used to study the rennet and acid gelation properties of milk from a OAD and milk from a TAD milking system. The effects of both milking frequency and stage of lactation are explored. The correlation between potential differences in the gelation properties and the results obtained in Chapters 6 and 7 will also be discussed. This study is the first to compare the rennet and acid gelation properties of milk from a OAD milking system and a TAD milking system,

8.3 Methods

A detailed description of the analytical methods is given in Chapter 3. Below, a summary of each method is provided.

8.3.1 Preparation of mixed milk samples

Milk was sampled three times in each of the early, mid- and late lactations from individual cows that met the study criteria described in Chapters 3 and 4. Only milk from Holstein-Friesian (F) and Holstein-Friesian×Jersey crossbred (F×J) cows were used as the Jersey cows from one of the farms did not strictly meet the parity criteria for the study. Three cows from each breed on each farm were selected. The yield of milk from each animal was recorded on farm by weight. Pooled milk was used for further characterisation; this mixed milk sample was created as follows. In the laboratory, the milk was mixed proportionally to the yields of each cow. The average proportion of milk from F cows was 59.9% and 54.1% in OAD and TAD milk, respectively. For F×J cows, the average proportion of milk from this breed in the mixed milk sample was 40.1% and 45.9% in OAD and TAD milk, respectively. The milk was mixed within hours of the collection and stored at 4 °C until used for processing. The heat treatments were performed in the FoodPilot (Massey University, Palmerston North, New Zealand) within 36 hours of collection.

All milk was pasteurised at 72 °C for 15 seconds. Part of this milk was also homogenised (200/50 bar) afterwards. Part of the homogenised milk was then heated at 95 °C for 5 minutes. The pasteurised homogenised and pasteurised non-homogenised milk were used for rennet gelation. Homogenised milk heated at 95 °C was used for the acid gelation experiments because this heat treatment is representative of that of yoghurt milk base.

8.3.2 Characterisation

8.3.2.1 Composition

The proximate composition was determined using the MilkoScan FT1 (Foss Analytics, Hillerød, Denmark). The pH was measured using an Edge Blu pH meter (Hanna Instruments, Woonsocket, RI, USA). The somatic cell count (SCC) of each mixed milk sample was determined by MilkTestNZ (Hamilton, New Zealand) using the CombiFoss (Foss Analytics, Hillerød, Denmark). The protein composition of skim milk samples and milk serum samples was analysed using a high-performance liquid chromatography (HPLC) method modified from the method by Bobe et al. (1998). The mineral composition of raw milk was determined by Hill Laboratories (Hamilton, New Zealand). The ionic calcium was determined using a calcium electrode (Orion 9720BNWP Thermo Scientific, Singapore) together with a CyberScan pH 510 pH/mV Meter (Eutech Instruments-Thermo Scientific, Singapore). The electrode was calibrated using 1-10 mM of $CaCl_2$, of which the ionic strengths were adjusted to 80 mM with potassium chloride, as described by Li et al. (2019).

8.3.2.2 Particle size

The casein micelle size was determined using the Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). Skim milk samples were diluted 50x in an imidazole buffer and filtered before analysis. The measurement was performed at 25 °C; each measurement was performed in triplicate, each consisting of 13 sub-measurements.

The fat globule size of the raw and heat-treated samples was determined using the Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK). Each milk sample was mixed 1:1 with a 2% SDS and 50 mM EDTA solution (pH 6.7) prior to analysis.

8.3.2.3 Heat-induced changes

Heat-induced changes were studied in more detail in Chapter 7. In summary, the protein composition of skim milk, the serum phase and native whey isolates were determined to analyse the denaturation degree, serum proteins and whey protein aggregation behaviour. The serum phase was separated by ultracentrifugation (63,000 g for 1 hour at 20 °C). The native whey fraction was isolated using acid precipitation according to a method described by Vasbinder and de Kruif (2003).

8.3.3 Rennet gelation

Rennet-induced gelation properties were analysed according to the method described by Glantz et al. (2010). Both homogenised and non-homogenised pasteurised milk were analysed. A microbial rennet (HANNILASE® XP 1050 NB, Christian Hansen A/S, Hørsholm, Denmark) was used for rennet gelation. Milk was heated to 36-37 °C, inoculated at 38 international milk clotting units per litre and mixed for approximately 1 minute before being transferred into the rheometer cup. Gelation analyses were carried out at 32 °C for 40 minutes, using 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 a radius of 15 mm and 14 mm, respectively) . The gelation time was defined as the time from the addition of the rennet until the time when the storage modulus (G′) had reached 1.0 Pa.

8.3.4 Acid gelation

The acid gelation properties were analysed according to the method described by Li et al. (2020). Milk heated at 95 °C for 5 minutes was used. This type of heat treatment results in the level of whey protein denaturation and consequent aggregation with casein micelles required for yoghurt productions. 2 g of glucono-δ-lactone (GDL) was added to 100 mL of milk at 36-37 °C followed by stirring for 2 minutes. 20 mL of sample was transferred to the rheometer cup. The gelation properties were measured for 8 hours at 30 °C using 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 a radius of 15 mm and 14 mm, respectively). The remaining of the sample was used to track the pH in a jacketed beaker connected to a waterbath at 30 °C. pH measurements were taken at 1-minute intervals using an Edge Blu pH meter (Hanna Instruments, Woonsocket, RI, USA), alongside the rheology analysis. The gelation time and gelation pH were defined as the time from the addition of the GDL and the pH at that time, at which the G′ was 1.0 Pa. The final gel strength was determined at 480 minutes.

8.3.5 Statistical analysis

A different statistical model was used for the rennet gelation results than for the acid gelation results.

For the rennet gelation results, marginal means of the dependent variables for each level of milking frequency (OAD and TAD), stage of lactation (early, mid, and late), homogenisation (non-homogenised and homogenised), combinations of milking frequency and homogenisation and combinations of milking frequency, stage of lactation and homogenisation were obtained using the MIXED procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) with the following linear model:

$$y_{ijk} = \mu + M_i + S_j + H_k + MH_{ik} + MSH_{ijk} + e_{ijkl}$$

Y_{ijk} is the observation for the trait for milking frequency i, lactation stage j and homogenisation k

μ is the population mean

M_i is the fixed effect of milking frequency i (i = OAD and TAD)

S_j is the fixed effect of stage of lactation j (j = early, mid, and late)

H_k is the fixed effect of homogenisation k (k = non-homogenised and homogenised)

MH_{ik} is the fixed effect of milking frequency i and homogenisation k

MSH_{ijk} if the fixed effect of interaction between milking frequency i, stage of lactation j and homogenisation k

e_{ijkl} is the residual random error associated with observation y_{ijkl}.

For the acid gelation results, marginal means of the dependent variables for each level of milking frequency (OAD and TAD) and stage of lactation (early, mid, and late) and combinations of milking frequency and stage of lactation were obtained using the MIXED procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) with the following linear model:

$$y_{ijk} = \mu + M_i + S_j + MS_{ij} + e_{ijk}$$

Y_{ijk} is the observation for the trait for milking frequency i, lactation stage j and homogenisation k

μ is the population mean

M_i is the fixed effect of milking frequency i (i = OAD and TAD)

S_j is the fixed effect of stage of lactation j (j = early, mid, and late)

MS_{ij} if the fixed effect of interaction between milking frequency i and stage of lactation j

e_{ijk} is the residual random error associated with observation y_{ijk}.

The residuals were modelled with heterogeneous variance across the combinations of milking frequency and stage of lactation. The partial correlation coefficients between dependent traits were estimated using the GLM procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) with the option MANOVA. Significant differences were declared at $P < 0.05$. Trends were declared at $P < 0.1$.

8.4 Results

8.4.1 Milk composition and physicochemical properties

The major components, as well as the protein composition and mineral composition of the mixed milk samples used for the gelation experiments, are discussed in detail in Chapter 6. Heat-induced changes to the proteins as a result of pasteurisation (72 °C, 15 seconds) and heat treatment at 95 °C for 5 minutes are discussed in Chapter 7. A summary of the findings in these chapters is given below.

The protein and fat contents were higher in OAD milk than in TAD milk. Both were affected by stage of lactation and increased in late lactation. The lactose content was similar between the two milking frequencies and decreased towards the end of the season. The somatic cell counts were higher in OAD milk but still well within the acceptable range for the milk supply. The pH did not differ between OAD milk and TAD milk.

Although casein micelles were smaller in raw OAD milk than in raw TAD milk, the difference was not significant ($P > 0.05$) after the heat treatments prior to the gelation experiments. Pasteurisation increased the casein micelle size by approximately 10 nm in early lactation, in OAD milk and TAD milk, but not in mid- and late lactation. The casein micelle size in pasteurised OAD and TAD milk decreased between early and mid-lactation but did not change significantly in late lactation. In milk heated at 95 °C, the casein micelle size increased in late lactation. Finally, the fat globule size did not differ in non-homogenised pasteurised milk but was larger in OAD (0.9 ± 0.07) than in TAD (0.6 ± 0.03) milk after homogenisation.

All major proteins except β-CN were affected by the milking frequency. The proportions of α_{s2}-CN, total whey protein, β-LG, and α-LA were lower, while the proportions of total casein, α_{s2}-CN, κ-CN, and κ-CN glycosylation were higher in OAD than in TAD milk. Stage of lactation affected all proteins in the milk, with the percentages of total casein, α_{s1}-CN, β-CN, and α-LA decreasing throughout the lactation. In contrast, the percentages of α_{s2}-CN, β-LG, and the degree of κ-CN glycosylation increased between early and late lactation. The proportion of κ-CN in the milk followed a different lactation pattern in OAD than in TAD milk. In OAD milk, the κ-CN percentage was lowest in mid-lactation and increased again in late lactation, while in TAD milk, the κ-CN decreased throughout lactation.

None of the major minerals (calcium, phosphorus, and magnesium) were affected by the milking frequency. The total calcium content was lowest in mid-lactation and highest in late lactation, but the distribution of calcium did not change significantly throughout the lactation. Stage of lactation also affected the content and distribution of phosphorus which was lowest in mid-lactation. The magnesium content was highest in late lactation, while the proportion of magnesium in the serum decreased throughout the lactation.

8.4.1.1 Heat-induced changes

In pasteurised milk, the denaturation degree did not differ between OAD and TAD milk but was affected by stage of lactation. The highest degree of denaturation (OAD 35.1%, TAD 32.5%) was found in mid-lactation and the lowest degree of denaturation (OAD 16.5%, TAD 10.9%) in early lactation. In milk heated at 95 °C for 5 minutes, the denaturation degree increased significantly compared to pasteurised milk. The milking frequency and stage of lactation effects were not significant at this heat treatment. The aggregation behaviour in heated (95 °C) milk was also not affected by milking frequency, but there was a stage of lactation effect. The proportion of whey protein associated with the casein micelle increased from 80.9% and 77.4% to 87.2% and 86.1% in OAD and TAD milk, respectively. The proportion of whey protein present in serum aggregates changed throughout the lactation inversely to the casein micelle-associated whey protein.

In raw milk, approximately 16.2% of κ-CN was present in the serum of OAD milk; in TAD milk, this was 13.9% which was significantly ($P = 0.009$) lower than in OAD milk. Pasteurisation did not cause significant dissociation of κ-CN compared to raw milk, but heat treatment at 95 °C for 5 minutes did. After heat treatment at 95 °C, OAD and TAD milk no longer differ significantly, but stage of lactation did affect the proportion of κ-CN in the serum phase. At both milking frequencies, the proportion of κ-CN in the serum was lower in early (OAD, 10.9%; TAD, 8.9%) than in mid- (OAD, 13.8%; TAD, 15.4%) and late (OAD, 14.7%; TAD, 12.3%) lactation.

8.4.2 Rennet gelation

The rennet gelation properties of pasteurised non-homogenised milk from a OAD milking system and TAD milking system are shown in Table 8.1. Milking frequency did not significantly affect the gelation time, G′ or loss tangent at 40 minutes. However, stage of lactation did affect the gelation time ($P < 0.001$), G′ ($P = 0.003$) and loss tangent ($P = 0.001$) at 40 minutes (Table 8.2). Homogenisation also significantly affected the gelation time ($P = 0.002$) and G′ ($P = 0.002$), but not the loss tangent. There was no interaction effect between milking frequency and homogenisation.

Table 8.1. Rennet gelation properties of non-homogenised and homogenised milk from cows milked once-day (OAD) and twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

	Non-homogenised		Homogenised		*P*-value[1]		
	OAD	TAD	OAD	TAD	M	H	M×H
Gelation time (min)	37 ± 1.1	36 ± 1.5	31 ± 2.0	30 ± 2.3	0.598	0.002	0.851
Storage modulus (Pa) at 40 min	3.4 ± 0.68	3.7 ± 1.11	10.9 ± 3.01	8.9 ± 1.72	0.657	0.002	0.552
Tan (δ) at 40 min	0.356 ± 0.0636	0.370 ± 0.0289	0.371 ± 0.0183	0.362 ± 0.0205	0.946	0.932	0.755

[1] M = milking frequency (OAD, TAD); H = homogenisation (non-homogenised, homogenised)

$^{a, b, c}$ means with different superscripts within each trait are significantly different ($P < 0.05$).

Table 8.2 shows the rennet gelation properties of OAD milk and TAD milk in early, mid- and late lactation. The longest gelation time was found in mid-lactation in both milking systems. The average gelation time in mid-lactation was 45 minutes for OAD milk, significantly higher than those in early and late lactation. In TAD milk, the average gelation time in mid-lactation was 42 minutes which was higher than early lactation ($P < 0.05$). A similar trend as in OAD milk was observed between mid- and late lactation in TAD milk ($P = 0.052$). Milk from the OAD milking system had a longer gelation time in late lactation than in early lactation, which did not significantly differ for TAD milk. As a result of the longer gelation time, the G′ was below 1 Pa at 40 minutes in mid-lactation. G′ values were similar in early and late lactation in both milking systems, ranging from 3.2 to 6.5 Pa. The loss tangent ranged from 0.314 to 0.380 in OAD milk gels but did not differ significantly between stages of lactation. In rennet gels from TAD milk, the loss tangent in late lactation was higher than in early lactation ($P = 0.006$).

