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Variation in raw milk quality

Impact on milk coagulation and cheese ripening

Hasitha Priyashantha

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Cover: Key areas of this thesis
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Abstract

This thesis examined some of the causes behind recent increasing variation in ripening time of a traditional, Swedish long-ripening hard cheese. Variations in the composition and properties of farm and dairy silo milk intended for production of the long-ripening cheese were recorded during one year, and the resulting cheese was characterised. Two major types of dairy farming systems associated with differences in milk quality attributes of importance in cheese making were observed. Farms characterised by loose-housing, milking parlour or automatic milking system (AMS), and Swedish Holstein as the dominant breed, were found to be larger than tiestall farms with Swedish Red and other dairy breeds. Levels of free fatty acids, plasminogen-derived activity and gel strength were higher in tank milk from tiestall compared with AMS farms. Some milk quality attributes were influenced by sampling month, *e.g*. casein micelle size was smaller and total proteolysis higher in milk during outdoor compared with indoor months. The effect of variation in casein micelle size and in calcium and citrate content on milk coagulation was investigated in an experimentally designed study. The results showed that elevated levels of calcium and citrate in the milk altered casein micelle size, while modifying the coagulation properties of the milk. Larger micelles with moderate citrate level led to a firmer gel than smaller micelles with higher citrate level. The process of cheese ripening was visualised using rapid and non-destructive NIR-hyperspectral imaging, through which cheese maturity could be predicted with 76% accuracy. The predictive model can function as a supplementary quality assurance tool for *e.g*. efficient planning and optimisation of the logistics in cheese ripening facilities. Overall, variation in the quality attributes of dairy silo milk had little impact on cheese ripening time, contradicting expectations. However, sensory and texture scores of the resulting cheese were influenced by plasmin and plasminogen activity. These findings suggest that associations between raw milk characteristics and cheese ripening are weak if the milk is of high quality.

Keywords: Raw milk quality, rennet-induced coagulation, dominant breed, milking system, season, hyperspectral image analysis, cheese-ripening time

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Variation i mjölkråvarans kvalitet

Sammanfattning

Avhandlingen ingår i ett större forskningsprojekt med syfte att öka kunskapen om orsakerna till den ökande variationen i mognadstid hos en svensk traditionell, långlagrad hårdost. Kvalitetsegenskaper hos mjölkråvaran studerades på gårds- och mejerinivå under ett år, och den resulterande osten karakteriserades. Två huvudtyper av gårdar med mjölkproduktion kunde urskiljas, varvid tankmjölken från dessa uppvisade skillnader i kvalitetsegenskaper. Gårdar som kännetecknades av lösdrift, mjölkning i grop eller med robot (AMS) och svensk Holstein som dominerande ras var generellt större än gårdar med uppbundna system, som dominerades av SRB och andra raser. Nivåerna av fria fettsyror, plasminogenaktivitet och gelstyrka var högre i tankmjölk från gårdarna med uppbundna system jämfört med AMS-gårdarna.Vissa mjölkkvalitetsparametrar påverkades av provtagningsmånad, varvid mjölkens kaseinmiceller var mindre, och totalproteolysen högre under betesmånaderna jämfört med stallperioden. I en experimentell studie studerades interaktionseffekter av varierande kaseinmicellstorlek, kalcium- och citratinnehåll på mjölkens koaguleringsegenskaper. Förhöjda halter av kalcium och citrat förändrade såväl micellstorlek som mjölkens koaguleringsegenskaper. Större miceller och måttlig citratnivå gav en fastare gel än högre citratnivå och små miceller. Ostmognadsprocessen visualiserades med hjälp av snabb och icke-destruktiv teknik baserad på NIR-hyperspektral bildanalys. Ostmognadsgraden kunde predikteras med 76% noggrannhet, och den prediktiva modellen erbjuder ett kompletterande verktyg vid planering och optimering av logistiken i ostmognadslager. I motsats till vår hypotes visade variationen i mjölkens kvalitetsegenskaper liten inverkan på ostens lagringstid. Däremot visade den sensoriska utvärderingen av osten att mjölkens plasmin/ plasminogenaktivitet hade betydelse för ostens textur. Resultaten i avhandlingen tyder på att sambanden mellan mjölkråvarans egenskaper och ostmognadsprocessen är svaga under förutsättning att ystningsmjölken är av hög kvalitet.

Nyckelord: Mjölkråvarans kvalitet, koaguleringsegenskaper, dominerande ras, mjölkningssystem, säsong, hyperspektral bildanalys, ostmognadstid

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Preface

The work described in this thesis was part of a larger project investigating factors associated with uncontrolled variation in ripening time (between 16-24 months) of a Swedish long-ripening hard cheese. The project was conducted in a collaboration between SLU (Uppsala and Umeå) and a cheese producer (Norrmejerier, Umeå, Sweden) and investigated the causes of the observed variation in cheese ripening. The work described in this thesis encompassed the whole dairy value chain, from dairy farms to marketing of the fully ripened cheese. It involved industrial-scale cheese production, on-farm studies, an experimentally designed study, and work to develop predictive tools to study cheese ripening.

Materials (milk and cheese) and participating farms in the study were from the sub-arctic region of Northern Sweden, offering unique perspectives and an interesting case to be compared globally with other dairy farming systems. I would like to thank all contributors, especially my supervisors Åse Lundh, Monika Johansson, Maud Langton, Mårten Hetta and Annika Höjer, for their great support in completion of this thesis. My parents, family and wife deserve a particular note of thanks for their continuous support.

I hope this thesis can be of benefit to a wide readership and inspire future research work in this subject area.

Hasitha Priyashantha
Uppsala, Sweden, 27th July 2021

Dedication

To our beautiful newborn daughter, **Samahi Netalya,** for giving the best possible cooperation at the time of writing most of the manuscripts and this thesis.

“Age is something that doesn't matter, unless you are a cheese”

Billie Burke

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Priyashantha, H.***, Lundh, Å., Höjer, A., Hetta, M., Johansson, M. & Langton, M. 2019. Interactive effects of casein micelle size and calcium and citrate content on rennet-induced coagulation in bovine milk. *Journal of Texture Studies* 50 (6):508-519.

II. **Priyashantha, H.*,** Lundh, Å., Höjer, A., Bernes, G., Nilsson, D., Hetta, M., Saedén, K.H., Gustafsson, A.H. & Johansson, M. 2021. Composition and properties of bovine milk: A case study from dairy farms in northern Sweden; Part I. Impact of dairy farming system. *Journal of Dairy Science* 104 (8):8582-8594

III. **Priyashantha, H.*,** Lundh, Å., Höjer, A., Bernes, G., Nilsson, D., Hetta, M., Saedén, K.H., Gustafsson, A.H. & Johansson, M. 2021. Composition and properties of bovine milk: A case study from dairy farms in Northern Sweden; Part II. Effect of monthly variation. *Journal of Dairy Science* 104 (8):8595-8609

IV. **Priyashantha, H.*,** Johansson, M., Langton, M., Samples, S., Jayarathna, S., Hetta, M., Saedén, K.H., Höjer, A. & Lundh, Å. 2021. Variation in dairy milk composition and properties has little impact on cheese ripening: insights from a traditional Swedish long-ripening cheese. *Dairy* 2(3):336-355.

V. **Priyashantha, H.*,** Höjer, A., Saedén, K.H., Lundh, Å., Johansson, M., Bernes, G., Geladi, P. & Hetta, M. 2020. Use of near-infrared hyperspectral (NIR-HS) imaging to visualize and model the maturity of long-ripening hard cheeses. *Journal of Food Engineering* 264. 109687.

 *Corresponding author

The contribution of Hasitha Priyashantha to Papers I-V was as follows:

I. Contributed to designing the study, acquisition of data and analysis, data curation, interpretation of data, visualisation, writing the original draft, review and editing of the manuscript. Submitting author.

II. Conceptualisation of the structure of the study, extraction and formal analysis of data, data curation, interpretation of data, visualisation, writing the original draft, review and editing of the manuscript. Submitting author.

III. Conceptualisation of the structure of the study, extraction and formal analysis of data, data curation, interpretation of data, visualisation, writing the original draft, review and editing of the manuscript. Submitting author.

IV. Conceptualisation of the design and structure of the study, extraction of data and analysis, interpretation of data, visualisation, writing the original draft, review and editing of the manuscript. Submitting author.

V. Contributed to designing the structure of the study, performed NIR-hyperspectral image analysis, model development, interpretation of data, visualisation, writing the original draft, review and editing of the manuscript. Submitting author.

Other academic contributions within the PhD project's scope

Poster, International Dairy Federation's (IDF) World Dairy Summit (2017), Belfast, UK: *"NIR hyperspectral imaging for predicting maturity of cheese"*
Poster, Food Science Sweden conference (2017) in Uppsala, Sweden: *"Near-infrared hyperspectral imaging"*
Popular science article: *"Forskning pågår - från foder till ost"* (2018), Institutionen för norrländsk jordbruksvetenskap (12609821), SLU
Oral presentation describing the PhD project during the study visit to Japan (2018) at the University of Tokyo, Japan
Poster, 32nd European Federation of Food Science and Technology International Conference (2018), Nantes, France: *"Interaction between casein micelle size, calcium and citrate in milk coagulation"*
Seminar and poster, Faculty of Veterinary Medicine and Animal Science's faculty day (2018): "*Visualization and modelling the maturity of long ripening hard cheese using NIR hyperspectral imaging"*
Poster, 7th Advanced Food Analysis International PhD course (2019), The Netherlands: "*Hyperspectral imaging in cheese ripening"*. Awarded 2nd place for the poster
Cover Image based on Paper I, *Journal of Texture Studies*, Volume 50, Issue 6, 2019
Oral presentation and poster, Chemical Side of SLU IV (2019), Uppsala, Sweden: "*NIR hyperspectral imaging for studying cheese maturation*"
Oral presentation (pitch) describing the PhD project at the Food Science Sweden Conference (2019) in Lund, Sweden
Oral short presentation describing the PhD project during a study visit to Norway (2019) at the Norwegian Institute for Sustainability Research (Østfoldforskning), Fredrikstad, Norway
Oral presentation describing the PhD project at the interdisciplinary and multidisciplinary PhD workshop (2019), Baltic University network, Poland

Popular science article in New Food magazine (2019): *"Can cheese maturity be measured using images?"* (Article number 90979)
Oral presentation to the Nordic Joint Committee for Agricultural and Food Research (2020), online: *"raw milk quality in cheese making"*
Oral presentation describing the PhD project in the virtual internship programme (2021) for graduate students at S. Seifullin Kazakh Agro Technical University, Kazakhstan
Oral presentation describing the PhD project at LiFT (Swedish Industrial PhD School) virtual mini-symposium (2021)
Popular science web-article at SLU's knowledge bank based on Papers II & III (2021): *"How milk quality is affected by dairy farming system and sampling month"*
Popular science web-article at SLU's knowledge bank based on Paper V (2021): *"Development of a non-destructive tool for quality assurance of cheese ripening"*
Oral presentation at IDF International Cheese Science and Technology Virtual Symposium (2021). Hosted by IDF-Canada and Université Laval, Canada: *"NIR-hyperspectral imaging enables rapid and non-destructive characterization of long-ripening cheeses based on maturity"* (1st place in the student oral presentation competition)
Flash oral presentation, Dairy Science and Technology Virtual Symposium (2021). Hosted by Arhus University, Denmark: *"Impact of on-farm factors and sampling month on the composition and properties of bovine milk from dairy farms in Northern Sweden"*
Short communication: Priyashantha, H., Höjer, A., Saedén, K. H., Lundh, Å., Johansson, M., Bernes, G., Geladi, P. & Hetta, M. (2021) in *Food Control*, Volume 130, 108316: *"Determining the end-date of long-ripening cheese maturation using NIR hyperspectral image modelling: A feasibility study"*
Graduate student literature review: Priyashantha, H. & Lundh, Å. (2021) in *Journal of Dairy Science*: *"Current understanding of the influence of on-farm factors on bovine raw milk and its suitability for cheese making."* Accepted on 17-July 2021

Abbreviations

AMS	Automatic milking system
ANOVA	Analysis of variance
CFU	Colony-forming units
FA	Fatty acids
FFA	Free fatty acids
FTIR	Fourier-transform infrared
G20	Gel strength at 20 min after rennet addition
ICP-OES	Inductively coupled plasma optical emission spectrometry
LAB	Lactic acid bacteria
MP	Milking parlour
NIR-HS	Near-infrared hyperspectral
NSLAB	Non-starter lactic acid bacteria
NTA	Nanoparticle tracking analysis
OPLS	Orthogonal projections to latent structures
PCA	Principal component analysis
PLS	Projection to latent structures (partial least squares)
RCT	Rennet coagulation time
RMSEC	Root mean square error of calibration
ROI	Region of interest
R^2	Coefficient of determination for calibration
SCC	Somatic cell count
SH	Swedish Holstein
SJB	Swedish Jersey Cattle
SKB	Swedish Polled Cattle
SRB	Swedish Red Cattle

1. Introduction

The composition and properties of raw milk are crucial for cheese quality, affecting the functional properties of both milk and cheese (Kailasapathy, 2015; Skeie, 2007). Milk quality is influenced by on-farm factors and season (Bittante *et al*., 2015), emphasising the importance of the dairy farming system to the raw milk and the quality of the resulting cheese.

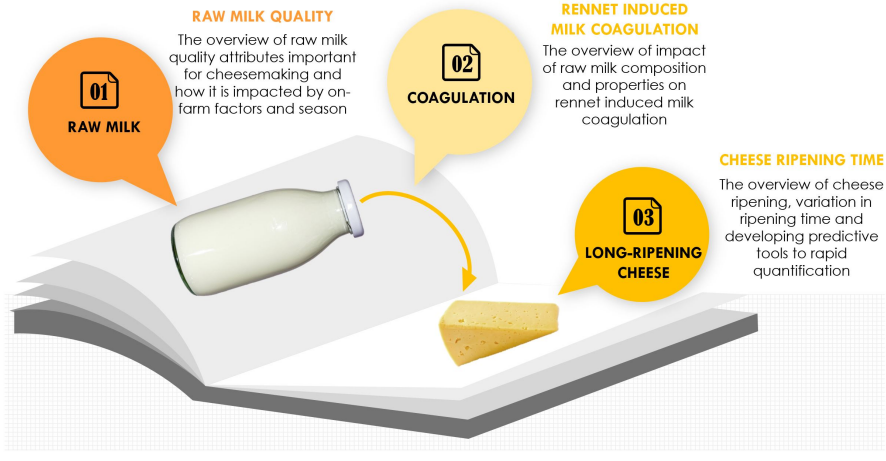

Figure 1. Three key focus areas of this thesis: (1) Raw milk quality, (2) rennet-induced milk coagulation and (3) ripening time of cheese.

In this thesis, three major focus areas (raw milk quality, milk coagulation and cheese ripening time) were studied, to elucidate the crucial link between raw milk and cheese (Figure 1). The conditions for raw milk quality are determined already on the dairy farm, influenced by the dairy farming system, and affect cheese ripening, as evaluated within this thesis.

1.1 Raw milk quality

1.1.1 A brief overview of milk quality in cheese making

Raw milk quality, including its composition and properties, is important in cheese making and for the quality of the resulting cheese. The most important milk quality parameters in cheese making are summarised in Table 1. Cheese milk should meet at least some of the expectations discussed in Table 1, and demonstrate good coagulation properties, host a healthy microbial community and be free from adulterants.

Table 1. *Overview of milk quality parameters, requirements and significance for cheese making and/or final cheese quality*

Milk quality parameter	Requirement/expectation	Significance / influence / importance	Reference
Fat	Usually standardised for protein:fat ratio depending on the type of cheese	For texture, microstructure, yield, rheology, aroma, flavour, biochemical properties, cheese cooking properties	Guinee & McSweeney (2006)
Protein content and genetic variants	Milk with high casein content (≥2.55 g/100g), especially high κ-casein, is preferred in cheese making	Curd firmness and cheese yield in general increase with protein content	Hallén *et al*., (2007); Law & Tamime (2010)
Lactose	Milk with average lactose (4.6 g/100g) concentration	Primary energy source in milk used by lactic acid bacteria (LAB). Hard cheese contains very little lactose, as it is removed with whey and used by LAB during ripening	McSweeney & Fox (2009)
pH	The normal pH of milk is 6.5-6.7.	Influences the equilibrium between colloidal CaP and free Ca^{2+}/PO_4^-. Affects the activity of rennet enzyme	Pandey *et al*. (2003)
Casein micelle size	Micelle size appears to be critical in coagulation, but further research is needed	Some studies show smaller micelles have beneficial properties, others show the opposite.	Glantz *et al.* (2010); Ekstrand *et al*. (1980)
Milk fatty acid (FA) composition	No special requirements based on FA composition, but preferably low variation between batches	Associated with the feeding of dairy cows. Unsaturated FA reduce the firmness of the cheese	Jaros *et al.* (2001)
Free fatty acids (FFA) (mmol/100g fat)	FFA levels <1.0 are considered optimal and FFA levels >1.2 as high risk	Elevated FFA levels indicate the action of milk lipases and may contribute to the development of rancid flavour in cheese	Jenkins (1984)

Milk quality parameter	Requirement/expectation	Significance / influence / importance	Reference
Calcium	The average concentration in milk is approximately 1072 mg/100kg	Important for the integrity of casein micelle and for coagulation (influences gel strength and coagulation time). Influenced by mineral supplementation and concentrate feeding of cows	Dunshea *et al*. (2019)
Plasmin and plasminogen	Importance varies with the type of cheese, although no requirements are specified at raw milk procurement	Plasmin-induced proteolysis has a positive effect on cheese (with high cooking temperatures) ripening (*e.g*. texture and flavour development)	France *et al.* (2021); Ismail & Nielsen (2010)
Total bacteria count	$<10^4$ CFU/mL milk, in general	The release of proteolytic enzymes breaks the casein micelles into smaller products (lost in whey and reduces cheese yield). For some cheese types, NSLAB affect cheese ripening/ flavour development	Skeie (2007)
Lactic acid bacteria	The presence of non-starter lactic acid bacteria (NSLAB) is essential for some cheeses	Important for final cheese (sensory) quality in certain cheese types. NSLAB proliferate during ripening and influence flavour development (both typical and atypical)	Beresford & Williams (2004)
Pathogenic bacteria	Absence of *Escherichia coli, Campylobacter jejuni, Staphylococcus aureus, Salmonella enterica, Listeria monocytogenes*	Killed during pasteurisation. Milk hygiene important for the safety of cheese, especially when cheese is made from unpasteurised milk	Donnelly (2004)
Psychrotrophic bacteria	Low numbers of bacteria from the genera *Pseudomonas, Bacillus, Streptococcus* and *Corynebacterium*	Release of heat-resistant proteases and lipases that may reduce yield and result in undesirable flavours in cheese. Major spoilage contributors of unprocessed and stored milk.	de Oliveira *et al*. (2015); Sørhaug & Stepaniak (1997)
Somatic cell count (SCC)	200-250 000 cells/mL	Higher SCC is associated with detrimental changes in the quality of raw milk, resulting in decreased gel firmness and cheese yield, and increased proteolytic activity in milk	Norrgården, (2020) Barbano *et al.* (2006)
The flavour of the milk	No off-flavours	Important for optimal sensory quality of resultant cheese	Skeie (2007)
Antimicrobials agents	Absence of antibiotics	Reduce/inhibit the activity of lactic acid bacteria	Skeie (2007)

1.1.2 Impact of on-farm factors and season

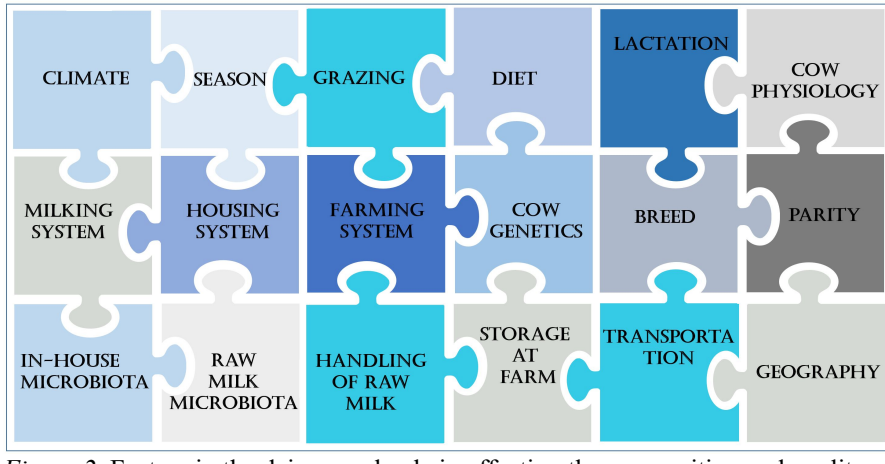

Figure 2. Factors in the dairy supply chain affecting the composition and quality of raw milk and resulting cheese.

Multiple and interconnected factors (represented in Figure 2 as pieces of jigsaw puzzle) in dairy farming and the supply chain influence the overall quality and suitability of raw milk for cheese making. Some of these factors are discussed in detail below.

Milking system and housing system

Milk composition and properties are known to depend on milking and housing system on the farm (Kunes *et al*., 2021; Tse *et al*., 2018). The confounding nature of the two factors is explained by the fact that cows milked in automatic milking systems (AMS) or milking parlours are kept in loose housing systems, rather than in tiestall barns. Hence, interpretation of milk quality attributes for a particular milking system goes hand in hand with the housing system. The major reason behind milk compositional differences between milking systems is the difference in milking frequency, with generally higher milking frequency in AMS than in tiestall systems. Milk yield is positively associated with AMS (Speroni *et al*., 2006), whereas fat and protein content in bulk milk is lower in milk from AMS than milking parlours (Johansson *et al*., 2017). Longer rennet coagulation time and higher free fatty acid (FFA) content have been observed in milk from Holstein-Friesian herds on farms with AMS compared with milking parlours (De Marchi *et al*., 2017). Total proteolytic activity is reported to be higher in milk from AMS herds, whereas plasmin activity and plasminogen-derived activity

are higher in milk from tiestalls than in milk from AMS systems (Johansson *et al*., 2017). This is suggested to be due to more frequent udder emptying in AMS, with a relatively shorter time for plasminogen to move from blood to milk and to be activated into plasmin between milkings. Svennersten-Sjaunja & Pettersson (2008) showed that total bacteria content and somatic cell count (SCC) are higher in AMS, most likely due to insufficiencies in teat cleaning and variations in milking interval.

Breeds and genetics

Milk composition is strongly influenced by breed, *e.g.* milk from Swedish Red Cattle (SRB) has higher fat and protein content than milk from Swedish Holstein (SH) (Larsen *et al*., 2010). Milk coagulation properties (important for cheese manufacturers), also differ between breeds (De Marchi *et al*., 2007), *e.g*. Jersey cow milk shows better coagulation properties than Danish Red or Holstein milk (Frederiksen *et al*., 2011).

Figure 3. Common dairy cow breeds in Sweden. Swedish Holstein (SH) is the most common (50% of the dairy cow population), followed by Swedish Red Cattle (SRB) (44%) (Frössling *et al*., 2017). Swedish Jersey (SJB) and Swedish polled (SKB) are minor dairy breeds in Sweden (Photos: Gun Bernes and Hasitha Priyashantha).

However, the effect of genetics on milk coagulation properties is more decisive than the effect of breed. Studies on SRB cows suggest that high frequency of the composite genotypes A1A2/AE and A2A2/AA results in non- or poor-coagulating milk (Gustavsson *et al*., 2014). Milk from SH cows has larger average casein micelle size (200 nm) than milk from SRB cows (191 nm), confirming differences in casein micelles between breeds (Glantz *et al*., 2010). Moreover, casein micelle size varies between individual cows of the same breed, independent of age, milk production or lactation stage (de Kruif & Huppertz, 2012).

Season

In countries with prominent seasonality, milk compositional changes are mainly induced by calving pattern, feeding regime and health status of the udder (Guinee & O'Brien, 2010). In Sweden, continuous calving is practised and therefore lactation does not contribute to a seasonal effect on milk composition. In general, milk fat and protein content are lower during summer than in winter months, as also shown in a Dutch study (Heck *et al*., 2009). Those authors attributed the higher protein content during the indoor feeding period to modification of hormonal signalling in protein synthesis, resulting from an increased glucose supply. This was in turn due to production of propionic acid with ingestion of high starch levels by feeding a higher concentrate-to-forage ratio in the indoor feed in winter. Milk fat synthesis was inhibited due to uptake of specific long-chain unsaturated fatty acids during pasture feeding, explained by the high levels of linoleic acid in fresh grass (Heck *et al*., 2009). Smaller casein micelles have been observed in summer (May-August) than in winter in Scottish cow's milk, confirming the effect of season on micelle size (Holt & Muir, 1978). Elevated SCC has been reported in Swedish bulk milk samples toward the end of the pasture season (Frössling *et al*., 2017). Higher SCC may contribute to elevated total proteolytic activity in milk (Senyk *et al*., 1985). Glantz *et al*. (2020) showed that lipase activity in Swedish dairy silo milk is higher in the outdoor season, but that protease activity does not differ between the seasons.

This trend of compositional variation has also been reported in Swedish bulk milk, with zero contribution from grazing to the diet during winter months and year-round calving (Larsen *et al*., 2010). During the indoor period, Swedish dairy cows mainly consume grass silage and concentrate, while they consume fresh grasses and concentrate in summer (Figure 4).

A study on dairy silo milk composition in Sweden by Lindmark-Månsson (2012) showed that most milk components varied significantly during the year, with milk fat composition showing the largest variation due to outdoor grazing in summer. In some cases, seasonal variation in milk composition poses challenges in maintaining consistent product quality. Season and year of production influence the final quality of Protected Designation of Origin cheeses, with cheese resulting from milk produced in spring and summer generally being of higher quality than cheese produced during autumn and winter (Bittante *et al*., 2011). This difference in cheese quality may be due to variations in climate factors, management and characteristics of the dairy herds.

Figure 4. (A) Grass-clover (mixed ley) silage preserved in round bales and (B) fed to dairy cows during winter (indoor period). (C) Dairy cows on pasture, which provides a major proportion of forage intake in Swedish cows during summer, as required by Swedish animal welfare legislation (Jordbruksverket, Swedish Board of Agriculture, 2019) (Photo: Gun Bernes).

