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University College Cork, Ireland

Effects of herd diet, protein fortification and coagulation conditions on in-vat curd syneretic properties and ripening profiles of Maasdam cheese

A Thesis Presented to the National University of Ireland for the Degree
of Doctor of Philosophy

By

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November 2018

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Signature:

Date:

Ram Raj Panthi

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Abstract

This thesis describes a series of studies undertaken to underpin developments in the Irish cheese industry, particularly an increased interest in the potential of achieving market advantage through the promotion of grass- or pasture-fed products. However, a knowledge gap exists in terms of the impact of herd diet on the processing characteristics and on the ripening and sensory properties of resultant cheeses. Similarly, the industry has moved to produce more continental-type cheeses in an effort to diversify from predominantly Cheddar manufacture. Thus, there is a further research requirement for information regarding the manufacture of continental-type cheese such as Maasdam from a seasonal Irish milk supply, and the application of process modifications such as membrane filtration in the manufacture of such cheeses.

This research focused initially on characterizing the cheesemaking properties of milk and the physico-chemical, ripening and sensory properties of Maasdam cheese derived from a herd fed indoors on total mixed ration (TMR) and herds fed outdoors on either perennial rye grass (GRA) or perennial rye grass with white clover (CLO). Furthermore, the effects of protein-standardization of milk on cheesemaking properties, e.g., rennet coagulation, in-vat curd moisture loss kinetics and curd microstructure at different set temperatures were also investigated.

There was a minimal feed-induced variation on in-vat curd moisture loss kinetics, final Maasdam cheese yield, recovery of fat and protein, physico-chemical composition, and proteolysis and biochemical changes (such as propionate and lactate) during 150 d of ripening. The principal differences noted were in colour, with GRA or CLO cheeses being less white and more yellow, and TMR cheeses being more white. Maasdam cheeses made from the milk of the TMR-fed herd had higher levels

of linoleic acid (C18:2) and palmitic acid (although the difference in palmitic acid content was not significant), compared to pasture-derived cheese and this translated into a firmer texture in the mouth from TMR cheeses. A detailed investigation of the metabolic profile of cheese samples using nuclear magnetic resonance and gas chromatography-mass spectrometry showed that TMR-derived cheese samples had higher levels of citrate, while GRA or CLO cheeses had higher levels of toluene, thus providing a potential chemical fingerprint for differentiation between cheeses based on feeding systems.

The coagulation properties of milk concentrated to protein levels of 4, 5 or 6% at 28, 32 or 36°C were also characterized. It was concluded that cheese producers would need to focus on optimizing the cutting window, based on milk protein level and coagulation temperature, to attain uniform curd rigidity on completion of the cutting cycle. However, the coagulation time and cutting process for milk with 5% protein at 28°C was similar to that observed for milk of 4% protein coagulated at 32°C, due to similar levels of curd firmness and curd firming rate in these gels. Curd from milk concentrated to 6% protein had a dense protein network compared to that with 4% protein, as observed by transmission and scanning electron microscopy, and the curds from the former milk contained lower levels of moisture during stirring compared to the latter. An increased coagulum cut size is required to achieve a uniform curd moisture content when milk protein levels are increased by standardization (4 to 6%). However, breakage of curd particles of size $>6\text{ mm}^3$ from milk concentrated to $>5\%$ milk was excessive, which significantly influenced the curd moisture loss kinetics during stirring.

Overall, a fundamental understanding of coagulation and curd syneresis properties was gained in terms of the application of ultrafiltration for milk protein concentration, which can be applied to reduce the impact of seasonal variability in milk composition for cheesemaking. This research generated fundamental knowledge which may be useful to achieve greater consistency in cheese manufacture and in achieving diversification of the Irish cheese industry through the manufacture of Maasdam type and other continental cheese types of consistent quality from a predominantly grass-fed milk production system.

Publications

Book chapter and peer-reviewed articles:

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Chapter 1

Selection and treatment of milk for cheesemaking: a review

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1.1 Abstract

The quality of any type of cheese is without doubt influenced by the quality of the milk from which it is made, from microbiological, biochemical, sensory, and many other perspectives. Raw milk is an inherently variable starting point for cheese manufacture, with its composition, for example, being influenced by factors, such as stage of lactation, diet, and health status of the producing animal, which subsequently determines milk coagulation properties and cheese yield, composition and likely ripening. Protein standardization treatments may be applied to cheese milks to minimize resultant cheese compositional variability. Similarly, milk is also biochemically complex, with a wide range of indigenous enzymes, some of which, for example, plasmin and lipase, are variable in activity and susceptible to processes applied to the milk, while contributing to cheese ripening. Finally, raw milk may act as a vector for bacterial spores and also provides an excellent medium for growth of a wide range of microorganisms. As cheese milk is vulnerable to contamination at multiple points before entering the cheese vat, control of the same for cheese safety and quality is critical. This chapter reviews information from the published literature on factors influencing milk composition and subsequently on cheesemaking properties of milk and final cheese composition.

1.2 Introduction

The composition of milk may vary depending on season, lactation period, parity number, health status, feed, breed, age, and many other factors (Roupas, 2001; Amenu and Deeth, 2007; O'Brien and Guinee, 2011). Among these factors, herd feeding practices for milk production particularly causes large alterations in milk properties. In some countries (e.g., Ireland, New Zealand and parts of Australia) herds are allowed to graze in pasture, whereas in other countries (e.g., USA and North West Europe), herds are kept indoors and are supplied a controlled diet of mixed rations composed of silage, concentrate, grains and added vitamins (O'Callaghan et al., 2016). In pasture-feeding systems, the calving date of cows is also generally adjusted to a specific season (e.g., spring) so that milking cows in pasture can utilize the growing grass, subsequently reducing the cost of milk production (O'Brien and Guinee, 2011). However, pasture-based feeding systems result in large variation in milk composition compared to indoor feeding systems over a full year, due to changes in lactation stage of herd and feed quality (O'Callaghan et al., 2016).

The impact of compositional variation of milk on value-added products, e.g., cheese, is of particular interest in those countries (e.g. Ireland and New Zealand) where the feeding system is mainly pasture-based (Auldist et al., 1996; Guinee et al., 2001). Fluctuations in milk composition can result in inconsistencies in product properties, such as final cheese composition and quality (Amenu and Deeth, 2007). Poor quality herd diet and milk in late lactation, in a pasture-fed seasonal milk production system, further accentuates compositional variation of milk and may even render it unsuitable for cheesemaking, due to reductions in the levels of total protein, casein and casein number (O'Brien and Guinee, 2011; O'Brien et al., 1999a). For instance, in Ireland,

many industrial cheese makers do not manufacture cheese during the period from November until February, since cows are in late lactation and grass growth is minimal, and also there is less milk volume available for processing.

The removal of milk quotas by European Union in April 2015 is projected to lead to increase in milk production by a 2.5 billion litres, from 5 billion litres in 2014 to 7.5 billion litres by 2020 in Ireland (Sheehan, 2013). Irish milk production volumes in 2017 already reached 7.2 billion litres, a 13% increase on 2015 levels (Central Statistics Office, 2018) and was significantly higher compared to the average increase within EU countries. Utilization of additional milk volumes will further present challenges as the human consumption of liquid milk has been fairly constant (Central Statistics Office, 2018) and production of dairy products is expected to grow in slower pace. Ireland produced 196.2 thousand tonnes of cheese in 2017 (Central Statistics Office, 2018), of which nearly 170 thousand tonnes were Cheddar cheese (Personal communication). The vast majority of Irish Cheddar cheese is exported to the UK. Over-reliance of Ireland on the UK export market has been taken as a threat to the Irish agri-food industry (Enterprise Ireland, 2018). The ability to successfully diversify the cheese portfolio from Cheddar cheese would leverage the increased milk pool and secure export market. For example, there is a growing interest to diversify cheese production into cheeses such as Maasdam cheese, which is a semi-hard cheese with holes and with typical sweet and nutty flavour (Fröhlich-Wyder et al., 2017), due to its wide international market opportunities (Sheehan, 2013). The Irish dairy or cheese industry has a potential of achieving market advantage through the promotion of grass- or pasture-fed products in the context of growing consumer demand for such dairy products.

As mentioned, the seasonal Irish pasture-based milk production systems further present challenges in achieving consistency in cheese manufacturing procedures and in final cheese quality. The basic process of cheesemaking includes hydrolysis of κ -casein ‘hairs’ from casein micelles by rennet and subsequent formation of a gel (Dalglish, 1993; Amenu and Deeth, 2007). The resultant three-dimensional protein gel simultaneously occludes fat, lactose, minerals and moisture in the form of a coagulum. Syneresis (expulsion of moisture) from the curd takes place in the series of processing and these include cutting, stirring, heat treatment, acidification, draining and pressing during cheese making, which transforms milk into curds and subsequently into cheese mass (Amenu and Deeth, 2007). Variation in milk composition leads to inconsistencies in the major processing steps such as coagulation and syneretic properties of cheese curd, and consequently to the final cheese composition.

This review provides the necessary information on factors associated with the variation of bovine milk composition, milk coagulation properties and cheese composition, to facilitate selection of appropriate milk for improved cheese consistency, quality and diversification.

1.3 Effect of milk composition on cheesemaking

For bovine milk, the major solids constituents are protein (~ 3.4%), fat (~ 4%) and lactose (~ 4.8%), while minor solids include calcium (~120 mg/100 g) and phosphorus (~ 120 mg/100 g). Given that cheese is produced by concentrating milk solids, the level of milk solids present has a major influence on coagulation and syneretic properties, as well as on final cheese yield. Many studies have examined the effect of factors such as protein level, casein composition (Robitaille et al., 1993;

Guinee et al., 1997; Wedholm et al., 2006), fat level, milk salts and size of casein micelles (Ford and Grandison, 1986) on cheesemaking properties, with the objective of defining the optimum properties (O'Keeffe, 1984; Auldist et al., 2004). In particular, a number of studies on natural variation in Irish milk composition were undertaken in relation to the manufacture of Cheddar (Guinee et al., 1994, 2006, 2007a) and Mozzarella cheese (Guinee et al., 2007b). It has been well documented that higher levels of milk solids (protein and fat) directly improve milk coagulation properties, for instance, through reduced rennet coagulation time, increased curd firmness, and increased cheese yield during cheesemaking (Guinee et al., 1994, 1997; Jõudu et al., 2008). The effects of milk composition on Cheddar cheese were reviewed by Amenu and Deeth (2007).

1.3.1 Effect of protein level on cheesemaking

In cheese milk, rennet coagulation does not occur within an hour of rennet addition to milk containing < 2% milk protein (Guinee et al., 1997). Significant improvement in milk coagulation properties (shorter coagulation and cutting time, higher curd firming rate and curd firmness) occurs with increasing protein level in the range 2-4%, although coagulation and set-to-cut time changes little thereafter (4-7.5%) (Guinee et al., 1997). An increased level of milk protein or casein number decreases rennet coagulation time (RCT) and increases curd firmness (Jõudu et al., 2008). Furthermore, higher protein content, particularly casein content, in milk leads to decreased cheese moisture and increased protein contents, cheesemaking efficiency, and fat recovery, and hence yield (Guinee et al., 2006; Lou and Ng-Kwai-Hang, 1992). However, increasing protein content in cheese milk above 5-8 % may cause tearing of

curds while cutting due to extra firmness, and may induce more protein and fat losses in whey and result in reduced yield and cheese quality (Guinee et al., 1994).

1.3.2 Effect of casein composition on cheesemaking

Casein micelles are comprised of different types of casein, α_{s1} -, α_{s2} -, β - and κ -casein, in a spherical structure, with κ -casein extending outwards from the micelles (Eigel et al., 1984; Dalgleish, 2011); these are central to the cheesemaking properties of milk. Understanding the individual effect of casein components on rennet coagulation through using milk of different cow breed (Auldist et al., 2004) or stage of lactation (Jõudu et al., 2008) or added β -casein powder (St-Gelais and Haché, 2005) was a focus of previous studies. Various studies relating to the correlation between milk components (fat, protein, casein, calcium and κ -casein) have found contradictory results on RCT, a negative correlation with K_{20} and a positive correlation with curd firmness (A_{30}) (Table 1.1, Figure 1.1).

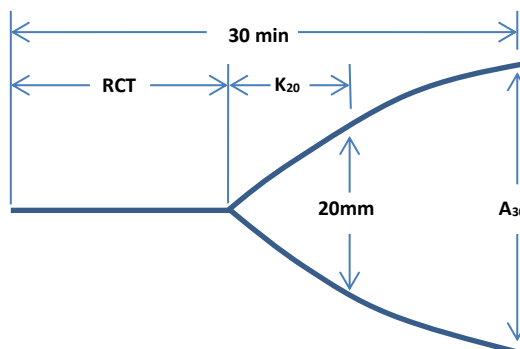


Figure. 1.1 Schematic presentation of milk coagulation properties (RCT, K_{20} , A_{30}) measured by lactodynamography. Redrawn with the permission from McMahon and Brown (1982). RCT is the time from the addition of the enzyme until the viscosity of milk just starts changing from normal. K_{20} is the time from end point of RCT until a curd firmness of 20 mm reached, and A_{30} is the firmness of curd in mm reached 30 min after the addition of rennet.

Table 1.1 Influence of milk components on rennet coagulation properties.

	RCT	K ₂₀	A ₃₀ or A ₆₀	References
Milk composition				
Fat	?	-	+	Auldist et al., 2004; Chen et al., 2014; Jõudu et al., 2009; Bland et al., 2015
Protein	?	-	+	Auldist et al., 2004; Auldist et al., 2002; Chen et al., 2014; Jõudu et al., 2008; Bland et al., 2015
Casein	?	-	+	Auldist et al., 2004; Auldist et al., 2002; Chen et al., 2014; Jõudu et al., 2008; Bland et al., 2015
β-casein	-	-	+	Auldist et al., 2004;; Jõudu et al., 2008
κ-casein	?	-	+	Auldist et al., 2004; Auldist et al., 2002; Jõudu et al., 2008
α _{S1} -casein	-		+	Jõudu et al., 2008
α _{S2} -casein	-		+	Jõudu et al., 2008
β-casein:total Cn	+		-	Jõudu et al., 2008
α _{S2} -Cn:total Cn	+		-	Jõudu et al., 2008
Lactose	-			Jõudu et al., 2008; Bland et al., 2015; Glantz et al., 2010
Total solids	+	-	+	Auldist et al., 2004
Casein number	-		+	Jõudu et al., 2008
Micelle size	+		-	Bland et al., 2015; Auldist et al., 2002; Glantz et al., 2010
Minerals				
Ca ⁺⁺	?	-	+	Auldist et al., 2004; Chen et al., 2014; Jõudu et al., 2008; Bland et al., 2015
Na	+	+	-	Auldist et al., 2004
K	-	+	-	Auldist et al., 2004

?: conflicting correlation, -: negative correlation, +: positive correlation. RCT denotes rennet coagulation properties, K₂₀ denotes time to develop 20 mm firmness and A₃₀ denotes the curd firmness at 30 minutes and A₆₀ denotes curd firmness at 60 min after rennet addition.

The discrepancies in RCT could be due to differences in casein composition (e.g., κ-casein/total casein ratio) and specific milk properties (e.g., Ca: protein ratio) in different studies (Auldist et al., 2004). Milk containing a higher proportion of α_{S2}- and β-caseins and a lower proportion of κ-casein with respect to total casein clotted

more slowly and sometimes was found to have non-coagulating (not exhibiting any firmness >30 min after rennet addition) properties compared to milk samples with higher levels of these proteins (Jõudu et al., 2009; Wedholm et al., 2006). Prediction of rennet coagulation time alone, as an indicator of the suitability of milk for cheese, may not provide accurate information (Auldist et al., 2002) as its correlation with milk solids level is weak (Auldist et al., 2004).

1.3.3 Effect of casein micelle sizes on cheesemaking

Besides casein composition, casein micelle size (CMS) has been found to be highly influential on cheesemaking properties. The size of casein micelles of individual cows lies within the range 154-230 nm (de Kruif et al., 2012). In general, factors such as breed, feed and post-translational modification cause variation in casein micelle size (Lodes et al., 1996; Glantz et al., 2010; Bijl et al., 2014a). Many studies have been carried out to explore the relationship between cheesemaking efficiency and size-related differences in casein micelles (Ford and Grandison, 1986; Lodes et al., 1996; Auldist et al., 2002; Glantz et al., 2010; Logan et al., 2014; Gustavsson et al., 2014b). There is general agreement that milk with smaller casein micelles (diameter of 147 -183 nm) coagulates faster and gives a firmer coagulum, than milk having larger casein micelles (200- 266 nm) (Walsh et al., 1998; Auldist et al., 2002; Glantz et al., 2010; Gustavsson et al., 2014b; Logan et al., 2014; Bland et al., 2015). Smaller casein micelles have a higher level of κ -casein than larger micelles, and could probably form more bonds *in para*-casein micelles during coagulation, resulting in a firmer coagulum. Hence, smaller casein micelles have attracted attention in selecting milk for cheesemaking (Glantz et al., 2010). However, various studies have classified casein micelle sizes as being within different ranges (Table 1.2) thus

leading to difficulty in defining size range. Nevertheless, it has been shown that there are no significant differences (except glycosylation of κ -casein, and levels of soluble phosphate) between larger (average 206 nm) and smaller (average 170 nm) casein micelles (Bijl et al., 2014a). Similarly, there were no significant differences in net negative charge (ζ potential) between larger (diameter 204 nm) and smaller casein micelles (diameter 178 nm) and this was found to be in the range -19 to -20 mV, indicating no significant differences in micelle stability as influenced by micelle size (Glantz et al., 2010). Auldist et al., (2002) showed that micelle size and the extent of glycosylation of κ -casein are the most important factors that lead to variations in milk coagulation properties.

Table 1.2 Categorization of casein micelles size in different studies

Small micelles, nm	Large micelles, nm	References
170	206	Bijl et al. (2014a)
183.5	266	Walsh et al. (1998)
153.4-159.3	181.2-202.6	Logan et al. (2014)
170-185	205-220	Glantz et al, (2010)

nm = nanometer

1.3.4 Effect of κ -casein glycosylation on cheesemaking

κ -Casein exists as variants, AA, AB, BB, AC BC and AE in bovine milk. Milk samples with κ -casein variants AB, BB or BC had casein micelle diameters less than 200 nm, whereas those with AA, AC and AE had micelle diameters greater than 200 nm (Lodes et al., 1996; Bijl et al., 2014a). Many studies have confirmed that milk containing the BB variant of κ -casein has faster and firmer gelling ability and better suitability for cheese production than other variants (Walsh et al., 1998; Ikonen et al., 1999; Auldist et al., 2002; Amenu et al., 2006; Jõudu et al., 2009). According to Ng-

Kwai-Hang (1998), milk with the BB variant of κ -casein has a reduced coagulation time (by 10-40%) and increased curd firmness (by 20-140%) compared to milk with the AA variant of κ -casein. The improved rennet coagulation properties with the BB variant of κ -casein are due to the higher level of κ -casein (Bijl et al., 2014a; Walsh et al., 1998) which could probably result in smaller casein micelles with more κ -casein at their surface. Hence, a higher κ -casein/casein ratio may favour the bridging of proteins by calcium, resulting in faster coagulation and a firmer gel (Robitaille et al., 1993).

Furthermore, cheese prepared from milk containing the BB variant of κ -casein has been shown to have higher fat recovery and yield compared to that with the AA variant (Walsh et al., 1998). Cows that produce milk containing the BB variant of κ -casein are economically important from a cheesemaking perspective, owing to the micelle size-related benefits of this protein type.

1.3.5 Effect of variation in fat level on cheesemaking

Guinee et al. (1997) reported that increasing fat levels in the range 0.1- 10 % (w/w) at a constant protein level of 3.3 % (w/w) resulted in reduction in gel time and in set-to-cut time at 20 Pa and increased curd firming rate and curd firmness. However, when maintaining fat plus protein contents at 7.1 %, increasing fat content resulted in reduced curd firming rate and firmness and the set-to-cut time at 20 Pa increased notably as fat levels exceeded 2.5 % (w/w). It is suggested that excessive fat in milk may dilute the caseins and hence hinder the formation of a *para*-casein network, impairing rennet coagulation ability (Guinee et al., 1997). For a seasonal milk supply, the protein to fat ratio changes from 0.84 to 1.02 over a complete lactation (Guinee et al., 2007a). Where milk composition is not standardized, the seasonal variation may

lead to product and process variations during cheesemaking. Fat standardization of cheese milk based on a protein to fat ratio (PFR) overcomes the problem associated with seasonal variation of milk composition to some extent. For instance, varying PFR in the range 0.70 to 1.15 did not significantly affect coagulation properties (Guinee et al., 2007a). However, such variations could significantly affect cheese composition; it has been reported that an increase of PFR by 0.1 leads to increased protein (0.6 %, w/w) and reduced fat in dry matter (1.9 %, w/w) and moisture in non-fat substance (0.7, % w/w) in Cheddar cheese prepared from milk with a fat content of 3.4-4.8 %.

Table 1.3 Cheese composition at different protein and protein to fat ratios in milk.

Cheese composition	At constant PFR (0.96)			At constant protein 3.6		
	Protein level			PFR ratio		
	3.3	3.6	4	0.70-.085	0.88-1.0	1.10-1.12
Moisture (% , w/w)	37.16	36.39	36.2	37.06	37.84	38.91
Fat (% , w/w)	31.37	31.9	31.8	34.53	31.36	28.76
Protein (% , w/w)	26.0	26.06	26.45	23.14	25.08	26.4
MNFS (% , w/w)	54.14	53.44	53.18	56.63	55.12	54.61
Ca (mg/g of protein)	29.3	29.7	29.47	28.89	29.4	28.45
P (mg/g of protein)	20.2	20.6	20.97	20.46	20.49	20.49

MNFS denotes moisture in non-fat substances; PFR denotes protein to fat ratio (Guinee et al. 2006; 2007a)

Increasing protein (3.3-4 %, w/w) and fat (3-4 %, w/w) level at constant PFR in milk led to reduced cheese moisture, and similarly decreasing the fat level at constant protein level (3.6 %) in milk led to increased cheese moisture (Table 1.3).

The influence of fat on the syneresis of cheese curd was studied extensively by Mateo et al. (2009). According to that study, higher levels of protein and fat in cheese milk and thus a higher level of total solids results in curd with a lower moisture content than curds prepared from milks with lower level of total solids (Mateo et al., 2009). On the other hand, milk with a higher solids content due to increased protein level from

3.3 to 4% at a uniform PFR may result in more porous curd particles, which could exude more moisture than particles from milk with a lower total solid contents (Guinee et al., 2006). Furthermore, processing steps (e.g., stirring) could induce a higher frequency of collision between dense curds particles, if produced from higher total solids milk, which could deform the curds, resulting in more whey expulsion than curd produced from milk with lower total solids. In contrast, cutting the gel at a higher gel strength could lead to higher moisture content than cutting the gel at a lower strength, possibly due to difficulty in contraction of the matrix. Surprisingly, the magnitude of the decrease in moisture in non-fat substances in cheese has been observed to be lower at a higher fat level in milk in comparison to increasing PFR (lower fat level) (Table 1.3). This is explained by the concepts that fat acts as a ‘stopper’, slowing down syneresis from the cheese curds (Guinee et al., 2006, 2007; Maeto et al., 2009).

1.3.6 Effect of interactions between fat globule and casein micelle size

Logan et al. (2014) reported an interactive effect of fat globule size and casein micelle size on the rennet coagulation properties of milk. It was found that milk with smaller casein micelles (153-159 nm) and larger fat globules (3.88-5.78 μm) resulted in faster coagulation and gave a firmer curd than milk with larger fat globules and large casein micelles (181-202 nm). The study clearly demonstrates the importance of smaller casein micelles for cheesemaking. It is expected that larger fat globules (3.88 to 5.78 μm) are tightly packed in the pores generated by smaller casein micelles (153 to 159 nm), giving a structural rigidity and hence firmer curds compared to curds with larger casein micelles and smaller fat globules (Logan et al., 2014, 2015). A subsequent study on the interactive effects of fat globule and casein micelle sizes in bovine milk has also given similar results (Logan et al., 2015). Previously, O’Mahony

et al. (2005) observed weaker gels with larger fat globules size (4.68 μm) compared to smaller fat globule size (3.45 μm). The contradiction between these studies could be due to differences caused by casein micelle sizes, emphasizing the interactive effects of casein micelle size with fat globules size in milk gels. The size of fat globules has been reported to vary depending on feed type (Argov-Argaman et al., 2014; Couvreur et al., 2007) which could also have an influence on rennet coagulation properties in different studies.

1.3.7 Composition of poorly coagulating milk

When milk from some cows does not coagulate within 30 min (at 35°C) of adding rennet, this milk is considered to have a non-coagulating property. Similarly, milk which coagulates but reaches a firmness of less than 20 mm, 30 min after rennet addition has been categorized as poorly coagulating milk (Jōudu et al., 2009) (Figure 1.1). Industrially, poorly coagulating or non-coagulating milk could lead to huge economic loss during cheesemaking. It is not clear why some milk shows this type of property. However, it has been proposed that milk with the AA variant of κ -casein, a higher concentration of α_{s1} - and β -caseins and/or larger casein micelles show poor coagulation properties (Wedholm et al., 2006). Moreover, a lower concentration of milk salts has a negative effect on the suitability of milk for cheesemaking. For instance, Wedholm et al. (2006) reported that milk containing less than 0.10 g/100 g calcium had a longer rennet coagulation time than milk with a higher calcium content (0.12 g/100 g). The availability of free (ionic) calcium in milk is important for bridging casein micelles after rennet-induced hydrolysis; Hallén et al. (2007) showed improved curd firmness in poorly coagulating milk after the addition of 0.05% CaCl_2 . However, this improvement may not occur in very late lactation milk because of decreased in

casein number due to enzymatic action and higher pH, leading to alterations in casein micelle integrity and mineral balance (Lucey and Fox, 1992). There has been much interest in understanding the causes of poorly or non-coagulating property of milk in genetic level. Jensen et al. (2012) reported the genetic constituent of BB, AA and AA variant of α_{s1} -, β - and κ -casein, respectively, contribute to impaired milk coagulation properties. It has been suggested that selecting milk on the basis of casein composition for cheesemaking could reduce the uncertainty in milk coagulation properties and hence optimize cheese composition (Glantz et al., 2010).

1.4 Factors that influence milk composition, rennet coagulation properties and cheese composition

1.4.1 Impact of seasonality on milk quality

The composition of milk changes depending on season, lactation period, parity number, health status, feed, breed, age, and many other factors (Roupas, 2001; Amenu and Deeth, 2007; O'Brien and Guinee, 2011) which could influence eye formation and flavour development in continental-type cheeses. Furthermore, in a seasonal milk production system, spring-calved herds yield milk with protein and fat levels in the range of 3.29 to 4.02 %, w/w, and 4.40 to 5.12%, w/w, respectively, during a year (O'Callaghan et al., 2016).

Recently, Chen et al. (2015) reported no significant differences in the composition of soft cheese produced from milk with compositional variations over the course of a production year. This lack of variation in cheese composition could be attributed to the calving pattern and controlled feed supply in milk production. A similar study in highly seasonal milk producing countries may have a different outcome.

Different sampling intervals have been used in different studies on seasonal variations in milk composition. Chen et al. (2014) examined compositional variation at intervals of two weeks, Heck et al. (2009) at weekly intervals, O'Brien et al. (1999b) and Mehra et al. (1999) at 3 week intervals and Lindmark-Mansson et al. (2003) at monthly intervals.

1.4.2 Impact of lactation stage on milk composition

Much emphasis has been given to late-lactation milk (>250 days after calving) in pasture-based milk-producing countries. Late-lactation milk has higher levels of fat, protein, casein, whey proteins, non-casein nitrogen, bovine serum albumin, sodium and casein hydrolysis products than other stages of lactation (Donnelly and Barry, 1983; Auldist et al., 1998; Mehra et al., 1999; O'Brien et al., 1999b). Auldist et al. (1998) studied seasonal variations of bovine milk composition at different stages of lactation in different seasons under similar husbandry management practices. It was found that, regardless of the season, milk yield and lactose level decreased and the level of milk fat, protein, casein and whey proteins increased in late lactation milk (Auldist et al., 1998). This change in milk composition could be due partly to reduced milk volume at late-lactation (Auldist et al., 1998; Hickey et al., 2006; Hinz et al., 2012), resulting in the concentration of milk constituents within the udder.

In addition, milk in late lactation shows impaired rennet coagulation and synergetic properties during cheesemaking (O'Keffee, 1984). Some studies have highlighted the importance of high quality nutrition to lactating cows to overcome the problem (Kefford et al., 1995; O'Keffee, 1984; Lucey, 1996; Guinee et al., 2007b). For instance, studies have shown no significant differences in the levels of fat and lactose, RCT, K₂₀, A₆₀ of milk samples taken during days 218-307 of lactation

(O'Brien et al., 2006; Guinee et al., 2007b). Such improvement in milk even in late lactation was attributed to high quality feed (pasture and 2 kg concentrate, or silage when cows were indoors) and a strict management policy (stocking rate 2.48/ha, drying off at 6 kg milk/day) was used. A subsequent study on Mozzarella cheese made from milk obtained during days 218-284 of lactation has also reported similar ($P < 0.05$) moisture, fat, protein, MNFS, NaCl, ash, calcium, phosphorus in the resultant cheese (Guinee et al., 2007b). These studies suggested that late lactation milk can be used to produce low moisture part-skim Mozzarella cheese without defects, if cows are fed with a high quality diet and milk from cows with a low milk yield (< 6 kg/day) is excluded.

The level of FFAs in raw or pasteurized milk in late lactation has been found to increase significantly (1.677 to 6.812 gm/kg fat) compared to early lactation (Hickey et al., 2006; O'Brien et al., 2006). There is a linear increase of (0.1-0.2 gm/kg fat) FFA level during ripening of Cheddar cheese (Hickey et al., 2006). Hence, a higher level of FFAs in milk is expected to lead to higher levels of FFAs in ripening cheese. Generally, plasmin activity is up to 8 times higher in young cheese than in the original milk (Hinz et al., 2012). Plasmin activity is critically important in protein breakdown (particularly β -casein) which produces γ -caseins. A higher proportion of protein breakdown occurs in cheese when it prepared from late lactation milk (Hickey et al., 2006; Hinz et al., 2012), possibly due to presence of higher levels of plasminogen activators in that milk (O'Brien et al., 2006). It is suggested that when using late lactation milk compared to early or mid lactation milks, consideration should be made for the cheese type to be produced and their intrinsic characteristics, as different cheese types may be more or less impacted by increased levels FFA and plasmin activity. Various studies have shown that the moisture level in Cheddar cheese

increases when it is manufactured from late-lactation milk, in comparison to mid or early-lactation milk (O'Keeffe, 1984; Lucey and Fox, 1992; Kefford et al., 1995; Hickey et al., 2006; Hinz et al., 2012). Similarly, there is a tendency for more slit (crack) formation in Swiss-type cheese when it is prepared from late-lactation milk (Daly et al., 2010), probably because of extensive proteolysis of β -casein in cheese during ripening (Hinz et al., 2012) although the mechanism for this has not yet been fully determined.

1.4.3 Effects of combining spring or autumn-calved herds on milk composition

There are contradictory reports as to whether variations in milk composition are more influenced by feed or by stage of lactation. Some studies (Auldist et al., 1998; Mehra et al., 1999; O'Connell et al., 2015) have discussed the possibility of splitting calving between spring and autumn to achieve a more consistent milk quality throughout the year. Seasonal variations in milk composition (Auldist et al., 1998; Mehra et al., 1999) and quantity (O'Connell et al., 2015) have been reported to be lower when increasing the proportion of autumn-calving cows in pasture-based milk producing countries. Mehra et al. (1999) also observed less variation in milk composition in retail milk (produced from a higher proportion of autumn-calving cows) than in manufacturing milk, produced from mainly spring-calved cows, in Ireland. For example, retail milk contained a higher percentage of casein (average 26.5 vs 25.6 g/L milk) than manufacturing milk during outdoor grazing of cows from June to October, indicating that year-round calving pattern may result in milk with better cheesemaking properties.

1.5 Impact of feed on milk composition

Offering feed that fulfils the nutritional requirements of cows has been shown to increase the concentration of protein, casein and mineral content in milk (Bovelenta et al., 2009; Dillon et al., 2002; O'Brien et al., 1999a). For instance, increased levels of grass intake by cows in the mid-lactation (16-24 kg DM) resulted in an increased casein number, protein and casein and lactose levels in milk (Guinee et al., 1998). Conversely, O'Brien et al. (1999a) found that limited feed supply at higher stocking rates results in lower levels of protein, lactose, fat, casein, and higher levels of FFAs, in bovine milk compared to milk from cows fed *ad libitum* at a lower stocking rate. Higher stocking density also coincides with increased somatic cell count (SCC) and activity of proteolytic enzymes in milk (O'Brien et al., 1999a). It has been reported that supplementing the diet of pasture-fed cows with concentrate (2-4 kg) as a protein and energy source in cow's feed reduces the production of sub-optimal milk quality, when accessibility of grass intake by cows is limited (Dillon et al., 1997). However, supplementation of feed with concentrate may not show similar results when cows have a good plane of nutrition throughout lactation (O'Brien et al., 1996; Dillon et al., 1997). The change in milk composition depends on the composition of feed materials (Macheboeuf et al., 1993; Kefford et al., 1995; Verdier-Mertz et al., 2005). Generally, cows grazing in natural pasture yield milk of almost similar composition (except free fatty acid level, casein number) to milk from cows diet supplemented with silage or concentrate when their energy requirement is met. Supplementation of cow's diet with silage or concentrate reduces the levels of FFA (O'Brien et al., 1996; Dillon et al., 1997) in milk compared to milk from cows exclusively pasture fed.

1.5.1 Impact of feed on casein composition

Although studies are limited in number, the effect of feed type on casein composition has shown that increasing the proportion of silage and hay in cows' feed results in a lower amount of β -casein, and κ -casein and higher levels of α_{s1} -casein, α_{s2} -casein and γ -casein in milk than feeding grass (Table 1.4). Apparently, there was little or no influence on the proportion of different caseins as influenced by different diets. Casein degradation products, e.g., γ -caseins, have been reported to be present at higher levels in milk when cows were fed insufficient amounts of protein and energy source (O'Brien et al., 1996). In the absence of grazing *ad libitum*, Dillon et al. (2002) suggested that allowing cows at least 5-6 h/day on pasture significantly improves the milk protein composition for cheesemaking. This improvement could be due to increased levels of β - and κ -caseins and reduced levels of γ -casein, and changes in Ca, P, and Mg salts in milk (Christian et al., 1999a,b).

Table 1.4 Effect of feed in casein composition.

Feed type	α_{s1} - casein	α_{s2} - casein	β -casein	κ -casein	γ -casein
100% Spring pasture	400-410	85-90	380-390	82-86	15-25
Spring pasture (50%) +silage (22%) +hay (28%)	420-430	75-85	360-380	77-81	11-25
Spring pasture (24%) +silage (33%) +hay (42%)	405-418	80-90	340-370	77-82	35-45
Grain (40%) +silage (33%) +hay (26%)	412-420	91-97	335-350	78-81	32-41
Silage (44%) + Pasture hay (56%)	420-430	81-87	335-345	76-79	40-50

Data expressed as g/kg casein (Christian et al., 1999a, b; O'Brien et al., 1996)

1.5.2 Impact of feed on the rennet coagulation properties of milk

Various studies have shown effects of feed on the cheesemaking quality of milk (Macheboeuf et al., 1993; Kefford et al., 1995; Christian et al., 1999a; Dillon et al., 2002; Kalač, 2011). There is broad agreement on the improvement of the cheesemaking properties of milk produced from cows fed sufficient energy (Malossini et al., 1996; O'Brien et al., 1996; Guinee et al., 1998; O'Brien et al., 1999a; Dillon et al., 2002). Enhanced cows' diet has been shown to result in milk having a shorter rennet coagulating time, firmer curd during cheesemaking, and subsequently a lower moisture level in cheese (Macheboeuf et al., 1993; Kefford et al., 1995; Guinee et al., 2001; Dillon et al., 2002; Bovolenta et al., 2008). No difference in the cheesemaking properties of milk from cows during 218-284 days of lactation was noted compared to cows in mid-lactation when cows were offered good nutrition throughout the lactation cycle (O'Brien et al., 1996; O'Brien et al., 2006). As discussed in section "Effect of feed on milk composition", feeding concentrate to milking cows has been suggested as a means to maintain the properties of milk for cheesemaking, especially in late-lactation when the grass availability is low (Lucey, 1996). The type of diet supplied to milking cows needs special consideration when producing milk for cheesemaking. For instance, cows fed buckwheat silage yielded milk that coagulated faster, and gave a firmer gel, in comparison to milk from cows offered rye grass silage (Kälber et al., 2013), possibly due to compositional differences. On the other hand, cows that were on a suboptimal diet, particularly in the late-lactation, yielded milk having undergone extensive proteolysis (Hinz et al., 2012), which resulted in impaired renneting and syneresis properties (O'Keeffe, 1984; O'Brien et al., 1996; Bovolenta et al., 2008). The mechanism of this impairment has not been elucidated clearly.

1.5.3 Impact of feed on cheese composition

Various studies have shown that cows fed low quality (silage, pasture hay and oaten straw, 12 kg DM/cow per day) feed yield milk that gives undesirably high moisture in Cheddar cheese made therefrom compared to cheese made from cows fed a high quality diet supplemented with maize grain and sunflower meal (Kefford et al., 1995). These studies have also shown reduced recovery of total solids and hence cheese yield when manufactured from milk, which is produced from inadequate feed (Christian et al., 1999a,b; Bovolenta et al., 2009; Guinee et al., 1998; Kefford et al., 1995). Moreover, Bovolenta et al. (2008) reported that milk from cows fed high level of supplements at a high stocking density did not show improved milk coagulation properties and gave cheeses with a hard and gummy texture.

Milk coagulation properties may differ between milk samples as influenced by feeding regimes (Grimley et al., 2009; Coppa et al., 2011a,b) or due to inclusion of silage (Verdier-Metz et al., 1998; Verdier-Metz et al., 2005; Kälber et al., 2013), due to the diversity of feed composition (Coulon et al., 2004). Milk produced by cows on a higher level of supplements gave cheese with better textural and sensory properties compared to milk from cows on low levels of supplement (Bovolenta et al., 2009). Silage-fed cows produce milk containing more volatile and colour compounds than cows fed hay; this contributed a yellowish colour to the cheese, probably due to greater retention of carotenoids in silage compared to hay during storage (Verdier-Metz et al., 1998, 2005; Kalač, 2011).

1.6 Impact of somatic cell count on milk composition

The quality and suitability of milk for cheese manufacture has been linked directly to somatic cells count (SCC). Milk with $>4 \times 10^5$ cells /ml has been deemed

unfit for human consumption or further processing within the EU (Council Directive 92/46/EEC, 1992). Somatic cells are comprised of different cell types, e.g., macrophages, polymorphonuclear leucocytes (PMN), lymphocytes and epithelial cells (Li et al., 2014). Generally, in milk from healthy cows, somatic cells are mainly macrophages, whereas milk from unhealthy cows contains a high proportion of PMN (Lietner et al., 2006). Macrophages and PMNs have a high level of proteolytic enzymes (cathepsins, B, D, H, L, G, S and elastase) (Li et al., 2014). Moreover, with an increase in SCC due either to mastitis-related pathogens (e.g. *Streptococcus aureus*, *Streptococcus dysgalactiae*, and *E.coli*) or when produced in late lactation has increased levels of associated enzymes (Leitner et al., 2006; Merin et al., 2008).

Kelly et al. (2006) reviewed the proteolytic enzymes in milk. The activity of plasmin is commonly found to be 2-4 fold higher in milk from mastitis-infected cows than healthy cows (Leitner et al., 2006; O'Brien et al., 2001). Conflicting results have been reported with respect to SCC and plasmin activity. Some studies have claimed that there is no influence of SCC up to 5.2×10^5 cells/ml on plasmin activity (Cooney et al., 2000; Hinz et al., 2012) whereas others have observed significantly higher plasmin activity when the count is $>5 \times 10^5$ cells/ml (Auldish et al., 1996; O'Brien et al., 2001; Somers et al., 2003). The activation of plasmin in milk is a complex phenomenon as it depends on the effect of plasminogen activators and their inhibitors (Leitner et al., 2006), which could probably depend on the presence of PMNs. Nevertheless, a study on the proteolysis by plasmin alone in milk showed extensive protein breakdown in milk, especially of β -casein (Srinivasan and Lucey, 2002).

Milk with a higher SCC reduced levels of total casein, β -casein, α -casein and increased levels of γ -casein and proteose peptones (Klei et al., 1998; Cooney et al.,

2000; Marino et al., 2005; Mazal et al., 2007; Forsbäck et al., 2011; O'Brien et al., 2001). Various studies have reported a longer rennet coagulation time and weaker curd with increasing milk SCC (Somers et al., 2003; O'Brien et al., 2001; Forsbäck et al., 2011). Srinivasan and Lucey (2002) showed that on addition of plasmin to milk, a decrease to < 40% of intact casein drastically altered the microstructure of rennet-induced gels and they reported that even with low levels of casein hydrolysis, the rheological properties of rennet gels were altered, which could have negative impacts on cheese yield and texture.

Changes in the SCC in milk depend on multiple factors such as breed and parity (McParland et al., 2013), stage of lactation (Auldist et al., 1996) and udder health (Green et al., 2006; Leitner et al., 2006). In healthy herds, milk from Montbeliarde or Jersey cows has a lower SCC than milk from Holstein or Friesian cows (McParland et al., 2013). Furthermore, cows in first lactation give milk with a lower SCC than older animals. In Ireland, the SCC in milk produced from a spring-calved herd showed a decreasing trend from January until April ($\sim 196 \times 10^3$ to 162×10^3 cells/ml) and increasing trend from June until November (162×10^3 cells/ml to 206×10^3) (O'Connell et al., 2015; McParland et al., 2013). In October and November, a rapid increase in SCC has been reported because the majority of cows were entering into late lactation. However, a different trend in the change of SCC in milk has been reported for milk obtained from herds containing spring- and autumn-calving cows compared to spring-calving alone. For instance, milk from herds calved at different season had a higher average SCC in early and mid-lactation and lower in late-lactation than milk from spring-calved cows alone; this has been explained due to mixing of milk from different stages of lactation (O'Connell et al., 2015).

The influence of SCC on milk composition has been reviewed widely (Li et al., 2014; Ruegg and Pantoja 2013; Le Maréchal et al., 2013). Geary et al., (2013) found that each \log_{10} unit rise of somatic cells count in milk correlated positively with true protein (0.082%), non-protein nitrogen (0.006%), casein (0.047%), whey protein (0.041%), fat (0.11%), and negatively with lactose (-0.14%) and casein levels as a percentage of total protein (-0.96%). Hence, presence of higher SCC in milk negatively influences the final yield. The loss of casein products into whey is responsible for reducing the final yield (Politis and Ng-Kwai-Hang, 1988; Klei et al., 1998). Therefore, milk with high SCC, e.g., mastitic or late lactation milk, may show hydrolysis of the native casein structure in milk with deleterious effects on the cheesemaking quality.

1.6.1 Impact of SCC on cheese composition

Each \log_{10} unit increase in the value of SCC (6×10^3 to 1.4×10^6 cfu/ml) in milk results in an increased moisture content of 0.5% in subsequent Cheddar cheese, according to a meta-analysis study from previously published literature (Geary et al., 2013). In addition, cheese made from milk with a high SCC contains a higher level of water-soluble nitrogen than cheese made from low SCC milk (Cooney et al., 2000; Marino et al., 2005; Mazal et al., 2007). For example, increasing somatic cell count from 3×10^5 to 6×10^5 in milk prior to cheesemaking resulted in increased primary proteolysis (% pH 4.6 SN/TN) from 19.4 to 21.0 in 2 months old ripened Cheddar cheese (Marino et al., 2005). This could be due to proteolysis during ripening by proteolytic enzymes (Hinz et al., 2012) which negatively affects sensory properties such as firmness, texture, and flavour in Cheddar cheese (Grandison and Ford, 1986; Auldist et al., 1996). Studies have suggested that milk exceeding SCC $1\text{--}3 \times 10^5$ cells/ml

may not be suitable for cheesemaking (Barbano et al., 1991) after 72 hours of cold storage (O'Brien et al., 2001).

However, it has been suggested that the effect of SCC in different studies has been ill-defined due to the association of various cofactors such as other endogenous enzymes, bacterial enzymes and plasmin (Li et al., 2014; Le Maréchal, 2011). Li et al. (2014) suggested that the appropriate method to study the impact of SCC in milk could be by concentrating or isolating somatic cells from healthy cows, and adding these somatic cells to somatic cell-free milk in different proportions.

Controlling the SCC in milk for cheesemaking is important at farm level. Kelly et al. (2009) emphasized on-farm management practices for cow hygiene, post milking teat disinfection and data recording systems as critical points at which a farmer can control SCC in milk that is destined for cheese production.

1.7 Impact of breed on milk composition and cheesemaking properties

The cheesemaking properties of milk from different breeds have been explored in order to customize cheese milk for efficient cheese production (Auldist et al., 2004; De Marchi et al., 2007; De Marchi et al., 2008; Frederiksen et al., 2011; Penasa et al., 2014). As already discussed, milk with high solids, casein, smaller casein micelles and the BB variant of κ -casein have improved cheesemaking characteristics (Auldist et al., 2002; Macheboeuf et al., 1993). Milk composition, casein composition and rennet coagulation properties are presented using the data from different studies in Table 1.5. Milk from Jersey cows shows superior properties in terms of milk and casein composition in comparison to that from Montbeliarde, Normande, Friesian-Holstein, Tarentaise and Brown Swiss cows. The superiority in terms of rennet coagulation properties (curd firmness) is in the order of Jersey > Normande > Montbeliarde >

Tarentaise > Brown Swiss > Friesian-Holstein. Interestingly, milk from Montbeliarde cows contains the smallest casein micelle size in comparison to other breeds.