Similar to non-homogenised milk, the rennet gelation properties of homogenised milk were unaffected by the milking frequency. Stage of lactation did affect the gelation properties of homogenised milk. The rennet gelation times of homogenised milk samples were significantly lower than for non-homogenised milk (Table 8.1). As a result, the storage moduli of gels from homogenised milk were also higher at 40 minutes than for gels from non-homogenised milk. The gelation times ranged from 23 to 36 minutes, compared to 31 to 45 minutes for non-homogenised milk. For OAD milk, the gelation time was significantly lower in early than mid- and late lactation. This trend was similar for TAD milk, but the difference between early and mid-lactation was not significant ($P = 0.062$). The G′ was higher in early lactation than in mid-lactation for the TAD milk gels but did not differ significantly between stages of lactation for OAD milk gels. Finally, the loss tangent of OAD milk gels was higher in late than early lactation and did not change significantly throughout the lactation for TAD milk.

Table 8.2. Rennet gelation properties of non-homogenised pasteurised milk from a once-a-day (OAD) and a twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

		Non-homogenised		Homogenised		*P-value*[1]			
	SOL	OAD	TAD	OAD	TAD	M	S	H	M×S×H
Gelation time (min)	Early	31 ± 1.6^{c}	32 ± 2.8^{b}	23 ± 3.9^{b}	25 ± 1.0	0.598	< 0.001	0.002	0.626
	Mid	45 ± 2.7^{a}	42 ± 1.2^{a}	36 ± 4.0^{a}	32 ± 3.7				
	Late	37 ± 1.1^{b}	35 ± 3.3^{ab}	34 ± 2.3^{a}	34 ± 5.8				
Storage modulus (Pa) at 40 min	Early	6.5 ± 1.92^{a}	5.4 ± 2.14^{a}	20.7 ± 8.25	12.1 ± 0.50^{a}	0.657	0.003	0.002	0.884
	Mid	0.6 ± 0.35^{b}	0.7 ± 0.21^{b}	4.3 ± 2.03	5.8 ± 2.39^{b}				
	Late	3.2 ± 0.57^{a}	5.0 ± 2.55^{ab}	7.7 ± 3.03	8.9 ± 4.55^{ab}				
Tan (δ) at 40 min	Early	0.380 ± 0.0236	0.345 ± 0.0030^{b}	0.337 ± 0.0115^{b}	0.330 ± 0.0036	0.946	0.155	0.932	0.714
	Mid	0.314 ± 0.1890	0.397 ± 0.0864^{ab}	0.401 ± 0.0530^{ab}	0.347 ± 0.0104				
	Late	0.375 ± 0.0120	0.369 ± 0.0074^{a}	0.376 ± 0.0086^{a}	0.408 ± 0.0605				

[1] M = milking frequency (OAD and TAD), S = stage of lactation (early, mid, and late), H = homogenisation (non-homogenised, homogenised)

[a, b, c] Means with different superscripts within each trait, milk type and milking frequency are significantly different ($P < 0.05$)

[A, B, C] Means with different superscripts within each trait and stage of lactation are significantly different ($P < 0.05$)

8.4.3 Acid gelation

The acid gelation properties of heated (95 °C, 5 minutes) milk from the OAD and TAD milking systems are shown in Table 8.3. The milking frequency had a significant effect on the gelation pH, but none of the other gelation properties. The gelation pH was lower for OAD milk than for TAD milk. For OAD milk, the gelation pH ranged from 5.15 up to 5.23; for TAD milk, the gelation pH ranged from 5.21 to 5.25 (Table 8.4). Stage of lactation significantly affected the gelation pH and time (Table 8.3). For both milking frequencies, the gelation pH was lowest in late lactation and the highest in early lactation. The gelation time increased as the lactation progressed. In OAD milk, the gelation time increased from 41 to 56 minutes; in TAD milk, the gelation time increased from 39 minutes to 53 minutes. The final G′ was not significantly affected by milking frequency or stage of lactation. Overall, there was a trend (*P* = 0.090) of higher loss tangents in acid gels from OAD than from TAD, which was significant in late lactation (*P* = 0.017). No interaction effect between milking frequency and stage of lactation was found for any acid gelation properties.

Table 8.3. Acid gelation properties of milk from a once-a-day (OAD) and a twice-a-day (TAD) at Massey University dairy farms in New Zealand during production season 2020-2021.

	OAD	TAD	M[1] effect (*P-value)*
Gelation pH	5.19 ± 0.005^b	5.24 ± 0.007^a	< 0.001
Gelation time (min)	48 ± 1.1	47 ± 3.1	0.956
Final storage modulus (Pa)	495.1 ± 39.77	538.4 ± 23.34	0.366
Final tan (δ)	0.226 ± 0.0082	0.211 ± 0.0013	0.090

[1] M = milking frequency (OAD, TAD). $^{a, b}$ Means with different superscript within each trait are significantly different (*P* < 0.05).

Table 8.4. Acid gelation properties of milk from a once-a-day (OAD) and a twice-a-day (TAD) at Massey University dairy farms in New Zealand at different stages of lactation during production season 2020-2021.

	S[1]	OAD	TAD	*P-value*[1]	
				S	M×S
Gelation pH	Early	5.23 ± 0.009^{a}	5.25 ± 0.018^{ab}	< 0.001	0.071
	Mid	5.21 ± 0.011^{aB}	5.24 ± 0.004^{bA}		
	Late	5.15 ± 0.004^{bB}	5.21 ± 0.009^{aA}		
Gelation time (min)	Early	41 ± 2.0^{b}	39 ± 2.0^{b}	0.005	0.409
	Mid	46 ± 1.4^{ab}	51 ± 5.0^{a}		
	Late	56 ± 2.0^{a}	53 ± 7.7^{a}		
Final storage modulus (Pa)	Early	555.1 ± 22.12	565.5 ± 32.16	0.092	0.795
	Mid	368.3 ± 111.94	464.3 ± 41.99		
	Late	561.9 ± 34.90	585.4 ± 45.89		
Final tan (δ)	Early	0.236 ± 0.0213	0.205 ± 0.0020^{b}	0.926	0.557
	Mid	0.221 ± 0.0125	0.213 ± 0.0022^{a}		
	Late	0.223 ± 0.0005^{A}	0.215 ± 0.0026^{aB}		

[1] M = milking frequency (OAD and TAD); S = stage of lactation (early, mid, and late). $^{a, b, c}$ Means with different superscripts within each trait and milking frequency are significantly different ($P < 0.05$). $^{A, B, C}$ Means with different superscripts within each trait and stage of lactation are significantly different ($P < 0.05$).

8.4.4 Partial correlations

In rennet-induced gels, the serum calcium content correlated negatively with the gelation time (*r* = -0.462, *P* = 0.027) and loss tangent at 40 minutes (*r* = -0.659, *P* = 0.001). No significant correlations were found between the rennet gelation properties and other milk characteristics, including heat-induced changes observed in Chapter 7.

In acid gels, the protein content correlated negatively with the gelation time (*r* = -0.614, *P* = 0.034), and the lactose content correlated positively with the gelation pH (*r* = 0.589, *P* = 0.044). Additionally, the proportion of calcium in the serum phase correlated positively with the final G′ (*r* = 0.604, *P* = 0.038). The ionic calcium, as a percentage of the total calcium, correlated negatively with the final G′ (*r* = -0.584, *P* = 0.046).

8.5 Discussion

8.5.1 Rennet gelation

The milking frequency did not affect the rennet gelation properties (gelation time, G′ and loss tangent) of pasteurised milk. O'Brien et al. (2002) also did not find a difference in the coagulation properties of OAD and TAD milk, nor did a more recent study (Sanjayaranj et al., 2023). Previously, a study showed a positive correlation between κ-CN glycosylation and the rennet coagulation time (Bonfatti et al., 2014). Although OAD milk has a higher κ-CN glycosylation degree, this increase was insufficient to increase the gelation time and decrease G′. Some studies have found increased protein and casein contents beneficial for rennet gelation properties (Auldist et al., 2004). The higher protein and casein contents, in this case, may have offset the negative effect of glycosylated κ-CN.

Stage of lactation affected the gelation time and G′ of rennet gels. The longest gelation time and lowest G′ were found in mid-lactation. Bittante et al. (2015) also found a steep increase in gelation time early in lactation, which stabilised after. In the present study, in OAD milk, the gelation time in late lactation was shorter than in mid-lactation. A similar trend was observed between mid- and late lactation in TAD milk. In mid-lactation, whey protein denaturation was highest in both milking frequencies (Chapter 7). Intense heating impairs gel formation in rennet-induced coagulation (Britten and Giroux, 2022; Dinkov and Dushkova, 2007; Kethireddipalli et al., 2010). Denatured whey proteins associate with the casein micelles, inhibiting the rennet action on the κ-CN and delaying the aggregation between casein micelles.

Homogenisation had pronounced effects on the rennet gelation properties, decreasing the gelation time and increasing the gel strength. During homogenisation, the fat globule membrane is disrupted and replaced by proteins to cover the increased surface area of the smaller fat globules (Walstra et al., 2005). Native fat globules do not actively participate in the gel network, but the smaller fat globules covered in mostly casein are integrated into the network (Michalski et al., 2002). Homogenisation has previously been shown to increase gel strength (Michalski et al., 2002). Generally, lower gelation times also result in a more advanced network formation at a certain timepoint leading to increased gel strength at a given time compared to samples that take longer to form a gel network.

The amount of calcium in the serum phase of the milk correlated negatively with both the gelation time and loss tangent. Calcium participates in the network formation, forming bridges between destabilised casein micelles (Lucey, 2002). Additionally, ionic calcium screens charges on the micelles thereby reducing repulsive forces and improving aggregation (Dalgleish, 1983). Results align with previous studies showing that adding soluble calcium decreases gelation time and increases gel strength (Sandra et al., 2012; Udabage et al., 2001). The increased amount of calcium in the serum does not actually provide information on the form the calcium is present in the serum in, and the ionic calcium did not correlate with the rennet gelation properties.

8.5.2 Acid gelation properties

The OAD milk had lower gelation pH than TAD milk, but milking frequency did not affect other acid gelation properties. The TAD milk also had a lower casein-to-whey ratio than OAD milk (Chapter 6). Since denatured whey proteins increase the gelation pH (Lucey et al., 2022; Morand et al., 2012), the higher proportion of casein in OAD milk might explain the lower gelation pH compared to TAD milk. The final loss tangent tended to be higher in acid gels from OAD than from TAD, which was significant in late lactation. Higher loss tangents could result in higher levels of syneresis which is undesirable in yoghurt (Lucey, 2001).

Additionally, the gelation pH decreased throughout lactation, most notably in late lactation. The gelation time also increased as the lactation progressed, which was likely due to the lower gelation pH. Milk samples with a lower gelation pH would take longer to form a gel. Li et al. (2020) found a similar stage of lactation effect. They attributed the inferior gelation properties of late lactation milk to an increase in the κ-CN glycosylation degree. The glycosylation of κ-CN, especially on the micellar surface, decreases the hydrophobicity, which plays an important role in the formation of the gel network (Cases et al., 2003). In the present study, the κ-CN glycosylation degree also increased in late lactation (Chapter 6). However, no correlation was found between the glycosylation degree and any of the gelation properties. Inferior acid gelation properties (lower gel strength and longer gelation times) in late lactation were also found by Underwood and Augustin (1997).

The present study found a positive correlation between the lactose content and gelation pH. Meletharayil et al. (2016) also found higher gelation pH in GDL-induced acid gelation with added lactose, although the lactose concentrations were much higher (up to 11.2%) than in the present study. The authors attributed this effect to the increased serum κ-CN and whey protein that occurred alongside the increased lactose content. Higher proportions of serum κ-CN and whey protein complexes generally improve acid gelation properties compared to milk with relatively more micellar κ-CN (Anema, 2008a; Anema et al., 2004; Lucey et al., 2022; Vasbinder et al., 2004). In late lactation, the proportion of whey proteins associated with casein micelle increased in heated (95 °C, 5 minutes) milk in both milking systems (Chapter 7, results not shown). This change in whey protein aggregation likely contributed to the decreased acid gelation properties in late lactation. Although the proportion of κ-CN in the serum was higher in raw OAD than in TAD milk, the distribution was similar after heating (95 °C, 5 minutes). The positive correlation between the lactose content and the gelation pH may have been caused by a stage of lactation effect on both characteristics, with the gelation pH and the lactose content both decreasing in late lactation.

The protein content correlated negatively with the acid gelation time which is in line with results from Meletharayil et al. (2015). Higher protein content contributes to a greater buffering capacity of the milk and a lower pH reduction rate during acidification. However, not all studies found a correlation between protein content and gelation time, and it has been highlighted that other factors also contribute to the acid gelation properties (Glantz et al., 2010; Li et al., 2020; Lucey et al., 2022), which may offset a potentially positive effect of the protein content.

Finally, across the lactation, the final G′ correlated negatively with the proportion of ionic calcium and correlated positively with the proportion of calcium in the serum. These findings seem contradictory since the ionic calcium is part of the serum calcium. The effect of the calcium content and distribution on the acid gelation properties of milk is unclear however (Barone et al., 2021). Ramasubramanian et al. (2008) adjusted the calcium content by adding calcium, both in the ionic and non-ionic form before heat treatment. They found that addition of ionic calcium decreased the firmness of stirred yoghurt, but the addition of non-ionic calcium increased the firmness of stirred yoghurt. However, in a different study where they added calcium chloride to milk, increased gel strength was found with increasing levels of added calcium chloride (Ramasubramanian et al., 2014). In the present study, the calcium content and distribution were measured in raw milk. During heat treatment and acidification, the calcium distribution would have changed. Ozcan-Yilsay et al. (2007) added trisodium citrate to milk after heat treatment, which chelates calcium and induces colloidal calcium phosphate dissolution into the milk serum phase. At low levels, chelation of calcium increased the G′ of acid-induced gels, but at higher levels, G′ decreased. Li et al. (2020) also found a negative correlation between the ionic calcium and the final gel strength but highlighted the inconsistency across literature. Previous research on the effect of calcium content and distribution on acid-induced gels remains inconclusive. The differences in the calcium distribution in the present study are also much smaller than in some of the studies above (Ozcan-Yilsay et al., 2007; Ramasubramanian et al., 2014).