1.2 Rennet-induced coagulation of bovine milk

1.2.1 A brief overview of the milk coagulation process

The process of milk coagulation is an essential component of cheese making, affecting the yield and quality of the resulting cheese (Harboe *et al*., 2010). The mechanism of rennet-induced aggregation of casein micelles is comparatively well understood. The action of proteolytic enzymes (*e.g*. chymosin) destabilises the casein micelles, which are sterically stabilised in their native form in milk due to the "hairy" κ-casein surface layer (Figure 5). Chymosin cleaves at the specific peptide bond Phe_{105}-Met_{106} of the protruding κ-casein, releasing glucomacropeptide (κ-casein peptide 106-169) to the aqueous phase of the milk, thereby weakening the steric and electrostatic stabilisation of micelles (Fox & McSweeney, 2003; Hyslop, 2003).

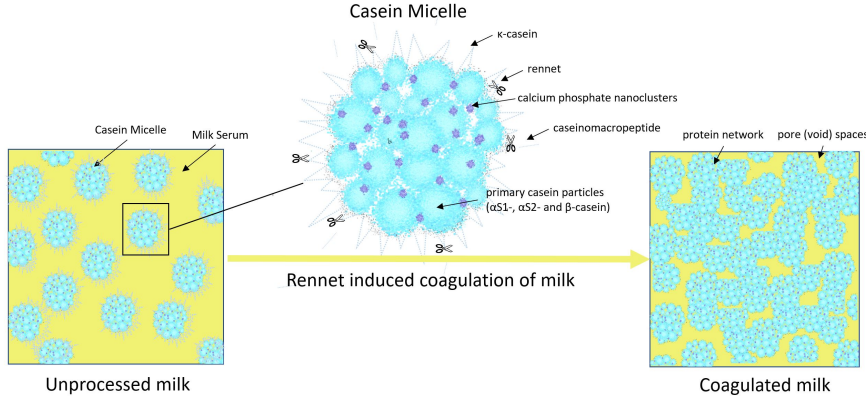

Figure 5. Schematic illustration of rennet-induced milk coagulation. The hydrophilic C-terminal region of κ-casein protrudes from the surface, giving a hairy appearance and stability to the micelle (Fox & Brodkorb, 2008). The action of rennet chymosin/pepsin cleaves the "hairy" κ-casein surface layer and destabilises the suspension of micelles, transforming milk into a gel.

1.2.2 Impact of raw milk composition and properties on coagulation

Milk coagulation properties are influenced by several milk attributes, such as lactose, casein and total protein content, pH and citrate level (Glantz *et al.*, 2010). This section focuses on the effect of casein micelle size, calcium content and citrate content on milk coagulation, due to the topical importance of the work described in this thesis.

Effect of casein micelle size

Casein micelles are nanoscale (50-600 nm in diameter), heterogeneous colloidal particles, composed of water, protein and calcium phosphate, with average molecular weight of ~7.6 x 109 Da. The dry matter contained in casein micelles consists of ~93% protein and ~7% inorganic material. Casein represents around 80% of the total protein content of milk and 20% is whey protein. The size of casein micelles in milk from individual cows is not influenced by the stage of lactation or protein content (Bijl *et al*., 2014; de Kruif & Huppertz, 2012). However, several other factors have been reported to influence the size of casein micelles, such as κ-casein content, casein:protein ratio, genetic variants of casein proteins, pH and season (Devold *et al*., 2000; Glantz *et al*., 2010; Holt & Muir, 1978). Various attempts have been made to investigate the effect of casein micelle size on milk coagulation. Some studies in the 1980s reported that milk with smaller casein micelles has a longer coagulation time (Ekstrand *et al*., 1980), although Dalgleish *et al*. (1981) found that the coagulation properties were not affected by micelle size. In more recent studies, improved coagulation (*e.g*. shorter coagulation time and firmer gels) has been observed with smaller micelles (Glantz *et al*., 2010; Logan *et al*., 2014).

Effect of calcium and citrate

Calcium is one of the important milk constituents known to have a great influence on milk coagulation. In milk, calcium exists in equilibrium between soluble and colloidal phases. Colloidal calcium phosphate linked to phosphorylated serine residues of casein plays an important role in stabilisation of the casein micelles in milk (Gaucheron, 2005). Calcium addition has been found to reduce the voluminosity of casein micelles (Van Hooydonk *et al*., 1986), improve milk coagulation (Horne & Lucey, 2017) and thereby increase cheese yield (Wolfschoon-Pombo, 1997). With added calcium, the average micelle size slightly decreases and the size of the smallest micelles increases, while larger aggregates of micelles develop in

the coagulum (Müller-Buschbaum *et al*., 2007). Calcium addition can also decrease rennet coagulation time (Gastaldi *et al*., 1994), explained by the reduction in pH and increased concentration of free Ca^{2+} ions (Van Hooydonk *et al*., 1986). Early research showed that total calcium content in milk varies with cow breed (Cerbulis & Farrell, 1976), udder health (Kitchen, 1981) and calcium in the feed (Chan *et al*., 2006).

In milk, citrate acts as a chelating agent (Gaucheron, 2005) and binds a portion of the free calcium ions in milk serum (Odagiri & Nickerson, 1965). The citrate level in cow milk is on average 1.7-1.9 g L^{-1} (Jenness, 1988), but is influenced by the extent of *de novo* synthesis of milk fatty acids. Higher citrate content in milk has thus been observed during the grazing season (Holt & Muir, 1978) and in early lactation (Braunschweig & Puhan, 1999), when *de novo* synthesis of fatty acids is reduced. Fluctuating citrate content in raw milk has been suggested to influence milk processing characteristics, due to alterations in citrate-mediated interactions with other milk components (Faulkner & Peaker, 1982; Garnsworthy *et al.*, 2006), and is thus also likely to play a role in coagulation. However, Akkerman *et al.* (2019) observed no differences in citrate level with sampling month and suggest that feeding has no influence on citrate content in milk.

1.2.3 Relationship with dairy farming conditions

Since milk coagulation is influenced by various milk compositional factors, it can be indirectly related to physiological factors, *e.g.* breed (Auldist *et al*., 2004), stage of lactation (Ostersen *et al*., 1997) and udder health (Grandison & Ford, 1986), and on-farm factors, *e.g.* feeding strategy (Macheboeuf *et al*., 1993). The quality of grass affects milk coagulation properties, with rennet coagulation time (RCT) being positively correlated with grass protein and negatively with fibre content (Bergamaschi *et al*., 2016). Bittante *et al*. (2015) found that parity influences coagulation properties, with milk from first-lactation cows showing a higher curd firming rate (considered as increase in curd firmness, % per min, from the point of RCT to infinite time) compared with milk from multiparous cows. Those authors attributed this to a decrease in protein and fat content as the number of lactations progressed.

1.3 Cheese production and ripening time

1.3.1 A brief overview of cheese production and ripening

At present, the vast majority of cheese factories make cheese on a year-round basis, thanks to advances in technology, *e.g*. process automation, and high-quality milk (Johnson, 2017). In general, the Swedish traditional long-ripened hard cheese that is the focus of this thesis is produced from pasteurised milk standardised with respect to its fat:protein ratio. Rennet (75/25 chymosin and pepsin, respectively, 180 International Milk Clotting Units) at a concentration of 0.3 mL L^{-1} milk is used to coagulate the milk. Cheese production includes long cooking periods with temperatures above 40 °C and the use of a specific starter culture (Rehn *et al*., 2010). In the production of long-ripened cheeses, *e.g*. Parmigiano Reggiano and Grana Padano, cooking of the curd by submerging in hot whey is critically important to exert heat stress on microorganisms and to regulate the amount of readily available energy and nutritional sources for the heat-resistant microorganisms (Gatti *et al*., 2014). Smaller grains are achieved by cutting the curd when the consistency is still soft, rapid cooking at 53-56 °C and letting the grains settle on the bottom of the vat for a long time (around one hour) in the hot whey (Iezzi *et al.*, 2012). The cheese is then pressed, brine-salted, waxed and stored in a ripening facility. Cheese maturation is a costly and often not fully predictable process (Fox *et al*., 1996), even though the conditions in the ripening facility are largely controllable. During maturation, the fresh cheese curd is converted into a cheese with characteristic flavours and texture. The characteristic flavours are associated with lipolytic activity on milk fat (Collins *et al*., 2003), proteolytic activity on milk proteins (Molina *et al*., 1999) and finally catabolic reactions, whereby compounds originating from fat, protein, lactate and citrate are combined into aroma components (Ardö *et al*., 2017). The characteristic textural changes taking place during maturation are mainly associated with proteolysis (Verdini & Rubiolo, 2002). Depending on cheese type, proteases of various origins in the cheese milk, such as indigenous enzymes, coagulants, starter culture and non-starter lactic acid bacteria (NSLAB), contribute to varying extents to these processes (Fox *et al*., 1996). Cheese maturation is influenced by storage conditions (temperature and humidity), the chemical composition of the curd and residual microflora in the curd (Robinson & Wilbey, 1998).

1.3.2 Cheese (sensory) evaluation for maturity

Long-ripening cheeses are premium products contributing to the profitability of the dairy sector (Ardö, 1993). The maturity of cheese is monitored using traditional and conventional methods based on sensory and chemical characterisation (Coker *et al*., 2005; O'Shea *et al*., 1996). The degree of maturity is often identified based on standards set by the manufacturer (Figure 6). The characteristic flavour and texture of a matured long-ripening cheese are used as a standard or reference to classify the maturity of the cheese and for the final approval, after which the cheese can be released to the market. Traditional approaches (*e.g*. using a sensory panel) are time-consuming and destructive, and therefore expensive. Moreover, due to uncontrolled variation in the cheese milk, resulting from on-farm factors and season, cheese ripening can vary both within and between batches. Monitoring the ripening of cheese may thus be very challenging (Benedito *et al*., 2001). Yet, identifying the appropriate maturity and delivering consistently matured cheese is crucially important and often driven by consumer demand. Thus, new knowledge of rapid and accurate tools for monitoring cheese ripening is urgently needed.

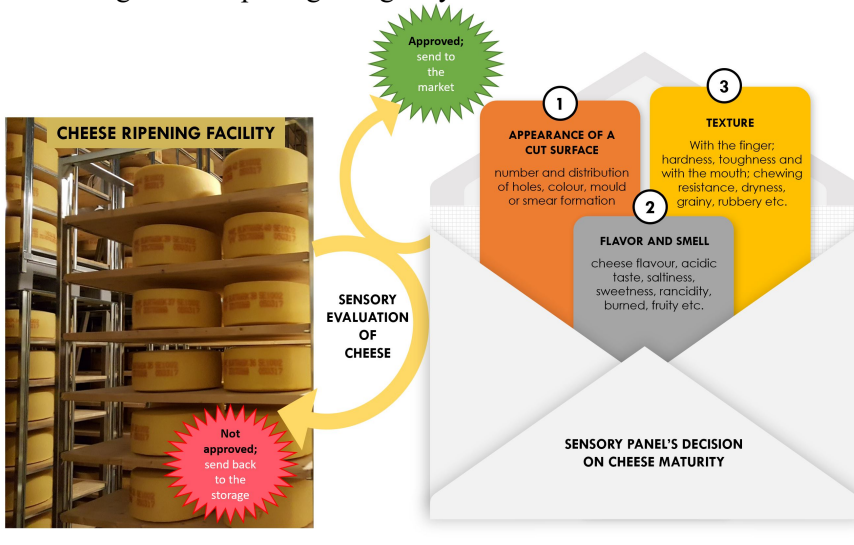

Figure 6. Cheeses are evaluated by a trained sensory panel against in-house standards for outer appearance, flavour/smell and texture. If the cheese meets the specified criteria, it is approved for distribution to the market. If not, the cheese is kept for additional ripening until the next sensory evaluation (for this specific long-ripening Swedish hard cheese, evaluation starts at 14 months and then takes place every two months).

1.3.3 Development of predictive tools for determining cheese ripening

Interest in applying novel non-destructive techniques to study and monitor cheese maturation is growing. With the use of online-process analytical techniques and quality assurance tools, a maximum value for the final cheese can be obtained and waste of resources can be minimised. Cheese ripening has been studied with various techniques over the years, *e.g.* ultrasound (Benedito *et al*., 2001), X-ray computed tomography (Huc *et al*., 2014a), confocal microscopic imaging (Soodam *et al*., 2014) and magnetic resonance imaging (Huc *et al*., 2014b). During the past decade, near-infrared hyperspectral (NIR-HS) imaging has gained attention as a novel non-destructive tool for quality and safety inspection in the food industry (Gowen *et al*., 2007; Liu *et al*., 2014; Sricharoonratana *et al*., 2021). The technology has also been evaluated recently for monitoring cheese ripening (Darnay *et al*., 2017; Malegori *et al.*, 2021).

Hyperspectral images consist of three-way data matrices (two pixel indices and a wavelength index) known as hypercubes or voxels. In a hypercube, a specific point is made with a pixel with a specific spectrum (*e.g.* 256 wavelength bands from 900-2500 nm) (Gowen *et al*., 2007). Hyperspectral images can be acquired using a line-scan pushbroom system, equipped with a moving belt, that consists of a HgCdTe 2-D array detector (386 pixels x 288 wavelengths) and a line-scan camera with 22.5 mm sisuChema short-wave infrared objective (Hetta *et al*., 2017) (Figure 7a). Obtaining a complete NIR spectrum of each pixel is a powerful technique to characterise complex biological matrices (Geladi *et al*., 2004). Wavelength selection is an important aspect of hyperspectral image processing (Liu *et al*., 2014). Identifying key wavelengths with multivariate methods improves the predictive capability and accuracy of the model, by reducing the size of the dataset (Burger & Gowen, 2011). Likewise, pre-processing data to improve the spectral information and prepare data for further processing is an important step in model development (Gowen *et al.*, 2007). The NIR-HS technology has the potential to describe the concentration and distribution of different components in the sample of interest, making both qualitative and quantitative analysis possible with the use of spectroscopic and multivariate calibration techniques (Burger & Geladi, 2005).

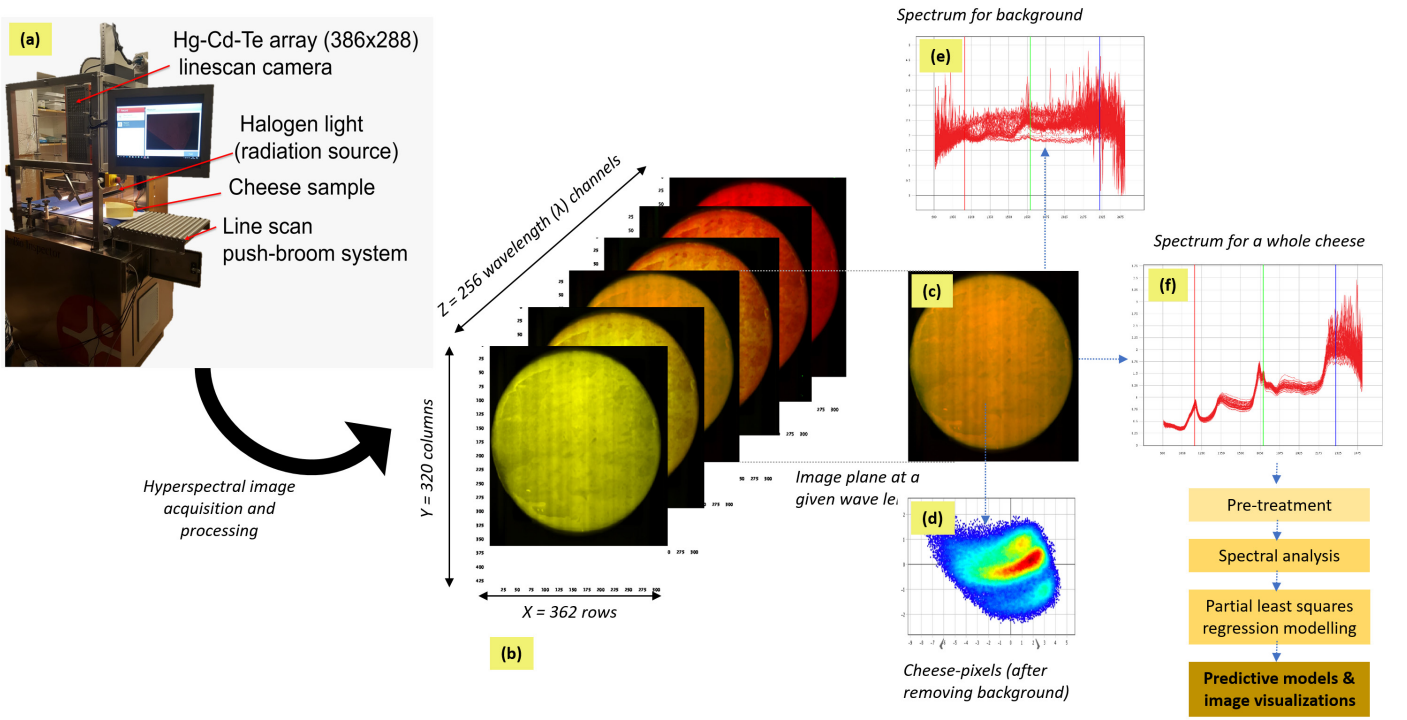

Figure 7. Examples of image structure, information and processing in hyperspectral imaging. (a) Hyperspectral imaging camera set-up, (b) stack of narrowband sub-images forming a 3-D hypercube, (c) single image at a given wavelength, (d) pixels of a whole cheese sample (region of interest: ROI), (e) reflectance spectrum from the background and (f) reflectance spectrum from a whole cheese used in modelling. The ROI spectra are then pre-treated and analysed using multivariate statistical tools to develop predictive models and visualisation maps.

2. Objectives and hypotheses

The aims of this thesis work were to obtain a better understanding of the causes of the variation in raw milk composition and properties, and to determine how this variation affects milk coagulation and cheese ripening time. For some decades, dairy farms in northern Sweden have been undergoing rapid intensification, and farm management practices are continually changing. During the same period, increasing variation has been observed in the ripening time of a traditional long-ripening hard cheese produced in northern Sweden. The hypothesis tested in this thesis was that both rennet-induced coagulation of milk and the ripening time of the resulting cheese are associated with the quality attributes of the raw milk used in production of the cheese, which is influenced in turn by dairy farm type and season. An additional aim was to evaluate NIR-HS as a rapid and non-destructive predictive tool to visualise and monitor cheese ripening. To achieve these aims, the work primarily focused on addressing the following key questions:

How are raw milk characteristics influenced by the type of dairy farm?
To what extent do quality characteristics of farm and dairy silo milk vary with month?
How is rennet-induced milk coagulation affected by variation in raw milk characteristics?
How is cheese ripening time affected by variation in dairy silo milk characteristics?
Can NIR-HS technology be used to visualise and predict cheese maturity?

2.1 Summary of studies

A summary of **Papers I-V** (short description of the study, rationale for its incorporation in the thesis, factors included and expected outcome) is provided in Table 2.

Table 2. *Overview of studies performed in Papers I-V in this thesis*

Paper	Short description	Rationale	Factors considered	Hypothesis	Expected outcome
I	Milk coagulation-an experimentally designed study	Investigate how co-variations in casein micelle size, citrate content and calcium contents interactively affect rennet-induced coagulation	Small and large casein micelle sizes, native calcium and citrate levels, and elevated concentrations by 10%	Variations in casein micelle size, calcium and citrate content of milk interactively affect the coagulation of milk	Improve understanding of the interactive effects of casein micelle size, citrate and calcium content on coagulation properties
II	Impact of dairy farm type on milk quality characteristics	Investigate the effect of different farm factors on milk quality traits of importance in cheese making	Among the numerous farm factors recorded in the study, breed, milking system, housing system, number of lactating cows, energy-corrected milk yield, were evaluated	Type of dairy farm (dominant breed, housing and milking systems) influences raw milk quality attributes	Improve understanding of the effects of various farm factors on the composition and properties of raw milk used for cheese making

Paper	Short description	Rationale	Factors considered	Hypothesis	Expected outcome
III	Monthly variation in farm milk quality characteristics	At dairy farm level, investigate how season/month influence milk quality traits important in cheese making	Farm milk samples collected monthly from February 2016 to February 2017 from 42 dairy farms in a cheese producing region in northern Sweden were used (same samples as in II)	Quality attributes of farm tank milk, of importance for cheese making, vary with month and/ or season	Generate data illustrating annual variation in detailed milk quality traits (composition, coagulation properties and other milk quality attributes)
IV	Monthly variation in dairy silo's milk and cheese ripening time	At dairy silo level, investigate seasonal/monthly variation in milk quality traits and the influence on cheese ripening time	Samples of dairy silo milk used to produce cheese, collected from February 2016 to February 2017, as well as data for the resulting cheese, describing sensory characteristics and final ripening time	Monthly variation in the quality of dairy silo milk influences the sensory properties of the resulting cheese and its ripening time	Contribute to the understanding of factors that influence the sensory characteristics and final ripening time of cheese
V	NIR-hyperspectral (NIR-HS) imaging in predicting cheese maturity	Investigate the feasibility of NIR-HS as a non-destructive tool to visualise and predict cheese ripening time	NIR-HS image spectra were modelled using the concept "maturity index", *i.e.* age of cheeses	A combination of NIR-HS imaging and chemometric techniques can be used to visualise and predict cheese maturity	In an exploratory study, visualise variation in the cheese ripening process and predict the degree of maturation using NIR-HS images

3. Materials and methods

3.1 Study designs

3.1.1 Experimental study

In **Paper I**, a laboratory experiment using a balanced factorial design with eight treatments was conducted, including biological duplicates of milk with small and large casein micelles from four SH cows, two for each micelle size class. The calcium and/or citrate content of fresh milk samples was increased by 10% based on the initial content and milk pH was adjusted back to the initial pH. To select the four cows for this study, individual milk samples from 60 mid-lactation cows (days in milk = 170 ± 46 days), of the SRB (n=32) and SH (n=28) breeds were screened for micelle size (screening data not shown). Fresh milk samples from the selected SH cows were analysed for their composition and rennet-induced coagulation properties.

3.1.2 Full-scale trials

Individual full-scale studies were conducted in **Papers II-V**, including a full-scale commercial cheese manufacturing trial in collaboration with a cheese-producing dairy cooperative in northern Sweden. The participating farms (n=42) were located in a region between 64°2'-65°0'N and 19°3'-21°5'E. Tank milk was collected every second day from the farms (Figure 8). On one of these collection occasions every month over a period of one year, tank milk samples were collected and sent for analysis at SLU (Uppsala). On each occasion, an identical sub-sample was sent to the official milk testing laboratory (Eurofins Steins Laboratory, Jönköping, Sweden) for routine quality analysis. Information about management practices was gathered through questionnaires filled out by participating farmers. Additional information was extracted from the Swedish cow recording

scheme and updates regarding management were collected during two farm visits. Farm factors were preliminarily screened using multivariate statistical tools to identify factors that were interesting to study further with respect to their effect on milk composition.

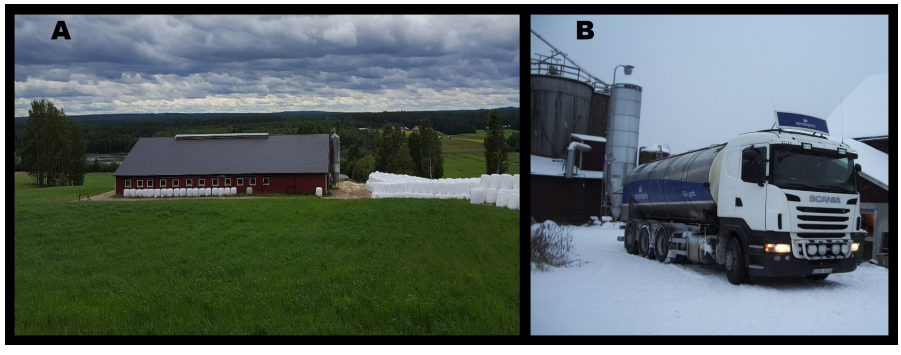

Figure 8. (A) One of the participating farms and (B) a milk truck making a collection. (Photos: Gun Bernes and Annika Höjer).

The sequence of the full-scale studies (and the resulting papers) is illustrated in Figure 9. In **Paper II,** associations between selected farm factors (*i.e*. dominant breed, milking system, housing system, number of lactating cows and energy-corrected milk yield) on different dairy farm types and milk quality were investigated. In **Paper III,** monthly variations in the same farm milk samples, collected over a year, were investigated. The participating farms and some other farms that were not part of the study, and which normally deliver their milk to the dairy processing plant, were also included in the dairy silos to maintain the required volume for producing cheese batches. The influence of the resulting variation in dairy silo milk on final sensory descriptions and ripening time is described in **Paper IV.** The cheese produced during the studies was monitored at the cheese ripening facility and evaluated for characteristic quality attributes by an industrial sensory panel at 14, 16, 18 and 20 months of ripening. In addition to the sensory evaluation, NIR-HS images of the cheeses (n=425) were captured. Acquired images were processed and chemometric models were developed to visualise the variation in ripening time. The chronological age (days) of each cheese was calculated as the difference between production date and imaging date and used in developing predictive models, as described in **Paper V**.