Moreover, a limited number of studies have shown the impact of milk produced from different breeds for cheesemaking. Some of the studies determined curd firmness as A_{60} (Auldist et al., 2002; Auldist et al., 2004), whereas others determined A_{30} (De Marchi et al., 2007; De Marchi et al., 2008; Macheboeuf et al., 1993; Bland et al. 2015), which makes it difficult to compare the trend for gel firmness between breeds reported in different studies.

It has been shown that the inferior properties of milk from Holstein cows may be improved by mixing with high quality milk from a superior breed, e.g., Jersey (De Marchi et al., 2008; Bland et al., 2015). Alternatively, improvement can be achieved by slightly increasing the concentration of milk solids by ultrafiltration (Auldist et al., 2004). For instance, De Marchi et al. (2008) made Italian soft cheese (Casolet, Vezzena, and Grana Trentino) from mixed milk from Holstein and Brown Swiss reported a better yield, coagulation time and curd firmness than milk from Holstein cows. Increases in moisture adjusted yield (9.7 to 12.7%), and fat (85.14 to 87.76%), and protein recovery (77.40 to 78.26%) were reported when increasing the proportion (25 to 75%) of Jersey milk in Holstein milk (Bland et al., 2015). However, milks used in the study Bland et al. (2015) were not standardized with respect to protein-to -fat ratio, for example, rennet-to-casein ratios, curd firmness at cut differed; hence, cheese composition may vary with respect to differences in moisture content.

Table 1.5 Effect of breed of cow on milk, casein profile and rennet coagulation properties.

Parameters	Friesian Holstein	Ref	Jersey	Ref	Montbeliarde	Ref	Brown Swiss	Ref	Tarentaise	Ref	Normande	Ref
Milk composition												
Fat %	3.77±0.32	1, 2,3,4,5,6,7,8, 9,11,12	5.43±0.38	1,5,7	3.67±0.22	8, 2,12	3.85 ±0.27	3,4,6,9,11	3.64 ±0.38	2,8,	3.81	12
Protein %	3.28±0.21	1,2,3,4,5,6,7,8 ,9, 10,11,12	3.84±0.12	1,5,7	3.26±0.04	8, 2,12	3.49 ±0.27	3,4,6,9,11	3.21±0.01	2,8	3.49	12
Casein %	2.57±0.23	1,2,5,6,7, 9, 10,11,12	2.96±0.17	1,5,7	2.63±0.02	2,12	2.77±0.29	6,9,11	2.61	2	2.77	12
Lactose%	4.78±0.19	1,2,4,5,7, 11,12	4.55±0.50	1,5,7	4.81±0.13	2,12	4.98±0.06	4,11	4.92	2	4.73	12
Casein no	78.42±2.77	1,5,6,7,8,10,1 1,12	76.66±2.3 1	1,5,7	80±1.41	8,12	79.1±0.28	6,11	81.7	8	79.4	12
Micelle size nm	164±10.12	5,12	158	5	138	12	N/a	-	N/a	-	151	12
Casein composition												
α_{s1} *	34.61±1.25	10,12	N/A	-	35.8	12	N/a	-	N/a	-	34.1	12
α_{s2} *	7.97±1.35	10,12	N/A	-	9.38	12	N/a	-	N/a	-	9.12	12
β -casein*	38.74±6.40	1, 10,12	49.27	1	34.8	12	N/a	-	N/a	-	35	12
κ -casein*	12.34±1.23	1, 10,12,	14.96	1	11.8	12	N/a	-	N/a		12.4	12

Continued

Continued **Table 1.5** Effect of breed of cow on milk, casein profile and rennet coagulation properties.

Parameters	Friesian Holstein	Ref	Jersey	Ref	Montbeliarde	Ref	Brown Swiss	Ref	Tarentaise	Ref	Normande	Ref
Rennet coagulation ability												
RCT min	19.03±5.29	1,2,3,4,6,7,9,1 2	32.2	1	18.9±5.65	2,12	32.76	3,4, 6,9	20.8	2	13.2	12
k ₂₀	12.44±2.63	1,2,3, 12	10.3	1	9.9±3.11	2,12	6.4	3	14.2	2	5.9	12
A _{60,30}	25.26±9.22 (A ₃₀)	2,3,4,6,9	52.7 (A ₆₀)	1	43.55±2.05 (A _{60,30})	2,12	27.5±4.87 (A ₃₀)	3,4,6,9,11	40.5 (A ₃₀)	2	48 (A ₆₀)	12

Figures with multiple references are presented with standard deviation. * concentration of individual caseins is expressed as a percentage of total casein. For RCT, K₂₀, A₆₀, please see Table no. 1.1, Ref stands for references, 1=Auldist et al. (2004), 2= Macheboeuf et al. (1993), 3=De Marchi et al. (2007), 4=De Marchi et al. (2008), 5= Bland et al. (2015), 6=Penasa et al., (2014), 7=Frederiksen et al. (2013), 8=Coulon et al. (1998), 9=Cecchinato et al. (2011), 10=Jōudu et al. (2008), 11=Malossini et al (1996) and 12= Auldist et al. (2002). N/A indicates data not available

Interestingly, concentration of Holstein milk to the level of that in Jersey milk showed no differences in cheesemaking properties between the two milk types (Auldist et al., 2004). Recent studies have also focused on milk from Finnish Ayrshire (Ikonen et al., 1999; Tyrisevä et al., 2004), Swedish red (Poulsen et al., 2013), Danish red (Frederiksen et al., 2011) to explore the cause of impaired coagulation characteristics.

1.7.1 Genomic variants

Recently, various attempts have been made to improve milk processability for cheesemaking through breeding. For instance, milk from Holsteins cross-bred with Montebelliarde bulls showed improved rennet coagulating properties in comparison to Holsteins cross-bred with Brown Swiss or Swedish red bulls (Malchiodi et al., 2014). Identification of appropriate breed based on genomic variants has also been attempted (Poulsen et al., 2013). For example, casein genes *CSN1S1 C*, *CSN2 B* and *CSN3 B* were associated with good coagulating and *CSN2AA* with poor coagulating milks (Poulsen et al., 2013). Genotype *CSN3BB* has been found to be associated with a high degree of κ -casein glycosylation (Bonfatti et al., 2014). In contrast, cow breeds that secreted genetic variants BB, AA and AA of α_{s1} -casein, β -casein, κ -casein, respectively were identified as being inappropriate for cheesemaking (Jensen et al., 2012; Gustavsson et al., 2014a).

Hence, cheesemaking could be more efficient if milk was selected on casein composition (Auldist et al., 2002; Wedholm et al., 2006; Bonfatti et al., 2014; Freitas et al., 2015) or by improving a breed having a particular casein genotype (Ikonen et al., 1999; Glantz et al., 2009; Gustavsson et al., 2014c). It is suggested that breeding

programmes for next generation cows should consider not only fertility but also milk compositional properties.

1.8 Protein standardization

Ultrafiltration of milk prior to cheesemaking allows the concentration of proteins and fat for cheesemaking and increases curd mass. Milk standardization allows for more consistent processing, reduced rennet costs and improved product quality (Guinee et al., 1994). Ultrafiltration of milk for cheesemaking to a protein level of >40 g/L results in a more dense curd, which tears easily. This problem is overcome by lowering the coagulation temperature from 31 to 27°C, which slows the secondary stage of coagulation, resulting in a softer, more workable curd (Guinee et al., 1994). A reduced moisture level associated with an increased protein level in cheese may pose problems for the cheese industry when using ultrafiltration. Cheese is sold based on legal moisture and FDM contents as opposed to a cheese solids basis. Cheesemaking methodologies that use ultrafiltered milk must therefore develop a method for increasing cheese moisture level in the presence of high protein concentrations. Decreasing set temperature, lowering maximum scald temperature, manipulation of cut size and a faster rate of cooking are all possible methods for increasing moisture level (Green et al., 1981; Guinee et al., 1994).

Ultrafiltration of milk prior to cheesemaking to a protein level of ~ 45 g/L or higher offers potential for increasing cheese yield and reducing seasonal variations associated with milk composition, allowing for a more consistent gel strength at cutting and improving the overall finished cheese (Guinee et al., 1994). However, cheese-makers need to understand the effect of changing coagulation conditions on rennet coagulation properties and curd moisture loss properties when using protein-

standardized milk. The rennet coagulation properties of protein-standardized milk results in increased curd firmness before cutting, and greater curd-to whey ratio in curd and whey mixture during stirring in comparison to the gel prepared from regular milk (Guinee et al., 2006) and alteration of these factors may entail optimizing the cheesemaking process. Therefore, a greater understanding of the coagulation properties of protein concentrated milk and the syneresis profile of the subsequent curds is necessary and should be focus of future research.

1.9 Conclusions

Undoubtedly, the quality of cheese depends on the milk from which it is made, and much of the variability in the composition and character of any cheese variety depends to a significant extent on the fact that raw milk is a rather variable material from compositional, microbiological, enzymatic, and other perspectives. Such variability may be exacerbated by factors which are unique to specific production contexts such as regions, which have a highly seasonal milk supply. In addition, variation in diet, season, somatic cell count, and other farm-level factors in any country can influence cheese composition and quality; in recent years, understanding of the relationship between compositional factors including specific protein fractions and cheesemaking has been greatly expanded. A key question then concerns the steps which cheese manufacturers can take to compensate such variability; key steps which have been practiced for decades include standardization of milk on a fat-protein basis, and addition of CaCl_2 to optimize the mineral balance of milk, while newer approaches include membrane filtration to adjust milk composition. This review considers how milk composition is influenced by various factors at farm level and how such variability in milk composition influences the cheesemaking properties and final

cheese quality. The information may be useful in the selection and treatment of milk for improving consistency and quality of cheese.

1.10 References

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Chapter 3

Kinetics of moisture loss during stirring of model cheese curds produced from standardized milks of cows on pasture or indoor feeding system

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3.1 Abstract

This study compared the in-vat moisture loss kinetics under fixed cheesemaking conditions during 75 min stirring of curds prepared from protein-standardized milks produced from indoor cows fed Total Mixed Ration (TMR), or outdoor cows fed Grass only (GRA) or Grass mixed with Clover (CLO). Relative Curd Moisture as a function of time was fitted to different empirical equations, of which a logarithmic function gave the best fit to the experimental data. The moisture loss rate constant ($k \text{ min}^{-1}$) was found to be similar for curds from protein-standardized TMR, CLO and GRA milks, showing minimal feed-induced variations in syneretic properties.

3.2 Introduction

Moisture levels in cheese-curds (hereafter curd) decrease due to whey expulsion (syneresis) after cutting of a rennet-induced coagulum during cheesemaking (Mateo et al., 2009b; Everard et al., 2008). The rate of reduction in curd moisture level depends on milk source, milk composition, e.g., protein and fat level, open spaces for whey movement (microstructure) and process conditions applied to the curds (Calvo and Balcones 2000; Mateo et al., 2009a; Everard et al., 2011). In modern plants, cheesemaking processes are mainly performed based on time-based standard operating procedures (Guinee et al., 2006), in which the effects of variation in milk source or composition on curd syneresis may not always be given sufficient consideration. Variations in milk composition have been reported to influence final cheese moisture (Auldist et al., 1996) and this could be (at least in part) due to differences in curd moisture contents before drainage (Dejmek and Walstra 2004).

Whey expulsion from curds is influenced by many intrinsic factors, e.g., pH (Piyasena and Chambers 2003; Piyasena et al., 2003; Grundelius et al., 2000; Lodaite et al., 2000; Giroux et al., 2014), fat (Mateo et al., 2009a; Talens et al., 2009), exopolysaccharide (Costa et al., 2012), whey proteins (Piyasena and Chambers 2003) and calcium levels (Geng et al., 2011; Caron et al., 2001; Casiraghi et al., 1987). Similarly, different processing conditions, e.g., cutting speed (Everard et al., 2008), curd size (Renault et al., 1997; Grundelius et al., 2000), stirring duration (Everard et al., 2011), temperature (Iezzi et al., 2012; Geng et al., 2011; Thomann et al., 2006; Giroux et al., 2014), homogenization (Thomann et al., 2008) and membrane filtration (Caron et al.,

2001), also influence the whey expulsion behaviour of curds and hence the curd moisture content.

Different feeding regimes for dairy cows have been reported to influence milk composition, e.g., protein and fat levels (O'Callaghan et al., 2016), and thereby final cheese composition (Christian et al., 1999; Martin et al., 2005). Countries following grass-based feeding regimes (e.g., Ireland and New Zealand) have larger seasonal variations in milk composition than countries where dairy herds are mainly fed indoor with mixed rations (O'Callaghan et al., 2016) which further emphasizes the importance of considering such variation for cheesemaking. Recently, Gulati et al. (2016) reported variations in composition and cheesemaking properties of milk produced under different feeding regimes, including indoor feeding of total mixed ration (TMR) and grazing on grass and grass plus white clover. feeding of grass only or grass mixed with clover in Ireland. Guinee et al. (2006) suggested that the impact of seasonal or feed-induced variation in milk composition on cheesemaking could be minimized by adjusting protein levels of milk to a uniform level followed by fat standardization. The effect of protein standardization of milks produced under different feeding regimes on curds moisture content dynamics during stirring in-vat have not, however, been investigated.

Traditionally, suitability of curds is evaluated by empirical methods, e.g., curd are squeezed by hand and released and if the curds segregate easily and show resilience against collapse, then curds are assumed to have the desired moisture content prior to drainage (Akkerman, 1992). Accurate in-process prediction of curd moisture levels would be a key development in determining curd drainage time based on milk composition (Mateo et al., 2009c). Furthermore, understanding changes in in-vat curd moisture content

as influenced by herd diet would help in reducing process variability during cheese manufacture.

The objective of this study is to examine curd moisture content and the kinetics thereof, from standardized milks produced from cows kept indoor and fed TMR or pasture-based cows grazed either on grass, or grass together with clover. The present study also aimed to compare different empirical models to monitor syneresis during cheese manufacture with experimental data to predict curd moisture contents in-vat. Previously it was reported that milk from pasture-fed cows during late lactation was suboptimal for cheesemaking, possibly because of a poor plane of nutrition (O’Keeffe, 1984; Kefford, et al., 1995). The present study investigates syneresis properties of curd using milk when spring-calved cows were supplied with a good plane of nutrition during late lactation stage.

3.3 Materials and Methods

3.3.1 Feeding regime of research herds

A spring-calved herd of cows at the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, was divided into three herds with 15 cows per group. The herds were each allocated to one of the following feeding systems: Total Mixed Ration (TMR), Grass (GRA) or Grass together with Clover (CLO) and the compositions of each feed are as described in the study of O’Callaghan et al. (2016). The current study focused on milk obtained from the cows over the period, 250-280 days in lactation (DIL). Briefly, the TMR feeding comprised of indoor feeding (grass silage, 7.2 kg DM; maize silage, 7.2 kg DM and concentrate, 8.7 kg DM, per cow per day). GRA and CLO feeding included

outdoor grazing (~13 hr), the former on land with perennial ryegrass (*Lolium perenne* L.) and the latter on land containing perennial ryegrass with white clover (*Trifolium repens* L.). The outdoor cows were also supplemented with 5.3 kg DM grass silage for the GRA treatment, 7.25 kg DM grass white clover silage for the CLO treatment, and 2.6 kg concentrate per cow on a daily basis for both treatments to fulfil the minimum nutritional requirement of the herd due to reduced grass/clover growth during the study period. Average clover content on the CLO treatment during the experimental period was 27% in the sward. The diet quantity was calculated on average data for one month.

3.3.2 Milk collection and preparation

Raw milks from each group of cows were collected at morning and afternoon milkings over 2 days, on each week of a three-week period. Milks were standardized to achieve protein-to-fat ratios (PFR) of ~1 after collection on each week. The PFR was chosen to represent a continental-type cheese with a PFR intermediate between Cheddar and a continental cheese-type (e.g., Maasdam cheese). GRA milk was standardized by adding skim milk prepared from GRA milk to the corresponding original raw milk, whereas CLO or TMR milks were standardized (to the same level of milk protein as in standardized GRA milk) by adding skim milk, ultrafiltration (UF) retentate and/or permeate prepared from the respective milks (Table 3.1). The objective of standardizing GRA, CLO and TMR milk to the same protein content was to minimize the differences in syneresis due to milk composition and relate the observed differences to feeding system. The point of using ultrafiltration to standardize protein content of milk from CLO or TMR feeding system was to achieve a similar casein composition and a mineral balance (in serum phase) in the protein-standardized milk as compared to the respective original

raw milk. Table 3.1 shows components used for standardization, which supports assumption that minerals in the standardized milk might not be influenced by the method of standardization. UF of skim milk was carried out as described by Kelly et al. (2000) using a spiral-wound membrane plant (Memtech Ltd, Swansea, UK, max surface area 144 m², molecular weight cut-off = 10 kDa) at 45°C to a milk protein level of ~5%. Standardized milks were pasteurized at 72°C for 15 s (MicroThermics, Inc. Wellington Ct, North Carolina, USA), and stored in 12-L sterile plastic jars overnight at 4°C.

3.3.3 Milk coagulation and curd cutting

The curd-making process was as described by Mateo et al. (2009a). Briefly, milk (11 kg) was transferred to a double-O mini cheese vat (Type CAL 10L, Pierre Guerin Technologies, Mauze, France) and heated to 32°C by circulating hot water in the jacket *via* a water bath (Grant Y28, Grant Instrument Ltd., Cambridge UK). The pH of the standardized milk was adjusted to 6.5 to reflect a typical pH during the coagulation step of cheese manufacture by adding a 4%, w/w, lactic acid solution and a milk sample (~20 g) was removed for compositional analysis. Coagulant (Chy-Max Plus, 205 IMCU/ml, Chr Hansen Ireland Ltd., Cork, Ireland) was then added at a level of 0.16 ml/kg (diluted as a 5%, w/w, solution) with constant stirring. Lactic acid cultures were not added in the present study to eliminate the effect of pH change on moisture loss from curds during stirring, which also minimizes the influence of internal factors such as buffering capacity, pH change and enzymatic activity and solubilization of colloidal calcium.

After three minutes of stirring, a sample of milk with coagulant-added (20 ml) was transferred to a rheometer (ER 2000 ex Rheometer, TA Instruments, Crawley, UK),

(cylindrical geometry), which was set up for small-amplitude oscillation, maintained at $32 \pm 0.1^\circ\text{C}$. The rheometer was operated at a frequency of 1 Hz and strain amplitude of 2% (bob diameter, 26 mm; cup diameter, 28 mm). Changes in storage modulus (G') of the gel were recorded every 30 s and the coagulum was cut at a G' value of 35 Pa as described in previous studies (Everard et al., 2008; Mateo et al., 2009a). The 3-min cutting programme included cutting at 10 rpm for 40 s and a healing period of 20 s, followed by two cycles of cutting at 22 rpm for 40 s and healing for 20 s. The cutter blades were replaced with stirrers operated at 22 rpm after 1 minute of healing.

3.3.4 Sampling of cheese-curds

Samples of the curd and whey mixture (~80 g to ~160 g as required for analysis) were withdrawn from the vat through an inline sampler at 10-min intervals from 5 to 75 min after cutting. The curd sampling method was followed according to previous studies (Mateo et al., 2009; Everard et al., 2011). Briefly, an inline sampler mounted on the wall of the cheese vat was used for sampling the mixture of curd and whey during in-vat stirring, after which the mixture was separated using a sieve (300 μm pore size) and pan. Since the curds were constantly stirred during sampling, the sample collection from the cheese vat was random. The duration of stirring time was chosen based on previous studies (Mateo et al., 2009a; Everard et al., 2008) which also reflects the residence time of curds in-vat from the start of cutting of the curds to vat drainage in commercial continental-type cheese manufacture. The moisture content of the curd sample was determined using hot-air oven-drying method as described in previous studies (Mateo et al., 2009a, b).

Table 3.1 Weight (kg), fat (g/100g) and protein (g/100g) contents of components used for milk standardization to a total weight of 100kg.

	Raw milk			Skim milk			Cream			Permeate			Retentate		
	Weight	Fat	Prot	Weigh	Fat	Prot	Weigh	Fat	Prot	Weigh	Fat	Prot	Weigh	Fat	Prot
Week 1															
TMR	-	-	-	-	-	-	13.4	29.39	3.89	18.75	0.13	0.25	67.85	0.1	5.22
CLO	-	-	-	-	-	-	8.93	42.75	N/A	16.59	0.14	0.26	74.48	0.1	5.22
GRA	79.64	5.0	4.12	20.36	0.13	3.89	-	-	-	-	-	-	-	-	-
Week 2															
TMR	37.91	4.9	3.77	-	-	-	5.57	40.54	2.17	0	0.16	0.23	56.25	0.1	4.6
CLO	68.67	4.7	4.07	-	-	-	2.95	27.12	2.5	5.13	0.15	0.28	23.26	0.1	5.32
GRA	78.6	5.2	4.21	21.56	0.18	4.05	-	-	-	-	-	-	-	-	-
Week 3															
TMR	80.12	5.0	3.89	-	-	-	-	-	-	0	0.15	0.21	19.88	0.1	4.83
CLO	80.61	4.9	3.88	-	-	-	-	-	-	0.89	0.17	0.26	18.5	0.2	5.19
GRA	77.34	5.2	4.01	22.66	0.19	3.98	-	-	-	-	-	-	-	-	-

N/A: Not available Weigh = Weight, Prot = Protein

TMR refers to milk produced from cows fed total mixed ration in indoor. CLO and GRA refer to milk produced from cows grazed in grassland containing grass with clover and grass, respectively.

3.3.5 Analytical methodologies

Gross milk compositions was determined by Fourier Transform Infrared (FTIR) spectroscopy, using a Milkoscan (Foss FT2® Foss Electric, Hillerod, Denmark) for raw milk, and using a Bentley Instruments Dairy Spec FT (Bentley Instruments, Inc. Minnesota, USA) for standardized milk. Levels of non-protein nitrogen (NPN), non-casein nitrogen (NCN) and whey protein were determined as described in O'Callaghan et al. (2016). Ionic calcium (Ca^{++}) was measured using a calcium ion selective electrode (SenSION+9660C) connected to a SensION+ MM 340 meter (Hach, Dublin, Ireland). A calibration curve was prepared using standard solutions of calcium chloride at concentrations of 0.5, 1, 2.5 and 5 mM at room temperature. Potassium chloride (3 M) was added to each standard solution as well as in to milk samples at the rate of 1% (v/v) prior to measurements. Samples were ashed using muffle furnace at 550°C for ash content.

3.3.6 Mathematical models

Curd moisture content at the point of cutting the curd (CMo) was assumed to be equivalent to the moisture levels of the milk (100 - total solids, g/100 g). The release of moisture from curd at time t equals $\text{CMo} - \text{CMt}$, where CMt refers to the moisture level in curd as a function of time.

The relative moisture content of the curd at time t after cutting (RCMt) was defined as the moisture content expressed as a % of the moisture content of the curd at time $t = 0$, and calculated as:

$$\text{RCMt} = \frac{\text{CMt}}{\text{CMo}} \times 100 \quad (1)$$

Thus, at $t=0$, $RCMo=100$.

Similarly, the relative whey expelled (RWE) from curd at time (t) is defined as:

$$RWE = \frac{CMo - CMt}{CMo} 100 \quad (2)$$

The Michaelis-Menten (MM) model for RWE can be presented as equation 3 (Thomann et al., 2006):

$$RWE = \frac{RWE_{max} \cdot t}{H + t} \quad (3)$$

Where, RWE_{max} is relative level of whey theoretically expelled at infinite time and H is the time for RWE to reach half of RWE_{max} .

The relative moisture levels in curd as a function of time can be predicted by substituting equations 1 and 2 in equation 3, to give

$$RCMt = RCMo \times \left(1 - \frac{RWE_{max}}{100(H+t)} \times t\right) \quad (4)$$

Different empirical models shown in Table 3.2 were fitted to the experimental data using a *PROC NLIN* procedure in SAS and goodness-of-fit statistics were determined.

3.3.7 Statistical analysis

A total of twenty seven vats of curd were manufactured within a three-week period, using milk samples (TMR, CLO and GRA) collected on each of 3 consecutive weeks, with 3 replicate vats prepared from each milk ($N = 27$).

Table 3.2. Description of models¹

Model	Order	Type	Equation	References
4	N/A	MM-model	$RCMt = RCM_o \times (1 - \frac{RWE_{max}}{100(H+t)} \times t)$	Thomann et al., 2006
5	First order	Exponential decay	$RCMt = RCM_{\infty} + a \times \exp(-kt)$	Giroux et al., 2014
6	Second order	Inverse time	$RCMt = RCM_{\infty} + a/(1 + akt)$	Giroux et al., 2014
7	First Order	Exponential decay	$RCMt = RCM_o \exp(-kt)$	Calvo and Balcones, 2000
8	N/A	Logarithmic function	$RCMt = RCM_o(1 - k \ln(t))$	-
9	First order	Exponential decay	$RCMt = RCM_o - RCM_5(1 - \exp(-kt))$	-
10	N/A	Power-law decay	$RCMt = RCM_o - RCM_5(1 - t^{-k})$	-

¹MM-model refers to Linear Michaelis-Menten model, RCMt refers to relative curd moisture at time t, RCM_o refers to initial relative curd moisture of milk; RCM₅ and RCM_∞ refers to relative curd moisture at 5 min of cutting and infinite time, respectively; k refers to rate constant for moisture loss and a corresponds to RCM_o – RCM_∞. RWE_{max} refers to maximum theoretical relative level of whey expelled; H refers to time calculated to expel half of RWE_{max}. Model no. 1-3 are described in materials and methods. N/A indicates not applicable.

Fat, protein and PFR levels of raw milk were compared with least square difference (LSD) at 95% significance level in one-way ANOVA using PASW Statistics for Windows, Version 18.0 (SPSS Inc. Released 2009, Chicago, US). A *PROC ANOVA* for curd moisture loss kinetic constant (k) and a *PROC MIXED* procedure were employed to compare means of standardized milk composition, curd moisture data with Tukey's multiple comparison at 95% significance level using SAS (version 9.3, SAS Institute Inc., Cary, NC). Boxplots were created using SPSS in which mild and extreme outliers were classified within and beyond the three quartile range, respectively. NPN, NCN, Ca⁺⁺, ash content, whey proteins were compared using independent sample t-test.

The goodness-of-fit of models was determined according to corrected Akaike's information criterion (AICc) statistics using the following equation (Symonds and Moussalli, 2011);

$$AIC_C = n * \left[\ln \left(\frac{SSE}{n} \right) \right] + 2p + 2p(p + 1)/(n - p - 1)$$

where, n= number of data points, SSE = sum of squared residuals; and p = number of model parameters.

Data from 27 trials were pooled according to the following equation and pooled AICc (AICcp) were calculated.

$$AIC_{Cp} = \sum n * \left[\ln \left(\frac{\sum SSE}{\sum n} \right) \right] + 2 \sum p + 2 \sum p (\sum p + 1) / (\sum n - \sum p - 1)$$

Models with the lowest AICcp values give the best-fit among the compared models.

3.4 Results and Discussion

3.4.1 Effects of feeding regimes on raw milk composition

Fat levels in raw milks, with respect to diet, were in the order GRA>TMR>CLO (Table 3.3) over the 3-week period, with significantly higher mean fat levels in GRA (5.15%) compared to CLO (4.74%) milk. Similarly, protein levels in raw milk in the present study were in the order GRA>CLO>TMR over a three-week period. The mean level of protein in GRA milk was significantly higher compared to that of TMR milk. The PFR ratio between milks from different feeding systems was not significantly different during the study period (Table 3.3).

Table 3.3 Fat, Protein, and PFR levels of raw milks by week of trial¹

Milk	Fat (g/100 g)	Protein (g/100 g)	PFR
Week 1			
TMR	4.68	3.82	0.82
CLO	4.53	3.95	0.87
GRA	5.0	4.12	0.82
Week 2			
TMR	4.92	3.77	0.76
CLO	4.74	4.07	0.85
GRA	5.23	4.21	0.80
Week 3			
TMR	5.01	3.89	0.77
CLO	4.97	3.88	0.78
GRA	5.22	4.01	0.76
Mean			
TMR	4.87 ^{a,b}	3.83 ^b	0.78 ^a
CLO	4.74 ^b	3.97 ^{a,b}	0.83 ^a
GRA	5.15 ^a	4.11 ^a	0.79 ^a

^{a,b}Means within a column sharing a common letter are not significantly different ($P > 0.05$)
Refer to Table 1 for the explanation of GRA, CLO and TMR. PFR refers to protein-to-fat ratio.

O’Callaghan et al. (2016) reported significantly higher fat levels in GRA milk than in milk from cows on TMR or CLO diets and significantly lower protein level in TMR milk compared to GRA or CLO during late lactation. Feeding systems of herds and days in lactation in this study differed from those of O’Callaghan et al. (2016), in that pasture-based cows in the present study were offered a low level supplementation to fulfil minimum nutritional requirements, necessitated by a seasonal reduction in sward growth; this may have somewhat reduced differences in protein levels in milks produced from the differently grouped cows. The lactose contents measured at the end of the lactation cycle (> 280 DIL) in all three types of raw milk were above 4.50 g/100g in raw milk, indicating that the level of nutrition supplied to the cows did not markedly influence milk composition. Kefford et al., (1995) reported that offering high energy diets to herds during late lactation helps to improve the nutritional status of herds and the composition of milk.

3.4.2 Effects of milk standardization on composition

Although variations occurred for individual milks between weeks, the mean fat levels, protein levels, PFRs, and set-times were similar each week for the standardized milks (Table 3.4). Lactose and total solids levels were not standardized in the present study and thus the levels of these components varied.

Table 3.4. Composition, Protein: Fat ratio and set times of standardized milks¹

Milk	Fat	Protein	Lactose	Total Solids	PFR	Set time
Week 1						
TMR	4.03	4.11	5.25	14.01	1.02	33.64
CLO	3.93	4.08	4.93	13.52	1.04	39.44
GRA	4.01	4.04	4.58	13.19	1.01	36.93
Week 2						
TMR	4.18	4.15	5.11	14.02	0.99	31.00
CLO	4.09	4.12	4.83	13.55	1.01	33.01
GRA	4.15	4.14	4.57	13.38	1.00	29.33
Week 3						
TMR	3.97	4.14	4.83	13.49	1.04	34.06
CLO	4.12	4.08	4.75	13.50	0.99	33.66
GRA	4.06	4.05	4.56	13.21	1.00	32.21
Mean						
TMR	4.05 ^a	4.13 ^a	5.04 ^a	13.81 ^a	1.02 ^a	33.20 ^a
CLO	4.05 ^a	4.09 ^a	4.83 ^a	13.52 ^{a,b}	1.01 ^a	35.56 ^a
GRA	4.08 ^a	4.08 ^a	4.56 ^b	13.23 ^b	0.99 ^a	32.96 ^a

^{a,b}Means within a column sharing a common letter are not significantly different ($P > 0.05$)

¹ Refer to Table 3.1 for the explanation of GRA, CLO and TMR. PFR refers to protein-to-fat ratio. Set time is time to reach G' values of 35 Pa.

In typical industrial practice, milk fat levels are standardized prior to curd-making to a target PFR. However, this may still result in variations in milk protein levels induced due to seasonal effects or feeding effects (Guinee et al., 2006), potentially resulting in fluctuations in the cheesemaking process or in the final cheese composition. In the present study, protein standardization of TMR and CLO milks minimized compositional variations in protein levels arising from different cows' feed, as suggested by Guinee et

al. (2006). While blending of different milk components resulted in increased lactose levels in some milk (Table 3.4), the levels of lactose were not considered to have influenced in-vat whey expulsion in the present study. Variation in the lactose content of milk used in commercial cheese manufacture may influence cheese pH and ripening profiles arising from cheese starter cultures. This may be controlled by employing different techniques, such as UF prior to cheesemaking followed by diafiltration or applying curd-washing during cheesemaking (Moynihan et al., 2016).

3.4.3 Effects on milk coagulation

Changes in gel firmness during coagulation of milk are shown in Figure 3.1 for a typical trial. The curds were cut at uniform firmness (35 Pa) and while some differences in set times occurred between weeks; there were no significant differences in set times between the standardized milks produced from herds on differing feeding regimes in each week (Table 3.4). The practice of cutting coagula at uniform G' values of gel firmness (Mateo et al., 2009a,b; Everard et al., 2011) gives uniformity in gel stiffness during cheesemaking when dealing with varying milk composition.

3.4.4 Effects of feeding systems on curd moisture in-vat

Curd moisture contents dropped significantly from ~81% to ~77% in the first 25 min of cutting, and the rate of decrease slowed thereafter to a final moisture level of ~72% after stirring for 75 min (Figure 3.2), consistent with previous studies (Caron et al., 2001; Everard et al., 2011). The initial moisture contents in the present study was lower (~81%) compared to the results obtained by Everard et al. (2008) (~86.9%, with skim milk) which could be due to differences in fat and protein contents of the milks used.

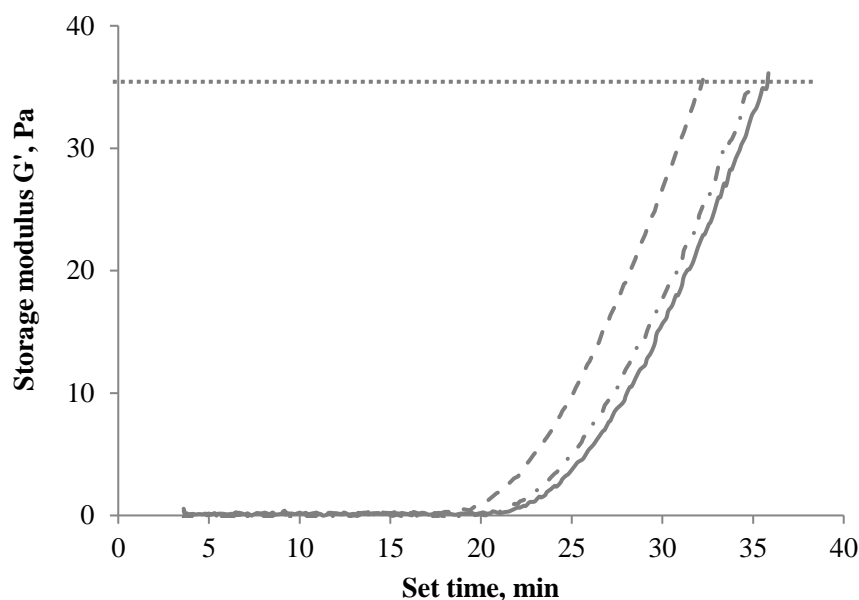


Figure 3.1 Gel firmness (Storage modulus, G') for a typical trial using GRA (— —), CLO (— · —) and TMR (——) milks. Each coagulum was cut at a uniform gel strength (G' , 35 Pa).

Mateo et al. (2009a) and Everard et al. (2011) showed that, with increasing fat content in milk, the initial moisture content of curd decreases. Mateo et al. (2009a), however, reported a complex relationship between whey expulsion and fat levels in milk, as fat may obstruct curd contraction or block pores for whey expulsion. Such complexities in the present study were probably minimized by standardizing fat and protein levels in milks to uniform levels.

The majority of whey expulsion took place within 25 min of cutting and with greater variation in data observed thereafter where some outlier data points were observed within the data (i.e. 2 extreme outliers and 6 mild outliers from the total data set of 216 points (Figure 3.2). Mateo et al. (2009a) observed similar trends while measuring syneresis under conditions similar to the present study. The outliers could be due to the presence of different curd particles sizes within the cheese vat, suggesting that different

levels of moisture within the curd can exist in a cheese vat and thus the data points were not removed in further analysis. Curds from GRA, CLO or TMR milk showed similar moisture levels at each sampling point during stirring, indicating that curds followed similar whey expulsion dynamics during stirring, irrespective of herd diets.

3.4.5 Comparison of models for moisture loss kinetics

A number of models were applied to the RCM data to determine the optimum model fit as defined by the lowest values for AICcp and root mean square error (RSME) (Table 3.5). AICc or RMSE values compare the goodness-of-fit for the various models (Symonds and Moussalli, 2011). The AICcp and RMSE values of model 8 (logarithmic function) or Model 10 were the lowest among the models (Table 3.5), indicating that these models fitted better compared to the other models, e.g., 1st order (model 2, 4, 6) or second order (model 3) or the MM equation (model 1), as indicated by higher AICcp values. The RCM decrease rate constant (k) of curds from different milk types was calculated using model 8. No significant differences in rate constant (k/min) were observed between curds produced from GRA (0.0369), CLO (0.0360) and TMR (0.0372) milks, confirming that curds from different feeding regimes followed the same extent and rate of moisture expulsion dynamics during cheesemaking (Figure 3.3).

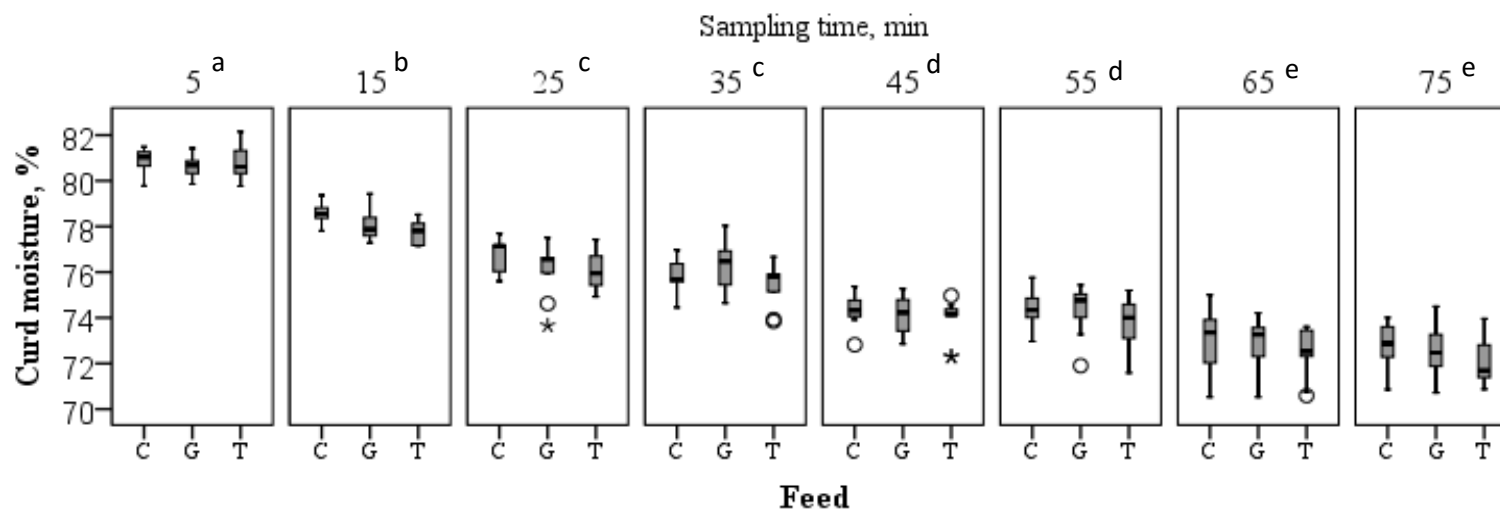


Figure 3.2 Boxplots of moisture content of cheese curd at intervals during stirring at 32°C, pH 6.5. The codes C, G, T refer to CLO, GRA and TMR milk, respectively. Each result is a mean of three repeated analyses in three weeks N=27. ○ indicates mild outliers, ★ indicates extreme outliers (greater than three quartile ranges). Sampling times with common superscript indicate statistically similar moisture levels in curds.

Table 3.5 Goodness-of model fit for Relative Curd Moisture (RCM)¹.

Model no	Type	Equation	p	Df	RMSE	AIC _{Cp}
4	MM-model	$RCMt = RCM_o \times (1 - \frac{RWE_{max}}{100(H+t)} \times t)$	2	161	1.071	111.42
5	Exponential decay	$RCMt = RCM_{\infty} + a \times \exp(-kt)$	2	161	1.420	234.37
6	Inverse time	$RCMt = RCM_{\infty} + a/(1 + akt)$	2	161	1.071	111.42
7	Exponential decay	$RCMt = RCM_o \exp(-kt)$	1	188	3.89	618.99
8	Logarithmic function	$RCMt = RCM_o(1 - k \ln(t))$	1	188	0.873	-26.33
9	Exponential decay	$RCMt = RCM_o - RCM_5(1 - \exp(-kt))$	1	188	3.87	616.83
10	Power-law	$RCMt = RCM_o - RCM_5(1 - t^{-k})$	1	188	0.894	-14.93

The number of time points in each model is n=216.

¹RMSE refers to root mean squared error, p refers to number of model parameters in the equation, df refers to degrees of freedom in model, AIC_{Cp} refers to pooled Akaike's information Criterion corrected for model comparison. Degrees of freedom (df), RMSE and AIC_C are pooled from 27 trials (n= 216).

The model parameters are explained in Table 3.2.

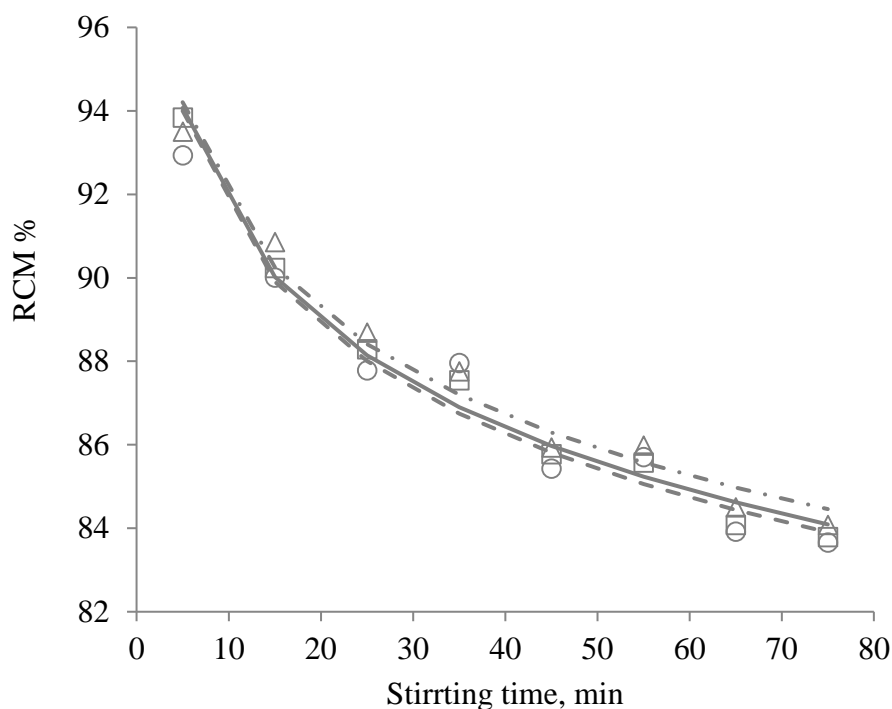


Figure 3.3 Relative curd moisture (RCM) in curds at 32°C, at pH 6.5 from TMR (\square), CLO (Δ) and GRA fed (\circ) milks fitted to a logarithmic function (model 8). The line plots are predicted values from model 8 for GRA (— —), CLO (— · —) and TMR (—). All data points shown are the mean of 9 values.

A good fit of model 8 or 10 signifies that the kinetics of in-vat curd moisture reduction may not perfectly follow first-order or second-order kinetics. However, studies have previously reported syneresis kinetics as fitting first-order (Grundelius et al., 2000; Kaytanli et al., 1994; Casiraghi et al., 1987; Calvo and Balcones, 2000), or second-order (Giroux et al., 2014) or higher models (Huber et al., 2001); in these studies, expulsion of whey was measured in small beakers (Piyasena and Chambers, 2003; Calvo and Balcones, 2000; Caron et al., 2001) or in tubes (Giroux et al., 2014; Thomann et al., 2008) or in one curd from one-dimensional perspective (Grundelius et al., 2000; Geng et al., 2011). The present study differs in that the coagulum formed in a 12-L cheese vat was cut using a rotating blade, which would have increased the frequency of curd collisions, thus leading

to a greater quantity of whey expulsion during cutting. A rapid decrease in curd moisture content in the vat may explain the poor-fit of first- or second-order equations with the RCM data.

Calvo and Balcones (2000) observed no differences in syneretic kinetics of curds made from bovine, caprine or ovine raw milks. However, the values of kinetic constants in the present study were higher than the value ($k=0.011 \text{ min}^{-1}$) observed by Calvo and Balcones (2000) in bovine milk, indicating that a faster release of moisture took place in the present study. In addition, the k value was close to that reported by Giroux et al. (2014), who measured whey expulsion from an uncut gel at 40°C.

Caron et al. (2001) reported change in whey expulsion rate in curds prepared from varying concentration of reconstituted milk powder made from UF-treated milk retentate. Addition of UF retentate to milks during standardization in the present study may have resulted in increased levels of colloidal calcium in the standardized milks; however, the different coagula were cut at uniform gel firmness to minimize such variations in whey expulsion dynamics. Levels of ash and ionic calcium in standardized milk samples and ash content in whey samples were similar, irrespective of milk type and their associated standardization history (Table 3.6).

3.4.6 Prediction of curd moisture

We predicted RCM during in-vat stirring using model 8, taking measured RCMo and the rate constant (k) as model parameters. The predictability of the model was satisfactory ($R^2 > 0.93$) (Figure 3.4). Application of theory-based models for determining moisture levels in cheese adds complexity (Jimenez-Marquez et al., 2005). Thus, recent

studies have explored the use of inline sensors to monitor syneresis during cheesemaking (Costa et al., 2012; Mateo et al., 2009b; Everard et al., 2007; Arango et al., 2015), which offers the possibility to determine curd moisture content in real-time (Mateo et al., 2009c). In this context, the prediction of curd moisture using empirical model 8 or 10 may provide valuable information for modelling of curd moisture and drainage times during cheesemaking using initial milk moisture. However, further validation of this model would be required for milks of differing compositions, or of differing stage of lactations or for curds produced under different process conditions, such as with the use of starter cultures.