8.6 Conclusion

This study showed the impact of OAD milking, compared to TAD milking, on the rennet and acid gelation properties of milk at different stages of lactation. Milking frequency did not affect the rennet gelation properties but decreased the gelation pH during acid gelation. Stage of lactation impacted the gelation properties of both rennet and acid-induced gels. The rennet gelation times of non-homogenised milk samples were highest in mid-lactation at both milking frequencies. In OAD milk, the gelation time was shorter in early than in mid-lactation which did not differ in TAD milk. Corresponding to the gelation times, the G′ of rennet gel was lowest in mid-lactation. Homogenisation resulted in shorter rennet gelation times and higher storage moduli in rennet gels. The rennet gelation times of homogenised milk were shortest in early lactation. The storage modulus was also highest in early lactation for TAD milk but not for OAD milk. Acid-induced gels had higher gelation pH in early than late lactation, and the gelation times increased throughout the lactation.

The results of this study showed that OAD milking may not have inferior cheese-making properties (rennet gelation) to TAD milk, but yoghurt production (acid gelation) may be negatively affected. The changes in composition and structural changes during heating between OAD and TAD milk were insufficient to affect most of the gelation properties.

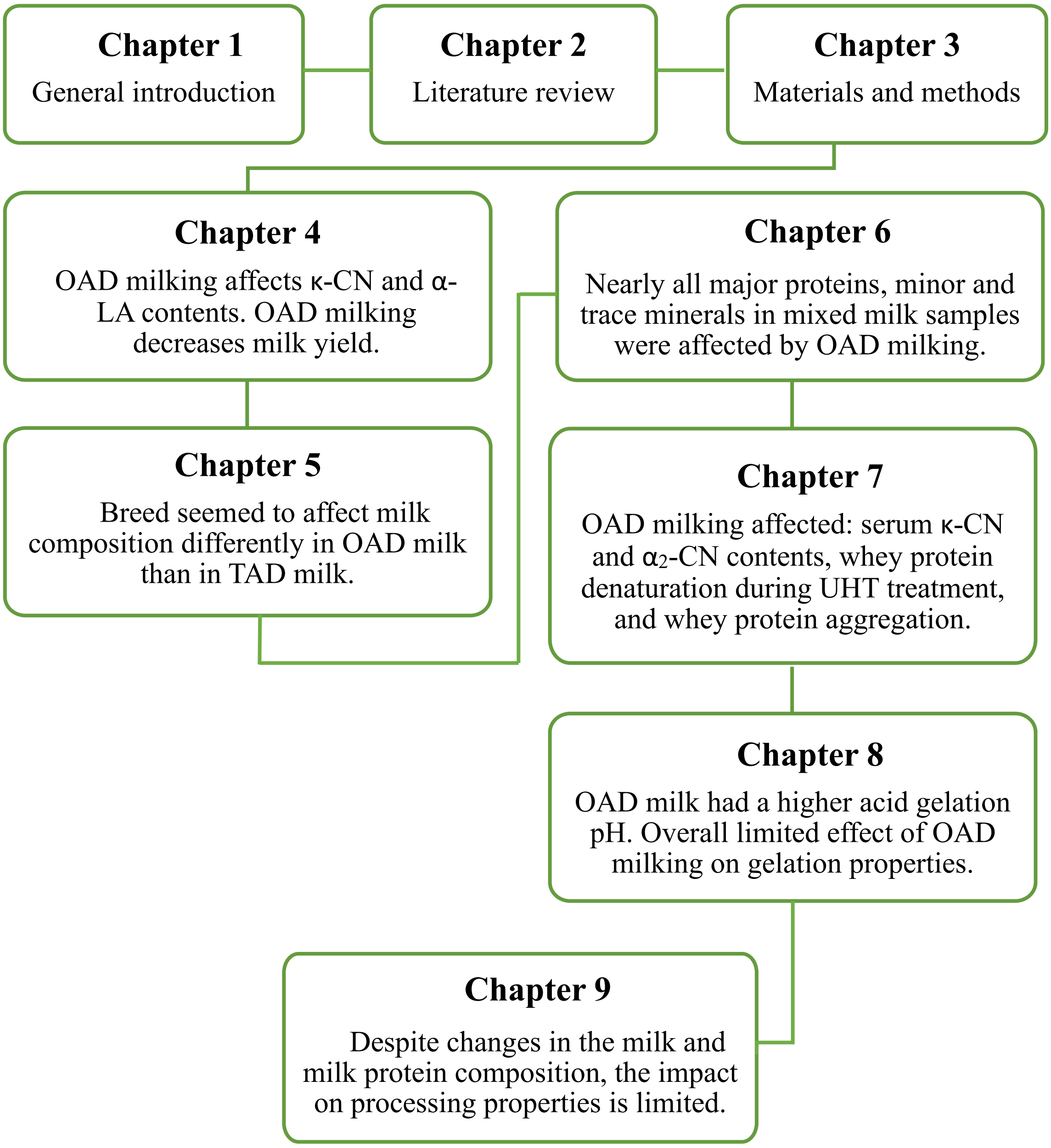
Chapter 9

General discussion

Dairy farming in New Zealand is pasture-based, with most cows calving in spring. Twice-a-day (TAD) milking during the whole lactation is practised in about 55% of New Zealand herds, and once-a-day (OAD) during the whole lactation in about 10% of herds, with the remainder of the farmers using a mixture of TAD and OAD (Edwards, 2018). Once-a-day milking provides several benefits, such as reduced labour cost, improved labour efficiency, improved health and reproductive performance of cows, and additional employment opportunities for farmers.

In general, OAD milking causes a reduction in milk yield and modifies milk composition by increasing the fat and protein percentages (Lopez-Villalobos et al., 2023). The modification of milk composition due to OAD milking may affect the processing properties of milk (O'Brien et al., 2002); however, there are only limited studies examining the effect of OAD milking on the processability of milk into cheese, yoghurt, and other dairy products.

This research aimed to study the effect of a OAD, compared to a TAD, milking production system on the protein composition and processing properties of bovine milk. Chapters 4 and 5 analysed the composition of milk from individual cows. Cows in the OAD milking system produced less milk and milk solids than cows in the TAD milking system. The fat, protein and lactose contents did not differ, but when milk was mixed in proportion to the respective yields of each cow (Chapter 6), OAD milk was higher in fat and protein contents. Interestingly, while data from individual cows only showed a milking frequency effect on the contents of κ-CN (κ-CN) and α-lactalbumin (α-LA), in the mixed milk samples, nearly all major proteins were impacted by OAD milking. The breed of the cows also seemed to affect the casein, α-LA, and β-lactoglobulin (β-LG) contents differently in a OAD milking system than in a TAD milking system (Chapter 5). The protein composition and the mineral composition (sodium, chloride, potassium, copper, iodine, and selenium) were also affected by the milking frequency (Chapter 6). Despite these compositional differences between OAD milk and TAD milk, the impact of milking frequency on the heat-induced changes (Chapter 7) and gelation properties (Chapter 8) was limited. The main findings are summarised in Figure 9.1.



Chapter 1
General introduction

Chapter 2
Literature review

Chapter 3
Materials and methods

Chapter 4
OAD milking affects κ-CN and α-LA contents. OAD milking decreases milk yield.

Chapter 5
Breed seemed to affect milk composition differently in OAD milk than in TAD milk.

Chapter 6
Nearly all major proteins, minor and trace minerals in mixed milk samples were affected by OAD milking.

Chapter 7
OAD milking affected: serum κ-CN and α_2-CN contents, whey protein denaturation during UHT treatment, and whey protein aggregation.

Chapter 8
OAD milk had a higher acid gelation pH. Overall limited effect of OAD milking on gelation properties.

Chapter 9
Despite changes in the milk and milk protein composition, the impact on processing properties is limited.

Figure 9.1. Main findings of the thesis. OAD = once-a-day, TAD = twice-a-day, α-LA = α-lactalbumin, α_{s2}-CN = α_{s2}-casein, κ-CN = κ-casein, UHT = ultra-high temperature.

There were some similarities but also differences between OAD milk and late lactation milk. The higher somatic cell count, protein, and fat contents, κ-CN glycosylation degree and lower α-LA content in OAD compared to TAD milk also occur in late lactation milk compared to milk from earlier stages of lactation. An enhanced involution process may give rise to the similarities between OAD milk and late lactation milk. In late lactation, the number and activity of the mammary epithelial cells decrease, which leads to changes in milk composition and reduced milk yield. A concentrating effect of the decreased volume as a result of decreased milking frequency is also likely to contribute to an increase in the somatic cell count, protein content, and fat content.

Despite these similarities, there were also some differences between OAD milk and late lactation milk. Chapter 6 showed that milk from cows milked OAD had a higher casein-to-whey ratio than milk from cows milked TAD. This finding is in contrast with previous research, of which many found increased whey protein concentrations due to an increased influx through weakened tight junctions between mammary epithelial cells. In many studies, however, the milking frequency is reduced temporarily, which would induce the involution process as observed in late lactation. Results suggest that the cows from the OAD milking system in the present study may have adapted to the decreased milking frequency. Additionally, although the somatic cell count was higher in OAD than in TAD milk, they were still within limits in which levels of subclinical mastitis are acceptable and do not infringe penalties by the milk processing plants (SmartSAMM, 2013). Other studies have discussed that the casein-to-whey ratio in milk only decreases with excessively high somatic cell counts (above 200,000 cells/mL) (Coulon et al., 1998; Li et al., 2019).

The study design was different from many of the other studies in two ways. Firstly, Massey University Dairy Farm No.1 switched to OAD milking in 2013. Since then, cows not suitable for OAD have been culled and replaced by superior cows for OAD milking and sires selected on a OAD selection index. As a result, the herd at Dairy No. 1 is more suitable for OAD milking. This herd offers a more realistic view into the effect of a OAD milking system on the milk composition because, in reality, cows unsuitable for OAD milking would be culled and replaced. Secondly, in many studies the milking frequency is reduced temporarily, which would provide relevant information for a situation where a farmer may switch to OAD mid-season, for example, because of unfavourable weather conditions or feed shortages.

However, the research question of the present research was whether milk from a OAD milking production system (long-term) has different properties than milk from a conventional TAD milking system. Thus, the present study provides a better understanding of how long-term OAD milking, in which cows are selected and bred for this lower milking frequency, impacts milk composition and processing properties than other studies investigating the effects and mechanisms of temporarily reducing the milking frequency.

One of the main limitations of this research was the sample size of the study. No other studies were done on the effect of OAD milking, compared to TAD milking, for an entire milking season on the protein composition and processing properties of bovine milk. The small sample size was highlighted in Chapter 5. Although the research was not designed to study the breed effect on the protein composition in a OAD and in a TAD milking system, this part of the data was explored, and the results are reported in Chapter 5. Large standard errors were found here.

Additionally, the diet of the cows differed between the two Massey University dairy farms which was a confounding factor. In Chapter 4 the diet composition was presented and discussed. Although there is no evidence that OAD milked cows need less feed, the animals Dairy No. 1 were offered less feed, based on the total DM, than the animals on Dairy No. 4. Both farms, however, are pasture-based and the relative amount of pasture in the diet was similar. There was some variation in the supplementary feeds offered to the cows. For example, Dairy No. 1 offered chicory and tapioca which was not fed at Dairy No. 4. At Dairy No.4, molasses, and dry roughage such as hay and straw as fed. Other supplementary feeds were similar, but the amounts and timing differed. Overall, differences in feed are inevitable when comparing animals on two different farms. To avoid this confounding factor, cows suitable for either OAD or TAD would have to be moved from one farm to the other to limit differences in farm management which is a costly experimental design.

Genetic polymorphism of the proteins occurs in milk. Multiple genetic variants are known to exist for each of the main proteins (α-casein, β-casein, κ-CN, α-LA, and β-LG). For κ-CN, variants A and B are the most common (Caroli et al., 2009). Milk can have either or both variants and κ-CN B has been associated with higher proportions of κ-CN in the milk (Ng-Kwai-Hang and Grosclaude, 2003) and higher degrees of glycosylation (Coolbear et al., 1996). Bonfatti et al. (2014) also showed that the κ-CN glycosylation degree has a high heritability. In the present study, the genetic polymorphism of proteins in the milk was not part of the selection criteria for the cows included in the study. Results showed a higher κ-CN content and κ-CN glycosylation degree in OAD compared to TAD milk. The higher κ-CN glycosylation degree might partly be explained by the enhanced involution process as compared to late lactation milk. However, since the cows at the OAD milking farm have been selected for this milking frequency for a number of years, there may be a genetic component to the change in the protein composition. It is recommended that future research ensures cows with similar genetic variants of these proteins are used. Additionally, future research could look at the prevalence of certain genetic variants in cows that are deemed more suitable for OAD milking. This point raised the question of whether there is a correlation between genetic traits that makes an animal more suitable for OAD milking and polymorphism in the milk proteins.