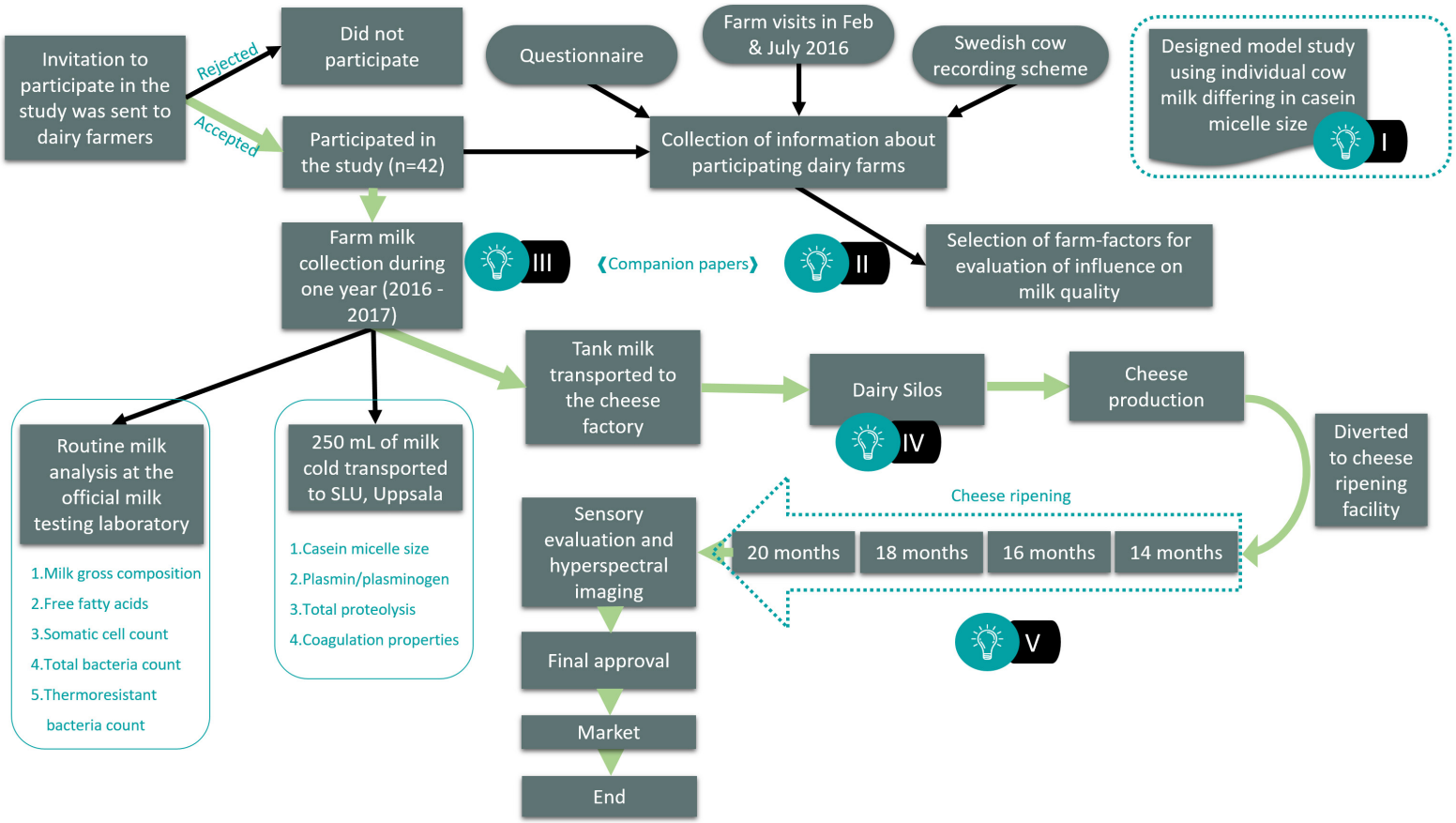

Figure 9. Schematic illustration of the full-scale study design and content of **Papers I-V**.

3.2 Milk and cheese characterisation

A summary (equipment, a short description of principle/technique, expected outcome and relevance to this study) of the analyses applied in the project and described in **Papers I-V** is presented in Table 3.

Table 3. *Overview of methods applied in the work described in this thesis*

Analysis	Analytical equipment	Technique	Expected outcome	Relevance to the study/project	Paper/s
Rheology	Bohlin CVOR-150-900 Rheometer (Malvern Instruments Nordic AB, Uppsala, Sweden)	Gel formation was studied with a small amplitude oscillation test at a frequency of 1 rad/s and a strain of 0.01	Rennet coagulation time in seconds from point of addition of rennet until gel strength reaching 1 Pa, and gel strength at 20 min after rennet addition	Studied the variation in gel formation dynamics, and evaluated the influence of milk composition and properties on rennet-induced coagulation properties	**I** **II** **III** **IV**
Micro-structures	Zeiss LSM780 inverted confocal microscope (Carl Zeiss, Germany)	Laser beam consisting of Argon 488 (505-560 nm emission spectrum) was used for sequential scanning of coagulum stained with Fast Green	Fast Green dye electrostatically attracted to the charged groups on the protein network allowed visualisation of microstructure differences in gel formation	Evaluated differences in gel microstructure due to varying casein micelle size, calcium and citrate contents and cross-checked rheological observations	**I**
Milk pH	pH meter (Seven Compact S210, Mettler-Toledo, Switzerland)	Measure the voltage differences and deduce pH values	Milk pH values	Evaluated pH for the purposes of quality control of the collected milk samples, *e.g.* for spoilage, and as milk quality variable, *e.g.* signs of mastitis infection (farm milk quality)	**I** **II** **III** **IV**

Analysis	Analytical equipment	Technique	Expected outcome	Relevance to the study/project	Paper/s
Milk composition	FTIR LactoScope with a CombiScope FTIR 300 device (Delta Instruments, The Netherlands)	Fourier-transform infrared (FTIR) spectral analysis (identifying molecular fingerprints of specific milk components based on the infrared absorption bands)	Total fat, protein, lactose, and total solids in experimental milk samples	Compared the composition of the selected milk samples differing in casein micelle size in the experimental study.	**I**
	Milkoscan CombiFoss 6000 (Foss, Hillerød, Denmark)		Total fat, protein, lactose, urea, and free fatty acids in milk samples (lactose was not analysed in silo milk)	Evaluated the effect of farm type and month on the gross composition of farm and dairy silo milk samples	**II** **III** **IV**
Citrate content	Milkoscan FT2 (Foss Electric, Hillerød, Denmark)	Fourier transform infrared (FTIR) spectroscopy	Native and elevated citrate content in experimental milk samples	Citrate content was one of the experimental parameters	**I**
Total calcium	FMS26 ICP-OES (Spectro Analytics, Germany)	Inductively coupled plasma optical emission spectrometry (ICP-OES)	Native and elevated calcium content in experimental milk samples	Calcium content was one of the experimental parameters	**I**
Total bacteria count	BactoScan FC (Foss Electric, Hillerød, Denmark)	Fluorescence intensity of individual bacteria cells, measured using flow cytometry technique	Total bacteria count in the milk samples	Important milk quality parameter and marker of milk hygiene, also associated with enzymatic activities in the raw milk	**II** **III**
	Colony count, incubation at 30°C for approximately 72 hours	Culturing technique			**IV**
Thermo-resistant bacteria	Colony count, incubation at 55°C for approximately 48 hours	Culturing technique	Thermoresistant bacteria count in the investigated milk samples	Indicator of cleanliness and suitability of raw milk for cheese making (bacteria survive pasteurisation)	**II** **III** **IV**
Psychro-trophic bacteria	Colony count, incubation at 21°C for approximately 25 hours	Culturing technique (ISO 8552 Milk – Estimation of psychrotrophic microorganism)	Psychrotrophic bacteria count in the investigated milk samples	Psychrotrophic bacteria release heat resistant enzymes that can degrade casein and fat and give rise to flavour defects in the cheese	**IV**

Analysis	Analytical equipment	Technique	Expected outcome	Relevance to the study/project	Paper/s
Somatic cell count (SCC)	CombiScope FTIR 300 (Delta Instruments, The Netherlands) and (Fossomatic, Foss, Hillerød, Denmark)	Based on flow cytometry and staining of somatic cell DNA. Light transmission is detected for the cell suspension on a laminar flow through a capillary tube	Number of somatic cells in the milk	Indicator of udder health and important milk quality parameter, also associated with changes in milk composition and enzymatic activities in the raw milk	**I** **II** **III** **IV**
Casein micelle size	NanoSight NS500 (Malvern Instruments, UK),	Nano-sized particles move by Brownian motion and scatter light when hit with a laser beam. Scattering is captured and nanoparticle tracking analysis calculates hydrodynamic diameter particle-by-particle, using the Stokes-Einstein equation	Quantification of the native size of casein micelles in milk and in experimental milk samples	Casein micelle size and the association with calcium and citrate levels in milk are of interest in milk coagulation. The natural variation in casein micelle size and its association to farm factors and season is of interest in evaluation of the coagulation properties of the milk	**I** **II** **III** **IV**
Capillary electro-phoresis	Agilent G 1600AX (Agilent Technologies Co., Kista, Sweden)	Electrokinetic separation of molecules according to size and charge inside a capillary tube	Individual milk proteins were identified and their relative concentration was expressed as a percentage of the total integrated area in the electropherogram	Evaluated and compared average relative concentrations of individual milk proteins in milk differing in casein micelle size	**I**
Plasmin and plasminogen derived activity	Multi-mode microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany)	Plasmin activity was measured using pyro-GLU-Phe-Lys-p-nitroanilide hydroxy chloride. Plasminogen-derived activity was derived after activation with urokinase	Plasmin and plasminogen-derived activities in raw milk (both farm and silo level)	Plasmin is an important proteolytic enzyme in the maturation of some cheese types. Evaluated the influence of farm factors and month on plasmin/ plasminogen activity in farm milk, and the association between plasmin activity in dairy silo milk and cheese ripening time	**II** **III** **IV**

Analysis	Analytical equipment	Technique	Expected outcome	Relevance to the study/project	Paper/s
Total proteolysis	Perkin-Elmer LS55 luminescence spectrometer (Waltham, MA, USA)	Determines the free amino terminals based on the reaction of primary amino groups with fluorescamine	Emitted fluorescence values are fitted to a leucine standard curve and related to the level of total proteolysis	The level of free amino terminals in milk is an indirect indication of total proteolysis, contributed by both indigenous and endogenous proteolytic enzymes	**II** **III** **IV**
Free fatty acids (FFA)	Milkoscan CombiFoss 6000 (Foss, Hillerød, Denmark)	FTIR spectral analysis	Concentrations of FFA levels in raw milk	FFA concentration is related to the level of lipolysis with an impact on raw milk and product quality	**II** **III** **IV**
Fatty acid (FA) composition	CP 3800 Gas chromatograph (Varian AB, Stockholm, Sweden)	Extraction (hexane: isopropanol, 3:2) and methylation with BF_3. Identify and quantify fatty acids from a flow-through column with Helium gas	Relative concentrations of different milk FA	Milk FA content is known to vary *e.g.* with feeding, and may impact on cheese aroma. Examined associations between dairy silo milk composition and sensory scores of the resulting cheese	**IV**
Near-infra-red hyper-spectral Imaging (NIR-HS)	Umbio Inspector (Prediktera AB, Umeå, Sweden) line-scan pushbroom system	SisuChema SWIR (short-wave infrared) objective (Specim, Spectral Imaging Ltd., Oulu, Finland) and equipped with a HgCdTe 2-D array detector.	Each pixel in the NIR-HS image provides information based on the spectrum of that pixel. Integrating NIR-HS and multivariate analysis tools, spatial and spectral information was derived	Developing rapid and non-destructive tools to predict maturity and end-point of ripening is of great interest for efficient planning of logistics related to cheese ripening facilities	**V**
Sensory analysis	By trained industrial sensory panel	Evaluating the cheese against a standard industrial protocol considering outer appearance, flavour, smell and texture	Determining the time point when the cheese meets the characteristic flavours /appearance for approval and can leave the ripening facility	Important in evaluating organoleptic properties of cheese and predicting maturation and final ripening time of cheese over various stages of the ripening process	**IV** **V**

3.3 Statistical designs and analysis

In **Paper I**, factorial mixed model statistical analysis was performed using SPSS statistical package, version 9.4 (SPSS Inc., Michigan, USA). Pairwise differences in the least-square means of three-factor combinations were evaluated with the Tukey-Kramer method. Calcium level (0 or 1), citrate level (0 or 1), micelle size (small or large) and all interactions between these were considered as fixed factors, while individual cow was considered as a random factor. Milk composition and relative proportions of milk protein fractions were evaluated by one-way analysis of variance (ANOVA), using Minitab® 18.1 (Minitab, Inc., USA). Differences were considered significant at $P<0.05$ using the Tukey-Kramer post hoc test in all univariate analysis described in **Papers I-IV**.

Univariate analysis (ANOVA) was performed using NCSS statistical program version 9.0 (NCSS, Kaysville, Utah, USA) in **Papers II** & **III**. In **Paper II**, milk quality parameters were evaluated based on dominant breed or milking system by accounting for the interaction with repetitive measurements collected monthly. In **Paper III**, the monthly variation in all milk quality parameters was evaluated by accounting for the interaction with the milking system on the farm. Variation between the 42 dairy farms and in their milk sample characteristics was explored by principal component analysis (PCA). Data from the farm with Swedish Jersey cows as the dominant breed were excluded from the multivariate analyses (except for characterisation of farms, as presented in **Paper II**), since that farm was deemed an outlier in the farm milk dataset. Milk quality attributes (15 variables) comprising all monthly values for the 41 farms were evaluated by PCA using the software Simca 16.0 (Sartorius Stedim Data Analytics AB, Umeå, Sweden). As the model was limited to carry only the variation associated with milk quality, farm factors were merely used for interpretation. PCA score and loading plots were used to assess similarities or differences between the milk quality attributes and to relate to study variables. In addition, orthogonal projections to latent structures (OPLS) was used for studying milk properties associated with season in **Paper III**, using Simca 16.0. For the OPLS models, loading plots were inspected to identify significant factors concerning the studied responses.

In **Paper IV**, the monthly variation in silo milk characteristics was analysed by ANOVA with the Tukey post hoc test, using Minitab® 18.1. After conducting the multivariate analysis using Simca 16.0, outliers deviating by more than four standard deviations were excluded from further analysis. All milk quality characteristics (unit variance scaled and auto-transformed) were displayed in the PCA loading plot, while milk samples were visualised in the score plot. Relationships between milk quality attributes, ripening time and sensory scores were studied using OPLS analysis. A loading plot (OPLS) was inspected to identify milk quality attributes that showed a significant influence on ripening time and sensory scores of the cheese.

In **Paper V**, NIR-HS image average spectra (cheese pixels) were calculated and analysed using Breeze and Evince software (Prediktera AB, Umeå, Sweden). Because predicting maturity was performed for the whole cheeses, average standard normal variate-corrected and mean-centred spectra per cheese were considered in the multivariate analysis. Only wavelengths 1000-2400 nm were included, as values outside this range were considered noise. A projection to latent structures (PLS) discriminant model was applied to a training dataset of NIR-HS images (n=100) in developing the model. Maturity of cheese, expressed in days, was used to make the PLS calibration model. The diagnostics used for evaluation of the PLS model were coefficient of determination for the calibration (R^2) and root mean square error of calibration (RMSEC) for better predictability.

4. Results and discussion

In this section, the five key questions defined in the objectives of this thesis (see section 2) are addressed in separate sub-sections (4.1-4.5).

4.1 How are raw milk characteristics influenced by the type of dairy farm?

In **Paper II**, the variation in composition and properties of farm tank milk as influenced by on-farm factors was investigated. During the study, farms were characterised and all possible on-farm factors (n=24 variables) were subjected to a preliminary screening using multivariate analysis to identify potentially important factors influencing milk composition and properties. From those, five factors were selected for in-depth studies: (i) dominant breed (defined as the breed comprising >70% of the total herd), (ii) milking system (AMS, tiestalls or milking parlour), (iii) housing (tied or loose), (iv) number of lactating cows, and (v) energy-corrected milk yield (kg) per cow.

In the study region in northern Sweden, two major types of dairy farms were identified: 1) dairy farms with a higher number of lactating cows, loose housing, AMS or milking parlour (MP), and predominantly cows of the SH breed; and 2) dairy farms with a lower number of lactating cows, tiestalls and cows of breeds other than SH, *e.g.* SRB, Swedish Jersey (SJB), Swedish Polled (SKB) and various cross-breeds. There was a clear effect of farm type on milk quality attributes, as illustrated in Figure 10A. Milk samples from farms with tiestall milking mainly clustered to the left on principal component 1, whereas milk samples from farms with AMS or milking parlour instead clustered more to the right. This variation can be elucidated based on differences in milk composition (Figure 10B).

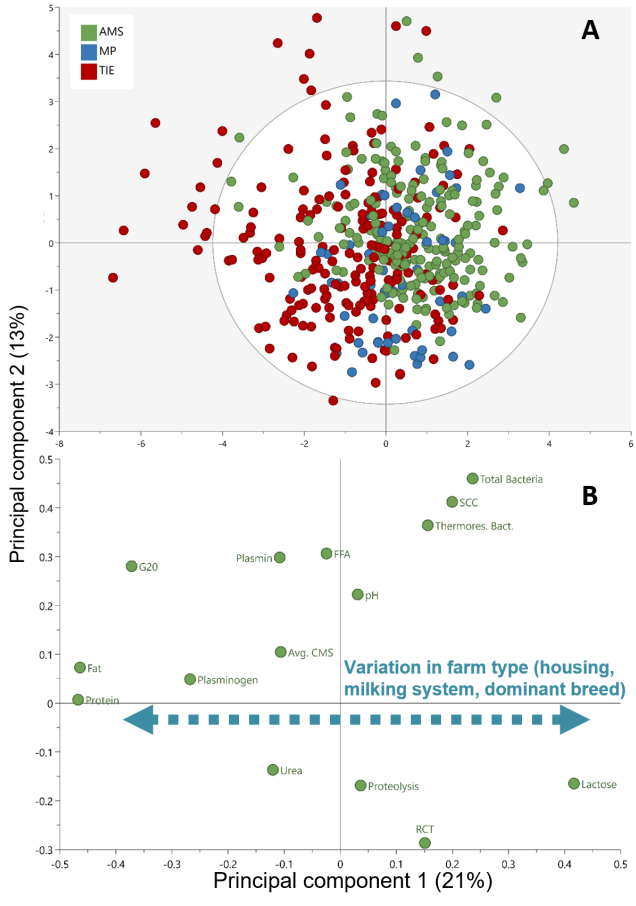

Figure 10. (A) Principal component analysis score plot and (B) loading plot of milk quality attributes (monthly data per farm). In the score plot, each dot represents a unique milk sample, with the colour of the dot indicating the type of dairy farm, represented by milking system. AMS: automatic milking system, MP: milking parlour, TIE: tiestall milking

Swedish Holstein was the dominant breed on participating farms, constituting the majority of the dairy cow population (Figure 11C). At the time of the studies, SH was the most common dairy cow breed in Sweden, according to Frössling *et al*. (2017), followed by SRB, representing 50% and 44%, respectively, of the Swedish dairy cow population. Three years later, in 2020, the dominance of the SH breed had increased to 57%, compared with 33% for SRB at national level (Växa Sverige, 2020), which has likely influenced Swedish dairy silo milk composition. Breed, housing and milking

system were cofounded factors, and individual effects of these or other factors related to the type of dairy farm, here categorised according to milking system, could not be separated in the analysis. The full diversity of cow breeds on the participating farms is shown in Figure 11D-11F. SRB and mixtures of breeds were dominant on farms with tiestall systems, while SH dominated on farms with AMS or MP.

Differences in fat and protein content were observed between the farm types (Figure 11G and 11H, respectively), with higher values for milk from tiestall farms compared with milk from farms with AMS or MP. Similarly, Johansson *et al*. (2017) found lower fat and protein content in bulk milk from AMS farms compared with farms milking twice daily using milking parlours, and attributed this to the higher milking frequency associated with automatic milking. Likewise, in **Paper II** the lower milking frequency on tiestall farms compared with AMS farms could explain the differences, since milking frequency has been shown to be negatively correlated to fat and protein content (Løvendahl & Chagunda, 2011). All farms characterised by tiestall milking or MP in **Paper II** milked their cows twice a day, while the daily milking frequency on AMS farms was on average 2.7, according to questionnaire responses. The observed differences in fat and protein content might also have been associated with the difference in dominant breed on the different farm types. For example, Larsen *et al*. (2010) and Wedholm *et al*. (2006) found higher fat and protein content in milk from SRB compared with milk from SH cows, which was the dominant breed in AMS and MP herds in **Paper II**. In contrast, lactose concentration in milk was lower in milk from tiestall farms (Figure 11I), probably due to the lower milk yield associated with this farm type. A strong genetic correlation between lactose yield and milk yield was reported by Haile-Mariam & Pryce (2017)

Higher FFA content is commonly associated with higher milking frequency (Klungel *et al*., 2000; Wiking *et al*., 2006), since disruption of the milk fat globule membrane as a result of mechanical stresses associated with AMS can lead to exposure of triglycerides to lipase (Hogenboom *et al*., 2019). Consequently, milk from farms using AMS could be expected to have a higher FFA content. However, it was found that the FFA content in milk from tiestall farms was similar to that in milk from AMS farms, but higher than that in milk from MP farms (Figure 11J). This could be due to differences in mechanical treatment of the milk on-farm, such as pumping, bubbling and post-handling time (Koning *et al*., 2003).

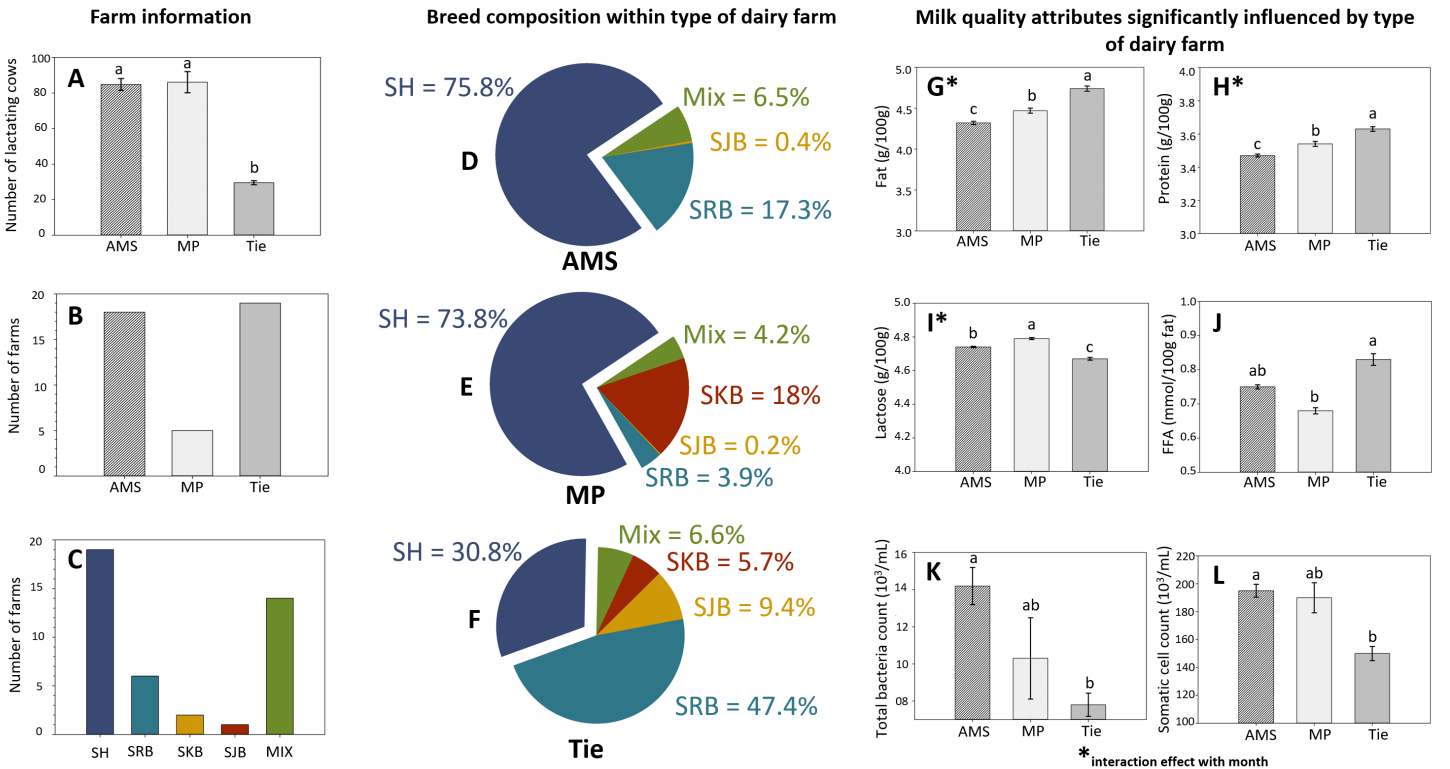

Figure 11. (A-C) Descriptive information about the participating farms, (D-F) distribution of breeds (Swedish Holstein (SH), Red (SRB), Polled (SKB), Jersey (SJB)) within type of dairy farm, here categorised according to milking system (automatic (AMP), parlour (MP), tiestall (Tie)), and (G-L) impact of dairy farm type on milk composition. *Significant interaction with month.

It could also be due to breed differences, since milk from SRB and other cross-breeds, which dominated on tiestall farms, showed elevated levels of FFA. In **Paper II**, it was not possible to distinguish whether the elevated FFA levels were an effect of milking system or breed. Other studies have also found no differences in FFA levels in bulk milk from AMS and milking parlours (*e.g.* Johansson *et al*., 2017).

Although total numbers of bacteria in farm milk were generally very low (**Paper II**), significantly higher numbers were observed in milk from herds with AMS than in milk from tiestall herds (Figure 11K). The SCC in milk was low on average, but higher in milk from farms with AMS than in milk from tiestall farms (Figure 11L). High SCC in bulk milk is associated with reduced curd firmness and cheese yield, increased losses of fat and protein in whey, and compromised sensory properties in cheese, so a premium is paid for raw milk with a low content of SCC (Barbano *et al*., 2006). A review by Svennersten-Sjaunja & Pettersson (2008) suggested that higher SCC is linked to several factors, some related to AMS *per se*, *e.g*. variation in the length of milking interval, and some related to herd management on AMS farms. Using data extracted from the Swedish Official Milk Recording Scheme, Frössling *et al*. (2017) showed increased incidence of elevated SCC associated with AMS.

The results for the milk quality attributes investigated, expressed as averages of monthly data from the participating herds and categorised according to the dominant breed (>70% of herd) on the farm, are shown in Table 4. Despite the interaction with month, milk from Swedish Jersey cows exhibited higher protein and fat percentages, 4.06% and 6.21%, respectively, compared with milk from SH cows (3.50% and 4.34%, respectively) and SRB cows (3.65% and 4.84%, respectively). These results are in agreement with Poulsen *et al*. (2013), who found higher fat and protein content in milk from Danish Jersey than from SRB or Holstein. Moreover, Bieber *et al*. (2019) and Larsen *et al*. (2010) found that milk from SRB contained higher fat and protein concentrations compared with milk from SH. Lactose content was highest in milk from Swedish polled (SKB) cows (Table 4), an unexpected finding since SKB is a small, indigenous cow breed with average milk yield around 50% of that in SH cows (Växa Sverige, 2021).