Table 3.6 Minor components in standardized milk and whey samples

Week	Standardized milk				Cheese whey	
	NPN (g/100g)	NCN (g/100g)	Total ash (g/100g)	Ca ⁺⁺ (mM/L)	PW (g/100g)	AW (g/100)
TMRW1	NA	NA	NA	NA	NA	NA
TMRW2	0.03	0.14	0.79	2.15	1.11	0.50
TMRW3	0.03	0.17	0.82	2.67	1.11	0.55
CLOW1	NA	NA	NA	NA	NA	NA
CLOW2	0.04	0.15	0.79	2.49	1.18	0.49
CLOW3	0.04	0.15	0.82	2.09	1.16	0.50
GRAW1	NA	NA	NA	NA	NA	NA
GRAW2	0.03	0.14	0.80	2.49	1.13	0.46
GRAW3	0.03	0.16	0.83	2.58	1.12	0.49
Mean						
TMR	0.03 ^b	0.16 ^a	0.81 ^a	2.41 ^a	1.11 ^b	0.53 ^a
CLO	0.04 ^a	0.15 ^a	0.81 ^a	2.29 ^a	1.17 ^a	0.50 ^a
GRA	0.03 ^b	0.15 ^a	0.82 ^a	2.54 ^a	1.13 ^b	0.48 ^a

NPN, NCN and Ca⁺⁺ refers to non-protein nitrogen, non-casein nitrogen and ionic calcium in standardized milk, PW and AW refers to protein and ash content in whey samples at 75 min of stirring respectively.

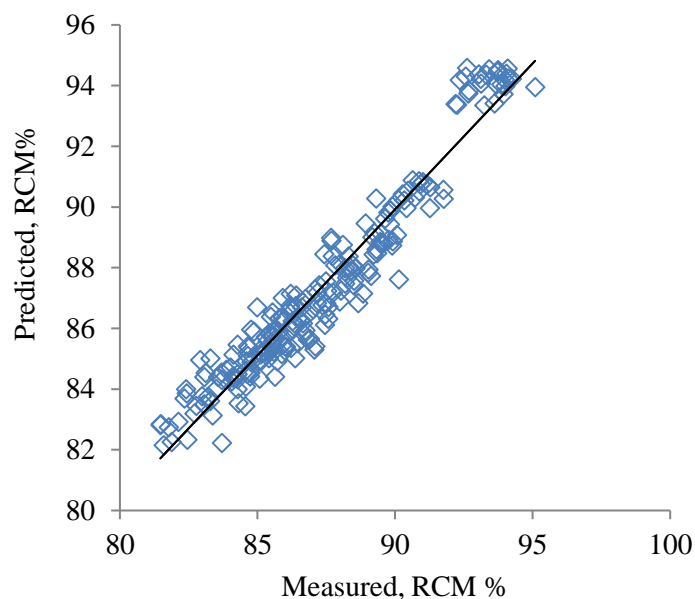


Figure 3.4 Scatterplot of RCM measured (x-axis) and predicted (y-axis) data using logarithmic model 8 (n= 216).

Natural variations in milk composition pose challenges in achieving a consistent cheesemaking process (Amenu and Deeth, 2007). Industrial cheesemaking has been largely dependent on standardization of fat, leaving protein levels unchanged, which may lead to variations in milk composition due to feeding practices, resulting in inconsistent cheese composition and quality (Guinee et al., 2006; Mateo et al., 2009a). Our study showed that, despite differences in milk composition due to herd diets (TMR, CLO or GRA), consistent coagulation and moisture levels in curds can be achieved by standardizing milk protein to uniform levels, for example using membrane filtration technology. However, the effect of this treatment on final cheese composition and quality requires further examination.

3.5 Conclusion

Herds fed with indoor (TMR) or outdoor (GRA or CLO) feeding regimes resulted in significant differences in raw milk compositions. However, this study found that protein and fat standardization of these milks to a uniform level minimized the impact of variation in milk composition arising from the differing herds' diets. This resulted in a consistent cheesemaking process, i.e., similar coagulation and curd moisture loss kinetics in-vat, resulting in consistent curd moisture levels before drainage. Either a logarithmic or power-law model provided a better fit compared to first- or second-order or exponential models for relative curd moisture data. Overall, application of these models could facilitate real-time prediction of curd moisture contents during in-vat stirring processes. This will enable cheese-makers to modify curd drainage times during cheese manufacture, to achieve optimum and consistent moisture levels in the final cheese, and thereby achieve optimum ripened cheese quality and consistency.

3.6 References

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Chapter 4

Influence of protein concentration and coagulation temperature on rennet-induced gelation characteristics and curd microstructure

Panthi, R. R., Kelly, A. L., Sheehan, J. J., Bulbul, K., Vollmer, A. H., and McMahon, D. J. (2019). Influence of protein concentration and coagulation temperature on rennet-induced gelation characteristics and curd microstructure. *Journal of Dairy Science*, 102, 177-189.

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4.1 Abstract

This study characterized the coagulation properties and defined the cutting window (CW, time between G' values of 35 and 70 Pa) using rheometry for milk standardized to 4, 5, or 6% protein and set at 28, 32, or 36°C. Milks were standardized to a protein-to-fat ratio of ~1 by blending ultrafiltration (UF) retentate, skim milk, and whole milk. The internal curd microstructure for selected curd samples was analysed with transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Lowering the coagulation temperature caused longer rennet coagulation time (RCT) and time to reach $G' 35$ Pa (K_{35}), translating into a wider CW. It also led to a lower maximum curd-firming rate (MCFR) with lower firmness at 40 min (A_{40}) at a given protein level. Increasing protein levels resulted in the opposite effect, although without a significant effect on RCT at a given temperature. On coagulation at 28°C, milk with 5% protein resulted in a similar MCFR (~4 Pa/min) and CW (~8.25 min) compared to milk with 4% protein at 32°C, which reflects more standard conditions, whereas increasing milk to 6% protein resulted in more than doubling of the curd-firming rate (MCFR, 9.20 Pa/min) and a shorter CW (4.60 min). Gels set at 28°C had lower levels of rearrangement of protein network after 40 min compared to those set at 36°C. Protein levels, on the other hand, had no significant influence on the levels of protein network rearrangement, as indicated by $\tan \delta$ values. The internal structure of curd particles, as investigated by both SEM and TEM appeared to have less cross-linking and smaller sized casein aggregates when coagulated at 28°C compared to 36°C, whereas varying protein levels did not show a marked effect on aggregate formation. Overall, this study showed a marked interactive effect between coagulation temperature and protein-standardization of milk on

coagulation properties, which subsequently requires adjustment of the CW during cheesemaking. Lowering of the coagulation temperature greatly altered the curd microstructure with a tendency for less syneresis during cutting. Further research is required to quantify the changes in syneresis and in fat and protein losses to whey due to changes in the microstructure of curd particles arising from the different coagulation conditions applied to the protein-fortified milk.

4.2 Introduction

In milk standardization for conventional cheesemaking practice, the protein level of milk is not altered, but the fat level is adjusted to achieve a specific protein-to-fat ratio (PFR), based on the type of cheese produced and on desired fat-on-dry-matter content. Concentrating milk using ultrafiltration (UF) offers an opportunity to increase protein levels with several benefits to the cheese industry; for example, reducing the impact of seasonal variation of milk composition in cheesemaking (Broome et al., 1998), increasing process efficiency and cheese yield, and ameliorating issues with poorly coagulating milk (Guinee et al., 1994, 1996a, 2006; Mistry and Maubois, 2017). Use of UF has been broadly categorized based on the extent of protein concentration from low concentration ratios (LCR; 1.2 to 2x) to medium concentration ratios (2 to 6x) or high concentration ratios (6 to 8x) (Pouliot, 2008). Protein levels in cheese milk can be increased directly using UF or by supplementing milk with UF retentate (Govindasamy-Lucey et al., 2011) or through supplementation by reconstitution of casein powders into milk (Guinee et al., 2006), depending on legal regulations in various countries. Using LCR-UF is the most frequent method for protein concentration, while applying higher concentration ratios adds greater complexity to the process (e.g., rennet coagulation properties) and to the equipment required (Lu et al., 2016; Mistry and Maubois, 2017). A summary of studies utilizing LCR-UF for different cheese varieties with maximum protein levels, PFR, and total solids content is presented in Table 4.1. It is evident from the studies reviewed that composition of milk (protein level) greatly influences subsequent steps in the cheesemaking process, with the most critical one being rennet coagulation.

Table 4.1 Cheese varieties produced from milk concentrated by ultrafiltration.

Cheese Variety	Maximum Protein Levels (%)	Protein-To-Fat Ratio	Total Solids (%)	Country ¹	Reference
Cheddar	4.6	0.86-1	-	Ireland	Guinee et al., 1996a
Cheddar	4.8	0.73	17.2	USA	Ozturk et al., 2015
Cheddar	4.81	0.82	16.2	Australia	Broome et al., 1998
Cheddar	5.93	0.88	18.2	USA	Oommen et al., 2000
Cheddar	6	0.93	17.5	USA	Acharya and Mistry, 2004
Cheddar	6	0.84	-	Australia	Ong et al., 2013
Cheddar	8.2	0.77-	-	Ireland	Guinee et al., 1994
Edam	4.2	1.26	-	Finland	Heino et al., 2010
Mozzarella	4	1	13.5	Italy	Francolino et al., 2010
Parmesan	5	1.4	14.2	USA	Govindasamy-Lucey et al., 2004
Pizza cheese	5.85	1.31	15.2	USA	Govindasamy-Lucey et al., 2005
Swiss	4.15	1.1	13.4	USA	Govindasamy-Lucey et al., 2011

¹ where the studies were performed.

In renneted milk systems, individual casein micelles flocculate after loss of the hydrophilic domain from κ -casein leading to the formation of casein aggregates and eventually a three-dimensional protein network, the gel/coagulum (Horne and Banks, 2004; Muñoz et al., 2017). On reaching a suitable firmness, the gel is cut (Govindasamy-Lucey et al., 2011) which then facilitates whey expulsion from the curd. It is well established that increased protein levels (Guinee et al., 2006; Sandra et al., 2011) in milk or higher coagulation temperature (Hussain et al., 2012; Muñoz et al., 2017) increases gel firmness and gel-firming rate (Guinee et al., 1996b; Waungana et al., 1998; Upreti et al., 2011). Guinee et al. (1994) reported that increasing protein to >5% caused difficulty in curd cutting using an existing cutting programme because of excessive gel strength. Lowering the temperature by 3 to 4°C standardizes the coagulation and cutting properties in the case of increased protein concentration (Guinee et al., 1994; Guinee et al., 1996a; Govindasamy-Lucey et al., 2011). Similarly, other factors, e.g., pH and CaCl₂ addition, are critically important for the development of the gel (Nájera et al., 2003; Mishra et al.,

2005; Muñoz et al., 2017). Thus, improved understanding on the interactive effects of altering coagulation temperature on the rennet coagulation characteristics and curd cutting properties for milk with increased protein content through use of LCR-UF is required.

The coagulation properties of a gel are typically characterized by the rheological determination of storage modulus (G') and loss modulus (G''). These parameters facilitate the calculation of rennet coagulation time (RCT), gel firmness at a given time, and gel-firming rate thereafter (Guinee et al., 1996a, b; Sandra et al., 2011). The ratio of G'' to G' gives the value for loss tangent ($\tan \delta$), which essentially measures differences in phase angle of stress and strain, indicative of breaking of protein-protein bonds in casein aggregates or clusters, leading to the formation of stronger bonds at new junctions (rearrangement) that mostly contributes to increased firmness of the gel (van Vliet et al., 1991; Mellema et al. 2002). This may then create an internal pressure for whey movement, causing endogenous syneresis or microsyneresis. Mellema et al. (2002) demonstrated that rearrangement was facilitated at higher temperature and with lower pH of the gel, influencing the internal structure of curds.

Structurally, rennet-induced gels entrap fat and whey in the protein network. Ong et al. (2011, 2013) investigated the rheological properties and microstructure of rennet gels at varying set temperatures (27, 30, 33 and 36 °C) and milk protein concentrations (3.7 to 5.8%) in separate studies using scanning electron microscopy (SEM) and confocal laser scanning microscopy. They reported that gels prepared at 5.8% protein were comprised of a dense protein network with fewer pores and unevenly distributed fat globules compared to gels prepared at 3.7 to 4.8% protein, and that lowering the temperature resulted in a finer network. However, those studies did not investigate the

interactive effect of protein content and temperature on rheological and subsequent curd microstructure.

Coagulation temperature in cheesemaking is usually between 20 to 36°C. For Mozzarella, which is considered a soft cheese, the milk is typically set at 35 to 36°C while 31 to 32°C is common for hard or semi-hard cheeses. Therefore, an understanding of the interactive effects of protein concentration and coagulation temperature on coagulation properties, and curd cutting characteristics, as well as resultant curd microstructure and syneresis, is desirable when using protein-fortified milk.

The first objective of this study was to characterize the interactive effects of protein level (4, 5, 6%) and set temperature (28, 32, 36°C) on rennet-induced gelation characteristics and on the cutting properties of gels formed. The second objective was to characterize the microstructure of the resultant curd as influenced by those changes in set temperature and protein concentration.

4.3 Materials and Methods

4.3.1 Milk collection and ultrafiltration

Bovine milk (fat 3.2% and protein 3.12%) was sourced from the George B. Caine Dairy Research and Teaching Centre (Wellsville, UT, USA) and transported to the Gary Haight Richardson Dairy Products Laboratory at Utah State University (Logan, UT, USA). After pasteurization (73°C for 15 s), UF of whole milk (~180 kg) was carried out at ~45°C to achieve ~3x concentration using a plant with spiral wound polyethersulfone membranes with a 10-kD molecular weight cut-off (Model ST-2-3838, 10 cm x 100 cm

with a 0.76-mm spacer, 7 m² surface area; Synder Filtration, Vacaville, CA, USA). Retentate and permeate were cooled and stored at ~5°C. Skim milk (~ 4 kg) was obtained from a local grocery shop in Logan, UT, USA.

4.3.2 Milk standardization and preparation

Milk was standardized to protein levels of 4, 5, and 6%, respectively, while maintaining a PFR of ~1.0 by combining skim milk, whole milk, and retentate. The protein-to-fat ratio was chosen to represent cheese varieties that contain 45% fat in dry matter. The pH of milk was 6.63 to 6.78 at ~8°C and this was adjusted to 6.5 with lactic acid (1:8 dilution, vol/vol). Milk was then heated to 45°C, cooled to the required coagulation temperature, and divided into 450-g-portions before rennet addition. The heating and cooling profile of milk used was performed to minimize the effect of cold storage on rennet coagulation properties (Qvist, 1979; Maciel et al., 2015).

4.3.3 Rheological properties

Viscoelastic properties of milk gels were measured using a rheometer (AR-G2, TA Instruments, New Castle, DE, USA) with cup and bob geometry as described in Chapter 3. Oscillation frequency was set to 1 Hz with strain 2% in time sweep mode. The gap was set at 5,920 µm. Temperature was controlled and adjusted using the water bath system attached to the rheometer. Low-amplitude oscillation conditions were within the linear viscoelastic region of milk gels (Guinee et al., 1996b).

Double-strength (~650 International Milk Clotting Units/mL, IMCU/mL) chymosin (Maxiren; DSM Food Specialties USA Inc., Eagleville, PA, USA) was used for

milk coagulation. A chymosin solution (2.6 ml) of a 1:100 (vol/vol) dilution was added to 450 mL of milk resulting in standardized addition rate of 37 IMCU/kg. The milk was stirred for 1 min, and then a 20 ml sample was transferred to the rheometer operated at the required set temperature (28, 32, or 36°C, respectively). Changes in G' and G'' were continuously recorded every 30 s between 42 and 71 min, depending on the rate of gel formation.

Starting time was defined as the point of addition of chymosin. Rennet coagulation time was defined as the time required for the first consistent increase in G' values. Storage modulus data was recorded at 1.5 and 2 times RCT ($G'_{1.5}$ and G'_2 , respectively). Curd-firming rate ($\Delta G'/\Delta t$) was calculated from the change in G' values per min. Maximum curd-firming rate (MCFR) was the maximum slope of the G' curve as a function of time. Storage modulus at 40 min after rennet addition (A_{40}) and loss tangent at the same time ($\tan \delta_{40}$) were recorded. Time taken to reach G' of 35 Pa (K_{35}) and 70 Pa (K_{70}) was recorded and were used to calculate the cutting window (CW) or the time thought appropriate for cutting of curd during cheesemaking.

4.3.4 Compositional analysis of milk

Gross composition was determined by Fourier Transform Infrared spectroscopy, using a Bentley Instruments Dairy Spec FT (Bentley Instruments, Inc., Chaska, MN, USA) for raw milk, standardized milk, and UF permeate and retentate. Calcium and phosphorus contents of milk were determined by inductively coupled plasma optical emission spectrometry (Thermo iCAP 6300, Thermo Fisher Scientific, MA USA) using

digested milk samples, as described by Gavlak et al. (2005). Phosphate was calculated as phosphorus content x 3.065.

4.3.5 Preparation of coagula for microstructure analysis

Milk (14 kg) was standardized in cheese vats as described above. Combinations of protein content and set temperature of 6% and 28°C, 4% and 36°C and, 6% and 36°C, were chosen so as to show the largest difference in the curd microstructure. Gels were cut at ~35 Pa using knives with 6 mm between cutting wires, followed by 4 min of healing and 1 min gentle stirring. Five min after cutting, 10 to 15 curd particles (6 mm³ size) per treatment were collected from the stainless-steel vat with a spatula and immediately transferred to glass vials (5 curds in each) with primary fixative (formaldehyde/glutaraldehyde 2.5% each in 0.1M sodium cacodylate buffer, pH 7.4, Electron Microscopy Sciences, Hatfield, PA, USA). Curd particles collected were of similar sizes having no sign of breakage, and these curd particles were firm in withstanding their shape. Large numbers (10-15) of curd particles were fixed in each treatment for adequate curd samples during sample preparation as well as for facilitating sample randomization, and extensive care was taken to avoid any particle deformation. The primary fixative was at room temperature at the time of sampling and curd samples in fixative were stored at 4°C overnight. A mixture of formaldehyde/glutaraldehyde was used as primary fixative for their fast penetration into the curd particles to stop further protein network rearrangement after sampling. Microscopic analysis of the curd samples were carried out in duplicate at pre-defined locations, so that images captured were representative

and comparable. The moisture contents of the curd samples that were not fixed were determined in triplicate using a gravimetric (hot air oven) method.

For transmission electron microscopy (TEM), curd particles were first cut into 1 mm-thick slices, from which small rectangles (3 x 1 x 1 mm) were cut perpendicularly to the skin. Samples were processed at the University of Utah (Salt Lake City, UT, USA) as described by Lu et al. (2015). Briefly, samples were treated twice with sodium cacodylate buffer followed by post-fixation in osmium tetroxide (2% in distilled H₂O) for 1 h at room temperature and received 2 more rinses with deionized water. Samples were dehydrated in an ascending ethanol series and transitioned to 100% acetone, gradually infiltrated with resin, embedded in flat-embedding molds, and cured overnight at 70°C. Thick sections (~0.5 µm) were cut and stained for light microscopy followed by thin sectioning (70 to 100 nm) using an ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL). Thin sections were contrasted with salts of uranyl acetate and lead citrate for 10 and 5 min, respectively. Sections were observed with a JEM 1400 Plus TEM (Jeol USA Inc., Peabody, MA) operated at 120 kV, and digital images were taken with a Gatan camera (Gatan, Inc., Pleasanton, CA, USA).

For SEM, intact curd particles were post-fixed and dehydrated as described above. After the last dehydration step in 100% ethanol, curd particles were frozen in liquid nitrogen and fractured before returning to 100% ethanol. The ethanol was replaced by hexamethyldisilazane followed by complete dehydration on filter paper. Dried samples were mounted onto aluminum stubs with double-sticky carbon tape and coated with ~15 nm of gold/palladium using a sputter coater (Gatan 682

Precision Etching and Coating System). Samples were viewed and imaged in an environmental SEM (Quanta 600 FEG, FEI, Hillsboro, OR, USA) at low vacuum (0.08 to 0.23 torr) and with an accelerating voltage of 15 kV.

4.3.6 Experimental design and statistical analysis

A split-plot factorial experimental design was used with protein concentration in the main plot and coagulation temperature in the sub plot. The experimental design focused on studying viscoelastic properties of renneted milk standardized at 4, 5 or 6% protein level (PFR ~1.0) at different set temperatures (28, 32, or 36°C). Each treatment was performed in duplicate.

Milk composition and viscoelastic parameters were compared using PROC GLM function in SAS software (version 9.4, SAS Institute, Inc., Cary, NC, USA) with Tukey adjustment for multiple comparisons of mean differences ($\alpha = 0.05$).

4.4 Results and Discussion

4.4.1 Milk composition

Milks were standardized to protein levels of 4, 5, or 6%, respectively, by blending different proportions of whole milk, UF retentate, and skim milk, while maintaining a PFR of ~1.0. As expected, levels of protein, fat, total solids, solids nonfat, total calcium, and phosphate increased significantly ($P < 0.05$) with increasing protein content, without a significant change in lactose and PFR levels (Table 4.2). The relative ratio of individual casein components in each standardized milk was presumed to be similar, as reported by Liu et al. (2014), since UF of whole milk was carried out with a 10-kDa membrane at

50°C, minimizing the dissociation of micellar casein components. The proportion of calcium to protein was significantly lower at higher protein levels (6%) compared to 4 or 5% protein. During UF, a portion of the soluble calcium partitions with the permeate while the calcium concentration in the milk serum phase remains constant (Salvatore et al, 2011; Sandra et al., 2011). Hence as protein (and fat) concentration is increased, there is a lower volume fraction of the milk serum phase, and the total calcium level in the concentrate decreases. Therefore, even though the overall calcium to protein ratio is lower, calcium to protein ratio within the casein micelles was not expected to change. A similar PFR in standardized milk samples indicated that, with increasing protein levels, fat levels also increased. A PFR~1 was selected in this study to represent semi-hard cheese types of 45% fat in dry matter. Milk standardized in the present study was concentrated ~1.25 to 1.9-fold, reflecting LCR-UF (Mistry and Maubois, 2017).

Table 4.2 Composition of standardized milks used in this study

Components	Standardized milk		
	4% Protein	5% Protein	6% Protein
Protein (g/100 g)	3.99 ^c	4.96 ^b	5.96 ^a
Fat (g/100 g)	3.87 ^c	4.85 ^b	5.89 ^a
Protein-to-fat ratio	1.03 ^a	1.02 ^a	1.01 ^a
Lactose (g/100 g)	4.83 ^a	4.80 ^a	4.84 ^a
Solids not fat (g/100 g)	9.75 ^c	10.77 ^b	11.88 ^a
Calcium (g/kg)	1.30 ^c	1.60 ^b	1.87 ^a
Phosphate ¹ (g/kg)	3.21 ^c	3.83 ^b	4.35 ^a
Calcium-to-protein ratio (mg/g)	32.54 ^a	32.35 ^a	31.31 ^b
Total solids (g/100 g)	13.64 ^c	15.63 ^b	17.77 ^a

^{abc} Means within rows with the same letter were not significantly different ($\alpha = 0.05$). Data presented are the mean of 6 measurements from duplicate trials.

¹ Calculated as PO₄ (phosphorus content times 3.065).

4.4.2 Rheological properties of milk gels as influenced by protein-standardization and coagulation temperature

The development of G' as a function of time in milk samples with different protein levels (4, 5, 6%) at different set temperatures (28, 32, 36°C) is shown in Figure 4.1. Gels showed higher G' values at a given time with increasing protein levels and, with increasing set temperature, the G' further increased (Figure 4.1A, B, and C) as did $\Delta G'/\Delta t$ (Figure 4.2). The latter graphically represents the slope of the gel formation curves (G') as shown in Figure 4.1.

After the rennet coagulation time is reached, $\Delta G'/\Delta t$ increased to a maximum value and then decreased. With increasing protein levels, there was an increase in $\Delta G'/\Delta t$ and, with increasing set temperature, a large increase in $\Delta G'/\Delta t$ was observed (Figure 4.2A, B, C).

The increase of $\Delta G'/\Delta t$ with respect to higher temperature and protein concentration indicates increased frequency of collisions between the casein micelles at higher protein levels, and this perhaps led to the formation of a stronger protein network, as indicated by the faster G' development at a given time (Sandra et al., 2011). The rheological properties of such gels (discussed below) at a given time were calculated, and the interactive effects on gel characteristics are illustrated in Figure 4.3.

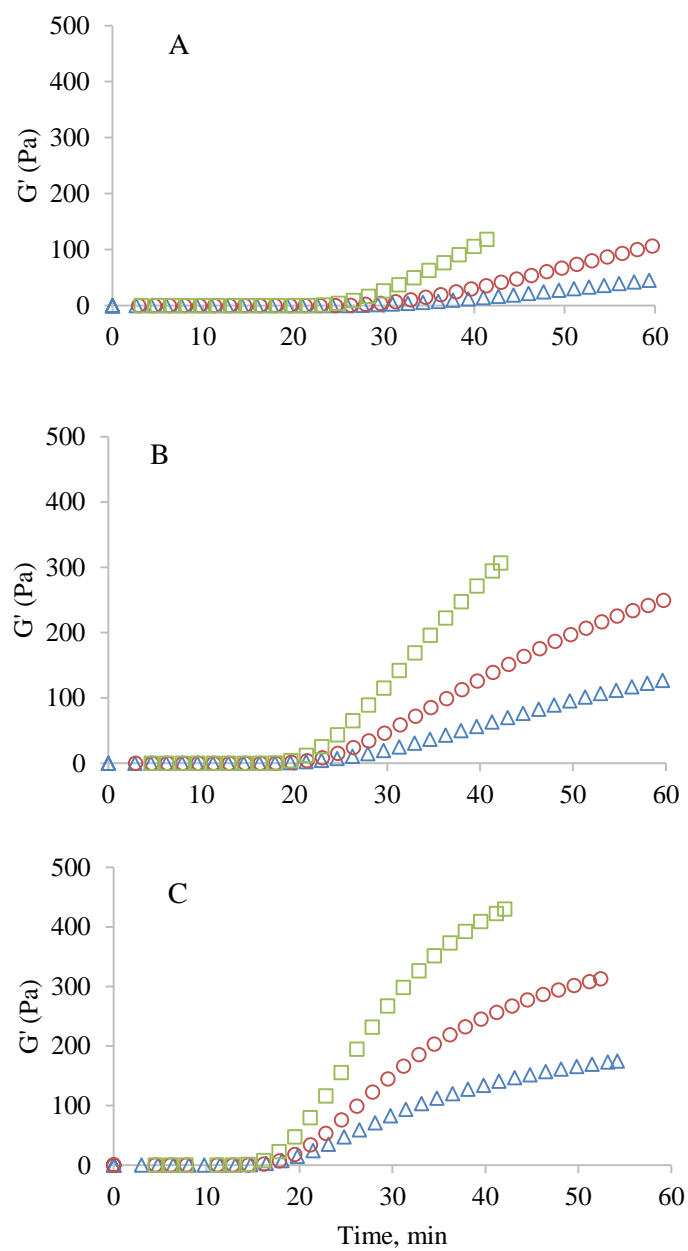


Figure 4.1 Storage modulus, G' , of rennet-induced gels from milk standardized using ultrafiltration retentate and containing 4% (Δ), 5% (\circ), or 6% (\square) protein, respectively, and set at 28°C (A), 32°C (B) or 36°C (C).

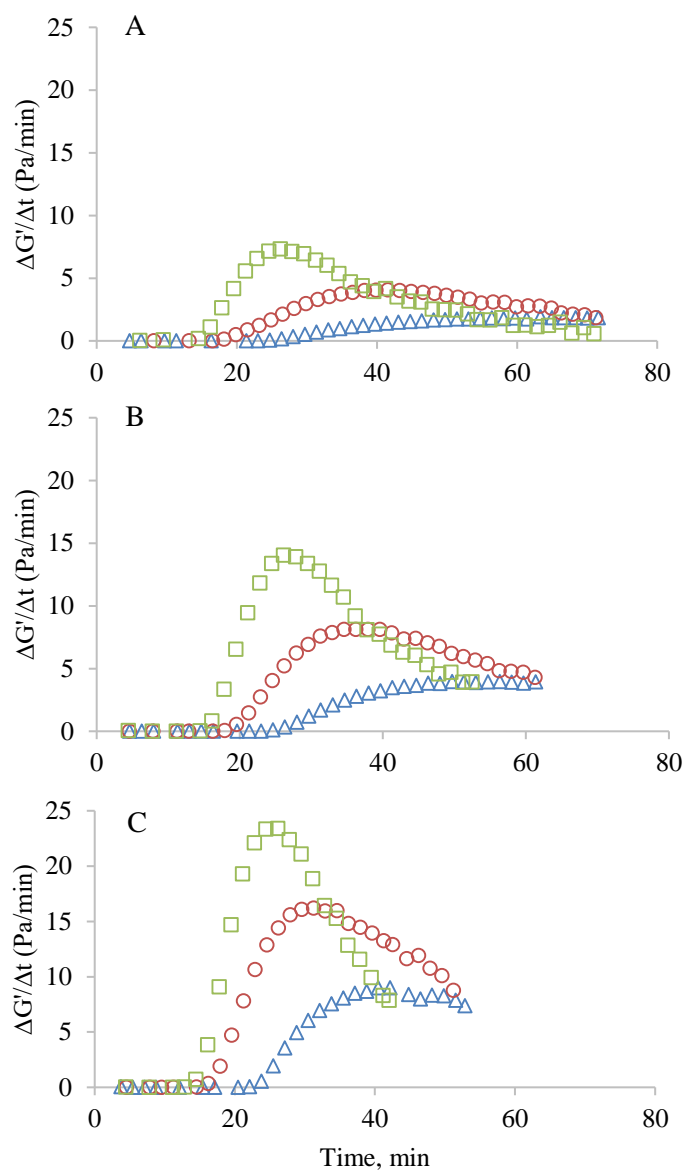


Figure 4.2 Curd-firming rate of rennet-induced milk gels from milk standardized using ultrafiltration retentate and containing 4% (A), 5% (B), or 6% (C) protein, respectively, and set at 28°C (Δ), 32°C (\circ), 36°C (\square) (Colour version available online.)

4.4.3 Rennet coagulation time

A significant influence of coagulation temperature on RCT, which decreased with increasing temperature, was observed, whereas protein concentration had no significant

influence, nor was there any significant interaction between temperature and protein levels (Table 4.3). Values of RCT are shown in Table 4.4. The definition of RCT varies and has included the time when G' equals G'' (Karlsson et al., 2007; Salvatore et al., 2011; Lu et al., 2017), the time when there is a consistent increase in viscosity (Nájera et al., 2003), and the time when G' value is above a fixed value such as 0.2 Pa (Guinee et al., 1996b) or 1 Pa (Waungana et al., 1998; Mishra et al., 2005). We chose to use the time when a consistent increase in G' was first observed, and this occurred between 0.25 to 0.75 Pa. On this basis, rennet coagulation time decreased as set temperature increased (Figure 4.3) probably because of increased hydrophobic interactions and enzymatic activity, which probably led to earlier formation of casein aggregates (Mishra et al., 2005). Rennet coagulation time was not influenced significantly by increasing protein levels from 4 to 6% at a given temperature (Table 4.3), which was consistent with the results of Sandra et al. (2011) who reported no influence of increasing protein concentration on RCT, while others (Waungana et al., 1998; Karlsson et al., 2007) reported longer RCT or even shorter RCT (Guinee et al., 2006; Govindasamy-Lucey et al., 2011; Upreti et al., 2011). Such differences may result from differences in the proportion of κ -casein hydrolysis arising from differences in substrate-to-enzyme ratios (Karlsson et al., 2007; Sandra et al., 2011), differences in methods and definition of RCT, or differences in milk pH (Waungana, et al., 1998). We used a constant concentration (37 IMCU/kg) of rennet at all protein levels, which was probably sufficient to hydrolyse κ -casein to form casein aggregates required for coagulation at protein levels between 4 to 6% at a given coagulation temperature. Sandra et al. (2011) observed ~90% hydrolysis of κ -casein in

skim milk gels regardless of concentration of protein to ~12.1% when using a similar concentration of rennet (34 IMCU/kg) at 30°C.

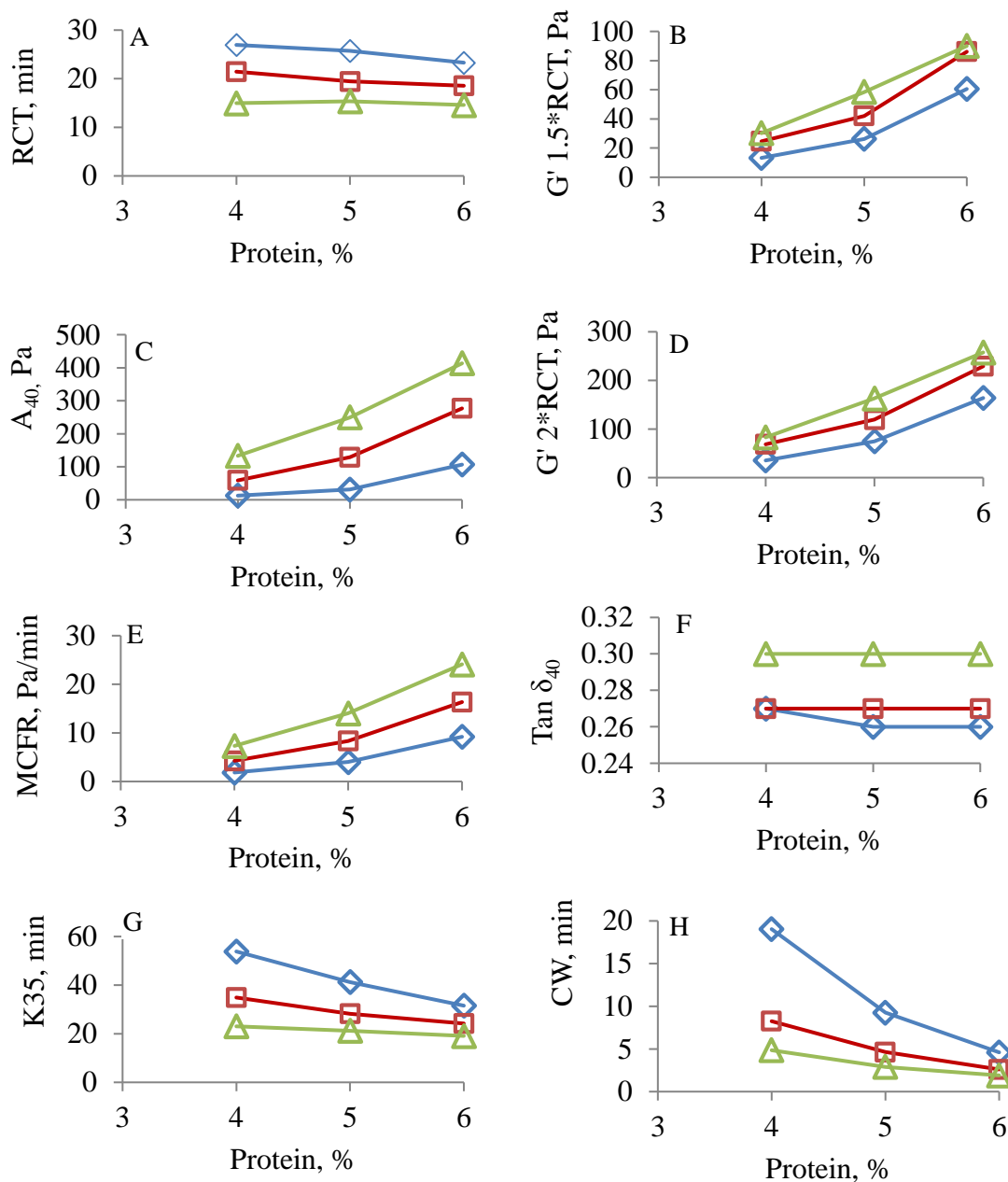


Figure 4.3 Interaction plot of rennet gelation properties as influenced by protein concentration and temperature: (A) rennet coagulation time; (B) storage modulus at 1.5 time RCT; (C) storage modulus at A_{40} ; (D) storage modulus at 2 times RCT; (E) maximum curd-firming rate; (F) loss tangent ($\tan \delta$); (G) Time taken to reach 35 Pa, and (H) cutting window. Symbols (\diamond), (\square) and (Δ) represent set temperatures of 28, 32 and 36°C, respectively.

4.4.4 Development of gel firmness

The values for $G'_{1.5}$ were considered to be equivalent to the gel firmness suitable for cutting of a coagulum 30 min after rennet addition. During cheesemaking with milk of normal protein concentration, on-set of gelation is observed ~20 min after renneting. Values of G'_2 were considered to represent the curd firmness when most of the κ -casein is cleaved by chymosin. While $G'_{1.5}$ and G'_2 are considered independent of RCT, A_{40} can be considered an expression of the rate at which coagulum formation occurs and is dependent on RCT (McMahon and Brown, 1982; Lu et al., 2017). The values of $G'_{1.5}$, G'_2 and A_{40} in renneted milk samples at different protein concentrations and set temperatures are shown in Table 4.4. There was a significant interaction between protein levels and set temperature for G'_2 ($P < 0.05$) and A_{40} ($P < 0.01$) but not for $G'_{1.5}$ (Table 4.3). Curd firmness, represented by $G'_{1.5}$, G'_2 and A_{40} , was highly influenced by coagulation temperature and protein concentration (Table 4.3). This indicates that the gel strength at $G'_{1.5}$ for cutting may not be suitable for protein-standardized milk, because $\Delta G'/\Delta t$ was highly dependent on protein level and temperature.

Log-linear associations of $G'_{1.5}$, G'_2 and A_{40} with protein levels at each coagulation temperature were observed (Figure 4.4), indicating that G' increased logarithmically with increasing protein levels, which is consistent with previously published studies (Guinee et al. 1996b; Lu et al., 2017). In comparison to milk with 4% protein renneted at 32°C, which most closely represents conditions occurring during cheesemaking when using protein-standardized milks, increasing temperature or protein concentration caused an increase in $G'_{1.5}$, G'_2 and A_{40} (Table 4.4). In contrast, when milk containing 5% protein

was renneted at 28°C, the values describing gel formation development (4.01 Pa/min) were similar to those of milk with 4% protein renneted at 32°C.

4.4.5 Maximum curd-firming rate

The MCFR can be considered a measure of the rate of aggregation of hydrolyzed casein micelles during coagulation, and MCFR increased with increased protein levels and with increasing coagulation temperature (Table 4.4). There was also a significant interactive effect of protein content and coagulation temperature on MCFR (Table 4.3). When comparing the MCFR of different conditions of coagulation temperature and protein concentration, gels set at 28°C with 5% protein showed values close to those of standard conditions (Table 4.4). Increased $\Delta G'/\Delta t$ at increasing temperature was probably because of increased hydrophobic interactions of casein micelles and protein rearrangement (van Vliet and Walstra, 1994). The hydrophobic domains in casein have a tendency to reduce contact with H₂O molecules, providing an entropic-related lowering of energy that favours self-association. Since changes in entropy have a temperature coefficient, such self-association increases with temperature, as there is increased mobility of protons as well as a reduction in electrostatic repulsive forces by binding with calcium (Horne, 1998). Increased $\Delta G'/\Delta t$ at increasing protein contents was probably because of a higher frequency of collisions between casein aggregates, as the mean distance between casein micelles is reduced when the volume fraction of casein in milk increases (Mishra et al., 2005; Sandra et al., 2011).

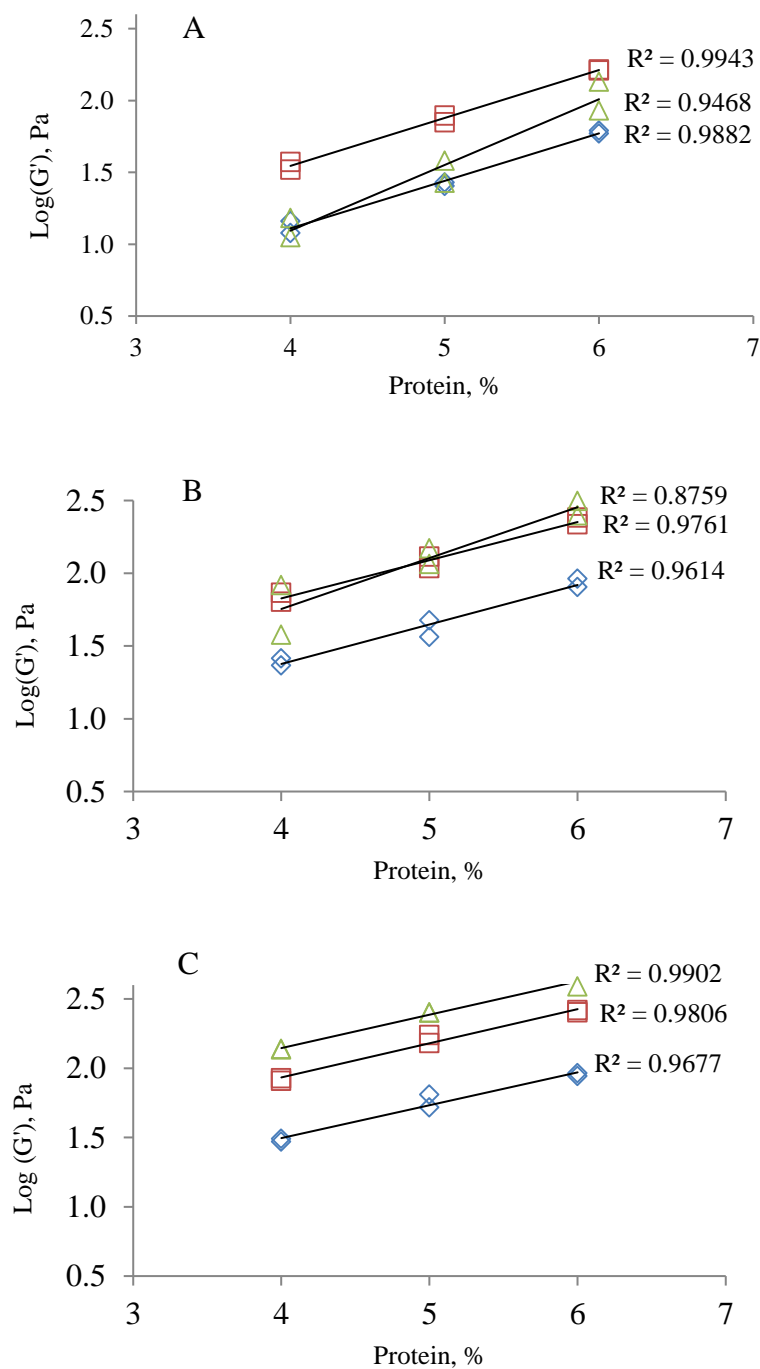


Figure 4.4. Log-linear association of $G'_{1.5}$ (\diamond), G'_2 (\square), or A_{40} (Δ) with protein concentrated milk during rennet gelation set at 28°C (A), 32°C (B) and 36°C (C).

Table 4.3 F values from analysis of variance of gelation properties influenced by milk protein concentration and set temperature.

Sources	RCT ¹ (min)	G' _{1.5} ² (Pa)	G' ₂ ² (Pa)	MCFR ³ (Pa/min)	A ₄₀ ⁴ (Pa)	Tan δ ₄₀ ⁴	k ₃₅ ⁵	k ₇₀ ⁵	CW ⁶
							----- (min) -----		
Protein	1.64 ^{NS}	198 ^{***}	372.5 ^{***}	188.5 ^{***}	86.39 ^{***}	0.12 ^{NS}	24.5 ^{***}	47.24 ^{***}	196.8 ^{***}
Temperature	32.95 ^{***}	43.5 ^{***}	90.7 ^{***}	130 ^{***}	100 ^{***}	413.5 ^{***}	73.0 ^{***}	100 ^{***}	209 ^{***}
Protein x Temperature	0.36 ^{NS}	2.2 ^{NS}	3.76 [*]	9.36 ^{**}	6.50 ^{**}	4.5 [*]	4.70 [*]	9.24 [*]	40.9 ^{***}

¹ Rennet coagulation time (RCT), time at first consistent increase in G'.

² Storage modulus (G') values at 1.5 x RCT (G'_{1.5}) and 2 x RCT (G'₂).

³ Maximum curd-firming rate (MCFR) based on change in G'.

⁴ A₄₀ and tan δ = Curd firmness measured as G' and ratio of G'' to G', respectively, 40 min after rennet addition.

⁵ k₃₅ and k₇₀ = time after addition of rennet to milk during gelation for reaching G' values of 35 and 70 Pa, respectively.

⁶ CW = cutting window in which the curd has a G' between 35 and 70 Pa.

NS indicates not significant. *, **, *** refers to significant main or interactive effects at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table 4.4 Effects of milk protein concentration and set temperature on gelation properties of cheese milk.