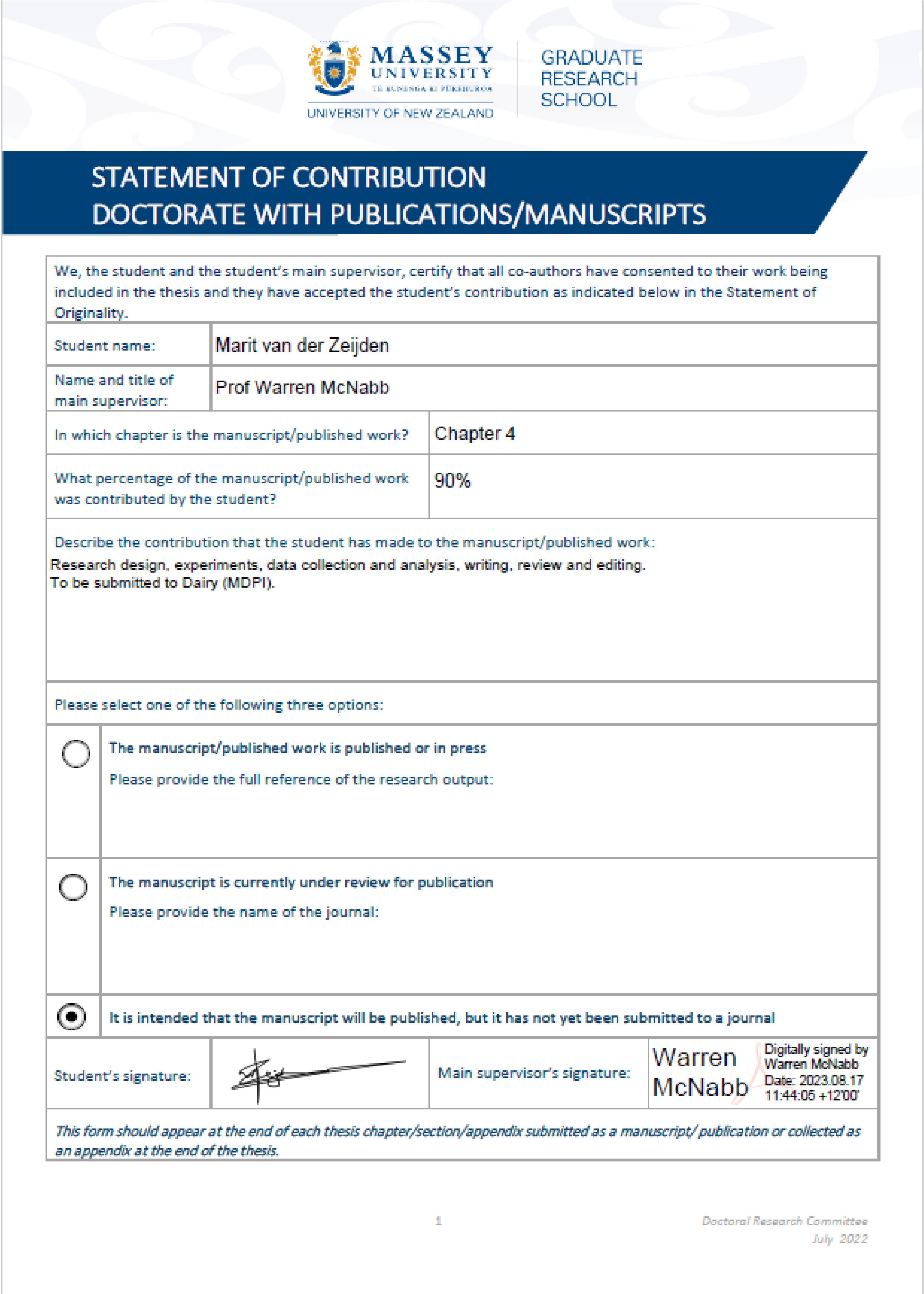
Changes in the milk composition, such as protein and fat contents along a change in the protein composition, may have confounded some of the results with the effect of one component counteracting the effect of another component. Although the results in Chapter 6 showed differences in the proximate and protein composition, these changes did not result in notable differences in the heating (Chapter 7) or gelation (Chapter 8) properties. In industry, it is common practice to standardise the fat and protein contents in the milk. For example, Li et al. (2019) found a seasonal effect on the storage moduli of acid gels from standardised milk, but not on those from non-standardised milk. In the present study, milk was not standardised prior to the gelation experiments. The difference in the protein and fat contents between OAD and TAD milk could have confounded the impact of differences in the protein composition. It is recommended that future research includes standardisation of the milk prior to gelation experiments to limit the confounding effect of changes in the protein and fat content as a result of OAD milking.

To summarise, this research was the first to report on the protein and mineral composition in milk from cows selected for OAD milking. The results showed that OAD milking affects protein composition and some, but not all, minerals in the milk. Despite the changes in the protein and mineral profile of the milk, the effect on heating and gelation properties was limited. However, other questions have been raised, and recommendations for future research have been made. Firstly, further research is needed to understand the genetic component in the observed changes in the protein composition. Secondly, the breed may affect the compositional and processing properties of the milk differently in OAD and TAD milking systems. For processing purposes, standardisation of milk in future studies is recommended. Overall, the results from the present study provided valuable insights into the implications of a OAD milking system for processing purposes.

Statements of contribution

Statements of contribution to chapters intended to be published.

(Chapters 4, 5, 6, 7 and 8)



MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE
RESEARCH
SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Marit van der Zeijden
Name and title of main supervisor:	Prof Warren McNabb
In which chapter is the manuscript/published work?	Chapter 4
What percentage of the manuscript/published work was contributed by the student?	90%

Describe the contribution that the student has made to the manuscript/published work:
Research design, experiments, data collection and analysis, writing, review and editing.
To be submitted to Dairy (MDPI).

Please select one of the following three options:

○ **The manuscript/published work is published or in press**
Please provide the full reference of the research output:

○ **The manuscript is currently under review for publication**
Please provide the name of the journal:

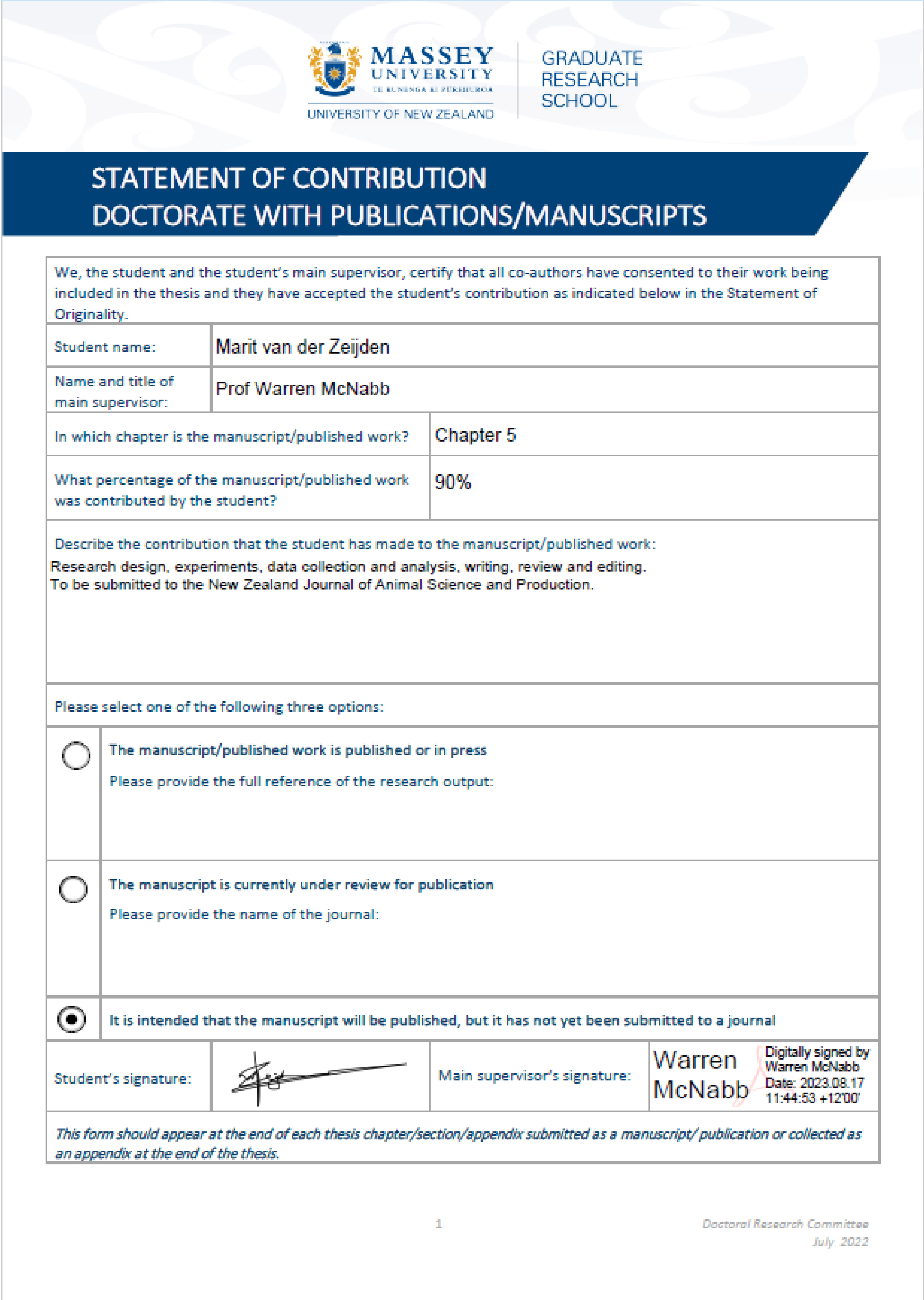
● **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:		Main supervisor's signature:	Warren McNabb Digitally signed by Warren McNabb Date: 2023.08.17 11:44:05 +12'00'

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

1

Doctoral Research Committee
July 2022



MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE
RESEARCH
SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Marit van der Zeijden
Name and title of main supervisor:	Prof Warren McNabb
In which chapter is the manuscript/published work?	Chapter 5
What percentage of the manuscript/published work was contributed by the student?	90%

Describe the contribution that the student has made to the manuscript/published work:
Research design, experiments, data collection and analysis, writing, review and editing.
To be submitted to the New Zealand Journal of Animal Science and Production.

Please select one of the following three options:

○ **The manuscript/published work is published or in press**
Please provide the full reference of the research output:

○ **The manuscript is currently under review for publication**
Please provide the name of the journal:

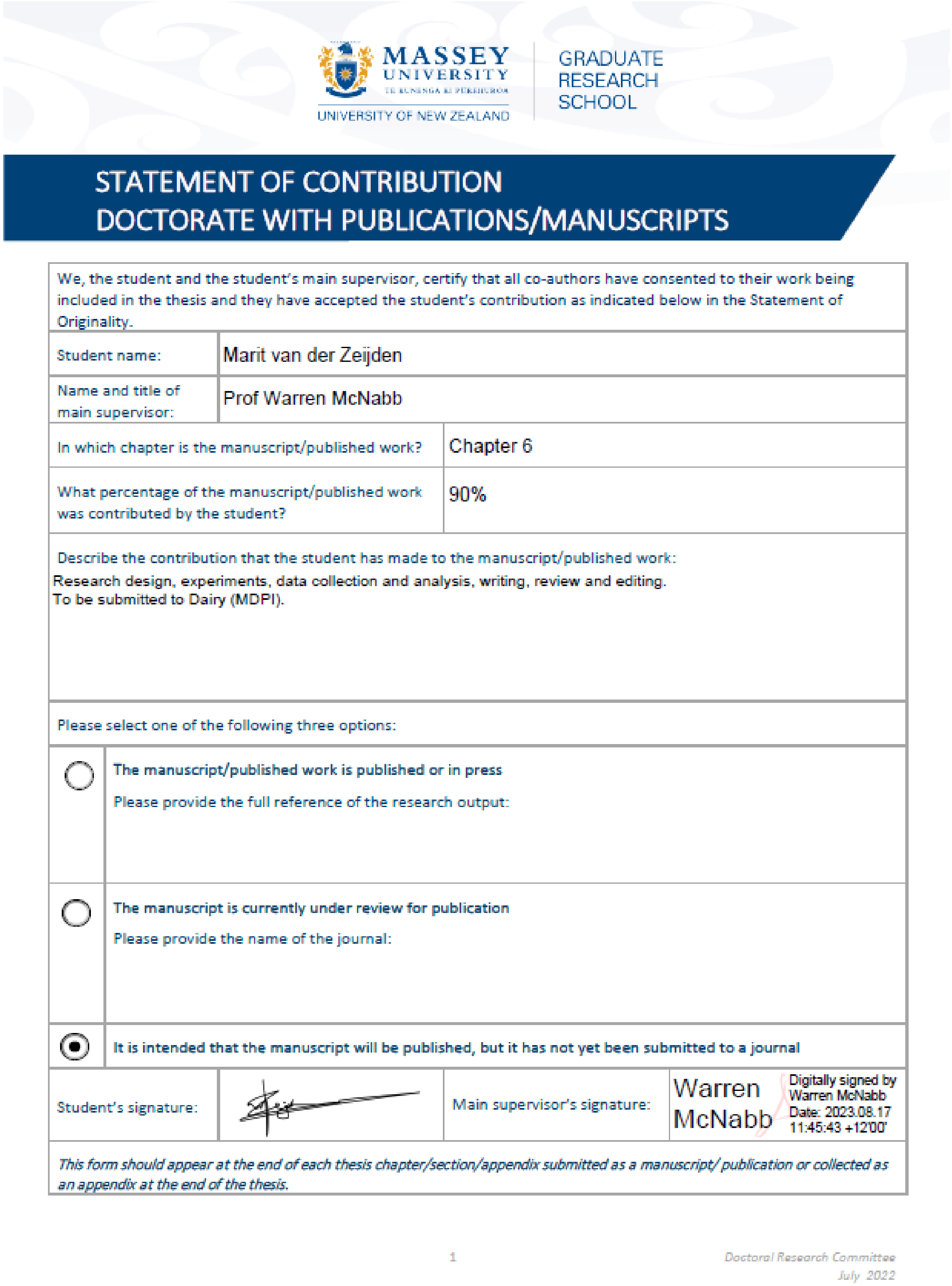
● **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:		Main supervisor's signature:	Warren McNabb Digitally signed by Warren McNabb Date: 2023.08.17 11:44:53 +12'00'

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

1

Doctoral Research Committee
July 2022



MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE
RESEARCH
SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Marit van der Zeijden
Name and title of main supervisor:	Prof Warren McNabb
In which chapter is the manuscript/published work?	Chapter 6
What percentage of the manuscript/published work was contributed by the student?	90%

Describe the contribution that the student has made to the manuscript/published work:
Research design, experiments, data collection and analysis, writing, review and editing.
To be submitted to Dairy (MDPI).