Table 4. *Quality attributes of farm tank milk. Averages of monthly data categorised according to the dominant breed on the farm*

	SH (n=19)		SRB (n=6)		SJB (n=1)		SKB (n=2)		MIX (n=14)		Main effect	Inter-action
	Mean ± S.E.	(N)	Mean ± S.E.	(N)	Mean ± S.E.	(N)	Mean ± S.E.	(N)	Mean ± S.E.	(N)	P	P
∑cows	86.1 ± 3.47	(200)	27.4 ± 1.47	(57)	49.7 ± 1.75	(12)	32.1 ± 2.71	(24)	41.2 ± 2.15	(157)	*	*
DMY	32.7 ± 0.25	(200)	31.7 ± 0.62	(57)	20.5 ± 0.54	(12)	16.5 ± 0.56	(24)	30.0 ± 0.45	(157)	0.23	0.22
Fat	4.33 ± 0.017	(227)	4.85 ± 0.038	(72)	6.21 ± 0.078	(11)	4.46 ± 0.026	(25)	4.54 ± 0.028	(170)	*	*
Protein	3.50 ± 0.008	(227)	3.66 ± 0.023	(72)	4.06 ± 0.027	(11)	3.47 ± 0.033	(25)	3.55 ± 0.014	(170)	0.07	*
Lactose	4.76 ± 0.004	(222)	4.64 ± 0.008	(71)	4.39 ± 0.013	(11)	4.83 ± 0.016	(25)	4.70 ± 0.008	(165)	*	*
Urea	3.98 ± 0.050	(227)	4.35 ± 0.099	(72)	3.58 ± 0.164	(11)	3.17 ± 0.174	(25)	4.12 ± 0.079	(170)	0.24	0.12
FFA	0.74 ± 0.006	(227)	0.82 ± 0.030	(72)	0.64 ± 0.011	(11)	0.78 ± 0.027	(25)	0.81 ± 0.018	(170)	0.47	0.21
PL	2.98 ± 0.090	(157)	3.00 ± 0.188	(47)	3.43 ± 0.352	(8)	2.97 ± 0.300	(17)	2.97 ± 0.097	(117)	0.99	0.25
PG	64.2 ± 0.90	(158)	69.6 ± 1.89	(50)	75.6 ± 4.64	(8)	63.9 ± 3.29	(17)	63.8 ± 1.09	(117)	0.43	*
Pro.lysis	32.6 ± 0.50	(188)	32.5 ± 1.12	(59)	34.9 ± 2.69	(8)	31.2 ± 1.07	(22)	32.1 ± 0.56	(143)	0.73	0.25
pH	6.72 ± 0.004	(205)	6.71 ± 0.008	(63)	6.73 ± 0.020	(10)	6.74 ± 0.015	(25)	6.72 ± 0.005	(158)	0.35	*
SCC	185 ± 4.5	(227)	134 ± 7.8	(72)	183 ± 18.1	(11)	208 ± 22.2	(25)	172 ± 6.3	(170)	0.39	*
TBC	11.5 ± 0.95	(206)	7.4 ± 0.77	(69)	7.5 ± 1.39	(11)	10.5 ± 2.30	(25)	12.0 ± 1.14	(159)	0.77	0.92
TRBC	1322 ± 203.3	(141)	1928 ± 527.3	(46)	387 ± 117.2	(8)	2947 ± 645.5	(19)	934 ± 158.1	(116)	0.55	0.58
CMS	140 ± 2.8	(184)	134 ± 5.1	(57)	130 ± 9.2	(9)	136 ± 7.3	(23)	136 ± 3.3	(144)	0.92	0.99

SH, SRB, SJB, SKB: >70% of cows on-farm Swedish Holstein, Swedish Red, Swedish Jersey, Swedish Polled, respectively. MIX: mixture of breeds with less than 70% of one breed, S.E.: standard error, n: number of farms based on dominant breed as average over the year, N: number of milk samples analysed, P: probability, Main and interaction effects with monthly repeated measurements are indicated by P-values and * indicates significance at ($P<0.05$), ∑cows = number of cows per farm, DMY: Daily milk yield (kg of ECM/cow), Fat, protein, lactose: g/100g. Urea: mmol/L, FFA: free fatty acids (mmol/100g fat), PL: plasmin (units/mL), PG: plasminogen (units/mL), Pro.lysis: total proteolysis (mM Leuc. Eq.), SCC: somatic cell count (10^3/mL), TBC: total bacteria count (10^3/mL), TRBC: thermo-resistant bacteria (number/mL), CMS: casein micelle size (nm).

In contrast to findings by Johansson *et al*. (2017), there were no significant differences in plasmin activity when comparing milk from farms categorised according to breed (Table 4). Bastian *et al*. (1991) also found no differences in plasmin activity on studying milk samples from Holstein and Jersey cows monthly over 10 months of lactation, and concluded that lactation number had the greatest influence on plasmin activity. Categorising participating farms according to milking system or dominant breed revealed no influence on total proteolytic activity (**Paper II**). Similarly, Karlsson *et al*. (2017) observed no differences in total proteolytic activity in dairy silo milk from outdoor (June and July) and indoor periods in northern Sweden. In **Paper II**, milk from SRB herds had lower numerical values of SCC (average 134 000 cells/mL, than milk from herds with other dominant breeds. Persson-Waller *et al*. (2009) also found that SRB cows had lower SCC in milk compared with SH cows, due to better udder health, inherent mastitis resistance and efficient immune defence.

The total number of bacteria in raw milk is typically known to be associated with microbial contamination within the udder, during and after milking, cleaning and sanitising practices, and raw milk storage temperature and time combination (Bramley & Mckinnon, 1990). According to the literature, SRB cows generally have a lower incidence of veterinary-treated clinical cases of mastitis than SH cows (Bieber *et al*., 2019; Nyman *et al.*, 2007). Since cows with mastitis can potentially shed higher numbers of bacteria into the milk compared with healthy cows (Bramley & Mckinnon, 1990), milk from SRB cows is likely to have a lower total bacteria count than milk from SH cows. Numerically lower total bacteria count in milk from SRB cows may also be associated with lower SCC. This is explained by the fact that elevated SCC in milk is typically caused by infection by mastitis bacteria, resulting in higher total bacteria count (Fenlon *et al*., 1995). The high levels of thermo-resistant bacteria in milk from farms with SKB as the dominant breed (Table 4) were probably a farm effect, rather than an effect of the breed. Since there were only two farms with SKB as the dominant breed, the results need to be interpreted with caution. There were no significant differences in casein micelle size between milk from the different breeds in **Paper II** (Table 4). Similarly, larger numerical values, but no significant differences in casein micelle size in milk from SH (200 nm) compared with SRB cows (191 nm) has been reported by *Glantz et al.* (2010).

4.2 To what extent do quality characteristics of farm and dairy silo milk vary with month?

The monthly variation in composition and properties of farm and dairy silo milk was determined. Although silo milk was a result of mixing milk from the participating farms, it was too complicated to derive direct relationships between farm milk and dairy silo milk. There were several reasons for this, *e.g*. the silo milk (described in **Paper IV**) also contained milk from additional farms apart from the farms participating in the project (as described in **Papers II** & **III**). Larger farms, delivering larger volumes of milk, typically had SH cows. This breed would thus have had a greater influence on the composition and properties of the silo milk. In addition, pumping, transportation and additional short-term refrigerated storage in dairy silos would have affected the quality characteristics of dairy silo milk, as they could have differed from those applied to farm tank milk.

There was clear variation in milk quality attributes with sampling month, based on separate averages for farm and dairy silo milk (Figure 12). Similarly, Lindmark-Månsson (2012) found that Swedish dairy silo milk composition was influenced by season. Protein concentrations in milk were lower during May-August (Figure 12A) than September-April (Figure 12B), with the highest and lowest protein content in farm milk being observed in November and June, respectively, despite interactions with milking system (**Paper III**). In dairy silo milk, the highest fat and protein concentrations were observed in November-December and the lowest in August (**Paper IV**). Karlsson *et al*. (2017) also observed higher protein content in November-December than in the rest of the year when studying unprocessed dairy silo milk in the same region. There was a tendency for fat content to be lower in farm milk delivered in May-August than in other months, but the differences were not significant. Within-year variation in dietary concentrate fraction on farms would affect milk production and synthesis of fat and protein, and thereby the composition and properties of the milk (Heck *et al*., 2009; Linn, 1988).

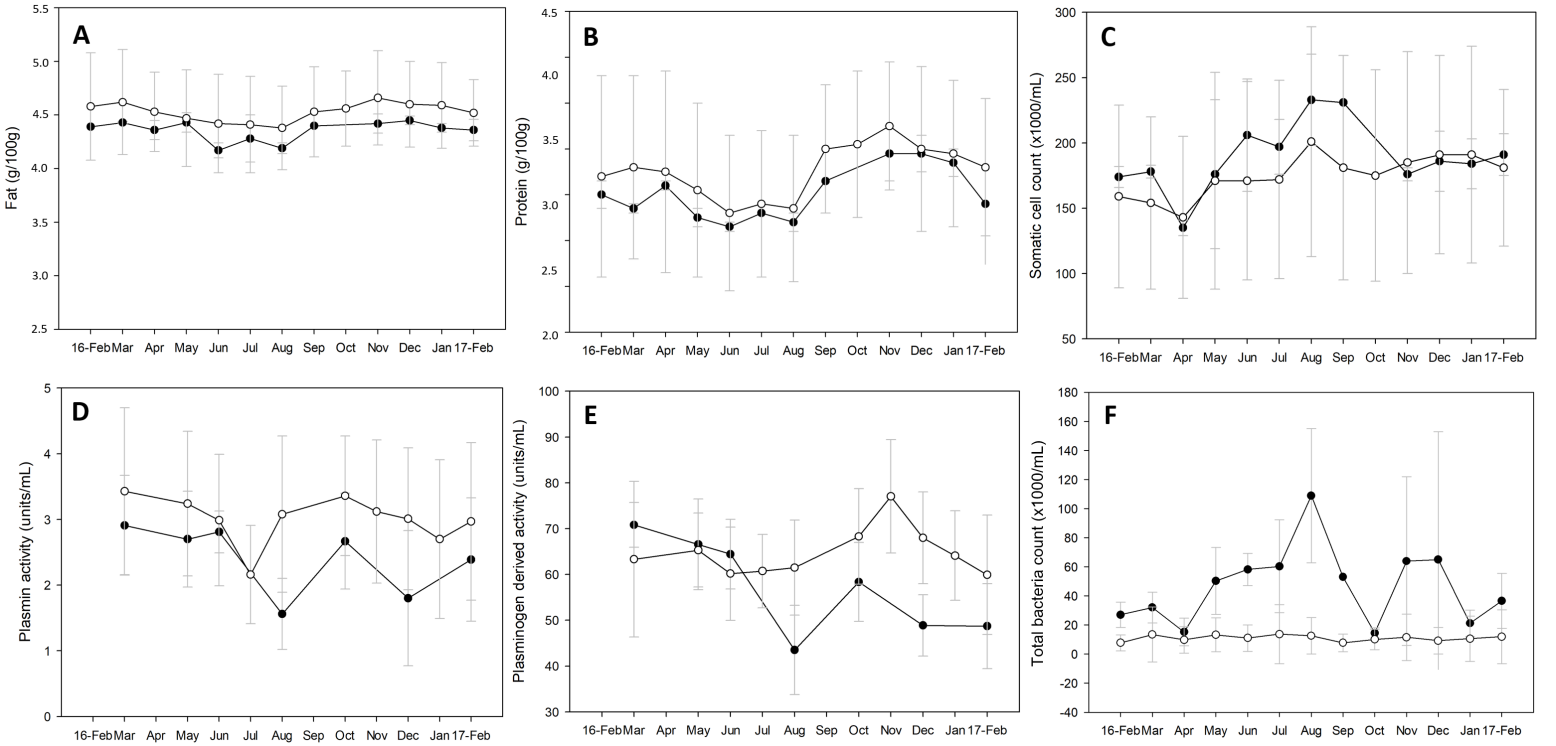

Figure 12. Variation in milk quality attributes that were significantly affected by month. (A) Fat content, (B) protein content, (C) somatic cell count, (D) plasmin activity, (E) plasminogen-derived activity and (F) total bacteria count. Filled circles indicate dairy silo milk and empty circles indicate farm milk. Bars indicate standard deviation.

Average urea concentration did not show any monthly variation in farm milk (**Paper III**), or dairy silo milk (**Paper IV**). The FFA concentration did not vary with month in dairy silo milk (**Paper IV**), but showed clear variation in farm milk (P**aper III**), with the highest FFA content observed in February-2017 (0.86 mmol/100g fat). The SCC values were generally low throughout, with the highest average SCC values in both farm and dairy silo milk observed in August (201 x10^3 and 233 x 10^3/mL, respectively) (Figure 12C). The SCC values were within the range (140-230 × 10^3 cells/mL) reported for dairy silo milk from the same region by Karlsson *et al*. (2017). Frössling *et al*. (2017) observed an increase in SCC during the latter part of the pasture season (August-September) in milk samples from dairy cow herds enrolled in the Swedish Official Milk Recording Scheme. Higher SCC during late summer may be due to higher incidence of sub-clinical mastitis, explained by seasonal differences in the occurrence of mastitis pathogens (Olde Riekerink *et al*., 2007).

Plasmin activity and plasminogen-derived activity varied with sampling month in both farm and dairy silo milk (Figure 12D and 12E, respectively). Plasmin activity in farm milk ranged from 2.16 units/mL in July to 3.43 units/mL in March, while plasminogen-derived activity varied from 59.93 units/mL in February 2017 to 77.06 units/mL in November (**Paper III**). Plasmin activity and plasminogen-derived activity in dairy silo milk showed a similar pattern, but the variation was more accentuated (**Paper IV**). The monthly variation in plasmin activity (Figure 12D) and plasminogen-derived activity (Figure 12E) did not show any clear seasonal pattern. Similarly, in a study examining the effect of season on plasmin activity in milk from pasture-fed dairy cows in New Zealand, Nicholas *et al*. (2002) found that time of year did not influence plasmin activity. However, they observed an effect of season on plasminogen-derived activity, with higher values in late spring, when cows were in late lactation. They concluded that plasmin activity and plasminogen-derived activity are not strongly influenced by milk yield or by feed quality and quantity, and that the effect of lactation stage is greater than that of time of year (Nicholas *et al*., 2002). Considering that year-round calving is applied in Sweden, the lack of a clear seasonal pattern in plasmin activity in **Papers III** & **IV** is not surprising. Similarly, Karlsson *et al*. (2017) did not observe a significant influence of season on plasmin/plasminogen-derived activities in dairy silo milk.

Total bacteria count in farm milk (Figure 12F) did not differ between sampling months (**Paper III**), indicating uniform and good hygiene conditions on the participating farms throughout the year. The variation was higher in dairy silo milk, with the highest value (109 x10^3 colony forming units (CFU)/mL) observed in August (**Paper IV**). Low numbers of bacteria result from proper handling of raw milk, as well as good hygiene and cleaning routines on-farm (Guerra *et al*., 2013). The higher total bacteria count in dairy silo milk is possibly due to longer cold storage of the milk compared with milk sampled on farms, which might particularly favour the growth of psychrotrophs during summer. The elevated total bacteria count could also stem from milk delivered from farms with SH as the dominant breed, since their milk contributed most to the dairy silo milk, as such farms were larger than farms with other breeds (**Paper II**). The average total bacteria count in dairy silo milk varied between 15 and 109 x10^3 CFU/mL, *i.e*. it was higher than that in farm milk (7-13 x10^3 CFU/mL).

To evaluate the effect of the month on different milk quality attributes, an OPLS analysis was performed (Figure 13A). The results showed that casein micelle size and proteolysis were the attributes most influenced by month, followed by lactose and protein content, pH, SCC and thermo-resistant bacteria count. Since the effect of sampling month was most pronounced for casein micelle size and total proteolysis, variation over months was plotted for these two variables (Figure 13).

In **Papers I-IV**, nanoparticle tracking analysis (NTA) was used to measure casein micelle size, whereas many other published studies use dynamic light scattering techniques. The NTA method detects particles in the nano-range, with less interference by larger particles, which is otherwise often a problem for commonly used techniques, *e.g*. dynamic light scattering, when used to measure the size of casein micelles. The NTA device counts each particle that moves through the laser beam and shows the distribution of particle concentrations of varying sizes. The individual particles moving in Brownian motion are detected in real-time and tracked by specific image tracking software to estimate particle size distribution by assuming the sphere shape of the particles. The particle concentration is derived by counting these tracks. Therefore, this technique enables detection of smaller particles without interference from larger particles, *e.g*. particle size distribution is not intensity-weighted, which is a common constraint in dynamic light scattering systems (Gross *et al*., 2016).

Average casein micelle size ranged from 72 nm in August to 184 nm in February 2017 (**Paper III**). There was a trend for smaller casein micelles between May and October than in other months (Figure 13C). However, results for the milk samples collected in September showed an abrupt increase, and very large variation, in casein micelle size. This is possibly explained by the large variation between farms in cows being indoors or outdoors (Figure 13B), with September being the transition month for many of the cows from outdoor to indoor feeding.

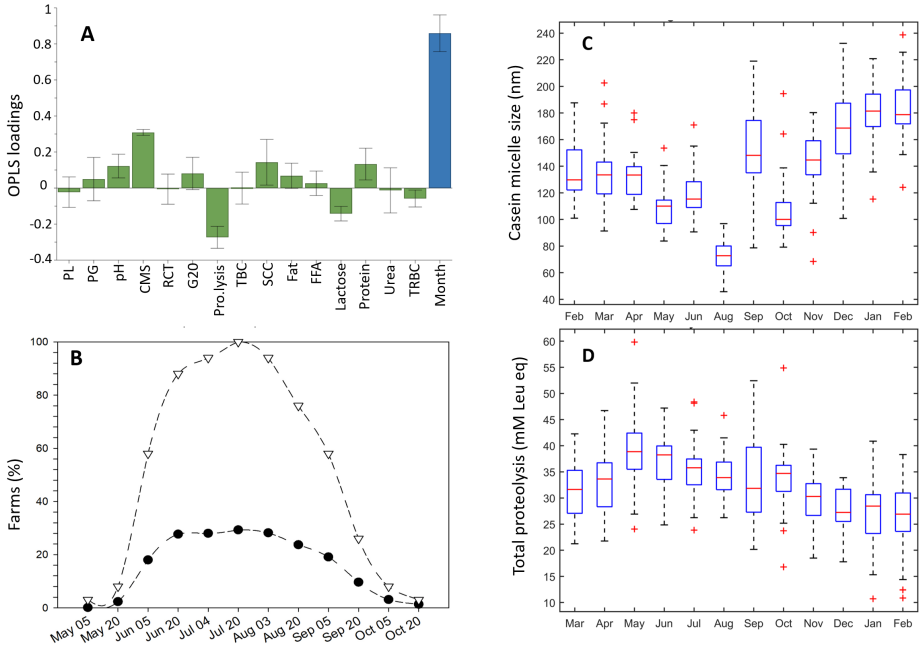

Figure 13. (A) Orthogonal projections to latent structures (OPLS) predictive component loadings for milk quality attributes (green bars, height indicates level of influence) in response to month (blue bar) (confidence intervals crossing 0-line indicate no association). PL: plasmin, PG: plasminogen, CMS: casein micelle size, RCT: rennet coagulation time, G20: gel strength, Pro.lysis: total proteolysis, TBC: total bacteria count, SCC: somatic cell count, FFA: free fatty acids, TRBC: thermo-resistant bacteria. (B) Percentage of farms with cows outdoors in different months (triangles) and contribution of pasture (%, circles) to total cow feed intake in the outdoor period. (C, D) Box and whisker plots of monthly variation in mean casein micelle size and total proteolysis (median, interquartile range, spread, outliers) for tank milk from participating farms (n=41).

Holt and Muir (1978) were the first to report seasonal variation in casein micelle size, with significantly smaller micelles in summer compared with other seasons. A similar trend, with smaller micelles in milk sampled during June, July and August, was observed by Chen *et al*. (2014) in a study with seasonal calving as the dominant farming system. Since the size of casein micelles in milk from individual cows is suggested not to be influenced by stage of lactation or protein content (Bijl *et al*., 2014; de Kruif & Huppertz, 2012), the variation recorded by Chen *et al*. (2014) and Holt and Muir (1978) must be associated with other factors. Casein content, casein:protein ratio, genetic variants of caseins and pH (Devold *et al.*, 2000; Glantz *et al*., 2010) have been reported to affect casein micelle size, while calcium and citrate concentrations had an influence in **Paper I**.

The difference in casein micelle size observed in **Paper III** may partly be explained by differences in casein-bound calcium, since micelle size is negatively correlated with casein-bound calcium and positively correlated with colloidal phosphate (Holt & Muir, 1978). A similar relationship was observed in the experiment in **Paper I,** where adding calcium to milk resulted in smaller casein micelles. The concentration of ionic calcium [Ca^{2+}] levels is generally higher in milk derived from pasture feeding than indoor feeding (Akkerman *et al.*, 2019). Organic phosphate levels are reported to be lower in small casein micelles compared with large micelles (Bijl *et al*., 2014). Moreover, Lin *et al*. (2017) observed lower levels of phosphorus in milk serum during summer compared with winter in spring-calving bulk milk. The levels of citrate in bulk milk are reported not to vary with sampling month or season (Akkerman *et al*., 2019; Chen *et al*., 2014; Karlsson *et al*., 2017). However, Garnsworthy *et al*. (2006) found that citrate content varied with stage of lactation and was related to *de novo* synthesis of fatty acids. In **Paper I**, increasing the level of citrate in milk by 10% increased the average casein micelle size by 6.7% for small-sized micelles (change not significant), whereas no noticeable effect was observed for large-sized micelles. Thus, increasing level of total calcium, decreasing level of phosphorus and relatively stable citrate level during summer months are likely explanations for the differences in casein micelle size observed in **Paper III**, although unfortunately these minerals were not analysed in that study.

Variation in total proteolysis in milk over one year is shown in Figure 13D. Total proteolysis was higher during May-September compared with the rest of the year, with a weak pattern suggesting that the variation in proteolysis was inversely related to casein micelle size. The values for total proteolysis in milk showed the largest variation in September, in parallel with the high variation in casein micelle size for that month. As previously discussed for casein micelle size, the higher variation in total proteolysis may be due to the greater variation between farms in the management of cows in September. In that month, some farms still had their cows outdoors with access to pasture, whereas other farms already had their cows indoors (Figure 13B). SCC was highest in farm milk in August (see Figure 12C), and slightly higher SCC in milk during the outdoor months may have contributed to the increase in total proteolysis, since elevated SCC is associated with proteolytic activity in milk (Senyk *et al*., 1985). In contrast, Karlsson *et al.* (2017) found no difference in total proteolytic activity in dairy silo milk between outdoor (June and July) and indoor periods in northern Sweden. However, this observation is likely to vary between summers, since a rainy and wet summer may perhaps have a different effect on SCC than a dry and warm summer (Alhussien & Dang, 2018).

In **Paper III**, the association between seasonal effects on milk quality attributes and pasture feeding was evaluated. There was a tendency for separation of milk samples originating from herds with cows fed indoors from milk samples originating from herds with cows with access to full or limited grazing (data not shown). However, on evaluating the data for milk samples collected only during the summer months, there was no clear separation of milk samples according to degree of grazing on participating farms (data not shown). Hence, grazing was not the only factor giving rise to the observed seasonal variation in **Paper III**. The lack of major variation in milk properties over the year confirmed that the raw milk sourced from farms in the region is suitable for cheese making on a year-round basis, at least from the perspective of the initial coagulation process. It is important to point out, however, that **Paper III** only examined milk composition and some enzymatic activities, not variation in milk microbiota, and overall conclusions are thus limited.

4.3 How is rennet-induced milk coagulation affected by variation in raw milk characteristics?

Milk coagulation, which is the first step in cheese making, has a significant impact on cheese yield and on the properties of the cheese. Hence, the effects of milk composition on milk coagulation were the main focus in **Paper I** and were also investigated in **Papers II-IV**. Repeated surveys have shown that raw milk composition of Swedish dairy milk has changed over the years (*e.g.* Lindmark-Månsson, 2012), *e.g.,* the content of calcium has increased, and the citrate content has decreased, over the years. However, the impact and interactions of these changing factors on the milk coagulation process had not been not studied prior to this thesis work. Therefore, **Paper I** investigated interactive effects of casein micelle size, calcium content and citrate content on rennet-induced coagulation in bovine milk.

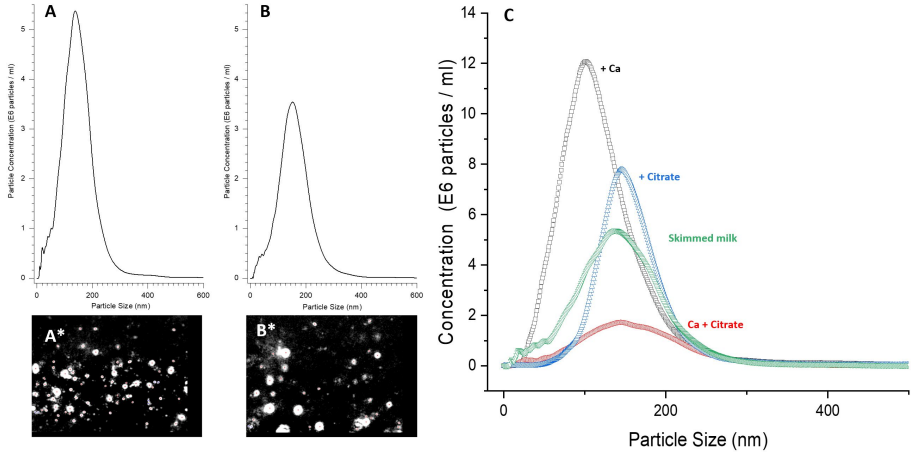

Figure 14. Measurement of casein micelle size using nanoparticle tracking analysis (NTA). Casein micelle size distribution and the corresponding NTA video frame (panels A* and B*) for milk with (A) smaller and (B) larger casein micelles and (C) effect of adding calcium, citrate and/or to skimmed milk on average casein micelle size distribution.

Particle concentration was higher in milk with smaller casein micelles than in milk with larger micelles (Figure 14A and 14B). Adding calcium and citrate, alone (10% increase related to original concentrations) or in combination, influenced the micelle size and micelle size distribution (Figure 14C). Addition of calcium decreased particle size, but increased the number of particles compared with addition of citrate

In practical cheese making, calcium is often added to the cheese milk, and therefore the inherent roles associated with functions and properties of calcium, citrate and casein micelles are likely to be influenced. The results of the experimental study described in **Paper I** can therefore improve understanding of the coagulation process as affected by variations in quality parameters in the milk used for cheese making.