Protein (%)	Temperature (°C)	RCT ¹ (min)	G' _{1.5} ² (Pa)	G' ₂ ² (Pa)	MCFR ³ (Pa/min)	A ₄₀ ⁴ (Pa)	Tan δ_{40} ⁴	k ₃₅ ⁵	k ₇₀ ⁵	CW ⁶
----- (min) -----										
4	28	27.0 ^a	13.3 ^d	35 ^e	1.85 ^f	12.6 ^d	0.27 ^{bc}	53.9 ^a	72.9 ^a	19.04 ^{7a}
4	32	21.5 ^{ab}	24.7 ^{cd}	69 ^e	4.27 ^{dfe}	58.7 ^{cd}	0.27 ^b	34.9 ^{cb}	43.2 ^{cb}	8.25 ^b
4	36	15.0 ^b	30.2 ^{cd}	83 ^{cd}	7.33 ^{cde}	133 ^c	0.30 ^a	23.1 ^{ced}	27.9 ^{de}	4.85 ^c
5	28	25.7 ^a	26.3 ^{cd}	74 ^d	4.01 ^{fe}	31.0 ^{cd}	0.26 ^c	41.3 ^b	50.6 ^b	9.25 ^b
5	32	19.5 ^{ab}	42.0 ^{cd}	119 ^c	8.35 ^{cd}	128 ^c	0.27 ^b	28.3 ^{ced}	32.9 ^{cde}	4.63 ^{cd}
5	36	15.4 ^b	58.4 ^b	163 ^b	14.0 ^b	249 ^b	0.30 ^a	21.3 ^{cd}	24.2 ^{de}	2.90 ^{cd}
6	28	23.3 ^{ab}	60.5 ^b	164 ^b	9.20 ^c	106 ^{cd}	0.26 ^c	31.6 ^{cbd}	36.2 ^{cd}	4.60 ^{cd}
6	32	18.6 ^{ab}	86.1 ^a	229 ^a	16.3 ^b	277 ^b	0.27 ^b	24.2 ^{ced}	26.8 ^{de}	2.60 ^{cd}
6	36	14.6 ^b	90.1 ^a	258 ^a	24.1 ^a	413 ^a	0.30 ^a	19.1 ^e	21.1 ^e	1.95 ^d
SEM ⁸		1.12	6.35	17.49	1.63	30.46	0.004	2.60	3.78	1.22

^{a-f} Means within column with the same letter were not significantly different ($\alpha = 0.05$).

¹⁻⁶ refer to Table 4.3

⁷ Projected value as G' only reached 45.5 Pa during the 60 min in which the gel was monitored.

⁸ Standard error of mean.

Lowering the set temperature to 28°C for milk with 5% protein content may counterbalance hydrophobic interactions and frequency of collisions compared to standard coagulation conditions, thus providing similar structural rigidity. Our results showed that development of gel-firmness for milk up to 5% protein can be normalized by lowering the set temperature, in close agreement with previous studies (Guinee et al., 1994; Govindasamy-Lucey et al., 2011). However, above 5% protein, the $\Delta G'/\Delta t$ was observed to be much higher than standard conditions. In contrast, when milk with 4% protein was set at 28°C, it took longer time to reach the appropriate firmness (K_{35} and K_{70}) as well as lower $G'_{1,2}$, G'_2 and A_{40} because of slow $\Delta G'/\Delta t$. Such conditions would have negative impacts on commercial-scale process efficiency.

4.4.6 Endogenous syneresis

Values for $\tan \delta_{40}$ increased significantly ($P < 0.001$) with increasing temperature, whereas protein levels had no significant influence (Table 4.4); there was also a slight interactive effect ($P < 0.05$) of coagulation temperature and protein levels (Table 4.3). At higher temperature, greater disruption of weak protein-protein bonds occurs, which results in rearrangement of casein aggregates and formation of stronger bonds in a new location (van Vliet, van Dijk, Zoon, & Walstra, 1991), and cause a contraction in the gel network. This creates pressure for whey to move internally, causing endogenous syneresis. This suggests that, by increasing coagulation temperature, curds are susceptible to greater syneresis, as indicated by higher values of $\tan \delta_{40}$ compared to gels set at lower temperature (Table 4.4). This result is consistent with findings of Mellema et al. (2002) and Mishra et al. (2005), who observed higher $\tan \delta$ values of milk gels at increased temperatures. A similar trend for $\tan \delta$ values at 28°C was also reported by Hussain et al.

(2012) at different protein levels for cow and buffalo milks. Furthermore, at increased set temperatures, caseins probably are less voluminous due to increased hydrophobic interactions, presumably leading to binding of caseins more tightly (van Vliet and Walstra, 1994; Horne, 1998), which may result in more reactive sites for fusion, conferring structural flexibility.

We propose that gels prepared at lower temperature have less rearrangement even at higher protein levels, causing a slower development in curd firmness. The lower $\tan \delta$ values also indicate that the curds set at lower temperature had less tendency for outward movement of whey compared to curd set at higher temperature because of less internal rearrangement (Mishra et al., 2005). Although cooking of curds in the later stages of cheesemaking may alter the structure, which favours rearrangement, the effect of having lower $\tan \delta$ values at lower set temperature during coagulation may have a bigger impact on initial whey expulsion during cutting and healing, depending on cutting programmes.

Since all milk samples were acidified to pH 6.5 (to minimize batch-to-batch variations in milk pH) we hypothesize that lowering set temperature could be combined with lowering the pH of cheese milk to give a more suitable curd formation when making cheese using LCR-UF concentrated milk. At lower pH, network rearrangement is enhanced (Mellema et al., 2002), favouring greater aggregation between casein micelles, which could possibly result in faster release of whey required for initial stirring. However, this again will lead to the faster gel formation, at higher $\Delta G'/\Delta t$, resulting in higher curd firmness.

4.4.7 Cutting window

In this study, CW (i.e., time between K_{35} and K_{70}) was 8.25 min for gels of 4% protein set at 32°C. Generally, in cheesemaking, curd is cut within a CW of 7 to 8 min (Guinee et al., 1994) although this depends on the size and type of the cheese vat used. With increased protein levels in milk, K_{35} and K_{70} decreased, and this further decreased with increased coagulation temperature (Table 4.4). There was a significant interactive effect of protein levels and coagulation temperature on K_{35} and K_{70} (Table 4.3), suggesting that set-to-cut time during cheesemaking process is reduced with increasing protein levels at a given temperature. Cutting of gels based on standard operating procedure (where gel is cut based on fixed time ~30 min) while using protein-standardized milk may result in excessive curd firmness because of higher $\Delta G'/\Delta t$. However, reducing the set temperature to 28°C for milk of ~5% protein resulted in statistically similar ($P = 0.53$; Table 4.4) K_{35} values (41.3 min) to standard conditions (34.9 min).

Guinee et al. (1994) reported difficulty in cutting curd while using milk with >4% protein set at 32°C, and excessive curd firmness was reported to show subsequent tearing of curds, leading to decreased cheese yield. In the present study, increasing protein levels and increasing set temperature markedly reduced CW, e.g., to 1.95 min for milk with 6% protein set at 36°C (Table 4.4), indicating the need for modification of the cutting programme when altering milk composition. A CW of 9.25 min for milk of 5% protein set at 28°C was close to that of the milk with 4% protein and set at 32°C, indicating that a similar cutting procedure can be employed to cut the gel under these conditions.

Such a reduction in set temperature on increasing protein levels would influence curd pH development during cheesemaking, because of slower starter culture growth as well as increased buffering capacity (Guinee et al., 1996a; Salvatore et al., 2011) of the curd. To ameliorate this situation, using higher starter inoculum levels has been suggested (Guinee et al., 2006). Seasonal variation in milk composition in some countries accounts for a large variation in processability of milk (O’Callaghan et al., 2016) for cheesemaking. We propose that protein and pH standardization of milk prior to renneting could compensate for variations in milk. Such standardization would result in increased cheese consistency, as it provides greater consistency in the rennet coagulation process, cutting of the curd, and acidification rates during cheesemaking, and coagulation temperature could be adjusted in tandem to achieve an appropriate $\Delta G'/\Delta t$.

4.4.8 Curd microstructure

4.4.8.1 Scanning electron microscopy

Scanning electron micrographs of curds 5 min after initiation of cutting are shown in Figure 4.5. For microstructural analysis, curd samples were collected after cutting, 3 min healing, and 1 min stirring. Micrographs shown are for gels of 4% protein set at 36°C (A, D, G), of 6% protein set at 36°C (B, E, H) and of 6% protein set at 28°C (C, F, I) at increasing nominal magnifications (2,000x, 50,000x and 100,000x). At lower magnification, curds set at 36°C and of 4% protein appeared to have a coarse protein network with regular voids, some of these containing small fat droplets (Figure 4.5A, white arrowhead), whereas curds set at 36°C at 6% protein appeared to have a dense casein network with void spaces filled with clusters of fat droplets (Figure 4.5B). Curds

set at 28°C and with 6% protein appeared to have an even denser protein network, also interspersed with void spaces of varying sizes (Figure 4.5C). Medium- and high-magnification micrographs showed that casein micelles formed a continuous network in all treatments, but strands appeared thicker at higher temperature in both curds with 4 or 6% protein (Figure 4.5D, E) compared to curds coagulated at 28°C (Figure 4.5F).

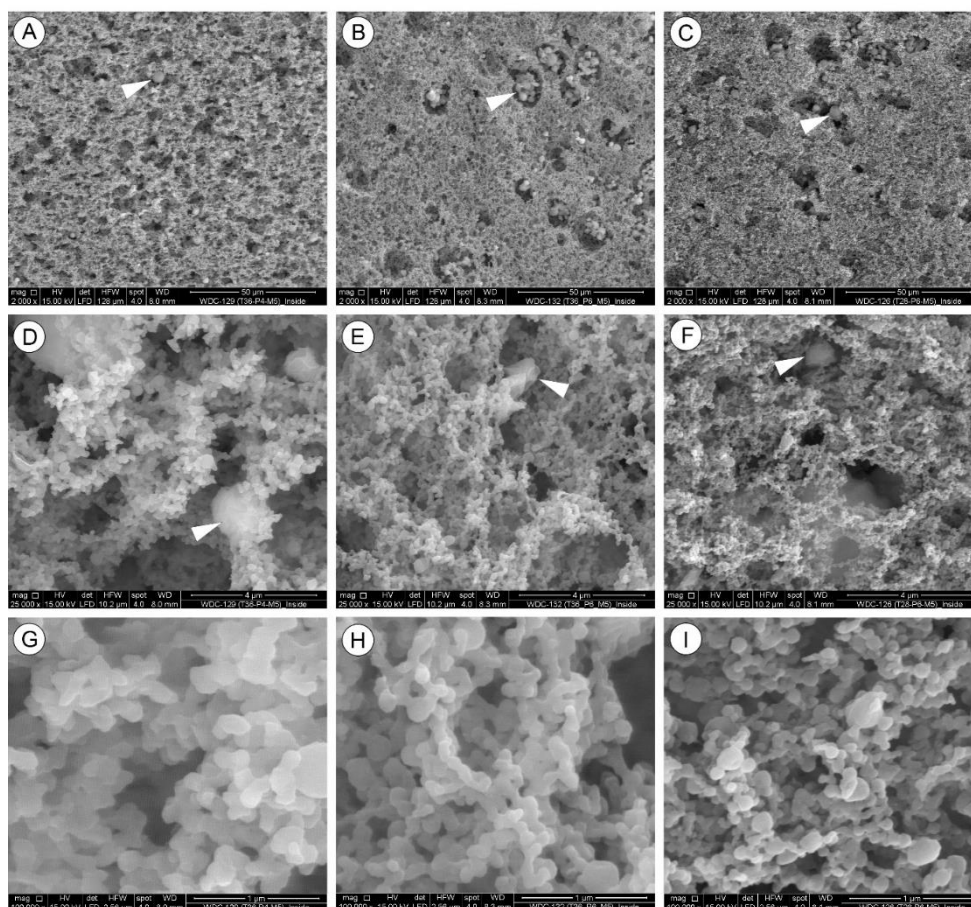


Figure 4.5 Internal microstructure of fractured curd particles prepared with milk of different protein concentrations and renneted at different set temperature, as analysed by scanning electron microscopy. A, D, G - 4% protein at 36°C; B, E, H - 6% protein at 36°C; C, F, I - 6% protein at 28°C. Micrographs were taken at a distance of ~500 µm from the outside of the particle. White arrowheads point to fat globules. Scale bars in A, B and C is 50 µm, D, E, F = 4 µm and G, H, I = 1 µm.

Casein micelle aggregates appeared smaller and less cross-linked in curds coagulated at low temperature treatment compared to higher temperature treatment (Figure 4.5I). This is consistent with the findings of Ong et al. (2011) who observed gels with a fine network when milk was set at 27°C compared to 36°C. It is evident that the set temperature had a significant effect on formation of curd microstructure, and curds with 6% protein had a more dense protein network compared to curds with 4% protein. Ong et al. (2013) reported that curds that gelled at higher protein levels had a denser protein network compared to those from curds prepared with lower proteins levels; similar results were obtained in our study.

It should be considered that, in the present study, milk with 6% protein had a higher total solids content (lower moisture) compared to that with 4% protein (Table 4.2) and, after cutting, curd samples set at 36°C with 4% protein, at 36°C with 6% protein, or at 28°C with 6% protein contained 81.8, 77.6 and 79.5 g moisture per 100g curd, respectively. These values, which differ to the standardized milk moisture content (Table 4.2), indicate that some syneresis occurred after cutting of the coagulum. The structural differences in curds from milk of 4% protein, with regular voids, could be due partly to a lower volume fraction of caseins, forming empty spaces which probably contained whey. Interestingly, fat globules were highly aggregated in the curds prepared from milk of protein 6% (Figure 4.5B, C). Fat in the standardized milk comes from whole milk and UF retentate. As the protein concentration is increased, the proportion of fat coming from UF also increases. In 4% protein milk, the proportion of fat from UF was ~33% (~67% from whole milk), while in 6% milk, the proportion of fat from UF retentate was ~71% (~28% from whole milk). It is possible that the UF of whole milk has induced clustering of fat

globules, which would be responsible for the aggregates observed on Figure 4.3B and C. Other investigators have also observed aggregated fat globules in gels prepared from concentrated milk (Ong et al., 2013). Fat globules of varying sizes were tightly entrapped in the protein network in curds with 28°C and 6% protein, whereas void spaces around fat globules were observed at 36°C. This could be because of greater microsyreresis that occurred by rearrangement of the casein network at higher temperatures (van Vliet et al., 1991). This is also supported by the higher $\tan \delta_{40}$ values of the gels when set at higher temperature (Table 4.3) and is in agreement with the findings of van Vliet et al. (1991) who proposed that greater rearrangement of the casein network results in endogenous syneresis because of protein network contraction. Thus, formation of highly cross-linked casein micelles in curds coagulated at higher temperatures forms the structural basis for the increased gel-firming rate (Figure 4.6).

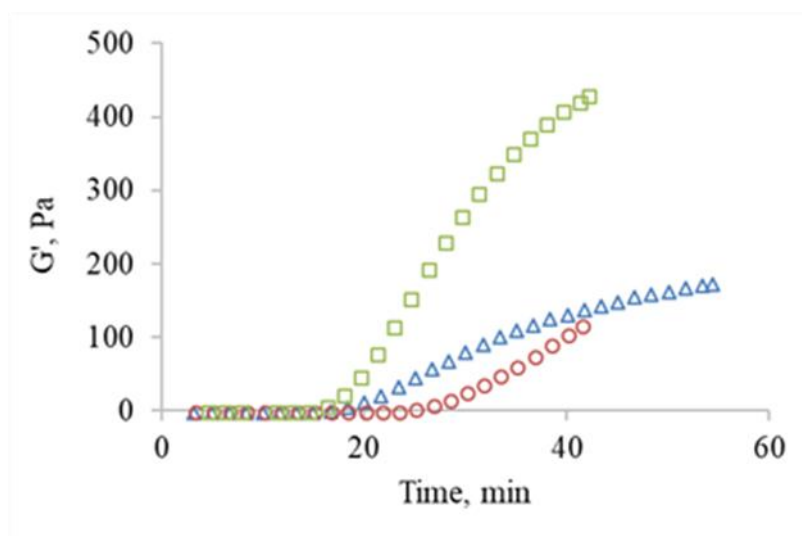


Figure 4.6. Gel formation at protein concentration and temperatures of (Δ) 4% - 36°C, (○) 6% -28°C and (□) 6%-36°C, respectively.

4.4.8.2 Transmission electron microscopy

Figure 4.7 shows transmission electron micrographs, with increasing magnification, of curds prepared from milk of 4% protein set at 36°C (A, D, G), of 6% protein set at 36°C (B, E, H) and of 6% protein set at 28°C (C, F, I). At low magnification, it was evident that casein micelles formed the protein network with interspersed fat globules. Milk with 6% protein had a dense casein network both at 36°C (Figure 4.7B) and 28°C (Figure 4.7C). Milk with 4% protein set at 36°C appeared to have a less dense casein network with casein micelles forming localized aggregates, which were not always connected to each other within the plane of the section that was imaged (Figure 4.4A). At medium magnification, casein micelles were observed in localized aggregates (Figure 4.7D, E, F) for all treatments but aggregates and casein micelles appeared smaller at 28°C (Figure 4.7F) compared to 36°C (Figure 4.7D and E). High-magnification images showed that the aggregates of casein micelles were indeed smaller with fewer cross-links between micelles at 28°C (Figure 4.7I) compared to 36°C, regardless of protein levels (Figure 4.7G and H). To summarize, at higher temperatures, extensive cross-linking between casein micelles occurred and thicker strands formed, making it difficult to discern individual casein micelles within aggregates.

The differences observed in curd microstructure using TEM parallels those observed with SEM (Figure 4.5), which indicated formation of bigger aggregates at 36°C compared to smaller and less cross-linked casein micelles at 28°C. The TEM microstructure images further provide a mechanism for slow and fast curd formation in gels set at 28°C and 36°C, respectively (Figure 4.1A and Figure 4.4), because of differences in formation of aggregate size and casein micelles cross-linking. Interestingly,

micelles with bigger aggregates and more cross-linking had more voids; this confirms the phenomenon of casein micelle rearrangement, which could possibly be because of bond relaxation at higher temperature that resulted in network rearrangement causing endogenous syneresis. These results agree with those of Mellema et al. (2002), who proposed that gel strength is increased because of rearrangement of the protein network, which increases at higher temperature. Muñoz et al. (2017) also observed rapid formation of bigger aggregates in gels prepared at 40°C compared to gels prepared at 30°C.

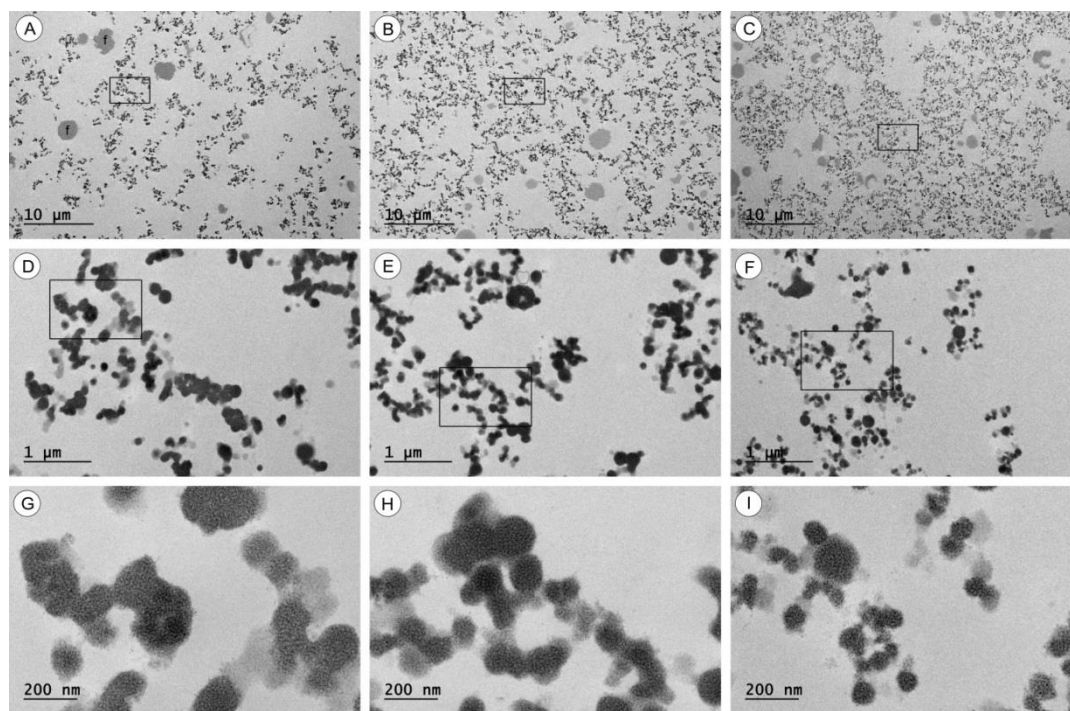


Figure 4.7 Internal microstructure of curd particles prepared with milk of different protein concentrations and renneted different set temperature as analysed by transmission electron microscopy. A, D, G - 4% protein at 36°C; B, E, H - 6% protein at 36°C; C, F, I - 6% protein at 28°C. Micrographs were taken at a distance of ~500-600 μm from the outside of the particle. Boxed areas are shown in increasing magnifications. f – fat globules.

As discussed above, gels set at lower temperatures had weaker casein micelle aggregates, producing curd with less capability to expel whey immediately after cutting compared with that at higher set temperatures. Less available whey during initial stirring

can lead to greater curd breakage, causing fat and protein losses during downstream processing. Contradictory results have been reported with respect to fat losses at lower set temperature during cheesemaking (Govindasamy-Lucey et al., 2011; Ong et al., 2013), partly because of differences in curd microstructure as induced by the organization of casein aggregates within the network and thereby syneresis. This suggests that different coagulation temperatures associated with different cheese varieties (Table 4.1) will have an important influence on the curd cutting and syneresis behaviour when using concentrated milk.

Curd samples used for microscopy were collected from where larger larger differences in treatments existed with an expectation to facilitate visualization of microstructural differences more clearly. Quantification of porosity within a microscopic image requires a large number of images at a specific location and images in the current study were taken at 4 different locations at different magnification levels to visualize curd microstructure in a wider area. However, quantification of microstructural changes in image using images processing software could also be used to compare curd porosity, length of casein micelles aggregate and numbers of junctions (Geng et al., 2011).

4.5 Conclusions

Application of UF to increase milk protein content for cheesemaking has various industrial benefits, e.g., reducing the impact of seasonal variation and increasing process efficiency. Increasing the protein concentration of milk to 6% resulted in faster development of curd firmness, which translates into a shorter cutting window. Although lowering of set temperature resulted in slower gel formation at all protein levels tested,

higher protein concentration (6%) at 28°C still produced excessively firmer coagula compared to milk with 4% protein set at 32°C (a condition close to standard cheesemaking practice). However, milk with a protein content of ~5% set at 28°C showed similar gel firmness, rate of gel formation and cutting window as compared to milk at 32°C with 4% protein. Based on observations from both SEM and TEM images of curd microstructure, it is concluded that lower set temperatures results in (1) smaller casein aggregates with less cross-linked caseins compared to the highly aggregated casein structure in gels set at higher temperatures and (2) such gels have a tendency for slow syneresis and expulsion of whey from within the gel network, as indicated by the $\tan \delta$ values observed. Further research is required to determine how this would influence losses of fat and protein that occur after cutting and stirring of curd during cheesemaking.

4.6 References

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Chapter 5

Effect of protein concentration, coagulum cut size and set temperature on curd moisture loss kinetics during curd stirring

Ram R. Panthi, Alan L. Kelly, Donald J. McMahon, Xin Dai, Almut H. Vollmer, and Jeremiah J. Sheehan. Response surface methodology modelling of protein concentration, coagulum cut size and set temperature on curd moisture loss kinetics during curd stirring. Accepted for publication in *Journal of Dairy Science*.

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5.1 Abstract

The effects of the independent variables protein concentration (4 to 6%), coagulum cut size (6 to 18 mm³) and coagulation temperature (28 to 36°C) on curd moisture loss during in-vat stirring was investigated using response surface methodology. Milk (14 kg) in a cheese vat was rennet-coagulated, cut, and stirred as per semi-hard cheesemaking conditions. During stirring, the moisture content of curd samples was determined every 10 min between 5 to 115 min after cutting. The moisture loss kinetics of curds cut to 6 mm³ followed a logarithmic trend, but the moisture loss from curds with larger cut sizes, 12 or 18 mm³, showed a linear trend. Response surface modelling showed that curd moisture level was positively correlated with cut size and negatively correlated with milk protein level. However, coagulation temperature had a significant negative effect on curd moisture up to 45 min of stirring, but not after 55 min, i.e., after cooking. Curds set at the lower temperature had a slower syneresis rate during the initial stirring compared to curds set at a higher temperature, which could be accelerated by reducing the cut size. This study shows that keeping a fixed cut size on increasing protein concentration decreased the level of curd moisture at a given time during stirring. Therefore, to obtain a uniform curd moisture content at a given stirring time at increasing protein levels, an increased coagulum cut size is required. It was also clear that breakage of the larger curd particles during initial stirring can also significantly influence the curd moisture loss kinetics. Both transmission and scanning electron micrographs of cooked curds (i.e., after 45 min of stirring) showed that the casein micelles were fused at a higher degree in curds coagulated at 36°C compared to 28°C, which confirmed that coagulation temperature causes a marked change in curd microstructure during the earlier stages of

stirring. The present study showed the dynamics of curd moisture content during stirring when using protein-concentrated milk at various set temperatures and cut sizes. This provides the basis for achieving a desired curd moisture loss during cheese manufacture using protein-concentrated milk, as a means of reducing the impact of seasonal variation in milk for cheesemaking.

5.2 Introduction

Application of membrane filtration systems to concentrate milk results in retention of the larger components, i.e., protein and fat, with removal of whey as permeate. Interest in the application of ultrafiltration (UF) in cheesemaking has increased in recent years (Heino et al., 2010; Govindasamy-Lucey et al., 2011; Ozturk et al., 2015) as it facilitates increased process plant throughput and helps to achieve greater cheese consistency despite seasonal variations in milk composition (Broome et al., 1998; Guinee et al., 2006;). Milk of ~3% protein can be concentrated to ~4 to 5% protein (Broome et al., 1998; Guinee et al., 1994), i.e., a low concentration ratio of 1.2 to 1.6 times the normal concentration, for cheesemaking using conventional industrial cheese vats and downstream equipment (Oommen et al., 2000; Govindasamy-Lucey et al., 2005). Higher concentration ratios add complexity to the process (Mistry and Maubois, 2017). Standardizing milk above 5% protein presents challenges during cheesemaking because of increased curd firmness and altered dynamics of rennet-induced coagulation and whey expulsion from curds (Guinee et al., 1994).

The underlying mechanism of rennet-induced gel formation in milk is related to the formation of inter-connected protein networks by the action of rennet. The resulting protein matrix enmeshes large components, e.g., fat globules, by stopping their mobility (Ong et al., 2011; Ong et al., 2013), whereas small-sized components, e.g., lactose and minerals, diffuse from areas of higher concentration towards areas of lower concentration. Cheesemaking includes cutting of a rennet-induced gel at suitable firmness into small curd pieces using cheese knives, followed by in-vat stirring to facilitate expulsion of entrapped water/whey (Dejmek and Walstra, 2004). In modern industrial cheesemaking

processes, the time allowed for stirring in-vat (vat residence time) is standardized for different cheese types.

Expulsion of whey from the curds during stirring occurs due to contraction of the protein network (Fagan et al., 2017). Curd moisture content decreases in line with the level of whey expulsion (Mateo et al., 2009a). Studies on the rates of whey expulsion during in-vat stirring suggest various orders of kinetics, e.g., first-order, second-order or even higher (Caron et al., 2001; Huber et al., 2001; Thomann et al., 2006; Giroux et al., 2014), probably because of multiple factors that influence protein network contraction (Janhøj and Qvist, 2010).

Whey expulsion from curd is a complex process influenced by multiple factors, such as milk composition, temperature, pH, rennet level, addition of CaCl_2 , and curd dimensions (Kaytanli et al., 1994; Thomann et al., 2006; Mateo et al., 2009a). Interactions between these factors critically affect the rate of moisture loss during in-vat stirring (Fagan et al., 2007; Giroux et al., 2014). At microscopic or macroscopic levels, moisture loss from a curd is related to breaking of protein-protein bonds, inducing microstructural changes in curds (Geng et al., 2011) and leading to the rearrangement of casein micelles in the protein network (Mellema et al., 2002). Cheesemaking from protein-standardized milk results in rapid gel-firming and excessive firmness; however, the rate of whey expulsion has been reported to either increase (Casiraghi et al., 1987), remain unchanged (Peri et al., 1985), or decrease (Calvo and Espinoza, 1999; Caron et al., 2001; Thomann et al., 2008) for such milk. Thus, this topic would benefit from more studies, leading to greater understanding of the effects of process parameters on whey expulsion.

Temperature is an important factor that influences coagulation and whey syneresis (Castillo et al., 2006). Although temperatures for semi-hard cheesemaking are generally between 31 to 32°C for coagulation and 37 to 38°C for cooking, curd contraction rate increases with increasing temperature, which results in increased whey expulsion (Huber et al., 2001; Giroux et al., 2014). Increasing protein concentration in milk further promotes rapid gel-firming rate and curd firmness, potentially causing difficulty in curd handling during in-vat stirring (Guinee et al., 1994). Lowering of coagulation temperature has been employed to normalize such coagulation properties in the case of UF-treated milk (Guinee et al., 1994; Govindasamy-Lucey et al., 2004; Govindasamy-Lucey et al., 2011).

Coagulum cut size is another important factor that influences whey expulsion. Curd particles of smaller size release whey faster in comparison to a bigger curd size (Grundelius et al., 2000). Coagula for hard cheese (which requires low moisture level in curds) are cut to cube sizes of 4 to 6 mm (Govindasamy-Lucey et al., 2004; Govindasamy-Lucey et al., 2011), while cube sizes vary between 11 and 21 mm (Renault et al., 1997; Grundelius et al., 2000) for medium- to high-moisture cheeses. Therefore, curd size can be manipulated to adjust the rate or extent of whey expulsion (Renault et al., 1997; Johnston et al., 1998; Everard et al., 2008).

Although various methods for syneresis measurement are available (Fagan et al., 2017), measurement of curd moisture content could offer more practical information for the selection of process conditions to achieve decision-making for appropriate curd moisture content prior to drainage. Previous studies have explored the possibility of real-time prediction of curd moisture, through sensors that are attached in-line to vats to achieve better control (Mateo et al., 2009a,b). Modelling of interactive effects of

temperature, cut size and protein concentration through response surface methodologies can provide insights to cheesemakers for optimization, minimization, or maximization of in-vat curd moisture content during stirring, as desired. An understanding of such interactive effects on moisture loss from curd when concentrated milk is used is particularly desirable, as this determines the combination of conditions that can be employed to achieve consistency in curd moisture levels during stirring.

Panthi et al. (2018) showed that, under constant pH and temperature conditions, curd moisture loss after cutting follows a power law trend. It is also known that less whey expulsion occurs at lower temperatures (Castillo et al., 2006; Giroux et al., 2014). To study the practical implications of raising or lowering the coagulation temperature, a study was designed to: (1) incorporate modern cheesemaking practices such as UF concentration of milk and standardization of pH; (2) minimize initial pH change so that prediction models could focus on protein concentration, cut size, and set temperature; (3) use a fixed cook temperature, and; (4) following a procedure that reflects the manufacturing process of cheeses such as Maasdam, Gouda, Swiss and other cheeses by including a whey dilution step, but separated from the cooking step. To achieve this, response surface methodology was used to investigate the interactive effects of protein level (4, 5, 6%), coagulum cut size (6, 12, 18 mm cubes), and coagulation temperature (28, 32, 36°C) on curd moisture loss kinetics during in-vat stirring, along with characterization of the microstructure of the curds using electron microscopy.

5.3 Materials and Methods

5.3.1 Milk collection and ultrafiltration

Milk collection, pasteurization, ultrafiltration, and standardization were as described in Chapter 4.

5.3.2 Milk preparation

Steps for milk preparation and cheesemaking procedure are schematically presented in Figure 5.1. Milks were standardized in 14-kg rectangular cheese vats. Standardized milk pH varied between 6.72 and 6.77, and this was adjusted to 6.5 by adding lactic acid (1:8 dilution, vol/vol). Milk was then heated to 45°C and cooled to the set temperature to minimize the effect of cold storage (Qvist, 1979).

5.3.3 Milk coagulation

Milks were adjusted to the required set temperature (28, 32 or 36°C) and inoculated (at 20 g starter /kg milk) with frozen pellets of *Lactococcus lactis* ssp. *lactis/cremoris* (DVS850, Chr. Hansen Inc., Milwaukee, WI). The starter culture was added only ~10 min prior to adding rennet to minimize any pH drop in the cheese vat prior to draining of whey, exploiting the long lag phase before exponential growth of frozen cultures (the normal ripening time allowed for frozen-direct-to-the-vat cultures would be 30 to 45 min). The experimental design for slowing the change in curd pH was used to represent Maasdam type cheesemaking process in which curd is drained at pH \geq 6.45, minimizing solubilization of colloidal calcium. Rennet concentration and rheometry conditions were as described in Chapter 4.

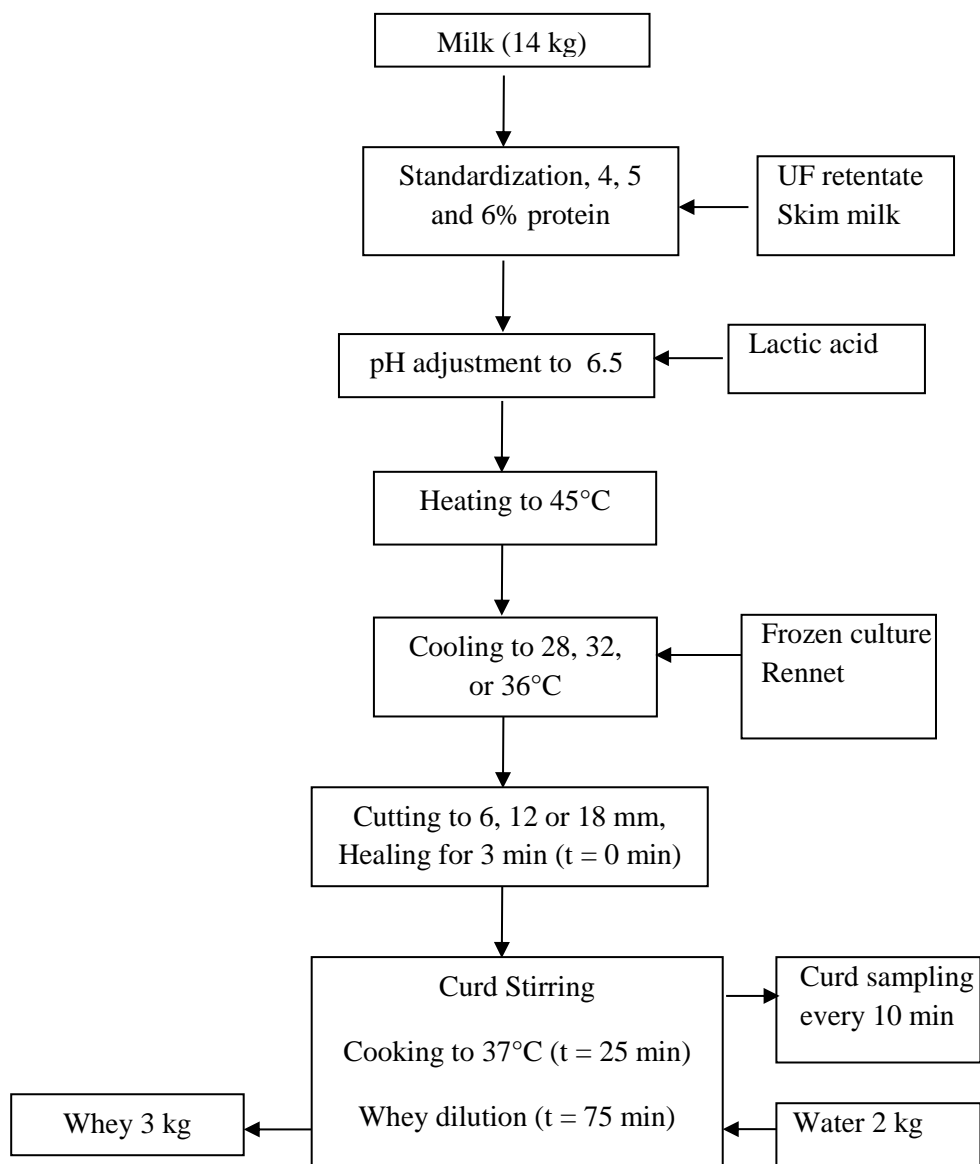


Figure 5.1. Schematic presentation of milk preparation and curd making.

5.3.4 Curd manufacture and sampling

The coagulated milk was cut based on the data from rheometer, when the storage modulus, G' , reached ~ 35 Pa, and coagulation was confirmed by visual observation. Cutting knives were fabricated to achieve curd cubes of size 6, 12, or 18 mm (hereafter cube is not mentioned before size). Coagula were cut in three dimensions within 1 min,

healed for ~3 min, and gently stirred manually from 4 min after the start of cutting. For curds prepared from milk of 5 and 6% protein with 12 and 18 mm cut size, a total of 4 kg of UF permeate produced from the original milk was overlaid on the top of coagula before and after cutting to avoid curd damage during stirring. The temperature of the permeate was raised to the coagulation temperature prior to overlaying.

Starting from 5 min after cutting, curds were sampled every 10 min for moisture content. At 25 min after cutting, the curd and whey were heated to 37°C over 20 min, and this temperature was maintained and stirring continued. This gave a temperature increase of 9, 4 or 1°C for curds set at 28, 32 or 36°C, respectively. At 75 min after cutting, 3 kg of whey was removed, 2 kg of water at 37°C was added to dilute the lactose content of whey, and curd stirring was continued until 115 min after cutting.

5.3.5 Transmission and scanning electron microscopy

To investigate differences in curd microstructure, samples of curd were obtained from milk containing 4 and 6% protein and set at 28 or 36°C from coagula cut into 6-mm sized curd pieces. Curd samples were removed at 45 min after cutting and fixed in glutaraldehyde/formaldehyde for curd microstructure analysis using Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) as described in Chapter 4.

5.3.6 Compositional analysis

The composition of milk, permeate, retentate, and skim milk was determined with a Fourier Transform Infrared Spectroscopy milk analyzer (Bentley Instruments, Inc.,

Chaska, MN, USA). Mineral contents of milk were determined using inductively coupled plasma optical emission spectrometry (Thermo iCAP 6300, Thermo Fisher Scientific, MA USA) after wet digestion, as described by Gavlak et al. (2005). Curd moisture contents were determined in triplicate using a hot air oven method, as described by Mateo et al. (2009a).

Relative curd moisture (RCM) content was calculated as described in Chapter 3 and RCM data were fitted to the following empirical equation:

$$RCM = RCM_o - \frac{(time^{k+a})}{k} \quad [1]$$

where RCM_o is relative curd moisture content at time 0 =100. k is the kinetic constant and a is another constant factor in the model. Values of k , a and the coefficient of determination (R^2) were calculated.

5.3.7 Statistical analysis and experimental design

Milk composition was analysed using one-way ANOVA with Tukey HSD (honest significance difference) for comparing means using IBM SPSS Statistics (version 24 for Windows) at a 95% significance level. Set-to-cut time was analysed using the PROC GLM function in SAS software (version 9.4, SAS Institute, Inc., Cary, NC, USA) with Tukey's adjustment for multiple comparisons of mean differences ($\alpha = 0.05$). Curd moisture loss kinetics data obtained using equation 1 were analysed using nonlinear least squares (NLS) function and plotted using the ggplot2 package using the R studio (version. 1.1.383) of the R program (version 3.4.2, The R foundation of statistical computing, Austria).

Table 5.1 Variable factors and conditions used in the response surface design

Conditions	Symbol		Level		
	Code	Unit	-1	0	+1
Temperature	x ₁	°C	28	32	36
Cut size	x ₂	mm ³	6	12	18
Protein	x ₃	% (g/100 g)	4	5	6

Influences of protein concentration, set temperature and curd size were analysed using response surface methodology with a central composite design ($\alpha = 1$; Table 5.1). Temperature and protein levels were randomized and batches with similar cut size were prepared on each day for consecutive runs. Initial trials were carried out to examine the cheesemaking and sampling variation within trials (these data were excluded from analysis), and then 21 cheesemaking trials were conducted for data analysis. The full polynomial model (equation 2) was used to determine the relationship between the three variables and curd moisture.

$$Y = b_o + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=i+1}^3 b_{ij} X_i X_j \quad [2]$$

where Y is the response variable, b_o is a model intercept, and X_i and X_j are the factor levels. The model comprises of linear (b_i), quadratic (b_{ii}), and cross-product (b_{ij}) terms. Response surface modelling was carried out using PROC RSREG in SAS/STAT (version 14.3; SAS Institute Inc., Cary, Nc, USA). Cross-product terms were removed from the full model for model parameter estimate. The same reduced model was employed to create contour plots using PROC REG and PROC TEMPLATE in SAS and surface plots using Design Expert (version 10, Stat-Ease, Inc, Minneapolis, MN, USA).

5.4 Results and Discussion

5.4.2 Composition of milk

The standardized milk compositions prior to cheesemaking are shown in Table 2. As expected, protein, fat, and total solids contents increased significantly ($P < 0.05$) with increased protein concentration in the standardized milk, with a uniform PFR (Table 5.2). Similarly, pH and lactose levels were not significantly affected by protein concentration. The levels of total calcium and phosphate increased significantly with increasing protein levels. Sandra et al. (2011) also reported a significant increase of total calcium in protein concentrated milk. Since one-third of the calcium in milk is contained in the serum phase, a portion of this soluble calcium passes into permeate during UF. Hence, the calcium-to-protein ratio decreased with increasing protein content.

Table 5.2 Composition of standardized milk prior to cheesemaking

Components	Milk composition		
	4% Protein (n = 6)	5% Protein (n = 7)	6% Protein (n = 8)
Protein (g/100 g)	3.94 ^c	5.03 ^b	6.02 ^a
Fat (g/100 g)	3.95 ^c	5.04 ^b	6.05 ^a
Protein to fat ratio	1.00 ^a	1.00 ^a	0.99 ^a
Lactose (g/100 g)	4.76 ^a	4.60 ^a	5.02 ^a
Total solids (g/100 g)	13.52 ^c	15.66 ^b	17.43 ^a
Total calcium (g/kg)	1.32 ^c	1.65 ^b	1.91 ^a
Total phosphate (g/kg)	2.96 ^c	3.60 ^b	4.24 ^a
Ca/Protein (mg/g)	33.6 ^a	31.7 ^b	32.05 ^b
pH	6.75 ^a	6.74 ^a	6.76 ^a

^{abc} means with same superscript letter were not different ($\alpha = 0.05$).

5.4.3 Curd breakage during stirring

There was a significant interactive effect of set temperature and protein level ($P = 0.015$; Table 5.3) on the time from renneting of milk to when the coagula was cut. Thus, cutting was performed at a storage modulus of ~ 35 Pa rather than at a specified time to obtain uniform curd rigidity.

Table 5.3 Effect of experimental conditions on set to cut time

Temperature	Protein %	Set to cut time \pm SD (min)
28°C	4	49.5 ± 0.7^a
	5	34.5 ± 0.7^{ab}
	6	26.5 ± 12^{bc}
32°C	4	25.0 ± 0.0^{bc}
	5	23.3 ± 0.6^{bc}
	6	24.7 ± 4.0^{bc}
36°C	4	24.5 ± 0.7^{bc}
	5	23.0 ± 4.2^{bc}
	6	20.0 ± 1.0^c

^{a-c} Means within column with the same letter were not significantly different ($\alpha = 0.05$).

During initial stirring after cutting, breakage of curds occurred due to resistance of the curd movement, when permeate was not overlaid. Breakage of curds can be illustrated by contour plots of curd moisture content over in-vat stirring (Figure 5.2). After 5 min of stirring, when the cut size used was increased from 6 to 12 mm, curds were observed to contain a greater moisture for all containing 4, 5 or 6 % protein, whereas such a trend in curd moisture content was not observed when the cut size used was 12 to 18 mm, indicating breakage of curd at the large cut size (Figure 5.2A). At 25 min of stirring, the effect of changing cut size was evident with curds of 4% protein, as indicated by a greater curd moisture content with higher cut size, from 6 to 18 mm (Figure 5.2B). However, applying the same change in cut size with curds of 6% protein resulted in

similar moisture contents (~72%). As the stirring continued to 45 min to 115 min, the effect of changing cut size was evident for 4% protein milk, whereas the moisture content of curds with 5% protein was observed to be less influenced when using cut size from 12 to 18 mm, and for 6% protein the impact of changing cut size on curd moisture was negligible (Figure 5.2C, D). Therefore, curds with 12 or 18 mm cut size and of 5 or 6% protein were highly impacted by the breakage of curds (See Table in Appendix, Table S1 and Table S2).