Please select one of the following three options:

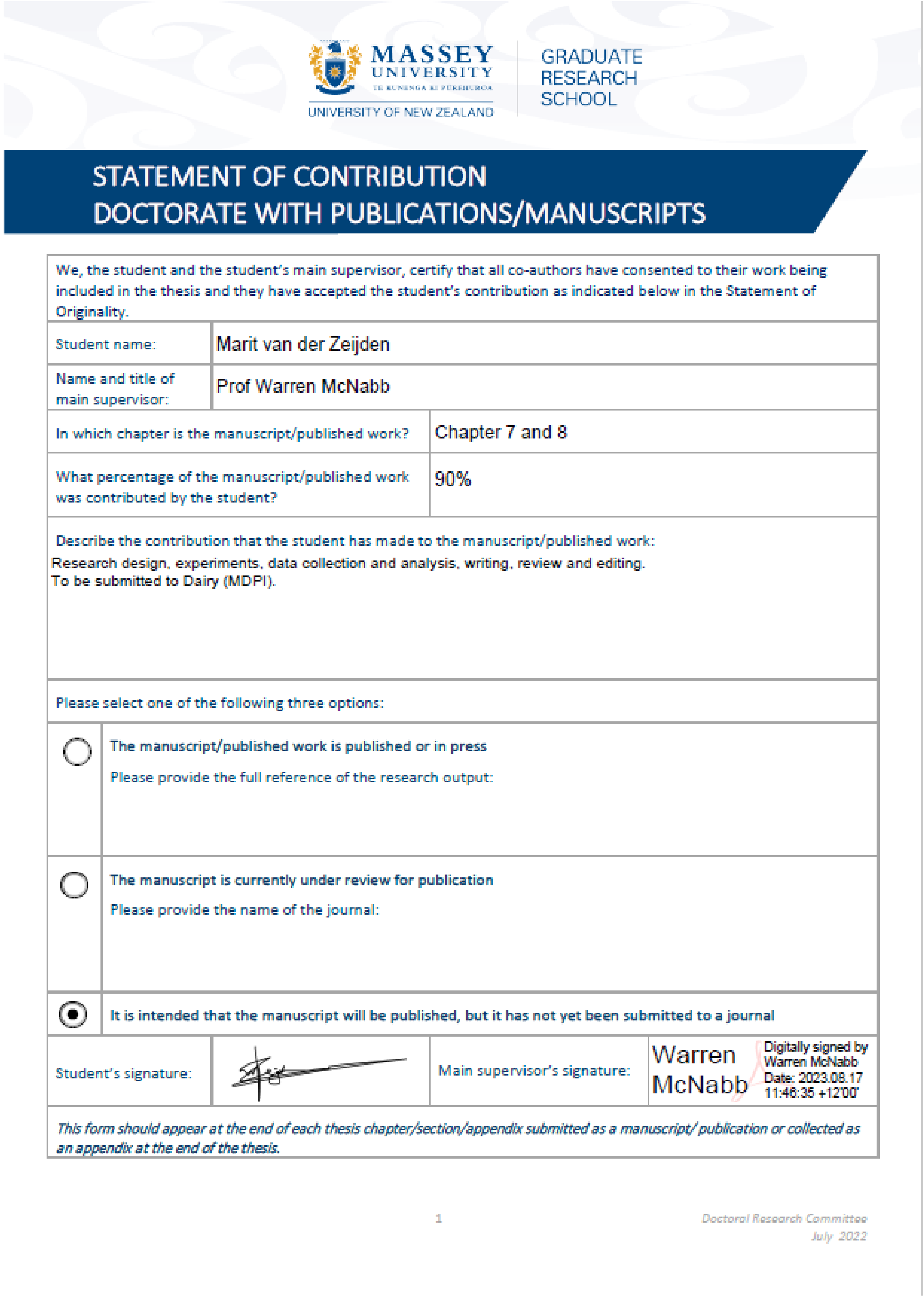
○	**The manuscript/published work is published or in press** Please provide the full reference of the research output:
○	**The manuscript is currently under review for publication** Please provide the name of the journal:
●	**It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:		Main supervisor's signature:	Warren McNabb Digitally signed by Warren McNabb Date: 2023.08.17 11:45:43 +12'00'

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

1

Doctoral Research Committee
July 2022



MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE
RESEARCH
SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Marit van der Zeijden
Name and title of main supervisor:	Prof Warren McNabb
In which chapter is the manuscript/published work?	Chapter 7 and 8
What percentage of the manuscript/published work was contributed by the student?	90%

Describe the contribution that the student has made to the manuscript/published work:
Research design, experiments, data collection and analysis, writing, review and editing.
To be submitted to Dairy (MDPI).

Please select one of the following three options:

○ **The manuscript/published work is published or in press**

Please provide the full reference of the research output:

○ **The manuscript is currently under review for publication**

Please provide the name of the journal:

● **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:		Main supervisor's signature:	Warren McNabb Digitally signed by Warren McNabb Date: 2023.08.17 11:46:35 +12'00'

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

1

Doctoral Research Committee
July 2022

References

Abeni F, Degano L, Calza F, Giangiacomo R, and Pirlo G 2005. Milk quality and automatic milking: Fat globule size, natural creaming, and lipolysis. Journal of Dairy Science 88: 3519-3529.

Ahlborn G, and Dempfle L 1992. Genetic parameters for milk production and body size in New Zealand Holstein-Friesian and Jersey. Livestock Production Science 31: 205-219.

Ahmad S, Piot M, Rousseau F, Grongnet JF, and Gaucheron F 2009. Physico-chemical changes in casein micelles of buffalo and cow milks as a function of alkalinisation. Dairy Science and Technology 89: 387-403.

Akkerman M, Johansen LB, Rauh V, Sørensen J, Larsen LB, and Poulsen NA 2021. Relationship between casein micelle size, protein composition and stability of UHT milk. International Dairy Journal 112, Article 104856.

Alex AP, Collier JL, Hadsell DL, and Collier RJ 2015. Milk yield differences between 1× and 4× milking are associated with changes in mammary mitochondrial number and milk protein gene expression, but not mammary cell apoptosis or SOCS gene expression. Journal of Dairy Science 98: 4439-4448.

Alichanidis E, Moatsou G, and Polychroniadou A 2016. Composition and properties of non-cow milk and products. In Non-bovine milk and milk products. pp. 81-116.

Amalfitano N, Cipolat-Gotet C, Cecchinato A, Malacarne M, Summer A, and Bittante G 2019. Milk protein fractions strongly affect the patterns of coagulation, curd firming, and syneresis. Journal of Dairy Science 102: 2903-2917.

Anema SG 1998. Effect of milk concentration on heat-induced, pH-dependent dissociation of casein from micelles in reconstituted skim milk at temperatures between 20 and 120 °C. Journal of Agricultural and Food Chemistry 46: 2299-2305.

Anema SG 2007. Role of κ-casein in the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. Journal of Agricultural and Food Chemistry 55: 3635-3642.

Anema SG 2008a. Effect of milk solids concentration on the gels formed by the acidification of heated pH-adjusted skim milk. Food Chemistry 108: 110-118.

Anema SG 2008b. On heating milk, the dissociation of κ-casein from the casein micelles can precede interactions with the denatured whey proteins. Journal of Dairy Research 75: 415-421.

Anema SG 2008c. The whey proteins in milk: Thermal denaturation, physical interactions and effects on the functional properties of milk. In Milk proteins. pp. 239-281.

Anema SG 2022. Denaturation of α-lactalbumin and bovine serum albumin in pressure-treated reconstituted skim milk. Food Chemistry Advances 1: 100002.

Anema SG, Lee SK, Lowe EK, and Klostermeyer H 2004. Rheological properties of acid gels prepared from heated pH -adjusted skim milk. Journal of Agricultural and Food Chemistry 52: 337-343.

Anema SG, and Li Y 2003. Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. Journal of Dairy Research 70: 73-83.

Anema SG, Siew KL, and Klostermeyer H 2006. Effect of protein, nonprotein-soluble components, and lactose concentrations on the irreversible thermal denaturation of β-lactoglobulin and α-lactalbumin in skim milk. Journal of Agricultural and Food Chemistry 54: 7339-7348.

Argov N, Lemay DG, and German JB 2008. Milk fat globule structure and function: Nanoscience comes to milk production. Trends in Food Science & Technology 19: 617-623.

Armand M, Pasquier B, André M, Borel P, Senft M, Peyrot J, Salducci J, Portugal H, Jaussan V, and Lairon D 1999. Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. American Journal of Clinical Nutrition 70: 1096-1106.

Auldist M 2011. Milk quality and udder health: Effect on processing characteristics. In Encyclopedia of dairy sciences: Second edition. pp. 902-907.

Auldist M, and Prosser C 1998. Differential effects of short-term once-daily milking on milk yield, milk composition and concentrations of selected blood metabolites in cows with low or high pasture intake. Proceedings of the New Zealand Society of Animal Production, 58: 41-43.

Auldist MJ, Coats S, Rogers GL, and McDowell GH 1995. Changes in the composition of milk from healthy and mastitic dairy cows during the lactation cycle. Australian Journal of Experimental Agriculture 35: 427-436.

Auldist MJ, Coats S, Sutherland BJ, Mayes JJ, McDowell GH, and Rogers GL 1996. Effects of somatic cell count and stage of lactation on raw milk composition and the yield and quality of cheddar cheese. Journal of Dairy Research 63: 269-280.

Auldist MJ, and Hubble IB 1998. Effects of mastitis on raw milk and dairy products. Australian Journal of Dairy Technology 53: 28-36.

Auldist MJ, Johnston KA, White NJ, Fitzsimons WP, and Boland MJ 2004. A comparison of the composition, coagulation characteristics and cheesemaking capacity of milk from Friesian and Jersey dairy cows. Journal of Dairy Research 71: 51-57.

Auldist MJ, Walsh BJ, and Thomson NA 1998. Seasonal and lactational influences on bovine milk composition in New Zealand. Journal of Dairy Research 65: 401-411.

Back P, and Lopez-Villalobos N 2007. Breed and heterosis effects for milk protein composition estimated in two stages of lactation in New Zealand dairy cows. Proceedings of the New Zealand Society of Animal Production. 67: 399.

Bansal N, and Bhandari B 2016. Functional milk proteins: Production and utilization—whey-based ingredients. In PLH McSweeney and JA O'Mahony (Eds.), Advanced dairy chemistry: Volume 1B: Proteins: Applied aspects. pp. 67-98. Springer New York

Barbano DM, Ma Y, and Santos MV 2006a. Influence of raw milk quality on fluid milk shelf life. Journal of Dairy Science 89 Suppl 1: E15-19.

Barbano DM, Ma Y, and Santos MV 2006b. Influence of raw milk quality on fluid milk shelf life. Journal of Dairy Science 89: E15-E19.

Barlowska J, Szwajkowska M, Litwińczuk Z, and Król J 2011. Nutritional value and technological suitability of milk from various animal species used for dairy production. Comprehensive Reviews in Food Science and Food Safety 10: 291-302.

Barone G, Yazdi SR, Lillevang SK, and Ahrné L 2021. Calcium: A comprehensive review on quantification, interaction with milk proteins and implications for processing of dairy products. Comprehensive Reviews in Food Science and Food Safety 20: 5616-5640.

Barry JG, and Donnelly WJ 1980. Casein compositional studies. The composition of casein from Friesian herd milks. Journal of Dairy Research 47: 71-81.

Beever DE, Sutton JD, and Reynolds CK 2001. Increasing the protein content of cow's milk. Australian Journal of Dairy Technology 56: 138-149.

Bernier-Dodier P, Delbecchi L, Wagner GF, Talbot BG, and Lacasse P 2010. Effect of milking frequency on lactation persistency and mammary gland remodeling in mid-lactation cows. Journal of Dairy Science 93: 555-564.

Bewsell D, Clark DA, and Dalley DE 2008. Understanding motivations to adopt once-a-day milking amongst New Zealand dairy farmers. The Journal of Agricultural Education and Extension 14: 69-80.

Bijl E, de Vries R, van Valenberg H, Huppertz T, and van Hooijdonk T 2014. Factors influencing casein micelle size in milk of individual cows: Genetic variants and glycosylation of κ-casein. International Dairy Journal 34: 135-141.

Bittante G, Cipolat-Gotet C, Malchiodi F, Sturaro E, Tagliapietra F, Schiavon S, and Cecchinato A 2015. Effect of dairy farming system, herd, season, parity, and days in milk on modeling of the coagulation, curd firming, and syneresis of bovine milk. Journal of Dairy Science 98: 2759-2774.

Bland JH, Grandison AS, and Fagan CC 2015. Effect of blending Jersey and Holstein-Friesian milk on cheddar cheese processing, composition, and quality. Journal of Dairy Science 98: 1-8.

Bobe G, Beitz DC, Freeman AE, and Lindberg GL 1998. Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. Journal of Agricultural and Food Chemistry 46: 458-463.

Bonfatti V, Chiarot G, and Carnier P 2014. Glycosylation of κ-casein: Genetic and nongenetic variation and effects on rennet coagulation properties of milk. Journal of Dairy Science 97: 1961-1969.

Brew K 2003. α-lactalbumin. In Advanced dairy chemistry—1 proteins. pp. 387-419. Springer

Britten M, and Giroux HJ 2022. Rennet coagulation of heated milk: A review. International Dairy Journal 124: 105179.

Broyard C, and Gaucheron F 2015. Modifications of structures and functions of caseins: A scientific and technological challenge. Dairy Science and Technology 95: 831-862.

Burton H 2012. Ultra-high-temperature processing of milk and milk products. Springer Science & Business Media.