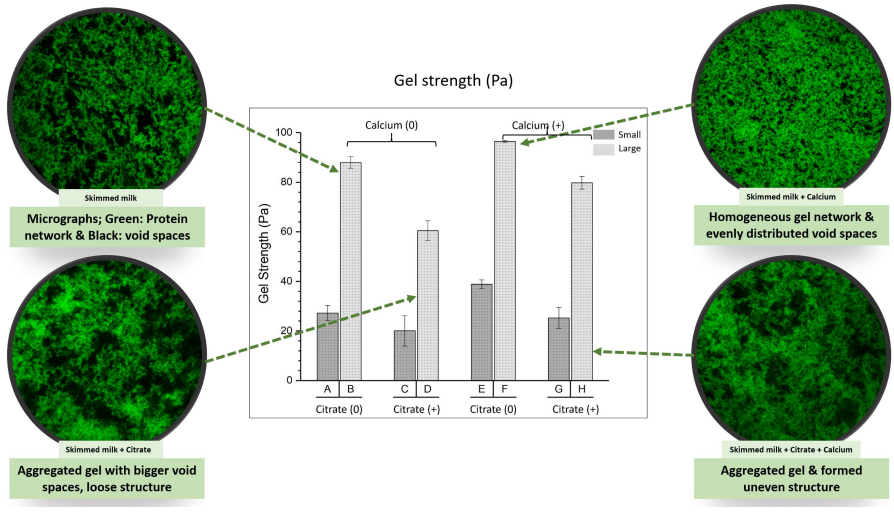

Figure 15. Effect of adding citrate, calcium and both citrate and calcium to skimmed milk with smaller and larger casein micelles on gel strength measured after 20 minutes of rennet addition. The respective confocal laser scanning micrographs of the resulting gels (left and right) show the protein network and pore spaces without protein network, visualised in green and black, respectively, for gels made with milk containing larger casein micelles.

Adding calcium resulted in a stronger gel, while adding citrate resulted in a weaker gel and longer RCT in skimmed milk (Figure 15). This is because addition of calcium creates bridges, facilitating interaction and aggregation of casein micelles and eventually increasing gel strength (Lucey & Horne, 2018). Addition of citrate instead results in chelation of free calcium in milk serum, shifting the calcium equilibrium into solution and thereby resulting in weaker gels (Griffin, Lyster & Price, 1988). In **Paper I**, it was found that milk with larger casein micelles resulted in stronger gels compared with milk with smaller micelles, in agreement with Ekstrand *et al*. (1980), but contradicting Glantz *et al*. (2010) and Logan *et al.* (2014). This inconsistency

in results might be associated with differences in measuring technique (NTA vs. dynamic light scattering), and type of sample (individual vs. bulk milk). Moreover, the results from the **Paper I** suggest that not only casein micelle size, but also particle concentrations, are important in determining the coagulation properties. Clear differences in micrographs were observed when calcium and citrate were added to the skimmed milk (Figure 15). In the presence of citrate, a loose gel network with large clumps was observed, while in the presence of calcium a denser gel network was observed. The observed differences in gel strength could be tracked from the micrographs, *i.e*. adding calcium resulted in an evenly distributed gel network with smaller open spaces, as well as a stronger gel. In **Paper I,** micelle size and citrate content also interacted, altering the resulting gel strength. Larger micelles in milk with native citrate levels formed a firmer gel than higher citrate levels in milk with smaller micelles. Thus, a higher citrate content may reduce gel strength to a greater degree for milk with larger micelles compared with milk with smaller micelles. Since firmer gels are likely to retain more protein and fat than less firm gels, this interaction could have an impact on the yield of the resulting cheese.

In **Paper II,** it was found that gel strength at 20 min after rennet addition (G20) was influenced particularly by breed. Milk from farms with Jersey cows had on average a G20 value of 139 Pa, in comparison with 65 Pa for milk from herds with SH as the dominant breed. Jensen *et al*. (2012) also found milk from Jersey cows to be associated with superior gelation properties compared with milk from Holstein-Friesian cows, possibly due to the high protein content (4.39 g/100 g) and total casein content (3.58 g/100 g) of Jersey milk. The effect of breed was also significant when comparing the G20 associated with milk from different farm types, categorised according to their milking system (**Paper II)**. Milk from tiestall farms, where cows of breeds other than SH dominated (see Figure 11), had significantly higher G20 (79.1 Pa) than milk from farms using AMS (65.9 Pa), where SH was the dominant breed. In contrast to the effect of farm type on G20, a breed effect was not observed with RCT. Coagulation time and G20 in general mirror each other, in the sense that milk which coagulates rapidly (short RCT) also results in a firmer gel (high G20), as reported in **Papers I-IV**. Hallén *et al*. (2007), investigating rheological properties of milk samples of individual cows of the SRB and SH breeds, observed that protein concentration was positively associated with G20, but not with RCT, which

was confirmed in **Paper II**. Problems previously reported to be associated with milk from SRB cows, *e.g*. non-coagulating properties or significantly lower gel strength, in individual cow milk samples (Nilsson *et al*., 2019) and in farm milk (Gustavsson *et al*., 2014), were not encountered in **Paper II**.

To evaluate the effect of milk composition on coagulation properties, an OPLS analysis was performed (Figure 16) using the milk samples described in **Papers II** & **III**. The RCT and G20 data were located on opposite sides of the loading plot (Figure 16A). Performing separate OPLS analyses for G20 and RCT, respectively, showed that it was only possible to model the former adequately. Thus, the effect of milk composition on G20 was evaluated further (Figure 16B). The results showed that fat content and protein content were positively associated with G20, while lactose content was negatively associated with G20. However, the positive association of milk fat content with G20 was due to an artefact. Considering that G20 was measured using skimmed milk, without the interference of milk fat, the observed association between milk fat and G20 is likely resulting from the positive association between fat and protein content in milk. The other milk quality attributes analysed in this study were of less importance for G20 (Figure 16). The OPLS analysis did not suggest any relationship between casein micelle size and gel strength as previously reported in the literature (*e.g*. Glantz *et al*., 2010; **Paper I)**. This lack of relationship is likely because bulk milk samples from farms were analysed, and in many cases these samples consisted of a mixture of milk from cows of different breeds (**Papers II** & **III**). It was thus a mixture of milk from cows with varying casein micelle sizes. Bulking of milk increases the polydispersity of micelle size compared with milk from individual cows (de Kruif & Huppertz, 2012).

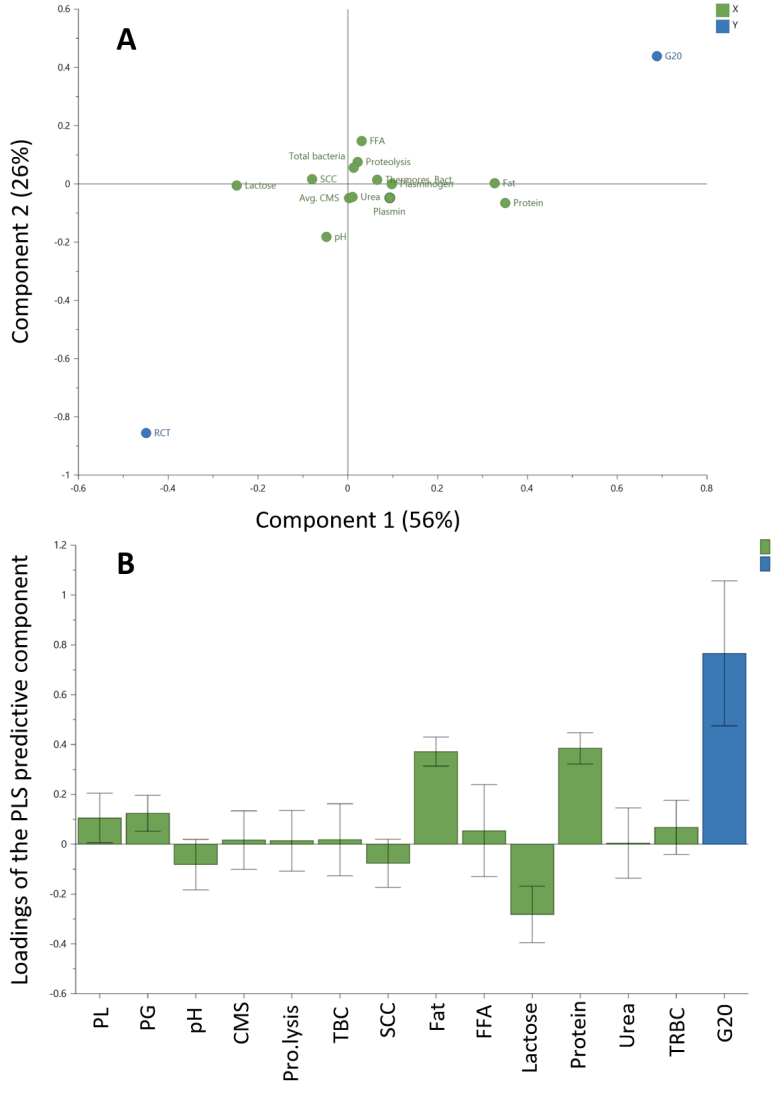

Figure 16. (A) Orthogonal projections to latent structures (OPLS) loading plot of milk quality attributes and their association with milk coagulation properties and (B) OPLS predictive component loadings of milk quality attributes (green bars, height indicates level of influence) associated with gel strength, G20 (blue bars) (confidence intervals crossing 0-line indicate no association). PL: plasmin, PG: plasminogen, CMS: casein micelle size, Pro.lysis: total proteolysis, TBC: total bacteria count, SCC: somatic cell count, FFA: free fatty acids, TRBC: thermo-resistant bacteria, G20: gel strength.

Coagulation properties of farm and dairy silo milk showed monthly variation, but no seasonality, in **Papers III** & **IV** (Figure 17). The highest average G20 value (88 Pa) for farm milk was recorded in December, and the lowest numerical value was associated with milk collected in July (64 Pa) (**Paper III)**. The shortest RCT for farm milk was also recorded in December (361 s), while the longest (529 s) was associated with milk collected in February 2017. In **Paper IV**, the highest G20 value was observed in dairy silo milk sampled in March (67 Pa), while the lowest G20 for dairy silo milk was associated with milk sampled in February 2017 (42 Pa) and July (52 Pa). In dairy silo milk, RCT ranged from 581 s in February 2017 to 450 s in May, but differences were not significant (**Paper IV)**.

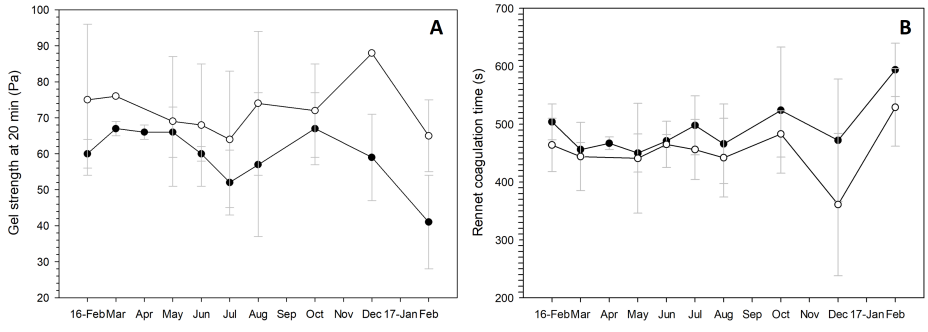

Figure 17. (A) Monthly variation in gel strength at 20 min (Pa) and (B) rennet coagulation time (s) with dairy silo milk (filled circles) and farm milk (empty circles).

In general, the higher gel strength values observed during the indoor period (Figure 17A) may result from the higher milk protein content found during the same period (Figure 12B), as explained by Panthi *et al*. (2019). In **Papers III & IV,** lower gel strength and longer coagulation time were observed for dairy silo milk than for farm milk. This was most likely due to the silo milk consisting of proportionally more milk from SH farms, since they deliver larger volumes, and this milk is associated with lower fat and protein, as reported in **Paper II**. The blending of milk with poor and good coagulating properties may have contributed to impaired coagulation properties of the dairy silo milk, in line with previous observations for individual cow milk samples of the Holstein-Friesian breed (Frederiksen *et al*., 2011).

4.4 How is cheese ripening time affected by variation in dairy silo milk characteristics?

The ripening time, *i.e.*, the time required for the cheese to develop its characteristic properties, varied between 485 and 721 days, with no clear pattern (Figure 18A). Although the variation showed no clear seasonality, the average cheese ripening time showed higher variation for cheese produced during June to October compared with cheese produced during the other months. This is the period between the transitions of cows to and from pasture feeding (see Figure 13B). It was expected that variation in the quality attributes of the dairy silo milk would be reflected in the ripening time of the resulting cheese. Relationships between dairy silo milk composition and ripening time of the resulting cheese were thus investigated using OPLS analysis in **Paper IV** (Figure 18B).

None of the milk quality parameters studied in **Paper IV** showed a significant effect on cheese ripening time. Hence, differentiated use of milk based on quality parameters for the production of premium cheese may add little benefit when the milk quality is of a high standard. However, several fatty acids showed weak influences, *e.g.* longer and shorter ripening time was associated with C14:1 and with C18:0 and C18:1, respectively. This might be because the relative concentration of C18:1 was highest in July (**Paper IV**) and the ripening time during July was comparatively shorter. C18:1 and C18:0 fatty acids are closely associated with pasture feeding. Cheese made from milk from pasture-fed cows shows elevated lipolytic activity, a more elastic and creamier texture and a yellower tone compared with cheese made from milk from silage-fed cows (Frétin *et al*., 2019). However, drawing a link between cheese ripening and milk quality aspects is challenging since, in industrial cheese production, there is a great span of process parameters and reasons for variation in cheese quality and ripening time between cheeses produced from the same vat, between vats produced within a day and between different production days. In addition, in **Paper IV** it was found that the "Smell & Taste" and "Texture" score values given in the sensory evaluation were positively associated with the plasmin activity and plasminogen-derived activity of the raw milk (data not shown). This probably due to the action of proteolytic enzymes on milk protein, resulting in flavour- and odour-active volatiles, resulting in higher sensory score values and beneficial effects on the cheese matrix, contributing to a preferred texture and higher texture score values.

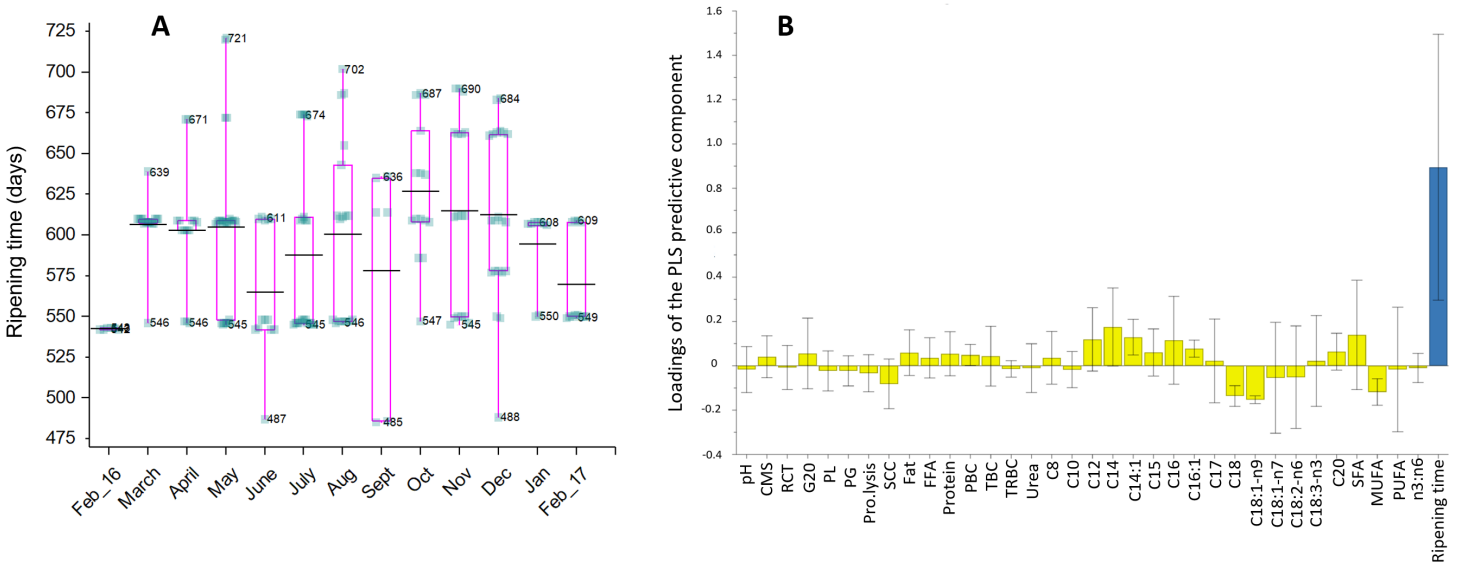

Figure 18. (A) Box and whisker plot of variation in ripening time (days) of long-ripening cheese, arranged according to production month, showing median values (black horizontal line), interquartile range (height of the box) and spread (blue squares and whiskers). (B) Orthogonal projections to latent structures (OPLS) predictive component loadings of milk quality attributes (yellow bars, height indicates level of influence) in response to ripening time (blue bars). Confidence intervals (95%) are given for each factor and the response (confidence intervals crossing 0-line indicate no association). CMS: casein micelle size, RCT: rennet coagulation time, G20: gel strength, PL: plasmin, PG: plasminogen, Pro.lysis: total proteolysis, SCC: somatic cell count, FFA: free fatty acids, PBC: psychrotrophic bacteria count TBC: total bacteria count, TRBC: thermo-resistant bacteria.

Cheese ripening is an amalgamation of the metabolism of residual lactose, lactate and citrate, lipolysis and proteolysis, which are known to co-vary with many factors. Hence, the ripening time of long-ripening cheeses is likely to vary greatly depending on the extent and rate of catabolic processes. In the case of long-ripening cheeses, hydrolytic enzymes, *e.g*. esterases, phosphatases and peptidases, are mainly responsible for the formation of specific flavour compounds, and thereby the ripening time (Ardö, 2021). Cheese ripening is a slow process and thus also an expensive step, due to the costs associated with the ripening facility (McSweeney, 2007a). Hence, considerable attention has been devoted to the ripening process and its importance for the characteristics of cheeses.

The literature provides some evidence to support the fact that milk quality is critically important for cheese ripening, *e.g*. development of aroma and texture. A review by Amenu and Deeth (2007) concluded that the composition of milk is an important parameter in determining processability and final product quality. The composition of milk is another key element regulating the profitability of the dairy industry (Lindmark-Månsson *et al*., 2003). The milk composition desired and the suitability of the raw milk will be dependent on the specific cheese variety. In most cases, the effect of altered raw milk quality on cheese ripening will be gradual and progressive, without abrupt changes in overall cheese quality. Fat content and casein content in milk have been shown to be positively associated with the ripening time of cheddar cheese (Młynek *et al*., 2018). However, in agreement with observations in **Paper IV**, Soodam *et al.* (2014) reported that milk protein content has very little effect on cheese ripening (texture and microstructure development) with adequate time for ripening. The fat to dry matter ratio of the cheese is governed by the standardisation of cheese milk to fat:protein ratio to maximise cheese yield and maintain uniform cheese quality. Elevated SCC in milk has deleterious effects on the quality of the cheese, since high SCC is associated with lower cheese yield and off-flavours (Auldist *et al*., 1996). Pasteurised and standardised milk is used for the production of the vast majority of cheeses. This enables high hygienic and microbiological quality of the milk to be maintained with minimal contribution to the variation in ripening time. This is important, since proteinases and lipases produced by psychrotrophic bacteria in raw milk can result in lower cheese yield and give rise to off-flavours during ripening (McSweeney, 2007b).

4.5 Can NIR-HS technology be used to visualise and predict maturity?

Using PLS regression, quantitative estimation of the relationships between targeted variables and the spectral response is possible. This can be used to predict concentrations of different components in each pixel of a NIR-HS image and visualise their spatial distribution in a sample (Vigneau *et al*., 2011). Part of the work in this thesis was to investigate whether the spectral profiles in cheeses can be related to cheese maturity using PLS analysis of NIR-HS images, for future potential applications in the prediction and control of the cheese ripening process **(Paper V**).

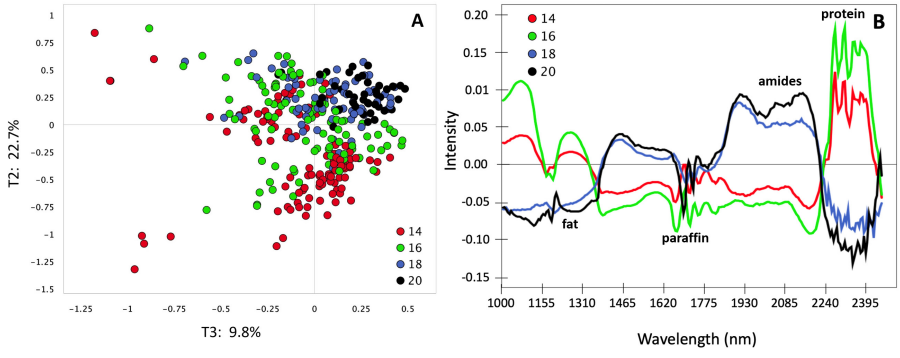

Figure 19. A) Projection to latent structures (PLS) score scatter plot of cheeses imaged at 14, 16, 18 and 20 months after production and (B) standard normal variate-transformed spectral data of one (random) cheese scanned at 14, 16, 18 and 20 months after production.

First, the PLS score plot based on principal components 2 and 3, describing the most meaningful variation in cheese maturity of all NIR-HS images, was investigated (Figure 19A). As can be seen, there was more variation between cheeses on earlier scanning occasions (young cheese) than on later scanning occasions (older cheese). This was also in agreement with the PLS sensory approval plot (not shown) presenting data from NIR-HS imaging concerning the approval of the sensory panel. To be approved by the sensory panel, a cheese must have achieved certain characteristic properties and most of the approved cheeses are among the older cheeses (*e.g*. scanning occasions 18 and 20 months). Variation was largest among the cheeses that were not approved, and likely to consist of young cheeses scanned at 14 and 16 months.

Using one cheese to illustrate, Figure 19B shows differences between younger (14 and 16 months) and more mature stages of a cheese (18 and 20 months) in standard normal variate-corrected absorbance intensities at various wavelengths. The differences reflect changes in the chemical composition of the cheese that occur during ripening. To identify the chemical components responsible for the differences in spectra, it is essential to refer to absorbances at the different wavelengths, as reported by Šašić and Ozaki (2000). Differences observed in band 1208 nm can be assigned to milk fat (CH, CH_2 and CH_3 bonds). According to Figure 19B, there were higher intensities at this band for the cheese at 14 and 16 months compared with 18 and 20 months of age. Due to the lipolysis of fat during ripening, less intact milk fat will be available in more mature cheeses than in young cheeses. Wavelengths 1645 to 1815 are mainly associated with paraffin wax (Palou *et al*., 2014). Consequently, the relatively small differences in absorbances between cheeses of various ages in Figure 19B were expected, since paraffin is applied several times (*i.e*. despite ageing, the composition and the thickness of paraffin layer is not changed). Band 2056 and 2160 nm have been assigned to amides (Šašić & Ozaki, 2000). As can be seen in Figure 19B, absorbance intensities at these bands were higher in older compared with younger cheeses, indicating the build-up of amides during ripening. Also, the bands at 2316, 2340 and 2368 nm arise from combinations of CH_2 stretching and bending modes of protein side-chain groups. In this region, intensities were higher for younger compared with older cheeses, probably due to a higher content of intact protein in the younger cheeses (**Paper V**). In agreement with these observations, Hickey *et al.* (2013) reported increasing proteolysis and reduced water activity during ripening.

The data generated were split into a training set (n=100 NIR-HS images) and a testing set (n=325 NIR-HS images) with the same distribution of scanning occasions. A PLS prediction model was then developed, using the training set with average spectra of all the cheeses and their respective age. Figure 20 shows the performance of the best PLS prediction model in assessing the maturity, *i.e*. the age, of the cheeses. The performance of the PLS prediction models was evaluated using the R^2 and root mean square error of calibration (RMSEC) values, where the higher the R^2 and lower the RMSEC, the more powerful a model is as a predicting tool (Vigneau *et al*., 2011). The best model had R^2 of 0.76 and RMSEC of 36.14 days. The model was then evaluated using the test set, resulting in a root mean square error of

prediction of 36.20 days. The maturity index and age of cheese showed a linear relationship up to 18 months (Figure 20). At this point, a bend in the curve was observed and cheese that needed a longer time to reach maturity seemed to follow a different pattern. Large variation in maturity index was apparent for the cheeses at each particular scanning occasion.

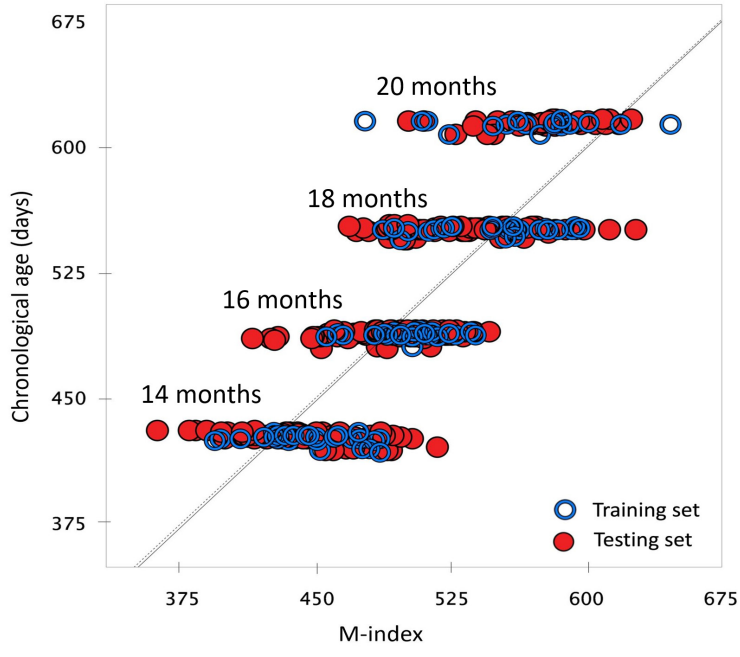

Figure 20. Performance of the best projection to latent structures (PLS) prediction model for predicting cheese maturity. Chronological age of the cheeses was calculated based on the difference (days) between production and imaging date. M-index: maturity index developed using NIR-HS image analysis.

The NIR-HS images are shown in Figure 21. These suggest that cheese maturation was non-homogenous, *i.e.,* within the same cheese some parts were more mature than others. Moreover, the images showed that ripening starts from the core of the cheese and moves on to its periphery. Cheeses scanned on a particular occasion after production were found to have reached different degrees of maturity and showed different maturity distributions. This reveals that cheeses mature in a non-uniform and uneven way, with variation arising both within and between cheeses. However, there is one major shortcoming with the data in Figure 21, namely that cheeses were scanned randomly, without exact orientation and side.