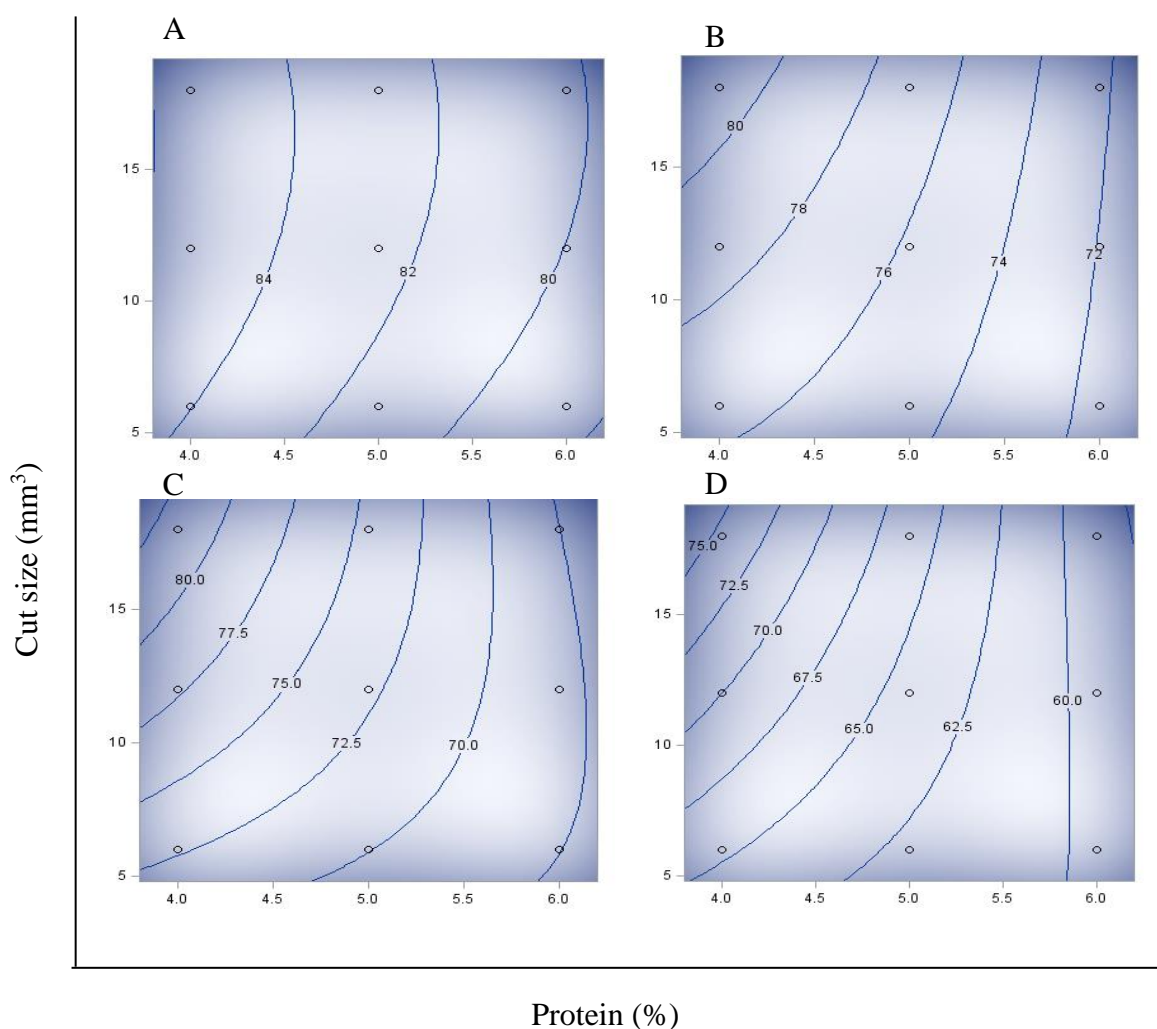


Figure 5.2 Contour plots of curd moisture content (g/100 g) as affected by cut size and protein level during stirring for (A) 5 min, (B) 25 min, (C) 45 min and (D) 115 min. These contour plots were for a fixed temperature of 32.2°C. No permeate overlay was performed.

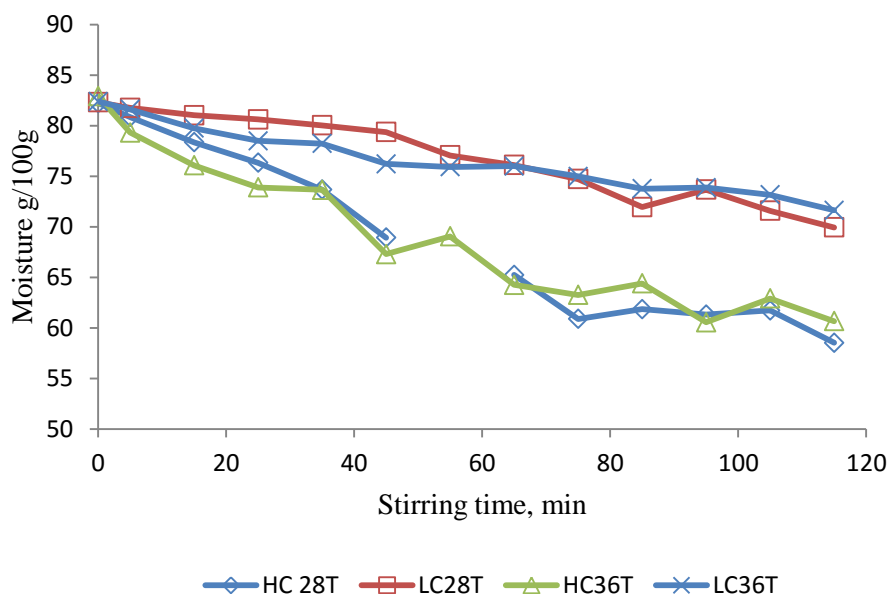


Figure 5.3 Curd moisture loss from large curds (18 mm) made from milk with 6% protein during stirring. HC refers to curd moisture during stirring with no permeate added (high collision resulted into more breakage). LC refers to curd moisture during stirring with permeate added (low collision resulted in retaining curd sizes). 28T and 36T refer to set temperatures of 28 and 36°C, respectively.

Curd requires whey to float during stirring, so that curd could move along with the motion of stirrer. However, slow release of whey from the curds from 5 or 6% protein with cut at large size did not allow curd to move during initial stirring, which subsequently led to the breakage of curds into small particles (See Figure S1 in Appendix). Maintaining the curd size during stirring was considered important in this study to establish the relationship between cut size and other variables. To avoid breakage of curds, a layer of UF permeate (total 4 kg) was overlaid on the coagula before and after cutting for trials conducted with milk of 5 or 6% protein and using a cut size of 12 and 18 mm. Overlaying permeate allowed the movement of curds during initial stirring and increased curd inter-particle distance, which resulted in the curds maintaining their shape and size. As expected, permeate-overlaid curds had a higher moisture content compared to those

prepared without overlay, because of the maintenannce of size (Figure 5.3). Hence, all experiments with 12 or 18 mm cut size with 5 or 6% protein were repeated with overlaying of permeate during cutting to establish the relation of cut size with other variables.

5.4.4 Curd moisture loss during stirring

Curd moisture contents during stirring from 5 to 115 min decreased under each experimental condition (Figure 5.4). Interestingly, the decrease in moisture content in curds of 6 mm cut size showed a logarithmic trend, whereas the decrease in curds of 12 mm or 18 mm cut size was linear. The profile of moisture loss from curds set at 28°C with cut size 6 mm was not logarithmic, due to a faster decrease in curd moisture content during cooking (25 to 45 min of stirring) compared to curds set at 32 or 36°C. Such a rapid decrease in moisture content loss was due to greater rise in temperature during cooking. However, a fast decrease in moisture even for curds set at 28°C and cut at the larger cut sizes (12 or 18 mm) was less evident. The contraction of the curds probably occurs at the curd surface and the presence of a larger proportion of moisture in the internal curd matrix minimizes the magnitude of moisture loss by temperature rise. Interestingly, the rate of moisture loss after 45 min of stirring showed a linear trend in each condition.

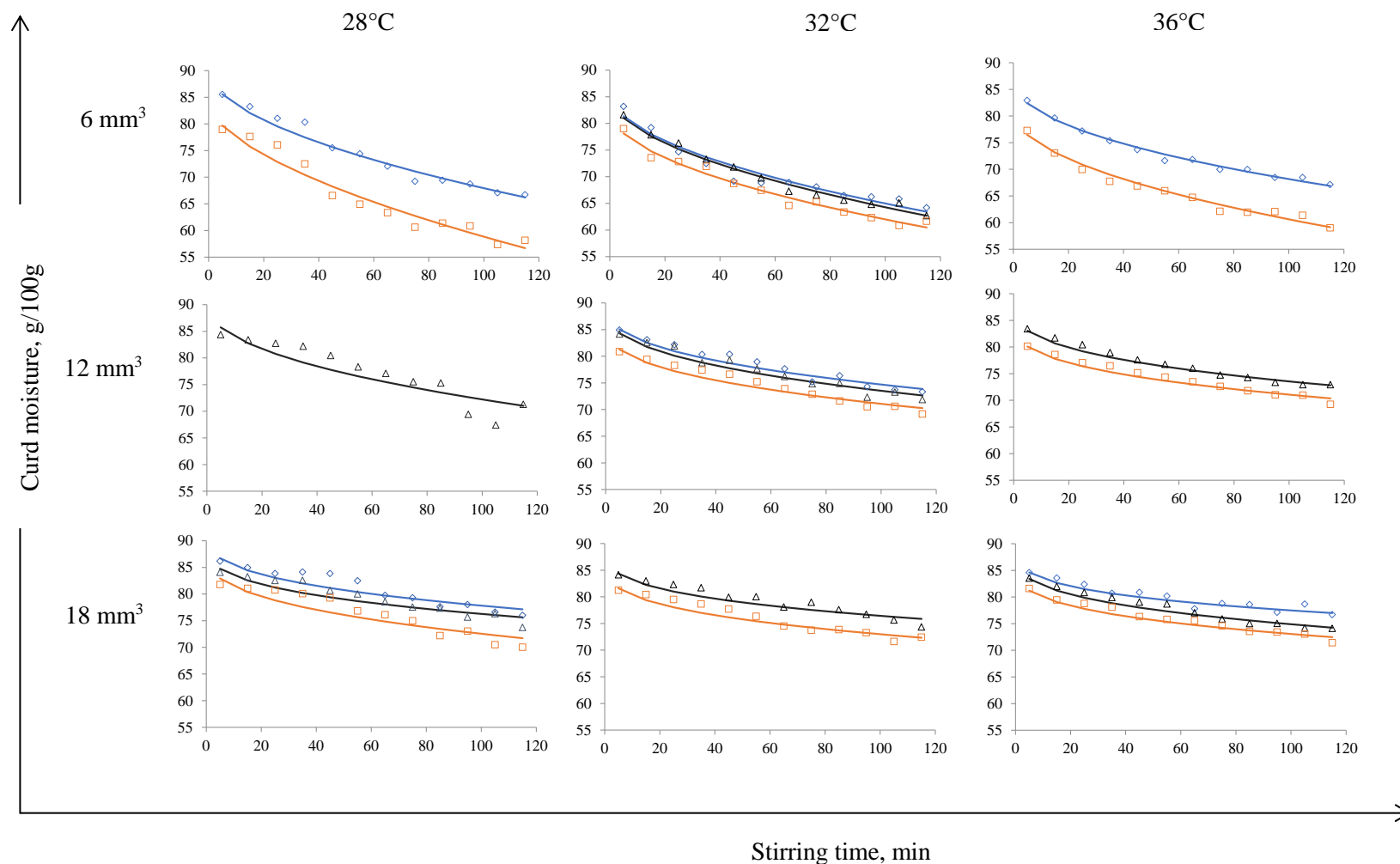


Figure 5.4 Moisture loss profile of curds (g/100 g) during stirring from 5 to 115 min after cutting at various conditions of set temperature (28-36°C), cut size (6-18 mm³), and protein concentration (◇; 4%, △; 5% and □; 6%). Trend lines were drawn using equation 1 for actual curd moisture content. Permeate was overlaid during cutting for coagulum of 5 and 6% protein with 12 and 18 mm cut size.

It is apparent that curd from milk with increased protein concentration attained a lower moisture content at a given time compared to the curd from milk with lower protein content. However, the rate of moisture loss was not influenced by protein concentration, as indicated by a similar slope of the moisture loss curve at similar conditions of set temperature and cut size (Figure 5.4). This finding is consistent with the results of Peri et al. (1985), who reported that expulsion of whey from curd was independent of protein concentration in milk, which was investigated at up to ~5x concentration without stirring curds. The results from the present study also agree with the finding of Thomann et al. (2008), who observed a greater volume of total liquid collected when combining permeate release during membrane filtration and whey expelled during curd stirring from concentrated curd, compared to total whey expelled from regular milk when 4 to 6 pieces of curds were shaken in a beaker.

5.4.5 Prediction of curd moisture contents

Prediction of in-vat curd moisture content using an empirical model is currently of interest (Panthi et al., 2018). The present study demonstrates various profiles of curd moisture loss depending on curd size and set temperature, emphasizing the requirement of a model which can explain the curd moisture loss under variable conditions. Relative curd moisture (RCM) data were fitted to equation 1; however, this did not strongly explain the moisture data from the curd cut at 18 mm at 28°C or 32°C ($R^2 = 0.79-0.88$). There was a better fit with data from curds set at 36°C ($R^2 > 0.90$), probably due to there being only a 1°C increase during cooking, resulting in an almost constant temperature during stirring. A representative graph of the model fit on RCM data is presented in Figure 5.5. The model explained the moisture loss profile of curds at small and large cut sizes, with R^2 values

being 0.98 and 0.90, respectively. As expected, curds coagulated at 36°C with 4% protein and cut at 6 mm had faster moisture loss kinetics ($k = 0.51$) compared to those of 18-mm cut sizes ($k = 0.30$). Curds of small particle size have a larger surface area and a shorter distance from center to edge for whey movement, compared to bigger curds particles, which have a much smaller surface-to-volume-ratio, resulting in restricted whey expulsion, with subsequent greater retention of moisture (Renault et al., 1997; Dejmek and Walstra, 2004).

In this study, curd size was varied based on spacing between the curd knives. Alternatively, the curd size can be varied depending on the number of rotations of the blades through the coagulum during cutting; as knives are of fixed dimensions, which results in wide range of curd size (Johnston et al., 1998; Everard et al., 2008; Mateo et al., 2009b). While actual whey expulsion rates may vary based on the method of cutting, (especially when curd particles are cut into irregular shapes), the same principles in general apply, in that overall moisture loss from the curd will be influenced by the size range of the curd particles.

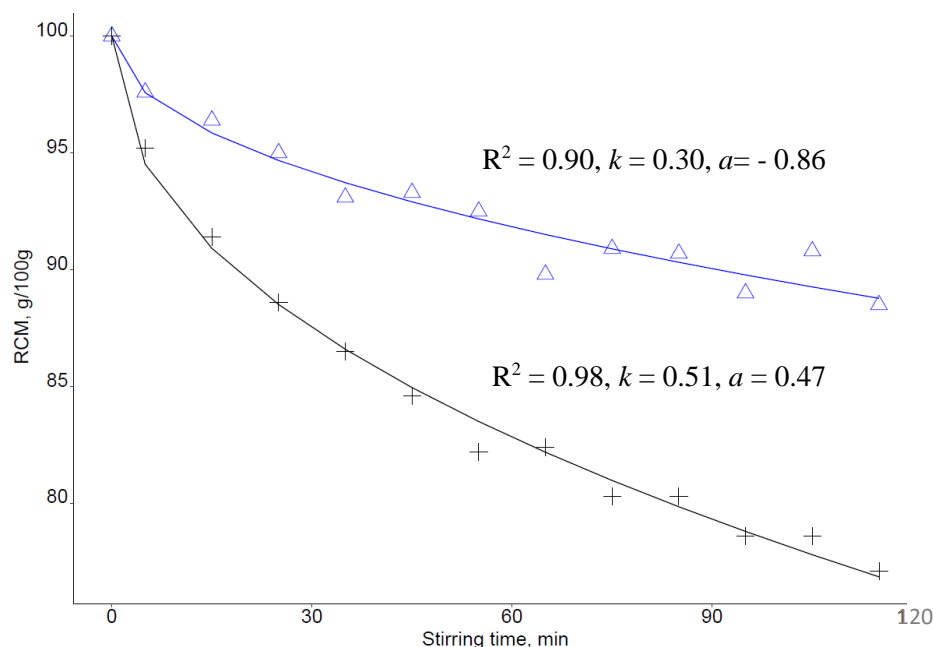


Figure 5.5 Representative plot of model fitted to the RCM data as a function of time during stirring. (+) 36°C, 6 mm, 4% protein, (Δ) 36°C, 18 mm and 4% protein. Coefficient of determination (R^2) and model parameters constants (k and a) are the values calculated using the model. The lines indicate model prediction.

5.4.6 Response surface modelling of curd moisture during stirring

Analysis of variance of the regression model fit showed that most variations in curd moisture were explained by the linear and quadratic regression terms of the model, whereas cross-product terms were non-significant (Table 5.3). To estimate the regression coefficients of the polynomial regression model, a reduced RSM without cross-product terms was used so that estimates could be more robust and more precise. Table 5.4 summarizes estimates and goodness of model-fit (R^2 and coefficient of variation) of the reduced model, which was satisfactory for prediction of curd moisture at a given stirring time.

Table 5.3 F values and associated probability (in parenthesis) for significance of polynomial model terms¹ on curd moisture throughout 115 min of curd stirring.

Model Terms	F value (<i>P</i>) for Curd Moisture at 5 to 115 min after cutting											
	5	15	25	35	45	55	65	75	85	95	105	115
Linear	165.97 (<0.0001)	125.75 (<0.0001)	61.54 (<0.0001)	51.10 (<0.0001)	56.18 (<0.0001)	98.53 (<0.0001)	213.73 (<0.0001)	86.68 (<0.0001)	106.84 (<0.0001)	123.37 (<0.0001)	67.40 (<0.0001)	127.22 (<0.0001)
Quadratic	10.24 (0.0016)	14.09 (0.0004)	9.04 (0.0026)	6.49 (0.0087)	7.49 (0.0043)	11.12 (0.0012)	30.27 (0.0001)	7.94 (0.0043)	13.48 (0.0005)	4.26 (0.0318)	3.28 (0.062)	11.43 (0.001)
Cross-product	5.94 (0.01)	5.60 (0.01)	2.51 (0.11)	1.08 (0.39)	0.41 (0.75)	0.88 (0.48)	5.85 (0.01)	0.85 (0.49)	0.37 (0.77)	1.09 (0.39)	0.75 (0.54)	0.34 (0.8)

¹ based on coded variable of each parameter (-1, 0, +1).

Coagulation temperature (X1) had a significant negative effect on curd moisture for the first 45 min, but not after the curd temperature reached 37°C, i.e., by the end of cooking (with the exception of 105 min, which could be attributed to random error). Cut size (X2) had a significant positive effect on curd moisture at all times, and this effect increased over stirring time, as indicated by increasing values of the coefficient for cut size (Table 5.4). The negative impact of protein concentration (X3) on curd moisture content was fairly uniform during stirring, indicating that protein concentration did not markedly influence the curd moisture loss. The significant quadratic effect of cut size (X2) indicates a non-linear correlation between cut size and curd moisture content.

5.4.7 Curd moisture loss during cutting and cooking

The effects of set temperature, cut size and protein content on the relative rate of initial curd moisture loss (during the first 5 min after cutting) compared to the phase of increasing temperature (25 to 45 min after cutting) are shown in Figure 5.6. The RSM contour plots show that the rate of moisture loss follows different patterns depending on whether during initial stirring or during cooking. As expected, the initial rate of moisture loss was proportional to the coagulation temperature (Figure 5.6A) and the rate of moisture loss during cooking was proportional to the temperature gradient (Figure 5.6 B). In both phases, the rate of moisture loss was inversely correlated with curd cut size.

Table 5.4 Estimates of selected model parameters¹ of set temperature (X1), cut size (X2) and protein concentration (X3) on curd moisture.

Curd moisture ¹	Curd stirring time (min)											
	5	15	25	35	45	55	65	75	85	95	105	115
Intercept	83.77	82.02	81.18	79.91	78.88	77.63	76.13	75.21	74.87	72.17	72.20	71.94
X1	-0.64*	-1.18*	-1.48*	-1.74*	-1.02*	-0.64	-0.33	-0.03	0.04	0.39	1.22*	0.46
X2	1.16*	2.26*	2.93*	3.65*	4.75*	4.97*	4.88*	5.49*	5.24*	5.46*	5.66*	5.56*
X3	-2.32*	-2.38*	-2.02*	-2.06*	-2.40*	-2.49*	-2.47*	-2.50*	-2.77*	-2.48*	-3.01*	-2.78*
X1.X1	0.03	0.66	0.61	0.81	0.35	0.19	0.57	-0.34	-0.07	-0.20	-0.67	-0.21
X2.X2	-0.87*	-1.89*	-2.36*	-2.70*	-3.58*	-3.21*	-3.66*	-3.00*	-3.56*	-1.99*	-2.29*	-3.22*
X3.X3	-0.63	-0.60	-0.94	-0.76	-0.49	-0.67	-0.44	-0.65	-0.68	0.19	-0.18	-0.29
R ²	0.98	0.93	0.91	0.92	0.94	0.96	0.96	0.95	0.97	0.96	0.94	0.97
CV	0.54	1.18	1.56	1.77	1.91	1.60	1.57	1.89	1.59	1.64	2.33	1.57

¹ predicted models parameters based on coded variables of each parameter (-1, 0, +1).

* Significant at $\alpha = 0.05$.

The contour plot shows that, when setting milk at 28°C, coagula should be cut to smaller sizes to achieve similar rates of initial moisture loss than under conditions where milk is set at higher temperature, e.g., 36°C (Figure 5.6A). The slow release of whey from a curd at lower temperature can be compensated with reducing the coagulum cut size. Curd cut to larger sized particles (12 to 18 mm) showed almost negligible moisture loss during initial stirring.

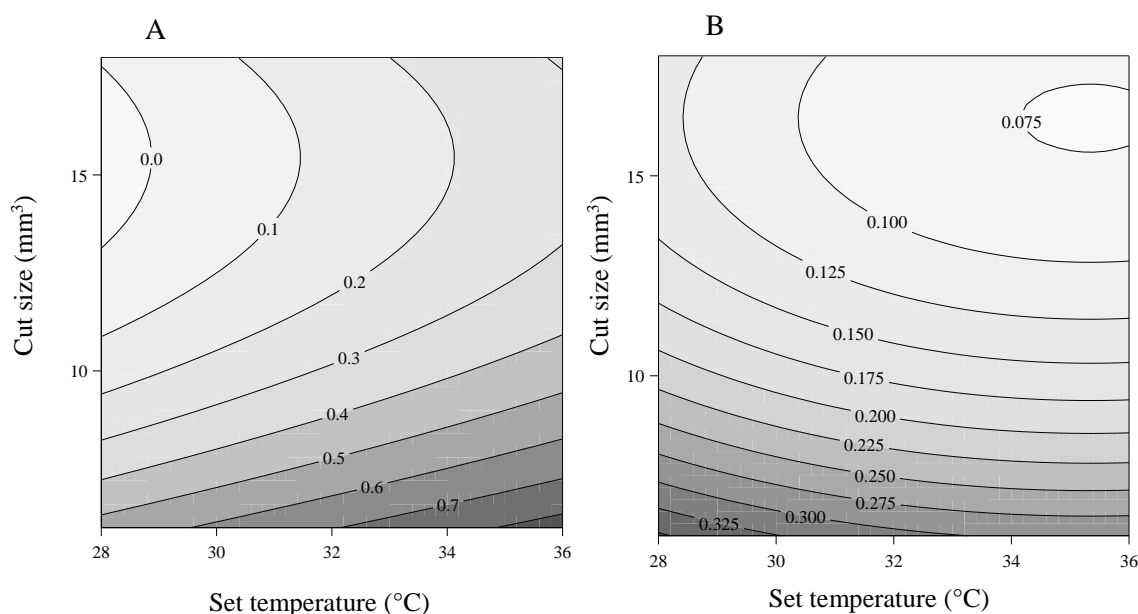


Figure 5.6 Contour plots of relative curd moisture loss (% /min) (A) after initial stirring and (B) during cooking, as affected by set temperature and cut size. Permeate was overlaid during cutting for coagulum of 5 and 6% protein with 12 and 18 mm cut size.

On the other hand, for curds set at 28°C (especially with smaller cut size) the rate of moisture loss during cooking was 0.32%/min and decreased with both increasing temperature or cut size to 0.07 %/min (Figure 5.6B). This occurs because the rise in temperature during cooking to 37°C is larger when the set temperature was 28°C compared to a set temperature of 36°C. At higher temperature, greater rearrangement of

casein micelles in the protein network occurs, creating a force for curd contraction (Mellema et al., 2002). Subjecting the curd to a temperature rise enhances contraction of the protein network, which causes concomitant whey expulsion. This perhaps minimized the impact of set temperatures on curd moisture content after cooking (Table 5.4). However, Fagan et al. (2007) observed a significant effect of coagulation temperature on curd moisture content during stirring (5 to 65 min) when cooking of curds was not undertaken.

In the present study, the impact of pH change was minimized by adding lactic acid and frozen culture and subsequently giving a shorter ripening time (~10 min) than in standard cheesemaking practice (~45 min). The pH value of whey samples measured after 75 min of stirring was not significantly influenced ($P = 0.426$) by protein concentration; however, set temperature did significantly influence pH ($P = 0.031$). The average pH of whey samples measured after 75 min was 6.31, 6.34, 6.36 from the curds coagulated at 28, 32 and 36°C, respectively. Slight differences in the pH at 75 min suggest that pH differences during the early stages of stirring, for example at 45 min, could be negligible. It is suggested that, with the growing use of direct-vat-inoculum cultures, little pH change is observed in the early stages of industrial cheesemaking, as was observed in the present study. However, changes in pH in the early stages may be more evident when bulk cultures are used, promoting earlier curd syneresis.

5.4.8 Curd moisture content during stirring.

Curd moisture contents at 75 min as influenced by cut size and protein concentration is presented in Figure 5.7A. It is evident that, on increasing protein

concentration of milk, moisture content of the curds decreased and curd moisture content increased with increasing coagulum cut size. Similar trends in the contour plots were observed at each sampling point with reduced curd moisture content over time (plots not shown).

Whey drainage occurs at ~75 min after cutting for many semi-hard cheese manufacturing processes. It is apparent that, at 75 min, cheese curds produced with a 6 mm cut size and from milk of 6% protein attained a low curd moisture content (60 g/100 g curds), whereas curds of 4% protein and with 18 mm cut size retained significantly more moisture (79 g/100 g curds). These results demonstrate that in-vat curd moisture content varies widely with respect to cut size and protein level in milk. It is proposed that these two factors can be exploited to control the moisture content of curds during stirring. For example, curds with 73% moisture can be produced with milk concentrated to 4% protein with a cut size of less than 9 mm while, at a milk protein concentration of 6%, a cut size of ~13 mm is required to obtain a similar moisture level. Therefore, manufacturing cheese from milk with increased protein concentrations requires increased curd sizes to achieve similar moisture to standard cheese manufacturing practices. Thus, cheesemaking from milk with increased protein concentration requires modified standard operating procedures to maintain an appropriate stirring time between cutting of the curd and curd drainage.

Achieving uniform curd moisture content prior to drainage of curds is a key control point for consistency in the cheesemaking process. To explore curd moisture content over stirring, response surface contour plots were generated with stirring time as a factor. For example, when cut size is fixed to 12 mm at a set temperature of 32°C, it will

take 95 min to attain 74% moisture for curds from 4% protein milk, which is reduced to 75 min for curds from 5% protein milk and 45 min for curds from 6% protein milk (Figure 5.7C). Likewise, curds with 70% moisture can be produced in 55 min when cutting a coagulum at 6 mm size, and more than 115 min is required to achieve similar levels of curd moisture when cut size is increased to 12 mm (Figure 5.7D). Therefore, increasing milk protein levels allows both processing of increased milk volume and a reduction in processing time, thus increasing efficiency in two ways.

Previous studies have shown that cheese moisture content decreases when increasing protein levels to ~4.5% protein (Guinee et al., 1996; Broome et al., 1998; Guinee et al., 2006) which could be because of lower curd moisture content prior to drainage. The negative correlation of moisture content with protein level can be explained partly by an increased level of protein and fat in standardized milk, due to the loss of serum phase by concentration (Thomann et al., 2008). Furthermore, increasing the volume fraction of the curd particles with respect to the serum phase leads to a subsequent increase in the frequency of collisions between curd particles, contributing to greater moisture loss during stirring, due to particle deformation (Guinee et al., 2006; Geng et al., 2011). Permeate overlay in the present study was expected to minimize such influences.

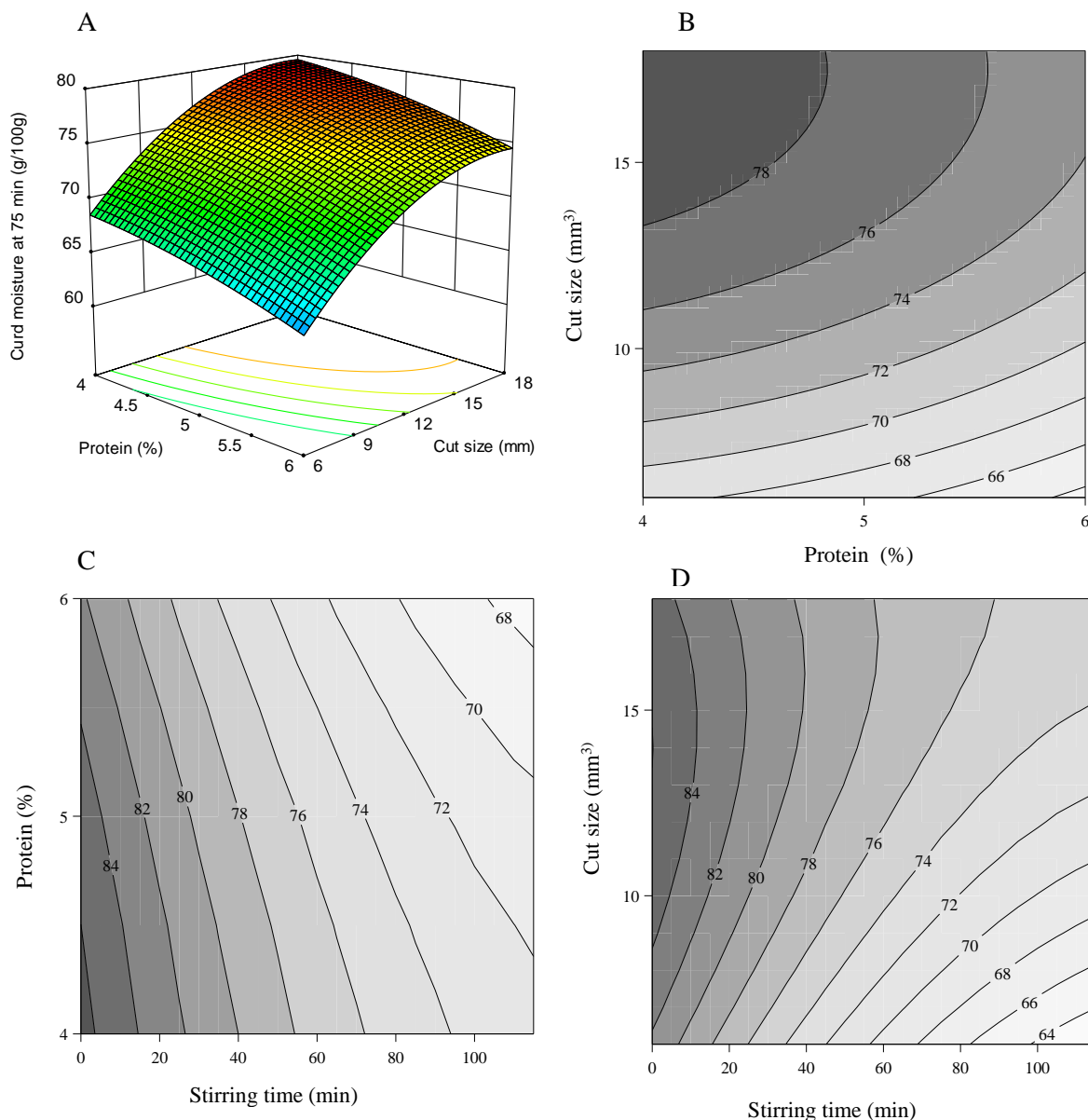


Figure 5.7 Surface plots (A) and contour plots (B) of curd moisture content (g/100 g) as affected by protein level and cut size at 75 min of stirring. Contour plots of curd moisture content as a function of stirring time as influenced by protein level (C) and cut sizes (D) are also shown. Permeate was overlaid during cutting for coagulum of 5 and 6% protein with 12 and 18 mm cut size.

Some studies have suggested a decrease in syneresis rate (Calvo and Espinoza, 1999; Caron et al., 2001; Thomann et al., 2008) with increasing milk concentration ratio, whereas other studies that expressed syneresis in terms of moisture retention reported increased syneresis rate with increasing protein levels in milk (Casiraghi et al., 1987).

Curds from concentrated milk expel less whey compared to those prepared from regular milk, due to the fact that the initial level of whey in the former is obviously lower (Thomann et al., 2008), resulting in lower moisture content in curds from concentrated milk at a given time compared to regular milk (Casiraghi et al., 1987). The results of the present study confirmed that curd moisture loss in-vat was independent of protein concentration of milks and that observed differences in curd moisture content were mainly attributed to difference in initial milk solid contents.

The current study was carried out to establish the relationship of curd size, protein level and set temperature on curd moisture loss kinetics. Addition of permeate to the coagulum was undertaken to reduce curd breakage during stirring as influenced by protein concentration and larger cut sizes. Indeed, when the permeate was not overlaid, the larger cut-size particles from milk with higher protein concentration disintegrated, which resulted in lower curd moisture compared to those curds cut at smaller cut size, showing confounding effects of protein levels in milk. For designing experiments with minimum breakage of curd, the concept of overlaying permeate was developed, which enabled establishment of the relationships between cut size and other parameters. However, overlaying permeate on coagula may not be a commercially viable technique due to use of permeate but this concept may lead to the incorporation of new steps such as overlaying water in the case of Maasdam type cheesemaking, where washing of the curd is carried out.

5.4.9 Curd microstructure

5.4.9.1 Scanning electron microscopy

Curd microstructure, as influenced by coagulation temperature, is shown in Figure 5.8 for milk with 6% protein concentration and cut at 6 mm and set at either 28°C or 36°C. At low magnification, curd particles at both temperatures appeared to have a loose structure with many large voids (Figure 5.8A, B). The medium magnification image shows that void spaces appeared bigger in curds set at 28°C (Figure 5.7C) compared to curds set at 36°C (Figure 5.8D) which had smaller voids, with a porous structure. Differences between curds were more apparent at higher magnification, with the appearance of a higher degree of fusion between casein particles in curds set at 36°C (Figure 5.8F) compared to curds set at 28°C (Figure 5.8E). The curd set at 36°C also showed casein aggregates with slightly thicker strands and a flattened shape compared to the curd formed at 28°C. Similar differences were observed at the time of cutting the coagulum (Chapter 4), but casein micelles were fused to a greater extent in the cooked curds in both treatments. For milk containing 3.3% protein, Ong et al. (2011) also observed a fine regular protein network in curds which had been set at 27°C and subsequently cooked to 38°C compared to curds set at 36°C and cooked to 38°C, the latter conditions resulted in a dense protein network with larger casein micelles aggregates.

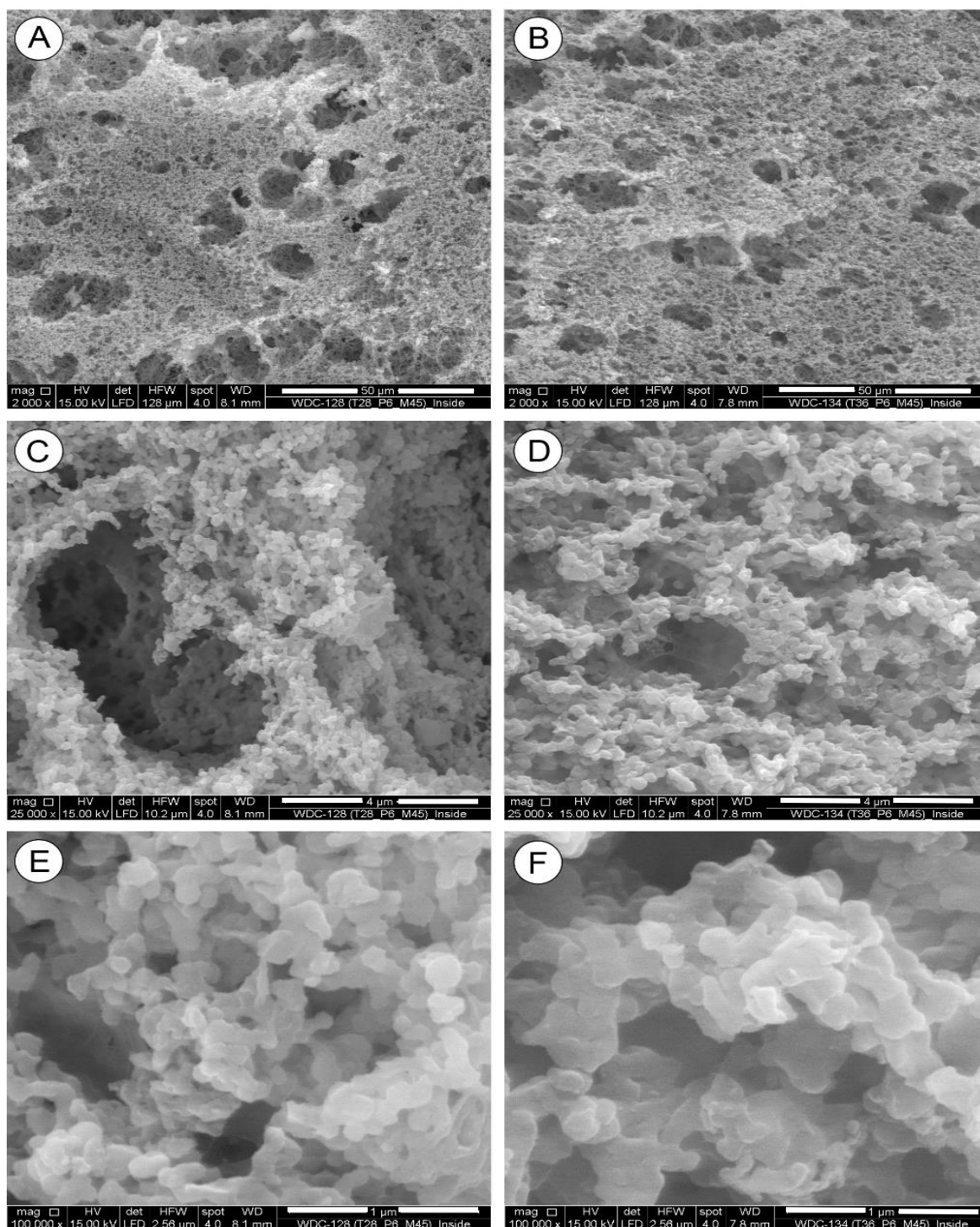


Figure 5.8 Representative scanning electron micrographs of cooked curd particles made from 6% protein-standardized milk that were rennetted at two different set temperatures (A, C, E: 28°C; B, D, F: 36°C). Micrographs were taken at a distance of ~500 µm from the outside of the particle. Scale bars A, B = 50 µm, C,D = 4 µm and E,F = 1 µm.

5.4.9.2 Transmission electron microscopy

Transmission electron micrographs of curds from different experimental treatments are shown in Figure 5.9. At low magnification, it could be seen that casein micelles formed a regular network with fat globules interspersed in the curds when set at 28°C (Figure 5.9A) or 36°C (Figure 5.9B), but the network appeared condensed with more connection points at the latter set temperature. Medium and high magnification images show that casein micelles were fused to thicker branches in the curds set at higher temperature (Figure 5.9D, F) compared to those set at lower temperature (Figure 5.9C, E). This correlates well with the SEM findings. Ong et al. (2013) reported no difference in the microstructure of cooked curds prepared from milk with increasing protein levels (4 to 5.8%) coagulated at 33°C, and thus it appears that the temperature at which coagulation occurs has a greater influence on internal structure than protein concentration.

It seemed that gels set at 28°C probably had continuous casein micelle rearrangement during cooking, because of less cross-linking of casein micelle aggregates (Figure 5.8E) whereas, in curds set at 36°C, such phenomena probably occurred before cooking, as observed by flattened and fused casein micelle aggregates (Figure 5.9F). Void spaces (probably filled with whey) observed in Figure 5.8A and B with curds set 28°C or 36°C are probably due to rearrangement of casein micelles in the protein network, which exerted endogenous pressure on whey, leading to accumulation of whey between casein clusters (Van Vliet et al., 1991). Larger void spaces in curds set at 28°C indicates protein network rearrangement was greater during cooking compared to curds set at 36°C, which probably explains the greater moisture loss during cooking from curds set at 28°C compared to those set at 36°C.

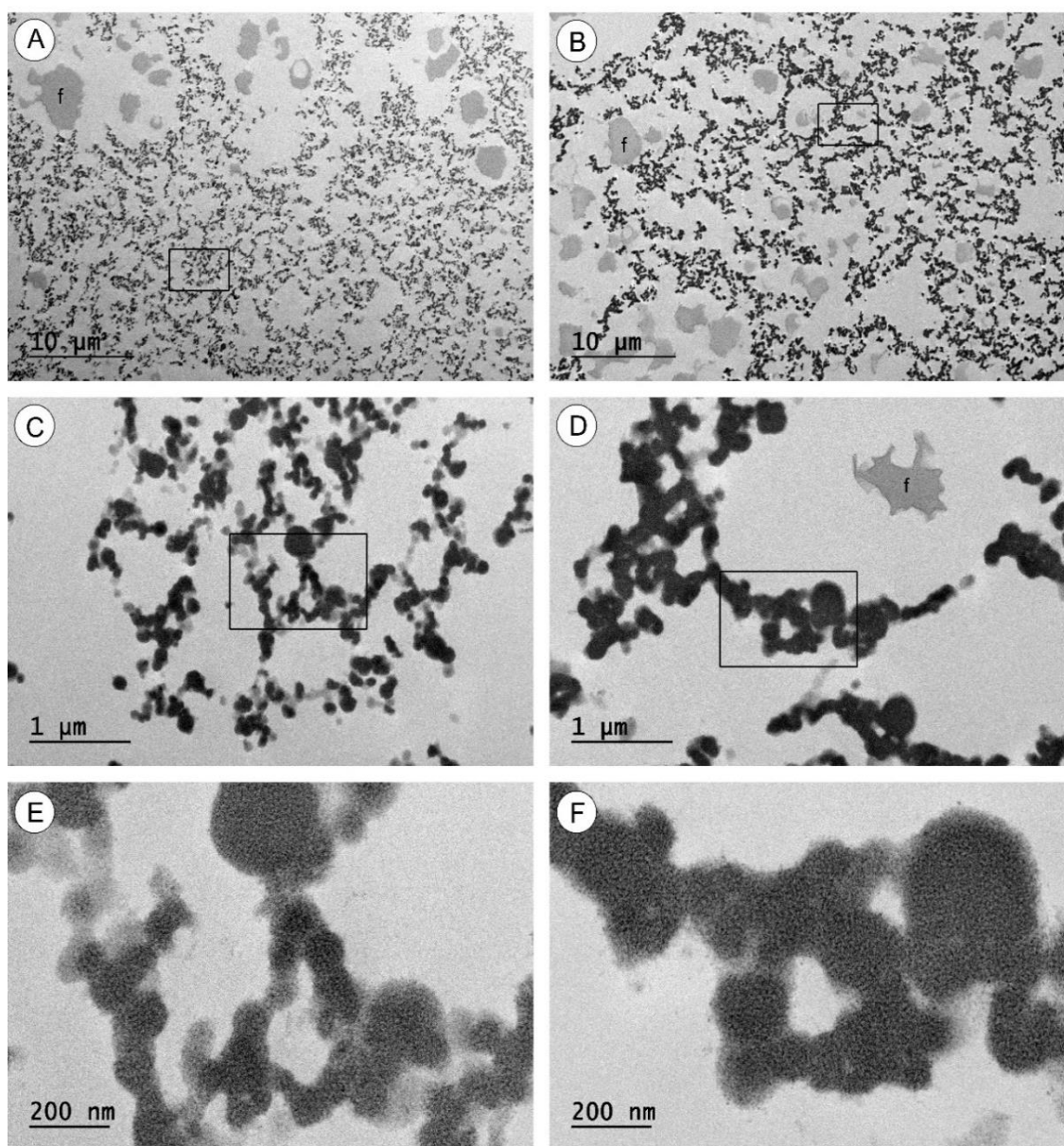


Figure 5.9 Internal microstructure of cooked curd particles manufactured with protein-standardized milk (6% protein) renneted at different set temperatures (A, C, E ; 28 °C; B, D, F; 36 °C), as analysed by transmission electron microscopy. Micrographs were taken at a distance of ~500-600 µm from the outside of the particle. Boxed areas are shown in increasing magnifications. f – fat globules.

5.5 Conclusions

Interactive effects of milk protein concentration, cut size, and coagulation temperature on curd moisture loss property were evaluated. The trend for moisture loss from curd cubes of 6 mm was logarithmic, whereas moisture loss for larger curds was of a linear pattern. Curds set at a lower temperature released less moisture immediately after cutting, which can be accelerated by reducing the cut size. Response surface modelling showed that cut size and protein levels were the most important factors influencing moisture content during stirring, whereas coagulation temperature did not have a significant influence, particularly after cooking (heating to 37°C). When protein concentration in the milk was increased, excessive curd breakage occurred when a large cut size (12 to 18 mm) was used. This suggested that, cutting to smaller cut sizes would be required for cutting the coagulum from protein-concentrated milk. It was shown that overlaying permeate on the coagula from protein-concentrated milk will prevented breakage of curds particles. However, further research would be required to investigate the impact of application of permeate on coagula on cheese quality and composition in an industrial cheese manufacturing process including economical aspects. This study shows that, if the cut size cannot be increased during cheesemaking with milk of increased protein level, it is necessary to shorten the vat residence time before draining the whey. The microstructure of the cheese curd after cooking (as shown using both transmission and scanning electron microscopy) retained the differences initiated by coagulating the milk at different temperatures, with curd set at 36°C having thicker protein network strands with more fusion of casein particles compared to curd coagulated at 28°C. A fundamental understanding of curd moisture loss properties as influenced by cut size,

protein concentration and coagulation temperature gained from this research may be useful in optimizing cheesemaking processes, predicting curd moisture content from protein-standardized milk, reducing the impact of seasonal variability in milk composition, and increasing process efficiency.

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Chapter 6

Effect of pasture versus indoor feeding regimes on the yield, composition, ripening and sensory characteristics of Maasdam cheese

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6.1 Abstract

Maasdam cheese was manufactured from standardized milk derived from each of three feeding systems: grass (GRA), grass and clover pasture (CLO), and indoor feeding of total mixed ration (TMR). Pasture-derived cheeses had significantly lower L^* (whiteness) and higher b^* values (yellowness) compared to TMR-derived cheeses. Acetate levels were significantly lower in CLO and butyrate levels significantly higher in TMR compared to the other cheeses. GRA cheese had significantly higher scores for smooth texture, ivory colour and shiny appearance compared to TMR. The influence of feed-type was minimal on cheese yield, composition and on glycolysis, lipolysis and proteolysis during ripening.