Caroli AM, Chessa S, and Erhardt GJ 2009. Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. Journal of Dairy Science 92: 5335-5352.

Carruthers VR, Davis SR, Bryant AM, and Copeman PJ 1993. Response of Jersey and Friesian cows to once a day milking and prediction of response based on udder characteristics and milk composition. Journal of Dairy Research 60: 1-11.

Cases E, Vidal V, and Cuq JL 2003. Effect of κ-casein deglycosylation on the acid coagulability of milk. Journal of Food Science 68: 2406-2410.

Ceballos LS, Morales ER, de la Torre Adarve G, Castro JD, Martínez LP, and Sampelayo MRS 2009. Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. Journal of Food Composition and Analysis 22: 322-329.

Cerbulis J, and Farrell HM, Jr. 1975. Composition of milks of dairy cattle. I. Protein, lactose, and fat contents and distribution of protein fraction. Journal of Dairy Science 58: 817-827.

Chen S, Bobe G, Zimmerman S, Hammond EG, Luhman CM, Boylston TD, Freeman AE, and Beitz DC 2004. Physical and sensory properties of dairy products from cows with various milk fatty acid compositions. Journal of Agricultural and Food Chemistry 52: 3422-3428.

Cipolat-Gotet C, Cecchinato A, Malacarne M, Bittante G, and Summer A 2018. Variations in milk protein fractions affect the efficiency of the cheese-making process. Journal of Dairy Science 101: 8788-8804.

Claeys WL, Verraes C, Cardoen S, De Block J, Huyghebaert A, Raes K, Dewettinck K, and Herman L 2014. Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. Food Control 42: 188-201.

Clark DA, Phyn CVC, Tong MJ, Collis SJ, and Dalley DE 2006. A systems comparison of once- versus twice-daily milking of pastured dairy cows. Journal of Dairy Science 89: 1854-1862.

Colahan-Sederstrom PM, and Peterson DG 2005. Inhibition of key aroma compound generated during ultrahigh-temperature processing of bovine milk via epicatechin addition. Journal of Agricultural and Food Chemistry 53: 398-402.

Coolbear KP, Elgar DF, and Ayers JS 1996. Profiling of genetic variants of bovine κ-casein macropeptide by electrophoretic and chromatographic techniques. International Dairy Journal 6: 1055-1068.

Correa-Luna M, Donaghy D, Kemp P, Shalloo L, Ruelle E, Hennessy D, and López-Villalobos N 2021. Productivity, profitability and nitrogen utilisation efficiency of two pasture-based milk production systems differing in the milking frequency and feeding level. Sustainability (Switzerland) 13: 1-15, Article 2098.

Corredig M, and Dalgleish DG 1996. Effect of different heat treatments on the strong binding interactions between whey proteins and milk fat globules in whole milk. Journal of Dairy Research 63: 441-449.

Costa A, Lopez-Villalobos N, Sneddon NW, Shalloo L, Franzoi M, De Marchi M, and Penasa M 2019. Invited review: Milk lactose—current status and future challenges in dairy cattle. Journal of Dairy Science 102: 5883-5898.

Coulon J-B, Hurtaud C, Rémond B, and Verite R 1998. Factors contributing to variation in the proportion of casein in cows' milk true protein: A review of recent inra experiments. Journal of Dairy Research 65: 375-387.

Cowley FC, Barber DG, Houlihan AV, and Poppi DP 2015. Immediate and residual effects of heat stress and restricted intake on milk protein and casein composition and energy metabolism. Journal of Dairy Science 98: 2356-2368.

Cressey PJ 2003. Iodine content of New Zealand dairy products. Journal of Food Composition and Analysis 16: 25-36.

Crowley SV, Megemont M, Gazi I, Kelly AL, Huppertz T, and O'Mahony JA 2014. Heat stability of reconstituted milk protein concentrate powders. International Dairy Journal 37: 104-110.

DairyNZ 2020. *Once-a-day milking*. Retrieved 21 May 2020 from www.dairynz.co.nz/milking/once-a-day-milking

DairyNZ 2021. *Breeding worth*. Retrieved 18 June 2021 from www.dairynz.co.nz/animal/animal-evaluation/interpreting-the-info/all-about-bw

DairyNZ 2022. *Feed values*. Retrieved 15 June 2022 from www.dairynz.co.nz/feed/supplements/feed-values

Dalgleish D, and Sharma S 1993. Interactions between milkfat and milk proteins. The effect of heat on the nature of the complexes formed. Protein and fat globule modifications by heat treatment, homogenization and other technological means for high quality dairy products. IDF Seminar, Munich (Germany), 25-28 Aug 1992.

Dalgleish DG 1983. Coagulation of renneted bovine casein micelles: Dependence on temperature, calcium ion concentration and ionic strength. Journal of Dairy Research 50: 331-340.

Dalgleish DG 2011. On the structural models of bovine casein micelles—review and possible improvements. Soft Matter 7: 2265-2272.

Dalgleish DG, and Pouliot Y 1987. Studies on the heat stability of milk ii. Association and dissociation of particles and the effect of added urea. Journal of Dairy Research 54: 39-49.

Dannenberg F, and Kessler HG 1988. Reaction kinetics of the denaturation of whey proteins in milk. Journal of Food Science 53: 258-263.

Davis SR, Farr VC, and Stelwagen K 1999. Regulation of yield loss and milk composition during once-daily milking: A review. Livestock Production Science 59: 77-94.

De Kruif CG, and Huppertz T 2012. Casein micelles: Size distribution in milks from individual cows. Journal of Agricultural and Food Chemistry 60: 4649-4655.

De Maria CG, and Angelucci M 1978. Trace element content in colostrum of different ruminant species at various post-partum intervals Annales de Recherches Vétérinaires, 9: 277-280.

De Villiers PA 1976. Die invloed van die melkinteval op melkprodukksie. South African Journal of Animal Science 6: 181-185.

Deeth HC, and Lewis MJ 2015. Practical consequences of calcium addition to and removal from milk and milk products. International Journal of Dairy Technology 68: 1-10.

Del Angel CR, and Dalgleish DG 2006. Structures and some properties of soluble protein complexes formed by the heating of reconstituted skim milk powder. Food Research International 39: 472-479.

Devold TG, Brovold MJ, Langsrud T, and Vegarud GE 2000. Size of native and heated casein micelles, content of protein and minerals in milk from Norwegian Red cattle - effect of milk protein polymorphism and different feeding regimes. International Dairy Journal 10: 313-323.

Dickinson E, and Matsumura Y 1991. Time-dependent polymerization of β-lactoglobulin through disulphide bonds at the oil-water interface in emulsions. International Journal of Biological Macromolecules 13: 26-30.

Dillon P, Roche JR, Shalloo L, and Horan B 2005. Optimising financial return from grazing in temperate pastures. In Utilisation of grazed grass in temperate animal systems: Proceedings of a satellite workshop of the xxth international grassland congress, july 2005, cork, ireland. pp. 131-147.

Dinkov K, and Dushkova M 2007. Effect of heat treatment of milk on the rennet coagulation and rheological properties of milk gels. Milchwissenschaft 62: 133-135.

Donato L, Alexander M, and Dalgleish DG 2007. Acid gelation in heated and unheated milks: Interactions between serum protein complexes and the surfaces of casein micelles. Journal of Agricultural and Food Chemistry. 55: 4160-4168.

Donato L, and Guyomarc'h F 2009. Formation and properties of the whey protein/κ-casein complexes in heated skim milk - a review. Dairy Science and Technology 89: 3-29.

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

Donnelly WJ, McNeill GP, Buchheim W, and McGann TCA 1984. A comprehensive study of the relationship between size and protein composition in natural bovine casein micelles. Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular 789: 136-143.

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

Edwards JP 2018. Comparison of milk production and herd characteristics in New Zealand herds milked once or twice a day. Animal Production Science 59: 570-580.

Edwards JP 2019. A comparison of profitability between farms that milk once or twice a day. Animal Production Science 60: 102-106.

Elfagm AA, and Wheelock JV 1978. Heat interaction between α-lactalbumin, β-lactoglobulin and casein in bovine milk. Journal of Dairy Science 61: 159-163.

Elgersma A 2015. Grazing increases the unsaturated fatty acid concentration of milk from grass-fed cows: A review of the contributing factors, challenges and future perspectives. European Journal of Lipid Science and Technology 117: 1345-1369.

Farrell Jr HM, Jimenez-Flores R, Bleck GT, Brown EM, Butler JE, Creamer LK, Hicks CL, Hollar CM, Ng-Kwai-Hang KF, and Swaisgood HE 2004. Nomenclature of the proteins of cows' milk - sixth revision. Journal of Dairy Science 87: 1641-1674.

Fenaille F, Parisod V, Visani P, Populaire S, Tabet J-C, and Guy PA 2006. Modifications of milk constituents during processing: A preliminary benchmarking study. International Dairy Journal 16: 728-739.

Flachowsky G, Franke K, Meyer U, Leiterer M, and Schöne F 2014. Influencing factors on iodine content of cow milk. European Journal of Nutrition 53: 351-365.

Fleming A, Schenkel FS, Ali RA, Corredig M, Carta S, Gregu CM, Malchiodi F, Macciotta NPP, and Miglior F 2019. Phenotypic investigation of fine milk components in bovine milk and their prediction using mid-infrared spectroscopy. Canadian Journal of Animal Science 99: 218-227.

Fleming A, Schenkel FS, Chen J, Malchiodi F, Ali RA, Mallard B, Sargolzaei M, Corredig M, and Miglior F 2017. Variation in fat globule size in bovine milk and its prediction using mid-infrared spectroscopy. Journal of Dairy Science 100: 1640-1649.

Fong BY, Norris CS, and MacGibbon AKH 2007. Protein and lipid composition of bovine milk-fat-globule membrane. International Dairy Journal 17: 275-288.

Fox PF, Guinee TP, Cogan TM, and McSweeney PL 2017. Fundamentals of cheese science. (Vol. 1) Springer.

Fox PF, Uniacke-Lowe T, McSweeney PLH, and O'Mahony JA 2015. Dairy chemistry and biochemistry. (Second edition ed.) Springer.

Franke AA, Bruhn JC, and Osland RB 1983. Factors affecting iodine concentration of milk of individual cows. Journal of Dairy Science 66: 997-1002.

French EA, Mukai M, Zurakowski M, Rauch B, Gioia G, Hillebrandt JR, Henderson M, Schukken YH, and Hemling TC 2016. Iodide residues in milk vary between iodine-based teat disinfectants. Journal of Food Science 81: T1864-T1870.

Gallier S, Vocking K, Post JA, Van De Heijning B, Acton D, Van Der Beek EM, and Van Baalen T 2015. A novel infant milk formula concept: Mimicking the human milk fat globule structure. Colloids and Surfaces B: Biointerfaces 136: 329-339.

Gaucheron F 2005. The minerals of milk. Reproduction Nutrition Development 45: 473-483.

Gaucheron F 2013. Milk minerals, trace elements, and macroelements. In Milk and dairy products in human nutrition: Production, composition and health. pp. 172-199.

Gaur V, Schalk J, and Anema SG 2018. Sedimentation in UHT milk. International Dairy Journal 78: 92-102.

Gedye K, Notcovich S, Correa-Luna M, Ariyarathne P, Heiser A, Lopez-Lozano R, and Lopez-Villalobos N 2020. Lactoferrin concentration and expression in New Zealand cows milked once or twice a day. Animal Science Journal 91, Article e13331.

Gezimati J, Singh H, and Creamer LK 1996. Aggregation and gelation of bovine β-lactoglobulin, α-lactalbumin, and serum albumin. ACS Symposium Series 650: 113-123.

Glantz M, Devold TG, Vegarud GE, Lindmark Månsson H, Stålhammar H, and Paulsson M 2010. Importance of casein micelle size and milk composition for milk gelation. Journal of Dairy Science 93: 1444-1451.

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

Graveland-Bikker JF, and Anema SG 2003. Effect of individual whey proteins on the rheological properties of acid gels prepared from heated skim milk. International Dairy Journal 13: 401-408.

Guyomarc'h F 2006. Formation of heat-induced protein aggregates in milk as a means to recover the whey protein fraction in cheese manufacture, and potential of heat-treating milk at alkaline pH values in order to keep its rennet coagulation properties. A review. Lait 86: 1-20.

Guyomarc'h F, Nono M, Nicolai T, and Durand D 2009. Heat-induced aggregation of whey proteins in the presence of κ-casein or sodium caseinate. Food Hydrocolloids 23: 1103-1110.

Handcock RC, Lopez-Villalobos N, McNaughton LR, Back PJ, Edwards GR, and Hickson RE 2019. Positive relationships between body weight of dairy heifers and their first-lactation and accumulated three-parity lactation production. Journal of Dairy Science 102: 4577-4589.

Havea P, Singh H, and Creamer LK 2000. Formation of new protein structures in heated mixtures of BSA and α-lactalbumin. Journal of Agricultural and Food Chemistry 48: 1548-1556.

Havea P, Singh H, and Creamer LK 2001. Characterization of heat-induced aggregates of β-lactoglobulin, α-lactalbumin and bovine serum albumin in a whey protein concentrate environment. Journal of Dairy Research 68: 483-497.

Heine WE, Klein PD, and Reeds PJ 1991. The importance of α-lactalbumin in infant nutrition. Journal of Nutrition 121: 277-283.

Hickson RE, Lopez-Villalobos N, Dalley DE, Clark DA, and Holmes CW 2006. Yields and persistency of lactation in Friesian and Jersey cows milked once daily. Journal of Dairy Science 89: 2017-2024.

Holmes C, Wilson G, Mackenzie D, and Purchas J 1992. The effects of milking once-daily throughout lactation on the performance of dairy cows grazing on pasture. Proceedings of the New Zealand Society Of Animal Production. 52: 13-13.

Holmes CW 2002. Milk production from pasture. Principles and practices. Massey University.

Huang W, Peñagaricano F, Ahmad KR, Lucey JA, Weigel KA, and Khatib H 2012. Association between milk protein gene variants and protein composition traits in dairy cattle. Journal of Dairy Science 95: 440-449.