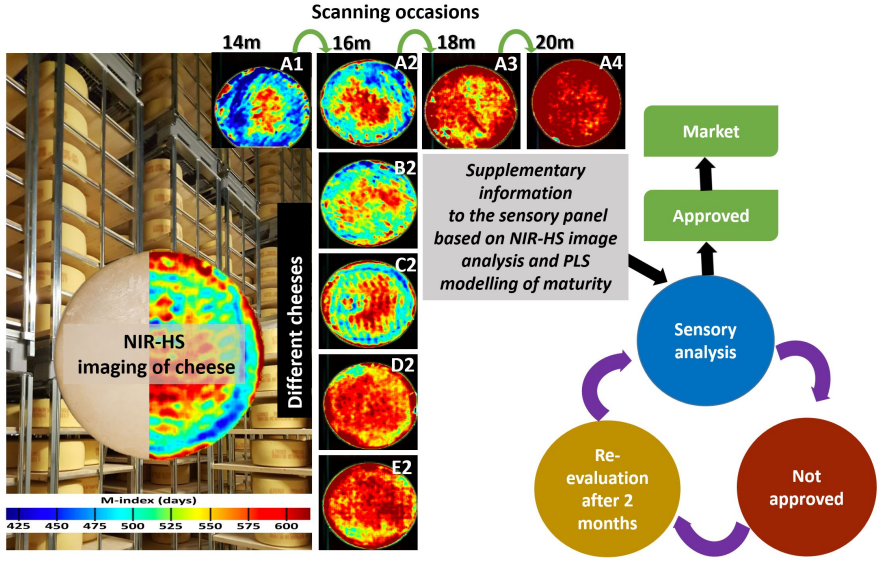

Figure 21. Spatial distribution of maturity index in the same cheese on scanning occasions at (A1) 14 months, (A2) 16 months, (A3) 18 months and (A4) 20 months), and (A2-E2) in different cheeses at 16 months. Colour key: maturity index in days.

Driven by economic and market forces, the cheese industry is considering alternative strategies to optimise logistics related to the use of costly ripening facilities. The NIR-HS imaging tool used in this thesis, which predicts M-index during the ripening process, is viewed as a feasible alternative, although further improvements of the model are needed. Cheese that is placed on the market should display uniform characteristics. To ensure that the cheese meets set criteria at the end of the ripening process, the producer evaluates the cheese by a sensory panel, a quality control step that is both costly and laborious. Thus, supplementary tools, such as computer-based assessment systems coupled with NIR-HS image modelling to predict cheese maturity, are expected to have great potential in the cheese industry. This was the conclusion reached by Khattab *et al*. (2019), who indicated the need for complementary analytical tools for effective quality control in cheese production. This will reduce the risk of mismanagement of the ripening facility and optimise the use of cheese wheels, while reducing the manual workforce needed to perform sensory evaluations.

5. Conclusions

Dairy farming intensification in northern Sweden has resulted in farms increasing in size, widespread use of milking robots and changes in the dairy breed used, with SH becoming dominant. These changes are reflected in farm milk quality. In this thesis, it was found that milk from farms with SH as the dominant breed had lower gel strength than milk from farms with other dominant breeds. Type of dairy farm, distinguished by dominant breed and milking system, also affected farm milk characteristics. The raw milk quality of farm and dairy silo milk varied between months. The most pronounced monthly-induced variations in milk properties were observed for casein micelle size (smaller during the outdoor period) and level of total proteolysis (higher during the outdoor period). The change to pasture was expected to be the sole explanation for the observed seasonal differences but, based on the data, it appeared that variations in factors not included in the analysis contributed more to the seasonal differences. The milk quality parameters investigated showed a weak relationship with the ripening time of the resulting cheese. Hence, as long as milk quality is high, a concept of differentiated use of milk for cheese production based on milk quality parameters might deliver little benefit to the cheese industry. The sensory and texture scores of the cheese were mainly influenced by plasmin and plasminogen-derived activities, suggesting an important role of the native proteolytic system in the ripening of the particular cheese studied. NIR-HS imaging can be used as a non-destructive technique to illustrate the onset and progress of cheese maturation, as well as the within- and between-batch variation. The model developed in this thesis achieved predictability of 76.4% in determining maturity. Thus, the technique has the potential to become an important tool in optimising logistics and securing efficient use of costly cheese ripening facilities.

6. Practical applications of results

Swedish raw milk quality has changed over the years, due to structural changes in dairy farming. However, the consequences of such changes on the technological properties and quality of raw milk are not fully understood. The work described in this thesis provided a better understanding of the consequences for dairy farms in northern Sweden. The interactive effect of casein micelle size and citrate observed in **Paper I** suggests that larger micelles with moderate citrate level will result in firmer gels. In **Papers II** & **III**, the natural variation in milk composition as influenced by factors in the dairy value chain, *e.g*. on-farm factors and season, was determined. Accurate knowledge about the influence of ongoing changes in dairy farming on variation in quality attributes and influencing factors is of major importance in anticipating changes in raw milk functional properties and in the resulting products, *e.g*. those observed by the cheese industry. However, the study described in **Paper IV** showed that the main milk quality parameters analysed were not of major importance for the ripening time of the resulting cheese. This information is of major practical value for the cheese making industry, since on knowing that the observed variation in cheese ripening is not linked to variation in milk composition, more effort can be spent on aspects related to the process, *e.g*. activity and composition of starter cultures and their interaction with milk microbiota. **Paper V** provided insights into use of NIR-HS imaging as a non-destructive predictive tool for efficient planning of ripening facilities and as a supplement information to the sensory panel. Overall, the results in this thesis are of direct practical benefit to the cheese industry, since the work was conducted in collaboration with a cheese manufacturer. It also generated valuable knowledge that the dairy industry can use as a basis in its decisions on future focus areas.

7. Future research

To gain deeper insights into the causes of variation in raw farm milk and potential associations with cheese ripening, future studies would need to span several years and consider additional parameters, some of which may be associated with farm and season, *e.g*. feeding and raw milk microbiota. More importantly, processing parameters at the dairy, *e.g*. activity and composition of starter culture, dynamics of the starter and NSLAB cultures during cheese ripening, cheese physicochemical properties, detailed sensory characterisation *etc*., *i.e.* factors that were not part of this thesis work, require attention. This is especially true considering the finding in this thesis that variation in raw milk quality has little influence on cheese quality and ripening time. This suggests a need for more in-depth and holistic study designs. In such studies, variation in ripening time should be examined by accounting for the complex and interactive effects of factors associated with the dairy supply chain.

Future studies should expand the scope by choosing various size classes of micelles and differing levels of calcium and citrate concentrations, preferably using different dairy breeds. It would also be interesting to study this in milk with the fat globules present, as opposed to the skimmed milk used in this thesis, to mimic industrial cheese making.

The results in this thesis provide a strong background and foundation for future studies aiming to develop predictive tools using NIR-HS images or other innovative and emerging techniques, *e.g*. ultrasonics, X-ray computed tomography scanning and artificial intelligence tools to optimise the use of cheese ripening facilities. Development of novel online tools will contribute to the digitalisation of the dairy value chain, minimising waste through improved resource utilisation and enhancing the efficiency of production of added-value cheese. Tests on more cheeses at different levels of maturity will

likely improve model performance further. The predictive capacity of the model could be improved if data varied more and if various types of data were included during model development. Examples of valuable data include data from a systematic sensory grading by multiple sensory panellists for the same cheese wheel, use of wet chemistry-based compositional data, analysis of chemical compounds (*e.g.* aroma, peptides, triglycerides, FFA and amino acids) resulting from the ripening process, identification of cheese volatiles together with their spectral changes, instrumental measurement of cheese texture and colour, use of cheese produced in various years and factories *etc*. The present work can also be of benefit in research related to the production of other types of long-ripening cheese. In such scenarios, the impact of raw milk quality on specific cheese types must be assessed by accounting for the whole dairy value chain.

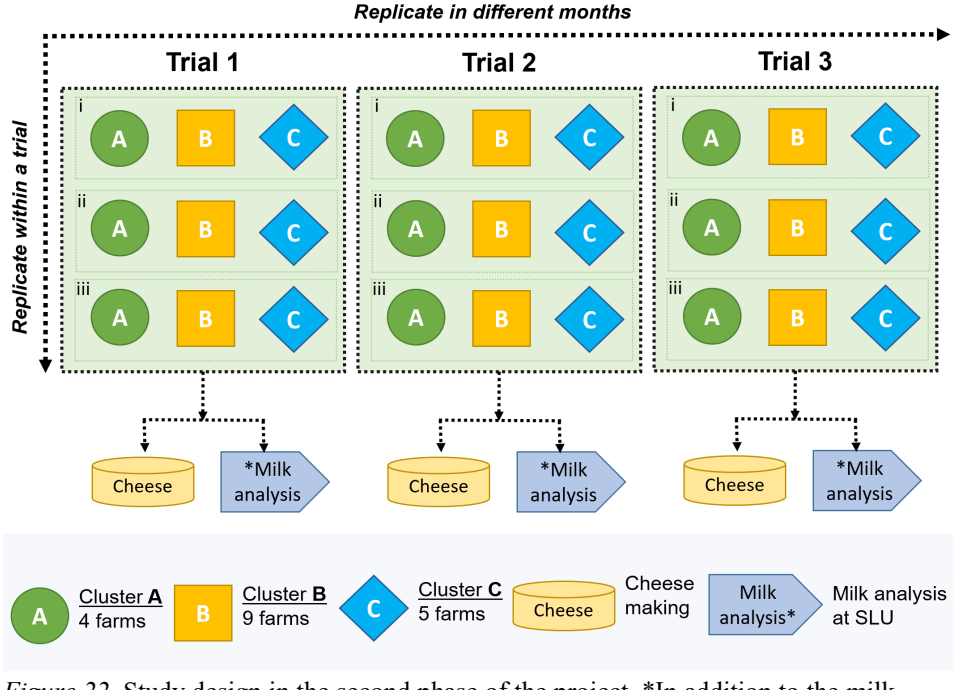

Figure 22. Study design in the second phase of the project. *In addition to the milk analysis described in this thesis, minerals, milk protein profile, fat globule size and milk microbiota were also analysed.

The knowledge gained from the work described in this thesis has already been applied in a second phase of the project, where I participated in planning, implementation, milk and cheese characterisation, and data curation. The aim in the second phase was to deepen understanding of the influence of milk composition, including milk microbiota, on cheese ripening. A full-scale cheese making plant was used and the cheese was made from milk collected repeatedly from 18 farms (subdivided into three clusters as shown in Figure 22), selected from among the 42 farms described in **Paper II**. The reason for creating these clusters was to achieve as much variation as possible in milk composition and make cheese using only milk from the participating farms on repeated occasions in three trials. The data generated in the thesis and from characterisation of the microbial community structure in the milk samples (not included in this thesis work) were used to select the farms. Milk was collected separately for each cluster of farms and cheese was made from milk delivered by the respective clusters. During ripening, cheese was sampled repeatedly from 2 to 20 months of age and the ripening process was characterised by analysis of texture, microstructure and aroma components (Figure 23). The results are being processed and I believe that this second phase of the project will provide deeper insights, but also raise questions that need to be addressed.

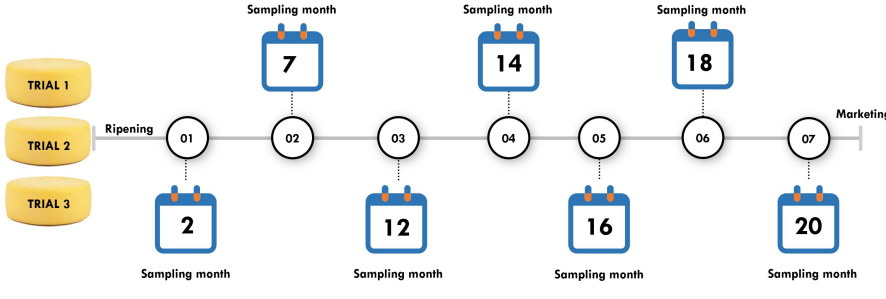

Figure 23. Cheese sampling schedule in the second phase of the project. The resulting cheese from three trials was matured and sampled at defined intervals from 2 months. Texture, colour, microstructure, volatile aroma compounds, amino acids (analysis scheduled) and NIR-hyperspectral images were determined at all sampling points. Sensory analysis of cheese samples was performed starting from 14 months. The microbiota of cheese samples from the same sampling occasions is being investigated in a parallel project.

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Popular science summary

Factors behind variation in raw milk quality and its impact on cheese making properties

Figure 24. Word cloud associated with the keywords in this thesis. Word size is proportionate to its relative importance in the work.

Close monitoring of raw milk quality is vital, as high quality in the final product can only be achieved using high-quality raw milk. Hence, approaches to characterise the raw milk, factors influencing the quality of milk and the association with final product characteristics are of great interest. A group of researchers from SLU (Uppsala and Umeå), a dairy factory in northern Sweden and a dairy farm advisory organisation investigated causes behind observed variation in the ripening time of a traditional long-ripening Swedish cheese. The aim was to evaluate the variation in raw milk quality, reasons for its variation and how it influenced the ripening time of the resulting long-ripening cheese. For this purpose, the composition and properties of milk from participating dairy farms, used for cheese making by the dairy partner in this project, were evaluated, to determine the influence of various factors on dairy farms and the influence of season or month. The cheeses produced from the milk were then monitored until they acquired the characteristic flavours and texture defining their ripening time.

Since raw milk quality is of great importance in cheese making, an experiment was conducted to examine the effects of three selected milk quality parameters (casein micelle size, calcium content and citrate content), and their interactive effects, on milk coagulation, which is the first step in cheese making. Work was also carried out to develop a predictive tool for rapid monitoring of cheese maturation without having to destroy the cheese wheel. For this purpose, an image analysis technique, near-infrared hyperspectral (NIR-HS) imaging was applied. This technique can be used to relate the NIR spectrum of a sample with pixels in the image, by applying predictive models using advanced statistical methods.

In industrial cheese making, calcium is added to the cheese milk and this will most likely induce interactions with citrate and casein micelles. The research group found that casein micelle size and citrate content interacted, influencing the resulting gel strength. Larger micelles with moderate citrate levels resulted in the formation of a firmer gel than small micelles with higher citrate levels. Field studies showed that casein micelle size was subject to seasonal variation, with smaller micelles during the summer months. Total proteolysis in farm milk showed higher values during the summer compared with the rest of the year. Dairy silo milk quality was also subject to monthly variation. Cheese ripening time showed a weak association with all milk quality parameters studied, but the sensory and

texture scores of the cheeses were affected by the activity of plasmin and plasminogen in the silo milk. This illustrates the important role of the native proteolytic system in milk for the ripening of this Swedish long-ripening cheese. An impact of the milking system and dominant breed on-farm on milk quality attributes was demonstrated. Two major types of dairy farms were distinguished in the study region: (I) smaller tiestall farms with cows of Swedish Red Cattle and multiple other breed types and (II) larger farms with loose-housing, milking parlour or automatic milking and Swedish Holstein as the dominant breed. These dairy farm types resulted in milk that differed in composition and properties, with *e.g.*, higher fat and protein content and lower cell count in milk from type I farms. Predicting and monitoring the cheese ripening process is crucial for the cheese industry, and for this, the use of non-invasive techniques is of great interest. Use of NIR-HS images in predicting and understanding the variation in cheese maturation (age-related) through visualising the maturity of cheeses was tested. The model developed achieved 76% accuracy in prediction of maturity and made it possible to visualise variation in cheese maturation both within and between cheeses. Cheese maturation was initiated in the core of the cheese wheel and then spread to the periphery. Variation in maturation also observed among cheeses within a batch.

Summing up, this work demonstrated that raw milk quality in northern Sweden varies with farm type and month and that the monthly variation is reflected at dairy silo level. However, the monthly variation in raw milk quality was not linked to observed variations in cheese ripening time, suggesting that factors not covered in this study, *e.g.*, processing parameters, are of greater importance. Thus, when the raw milk is of high quality, as it was in this study, the development of aroma and texture, which governs the cheese ripening time, is not influenced by the variation in raw milk quality parameters. Consequently, approaches to differentiate raw milk based on quality parameters when producing this specific traditional Swedish cheese might provide very little economic benefit. This research provided multiple insights on variations in raw milk quality and factors, which can influence cheese quality. It also raised many questions to be addressed in future work, such as identifying major causative factors for the variation in ripening time. The results obtained to date can be valuable in future studies working to solve the challenge of variation in cheese ripening time and increase the sustainability of cheese production.

Populärvetenskaplig sammanfattning

Faktorer bakom variationen i mjölkråvarans sammansättning samt inverkan på mjölkens ystningsegenskaper

I mejeriernas kvalitetsprogram för mjölkråvaran kontrolleras leverantörsmjölkens sammansättning regelbundet, eftersom hög kvalitet hos de resulterande produkterna endast kan uppnås med en högklassig mjölkråvara. I takt med den ökande intensifieringen på svenska gårdar är det viktigt att känna till hur olika gårdsfaktorer bidrar till variationen i mjölkråvarans sammansättning samt hur denna variation påverkar slutprodukterna. I denna avhandling har forskare vid SLU (Uppsala och Umeå), mejeriföreningen Norrmejerier och rådgivningsorganisationen Växa Sverige undersökt hur variationen i mjölkråvarans sammansättning och egenskaper hänger samman med olika gårdfaktorer och årstid. Med syfte att testa vår hypotes, dvs. ”mjölkråvarans sammansättning och egenskaper har stor effekt på ostens kvalitet och lagringstid” studerade vi även vilken betydelse variationen i mejeriets silomjölk har för den resulterande ostens lagringstid. Vi genomförde även en experimentell studie för att undersöka effekterna av tre utvalda mjölkkvalitetsparametrar på mjölkens koaguleringsegenskaper, det första steget vid osttillverkningen. Slutligen utvärderades lämpligheten hos en bildanalysteknik (NIR-hyperspektral imaging) vid övervakning och prediktering av ostmognad, varvid avancerade statistiska metoder användes för att relatera NIR-spektra och bildpixlar för ostprov.

Vid osttillverkning tillsätts ofta kalcium till ystmjölken, vilket sannolikt inducerar interaktioner med mjölkens citrat och kaseinmiceller. I våra studier fann vi att kaseinmicellstorlek och citratinnehåll interagerade vid mjölkens

koagulering, vilket påverkade den resulterande gelstyrkan. Mjölk med större miceller och måttliga citratnivåer resulterade i en fastare gel än högre citratnivåer och små miceller. I vår fältstudie fann vi att kaseinmicellernas storlek varierade med årstid, med mindre miceller under sommarmånaderna. Den totala proteolytiska aktiviteten i tankmjölken på de medverkande gårdarna var högre under sommaren jämfört med resten av året. Silomjölkens sammansättning och egenskaper varierade också mellan månader, emedan ostmognadstiden endast visade en svag koppling till variationen i de studerade mjölkkvalitetsparametrarna. Ostens lukt, smak, och textur påverkades positivt av aktiviteten av plasmin/ plasminogen i silomjölken, vilket illustrerar betydelsen av mjölkens primära proteolytiska enzym för denna svenska långlagrade osttyp. Vår studie visade att typ av mjölkgård, dvs. inhysning-/mjölkningssystem och dominerande ras hade betydelse för en rad mjölkkvalitetsparametrar. Vi kunde urskilja 2 huvudtyper av mjölkgårdar i den studerade regionen, dvs (I) mindre uppbundna gårdar med SRB-kor och flera andra rastyper och (II) större gårdar med lösdrift, mjölkgrop eller robotmjölkning, och svensk Holstein som dominerande ras. Mjölken som producerades på gårdar av respektive typ skilde sig åt avseende sammansättning och egenskaper, t.ex. fett- och proteinhalter var högre och celltalet lägre i mjölk från gårdstyp I.

Övervakning och prediktering av ostmognaden är centrala processteg för ysterierna, och användningen av nya, icke-invasiva tekniker av stort intresse. I projektet utvärderade vi användningen av NIR-hyperspektral bildanalys för att visualisera och prediktera ostmognaden. Genom modellering kunde vi förutsäga ostens mognadsgrad med 76% korrekthet, och vi kunde visualisera variationen i ostmognad både inom ost och mellan ostar. Våra bilder visade att mognadsprocessen initierades i ostens kärna och spreds därefter till dess periferi. Variation i mognadsgrad observerades mellan ystningar, men även bland ostar tillverkade i samma ystning.

Sammanfattningsvis visar våra studier att mjölkråvarans sammansättning varierade beroende på typ av mjölkgård och månad. Den månatliga variationen återspeglades också i silomjölken på mejeriet. Variationen i silomjölkens sammansättning var dock inte starkt kopplad till den observerade variationen i ostmognadstid, vilket tyder på att faktorer som inte ingick i studien, t.ex. olika processparametrar på mejeriet, hade större betydelse för ostens lagringstid. Vår slutsats blir att utvecklingen av ostens arom och konsistens, vilket avgör ostens lagringstid, är inte associerad med

mjölkråvarans sammansättning så länge mjölkråvaran är av hög kvalitet. Ett koncept med differentierad användning av mjölkråvaran för produktion av den långlagrade hårdosten, baserat på de studerade kvalitetsparametrarna, skulle därför sannolikt inte medföra några större fördelar för mejeriet.

Vår forskning har bidragit med fördjupade insikter i variationen i mjölkråvarans kvalitet och vilka faktorer som bidrar till denna. Det finns dock fortfarande många viktiga frågor att belysa i vårt framtida arbete, som att utforska vilka faktorer som ligger bakom variationen i ostens mognadstid. Resultaten i denna avhandling kommer att vara värdefulla för att staka ut riktningen för framtida studier, för att lösa utmaningen med en varierande ostmognadstid och bidra till en hållbarare framtida ostproduktion.

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RESEARCH ARTICLE

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Interactive effects of casein micelle size and calcium and citrate content on rennet-induced coagulation in bovine milk

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Abstract

Interactive effects of casein micelle size and milk calcium and citrate content on rennet-induced coagulation were investigated. Milk samples containing small (SM) and large (LM) micelles, obtained from individual Holstein cows, were modified by addition of calcium and/or citrate and milk coagulation properties were evaluated in a full factorial design. The results showed that LM milk had a higher relative proportion of casein, coagulated faster, and resulted in a stronger gel than SM milk. Addition of calcium slightly decreased casein micelle size, while addition of citrate slightly increased micelle size. Calcium addition resulted in a shorter coagulation time and the strongest gels, while citrate addition increased the coagulation time and resulted in the weakest gels. Addition of calcium and citrate in combination resulted in intermediate coagulation properties. The interactive effect of micelle size and citrate was significant for gel strength. Microstructural differences between the milk gels were consistent with the rheological properties, for example, the micrographs revealed that a more homogeneous network was formed when calcium was added, resulting in a stronger gel. A more inhomogeneous network structure was formed when citrate was added, resulting in a weaker gel. Thus, variations in casein micelle size and in calcium and citrate content influence rennet-induced coagulation in bovine milk. The calcium and citrate contents in Swedish milk have changed over time, whereby calcium content has increased and citrate content has decreased. In practical cheese making, calcium is added to cheese milk, most likely altering the role of inherent citrate and possibly influencing casein micelle size. The observed interaction effect between casein micelle size and citrate in this study, suggests that larger micelles with moderate citrate level will result in firmer gels, whereas a higher citrate content reduced gel strength more in case of large than SM. Since firmer gels are likely to retain more protein and fat than less firmer gels, this interaction effect could have implications in practical cheese production.

KEYWORDS
calcium, casein micelle size, citrate, gel network, gel strength, rennet coagulation time

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1 | INTRODUCTION

Milk coagulation is an essential step in cheese making, affecting the yield and quality of the cheese (Harboe, Broe, & Qvist, 2010). The mechanism behind rennet-induced aggregation of casein micelles is comparatively well understood. Coagulation is influenced by various milk compositional factors, which in turn vary with dairy cow breed (Auldist, Johnston, White, Fitzsimons, & Boland, 2004), stage of lactation (Ostersen, Foldager, & Hermansen, 1997), milk protein genetic variant (McLean, Graham, Ponzoni, & McKenzie, 1984), feeding strategy (Macheboeuf, Coulon, & D'Hour, 1993), and udder health (Grandison & Ford, 1986).

Calcium is an important milk constituent known to have a great influence on milk coagulation. In milk, calcium exists in equilibrium between soluble and colloidal phases (Gastaldi, Pellegrini, Lagaude, & Fuente, 1994). Colloidal calcium phosphate linked to phosphorylated serine residues of casein plays an important role in the stability of casein micelles in milk (Gaucheron, 2005). Calcium addition has been reported to reduce the volume of casein micelles (Van Hooydonk, Hagedoorn, & Boerrigter, 1986) and improve milk coagulation (Horne & Lucey, 2017), and thereby increase cheese yield (Wolfschoon-Pombo, 1997). With added calcium, the average micelle size slightly decreases and the size of the smallest micelles increases, while larger aggregates of micelles form in the coagulum (Müller-Buschbaum, Gebhardt, Roth, Metwalli, & Doster, 2007). Calcium addition has also been observed to decrease the rennet coagulation time (RCT; Gastaldi et al., 1994), an effect attributed to decreasing pH and increasing concentration of free Ca^{2+} ions (Van Hooydonk et al., 1986). Thus, variations in the calcium content in raw milk will have an impact on milk coagulation properties, both directly and indirectly. The calcium content in milk varies with breed (Cerbulis & Farrell, 1976), udder health (Kitchen, 1981), and the calcium content in feed (Chan, West, & Bernard, 2006).

Citrate acts as a chelating agent in milk (Gaucheron, 2005), and binds a proportion of the free calcium ions in milk serum (Odagiri & Nickerson, 1965). The citrate level in bovine milk is on average 1.7–1.9 g/L (Jenness, 1988), but is influenced by the extent of de novo synthesis of milk fatty acids (Faulkner & Peaker, 1982). The citrate content in milk is highest during the grazing season (Holt & Muir, 1979) and in early stages of lactation (Braunschweig & Puhan, 1999), when de novo synthesis of fatty acids is lowest. Fluctuating citrate content in raw milk has been suggested to influence milk processing characteristics, due to changes in citrate-mediated interactions with other milk components (Faulkner & Peaker, 1982; Garnsworthy, Masson, Lock, & Mottram, 2006), and citrate is thus also likely to play a role in coagulation.