6.2 Introduction

Dairy farming systems in Ireland, parts of North West Europe, New Zealand and some parts of Australia are unique in that milking herds are mainly fed on grazed pasture whereas, in the majority of countries (e.g., USA or parts of Europe), herds are kept indoors and supplied with total mixed ration (TMR) consisting of silage, grains, added minerals and vitamins (O'Callaghan et al., 2016). Pasture-based feeding systems could be regarded as “more natural” by facilitating cow's access to fresh forage. This system also reduces the cost of milk production as well as enteric methane (CH₄) emissions compared to TMR feeding systems (O'Neill et al., 2011). Emissions of CH₄ from grazing cows with increasing proportions of white clover (*Trifolium repens*) is also less compared to full grazing of ryegrass grass (*Lolium perenne* L.; Lee et al., 2004), making for more environmentally sustainable production systems, e.g., low nitrous oxide and greenhouse gas emissions.

However, herd feeding systems can influence milk properties (Gulati et al., 2017; O'Callaghan et al., 2017) and thereby product properties. Christian et al. (1999) studied the composition of milk and Cheddar cheese derived from cows fed with an increasing proportion of spring pasture or grains in a control diet of hay and silage, and found that increasing the diet quality (spring pasture and lupin-wheat) resulted in lower cheese moisture levels and improved cheese functionality compared to the control diet. Amenu et al. (2006) reported that altering the herd feeding systems influence Cheddar cheese yield and quality little. O'Callaghan et al. (2017) demonstrated differences in physico-chemical and sensory properties of Cheddar cheese manufactured using milk derived from indoor vs outdoor feeding systems.

A significant increase in milk production (from 5.51 billion litres in 2014 to 7.27 billion litres in 2017) has occurred in Ireland (Central Statistics Office, 2018) since abolition of EU milk quotas in 2015. Cheese is a vital value-added dairy product, and diversification of the cheese portfolio offers a strategic expansion approach to cheese manufacturers. Studies on development of new cheese types for diversification of the cheese industry have been undertaken, particularly focusing on semi-hard cheeses (Sheehan et al., 2008; Sheehan, 2013) or on cheeses having tailored sensory properties (Bertuzzi et al., 2017), which would be feasible to manufacture on traditional Cheddar cheese plants without extensive capital investment (Sheehan and Wilkinson, 2001).

Unlike Cheddar, Maasdam is an intermediate cheese between Gouda and Emmenthal manufactured with respect to manufacturing procedure and starter cultures. For example, mesophilic starter cultures (*Lactococcus* and *Leuconostoc*) along with some thermophilic adjuncts and Propionic Acid Bacteria (PAB) as a secondary culture (Lamichhane et al., 2018a,b) are inoculated to cheese milk. During cheesemaking, curd washing is utilized and cheeses are ripened in a warm room (20-21°C for 3-6 weeks) to achieve PAB growth, which leads to the development of a sweet and nutty taste as well as round eyes (Fröhlich-Wyder et al., 2017). However, little information has been published characterizing Maasdam cheesemaking, cheese yield, cheese ripening, and sensory attributes, particularly those made from milk of herds on different feed types.

The objective of this research was to investigate effect of three feeding systems, perennial rye-grass (GRA), perennial rye-grass and white clover (CLO) or TMR, on the physico-chemical properties, yield, ripening and sensory characteristics of Maasdam cheese over 150 d of ripening. Herds fed GRA or CLO were kept outdoor on pasture,

whereas TMR fed herds were kept indoor, as described in the study of O'Callaghan et al. (2016).

6.3 Materials and Methods

The composition of GRA, CLO or TMR feed and allocation of each group of 18 cows based on each feeding system was described previously (O'Callaghan et al., 2016). Milk samples were collected from each group of cows during mid-lactation over three week period. Methods of collection and milk standardization at protein to fat ratio (PFR) of 1.17 were as described in Chapter 3. The Maasdam cheesemaking process from the standardized and pasteurized milk was as described by Lamichhane et al. (2018a). Briefly, cheese milk (380 kg, 32°C) in each vat was inoculated with frozen pellets (Chr. Hansen Ltd, Denmark) of mesophilic (*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris* and *Lueconostoc*), thermophilic (*Lactobacillus helveticus*) and propionic acid bacteria (PAB) at the rate of 18 mg/kg of milk, 4.8 mg/kg of milk, and 7 mg/kg of milk, respectively. This was followed by the addition of calcium chloride (34% wt/vol) at level of 0.3 ml/kg and rennet (0.18 ml/ kg of milk, Chymax plus, 200 IMCU/ml; Chr. Hansen, Denmark) after diluting (~1:10) in deionized water. Cutting, stirring, cooking, curd washing, pre-pressing and brining was as described by Lamichhane et al. (2018a). Cheeses wheels (~12 kg) were wrapped under vacuum and pre-ripened at 8°C for 10 days, transferred to a warm room at ~23°C for 21 days and finally ripened under refrigeration (2-4°C) up to 150 d from the date of manufacture. Cheeses were manufactured in triplicate over a three-week period.

6.3.1 Compositional analysis of milk and cheese

Gross composition of raw and standardized milk (fat, total protein and lactose) was determined by Fourier Transform Infrared (FTIR) spectroscopy (Milkoscan; Foss FT2 Foss Electric, Hillerød, Denmark). The levels of non-casein nitrogen (NCN), non-protein nitrogen (NPN), and casein number were calculated as described previously (O'Callaghan et al., 2016). Calcium content in standardized milk was determined as described by Lin et al. (2016).

Cheese samples (wedges ~200 g of length equivalent to half of the diameter of a circular cheese) were removed at prior to brining and 1d of ripening, grated and analysed for protein, fat, pH, ash, salt, calcium and moisture content using the standard International Dairy Federation methods (Guinee et al., 2006). Salt in moisture (S/M), fat in dry matter (FDM), moisture in non-fat substances (MNFS), PFR, Ca/protein were calculated from the compositional data.

6.3.2 Whey composition, fat and protein recovery and cheese yield

The levels of fat, protein, casein fines, and total solids in whey samples, recovery of fat and protein based on the composition of milk, actual cheese yield and yield adjusted to 45% moisture level were determined as described by Guinee et al. (2006).

6.3.3 Determination of colour

The colour parameters (L^* , a^* b^*) of fresh cheese samples between 65 and 150 d of ripening were measured using a Minolta Chroma-Meter CR 400 (Masontec, Dublin Ireland) as described by Bertuzzi et al. (2017).

6.3.4 Biochemical analysis

Samples for biochemical analyses were taken after 1, 10, 35, 97, and 150 d of ripening and stored at -20°C until analysed. The levels of D (-), L (+) and total lactate were analysed as described by Lamichhane et al. (2018a). Changes in nitrogen soluble at pH 4.6 (pH 4.6-SN)/total nitrogen (TN), total amino acids (AA) in the pH 4.6-soluble extract were determined as described by Lamichhane et al. (2018a). Levels of acetate, propionate, and butyrate (C2:0, C3:0, C4:0) were quantified, as described by Sheehan et al. (2008).

6.3.5 Determination of free fatty acids (FFA)

Free fatty acids (FFA) levels in Maasdam cheeses were determined at 97 d of ripening using gas chromatography (GC) with a flame ionized detector as described by McCarthy et al. (2017) and sample extraction was as described by De Jong and Badings (1990) on grated cheese (4 g). Results were expressed as mg/kg fat in cheese.

6.3.6 Sensory analysis

Sensory characteristics of ripened Maasdam cheese (~97 d) were assessed by twenty-seven naïve assessors (21 to 48 years old) recruited in University College Cork, Ireland. Samples were served at room temperature. Selection criteria for assessors were availability and motivation to participate on all days of the experiment and they were cheese consumers (Swiss-type). The age of the assessors was between 21-48 years old. Cheese samples were held at refrigeration temperatures overnight (4°C), before monadic presentation to the naïve assessor panel at ambient temperatures (21°C) and coded with a

randomly selected 3 digit code. Samples were presented blind and the order of the presentation of all test samples was randomized to prevent first order and carry-over effects. Each assessor was asked to indicate their degree of liking on a 10-cm line scale ranging from 0 (extremely dislike) at the left to 10 (extremely like) at the right (O'Sullivan, 2017). Ranking descriptive analysis (RDA) quantifies the intensity of the sensory attributes of a product using a trained sensory panel. A trained expert panel with extensive experience in cheese tasting including Maasdam type was used for RDA. Eight assessors (4 male and 4 female) participated in a ranking descriptive analysis (RDA) using the list of sensory descriptors of Swiss-type cheese and measured on a 10 cm line scale. All samples were presented in triplicate. Since the assessors were trained, lower numbers were used in this case compared to tasting for hedonic characteristics.

6.3.7 Statistical analysis

One-way ANOVA was performed to compare the effects of treatment on the composition of raw milk, standardized milk, cheese composition before and after brining, whey composition, fat and protein recovery, cheese yield, cheese colour, and free fatty acids, using SPSS statistics version 24 (IBM Corp., 2016). The effect of treatment, ripening time and their interaction on levels of pH, % pH 4.6-SN/TN, lactates, total AA, colour, propionate, acetate and butyrate were analysed with a split-plot design using a PROC MIXED procedure in SAS version 9.4 (SAS Institute Inc., 2011). Pairwise comparisons of means were performed using Tukey's multiple-comparison at $\alpha = 0.05$. Sensory scores were compared using a two-independent-sample t-test in Excel.

6.4 Results and Discussion

6.4.1 Composition of raw and standardized Milk

The mean compositions of raw and standardized milks are shown in Table 6.1. The levels of fat, lactose, NCN, and NPN were similar between GRA, CLO and TMR milks. Protein content was significantly lower in TMR milk (3.45 %, wt/wt) compared to GRA (3.60 %, wt/wt), or CLO (3.56 %, wt/wt) milk, similar to the observation of O'Callaghan et al. (2016). Because of the lower protein content, the PFR also decreased (although not significantly) in the raw TMR milk compared to GRA or CLO. The composition of raw milk was typical for an Irish milking herd at mid-lactation (Gulati et al., 2017). Standardization of milk protein level was undertaken by increasing the protein level of TMR or CLO milk to similar to that of GRA. This eliminates the confounding effect of compositional differences of milk on cheesemaking and on the final cheese composition. Standardization was performed by combining skim milk and retentate and permeate produced by ultrafiltration from the original milk from the same feeding regime with addition of cream from each raw milk to achieve the desired standardization as described in Chapter 3.

As expected, the composition of each standardized milk (TMR, CLO and GRA) prior to cheesemaking were similar (Table 6.1), having a protein content of ~3.6 %. Total calcium level in milk (~140 mg/ 100 g) was in close agreement with that reported by Gulati et al. (2017) and was not significantly influenced by the method of standardization, probably because of similar casein contents in the standardized milks. Lactose levels were also similar between the standardized milk samples, in agreement with the results of

Govindasamy-Lucey et al. (2011), who blended skim milk retentate with milk prior to cheesemaking.

Table 6.1 The composition of raw and standardized milks derived from cows fed on pasture (GRA: perennial ryegrass or CLO: perennial ryegrass and white clover) or indoor (TMR) feeding regimes.

Components	GRA	CLO	TMR	SED ¹	P -Value
Raw milk					
Fat (g/100 g)	4.25 ^a	4.13 ^a	4.29 ^a	0.15	0.59
Protein (g/100 g)	3.60 ^a	3.56 ^a	3.45 ^b	0.03	0.03
Lactose (g/100 g)	4.63 ^b	4.72 ^a	4.74 ^a	0.03	0.32
Non-casein nitrogen (mg/100 g)	116 ^a	120 ^a	112 ^a	4.56	0.31
Non-protein nitrogen (mg/100 g)	27 ^a	33 ^a	29 ^a	2.06	0.05*
Casein no.	79.30 ^a	78.50 ^a	79.17 ^a	0.73	0.53
Protein to fat ratio	0.85 ^a	0.86 ^a	0.80 ^a	0.03	0.21
Standardized milk					
Fat (g/100 g)	3.06 ^a	3.10 ^a	3.04 ^a	0.05	0.35
Protein (g/100 g)	3.63 ^a	3.62 ^a	3.60 ^a	0.04	0.91
Lactose (g/100 g)	4.67 ^a	4.69 ^a	4.73 ^a	0.09	0.89
Non-casein nitrogen (mg/100 g)	114 ^a	116 ^a	111 ^a	5.6	0.70
Non-protein nitrogen (mg/100 g)	26 ^a	32 ^a	28 ^a	2	0.06
Casein no.	78.90 ^a	78.10 ^a	79.40 ^a	0.86	0.61
Protein to fat ratio	1.19 ^a	1.17 ^a	1.18 ^a	0.02	0.48
Total Ca (mg/100 g)	138.97 ^a	140.96 ^a	139.47 ^a	11.53	0.97

^{ab}Different superscript letters in a row indicates significant differences ($\alpha = 0.05$). Data are the means of triplicate trials. * $P = 0.053$, ¹ Standard error of mean difference.

The level of NPN for both raw and standardized milk was slightly higher (at a level close to being significantly different) in CLO milk than in TMR milk. Higher NPN content in milk has a negative influence on cheese yield, due to these nitrogenous compounds not participating in the cheese coagulum and being lost to whey. Milk from cows fed grass and white clover has been reported to contain more non-protein nitrogenous compounds, e.g., urea, than other feeding regimes (O’Callaghan et al., 2016; 2018), which probably led to a higher NPN content in the CLO milk. This is possibly

related to the level of clover present in the sward, with greater concentrations of clover in the diet leading to higher protein metabolism in the rumen, which potentially results in the formation of urea from ammonia in the liver before diffusing into the blood and milk (O'Callaghan et al., 2018).

6.4.2 Composition of cheeses before brining and post-brining

The mean compositions of cheese samples before and after brining are shown in Table 6.2. The composition (moisture, protein, fat, FDM, MNFS, ash, total calcium) and pH (~5.4) of cheese analysed before brining were similar between treatments. The similar composition of cheeses measured before brining indicates that cheeses prepared from milk of different feeding systems underwent similar changes during the cheesemaking process and that any compositional differences in milk were negated by milk standardization.

The mean composition (moisture, protein, fat, FDM, MNFS, salt, ash, calcium) and pH (~5.26) of Maasdam cheese measured after brining was also similar between cheese samples derived from different feeding systems; the PFR in all Maasdam cheeses were close to 1 and the FDM content in cheese samples were ~45 to 46%. Acerbi et al. (2015) reported a composition of 26.5% fat, 42% moisture, 45.6% FDM in semi-hard model cheeses with eyes. Maasdam cheese prepared in the present study had a slightly higher (~3-4%) moisture compared to commercial Maasdam cheese samples (Botosoa and Karoui, 2013). Cheese moisture and MNFS level decreased during brining (significant decreases in GRA and TMR but not significant in CLO), by ~2%, whereas the level of protein and fat was similar in all brined cheese samples.

Table 6.2 The mean composition of Maasdam cheese produced from standardized milk derived from cows fed on pasture (GRA: perennial ryegrass or CLO: perennial ryegrass and white clover) or indoor (TMR) feeding regimes.

Composition	GRA	CLO	TMR	SED ¹	P-value
Before-brining					
Moisture (g/100 g)	47.57 ^{aA}	47.37 ^{aA}	47.24 ^{aA}	0.74	0.91
Protein (g/100 g)	24.76 ^{aA}	24.32 ^{aA}	24.12 ^{aA}	0.57	0.55
Fat (g/100 g)	25.06 ^{aA}	24.51 ^{aA}	24.70 ^{aA}	0.29	0.24
Fat in dry matter (g/100 g)	47.79 ^{aA}	46.56 ^{aA}	46.81 ^{aA}	0.40	0.05
MSNF ² (g/100 g)	63.47 ^{aA}	62.74 ^{aA}	62.73 ^{aA}	0.81	0.60
Protein to fat ratio	0.99 ^{aA}	0.99 ^{aA}	0.97 ^{aA}	0.02	0.62
Ash (g/100 g)	2.68 ^{aB}	2.55 ^{aB}	2.70 ^{aB}	0.12	0.49
Ca (mg/100 g)	890 ^{aA}	856 ^{aA}	898 ^{aA}	45.19	0.63
Ca (mg/ g protein)	35.93 ^{aA}	35.21 ^{aA}	37.29 ^{aA}	1.99	0.60
pH	5.49 ^{aA}	5.48 ^{aA}	5.46 ^{aA}	0.30	0.52
Post- brining					
Moisture (g/100 g)	45.63 ^{aB}	45.35 ^{aA}	45.45 ^{aB}	0.46	0.84
Protein (g/100 g)	25.12 ^{aA}	24.68 ^{aA}	24.32 ^{aA}	0.52	0.37
Fat (g/100 g)	24.95 ^{aA}	24.78 ^{aA}	25.17 ^{aA}	0.35	0.57
Fat in dry matter (g/100 g)	45.89 ^{aB}	45.34 ^{aA}	46.13 ^{aA}	0.50	0.33
MSNF ² (g/100 g)	60.80 ^{aB}	60.29 ^{aA}	60.73 ^{aB}	0.49	0.56
Salt (g/100 g)	1.21 ^a	1.49 ^a	1.30 ^a	0.11	0.09
Salt in moisture (g/100 g)	2.64 ^a	3.29 ^a	2.86 ^a	0.23	0.08
Protein to fat ratio	1.01 ^{aA}	1.00 ^{aA}	0.97 ^{aA}	0.02	0.21
Ash (g/100 g)	3.42 ^{aA}	3.46 ^{aA}	3.74 ^{aA}	0.21	0.34
Ca (mg/100 g)	950 ^{aA}	917 ^{aA}	970 ^{aA}	36.25	0.29
Ca (mg/ g protein)	37.55 ^{aA}	36.77 ^{aA}	39.88 ^{aA}	1.71	0.24
pH	5.29 ^{aB}	5.23 ^{aB}	5.26 ^{aB}	0.06	0.60

^{ab} Different lower-case superscript letters in a row and upper-case superscript letters ^{AB} in a column (for comparable parameters before or post-brining) indicate significant differences ($\alpha = 0.05$). Data are the means of triplicate trials. ¹ Standard error of mean difference, ² Moisture in Non-Fat Substances.

The predominant change that occurs during brining was absorption of salt and loss of moisture, which led to significantly increased ash content in the brined cheese samples. Although it was not significant, a slight increase in total calcium level was observed in brined cheeses compared to those pre-brined, possibly due to loss of moisture resulting in a reduction in MNFS levels, which also resulted in an increased (although not statistically significantly) Ca/protein ratio in the brined cheeses.

6.4.3 Whey composition, recovery of fat and protein in cheese and cheese yield

The composition of whey, recovery of fat and protein in cheese and cheese yield related to the different feeding regimes are presented in Table 6.3. The level of fat, protein, fines and total solids in bulk whey produced during experimental cheesemaking were similar. The percentage recovery of fat and protein were around 90% of milk fat and ~76% of milk protein during Maasdam cheesemaking.

Table 6.3 Composition of whey, recovery of fat and protein, and cheese yield during Maasdam cheesemaking from standardized milk derived from pasture (GRA: perennial ryegrass or CLO: perennial ryegrass and white clover) or indoor (TMR) feeding regimes.

Parameters	GRA	CLO	TMR	SED ¹	<i>P</i> -value
Whey composition					
Fat (% wt/wt)	0.40 ^a	0.40 ^a	0.37 ^a	0.04	0.67
Protein (% wt/wt)	0.96 ^a	1.01 ^a	1.02 ^a	0.06	0.65
Fines (mg/kg)	394.6 ^a	275.23 ^a	408.93 ^a	48.08	0.06
Total solids (% wt/wt)	6.63 ^a	6.55 ^a	6.63 ^a	0.37	0.97
Fat (% of milk fat)	11.42 ^a	11.10 ^a	10.68 ^a	1.26	0.85
Protein (% of milk protein)	23.35 ^a	24.16 ^a	24.60 ^a	0.38	0.67
Recovery					
Fat (% of milk fat)	89.41 ^a	89.60 ^a	91.89 ^a	1.68	0.33
Protein (% of milk protein)	76.49 ^a	76.78 ^a	74.61 ^a	1.65	0.42
Cheese yield					
Actual yield (%)	10.88 ^a	11.15 ^a	10.94 ^a	0.14	0.23
45% moisture adjusted yield (%)	10.76 ^a	11.08 ^a	10.85 ^a	0.20	0.35

^aSimilar superscript letters in a row indicates no significant differences ($\alpha = 0.05$).

¹Standard error of mean difference.

Milks were standardized to similar PFR and coagula were cut at uniform firmness in all cheese trials, thus minimizing curd tearing and shattering, and resulting in a similar level of fat and protein loss from the freshly-cut curd particle during early stirring (Guinee et al., 2006). Similarly, standardization of milk to a uniform PFR could potentially have led to a similar curd microstructure entrapping similar proportions of fat into the protein network, resulting in similar fat and protein recovery in all experimental cheeses. In

agreement with the recovery of the major constituents of cheese, the actual cheese yield (~11%) and with the moisture adjusted yield was similar between cheese derived from the different feeding regimes (Table 6.3).

6.4.4 Cheese pH

All Maasdam cheeses before brining had pH ~5.48, and this decreased significantly ($P < 0.01$) to ~5.23 after brining (Table 6.2). The pH measured in Maasdam cheese after brining (1 d) was within the range (5.1 to 5.3) reported by Govindasamy-Lucey et al. (2011) for Swiss-type cheese. The decrease in pH during brining was due to continued starter and non-starter lactic acid bacterial (NSLAB) metabolism of residual lactose and galactose, yielding lactic acid (O'Sullivan et al., 2016; Sheehan et al., 2008). As expected, lactose was not detected in experimental cheese samples after 10 d of ripening (data not shown). During ripening, the pH values significantly increased from ~5.2 at 1d to ~5.7 by 150 d of ripening (Figure 6.1), which was in agreement with previous studies undertaken for cheese with eyes (Govindasamy-Lucey et al., 2011; Lamichhane et al., 2018a) and for Cheddar cheese (pH 5.43 at 270 d; O'Callaghan et al., 2017).

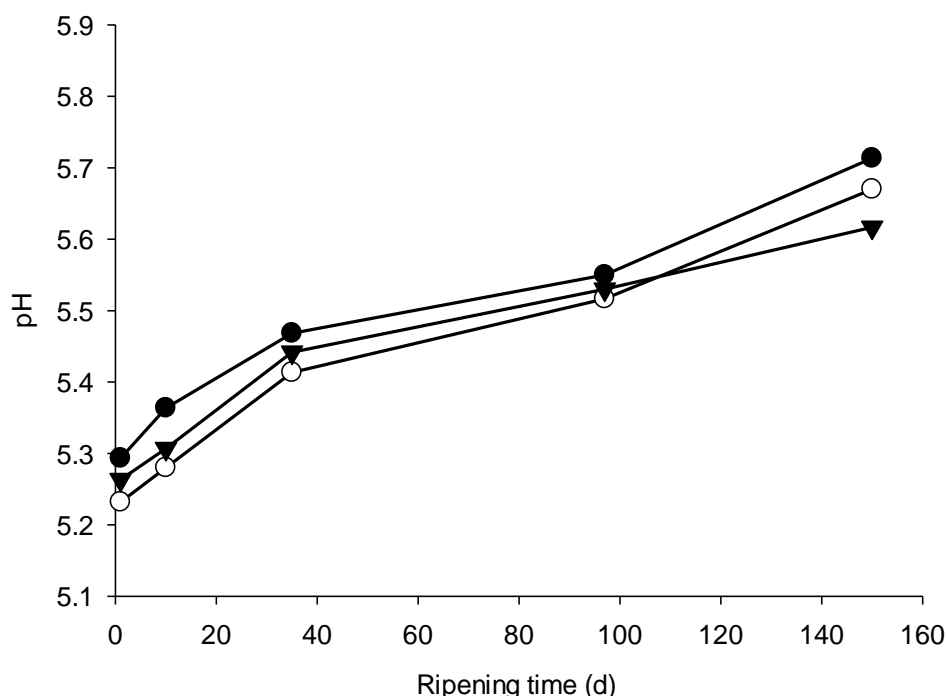


Figure 6.1 Changes in pH during Maasdam cheese ripening. GRA (●), CLO (○) or TMR (▼) represent cheeses made from milk of cows fed perennial ryegrass only, perennial ryegrass and white clover or total mixed ration, respectively.

Liberation of free amino acids and short chain peptides (McSweeney, 2004), along with utilization of lactate by PAB, results in this increase in pH during ripening. No significant effect of GRA, CLO and TMR feeding systems was observed on pH during ripening (Table 6.4), which indicates that all the experimental cheeses had similar metabolic, e.g., glycolysis, events during ripening. O'Callaghan et al. (2017) also reported no significant influence of feeding system on pH of Cheddar cheese during ripening.

Table 6.4 Statistical summary (*P* values) of feed, time and interaction on parameters during Maasdam cheese ripening.

Parameter	Feed	Time	Interaction
pH	0.09	<.0001	0.97
%pH 4.6-SN/TN	0.99	<.0001	0.69
L-lactate	0.07	<.0001	0.88
D-lactate	0.18	<0.001	0.71
Total-lactate	0.07	<.0001	0.89
Total free amino acid	0.96	<.0001	0.99
Colour*			
L	0.015*	0.86	0.98
A	0.08	0.21	0.99
B	0.0001*	0.17	0.89
Propionate	0.21	<.0001	0.91
Acetate	.0002*	<.0001	0.175
Butyrate	<.001*	<0.001	0.11

* indicates significant effect of feed

6.4.5 Levels of L- D- and Total Lactate of Maasdam cheese

There was a significant ($P < 0.001$) decrease in the level of L-lactate over the ripening period (Figure 6.2A), which concurs with the reports of Sheehan et al. (2008) and Lamichhane et al. (2018a). The decrease in L-lactate is due to its utilization by PAB, which produce acetate, propionate, CO₂, and H₂O (Beresford et al., 2001). D-lactate was observed after 10 d and its level increased during warm room ripening and then subsequently decreased up to 150 d of ripening (Figure 6.2B). Such trends for D-lactate levels in cheese have been previously reported and attributed in part to racemization by NSLAB at higher temperatures (Beresford et al., 2001). Total lactate levels followed a similar trend to that of L-lactate in all experimental cheeses (Figure 6.2C). No significant effect of treatment was observed for the levels of L-, D-and total lactate during ripening (Table 6.5).

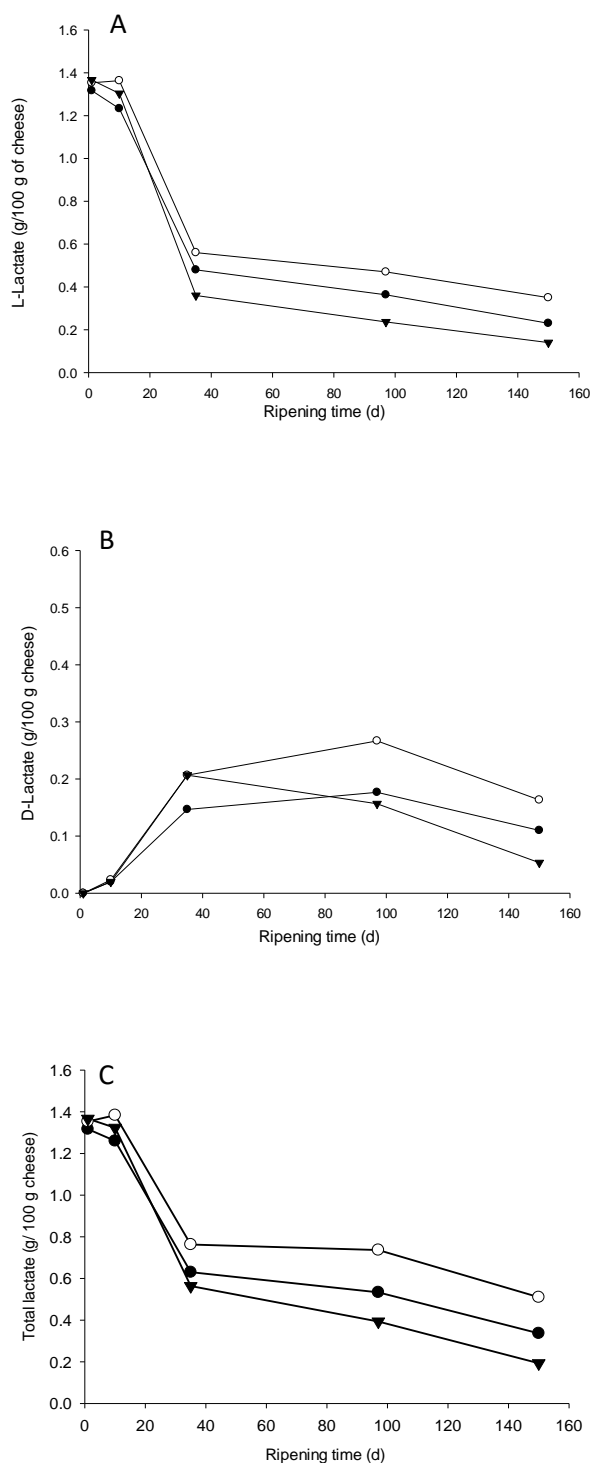


Figure 6.2 Changes in L-lactate (A), D-lactate, (B) and total lactate (C) levels during Maasdam cheese ripening. GRA (●), CLO (○) or TMR (▼) represent cheeses made from milk of cows fed perennial ryegrass only, perennial ryegrass and white clover or total mixed ration, respectively.

6.4.6 Colour

The L^* values measures visual lightness (within 0 to 100), a^* values measure redness (positive values) to greenness (negative values), and b^* values measure yellowness (positive value) to blueness (negative value). The colour parameters (L^* , a^* , b^* values) in Maasdam cheese samples derived from different feeding systems are presented in Table 6.5.

Table 6.5 Effect of feeding systems on colour parameters of Maasdam cheese samples produced from standardized milk derived from pasture (GRA: perennial ryegrass or CLO: perennial ryegrass and white clover) or indoor (TMR) feeding regimes.

Colour components	GRA	CLO	TMR	SED ¹	<i>P</i> -value
L^* (lightness)	79.36 ^b	80.37 ^b	82.17 ^a	0.57	<.0001
a^* (green to red)	-2.83 ^{ab}	-3.29 ^a	-2.72 ^b	0.20	<0.05
b^* (blue to yellow)	28.89 ^a	29.10 ^a	24.19 ^b	0.30	<.0001

^{ab} Different superscript letters in a row indicates significant differences ($P < 0.05$). Data are the means of 10 measurements from 65 to 150 d ripening.

¹Standard error of mean difference

Cheeses derived from the TMR feeding system had a significantly ($P < 0.001$) higher value (82.17) for L^* compared to the CLO (80.37) or GRA (79.36) feeding systems. There was no significant difference in L^* value between GRA or CLO cheese samples. The a^* value was higher ($P < 0.05$) in TMR (-2.72) compared to GRA (-2.83) or CLO (-3.29) cheese samples, with no significant difference between GRA or CLO cheese samples. Similarly, TMR cheese samples had a significantly ($P < 0.001$) lower b^* value (24.19) compared to GRA (28.89) or CLO (29.10) cheese samples, with no significant difference between GRA or CLO cheese samples. Therefore, the colour parameters showed that pasture-derived cheeses were more yellow and TMR-derived

cheeses were paler, in agreement with previous studies (Coppa et al., 2011; O’Callaghan et al., 2017).

Pasture-derived cheese has been reported to contain more than two-fold higher β -carotene levels compared to those cheeses derived from TMR feeding systems (O’Callaghan et al., 2017), resulting in more yellow colour in the former (Carpino et al., 2004). Cheese colour during 65 to 150 d of ripening period was not significantly influenced by the ripening time, nor was any interaction of feeding system with ripening time observed (Table 6.4). However, O’Callaghan et al. (2017) reported a significant decrease in a^* and b^* values over the ripening time in Cheddar cheese. O’Callaghan et al. (2017) measured colour parameters at the surface of the cheese block, whereas in the present study, colour was measured on the surface of freshly cut cheese wedges, where light-induced degradation of β -carotene was minimal (Juric et al., 2003).

6.4.7 Proteolysis

6.4.7.1 % pH 4.6-SN/TN

The proportion of nitrogen soluble at pH 4.6 as percentage of total nitrogen indicates primary proteolysis of casein fractions by the proteolytic action of enzymes (Fenelon et al., 2000). A significant ($P < 0.001$) increase in levels of % pH 4.6-SN/TN occurred over the course of ripening (Figure 6.3), similar to that reported for Maasdam cheese (Lamichhane et al., 2018a) and Swiss cheese (reported as 12% TCA-SN; Govindasamy-Lucey et al., 2011). Overall, levels of primary proteolysis in Maasdam cheese were not influenced by the feeding system of the herd (Table 6.4), as reported by O’Callaghan et al. (2017) for Cheddar cheese.

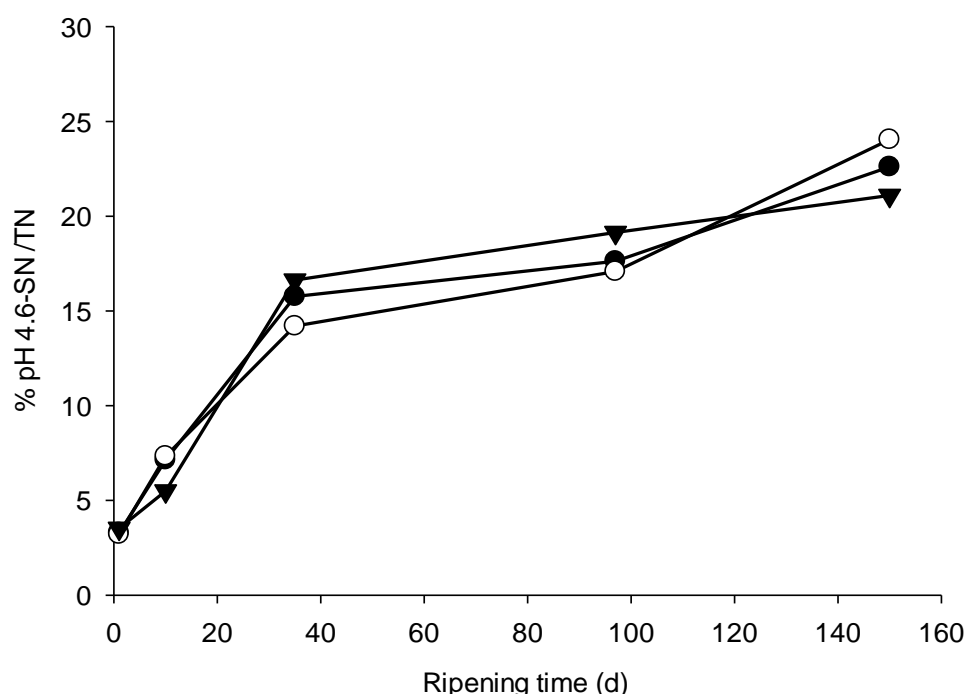


Figure 6.3 Changes in pH 4.6 SN/TN levels during Maasdam cheese ripening. GRA (●), CLO (○) or TMR (▼) represent cheeses made from milk of cows fed perennial ryegrass only, perennial ryegrass and white clover or total mixed ration, respectively.

6.4.7.2 Total AA levels

Total AA levels in Maasdam cheese samples during ripening are shown in Figure 6.4. The mean level of total AA significantly increased ($P < 0.001$) during ripening and an effect of feeding system on total AA was not observed (Table 6.4). Total AA content was ~1200 mg/kg at 10 d which markedly increased to ~4700 to 5000 mg/kg after warm room ripening at 35 d. Similarly, no effect of the different feeding systems on total AA was also observed by O’Callaghan et al. (2017) for Cheddar cheese. The trend of increasing total AA in Maasdam cheese samples during ripening were in agreement with those reported by Lamichhane et al. (2018a), and the increase of total AA is consistent with the increase in % pH 4.6-SN/TN (Figure 6.3).

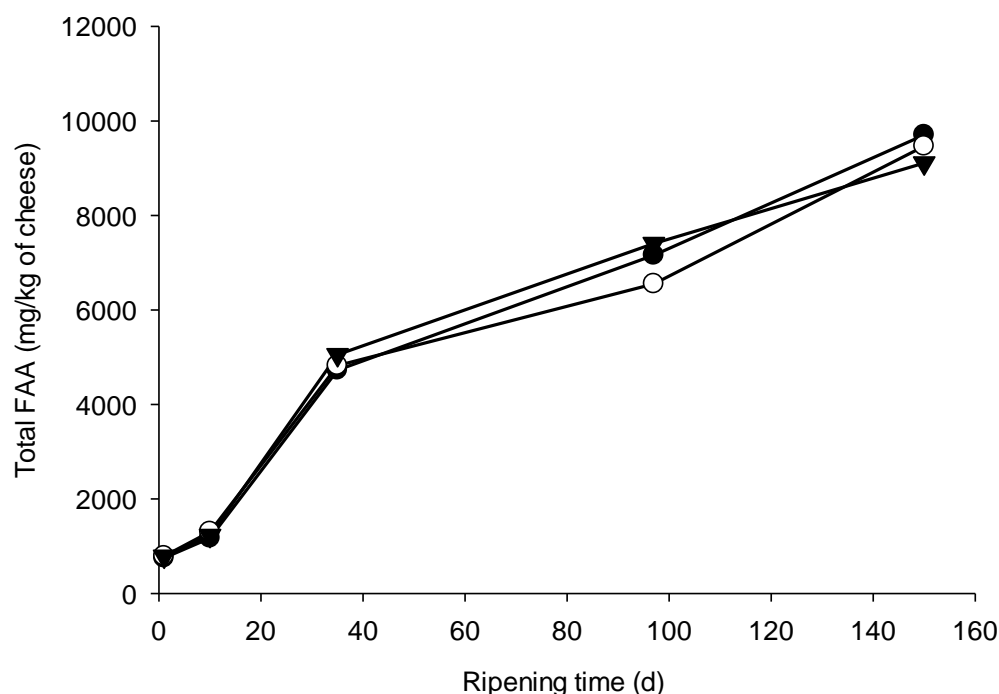


Figure 6.4 Changes in total free amino acids levels during Maasdam cheese ripening. GRA (—●—), CLO (—○—) or TMR (—▼—) represents cheeses made from milk of cows fed perennial ryegrass only, perennial ryegrass and white clover or total mixed ration, respectively.

6.4.8 Short-chain volatile acids

6.4.8.1 Propionate and Acetate

Significant increases in mean levels of propionate were observed in all experimental cheese samples during ripening, especially during warm room ripening (Figure 6.5A). No significant effect of feeding systems on cheese propionate level was observed (Table 6.4). The mean propionate level in Maasdam cheese reached ~5000 mg/kg after 150 d, and this trend was in agreement with previous results reported at similar ripening times (Sheehan et al., 2008; Lamichhane et al., 2018b).

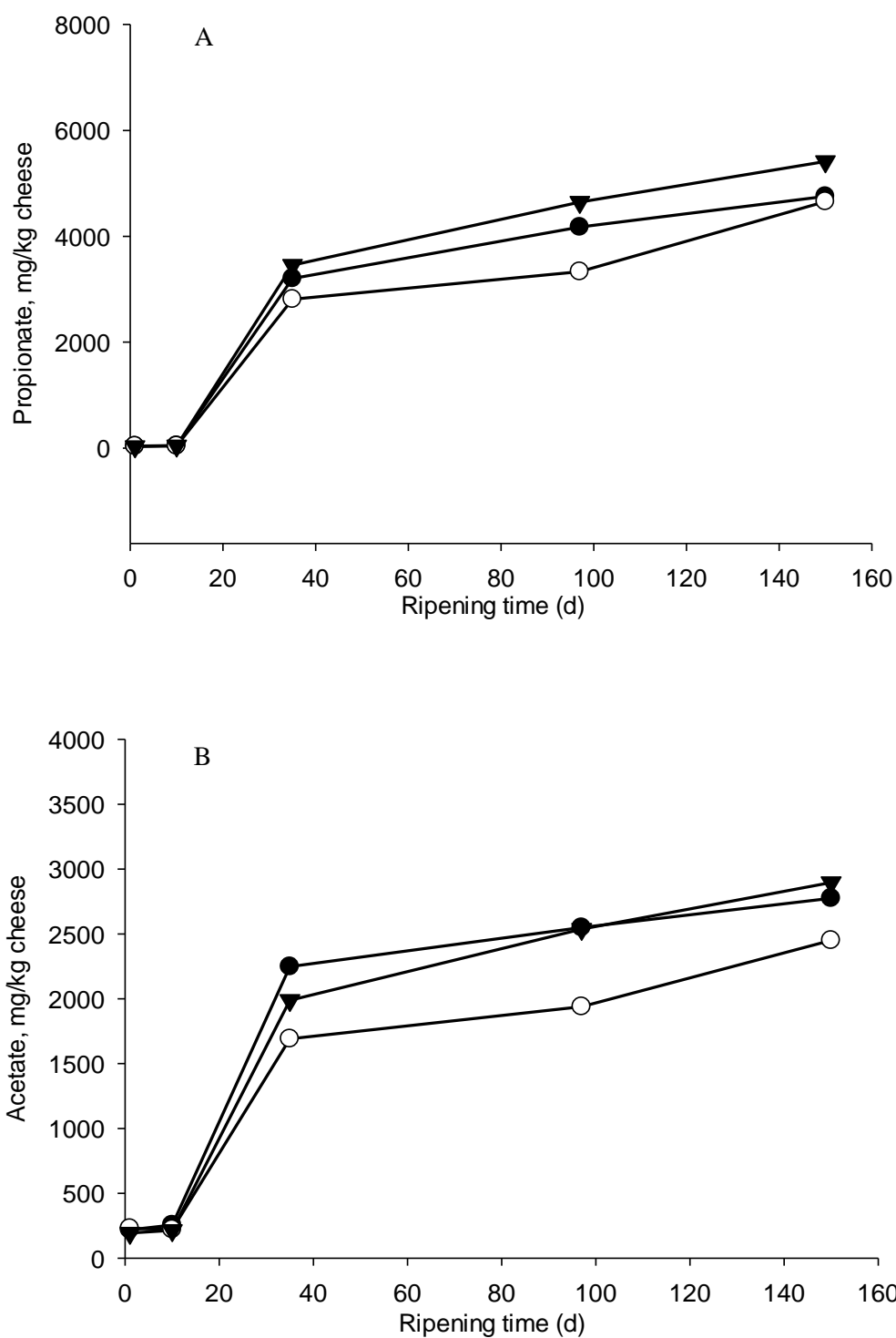


Figure 6.5 Changes in (A) propionate, (B) acetate, and during Maasdam cheese ripening. GRA (—●—), CLO (—○—) or TMR (—▼—) represent cheeses made from milk of cows fed perennial ryegrass only, perennial ryegrass and white clover or total mixed ration, respectively. Mean values are the mean of triplicate trials.

Levels of acetate also followed a similar trend to that of propionate during ripening (Figure 6.5B). Interestingly, a significant effect of feeding system on acetate level was observed (Table 6.4). The CLO cheese had less acetate formation compared to GRA or TMR cheese, especially after the warm-room ripening stage. O’Callaghan et al. (2018) observed that acetate level of Cheddar cheese was positively correlated with TMR-derived milk; albeit its level was not significantly different between milk from pasture or TMR feeding systems.

Production of propionate and acetate occurs in Swiss-type cheeses that are inoculated with PAB, which converts lactate to propionate, acetate, carbon dioxide and H₂O (Fröhlich-Wyder et al., 2017). In addition, production of acetate in cheese may also be attributed to NSLAB converting citrate, amino acids, nucleotides or lactate to pyruvate (Lazzi et al., 2014). The pathway for the conversion of pyruvate to acetate by NSLAB involves two enzyme systems. Pyruvate oxidase (POX) converts pyruvate to acetyl-phosphate whereas acetyl kinase (ACK) converts acetyl-phosphate to acetate (Lazzi et al., 2014). The expression of genes in NSLAB that regulates the POX-ACK pathway can vary between batches of cheese and over ripening (Levante et al., 2017), suggesting adaptation of NSLAB in stress conditions is strain specific. Recently, some of the microbiota in raw milk and in ripened cheese has been associated with microbiota in teat skin of cows (Frétin et al., 2018). Although semi grazing or extensive grazing of herd was reported to have minimal effect on microbial taxa in milk, large botanical diversity and feeding practices (e.g., indoor or outdoor) would have marked effect on milk microbiota originated from teat skin (Frétin et al., 2018). It may be a consideration for future studies to determine whether herd feeding system influences the evolution of microbiota in Maasdam cheese

and gene expression profiles of NSLAB that contributes in the production of organic acids (e.g., acetate) during warm room ripening and maturation.

6.4.8.2 Butyrate

A significant increase in mean levels of butyrate was observed in all experimental cheeses during ripening (Figure 6.6) and a significant effect of treatment was also observed (Table 6.4). Butyrate levels in TMR cheese samples were significantly higher than in GRA and CLO samples. Butyrate in cheese could possibly be produced by *Clostridium* spp, the spores of which may germinate during warm room ripening, subsequently resulting in gas production and leading to a late-blowing defect in cheese (Sheehan, 2013) or by hydrolysis of lipids through milk lipase/esterase activity. However, levels of butyrate in the present study were < 200 mg/kg, a level which is commonly detected in Swiss-type cheese without any blowing defects (Lamichhane et al., 2018b; O’Sullivan et al., 2016).

The observation of higher butyrate levels in TMR cheeses could be attributed to individual components of the indoor feeding system of cows, in which higher numbers of butyrate-fermenting spores are likely to be contaminants, compared to pasture grazing (Sheehan, 2013). A similar trend for butyrate was observed when profiling metabolites using gas chromatography-mass spectrometry (See Chapter 7).