Ianni A, Innosa D, Martino C, Grotta L, Bennato F, and Martino G 2019. Zinc supplementation of Friesian cows: Effect on chemical-nutritional composition and aromatic profile of dairy products. Journal of Dairy Science 102: 2918-2927.

Ikonen T, Morri S, Tyrisevä AM, Ruottinen O, and Ojala M 2004. Genetic and phenotypic correlations between milk coagulation properties, milk production traits, somatic cell count, casein content, and pH of milk. Journal of Dairy Science 87: 458-467.

Ingham B, Smialowska A, Kirby NM, Wang C, and Carr AJ 2018. A structural comparison of casein micelles in cow, goat and sheep milk using x-ray scattering. Soft Matter 14: 3336-3343.

Inglingstad RA, Devold TG, Eriksen EK, Holm H, Jacobsen M, Liland KH, Rukke EO, and Vegarud GE 2010. Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. Dairy Science and Technology 90: 549-563.

Jensen RG 2002. The composition of bovine milk lipids: January 1995 to december 2000. Journal of Dairy Science 85: 295-350.

Jørgensen CE, Abrahamsen RK, Rukke EO, Johansen AG, and Skeie SB 2017. Fractionation by microfiltration: Effect of casein micelle size on composition and rheology of high protein, low fat set yoghurt. International Dairy Journal 74: 12-20.

Kalač P, and Samková E 2010. The effects of feeding various forages on fatty acid composition of bovine milk fat: A review. Czech Journal of Animal Science 55: 521-537.

Kedzierska-Matysek M, Litwinczuk Z, Florek M, and Barlowska J 2011. The effects of breed and other factors on the composition and freezing point of cow's milk in poland. International Journal of Dairy Technology 64: 336-342.

Kelly AL, Huppertz T, and Sheehan JJ 2008. Pre-treatment of cheese milk: Principles and developments. Dairy Science and Technology. 88: 549-572.

Kelly AL, Reid S, Joyce P, Meaney WJ, and Foley J 1998. Effect of decreased milking frequency of cows in late lactation on milk somatic cell count, polymorphonuclear leucocyte numbers, composition and proteolytic activity. Journal of Dairy Research 65: 365-373.

Kendall PE, Tucker CB, Dalley DE, Clark DA, and Webster JR 2008. Milking frequency affects the circadian body temperature rhythm in dairy cows. Livestock Science 117: 130-138.

Kethireddipalli P, Hill AR, and Dalgleish DG 2010. Protein interactions in heat-treated milk and effect on rennet coagulation. International Dairy Journal 20: 838-843.

Kitchen BJ, Middleton G, Durward IG, Andrews RJ, and Salmon MC 1980. Mastitis diagnostic tests to estimate mammary gland epithelial cell damage. Journal of Dairy Science 63: 978-983.

Klei LR, Lynch JM, Barbano DM, Oltenacu PA, Lednor AJ, and Bandler DK 1997. Influence of milking three times a day on milk quality. Journal of Dairy Science 80: 427-436.

Knight CH, and Dewhurst R 1994. Once daily milking of dairy cows: Relationship between yield loss and cisternal-milk storage. Journal of Dairy Research 61: 441-449.

Komara M, Boutinaud M, Ben Chedly H, Guinard-Flament J, and Marnet PG 2009. Once-daily milking effects in high-yielding alpine dairy goats. Journal of Dairy Science 92: 5447-5455.

Kontopidis G, Holt C, and Sawyer L 2004. Invited review: β-lactoglobulin: Binding properties, structure, and function. Journal of Dairy Science 87: 785-796.

Kücükcetin A 2008. Effect of heat treatment and casein to whey protein ratio of skim milk on graininess and roughness of stirred yoghurt. Food Research International 41: 165-171.

Lacy-Hulbert SJ, Woolford MW, Nicholas GD, Prosser CG, and Stelwagen K 1999. Effect of milking frequency and pasture intake on milk yield and composition of late lactation cows. Journal of Dairy Science 82: 1232-1239.

Lakemond CMM, and van Vliet T 2008. Acid skim milk gels: The gelation process as affected by preheating pH. International Dairy Journal 18: 574-584.

Law AJR, and Leaver J 1997. Effect of protein concentration on rates of thermal denaturation of whey proteins in milk. Journal of Agricultural and Food Chemistry 45: 4255-4261.

Layman DK, Lönnerdal B, and Fernstrom JD 2018. Applications for α-lactalbumin in human nutrition. Nutrition Reviews 76: 444-460.

Lazzarini B, Lopez-Villalobos N, Lyons N, Hendrikse L, and Baudracco J 2018. Productive, economic and risk assessment of grazing dairy systems with supplemented cows milked once a day. animal 12: 1077-1083.

Lee SJ, and Sherbon JW 2002. Chemical changes in bovine milk fat globule membrane caused by heat treatment and homogenization of whole milk. Journal of Dairy Research 69: 555-567.

Lefèvre T, and Subirade M 2003. Formation of intermolecular β-sheet structures: A phenomenon relevant to protein film structure at oil–water interfaces of emulsions. Journal of Colloid and Interface Science 263: 59-67.

Lembeye F, Lopez-Villalobos N, Burke JL, and Davis SR 2016a. Breed and heterosis effects for milk yield traits at different production levels, lactation number and milking frequencies. New Zealand Journal of Agricultural Research 59: 156-164.

Lembeye F, Lopez-Villalobos N, Burke JL, and Davis SR 2016b. Estimation of genetic parameters for milk traits in cows milked once- or twice-daily in New Zealand. Livestock Science 185: 142-147.

Lembeye F, Lopez-Villalobos N, Burke JL, and Davis SR 2016c. Milk production of Holstein-Friesian, Jersey and crossbred cows milked once-a-day or twice-a-day in New Zealand. New Zealand Journal of Agricultural Research 59: 50-64.

Lembeye F, Lopez-Villalobos N, Burke JL, Davis SR, Richardson J, Sneddon NW, and Donaghy DJ 2016. Comparative performance in Holstein-Friesian, Jersey and crossbred cows milked once daily under a pasture-based system in New Zealand. New Zealand Journal of Agricultural Research 59: 351-362.

Li S, Ye A, and Singh H 2019. Seasonal variations in composition, properties, and heat-induced changes in bovine milk in a seasonal calving system. Journal of Dairy Science 102: 7747-7759.

Li S, Ye A, and Singh H 2020. Effect of seasonal variations on the acid gelation of milk. Journal of Dairy Science 103: 4965-4974.

Linzell J, and Peaker M 1971. Intracellular concentrations of sodium, potassium and chloride in the lactating mammary gland and their relation to the secretory mechanism. The Journal of physiology 216: 683-700.

Liu Z, Logan A, Cocks BG, and Rochfort S 2017. Seasonal variation of polar lipid content in bovine milk. Food Chemistry 237: 865-869.

Livestock Improvement Corporation Ltd. (LIC), and DairyNZ. (2021). New Zealand dairy statistics 2020-2021.

Logan A, Leis A, Day L, Øiseth SK, Puvanenthiran A, and Augustin MA 2015. Rennet gelation properties of milk: Influence of natural variation in milk fat globule size and casein micelle size. International Dairy Journal 46: 71-77.

Lopez-Villalobos N, Garrick DJ, Holmes CW, Blair HT, and Spelman RJ 2000. Profitabilities of some mating systems for dairy herds in New Zealand. Journal of Dairy Science 83: 144-153.

Lopez-Villalobos N, Jayawardana JMDR, McNaughton LR, and Hickson RE 2023. A review of once-a-day milking in dairy cow grazing systems. Presented as part of the joint ADSA midwest branch/forages and pastures symposium: Grazing to improve profitability of midwest dairy farms held at the adsa annual meeting, june 2022. JDS Communications 4: 329-333.

Lopez C 2011. Milk fat globules enveloped by their biological membrane: Unique colloidal assemblies with a specific composition and structure. Current Opinion in Colloid & Interface Science 16: 391-404.

Lopez C, Bourgaux C, Lesieur P, Bernadou S, Keller G, and Ollivon M 2002. Thermal and structural behavior of milk fat - 3. Influence of cooling rate and droplet size on cream crystallization. Journal of Colloid and Interface Science 254: 64-78.

Lucey JA 2001. The relationship between rheological parameters and whey separation in milk gels. Food Hydrocolloids. 15: 603-608.

Lucey JA 2002. Formation and physical properties of milk protein gels. Journal of Dairy Science 85: 281-294.

Lucey JA 2016. Acid coagulation of milk. In P. L. H. McSweeney & J. A. O'Mahony (Eds.), Advanced dairy chemistry: Volume 1b: Proteins: Applied aspects. pp. 309-328. Springer New York

Lucey JA, and Singh H 1997. Formation and physical properties of acid milk gels: A review. Food Research International 30: 529-542.

Lucey JA, Tamehana M, Singh H, and Munro PA 1998. Effect of interactions between denatured whey proteins and casein micelles on the formation and rheological properties of acid skim milk gels. Journal of Dairy Research 65: 555-567.

Lucey JA, Wilbanks DJ, and Horne DS 2022. Impact of heat treatment of milk on acid gelation. International Dairy Journal 125: 105222.

Ma Y, Ryan C, Barbano DM, Galton DM, Rudan MA, and Boor KJ 2000. Effects of somatic cell count on quality and shelf-life of pasteurized fluid milk. Journal of Dairy Science 83: 264-274.

Malacarne M, Franceschi P, Formaggioni P, Sandri S, Mariani P, and Summer A 2014. Influence of micellar calcium and phosphorus on rennet coagulation properties of cows milk. Journal of Dairy Research 81: 129-136.

Marchin S, Putaux J-L, Pignon F, and Léonil J 2007. Effects of the environmental factors on the casein micelle structure studied by cryo transmission electron microscopy and small-angle x-ray scattering/ultrasmall-angle x-ray scattering. The Journal of Chemical Physics 126: 045101.

Martin B, Pomiès D, Pradel P, Verdier-Metz L, and Rémond B 2009. Yield and sensory properties of cheese made with milk from Holstein or Montbéliarde cows milked twice or once daily. Journal of Dairy Science 92: 4730-4737.

Martini M, Salari F, and Altomonte I 2016. The macrostructure of milk lipids: The fat globules. Critical Reviews in Food Science and Nutrition 56: 1209-1221.

Matumoto-Pintro PT, Rabiey L, Robitaille G, and Britten M 2011. Use of modified whey protein in yoghurt formulations. International Dairy Journal 21: 21-26.

McClements DJ, Monahan FJ, and Kinsella JE 1993. Disulfide bond formation affects stability of whey protein isolate emulsions. Journal of Food Science 58: 1036-1039.

McPherson AV, and Kitchen BJ 1983. Reviews of the progress of dairy science: The bovine milk fat globule membrane-its formation, composition, structure and behaviour in milk and dairy products. Journal of Dairy Research 50: 107-133.

McSweeney PLH, and Fox PF 2013. Advanced dairy chemistry : Volume 1a: Proteins: Basic aspects, 4th edition Springer.

McSweeney PLH, and O'Mahony JA 2016. Advanced dairy chemistry (Fourth edition ed.) Springer.

Mehdi Y, and Dufrasne I 2016. Selenium in cattle: A review. Molecules 21, Article 545.

Meletharayil GH, Patel HA, and Huppertz T 2015. Rheological properties and microstructure of high protein acid gels prepared from reconstituted milk protein concentrate powders of different protein contents. International Dairy Journal 47: 64-71.

Meletharayil GH, Patel HA, Metzger LE, and Huppertz T 2016. Acid gelation of reconstituted milk protein concentrate suspensions: Influence of lactose addition. International Dairy Journal 61: 107-113.

Michalski MC, Camier B, Briard V, Leconte N, Gassi JY, Goudédranche H, Michel F, and Fauquant J 2004. The size of native milk fat globules affects physico-chemical and functional properties of emmental cheese. Lait 84: 343-358.

Michalski MC, Cariou R, Michel F, and Garnier C 2002. Native vs. damaged milk fat globules: Membrane properties affect the viscoelasticity of milk gels. Journal of Dairy Science 85: 2451-2461.

Michalski MC, Gassi JY, Famelart MH, Leconte N, Camier B, Michel F, and Briard V 2003. The size of native milk fat globules affects physico-chemical and sensory properties of camembert cheese. Lait 83: 131-143.

Michalski MC, and Januel C 2006. Does homogenization affect the human health properties of cow's milk? Trends in Food Science and Technology 17: 423-437.

Moatsou G 2013. Sanitary procedures, heat treatments and packaging. In Milk and dairy products in human nutrition: Production, composition and health. pp. 288-309.

Moatsou G, Hatzinaki A, Samolada M, and Anifantakis E 2005. Major whey proteins in ovine and caprine acid wheys from indigenous greek breeds. International Dairy Journal 15: 123-131.

Morand M, Guyomarc'h F, Legland D, and Famelart MH 2012. Changing the isoelectric point of the heat-induced whey protein complexes affects the acid gelation of skim milk. International Dairy Journal 23: 9-17.

Muir DD, and Sweetsur AWM 1976. The influence of naturally occurring levels of urea on the heat stability of bulk milk. Journal of Dairy Research 43: 495-499.

Murney R, Stelwagen K, Wheeler TT, Margerison JK, and Singh K 2015. The effects of milking frequency in early lactation on milk yield, mammary cell turnover, and secretory activity in grazing dairy cows. Journal of Dairy Science 98: 305-311.

Murphy SC, Martin NH, Barbano DM, and Wiedmann M 2016. Influence of raw milk quality on processed dairy products: How do raw milk quality test results relate to product quality and yield? Journal of Dairy Science 99: 10128-10149.

Neville MC, Zhang P, and Allen JC 1995. Minerals, ions and trace elements in milk. Handbook of Milk Composition: pp 577-592.

Ng-Kwai-Hang K, Hayes J, Moxley J, and Monardes H 1982. Environmental influences on protein content and composition of bovine milk. Journal of Dairy Science 65: 1993-1998.