The structure and stability of casein micelles have attracted great research interest in recent decades (Dalgleish, Spagnuolo, & Goff, 2004; Farrell, Malin, Brown, & Qi, 2006; Holt, Carver, Ecroyd, & Thorn, 2013; Horne, 2006; Huppertz et al., 2017; McMahon & McManus, 1998; Walstra, 1990). Different models describing the interactions and aggregation of different types of casein micelle have been suggested, and attempts have been made to investigate the effect of casein micelle size on milk coagulation. Some studies in the 1980s reported that milk with smaller casein micelles have a longer coagulation time (Ekstrand, Larsson-Raźnikiewicz, & Perlmann, 1980), whereas others, for example, that by Dalgleish, Brinkhuis, and Payens (1981), reported that the coagulation properties are independent of micelle size. In more recent studies, improved coagulation with smaller micelle sizes has been reported (Glantz et al., 2010; Logan et al., 2014). Micelle size is suggested to be influenced by factors such as milk protein polymorphism and feeding regime (Devold, Brovold, Langsrud, & Vegarud, 2000), season (Holt & Muir, 1978), and content of casein and whey protein in milk (Devold et al., 2000). In a study by Bijl, de Vries, van Valenberg, Huppertz, and van Hooijdonk (2014), average micelle size was found to be associated with genetic variants of κ-casein and explained by differences in the concentration of glycosylated κ-casein.

In the present study, we evaluated interactions between casein micelle size and milk calcium and citrate content in rennet-induced coagulation. The hypothesis tested was that variations in the size of casein micelles and in the calcium and citrate content of milk affect interactions between micelles, and consequently also RCT and gel strength.

2 | MATERIALS AND METHODS

2.1 | Study design

In order to evaluate both individual and interactive effects, the experiment was designed and conducted as a fully balanced factorial study, as schematically illustrated in Figure 1. A total of eight treatments, including biological duplicates, were applied to milk from four cows, two producing milk with small (SM) casein micelles and two producing

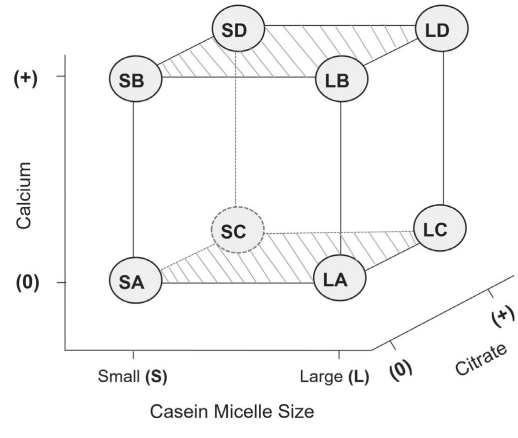

FIGURE 1 Schematic illustration of the two-level factorial experimental design, with the design factors casein micelle size, milk citrate content, and milk calcium content

milk with large (LM) micelles. The fresh SM and LM milk samples were treated by increasing their level of calcium or citrate, or both, by up to 10% of the initial level, using 1 M $CaCl_2$ and 1 M citric acid trisodium salt dehydrate (Sigma–Aldrich Gmbh, Germany). Milk pH was adjusted back to that in the original milk sample by adding 1 M NaOH.

2.2 | Milk sample collection

Morning milk samples from four individual cows known to produce SM (n = 2) and LM (n = 2) were collected from the experimental dairy herd at the Swedish Livestock Research Centre, Lövsta, Uppsala, Sweden. The cows are kept in an indoor loose housing system, fed silage ad libitum and concentrate according to their milk production, and milked using an automatic milking rotary parlor (DeLaval, Tumba, Sweden). To select the four cows used in the study, individual milk samples from 60 mid-lactation cows (days in milk, DIM = 170 ± 46 days), of the Swedish Red (n = 32) and Swedish Holstein (n = 28) breeds, were screened for micelle size. Within 3 weeks of screening, a subset of four Swedish Holstein cows in mid-lactation (DIM 195 ± 41 days) was selected. The criterion applied in selection of the cows was to have the largest possible difference in micelle size, but the smallest possible difference in milk composition (screening data not shown). Milk samples were handled without preservatives and all analyses were performed on fresh milk, within 3 days of milking. The composition of the SM and LM milk samples is described in subsection 2.3.

2.3 | Milk gross composition analysis

The experiments were generally performed using defatted milk, to avoid the interference of fat globules. The only exception was somatic cell count (SCC) analysis, which was performed on whole milk by LED flow cytometry (SomaScope) of CombiScope FTIR 300 device (Delta Instruments, The Netherlands). Fresh milk samples were centrifuged at 3,400g and 4°C for 10 min (Sorvall Super T21, Sorvall Products L.P., Newton, CT). Citrate content in skimmed milk was measured using Fourier transform infrared (FTIR) spectroscopy (Milkoscan FT2, Foss Electric, Hillerød, Denmark). Skimmed milk composition (fat, protein, lactose, and solids) was analyzed using FTIR rotation scanning (FTIR LactoScope) with a CombiScope FTIR 300 device. Total calcium content was measured by inductively coupled plasma optical emission spectrometry (ICP-OES) using an FMS26 device (Spectro Analytics, Germany). Skimmed milk samples were analyzed for calcium at 1:100 (vol:vol) dilution using a Hamilton MicroLab 500 series auto-dilutor and spectro-blue FMS26 ICP-OES at 183.801 nm. The pH of skimmed milk and modified milk samples was measured using a pH meter (Seven Compact S210, Mettler-Toledo, Switzerland). Descriptive data on the composition of LM and SM skimmed milk samples from individual cows are presented in Table 1.

The observed difference in average casein micelle size between SM and LM samples was not significant (Table 1), but micelle size was still included as one of the variables in the model.

The fat content in whole milk samples varied between 2.54 and 3.96 g/100 g, while skimming resulted in a fat content of 0.08–0.09 g/100 g (Table 1). The protein content was significantly higher in LM milk. SCC, determined in whole milk samples, ranged from 2.87×10^3 to 24.5×10^3 cells/mL (data not shown), indicating that the milk was collected from cows with good udder health, and there was no difference in pH between SM and LM milk samples.

TABLE 1 Descriptive data (mean and SD) on quality traits of cow's milk samples with small and large casein micelles

	SM (n = 2)		LM (n = 2)	
Quality trait	Mean	SD	Mean	SD
Micelle size (nm)	149.8	4.15	161.6	6.63
Calcium (g/L)	1.4	0.02	1.22	0.01
Citrate (g/L)	0.23	0.01	0.15	0.01
Fat (%)	0.09	0.01	0.08	0.01
Protein (%)	3.47[a]	0.01	3.72[b]	0.01
Lactose (%)	4.68	0.01	4.84	0
Solids (%)	9.01[c]	0.01	9.45[d]	0.02
pH	6.68	0.02	6.66	0.01

Note: Values obtained for skimmed milk samples. Mean values within rows with different superscripts are significantly significant ($p \leq .05$).
Abbreviations: LM, large micelles; SD, standard deviation; SM, small micelles.

2.4 | Protein profiling of milk

The milk protein profile of individual milk samples was determined by capillary electrophoresis (Agilent G 1600AX, Agilent Technologies Co., Kista, Sweden), controlled by Chemstation software (version A 10.02) as described by Johansson, Åkerstedt, Li, Zamaratskaia, and Sternesjö Lundh (2013). Separation of proteins was performed on an unfused silica standard capillary column with 50 μm inner diameter and 40 cm active length (Chrom Tech, SE-195 30, Märsta, Sweden). Relative concentrations of individual proteins were calculated based on peak area and expressed as percentage of total integrated area in the electropherogram. The mean relative proportions (%) of different protein fractions in SM and LM samples are shown in Figure 2. Milk with LM had a higher relative proportion of total casein in total protein and a higher relative proportion of β-casein than SM milk. These results contradict de Kruif & Huppertz (2012), who found no strong correlation between casein micelle size and protein composition.

2.5 | Casein micelle size

Casein micelle size in SM and LM milk samples, before and after modification, was analyzed using nanoparticle tracking analysis (NTA), assuming spherical shape of the particles. NTA was conducted using NanoSight NS500 (Malvern Instruments, UK), coupled with a temperature sensor (NTA Temperature Comms) and automatic stage controller (NTA Stage Comms). A syringe pump (Harvard apparatus, MA), fitted with a 1 mL syringe, was connected to the NanoSight device by

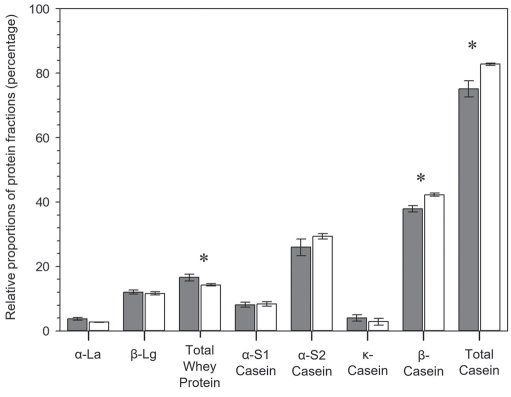

FIGURE 2 Relative proportions of whey protein and casein fractions (%) in milk samples with large micelles (LM, light color) and small micelles (SM, dark color). * indicates significant difference ($p \leq .05$) between SM and LM milk. Abbreviations: α-La, α-lactalbumin; β-Lg, β-lactoglobulin. Standard error bars are included for each column

a four-way connector. Skimmed milk, diluted 2,000-fold with distilled water, was injected into the NanoSight using the syringe pump at a constant speed of 150 (~15.6 μL/min). A scientific complementary metal-oxide semiconductor (sCMOS) camera, fixed at a 90° angle and a wavelength of 658 nm, was used to video-record the particle flow. Sample flow was captured at a camera level of 13 for 90 s and measurements were repeated to give an average of four consecutive measurements at 2 s intervals. The NTA was performed at constant temperature (35°C) equivalent to the temperature used for the rheological measurements. The video sequence captured was batch-processed using NanoSight 2.3 NTA software by setting the screening gain to 17 and the detection threshold to 14.

2.6 | Rennet-induced coagulation properties

Rennet-induced coagulation properties of skimmed SM and LM milk samples were evaluated using a Bohlin CVOR-150-900 rheometer (Malvern Instruments Nordic AB, Uppsala, Sweden). The rheometer was equipped with a cup and bob geometry (25 and 28 mm in diameter, height 40 mm), and a Peltier element for temperature control. The method used was as previously described by Johansson, Högberg, and Andrén (2015). In brief, calf rennet (75/25 chymosin/bovine pepsin, 180 IMCU, obtained from Kemikalia, Skurup, Sweden) was added to the skimmed milk at a concentration of 0.18 IMCU/mL. Time of rennet addition was taken as the starting time and gel formation was monitored. Coagulation properties were measured as RCT, that is, time from the point of the enzyme addition until a gel strength of 1 Pa was reached, and gel strength (Pa) at 20 min (G20). Gel formation rate (GR) was calculated based on the relative change in elastic modulus (linear phase) during 800 to 1,000 s.

2.7 | Gel microstructures

Samples (2 mL) of SM and LM milk were coagulated by adding 60 μL of 0.18 IMCU rennet (Kemikalia, Skurup, Sweden) at 35°C. Prior to coagulation, the protein matrix was stained with 15 μL of Fast Green 1% wt/wt (Sigma–Aldrich Co, St. Louis, MO). After 20 min of coagulation, a few drops of curd were placed on a cover glass (24 × 60 × 0.13–0.17 mm, Knittle Gläser, Germany) and microstructures in the gel samples were studied under a Zeiss LSM780 inverted confocal microscope (Carl Zeiss, Germany) with ×60 numerical aperture, 1.2 oil objective. A laser beam consisting of Argon 488 (505–560 nm emission spectrum) was used for sequential scanning of images. Zen2012 software (Carl Zeiss, Germany) was used in image acquisition. Intensity of the laser beam was adjusted below 25% of power and smart gain was controlled to avoid noise and intensity saturation of pixels. Image background was monitored with smart set and kept at zero. A line average of 4 at 8-bit resolution of 1,024 × 1,024 pixels was used to acquire images with improved resolution. Micrographs were subjected to a noise-despeckle procedure using software ImageJ 1.33u (Wayne Rasband, National Institutes of Health), to evaluate the porous spaces (gel porosity, GP) entangled in the network.

2.8 | Statistical analysis

The statistical package for the social sciences (SPSS), statistical package, version 9.4 (SPSS Inc., MI), was used for statistical analysis of results for the treatments described in subsection 2.1. Compositional differences between SM and LM milk samples, differences in the relative proportions of different milk protein fractions, and the effects of the model factors on casein micelle size, GP, and GR were evaluated using one-way analysis of variance (ANOVA), followed by post hoc Tukey Test using Minitab 18.1 (Minitab, Inc.). The data were analyzed using factorial mixed models, as described in the literature (e.g., Littell, Milliken, Stroup, Wolfinger, & Schabenberger, 2006; Olsson, 2011). Calcium level (0 or 1), citrate level (0 or 1), micelle size (SM or LM), and all interactions between these were considered as fixed factors in the model, while the individual cows were considered as a random factor. The model took the form:

$$y_{jkl} = \mu + \alpha_j + \beta_k + \gamma_l + (\alpha\beta)_{jk} + (\gamma\alpha)_{jl} + (\gamma\beta)_{kl} + (\alpha\beta\gamma)_{jkl} + C_m + e_{jklm} \quad (1)$$

Fixed effects denoted with Greek characters, random effects with Roman characters. y = dependent variable; μ = general mean value; α = calcium level, index j = 0, 1; β = citrate level, index k = 0, 1; γ = mean micelle size, index l (regarded as numerical); C = cow; and e = random residual.

Mean square values of RCT, G20, GR, and porosity were calculated using Equation (1). Pairwise differences in least square means (LSM) of three factor combinations were evaluated with the Tukey–Kramer method. Denominator degrees of freedom were determined according to Kenward and Roger (1997). The assumptions underlying the analyses were checked using diagnostic plots. No apparent deviations from normality or homoscedasticity were detected. Post hoc

pairwise comparisons were adjusted for multiplicity using Tukey's method.

3 | RESULTS AND DISCUSSION

Effects of calcium and/or citrate addition on casein micelle size are reported in subsection 3.1, effects on gelation behavior in subsection 3.2, and interactive effects in subsection 3.3.

3.1 | Impact of calcium and citrate on casein micelle size and particle size distribution

3.1.1 | Casein micelle size

The NTA analysis of micelle size distribution in SM and LM milk samples revealed that the average micelle size in SM milk before treatment (SM-A) was 149.86 nm and that in LM milk with large micelles before treatment (LM-A) was 161.63 nm (Table 2). In a previous study by Glantz et al. (2010), an average micelle size of 200 nm was reported for Swedish Holstein and the same average size was recently reported by Ranadheera et al. (2019). However, both studies applied dynamic light scattering to measure volume weighted micelle size as opposed to the present study, using nano particle tracking.

The average hydrodynamic diameter was strongly influenced by the treatments (Table 2). Adding calcium alone decreased mean micelle size by 8% for SM (change not significant) and 18% for LM, in agreement with findings by Van Hooydonk et al. (1986). Calcium is essential for the stability of micelles and increasing the calcium content amplifies interactions within and between primary casein particles, thereby tightening the micelles, as described by Lucey and Horne (2018). This decrease in micelle size is due to shrinkage of the porous network present inside the micelles, as described by Huppertz et al. (2017).

Adding citrate alone increased mean micelle size by 6.7% for SM milk (change not significant), whereas no noticeable effect was observed for LM milk. Added citrate forms chelates with free Ca^{2+} ions, shifting the equilibrium from colloidal to dissociated calcium. As a consequence, the slightly calcium-deprived micelles will tend to dissociate and disintegrate, according to Griffin, Lyster, and Price (1988), resulting in an increment in the hydrodynamic average diameter of the micelle. Moreover, the water-binding ability of citrate most likely hydrates the casein micelles and thereby increases the average size.

Adding calcium and citrate in combination increased mean micelle size by 8.9% for SM milk and 5% for LM milk (change not significant). One possible explanation for this is that, given a sufficient amount of calcium to create rigid interactions between primary casein particles, added citrate will contribute to retention of more water molecules inside the porous micelle. Therefore, a hydrated and swollen micelle is a likely explanation for the increase in micelle size. In line with this, the comparatively smaller increase in micelle size when only adding citrate is explained by lower water-holding ability without the additional calcium creating interactions between primary casein particles. The D70 values for SM milk, that is, the size threshold below which 70% of the micelles are contained, increased significantly when citrate was added alone or in combination with calcium, whereas the increase for LM milk was not significant.

3.1.2 | Effect on casein micelle size distribution

The hydrodynamic diameter of untreated casein micelles and casein micelles treated with calcium and/or citrate showed an unimodal distribution (Figure 3). de Kruif & Huppertz (2012) observed lower polydispersity in casein micelle size distribution in milk from individual cows compared with bulk milk. This is in agreement with observations we made in a preliminary study, where the observed average micelle size deviated strongly from the calculated average micelle size of the bulk milk (data not shown). This is due to the effect of pooling individual, polydisperse cow milk samples with small and large average micelle sizes to form bulk milk. Therefore, in the present study, milk samples from two individual cows were used as biological duplicates,

TABLE 2 Average micelle size in milk with small micelles (denoted SM-) and large micelles (denoted LM-) before and after the different treatments: (a) no treatment, (b) addition of calcium, (c) addition of citrate, and (d) addition of both calcium and citrate

	Average micelle size							
Milk treatment	Mean (nm)	SD	Mode (nm)	SD	D50 (nm)	SD	D70 (nm)	SD
SM-A	149.86^{abc}	4.52	130.63^{abc}	5.88	139.50^{ab}	4.43	166.75^{a}	4.00
SM-B	137.75^{bc}	2.24	114.25^{c}	5.85	131.63^{b}	1.36	166.88^{a}	4.99
SM-C	159.63^{ab}	6.41	143.75^{a}	6.36	153.75^{ab}	5.53	185.13^{b}	4.51
SM-D	163.25^{ab}	7.36	140.50^{ab}	8.84	154.50^{a}	6.32	189.63^{b}	6.99
LM-A	161.63^{ab}	6.63	146.75^{a}	9.55	153.75^{ab}	5.48	182.13^{b}	4.12
LM-B	129.13^{c}	5.20	104.75^{c}	5.47	114.88^{b}	3.33	141.13^{c}	2.08
LM-C	161.50^{ab}	7.37	147.25^{a}	7.01	153.38^{ab}	6.66	186.25^{b}	7.02
LM-D	169.86^{a}	6.41	148.13^{a}	13.24	161.25^{a}	5.74	196.00^{b}	9.29

Note: Mean: mathematical average, mode: most likely value, D50 and D70 are the size values below which 50% and 70% of the micelles are contained, respectively. Mean values within columns with different superscripts are significantly different ($p < .05$).
Abbreviations: LM, large micelles; SD, standard deviation; SM, small micelles.

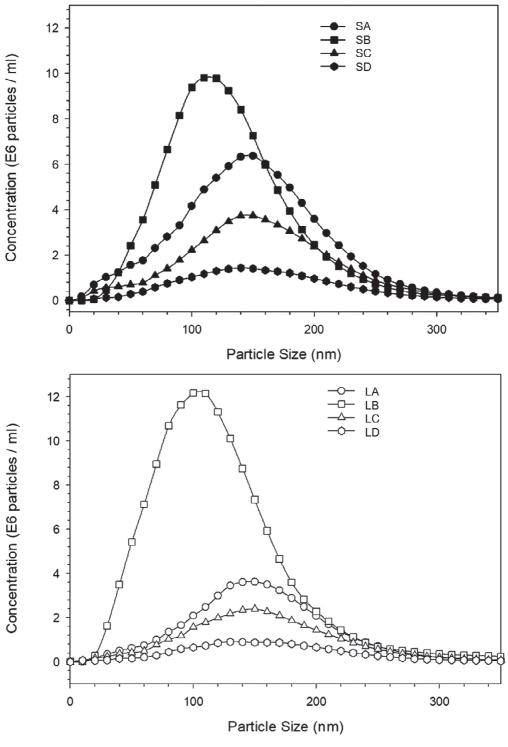

FIGURE 3 Effect of adding calcium (B), citrate (C), and both calcium and citrate (D) to skimmed milk (A) on average casein micelle size distribution of milk with small micelles (SM: upper panel) and large micelles (LM: lower panel), measured with nanoparticle tracking analysis. The values were obtained by averaging individual cow milk samples with small (SM, $n = 2$) and large (LM, $n = 2$) casein micelles

representing SM and LM milk, to reduce polydispersity in micelle size distribution in the respective sample.

In addition to altering mean micelle size, adding calcium and citrate either alone or in combination altered the micelle size distribution (Figure 3). Some NTA video snapshots of milk samples are shown in Figure 4. Concomitant evaluation of micelle size distribution and the respective video frames was used to assess the influence of treatments on mean particle (micelle) size and particle size distribution. The particles visualized in the video frame for LM milk were on average larger, and particle concentration was lower, than in SM (Figure 4).

Adding calcium alone gave a large increase in particle numbers per mL (particle concentration), while adding citrate alone or in combination with calcium reduced the particle concentration compared with the untreated control milk sample. One probable reason for the increased particle concentration upon addition of calcium is aggregation of primary casein particles available in the milk serum. As a result, smaller-sized particles, previously not detected by NTA, were detected and counted. This is in agreement with Müller-Buschbaum et al. (2007), who reported that the smallest micelles increased in size with addition of calcium. In contrast, adding citrate alone or in combination with calcium resulted in a reduction in particle concentration. This may be due to the effect of citrate, as formation of calcium chelates is likely to hinder association of primary casein particles and thereby reduce the detection of smaller-sized particles in the serum. Moreover, NTA video frames of these samples were found to be noisy, with aggregation of particles reducing the total number of micelles while increasing the average micelle size (Figure 4).

3.2 | Effects of treatments on gelation behavior

3.2.1 | Rennet-induced coagulation

The effects of micelle size and of the different treatments on rennet coagulation are presented in Figure 5. After adding rennet, the elastic or storage modulus (G′) increased and tan δ decreased with time. During coagulation, aggregates of casein increase in size over time and gradually form a gel and, as a consequence, G′ increases and tan δ decreases. In this study, LM milk was found to have a shorter RCT, that is, it coagulated faster than SM milk (Figure 5). Differences in coagulation time can be explained by two factors: (a) the efficacy of cleaving κ-casein into glycomacropeptide (GMP) and para-κ-casein by the action of rennet (Dalgleish, 1993), and (b) meeting the required critical coagulation concentration (CCC), that is, the lowest concentration of particles needed to induce coagulation (Hsu & Liu, 1998). After removal of the hydrophilic GMP, destabilized particles will aggregate in the milk serum, leading to formation of assemblages when the CCC is met. As a consequence of the larger relative surface area of SM, it may take comparatively longer to remove the amount of GMP required for aggregation to be initiated. According to Hsu and Liu (1998), smaller particles require a higher CCC to coagulate. The lower net coagulation rate is caused by a lower colloiding velocity near small particle surfaces and deflocculation of coagulated particles because of the shallow primary minimum of the interparticle potential (Higashitani, Kondo, & Hatade, 1991). For these reasons, it took a longer time to initiate instability of the colloidal dispersion by removing GMP and meeting the CCC in SM milk, explaining its longer RCT.

Milk with LM resulted in higher elastic modulus (measured as G20) and lower tan δ than milk with SM (Figure 5). The higher casein content (subsection 3.2) may have contributed to the stronger gels in LM milk. Similarly, Cassandro et al. (2008) observed faster coagulation for milk with a higher total casein content. A stronger gel resulting from LM milk in our study is thus likely to be explained by two factors. First, LM allow faster onset of coagulation in milk, providing a longer time for formation of protein aggregates and consequently a stronger gel at the point of measuring gel strength. Second, the higher total casein content in LM milk contributes to a stronger gel, as casein is the main component involved in formation of the protein network (van Vliet & Walstra, 1994). Consequently, in LM milk in the present study, growth of larger numbers of voluminous clusters, distributed in the spatial network of the coagulum, resulted in higher gel strength due to a more rigid gel network than in SM milk.

Addition of calcium only resulted in a stronger gel and shorter RCT, while addition of citrate resulted in a weaker gel and longer RCT, irrespective of micelle size (Figure 5). Similarly, Gastaldi et al. (1994)

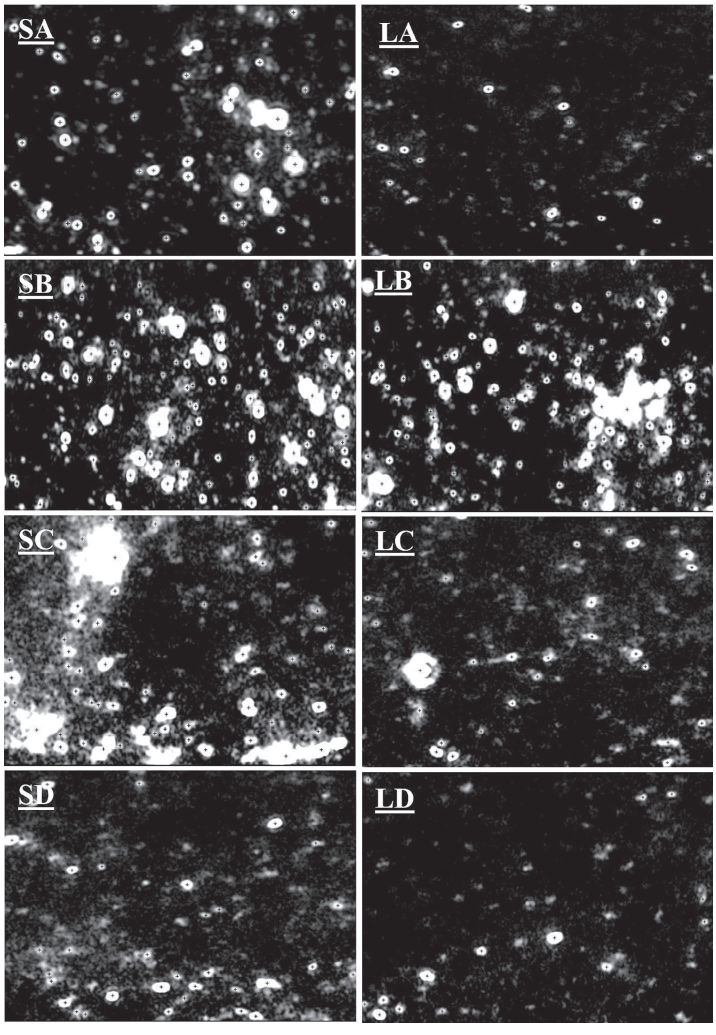

FIGURE 4 Nanoparticle tracking analysis video frames of milk samples with small micelles (left panel) and large micelles (right panel) in treatments with: (A) skimmed milk only, (B) skimmed milk with addition of calcium, (C) skimmed milk with addition of citrate, and (D) skimmed milk with addition of both calcium and citrate

reported that milk enriched with calcium had a shorter RCT. Due to added calcium, the repulsive properties of the κ-casein which stabilizes the micelle become weaker and added calcium creates calcium bridges, allowing adjacent micelles to interact and aggregate, resulting in an increase in gel strength (Lucey & Horne, 2018). The improved coagulation properties observed with added calcium are most likely due to formation of larger micelle clusters, contributing to initiation of aggregation at an earlier stage (Müller-Buschbaum et al., 2007). In contrast, addition of citrate would chelate any calcium available in the milk serum, thereby shifting the calcium equilibrium into solution and resulting in a longer RCT and a weaker gel.