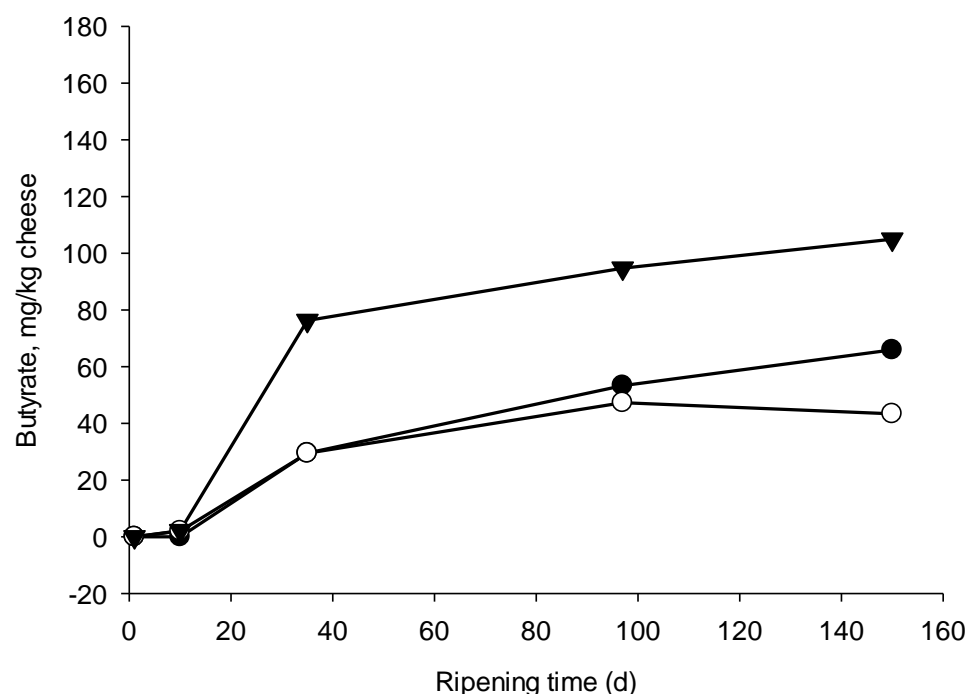


Figure 6.6 Changes in butyrate during Maasdam cheese ripening. GRA (—●—), CLO (—○—) or TMR (—▼—) represent cheeses made from milk of cows fed perennial ryegrass only, perennial ryegrass and white clover or total mixed ration, respectively. Mean values are the mean of triplicate trials.

6.4.9 Free fatty acid compositions

The mean composition of individual FFA and total FFA (mg/kg of cheese fat) in 97 d ripened Maasdam cheese samples derived from different feeding systems are presented in Table 6.6, which shows the extent of lipolysis in the experimental cheeses; the levels of FFA (C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, C18:2, C:18:3) were not statistically different, except in the case of C18:2.

Table 6.6 Mean composition of free fatty acids (mg/kg of cheese fat) in Maasdam cheese produced from standardized milk derived from pasture (GRA: perennial ryegrass or CLO: perennial ryegrass and white clover) or indoor (TMR) feeding regimes and ripened for 97 d.

Free fatty acids	Mean level of free fatty acid			SED ¹	P value
	GRA	CLO	TMR		
Saturated fatty acids					
Butyric acid (C4:0)	168 ^a	150 ^a	289 ^a	51	0.07
Caproic acid (C6:0)	113 ^a	108 ^a	174 ^a	24	0.06
Caprylic acid (C8:0)	139 ^a	124 ^a	176 ^a	24	0.16
Capric acid (C10:0)	269 ^a	232 ^a	340 ^a	42	0.10
Lauric acid (C12:0)	394 ^a	338 ^a	478 ^a	53	0.10
Myristic acid (C14:0)	1113 ^a	920 ^a	1239 ^a	191	0.31
Palmitic acid (C16:0)	3359 ^a	3012 ^a	4378 ^a	533	0.10
Stearic acid (C18:0)	1133 ^a	1002 ^a	1173 ^a	213	0.72
MUFA ²					
Oleic acid (C18:1)	2239 ^a	2018 ^a	2375 ^a	337	0.59
PUFA					
Linoleic acid (C18:2)	152 ^b	159 ^b	352 ^a	42	0.01
Linolenic acid (C18:3)	100 ^a	112 ^a	103 ^a	17	0.77
Total FFA	9178 ^a	8176 ^a	11076 ^a	1442	0.20

^{ab} Different superscript letters in a row indicates significant differences ($\alpha = 0.05$). Data are the means of triplicate trials. ¹Standard error of mean difference, ² Monounsaturated fatty acid

³ Polyunsaturated fatty acid

The effect of GRA, CLO or TMR feeding systems on fatty acid composition in milk (O’Callaghan et al., 2016) and Cheddar cheese (O’Callaghan et al., 2017) has been reported previously, whereas the present study levels of FFA in Maasdam cheese were reported for the first time. Dherbécourt et al. (2010) reported that a large proportion (80-90%) of short-chain free fatty acids originating in milk are lost into whey in Swiss-type cheese during drainage and that extensive lipolysis in cheese starts from the breakdown of those entrapped lipids (diglycerides or triglycerides) in curds. Therefore, feed-induced variations in the fatty acid profile are probably observed in terms of long-chain fatty acids. Indeed, the observation of a higher level of C18:2 in TMR cheese compared to GRA or CLO concurs with the findings of O’Callaghan et al. (2016) for FFA contents of milk.

Total free fatty acid levels in Maasdam cheeses were 9178, 8176, 11076 (mg/kg cheese fat) in GRA, CLO and TMR cheeses, respectively. The level of total fatty acids in Maasdam cheese was higher in comparison to mature Cheddar cheese, which ranged from 2547 to 3074 (mg/kg fat in cheese; McCarthy et al., 2017). *Propionibacterium freudenreichi* has been reported to markedly increase the lipolytic activity during warm room ripening in Swiss-type cheese, the action of which leads to increased FFA levels (Dherbécourt et al., 2010). Altering milk fatty acid profile through herd feeding systems for the production of poly-unsaturated fatty acid (PUFA) has been widely studied (Morales-Almaráz et al., 2010). In particular, grass feeding systems have been related to production of more conjugated linoleic acid compared to TMR feeding system (Morales-Almaráz et al., 2010; O'Callaghan et al., 2016); however, this was not investigated in the present study.

6.4.10 Sensory analysis of Maasdam cheese

The mean scores from sensory hedonic and ranking descriptive analysis (RDA) of Maasdam cheese after ~97 d of ripening are shown in Figure 6.7. The majority of sensory attributes were similar between GRA, CLO, and TMR cheese samples, except for ivory (yellow) colour, shiny appearance, smooth texture, rubbery texture, and firmness in mouth. Ivory colour was significantly higher ($P < 0.05$) in GRA or CLO cheese compared to TMR. Shiny appearance and smoothness scores were significantly higher ($P < 0.01$) in GRA compared to TMR cheese samples. The scores for CLO samples did not differ significantly between GRA or TMR cheese. The TMR cheese had significantly higher rubbery texture and firmness in mouth compared to CLO cheese, whereas GRA cheese did not differ significantly from TMR or CLO cheese.

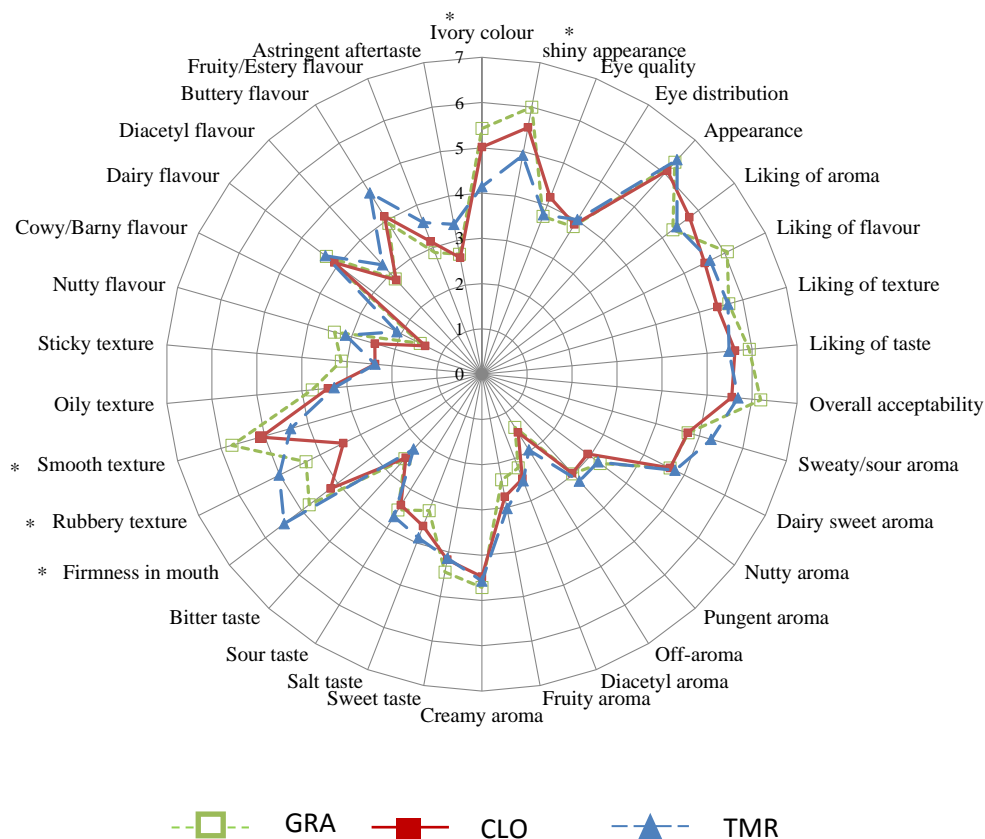


Figure 6.7 Mean sensory hedonic and ranking descriptive analysis of Maasdam cheese derived from perennial ryegrass only (GRA), perennial ryegrass and white clover (CLO) or total mixed ration (TMR) feeding regimes after 97 d of ripening. * Denotes significant difference. Colour version available online.

The overall acceptability of the cheese samples was numerically higher for GRA, compared to CLO or TMR, although a statistically significant difference was not observed. Interestingly, ivory colour and shiny appearance in GRA or CLO cheese is consistent with yellow colour measured by a colourimeter (Table 6.5).

6.5 Conclusions

Indoor or outdoor herd feeding systems had no effect on cheese yield, fat and protein recovery or gross chemical composition in Maasdam cheese. Maasdam cheese made from milk of pasture-fed cows (GRA or CLO) was more yellow compared to TMR-fed cheeses, as in the case of previous studies, which can be a basis for distinguishing

cheeses at consumer level. The levels of acetate were significantly lower in CLO and the levels of butyrate were significantly higher in TMR cheese, although this did not translate into differences in cheese sensory properties. Cheese derived from GRA milk had a softer mouth feel compared to TMR-derived cheese. The present study showed that the principal biochemical events such as glycolysis, proteolysis and lipolysis in Maasdam cheese were influenced little by the type of feed supplied to the cows. Future research on the influence of herd feeding systems on the sensory and metabolic profile of cheese produced therefrom would benefit from profiling both the milk microbiota and bacterial gene expression, particularly those associated with the teat skin, as influenced by feeding system.

6.6 References

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Chapter 7

Influence of herd diet on the metabolites of Maasdam cheeses

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7.1 Abstract

The untargeted metabolic profiles of ripened Maasdam cheese samples prepared from milk derived from three herd groups, fed: (1) indoors on total mixed ration (TMR), or outdoors on (2) grass only pasture (GRA) or (3) grass and white clover pasture (CLO) were studied using high resolution nuclear magnetic resonance (^1H NMR), high resolution magic angle spinning nuclear magnetic resonance (^1H HRMAS NMR) and headspace (HS) gas chromatography mass spectrometry (GC-MS). A total of 31 metabolites were detected by NMR and 32 by GC-MS. ^1H NMR showed that TMR cheese had higher levels of citrate compared to pasture-derived cheese. ^1H HRMAS NMR data showed less variability between cheese metabolites, when excluding lipids. The toluene content of cheese was higher in GRA or CLO compared to TMR cheeses and dimethyl sulfide was identified only in CLO-derived cheese samples as detected using HS GC-MS. Overall, this study identified specific metabolites which can discriminate between cheese samples manufactured from different herd-feeding systems.

7.2 Introduction

Maasdam cheese is manufactured with mesophilic starter cultures (*Lactococcus* and *Leuconostoc*) and thermophilic adjunct cultures (*Lactobacillus*) along with propionic acid bacteria (PAB) as a secondary culture. The latter contributes to the development of associated sensory properties (e.g., sweet and nutty flavour), along with the formation of eyes. Generally, in comparison to milk, cheeses are more complex biological systems because of the metabolism of fat, protein and lactose to organic acids, amino acids, peptides, and the subsequent formation of a large number of volatiles or non-volatiles metabolites (McSweeney, 2004).

Untargeted high-resolution proton nuclear magnetic resonance (^1H NMR) of cheese extract (liquid-state) has been used to characterize the metabolic profiles of different cheese types to establish *protected designation of origin* (PDO) based on geographical origin of manufacture, e.g., Parmigiano-Reggiano (Consonni & Cagliani, 2008) or buffalo Mozzarella cheese (Brescia et al., 2005). This approach has also been used to determine changes associated with bacterial cultures (Rodrigues et al., 2011), e.g., Fiore Sardo cheese from ovine milk (Piras et al., 2013). Moreover, ^1H -NMR has been used to differentiate cheese based on stage of ripening (Consonni & Cagliani, 2008; Piras et al., 2013; Rodrigues et al., 2011) or when brining conditions have been altered (Ruyssen et al., 2013), as indicated by changes in metabolite intensity and concentration. Similar interest has been shown for high resolution magic angle spinning (^1H HRMAS) NMR in which a solid sample (solid-state) is directly analysed without necessarily extracting metabolites from cheese samples into a liquid phase, as in ^1H NMR methods (Lamichhane et al., 2015; Mazzei et al., 2005).

Recently, interest has focused on the ability to differentiate between milk or milk products derived from cows fed on pasture or on total mixed rations (TMR) composed of silage, concentrate with added minerals and vitamins (Kilcawley et al., 2018). Demand for pasture-based dairy products has increased over those produced from controlled supplementation of silage and concentrate (Getter et al., 2015), further highlighting the importance of determining marker compounds which can differentiate products produced from different diets. The feeding regime of a herd partly contributes to the sensory properties of dairy products, depending on the concentration and odour threshold of metabolites therein (Kilcawley et al., 2018). O'Callaghan et al. (2017) reported that full-fat Cheddar cheese made from milk derived from pasture-fed cows had a higher concentration of toluene and lower concentration of 2,3-butanediol compared to cheese produced from TMR feeding systems. Profiling of Maasdam cheese using ^1H NMR or ^1H HRMAS NMR has not been reported previously, although cheese volatiles profiles using gas chromatography mass spectrometry (GC-MS) have been reported by Engels et al. (1997) and Lamichhane et al. (2018b). Identification of metabolites that could differentiate Maasdam cheeses based on herd feeding system are of particular interest.

The primary objective of this research was to apply ^1H NMR spectroscopy and GC-MS to establish the metabolic profiles of Maasdam cheeses produced from milks of cows kept indoors and fed TMR or cows kept outdoors and grazed in pastures of either grass only (GRA) or grass and white clover (CLO). A second objective of the present study was to generate a chemical fingerprint of Maasdam cheese samples using ^1H NMR and ^1H HRMAS NMR spectroscopy, to illustrate the differences in chemical fingerprints

between liquid-state and solid-state NMR. Cheese samples prepared in Chapter 6 were taken for the metabolomic analysis.

7.3 Materials and Methods

7.3.1 Sample preparation for NMR

Maasdam cheeses were sampled at 97 and 150 days of ripening, grated, and freeze dried (~30 g) for 24 hours and stored at -20°C. For analysis, samples were thawed in a cold room, and then ground using a mortar and pestle to make a fine powder.

7.3.1.1 Sample extraction for ^1H NMR

Cheese powder (50 mg) samples were weighed in 1.5 ml Eppendorf tubes in which a methanol-chloroform-water (4:3:3) extraction was carried out by the method of Folch et al. (1957), with over-night phase separation, and subsequent centrifugation ($1400 \times g$) for 30 min at 4°C. The supernatant (methanol –water-soluble phase) was transferred into a new Eppendorf tube and vacuum-dried in an Eppendorf Concentrator Plus at 30°C, stored at -80°C and analysed within a week. The soluble extract was dissolved with 700 μl Deuterium oxide (D_2O) containing 0.025% sodium trimethylsilyl[2,2,3,3- $^2\text{H}_4$]-1-propionate (TSP; Sigma-Aldrich A/S, Copenhagen, Denmark) as an internal standard for chemical shift reference. The dissolved and vortexed (~30 s) extract (700 μl) was transferred into 5-mm NMR tubes and capped. Samples were prepared in duplicate from a total of 18 cheese samples ($n = 36$).

7.3.2 ^1H NMR Spectroscopy

The instrumental conditions were as described by Sundekilde et al. (2013b), using Proton (^1H) NMR spectroscopy at 298 K (25°C) on a Bruker Avance III 600 spectrometer equipped with a TXI-probe (Bruker Biospin, Rheinstetten, Germany). Briefly, operating conditions were: ^1H frequency 600.13 MHz, 5 s relaxation delay, 0.1 s mixing time, 64 scans, 32 K data point and 12.15 ppm spectral width. Acquisition time was 2.25 s and standard 1D spectra were acquired using a NOESY sequence 90° pulse with water suppression by pre-saturation during relaxation delay and mixing time (Bruker pulse: `noesypr1d`). The Free Induction Decay (FID) data were multiplied by 0.3-Hz line-broadening function before Fourier transformation. Topspin 3.0 (Bruker BioSpin) was used for phase and baseline correction of ^1H NMR spectra. NMR signals were identified in accordance with the literature (Sundekilde et al., 2013a), the Human Metabolome Database (www.HMDB.ca) and the Chenomx NMR suite (Chenomx Inc., Alberta, Canada).

7.3.3 ^1H HRMAS NMR spectroscopy (solid state NMR)

The methods followed for ^1H HRMAS NMR analysis was as described by Lamichhane et al. (2015). Briefly, fine powder (7 ± 3 mg) of freeze-dried cheese samples was weighed ($n=18$) into 30 μL inserts (Bruker Biospin, Germany) in which 10 μL of D_2O (containing TSP for chemical shift reference) was added and centrifuged for 15 s to enable mixing of D_2O with the sample. The inserts were placed into a 4-mm Zirconium rotor (Bruker Biospin, Germany). Bruker Avance III 600 MHz was used for HRMAS in using $^1\text{H}/^{13}\text{C}/^{31}\text{P}$ MAS Probe with gradient (Bruker Biospin, Germany). Analysis was

performed at ^1H frequency 600.13 MHz, 5 KHz spin rate, 273 K, 17.36 ppm spectral width. Spectra were recorded in Carr-Purcell-Meiboom-Gill (CPMG) (Bruker pulse: cpmgpr1d) or Nuclear Overhauser Effect Spectroscopy (NOESY) pulse sequence. The CPMG pulse sequence suppresses the resonance signals from lipids whereas the NOESY pulse sequence does not suppress the lipid signals (Lamichhane et al., 2015; Shintu and Caldarelli, 2006). The number of scans was 128, relaxation delay of 3 s, T2 echo time was 400 ms, the acquisition time was 1.57 s and the collected data points were 32 K. The FID was multiplying with 0.3 Hz of line broadening prior to Fourier Transform. The phase and base-line was corrected using Topspin 3.0 software.

7.3.4 Volatile profiles by HS GC-MS

For profiling of volatile compounds in the cheese, grated samples (4 g) were weighed into screw-capped amber HS vials with a silicone/PTTE septum (Apex Scientific, Maynooth, Ireland). The temperature of vials was raised to 40°C for 10 min under the condition of pulsed agitation (5 s on and 2 s off at 500 rpm). Volatile extraction was performed using a single 50/30 μm Carboxen /divinylbenzene/polydimethylsiloxane (DVB/CAR/PDMS) solid-phase micro-extraction (SPME) fibre (Agilent Technologies Ireland Ltd, Cork, Ireland), which was exposed to the HS for 20 min at a depth of 54 mm at 40°C using a Shimadzu AOC-5000 injection system (Mason Technology, Dublin Ireland). The fibre was injected to a Shimadzu 2010 plus GC (Mason Technology) *via* a merlin microseal (Sigma-Aldrich A/S) at 250°C for 2 min in split-less mode. The column was an Agilent DB-5ms column (Agilent Technologies Ireland Ltd) and the oven temperature was set at 30°C for 30 min, increased to 230°C ($6.5^\circ\text{C min}^{-1}$), then increased to 320°C ($15^\circ\text{C min}^{-1}$) over 41.5 min, with helium as the carrier gas at a uniform pressure

of 1.58 bar. The detector was a Shimadzu TQ8030 MSD triple quadrupole mass spectrometer (Mason Technology) in single quadrupole mode. The temperature of ion source and interface were set at 220°C and 280°C, respectively. Electronic ionization (70 v) with the mass range between 35 and 250 amu were selected for MS mode. Total ion chromatograms were processed using TargetView (Markes International Ltd, Llantrisant, UK), and identification was carried out using mass spectra comparisons to the NIST 2014 mass spectral library, the Shimadzu flavour and fragrance library (FFNSC 2, Shimadzu Corporation, Japan) and an in-house library created in GC-MS Solutions software (Shimadzu, Japan) with target and qualifier ions. Kovat retention indices were also used aid identification as described by van Den Dool and Dec. Kratz (1963) and matched against peer-reviewed publications where possible to confirm compound identification. An auto-tune of the GC-MS was carried out prior to the analysis to ensure optimal GC-MS performance. A set of external standards was run at the start and end of the sample set and abundances were compared to known amounts to ensure that both the SPME extraction and MS detection were performing within specification.

7.3.5 Multivariate data analysis

Proton NMR spectra were aligned based on TSP using icoshift (Savorani, Tomasi, & Engelsen, 2010). In addition, resonance signals from water, urea and filtering procedures were removed using Matlab 7.13 (Mathworks, Inc., Natick, Ma, USA) generating variables ($n = 928$) for multivariate analysis. Firstly, principal component analysis (PCA) was applied to the unit variance-scaled data for identifying clustering based on feeding system of cow on 36 samples of Maasdam cheese. To discriminate cheese samples, an orthogonal partial least square discriminant analysis (OPLS-DA)

approach was exploited, which removes the variables that are systematically uncorrelated to the model, improving the model predictability (Bylesjö et al., 2006; Sundekilde et al., 2013b). Cross-validation of the OPLS-DA model was performed by dividing the samples into 4 groups and randomly leaving 1 group out of the model. Sample duplicates were left out together.

7.3.6 Statistical analysis

Integrals were calculated for carnitine, choline, succinate, 2,3-butanediol and citrate. Mean relative concentration of these metabolites with respect to GRA samples at 97 days were determined. One-way ANOVA was performed on the relative concentration of metabolites using IBM SPSS statistics (version 24, IBM Corp) to determine the effect of GRA, CLO or TMR feeding system at 5% significance level. Volatile compounds were compared using the Kruskal-Wallis test for independent samples using SPSS. Principal component analysis (PCA) of volatile compounds was carried out using Minitab 17 (Minitab Inc.).

7.4 Results and Discussion

7.4.1 ^1H NMR spectroscopic analysis of Maasdam cheese samples

The representative ^1H NMR spectra (δ 0.5 to 4.5, δ 6.7 to 8.5) for Maasdam cheese prepared from milk from cows fed on (A) GRA (B) CLO or (C) TMR is shown in Figure 7.1. The assigned metabolites are summarized in Table 7.1 with code numbers. The resonance signals from organic acids (lactate, propionate and acetate), and amino acids dominated the ^1H NMR spectra in the Maasdam cheese. The spectra also showed

resonance signals from other low molecular weight metabolites present in cheese samples; however, these could not be assigned. The resonance signals from lactose were absent in the spectra, as lactose was converted to lactic acid during ripening. Between the chemical shift from 0.5 to 4.5 ppm of the ^1H NMR spectrum, the signals from aliphatic group of organic acids, amino acids and alcohol were predominant, whereas resonances from aromatic group of amino acids were predominant between 6.9 to 8.5 ppm.

The Chenomx NMR suite databases were used for the comparison of the chemical shift values and the values were largely in accordance with those published previously (Sundekilde et al., 2013a). By comparing the spectra, it was shown that metabolic profiles of the ripened cheese samples from GRA, CLO or TMR feeding systems were not largely different, apart from the differences in intensities of some metabolites.

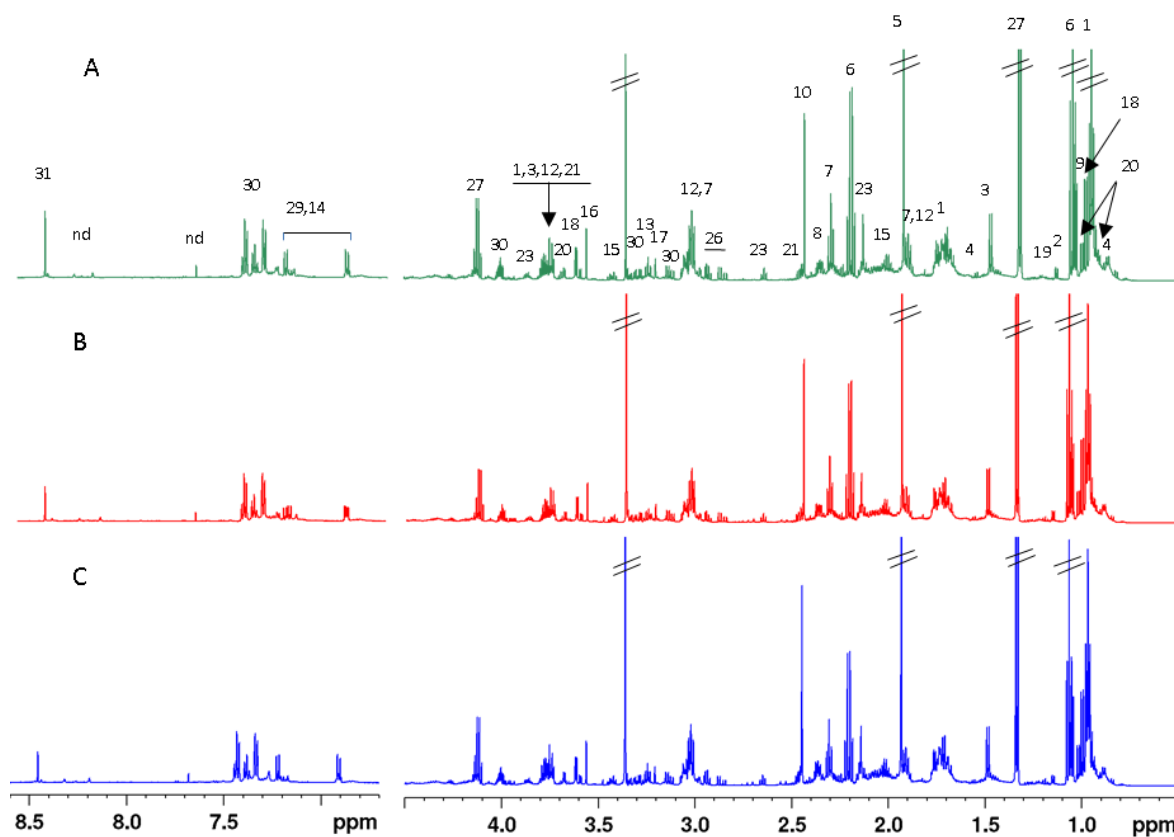


Figure 7.1 Representative ^1H NMR spectra of Maasdam cheese samples after 97 days of ripening. Spectrum A, B and C represents cheese made from cow milk fed on grass only pasture (GRA), fed on grass and clover (CLO) pasture and fed total mixed ration (TMR), respectively. The chemical shift between 0.5 and 4.5 ppm represents the aliphatic region. The aromatic region (6.7 and 8.8 ppm) is vertically expanded (2x). The name of assigned metabolites is shown in Table 7.1; numbers are nominal and nd refers to not identified.

Table 7.1 ^1H NMR chemical shift assignment for Maasdam cheese

SN	Code	Chemical shift (multiplicity)			
1	Leucine	0.95 (d)	1.7 (m)	0.96(d)	3.72(m)
2	2,3-Butanediol	1.14 (d)			
3	Alanine	1.47 (d)	3.76(m)		
4	Butyrate	1.54 (m)		0.87(t)	
5	Acetate	1.91(s)			
6	Propionate	2.19(m)	1.04(t)		
7	4-Aminobutyrate	2.30(m)	2.28(t)	1.88(m)	
8	Glutamate	2.33(m)			
9	Isobutyrate	1.03(d)			
10	Succinate	2.43 (s)			
11	Citrate	2.70 (d)	2.53(d)		
12	Lysine	3.74 (t)	1.72(m)	3.00 (t)	
13	Carnitine	3.22 (s)			
14	Tyramine	7.22 (d)	6.91(d)	2.95 (t)	
15	Proline	3.41 (m)	2.00(m)		
16	Glycine	3.54 (s)			
17	Choline	3.51(m)	3.19(s)		
18	Valine	3.60 (d)	2.26(m)	1.02(d)	0.98(d)
19	Ethanol	1.18(t)			
20	Isoleucine	3.66(d)	1.27(m)	1.00(d)	0.92(t)
21	Glutamine	3.77 (t)	2.46(m)		
22	Ornithine	3.05(t)			
23	Methionine	3.85(m)	2.64(t)		2.12 (s)
24	Arginine	3.24 (t)			
25	Betaine	3.89 (s)	3.25 (s)		
26	Asparagine	2.95(m)	2.84(m)	4.0(m)	
27	Lactic acid	4.11 (m)	1.31 (d)		
28	Tryptophan	7.73 (d)	7.53 (d)		
29	Tyrosine	7.19 (m)	6.89 (m)		
30	Phenylalanine	7.40 (m)	3.98 (t)	3.27(m)	3.10(m)
31	Formate	8.44 (s)			

S = singlet, d = duplet, t=triplet, m=multiplate

7.4.2 ^1H HR MAS NMR spectroscopic analysis of Maasdam cheese samples

Figure 7.2 shows the representative ^1H HRMAS NMR (CPMG pulse sequence) spectra of GRA, CLO and TMR cheese samples between chemical shifts 0 to 8.5 ppm. As expected, spectra with CPMG pulse sequence suppressed the signals from fatty acids and resonance signals from organic acids and amino acids dominated. The assigned metabolites are presented in Table 7.1. Spectra with NOESY pulse sequence of same sample were dominated by fatty acids, which superimposed the

signals of organic acids and amino acids (Figure 7.3). Cheese samples produced from GRA, CLO and TMR feeding systems appeared to have a similar resonance peak (in both CPMG or NOESY pulse sequence), indicating similar metabolic profile of experimental cheese samples despite the variation in feeding systems. The results of ^1H HRMAS NMR are consistent with the results of ^1H NMR in that feeding system did not show large effects on the spectral resonance peak of experimental Maasdam cheese samples. Therefore, it was necessary to apply multivariate data analysis on both ^1H NMR and HRMAS NMR spectra for differentiation of cheese samples.

7.4.3 Comparison of different feeding systems (^1H NMR spectra)

The ^1H NMR metabolomic data from 36 cheese samples (18 from 97 days and 18 from 150 days of ripening) was first treated with an unsupervised PCA (PCA without transformation of data) to investigate the clustering of the cheese samples based on the feeding systems of the cows. However, PCA did not show any clustering of the cheese samples, indicating that the source of variation in the spectrum was largely unexplained by the feeding system (plots not shown). The OPLS-DA score plots showed the separation of cheese samples based on feeding regimes of cows (Figure 7.4 A, B, C); however, variance within the samples was low, as indicated by smaller values of predictive components and first orthogonal components. It is worth noting that the coefficients of determination and Q^2 values of the models were satisfactory for predictive reliability of the OPLS-DA model.

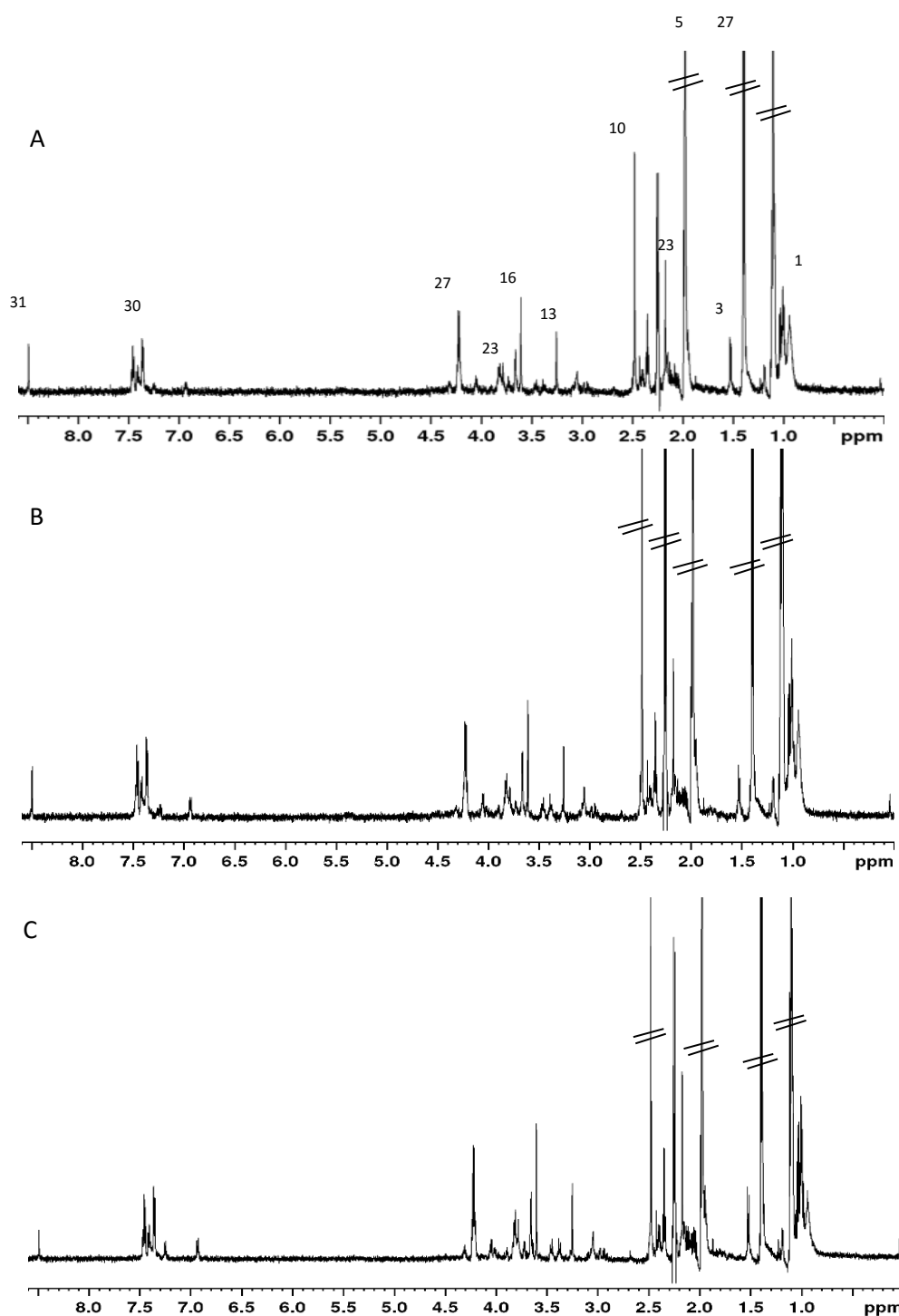


Figure 7.2 Representative ^1H RMAS NMR (CPMG pulse sequence) spectra of Maasdam cheese samples after 97 days of ripening. Spectrum A, B and C represents cheese made from cow milk fed on grass only pasture (GRA), fed on grass and clover (CLO) pasture and fed total mixed ration (TMR), respectively. The chemical shift between 0.5 and 4.5 ppm represents the aliphatic region. The names of assigned metabolites are shown in Table 7.1.

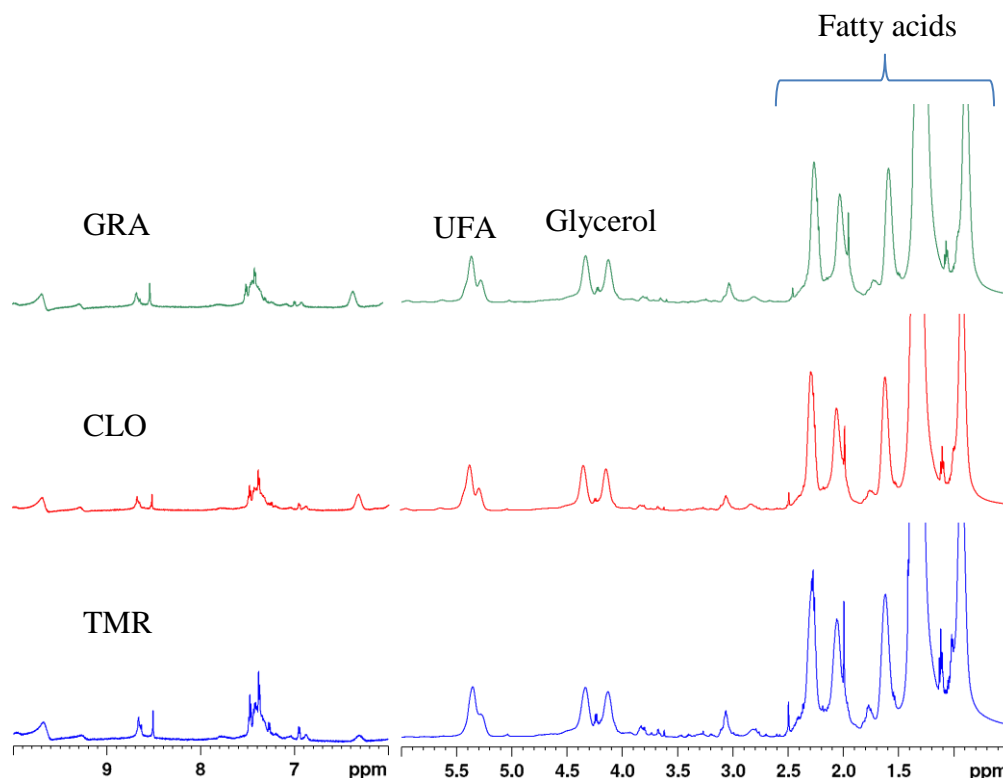


Figure 7.3 Representative ^1H MAS NMR (NOESY pulse sequence) spectra of Maasdam cheese samples after 97 days of ripening. Spectrum GRA, CLO or TMR represents cheese made from milk fed grass only, grass with clover or total mixed ration, respectively. Chemical shift from 6 to 10 ppm is vertically enlarged 8x times. Assignments were done according to Shintu and Caldarelli (2006). UFA refers to resonance peak from unsaturated fatty acids, and glycerol and acyl chain from saturated fatty acids.

For better interpretability, OPLS-DA coefficients were back-transformed from the unit variance scale by multiplying mean-centered data with the standard deviation of respective compounds and colour-coded based on OPLS-DA loading weight (Cloarec et al., 2005). Primarily, it was found that the resonance of organic acids had high correlation (lactate, acetate, and propionate), which minimized the impact of metabolites that are present in lower concentration (plots not shown). Therefore, the resonance signals of organic acids (e.g., propionate, lactate, acetate) were removed from the spectra and a reduced model for OPLS-DA coefficient plots was generated.

The corresponding coefficients plot of the OPLS-DA model shows the metabolites that are responsible for discrimination between cheese samples (Figure 7.4A, B, C).

For example, Maasdam cheese manufactured from grass-fed milk (GRA) correlated with 4-aminobutyrate (No. 7, Table 7.1; code number is shown with subsequent metabolites), phenylalanine (30), asparagine (26), formate (31), isoleucine (20) and 2, 3-butanediol (2), compared to those cheeses from TMR-fed milk. Interestingly, cheeses produced from TMR-fed milk had a greater proportion of amino acids, e.g., leucine (1), alanine (3), valine (18), proline (15), methionine (23) and tyrosine (29), and also citrate (11) and carnitine (13), than cheeses made from GRA-fed milk (Figure 7.4 A).

Similarly, cheese from CLO-fed milk had more lysine (12), ornithine (22), tyrosine (29), glycine (16), methionine (23) whereas, cheese from milk derived from TMR-feeding systems contained more butyrate (4), glutamine (21), glutamate (8) alanine (3) and carnitine (13) (Figure 5 B). On comparing feeding systems between GRA or CLO, it was shown that cheese derived from milk produced from cows fed GRA had a higher level of 2,3-butanediol (2), alanine (3), butyrate (4), and carnitine (13) (Figure 7.4 C).

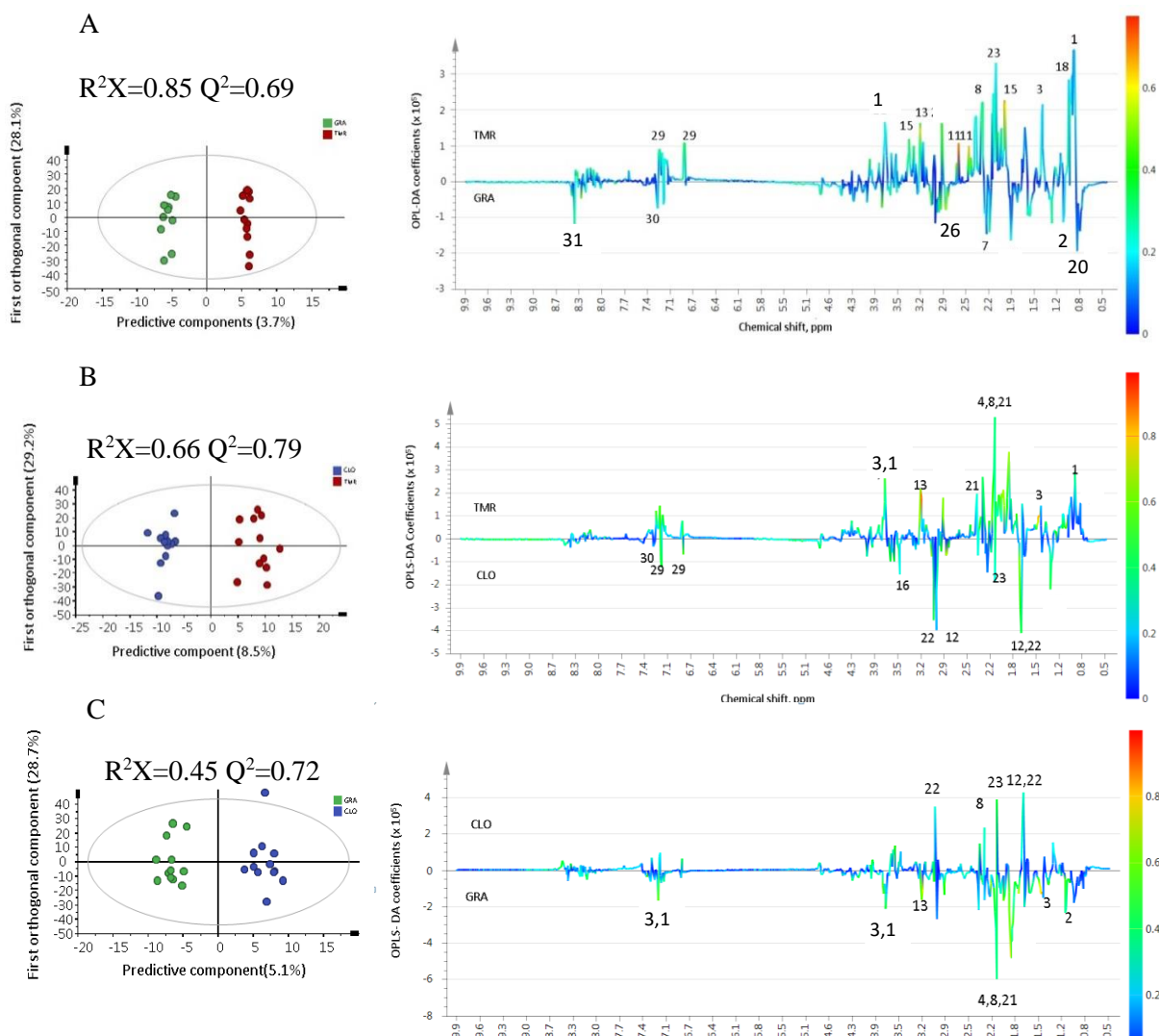


Figure 7.4 Orthogonal projection to latent squares discriminant analysis (OPLS-DA) scores plots and corresponding back-transformed coefficients plots of a cross-validated model of the unit variance scaled data; of cheese prepared from cow milk fed on (A) grass only in pasture (GRA) versus total mixed ration (TMR), (B) grass and clover in pasture (CLO) versus TMR, and (C) GRA versus CLO. The resonances from lactate, propionate, and acetate were excluded. Each variable has been coloured according to the OPLS-DA loadings (correlation between NMR variable and feeding systems). The list of metabolites with code number is shown in Table 7.1.

7.4.3.1 Relative quantification of ¹H NMR signals

The integrals of resonance signals of selected metabolites of ¹H NMR were generated. The concentration of metabolites were set to 1 in GRA Maasdam samples ripened for 97 days and relative concentrations of metabolites in CLO and TMR cheeses were scaled accordingly. Figure 7.5 shows the relative abundance of selected

metabolites. It is worth noting that different metabolites cannot be compared because of data transformation.