Ng-Kwai-Hang KF, and Grosclaude F 2003. Genetic polymorphism of milk proteins. In P. F. Fox & P. L. H. McSweeney (Eds.), Advanced Dairy Chemistry—1 proteins: Part a / part b. pp. 739-816. Springer US

Nguyen HTH, Ong L, Lefèvre C, Kentish SE, and Gras SL 2014. The microstructure and physicochemical properties of probiotic buffalo yoghurt during fermentation and storage: A comparison with bovine yoghurt. Food and Bioprocess Technology 7: 937-953.

O'Brien B, and Guinee TP 2016. Seasonal effects on processing properties of cows' milk. In Reference module in food science. Elsevier

O'Brien B, Ryan G, Meaney WJ, McDonagh D, and Kelly A 2002. Effect of frequency of milking on yield, composition and processing quality of milk. Journal of Dairy Research 69: 367-374.

O'Connell A, Kelly AL, Tobin J, Ruegg PL, and Gleeson D 2017. The effect of storage conditions on the composition and functional properties of blended bulk tank milk. Journal of Dairy Science 100: 991-1003.

Oldfield DJ, Singh H, and Taylor MW 1998. Association of β-lactoglobulin and α-lactalbumin with the casein micelles in skim milk heated in an ultra-high temperature plant. International Dairy Journal 8: 765-770.

Oldfield DJ, Singh H, and Taylor MW 2005. Kinetics of heat-induced whey protein denaturation and aggregation in skim milks with adjusted whey protein concentration. Journal of Dairy Research 72: 369-378.

Ono T, Kohno H, Odagiri S, and Takagi T 1989. Subunit components of casein micelles from bovine, ovine, caprine and equine milks. Journal of Dairy Research 56: 61-68.

Ormston S, Davis H, Butler G, Chatzidimitriou E, Gordon AW, Theodoridou K, Huws S, Yan T, Leifert C, and Stergiadis S 2022. Performance and milk quality parameters of Jersey crossbreds in low-input dairy systems. Scientific Reports 12, Article 7550.

Ostersen S, Foldager J, and Hermansen JE 1997. Effects of stage of lactation, milk protein genotype and body condition at calving on protein composition and renneting properties of bovine milk. Journal of Dairy Research 64: 207-219.

Ozcan-Yilsay T, Lee WJ, Horne D, and Lucey JA 2007. Effect of trisodium citrate on rheological and physical properties and microstructure of yogurt. Journal of Dairy Science 90: 1644-1652.

Park YW, Haenlein GFW, and Wendorff WL 2017. Overview of milk of non-bovine mammals (second edition). In Handbook of milk of non-bovine mammals: Second edition. pp. 1-9.

Park YW, Juárez M, Ramos M, and Haenlein GFW 2007. Physico-chemical characteristics of goat and sheep milk. Small Ruminant Research 68: 88-113.

Patiño EM, Pochon DO, Faisal EL, Cedrés JF, Mendez FI, Stefani CG, and Crudeli G 2007. Influence of breed, year, season and lactation stage on the buffalo milk mineral content. Italian Journal of Animal Science 6: 1046-1049.

Penasa M, Lopez-Villalobos N, Evans RD, Cromie AR, Dal Zotto R, and Cassandro M 2010. Crossbreeding effects on milk yield traits and calving interval in spring-calving dairy cows. Journal of Animal Breeding and Genetics 127: 300-307.

Philippe M, Gaucheron F, Le Graet Y, Michel F, and Garem A 2003. Physicochemical characterization of calcium-supplemented skim milk. Lait 83: 45-59.

Pintado ME, Lopes da Silva JA, and Malcata FX 1999. Comparative characterization of whey protein concentrates from ovine, caprine and bovine breeds. LWT - Food Science and Technology 32: 231-237.

Piredda G, and Pirisi A 2005. Detailed composition of sheep and goat milks and antimicrobial substances. International Dairy Federation special issue: 110-116.

Pomiès D, Martin B, Chilliard Y, Pradel P, and Rémond B 2007. Once-a-day milking of Holstein and Montbéliarde cows for 7 weeks in mid-lactation. animal 1: 1497-1505.

Prendiville R, Pierce KM, and Buckley F 2009. An evaluation of production efficiencies among lactating Holstein-Friesian, Jersey, and Jersey × Holstein-Friesian cows at pasture. Journal of Dairy Science 92: 6176-6185.

Prendiville R, Pierce KM, Delaby L, and Buckley F 2011. Animal performance and production efficiencies of Holstein-Friesian, Jersey and Jersey × Holstein-Friesian cows throughout lactation. Livestock Science 138: 25-33.

Pretto D, De Marchi M, Penasa M, and Cassandro M 2013. Effect of milk composition and coagulation traits on grana padano cheese yield under field conditions. Journal of Dairy Research 80: 1-5.

Qi PX, Ren D, Xiao Y, and Tomasula PM 2015. Effect of homogenization and pasteurization on the structure and stability of whey protein in milk. Journal of Dairy Science 98: 2884-2897.

Ramasubramanian L, D'Arcy BR, Deeth HC, and Oh HE 2014. The rheological properties of calcium-induced milk gels. Journal of Food Engineering 130: 45-51.

Ramasubramanian L, Restuccia C, and Deeth HC 2008. Effect of calcium on the physical properties of stirred probiotic yogurt. Journal of Dairy Science 91: 4164-4175.

Rémond B, and Pomiès D 2005. Once-daily milking of dairy cows: A review of recent french experiments. Animal Research 54: 427-442.

Rémond B, Pomiès D, Dupont D, and Chilliard Y 2004. Once-a-day milking of multiparous Holstein cows throughout the entire lactation: Milk yield and composition, and nutritional status. Animal Research 53: 201-212.

Robitaille G, Ng-Kwai-Hang KF, and Monardes HG 1991. Variation in the N-acetyl neuraminic acid content of bovine κ-casein. Journal of Dairy Research 58: 107-114.

Rocha JF, Lopez-Villalobos N, Burke JL, Sneddon NW, and Donaghy DJ 2018. Factors that influence the survival of dairy cows milked once a day. New Zealand Journal of Agricultural Research 61: 42-56.

Rüegg M, Moor U, and Blanc B 1977. A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. Journal of Dairy Research 44: 509-520.

Sandra S, Ho M, Alexander M, and Corredig M 2012. Effect of soluble calcium on the renneting properties of casein micelles as measured by rheology and diffusing wave spectroscopy. Journal of Dairy Science 95: 75-82.

Sanjayaranj I, Lopez-Villalobos N, Blair HT, Janssen PWM, Holroyd SE, and MacGibbon AKH 2023. A study of milk composition and coagulation properties of Holstein-Friesian, Jersey, and their cross milked once or twice a day. Dairy 4: 167-179.

Singh G, Arora S, Sharma GS, Sindhu JS, Kansal VK, and Sangwan RB 2007. Heat stability and calcium bioavailability of calcium-fortified milk. LWT - Food Science and Technology 40: 625-631.

Singh H, and Gallier S 2017. Nature's complex emulsion: The fat globules of milk. Food Hydrocolloids 68: 81-89.

SmartSAMM 2013. *Guideline 11: Monitor bulk milk SCC*. Retrieved 11 July 2023 from www.dairynz.co.nz/media/193850/SmartSAMM_Guideline_11_Monitor_bulk_milk_SCC_2013.pdf

Smith DL, Smith T, Rude BJ, and Ward SH 2013. Short communication: Comparison of the effects of heat stress on milk and component yields and somatic cell score in Holstein and Jersey cows. Journal of Dairy Science 96: 3028-3033.

Sorensen A, Muir DD, and Knight CH 2008. Extended lactation in dairy cows: Effects of milking frequency, calving season and nutrition on lactation persistency and milk quality. Journal of Dairy Research 75: 90-97.

Stelwagen K, Davis SR, Farr VC, Eichler SJ, and Politis I 1994. Effect of once daily milking and concurrent somatotropin on mammary tight junction permeability and yield of cows. Journal of Dairy Science 77: 2994-3001.

Stelwagen K, Davis SR, Farr VC, Prosser CG, and Sherlock RA 1994. Mammary epithelial cell tight junction integrity and mammary blood flow during an extended milking interval in goats. Journal of Dairy Science 77: 426-432.

Stelwagen K, and Knight CH 1997. Effect of unilateral once or twice daily milking of cows on milk yield and udder characteristics in early and late lactation. Journal of Dairy Research 64: 487-494.

Stelwagen K, and Lacy-Hulbert SJ 1996. Effect of milking frequency on milk somatic cell count characteristics and mammary secretory cell damage in cows. American Journal of Veterinary Research 57: 902-905.

Stelwagen K, Phyn CVC, Davis SR, Guinard-Flament J, Pomiès D, Roche JR, and Kay JK 2013. Invited review: Reduced milking frequency: Milk production and management implications. Journal of Dairy Science 96: 3401-3413.

Stockdale CR 2006. Influence of milking frequency on the productivity of dairy cows. Australian Journal of Experimental Agriculture 46: 965-974.

Storry JE, Grandison AS, Millard D, Owen AJ, and Ford GD 1983. Chemical composition and coagulating properties of renneted milks from different breeds and species of ruminant. Journal of Dairy Research 50: 215-229.

Strucken EM, Laurenson YC, and Brockmann GA 2015. Go with the flow—biology and genetics of the lactation cycle. Frontiers in genetics 6: 118.

Tallamy PT, and Randolph HE 1970. Influence of mastitis on properties of milk. V. Total and free concentrations of major minerals in skim milk. Journal of Dairy Science 53: 1386-1388.

Timlin M, Tobin JT, Brodkorb A, Murphy EG, Dillon P, Hennessy D, O'donovan M, Pierce KM, and O'callaghan TF 2021. The impact of seasonality in pasture-based production systems on milk composition and functionality. Foods 10, Article 607.

Tinggi CPCRU 2001. Selenium levels in cow's milk from different regions of australia. International Journal of Food Sciences and Nutrition 52: 43-51.

Udabage P, Augustin MA, Versteeg C, Puvanenthiran A, Yoo JA, Allen N, McKinnon I, Smiddy M, and Kelly AL 2010. Properties of low-fat stirred yoghurts made from high-pressure-processed skim milk. Innovative Food Science and Emerging Technologies 11: 32-38.

Udabage P, McKinnon IR, and Augustin MA 2001. Effects of mineral salts and calcium chelating agents on the gelation of renneted skim milk. Journal of Dairy Science 84: 1569-1575.

Underwood JE, and Augustin MA 1997. Seasonal variation in the rheological properties of acid and heat-induced gels made from reconstituted concentrated milk. Australian Journal of Dairy Technology 52: 83-87.

Vaia B, Smiddy MA, Kelly AL, and Huppertz T 2006. Solvent-mediated disruption of bovine casein micelles at alkaline pH. Journal of Agricultural and Food Chemistry 54: 8288-8293.

van Hulzen KJE, Sprong RC, van der Meer R, and van Arendonk JAM 2009. Genetic and nongenetic variation in concentration of selenium, calcium, potassium, zinc, magnesium, and phosphorus in milk of dutch Holstein-Friesian cows. Journal of Dairy Science 92: 5754-5759.

Vasbinder AJ, and de Kruif CG 2003. Casein–whey protein interactions in heated milk: The influence of pH. International Dairy Journal 13: 669-677.

Vasbinder AJ, van de Velde F, and de Kruif CG 2004. Gelation of casein-whey protein mixtures. Journal of Dairy Science 87: 1167-1176.

Visentin G, De Marchi M, Berry DP, McDermott A, Fenelon MA, Penasa M, and McParland S 2017. Factors associated with milk processing characteristics predicted by mid-infrared spectroscopy in a large database of dairy cows. Journal of Dairy Science 100: 3293-3304.

Vyas HK, and Tong PS 2004. Impact of source and level of calcium fortification on the heat stability of reconstituted skim milk powder. Journal of Dairy Science 87: 1177-1180.

Walker GP, Dunshea FR, and Doyle PT 2004. Effects of nutrition and management on the production and composition of milk fat and protein: A review. Australian Journal of Agricultural Research 55: 1009-1028.

Walker GP, Wijesundera C, Dunshea FR, and Doyle PT 2013. Seasonal and stage of lactation effects on milk fat composition in northern victoria. Animal Production Science 53: 560-572.

Walstra P, Wouters JTM, and Geurts TJ 2005. Dairy science and technology (Second edition ed.) CRC Press.Wedholm A, Larsen LB, Lindmark-Månsson H, Karlsson AH, and Andrén A 2006. Effect of protein composition on the cheese-making properties of milk from individual dairy cows. Journal of Dairy Science 89: 3296-3305.

Wegner TN, and Stull JW 1978. Relation between mastitis test score, mineral composition of milk, and blood electrolyte profiles in Holstein cows. Journal of Dairy Science 61: 1755-1759.

White JCD, and Davies DT 1958. 712. The relation between the chemical composition of milk and the stability of the caseinate complex: I. General introduction, description of samples, methods and chemical composition of samples. Journal of Dairy Research 25: 236-255.

Wijayanti HB, Bansal N, and Deeth HC 2014. Stability of whey proteins during thermal processing: A review. Comprehensive Reviews in Food Science and Food Safety 13: 1235-1251.

Wiking L, Nielsen JH, Båvius AK, Edvardsson A, and Svennersten-Sjaunja K 2006. Impact of milking frequencies on the level of free fatty acids in milk, fat globule size, and fatty acid composition. Journal of Dairy Science 89: 1004-1009.

Wiking L, Stagsted J, Björck L, and Nielsen JH 2004. Milk fat globule size is affected by fat production in dairy cows. International Dairy Journal 14: 909-913.

Zahar M, and Smith DE 1996. Adsorption of proteins at the lipid-serum interface in milk systems with various lipids. International Dairy Journal 6: 697-708.

Zhao FQ, and Keating AF 2007. Expression and regulation of glucose transporters in the bovine mammary gland. Journal of Dairy Science 90: E76-E86.