Different herd feeding systems did not significantly influence carnitine content in Maasdam cheese, but cheese from TMR appeared to contain more carnitine levels compared to CLO cheeses. Choline contents in the experimental cheese were also found to be similar. No significant differences in 2,3-butanediol content was observed between the experimental cheese samples, although a slightly higher level was observed in GRA cheese samples compared to CLO or TMR. In contrast, O'Callaghan et al. (2017) reported higher levels of 2,3-butanediol in Cheddar cheese derived from a TMR feeding system than from pasture grazing. The discrepancy could be due to citrate metabolism by starter or non-starter bacteria present in cheese milk that favours the metabolism of citric acid partly into 2,3-butanediol. Mesophilic cultures, e.g., *Lactococcus* spp and *Leuconostoc* spp. added into milk in the present study are known to catalyze citrate (McSweeney, 2004). Interestingly, the citric acid level was significantly higher in TMR compared to GRA cheeses after 97 days of ripening. However, significant differences in citric acid levels were not observed when cheese were ripened for 150 days, but the trend TMR>CLO>GRA remained. O'Callaghan et al. (2018) observed a greater level of citric acid in TMR-fed milk than in GRA- or CLO-fed milk. Results of the present study indicate that a higher citric acid level in TMR cheese could be due to a higher proportion of citrate in milk, possibly as a result of herd diet. The diet of the cow's did not influence succinate content in the experimental Maasdam cheese samples.

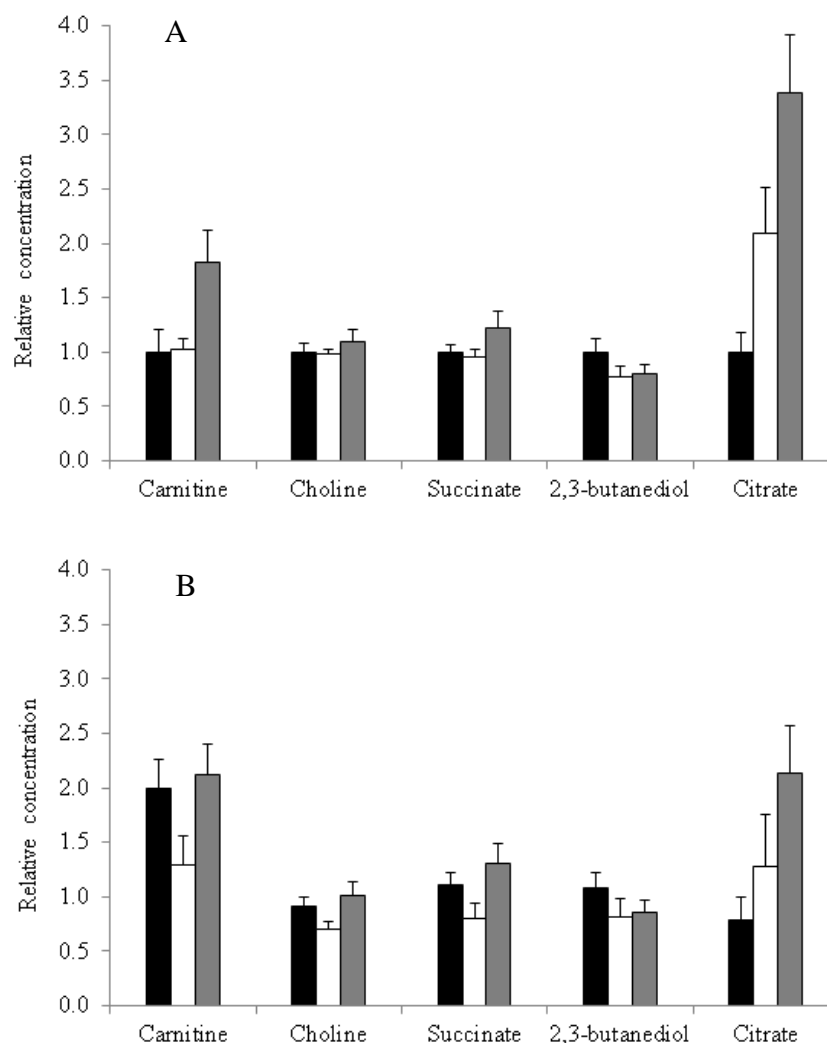


Figure 7.5 Relative quantification of metabolites from integration of nuclear magnetic resonance (NMR) signals (A) ripened for 97 days, (B) ripened for 150 days of Maasdam cheese samples prepared from milk of cows fed grass (■), grass with clover (□) or TMR (■). Relative concentrations of mean \pm standard error to GRA ripened for 97 days are shown.

7.4.4 ^1H HRMAS NMR spectral analysis of Maasdam cheese

The unsupervised PCA of HRMAS NMR resonance signal from 18 cheeses (9 from 97 days and 9 from 150 days) revealed a tendency towards grouping in spectra from CPMG pulse sequence and clear grouping in NOESY pulse sequence between outdoor (GRA and CLO) and indoor (TMR) feeding systems (Figure 7.6A, B). The PCA plot also showed that ^1H HRMAS data with CPMG pulse sequence was less variable (PC2, 12.9%; PC3 10.6%), this was in agreement with the result of ^1H NMR in which metabolites were extracted. However, the variability of ^1H HRMAS data with

NOESY pulse sequence was PC1 78.0%, PC2, 8.47%. The spectral signal from CPMG pulse sequence largely suppressed signals from lipids, which led the appearance of resonance signals from amino acids and organic acids (Figure 7.2) whereas, in spectra with NOESY pulse sequence, lipid signals masked the signals of small molecular weight metabolites (Figure 7.3). This indicates that Maasdam cheeses prepared from milk of herds kept indoors or outdoors on separate feeding systems largely differed due to lipid profile. Maasdam cheese prepared from milk derived from cows fed GRA or CLO could not be separated, indicating that metabolites were largely similar in the final cheeses. O'Callaghan et al. (2017) also reported less variability between GRA or CLO feeding systems in Cheddar cheese. The CPMG or NOESY ^1H HRMAS spectra obtained were largely similar to previously reported spectra for ripened cheese (Lamichhane et al., 2015; Shintu & Caldarelli, 2006).

The advantage of NOESY HRMAS data over ^1H NMR in the present study is that it can highlight possible differences caused by lipids, which indeed appeared between cheese samples prepared from milk of outdoor or indoor feeding systems. The lipid content in cheese has been reported to be highly influenced by the feeding system of cows (O'Callaghan et al., 2017). O'Callaghan et al. (2017) reported that cheese prepared using milk from a TMR feeding system had higher levels of saturated fatty acids compared to cheeses made using milk from a GRA or CLO feeding system. Our results suggest that most discrimination based on different feeding systems for Maasdam cheeses is related to lipids or metabolites soluble in lipids, since most of the unbound water-soluble metabolites may also be removed along with whey during cheesemaking, as indicated by low variation of ^1H NMR spectra during multivariate data analysis. For detecting lower molecular weight compounds that are masked by

lipids and amino acids, a specific sample extraction (e.g., exclusion of lipids) may be required.

7.4.5 Volatile compounds in cheese

The composition of herd feeds supplied and their volatiles profiles have been reported elsewhere (Faulkner et al., 2018; O’Callaghan et al., 2016). The profile of volatile compounds in GRA, CLO or TMR Maasdam cheeses ripenend after 97 days are shown in Table 7.2. Headspace GC-MS analysis of experimental cheese samples detected a total of 32 compounds, including 7 acids, 5 esters, 4 alcohols, 4 ketones, 4 sulphur compounds, 2 aldehydes, 3 hydrocarbons, 2 terpenes and a lactone. The contents of the majority of volatile compounds were similar between experimental cheeses, except for the levels of toluene and dimethyl sulfide.

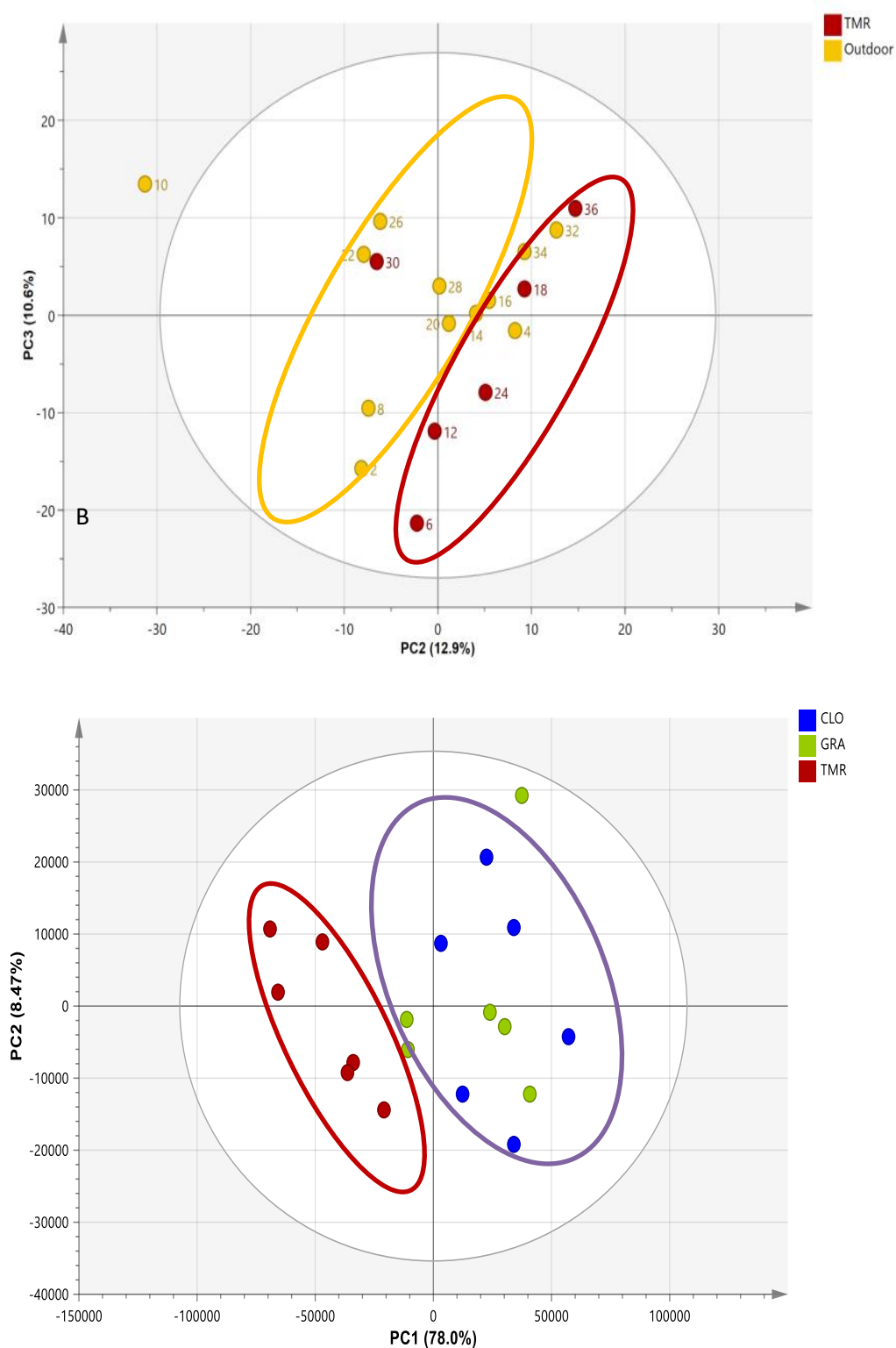


Figure 7.6 Principal component analysis of ^1H HRMAS NMR spectra with different pulse sequence, (A) CPMG or (B) NOESY, for spectra of ripened cheeses made from TMR-fed cow milk and combined data generated from outdoor feeding of grass only (GRA) and grass with clover (CLO). Circles indicate the grouping of metabolites. Numbers in A are sample label.

Principal component analysis was carried out on the volatile profiles with respect to GRA, CLO and TMR cheeses and PCA showed clustering of the samples between feeding systems, particularly between indoor or outdoor feeding systems (Figure 7.7); however, a trial effect was also apparent. The first, second and third principal components (PC) explained the variance of 27.4%, 21%, and 12%, respectively. Compounds that were positively correlated with PC1, PC2 and PC3 were not significantly different between cheese samples, indicating cheese samples were largely similar in terms of volatile compounds.

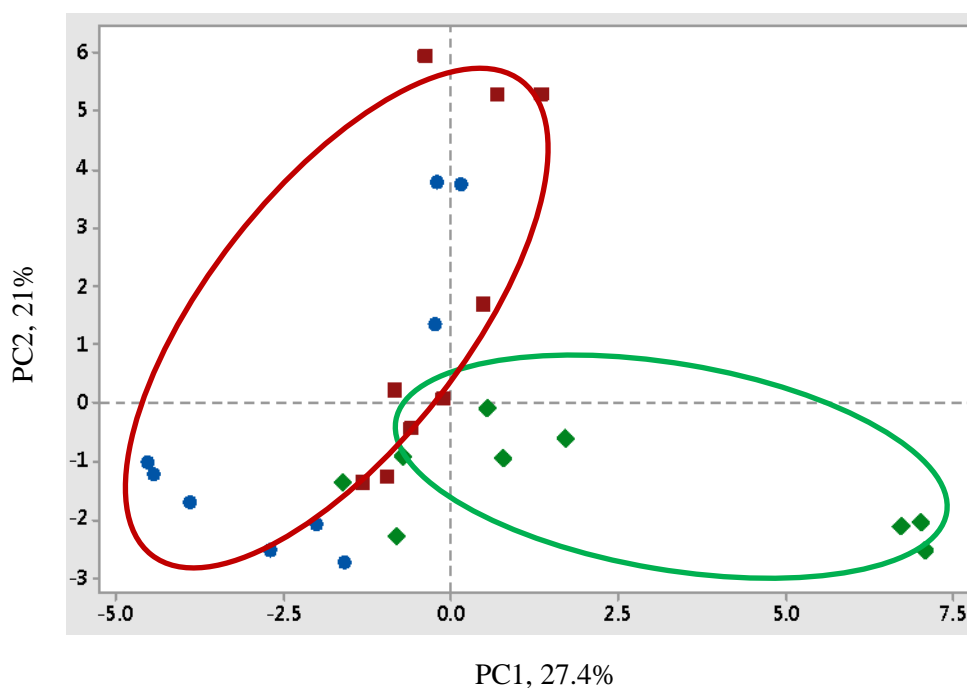


Figure 7.7 Principal component (PC) analysis of volatile compounds of Maasdam cheeses made from TMR (◆) fed cow milk and combined data generated from outdoor feeding of grass only (GRA; ■) and grass with clover (CLO; ●). Circles indicate the grouping of metabolites.

The profile of volatile compounds has a significant role on the flavour properties of cheeses and these are primarily produced as a consequence of the breakdown of citric acid, lactic acid, amino acids and fatty acids during cheese ripening, due to the action of enzymes associated with microorganisms or coagulant

(McSweeney, 2004). Maasdam cheeses are effectively intermediate between Gouda and Emmental, in which mesophilic cultures (*Lactococcus* and *Leuconostoc*) are added along with thermophilic cultures and PAB (Lammichhane et al., 2018) and their combined action results in the formation of a wide range of volatile compounds.

It is generally known that PAB primarily converts lactate to volatile compounds such as propionate and acetate, along with H₂O and CO₂. Propionic acid bacteria are known for accelerating lipolysis and catabolism of amino acids such as isoleucine during ripening (Thierry et al., 2005) and are the major contributors to flavour formation in Swiss-type cheeses (Kilcawley, 2017). A high abundance of acetic acid and propionic acids were detected in GRA, CLO or TMR cheeses. Butanoic acids (butyric acid) were also detected, which could possibly originate from the butyrate-fermenting *Clostridium* spores (Lammichhane et al., 2018b) or lipolysis by enzymes. Branched short-chain fatty acids such as 2-methylbutanoic acids are believed to originate from the breakdown of branched amino acids such as leucine and isoleucine (Urbach, 1997) mainly by PAB (Thierry et al., 2005). The presence of free fatty acids (hexanoic and octanoic acids) in experimental cheeses was attributed to the lipolytic activity of cheese microbiota (Collins et al., 2003).

Table 7.2 Effect of feeding systems¹ on volatile compounds in Maasdam cheese at 97 day of ripening.

Volatile compounds	Std	RI	Experimental cheese groups (Peak area)			
			GRA ¹	CLO ¹	TMR ¹	P-values
Acid						
Acetic acid	62	680	43,470,237	38,656,0	39,608,1	0.39
Propionic acid	72	720	41,160,997	31,657,3	35,199,2	0.58
Butanoic acid	78	809	13,918,967	8,756,36	15,855,9	0.06
3-Methylbutanoic	83	847	1,737,262	1,585,90	2,928,27	0.43
2-Methylbutanoic	83	853	2,232,382	2,139,82	3,918,51	0.19
Hexanoic acid	10	980	7,997,236	3,208,14	10,394,3	0.56
Octanoic acid	11	116	1,356,320	1,227,75	2,583,58	0.17
Alcohol						
1-Propanol	54	537	453,881	113,262	34,213	0.09
3-Methyl-1-butanol	73	740	607,258	451,976	494,280	0.83
2-Methyl-1-butanol	74	747	627,826	1,920,39	862,503	0.67
2,3-Butanediol	80	814	41,26,767	3,496,58	4,230,62	0.83
Aldehyde						
Benzaldehyde	99	971	2,913,084	1,737,66	2,115,76	0.87
Benzeneacetaldehyd	10	105	27,358	23,138	28,009	0.95
Ester						
Ethyl propionate	71	712	1,165,174	885,988	1,125,32	0.39
Ethyl isobutyrate	76	770	75,807	36,567	54,637	0.93
Ethyl butanoate	79	802	1,452,664	1,743,93	793,774	0.73
Ethyl hexanoate	10	998	160,181	153,632	241,967	0.19
Ethyl octanoate	11	119	74,716	66,830	131,898	0.17
Hydrocarbons						
Toluene	72	759	944,012	1,678,52	152,455	0.04
o-Xylene	87	875	57,739	51,971	47,247	0.87
1-Octene	78	799	337,143	656,130	171,012	0.48
Ketones						
2-Butanone	59	576	60,920,446	42,737,4	34,960,4	0.25
Acetoin	73	744	10,765,736	10,300,2	7,916,37	0.73
2-Heptanone	89	892	1,900,271	1,745,74	2,148,87	0.43
2-Nonanone	10	109	282,386	253,430	285,502	0.83
Lactone						
δ-Decalactone	15	150	15,298	25,005	35,206	0.17
Sulfer						
Dimethyl Sulfide	49	515	0	54,014	0	0.02
Carbon disulfide	53	530	145,628	131,109	109,414	0.06
Dimethyl disulfide	75	756	128,710	67,400	91,569	0.25
Dimethyl trisulfide	98	980	35,545	13,997	17,693	0.39

Continued

Continued Table 7.2 Effect of feeding systems¹ on volatile compounds in Maasdam cheese at 97 day of ripening.

Terpenes						
D-Limonene	10	103	29,575	32,399	29,604	0.73
α -Pinene	94	941	8,362	9,875	6,402	0.66

Volatile compounds with $P < 0.05$ significantly differ between each treatment. Data presented are the means of 3 replicate trials. ¹GRA = Grass only, CLO = Grass with clover, TMR = total mixed ration

Moreover, some compounds either present in the feed of cows, or produced in the rumen during digestion, may pass into milk and subsequently accumulate in cheese, forming a basis for differentiation according to feeding systems (Martin et al., 2007). β -carotene in feed decomposes to toluene in the rumen and is transferred to milk (Coppa et al., 2011; Faulkner et al., 2018). In the present study, GRA or CLO cheeses had a higher concentration of toluene compared to TMR cheeses, and a higher level of toluene was observed in CLO than in GRA cheeses, in agreement with the findings of O'Callaghan et al. (2017). Since fresh forage is likely to have higher level of β -carotene compared to mixed rations, cheeses prepared from pasture-fed milk may result with higher toluene levels (Kilcawley, et al., 2018). However, the impact of toluene on sensory perception is minimal because of a high odor threshold (Faulkner et al., 2018). The presence of *p*-xylene and 1-octene were also noted in the experimental cheeses. Xylene in milk has been reported to originate from feed samples (Faulkner et al., 2018); its level in cheese depends on the feed botanical diversity (Buchin et al., 1999).

Alcohols such as 1-propanol, 3-methyl 1-butanol, 2-methyl-1-butanol, 2,3-butanediol were detected but showed virtually no effect of feeding system on alcohol content between cheese samples. Thierry et al. (2005) reported that alcohols could be produced through a heterofermentative pathway by lactic acid bacteria. However, O'Callaghan et al. (2017) observed higher levels of 2,3-butanediol in TMR cheeses

compared to GRA or CLO Cheddar cheese, although such differences were not observed in the present study using both ^1H NMR and GC-MS techniques. The level of 2,3-butanediol could have been influenced in cheese through citrate metabolism by the mesophilic starter (*Lactococcus* and *Leuconostoc*) culture (McSweeney, 2004). The presence of alcohols in cheeses is vital for the formation of esters of free fatty acids (Kilcawley, 2017).

Esters of propionate are unique compounds present in Swiss-type cheeses and impart a sweet and fruity odor to the cheese (Liu et al., 2004). Coppa et al. (2011) reported higher levels of esters in pasture-derived milk compared to hay-derived milk. Esterases released from lactic acid bacteria or PAB probably lead to the formation of ethyl groups, along with free fatty acids, during ripening, influencing the ester content of cheese (Liu et al., 2004). The presence of primary alcohols and carboxylic acids in cheese has been reported to be critical factor for esterification rather than concentration of enzymes (Kilcawley et al., 2018). Ethyl isobutyrate, ethyl butanoate, ethyl hexanoate and ethyl octanoate were also detected in the experimental Maasdam cheeses, indicating the esterification of ethanol in the presence of lactic acid bacteria/ or PAB.

Four volatile sulfur compounds, namely carbon disulfide, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide were detected in the cheese samples. However, dimethyl sulfide was only detected in CLO cheeses. In contrast, O'Callaghan et al. (2017) reported similar levels of dimethyl sulfide levels in Cheddar cheese prepared from indoor or outdoor feeding systems. Dimethyl disulfide and dimethyl trisulfide are known to influence cheese flavour (Landaud et al., 2008); however, levels (e.g., dimethyl trisulfide) are probably influenced by the presence of

PAB (Thierry et al., 2005). Although dimethyl sulfone was previously highly correlated with pasture-based products (Kilcawley et al., 2018), it was not detected in this study, presumably due to metabolic activity of cheese microbiota.

Ketones, particularly methyl ketones, are commonly found in cheese and are important for aroma (Urbach, 1997). Acetoin (3-hydroxy 2-butanone), 2-butanone, 2-heptanone, and 2-nonanone were identified in the experimental cheeses. The levels of acetoin was higher in GRA or CLO compared to TMR cheese samples (albeit not significantly different). Acetoin is generally detected in high abundance in Maasdam cheeses mainly due to citrate metabolism by *Lactococcus Lactis* and *Leuconostoc spp.* (Engels et al., 1997; Lamichhane et al., 2018b) or due to conversion of aspartate by *L. lactis* (Le Bars & Yvon, 2008). The influence of PAB on acetoin production is unclear (Thierry et al., 2005). This indicates that acetoin most probably resulted from the metabolism of citrate by the added mesophilic lactic culture (McSweeney, 2004); and could possibly be reduced to butanone (Engels et al., 1997).

Aldehydes are highly abundant volatile compounds present in different cheese varieties, including Maasdam cheeses (Engels et al., 1997). Aldehydes are produced from α -keto acids, which are derived from transaminase reactions of amino acids (McSweeney, 2004). These are reported as an intermediate product, which eventually lead to the formation of alcohol or carboxylic acids (Singh et al., 2003). Only two aldehyde compounds, benzaldehyde and benzenecetaldehyde, were identified. Carpino et al. (2004) observed the presence of benzenecetaldehyde in pasture-derived cheeses and Aprea et al. (2016) showed higher levels of benzaldehyde in cheese with lower feed quality. Faulkner et al. (2018) reported that the level of benzaldehyde or benzenecetaldehyde could be influenced by milk pasteurization.

The levels of terpenes in milk and milk products are believed to be influenced by the feed supplied to cows and offers an opportunity to discriminate based on botanical composition of the feed. However, D-limonene and α -pinene were identified in the experimental cheese samples and were not influenced by feeding systems, in agreement with the findings of O'Callaghan et al. (2017), who reported a possible metabolism of terpene compounds by cheese microbiota.

In summary, volatile analysis of Maasdam cheese suggests that the influence of cheese bacterial (starter culture and secondary culture) metabolic pathways may be greater than the influences of differences in the constituents of the milks used for cheese manufacture, except for toluene and dimethyl sulphide.

7.5 Conclusions

An untargeted metabolomics approach was utilized to investigate metabolites (volatiles and non-volatiles) present in Maasdam cheese using one-dimensional ^1H NMR, ^1H HRMAS NMR, and HS-GC-MS. OPLS-DA analysis of ^1H NMR data revealed that Maasdam cheese prepared from TMR feeding systems correlated most with citrate and carnitine, whereas cheeses from GRA and CLO were correlated with 4 amino-butyrate, 2,3-butanediol, formate and asparagine. Resonance signals from solid-state NMR with NOESY pulse sequence appeared to dominate spectra by lipid signals, as compared to the liquid-state or solid-state when using CPMG pulse sequence. NOESY pulse sequence showed clear separation of Maasdam cheese in PCA plot between indoor TMR feeding system or outdoor grass/grass and clover feeding system, emphasizing that the difference was mainly due to lipid contents. However, cheese prepared from GRA- or CLO-fed milk was not largely different in terms of metabolic profile. Levels of toluene were higher in Maasdam cheeses

prepared from GRA- or CLO-fed milk compared to those prepared from TMR-fed milk. Overall, feed-induced variation in metabolite content of Maasdam cheese was low, suggesting that metabolites produced by starter cultures and secondary PAB cultures have a much greater role and can mask the majority of metabolites derived from milk/feed.

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Chapter 8

General discussion

8.1 General summary

Ireland has typically a bell-shaped milk production profile, with rapidly increasing milk volumes from February until May and decline slowly thereafter. At peak season, Ireland produces nearly 6 times more milk than in the lean (trough) season (Central Statistics Office, 2018), resulting in wide variation in the quantity of milk supply as well as in composition. Adequate processing capacity in peak times and redundant capacity in off-peak times is a primary issue for Irish dairy processors in coping with seasonal milk production. Similarly, inconsistencies in processing properties of milk resulting from seasonal milk production are also a key issue for the industry.

While average EU milk production figures have remained stable in recent years, a significant increase in milk production (from 5.51 billion litres in 2014 to 7.27 billion litres in 2017) has occurred in Ireland (Central Statistics Office, 2018) since the abolition of EU milk quotas in 2015. It has been projected that the initial target increase of 2.75 billion litres of milk production by the end of 2020 (Sheehan, 2013) will be greatly surpassed. As a result, Irish dairy producers may be processing further increased milk pools in coming years.

The Irish industry is exploring opportunities to deal with increased milk supply and changing markets by (1) diversifying the types of cheese made, including continental-type cheese and different cheese types capable of being manufactured on existing Cheddar plants, and (2) increasing plant efficiency. Studies on the development of new cheese types for diversification of the cheese industry have already been undertaken, particularly focusing on semi-hard cheeses such as Maasdam (Sheehan, 2013) and also cheeses with

tailored sensory characteristics, which would be feasible to manufacture in existing cheese plants without extensive capital investment (Sheehan and Wilkinson, 2001; Sheehan et al., 2008; Sheehan, 2013). The use of ultrafiltration systems for protein standardization of milk by the Irish cheese industry to mitigate the issues of seasonal milk production has also been advocated previously (Guinee et al., 1994; Guinee et al., 2006). This also facilitates increasing processing capacity using existing resources (Ong et al., 2013a; Kevany and Guinee, 2018).

In the context of utilizing the increased milk pool, diversifying cheese types, and enhancing cheese quality and consistency, this thesis has generated detailed knowledge in the area of selection of milk for cheesemaking, cheesemaking processes (rennet coagulation and curd syneretic properties), and on final cheese composition and quality.

It was clearly shown that protein standardization using ultrafiltration minimized the compositional variation in major milk components derived from GRA, CLO and TMR feeding systems (Chapter 3, 6). Cheesemaking with milk from different feeds but which was protein-standardized showed similar coagulation and kinetics values for curd moisture loss (Chapter 3). Similarly, yield, recovery of fat and protein, physico-chemical composition, proteolysis and biochemical properties of the resultant Maasdam cheese were also not influenced by the herd feeding systems (Chapter 6), indicating that cheese producers can manufacture cheese from such systems while adhering to standard operating procedures when protein levels in milk are standardized to a uniform level, leading to greater consistency in process and quality. Slightly higher levels of NPN in CLO-derived milk also did not influence the yield and final composition of Maasdam cheese. Such characterization of Maasdam cheese yield, composition, and ripening

changes provides an underpinning knowledge to help Irish cheese producers to produce ranges more diverse than conventional Cheddar cheese.

Pasture-derived cheeses (GRA or CLO) had significantly lower whiteness and higher yellowness values compared to TMR derived cheese (Chapter 6). Cheeses from different feeding system showed greater differences in terms of long-chain fatty acids, e.g., linoleic acid (C18:2) and palmitic acid (although the difference in palmitic acid content was not significant).

Differences in colour and fatty acid composition due to feeding regimes may translate into different textural perceptions in the mouth. Cheese derived from TMR feeding systems were firmer in the mouth compared to GRA- or CLO-derived cheeses, which could be attributed to a higher palmitic acid content in the former (Chapter 6). Although TMR-derived cheeses had significantly higher butyrate levels, and CLO cheeses had significantly lower acetate levels, this was not reflected in flavour perception. However, the difference in acetate and butyrate levels suggests that further attention is required to investigate changes in microbiota from milk to final cheese during processing; seven genera of microorganisms that are found on the teat skin of cows have been reported to be isolated from raw milk cheese (Frétin et al., 2018).

The investigation of cheese samples using HR MAS NMR showed that herd diet mainly resulted in different lipid profiles (Chapter 7), emphasizing a future direction for research to focus on the use of lipid profiles to differentiate cheeses based on feeding systems. Metabolites in the non-lipid phase were mainly similar, except for citrate level, which was significantly higher in TMR compared to GRA cheeses (Chapter 7). Likewise,

toluene content was higher in GRA or CLO compared to TMR cheese, and this could be a key metabolite for chemically discriminating milk products made from pasture or indoor feeding systems. However, its capability for differentiating product on sensory properties is low, because of its high-threshold value for odour (Kilcawley, 2018). Dimethyl sulphide was found to be present only in CLO-derived cheeses, which contrasts with the findings of O’Callaghan et al. (2017). However, feed-induced differences in Maasdam cheese metabolites were minimal, possibly because starter cultures and secondary PAB cultures also produce metabolites which may have a greater impact than the metabolites derived from milk/feed during fermentation. This probably results in a lower influence of herd diet in comparison to the influence of starter and NSLAB metabolism on the overall consumer acceptance. However, where cheesemaking occurs using milk from cows fed grass with large variations in botanical diversity in pasture land, the final cheese textural properties and levels of volatile compounds (e.g., terpene content), and thereby sensory properties, may be significantly influenced (Buchin et al., 1999). The level of terpene in milk has not been observed to be influenced by the feeding systems utilized in the current study (Faulkner et al., 2018), which might be the reason for not observing significant difference in terpene contents (D- Limonene and α -pinene) in Maasdam cheese samples (Chapter 6).

Pasture-based feeding systems could be perceived by consumers as “more natural” by facilitating cow’s access to fresh forage. Therefore, demand for pasture-based dairy products has increased over those produced from controlled supplementation of silage and concentrate (Getter et al., 2015) and the Irish dairy production system has the advantage of producing on predominantly pasture-based dairy products. Hence, the effect of indoor

or outdoor herd feeding systems on milk and product properties has become of increasing interest (Gulati et al., 2017; O’Callaghan et al., 2017) and these factors could increase Ireland’s competitiveness in the global export market. The results from this thesis provide significant knowledge as to the differences which may occur in cheeses made from milk derived from herds fed on different feed types.

Besides reducing the impact of seasonal variation of milk composition in cheesemaking (Broome et al., 1998), increasing protein levels during cheesemaking offers benefits through increasing process efficiency and cheese yield, and ameliorating issues with poorly coagulating milk (Guinee et al., 1994, 1996a, 2006; Mistry and Maubois, 2017). The impact of the extent of milk concentration on cheesemaking processes at different coagulation temperatures was investigated in Chapters 4 and 5. It was found that milk with 5% protein coagulated at 28°C had similar coagulation characteristics compared to milk with 4% protein coagulated at 32°C, whereas increasing milk protein content to 6% resulted in more than doubling of the curd-firming rate and a shorter cutting window (Chapter 4). This emphasized the requirement for cutting of the coagulum within a short period of time so as to maintain similar curd rigidity compared to cheesemaking from regular milk. Interestingly, concentrating milk to ~6% protein is equivalent to a reduction of nearly 50% milk volume by the release of permeate prior to cheesemaking, doubling the processing capacity of the vats in a cheese plant.

A significant issue observed for protein-concentrated gels was the breakage of the curds during stirring, particularly for milk of >5% protein with >6 mm cut size (Chapter 5). Results from this study suggested the requirement for a smaller cut size when increasing protein levels in milk, as curd from larger cut sizes disintegrated excessively.

An interesting approach for the prevention of the curd breakage was proposed, which included overlaying permeate during cutting of the coagula to float curds during stirring. If the breakage of curds is prevented, then manipulation of cut size can be important to control the rate of curd moisture loss during cheesemaking. It was shown that, to achieve a uniform curd moisture content at a given time, when cheesemaking from milk with increasing protein level, coagula should be cut at larger sizes to slow down the rate of moisture loss.

It was shown that, at higher temperature, extensive cross-linking of casein micelles occurs during coagulation (Chapter 4). On increasing protein concentration of milk, the network became more dense, which was reflected at higher $G'/\Delta t$ values in rheometry. Lowering coagulation temperature resulted in fewer linkages in casein micelles, with a smaller aggregate size, as evidence of less casein micelle rearrangement (Chapter 4). A loose microstructure of the curd at lower temperature was consistent with the lower values of $\tan\delta_{40}$, with a tendency towards less whey expulsion during initial stirring (Chapter 4). When curds were cooked to 37°C, both TEM and SEM micrographs showed that casein micelles were fused to a higher degree in curds coagulated at 36°C compared to those at 28°C (Chapter 5) and void spaces observed in curds coagulated at 28°C further indicated that occurrence of casein micelles rearrangement during cooking of the curds. Such differences in curd microstructure were probably responsible for altering curd moisture loss profiles prior to and during cooking. Micrographs of the curds showed that use of ultrafiltration of milk for protein concentration caused the formation of fat globule clusters, which were inhomogeneously distributed within curd particles (Chapter 4).

Prediction of curd moisture contents either in-line or off-line is a tool that facilitates cheese producers to make appropriate decisions about curd drainage parameters. Various empirical models are presented in this thesis (Chapter 3, 5), and it was found that coagula cut at smaller size follow a logarithmic trend for moisture loss, whereas coagula cut at larger sizes show a linear trend. It was also demonstrated that coagulum cut size determines the in-vat moisture loss profile, that this trend was independent of protein concentration of milk. Curd from protein-concentrated milk attained a lower moisture content at any given time because of the increased milk solids content of such milk. Set temperature largely influenced the moisture loss profile prior to cooking, and thus consideration of all these factors is important in developing more robust models for the prediction of changes in curd moisture during stirring.

Natural variations in milk composition pose challenges in achieving a consistent cheesemaking process (Amenu and Deeth, 2007). Although protein standardization of milk for addressing seasonal variation in milk composition has previously been recommended (Guinee et al., 1994, 2006), the present research investigated the impact of milk protein concentration on gel rheology and curd syneretic properties of such milk in detail. In typical industrial practice, milk fat levels are standardized prior to cheesemaking. However, there may still be variations in milk protein levels due to seasonal or feeding effects (Guinee et al., 2006), potentially resulting in fluctuations in the cheesemaking process or in the final cheese composition. Blending of UF retentate, skim milk, and whole milk to achieve a target protein level is thus important in modern cheesemaking processes where compositional variation of milk is large, and this also results in increased plant process efficiency.

8.2 Proposals for further research

Based on the knowledge gained from this thesis, further investigations of the following topics would add value in achieving a greater understanding of the influence of seasonal variation in milk compositions on cheesemaking processes:

- Studies on the influence of water overlay of the coagulum from protein-concentrated milk during cutting to replace the washing step in continental-type cheesemaking, and the impact of such a step on cheese composition and ripening profiles;
- Use of ^1H NMR metabolomics to investigate potential biomarkers influenced by different herd feeding systems in the lipid and non-lipid phases of young and mature cheese samples, to separate and identify hydrophilic and lipophilic metabolites originating from the milk and from bacterial fermentation;
- Use of metagenomic study, based on high-throughput shot-gun sequencing or other relevant methods such as third-generation sequencing, to evaluate the influence of herd feeding systems on microbiological population, evolution and cheese flavour profiles. High-throughput shot-gun sequencing identifies the bacterial community through a non-targeted approach at strain level (Bhagya, et al., 2018), which may be useful in identifying the bacterial populations and their evolution during the ripening of cheese. It may also be a consideration for future studies to determine whether herd feeding system influences the evolution of the microbiota in Maasdam cheese, and to examine gene expression profiles of NSLAB that contribute to the production of organic acids (e.g., acetate) during warm room ripening and maturation. Recently, it has been proposed that wide

botanical diversity and feeding practices (e.g., indoor or outdoor) may have marked effects on milk microbiota originating from teat skin (Frétin et al., 2018) which could pass through the cheesemaking steps to the final cheese.

The work in this thesis also provides greater insight into the impact of protein standardization of milk by ultrafiltration on process efficiency. Investigating the following parameters would complement in understanding cheesemaking process from protein-concentrated milk:

- During stirring, it was shown that set temperature does not have a significant effect on curd moisture content after cooking when pH of milk is standardized (Chapter 5). In a previous study (Guinee et al., 2006), no significant difference in total time required for achieving a specific final curd pH between milks with increasing protein concentration (up to a maximum of 4% protein) was observed when inoculating active starter culture at a fixed ratio with respect to protein level. However, Ong et al. (2013) observed high buffering capacity of curds prepared from milk with 6% protein, which resulted in longer time to reach desired pH value of cheese curds compared to curd prepared from milk with lower protein concentration. It would be important to investigate the effect of set temperature on curd moisture loss kinetics when acid production is promoted by adding different levels of active culture to protein-concentrated milk. Moisture loss kinetics of curd are expected to be influenced by both factors, so standardization of the rate of change of moisture with pH may facilitate achieving a desired curd moisture and pH level prior to curd drainage, by overcoming the increased buffering capacity with increased protein contents in milk;

- There is a growing interest on inline prediction of curd moisture using NIR sensors (Chapter 2). Application of NIR sensors for milk with increased protein concentration may demonstrate its potential use for cheesemaking from protein-standardized milk. However, breakage of curd particles prepared from such milk results in curd particles with large variation in their size and hence the in-vat curd moisture content (Chapter 6). Use of NIR sensors for the prediction of curd moisture content, including factors such as particle breakage, may further enhance model predictability for overall curd moisture content in a cheese vat;
- Modelling of the influence of protein concentration, set temperature and cut size on moisture release during pressing of the cheese curds would facilitate optimizing the pressing regime of cheese prepared from milk with increased protein concentration.
- Curd microstructure from SEM and TEM images could be further quantified using images analysis softwares (El-Bakry and Sheehan, 2014). However, the location of images collected within the curd from such technology should be identical so that they are comparable.

8.3 References

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Appendix

Supplementary Table S1. Moisture content (g/100 g) at 5 to 115 min after cutting (without overlaying coagulum with permeate)

Run	X1	X2	X3	Stirring time (min)											
				5	15	25	35	45	55	65	75	85	95	105	115
1	36	6	6	77.26	73.03	69.97	67.73	66.86	65.98	64.74	62.11	61.94	62.05	61.35	59.03
2	36	12	5	81.49	78.21	75.34	75.52	73.29	72.17	70.59	69.98	69.43	68.18	66.95	67.58
3	32	6	5	81.62	77.84	76.32	73.26	71.83	69.76	67.28	66.51	65.60	64.80	65.08	62.69
4	36	18	4	84.58	83.57	82.36	80.68	80.87	80.15	77.78	78.79	78.58	77.11	78.66	76.70
5	28	6	6	78.96	77.61	76.04	72.50	66.54	64.93	63.33	60.62	61.35	60.83	57.35	58.14
6	32	12	5	82.59	80.08	77.01	76.54	73.18	71.93	69.96	67.76	70.34	67.70	67.41	66.68
7	32	18	5	83.00	77.10	74.21	74.44	74.99	74.45	67.85	69.71	68.22	65.92	65.92	64.70
8	28	6	4	85.52	83.22	81.04	80.30	75.49	74.36	72.06	69.20	69.41	68.72	67.08	66.70
9	28	18	4	86.20	84.99	83.86	84.16	83.89	82.51	79.80	79.33	77.65	78.04	76.63	76.01
10	36	6	4	82.93	79.61	77.19	75.34	73.69	71.62	71.81	69.96	69.97	68.45	68.47	67.14
11	32	12	6	80.66	75.79	72.18	70.55	68.26	66.25	64.05	62.93	63.28	61.74	61.25	59.94
12	28	12	5	83.44	79.64	79.45	75.07	72.37	70.01	68.34	67.03	65.74	65.33	64.64	61.67
13	28	18	6	80.86	78.37	76.35	73.68	68.93	75.92	65.24	60.89	61.85	61.31	61.72	58.53
14	32	12	4	84.89	83.93	79.64	78.94	77.48	75.00	72.06	72.10	71.47	69.99	69.22	66.75
15	36	18	6	79.33	76.08	73.90	73.66	67.30	69.05	64.27	63.26	64.40	60.55	62.91	60.66
16	32	6	6	79.01	73.57	72.83	71.93	68.72	67.44	64.61	65.29	63.36	62.31	60.84	61.66
17	32	6	4	83.19	79.22	74.68	72.49	69.14	68.82	68.93	68.07	66.47	66.23	65.82	64.17
18	36	18	5	80.49	78.33	77.56	75.56	73.41	72.41	73.31	69.03	67.83	68.08	66.48	63.86
19	36	12	6	78.10	74.96	70.69	69.94	69.30	66.58	66.57	65.95	64.26	64.39	63.53	61.22
20	28	18	5	83.89	79.84	82.21	80.82	74.63	73.42	75.62	72.96	71.21	68.28	67.41	68.28
21	32	18	6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

See Supplementary Table S2 for the explanation of table parameters

Supplementary Table S2. Moisture content (g/100 g) at 5 to 115 min after cutting (*overlying coagulum with permeate)

Run	X1	X2	X3	Stirring time (min)											
				5	15	25	35	45	55	65	75	85	95	105	115
1	36	6	6	77.26	73.03	69.97	67.73	66.86	65.98	64.74	62.11	61.94	62.05	61.35	59.03
2*	36	12	5	83.40	81.71	80.43	78.95	77.57	76.76	76.00	74.73	74.26	73.36	72.92	72.92
3	32	6	5	81.62	77.84	76.32	73.26	71.83	69.76	67.28	66.51	65.60	64.80	65.08	62.69
4	36	18	4	84.58	83.57	82.36	80.68	80.87	80.15	77.78	78.79	78.58	77.11	78.66	76.70
5	28	6	6	78.96	77.61	76.04	72.50	66.54	64.93	63.33	60.62	61.35	60.83	57.35	58.14
6*	32	12	5	84.17	82.54	81.92	78.77	79.23	77.64	76.21	74.84	74.88	72.31	73.26	71.87
7*	32	18	5	84.14	82.97	82.31	81.69	79.90	80.03	78.09	78.98	77.60	76.73	75.64	74.34
8	28	6	4	85.52	83.22	81.04	80.30	75.49	74.36	72.06	69.20	69.41	68.72	67.08	66.70
9	28	18	4	86.20	84.99	83.86	84.16	83.89	82.51	79.80	79.33	77.65	78.04	76.63	76.01
10	36	6	4	82.93	79.61	77.19	75.34	73.69	71.62	71.81	69.96	69.97	68.45	68.47	67.14
11*	32	12	6	80.87	79.40	78.29	77.40	76.63	75.21	73.89	72.86	71.63	70.51	70.61	69.16
12*	28	12	5	84.38	83.41	82.76	82.18	80.46	78.32	77.09	75.54	75.30	69.41	67.42	71.31
13*	28	18	6	81.78	81.06	80.78	80.07	79.26	76.83	76.11	74.97	72.19	73.05	70.49	70.02
14*	32	12	4	84.91	83.09	82.16	80.33	80.38	78.96	77.65	75.18	76.34	74.28	73.71	73.34
15*	36	18	6	81.59	79.43	78.79	78.07	76.31	75.79	75.57	74.57	73.54	73.41	73.03	71.43
16	32	6	6	79.01	73.57	72.83	71.93	68.72	67.44	64.61	65.29	63.36	62.31	60.84	61.66
17	32	6	4	83.19	79.22	74.68	72.49	69.14	68.82	68.93	68.07	66.47	66.23	65.82	64.17
18*	36	18	5	83.53	82.00	80.84	79.90	79.02	78.70	77.04	75.87	75.00	75.04	74.18	74.10
19*	36	12	6	80.13	78.58	77.02	76.44	75.16	74.34	73.48	72.60	71.79	71.03	70.95	69.24
20*	28	18	5	84.11	83.28	82.56	82.60	80.72	80.06	78.56	77.60	77.39	75.69	76.34	73.75
21*	32	18	6	81.25	80.43	79.49	78.65	77.71	76.33	74.50	73.73	73.84	73.26	71.62	72.43

¹based on the response factor X1, X2, X3; set temperature (°C), cut size (mm) and target protein levels (%), respectively. Curds were stirred at set temperature between 5 and 25 min, then heated to 37°C from 25 to 45 min, and held at 37°C. Whey dilution occurred at 75 min. NA= not available.



Supplementary Figure S1. Initial in-vat stirring of curds of 18-mm cut size produced from (A) milk with 4% protein without permeate overlay, (B) milk with 6% protein without permeate overlay, and (C) milk with 6% protein with permeate overlay during cutting. Pictures were taken during initial stirring.