Our findings regarding the effect of micelle size on coagulation are in agreement with early results reported by Ekstrand et al. (1980) in a study in which micelles of different sizes were separated by controlled-pore glass. They found that smaller micelles showed longer coagulation time, while medium-sized micelles showed shorter coagulation time. However, our findings are in contrast to those reported by Glantz et al. (2010) and Logan et al. (2014), who observed improved coagulation in milk with smaller micelles. This discrepancy may be partly an effect of the different techniques used to measure the size of the casein micelles. In NTA, particles in solution are counted without the bias caused by larger particles associated with the commonly used light-scattering techniques. Using dynamic light scattering to study micelle size, Glantz et al. (2010) reported an average micelle size of 200 nm for the Swedish Holstein breed, compared with 149.8 and 161.63 nm for SM and LM milk, respectively, in our study.

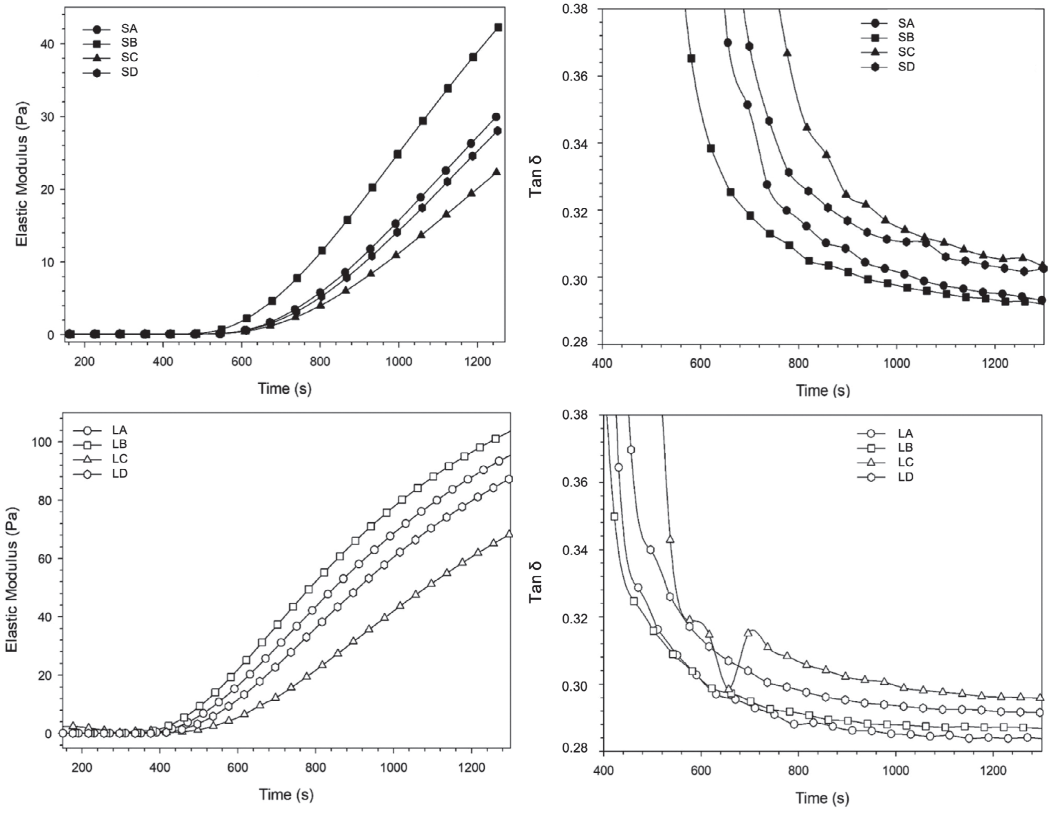

FIGURE 5 Effect of adding calcium (B), citrate (C), and both calcium and citrate (D) to skimmed milk (A) on elastic modulus (left panel) and tan δ (right panel) of renneted milk with small casein micelles (upper panel) and large casein micelles (lower panel). Note: Vertical axes of left panel are not identical

Moreover, our analyses were performed on milk samples from individual cows, whereas Glantz et al. (2010) and Logan et al. (2014) used bulk milk that probably contained a more polydisperse size distribution. Moreover, our results are not fully comparable with those reported by Logan et al. (2014) due to the interactions of fat globules in gelation in their study, whereas gels were made from skimmed milk in the present study. Our results suggest that micelle size, but also particle (micelle) concentration, are important in determining the coagulation properties of milk, but the latter has not been considered in previous studies.

3.2.2 | Microstructure formation of gels

The effects of adding calcium and/or citrate on rennet-induced gels were further analyzed by scrutiny of the gel micrographs. These revealed that the rennet gels formed a particular heterogeneous network consisting of a protein network surrounding void spaces that were probably filled with whey, as illustrated in Figure 6.

There was a clear distinction between the effects of the different treatments on the quality of the gels formed (Figure 6). In the presence of citrate, a loose gel network was observed, while in the presence of calcium a denser gel network was observed. Aggregation of larger clumps was visualized when citrate was added, creating comparatively larger void spaces than upon calcium addition. Gels made with added citrate had a more open microstructure, resulting in a more inhomogeneous network and, as a consequence, lower gel strength compared with gels formed with added calcium. Gels formed by adding only calcium consisted of thin protein strands, which formed a fine and continuous gel network (Figure 6). Gel microstructure was more homogeneous, with evenly distributed smaller open spaces, with the addition of calcium, resulting in stronger gels. The combination of calcium and citrate formed a more aggregated gel than observed in the untreated control. This is agreement with findings that particulate whey protein gels with larger pore size fall apart more easily (Langton & Hermansson, 1996). In general, SM milk resulted in a looser gel network (left panel, Figure 6) than seen in gels resulting

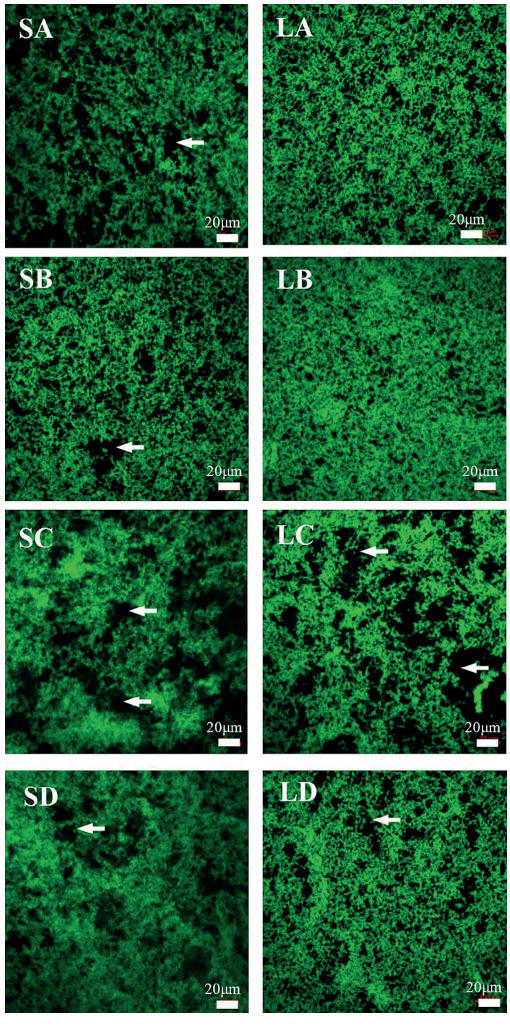

FIGURE 6 Confocal laser scanning micrographs of gels made from milk with small micelles (SM-, left panel) and large micelles (LM-, right panel) in treatments with (A) skimmed milk only, (B) skimmed milk with addition of calcium, (C) skimmed milk with addition of citrate, and (D) skimmed milk with addition of both citrate and calcium. Protein network is indicated in green, empty/pore spaces in black. Examples of void spaces are indicated with arrows. Scale bar: 20-μm

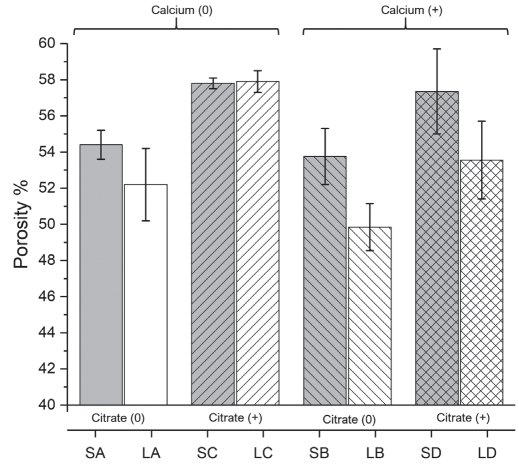

FIGURE 7 Porosity measurements on confocal micrographs of gels formed in milk with small micelles (SM-, dark) and large micelles (LM-, light) micelles in treatments with: (A) skimmed milk only, (B) skimmed milk with addition of calcium, (C) skimmed milk with addition of citrate, and (D) skimmed milk with addition of both citrate and calcium. (0) indicates no addition and (+) indicates addition of the respective compound. Standard error bars are included for each column

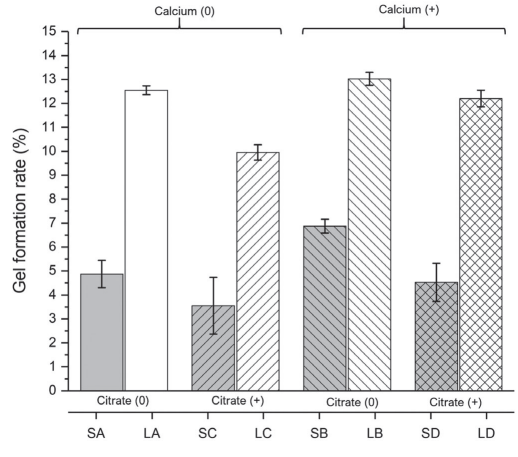

FIGURE 8 Formation rate of gels formed in milk with small micelles (dark) and large micelles (light) in treatments with: (A) skimmed milk only, (B) skimmed milk with addition of calcium, (C) skimmed milk with addition of citrate, and (D) skimmed milk with addition of both citrate and calcium. (0) indicates no addition and (+) indicates addition of the respective compound. Standard error bars are included for each column

from LM milk (right panel, Figure 6). The different effects of treatments on rheological parameters (subsection 3.2.1) were thus consistent with the microstructure observations.

Micrographs were evaluated for gel formation patterns (i.e., porous space) in a quantitative evaluation of the impact of treatments on gel formation (Figure 7). Larger void spaces were observed when only citrate was added and a decrease in porosity was observed when calcium was added. Calcium and citrate in combination resulted in intermediate values. In general, gels made with SM milk tended to result in higher porosity values than gels made with LM milk, except

TABLE 3 Results (significance levels) of three-way ANOVA on the effects of casein micelle size and addition of calcium (Ca) and/or citrate on milk gelation behavior

	Micelle size	Ca	Citrate	Micelle size × Ca	Micelle size × citrate	Ca × citrate	Micelle size × Ca × citrate
RCT	**	**	**	NS	NS	NS	NS
G20	**	**	**	NS	*	NS	NS
GR	**	**	**	NS	NS	NS	NS
GP	NS	NS	*	NS	NS	NS	NS

Abbreviations: ANOVA, analysis of variance; G20, gel firmness 20 min after rennet addition; GP, gel porosity; GR, gel formation rate; NS: not significant; RCT: rennet coagulation time.
*$p \leq .05$; **$p \leq .01$.

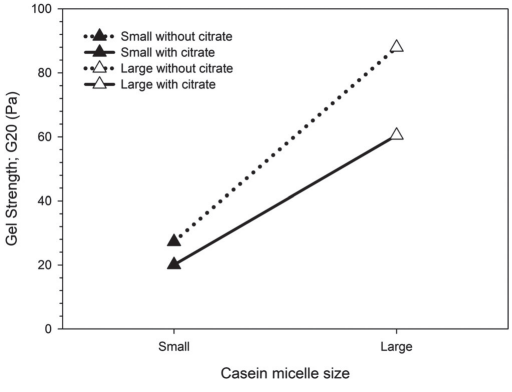

FIGURE 9 Interactive effects of casein micelle size and citrate addition on G20 (gel strength measured 20 min after rennet addition)

when only citrate was added. Thus image analysis confirmed the visual perceptions about the gels and supported the results reported in subsection 3.2.1.

3.2.3 | Gel formation rate

GRs were studied during the 800–1,000 s of the coagulation process (Figure 8). Milk with LM showed higher GRs than SM milk, most likely due to the formation of larger clusters, inducing instability of dispersion faster than in the case of SM milk. Milk with a higher GR thus also resulted in a stronger gel. Addition of calcium increased the GR, in contrast to addition of citrate, irrespective of micelle size.

3.3 | Effect of factor combinations in coagulation

The ANOVA results revealed the main and interactive effects of the treatments (Table 3). Micelle size and calcium and/or citrate addition all had a significant impact on the response variables RCT, gel firmness at 20 min (G20), and GR. However, GP was only influenced by citrate addition (Table 3).

Micelle size and citrate addition had a significant interactive effect on G20, with the reduction in G20 being greater with addition of citrate for larger micelles than small micelles (Figure 9). This interaction effect suggests that larger micelles with moderate citrate level will result in firmer gels, whereas an increase in citrate content will reduce gel strength more in case of large than small micelles. Since firmer gels are likely to retain more protein and fat than less firmer gels, this interaction effect could have implications in practical cheese production. There were no other significant two- or three-factor interactions. This demonstrates the importance of study design, as interactive effects can only be studied when many design parameters are analyzed together and individually.

4 | CONCLUSIONS

Changes in citrate and calcium content and in micelle size altered the properties of milk in rennet-induced coagulation. However, the only observed interactive effect of the factors was for micelle size and citrate on gel strength (G20). These results provide further evidence that addition of calcium and citrate affects the average size and size distribution of casein micelles in bovine milk.

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AUTHOR CONTRIBUTIONS

H.P.: contributed to designing the study, acquisition of data and analysis, interpretation of data, and drafting of the manuscript; Å.L.: contributed to designing the study, organization of the practicalities, supervision of the laboratory work, interpretation of data, and revision of the manuscript; A.H. and M.H.: contributed to interpretation of data and, revision of the manuscript; M.J.: contributed to organization of the practicalities, supervision of the laboratory work, interpretation of data, and revision of the manuscript; M.L.: contributed to

conception and designing of the study, discussion of the results and revision of the manuscript.

ETHICAL STATEMENTS

Conflict Of Interest: The authors declare no conflict of interest.
Ethical Review: This article does not contain any studies with human participants or animals performed by any of the authors.
Informed Consent: Written informed consent was obtained from all study participants.

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Composition and properties of bovine milk: A study from dairy farms in northern Sweden; Part I. Effect of dairy farming system

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ABSTRACT

This study was part of a larger project that aimed to understand the causes for increasing variation in cheese ripening in a cheese-producing region in northern Sweden. The influence of different on-farm factors on raw milk composition and properties was investigated and is described in this paper, whereas the monthly variation in the milk quality traits during 1 yr is described in our companion paper. The dairy farming systems on a total of 42 dairy farms were characterized through a questionnaire and farm visits. Milk from farm tanks was sampled monthly over 1 yr and analyzed for quality attributes important for cheese making. On applying principal component analyses to evaluate the variation in on-farm factors, different types of farms were distinguished. Farms with loose housing and automatic milking system (AMS) or milking parlor had a higher number of lactating cows, and predominantly Swedish Holstein (SH) breed. Farms associated with tiestalls had a lower number of lactating cows and breeds other than SH. Applying principal component analyses to study the variation in composition and properties of tank milk samples from farms revealed a tendency for the formation of 2 clusters: milk from farms with AMS or a milking parlor, and milk from farms with tiestall milking. The interaction between the milking system, housing system, and breed probably contributed to this grouping. Other factors that were used in the characterization of the farming systems only showed a minor influence on raw milk quality. Despite the interaction, milk from tiestall farms with various cow breeds had higher concentrations (g/100 g of milk) of fat (4.74) and protein (3.63), and lower lactose concentrations (4.67) than milk from farms with predominantly SH cows and AMS (4.32, 3.47, and 4.74 g/100 g of milk, respectively) or a milking parlor (4.47, 3.54, and 4.79 g/100 g of milk, respectively). Higher somatic cell count (195×10^3/mL) and lower free fatty acid concentration (0.75 mmol/100 g of fat) were observed in milk from farms with AMS than in milk from tiestall systems (150×10^3/mL and 0.83 mmol/100 g of fat, respectively). Type of farm influenced milk gel strength, with milk from farms with predominantly SH cows showing the lowest gel strength (65.0 Pa), but not a longer rennet coagulation time. Effects of dairy farming system (e.g., dominant breed, milking system, housing, and herd size) on milk quality attributes indicate a need for further studies to evaluate the in-depth effects of farm-related factors on milk quality attributes.

Key words: farm management factor, milking system, dominant breed, raw milk quality, milk coagulation property

INTRODUCTION

Raw milk composition and properties are crucial in controlling dairy product quality. Variation in the quality attributes of the raw milk affects different functional properties (Kailasapathy, 2015), and in cheese manufacturing, the characteristics of the resulting cheese rely on the composition and properties of the raw milk (Skeie, 2007; Guinee and O'Brien, 2010). During the last decades, dairy farming has been characterized by rapid intensification, moving toward fewer and larger farms, and, at the same time, increased efficiency and productivity (Clay et al., 2020). The intensification has been characterized by increasing adoption of novel technologies (e.g., for breeding), increased mechanization in feeding, and increased use of robotic milking systems. This transition is also characteristic for dairy production in northern Sweden, to an extent where it has become important to assess how changes in on-farm management and practices may have influenced

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the properties of the milk in this region. In a recent study, the increasing variation in maturation time of a traditional Swedish cheese produced in the region was visualized using hyperspectral image analysis, underlining a need to identify factors contributing to the variation in ripening (Priyashantha et al., 2020).

Milk coagulation properties are reported to be correlated with several milk quality attributes (Glantz et al., 2010; Priyashantha et al., 2019), which in turn are known to be influenced by breed and different on-farm factors (e.g., the type of milking system). The higher milking frequency associated with automated milking systems (**AMS**), has been suggested to influence several milk compositional parameters compared with milking parlor or tiestall milking (e.g., lower fat and protein content; Løvendahl and Chagunda, 2011), longer rennet coagulation time (**RCT**) and higher levels of free fatty acids (**FFA**; De Marchi et al., 2017; Wiking et al., 2019), as well as lower plasmin and plasminogen-derived proteolytic activity (Johansson et al., 2017).

The dairy farming system in northern Sweden is an interesting case for comparison with dairy farming systems in other regions in Sweden and Europe. The region where this study was conducted is characterized by boreal forests, scattered landscape, and forage-based agriculture. The region has a subarctic climate with short and fairly warm summers as well as lengthy and freezing winters. Dairy farms in the region deliver their milk to a cheese-making plant to produce a characteristic long-ripening Swedish cheese. Dairy farming is characterized by year-round calving, and during the last decade, the increase in the proportion of farms using AMS was above the average for Sweden. During the same period, there was a transition from tiestalls toward loose housing, as well as an increase in the proportion of farms with Swedish Holstein (SH) as the dominant breed. This study aimed to investigate the variation in the composition and properties of raw milk intended for the production of long-ripening hard cheese in a region in northern Sweden. This first part of the study evaluated the effects of different farm factors on the quality traits of the milk. In a companion paper (Priyashantha et al., 2021), we describe how the milk quality traits are influenced by monthly variation over 1 yr in the region, which was not well explored in previous studies.

MATERIALS AND METHODS

Experimental Design and Milk Sampling

The study was part of a full-scale commercial cheese manufacturing trial, using farm milk samples collected monthly during the period February 2016 to February 2017. The participating farms were located in a region between 64°2′ to 65°0′ N and 19°3′ to 21°5′ E in the county of Västerbotten. At the beginning of the study, all dairy farmers delivering milk to the participating cheese-making plant were asked about their willingness to participate in the study. A total of 37 farms agreed and were recruited for this study. An additional 5 organic milk production farms that delivered their milk to another milk-processing plant in the region were also included, making a final total of 42 farms. During autumn 2015, the participating farmers were asked to fill out a questionnaire about on-farm management factors, covering feed production and feeding facilities, housing and milking systems, and routines for milking and cleaning of the equipment. Herd and individual animal data (i.e., breed and milk yield) were obtained from the Swedish cow-recording scheme (Kokontrollen, 2016). The farms were visited on 2 occasions during the sampling period, in February and March (indoor period) and July (outdoor period), for an update on feeding, milking, and cleaning routines. The influence of monthly variation on the characteristics of the milk samples is described in a companion paper (Priyashantha et al., 2021). During the study, a few monthly values for 2 farms that decided to end their milk production were excluded. In total, between 296 and 505 of the collected farm milk samples were analyzed for the different milk quality attributes described in this paper. Not all samples were analyzed for all milk quality parameters at every sample collection due to various practical reasons, resulting in varying numbers of analyses per milk quality attribute.

Once every month over a period of 1 yr, an extra 250 mL of milk was sampled by the tanker driver from each farm on the same occasion as the sample routinely collected for raw milk quality control at the official milk testing laboratory (Eurofins Steins Laboratory, Jönköping, Sweden). The extra milk samples were transported separately at 4°C to the Department of Molecular Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala for analysis of additional quality traits. Upon arrival, the pH of milk samples was measured using a pH meter (Seven Compact S210, Mettler-Toledo). We conducted pH measurements at room temperature after letting samples equilibrate for 1 h. Casein micelle size and rennet-induced coagulation properties were analyzed in fresh skim milk samples. All analyses were performed in the same sequential order on all occasions. Milk samples were then aliquoted and stored at −80°C for analyses of plasmin and total proteolysis (measured as free amino terminals).

Raw Milk Gross Composition, SCC, and Bacteria

Raw milk samples were routinely analyzed for gross composition at the official milk testing laboratory, which used Fourier-transform infrared spectral analysis to measure the content of total fat, protein, lactose, urea, and FFA (CombiFoss 6000, Foss). The SCC was routinely analyzed by flow cytometry (Fossomatic, Foss) and total bacteria count using BactoScan FC (Foss Electric). Additionally, starting in May 2016, thermoresistant bacteria were analyzed at Eurofins by a culturing method (Wehr and Frank, 2004).

Casein Micelle Size

Casein micelle size was determined by nanoparticle tracking analysis (**NTA**) using NanoSight NS500 (Malvern Instruments) according to the method described by Priyashantha et al. (2019). The system was coupled to a temperature sensor, stage controller, and syringe pump (Harvard Apparatus) fitted with a 1-mL syringe filled with 2,000-fold diluted skim milk. Recorded video clips captured using a camera fixed at 90° angle and 658 nm wavelength were batch-processed using NanoSight 2.3 NTA. Average casein micelle size, mode, and standard deviation were determined for each sample analyzed.

Rennet-Induced Gelation

Rennet-induced coagulation properties of skim milk samples were evaluated using a Bohlin CVOR-150–900 rheometer (Malvern Instruments Nordic AB) according to the method described by Johansson et al. (2015). In brief, calf rennet (75/25 chymosin/bovine pepsin, 180 international milk clotting units (IMCU), Kemikalia) was added to the skim milk at a concentration of 0.18 IMCU/mL. The time of rennet addition was recorded as the start time, and gel formation was monitored. Coagulation properties were measured as RCT (i.e., time in seconds from the point of enzyme addition until a gel strength of 1 Pa was reached) and gel strength (in Pa) 20 min after rennet addition (**G20**). Each milk sample was analyzed in duplicate.

Plasmin Activity

Plasmin- and plasminogen-derived activities were determined according to the method described by de Vries et al. (2016). In brief, plasmin and plasminogen were dissociated from casein micelles by incubation of defatted milk with ε-amino-*n*-caproic acid, followed by ultracentrifugation (Beckman Coulter, Inc.). Plasmin activity was measured in the resulting milk serum using 2.5 mg/mL of a chromogenic substrate, pyro-Glu-Phe-Lys-*p*-nitroanilide hydroxy chloride (Aniara, West Chester). Plasminogen activity was derived after activation with urokinase (49.5 plow units) using a multimode microplate reader (FLUOstar Omega, BMG Labtech) at 37°C. Absorbance was recorded every 3 min for 120 min, and activity was expressed as a change in absorbance at 450 nm per unit time ($\Delta A405/\Delta t$). Each milk sample was analyzed in duplicate.

Total Proteolysis Measured as Free Amino Terminals

Total proteolysis was measured by a fluorescamine method based on the reaction of primary amino groups of trichloroacetic acid–soluble peptides and free AA with fluorescamine, as described by Wiking et al. (2002) and modified by Johansson et al. (2017). In short, milk samples were mixed with an equal volume of 24% trichloroacetic acid and kept on ice for 30 min before centrifugation at 16,000 × *g* for 20 min at 4°C. The supernatant (20 μL) was mixed with freshly made sodium tetraborate pH 8, fluorescamine was added, and the mixture was loaded in a 96-microwell plate. Fluorescence was measured after 23 min in a Perkin-Elmer LS55 luminescence spectrometer (Waltham) at excitation wavelength 390 nm and emission wavelength 480 nm. The extent of proteolysis was expressed as leucine equivalents (eq. m*M*), based on a standard curve with 5 different concentrations (1, 0.75, 0.5, 0.3, and 0.05 m*M*) of 0.1 *M* L-leucine dissolved in 1 m*M* HCl. Each milk sample was analyzed in triplicate.

Statistical Analysis

Principal component analysis (**PCA**; Wold et al., 1987) was used to explore the variation between the 42 farms and their milk sample characteristics. All farm data collected were subjected to a preliminary screening using multivariate analysis to identify the most influential on-farm factors. All types of variation (i.e., both farm and milk variables) were considered for the model, and representative on-farm factors were selected based on their location in the PCA loading plot (Figure 1). Remaining redundant factors were not further considered in statistical models; however, some of the factors were still of interest for the interpretation of overall results. One farm, the only one with Jersey as the main dairy breed, was excluded from the PCA (except when characterizing farms in Figure 2) and all further multivariate analyses. This herd was deemed as an outlier as all its observations were more than 2 standard deviations from the multivariate mean using the Hotelling T2 statistic (Jackson, 1991). One milk sample

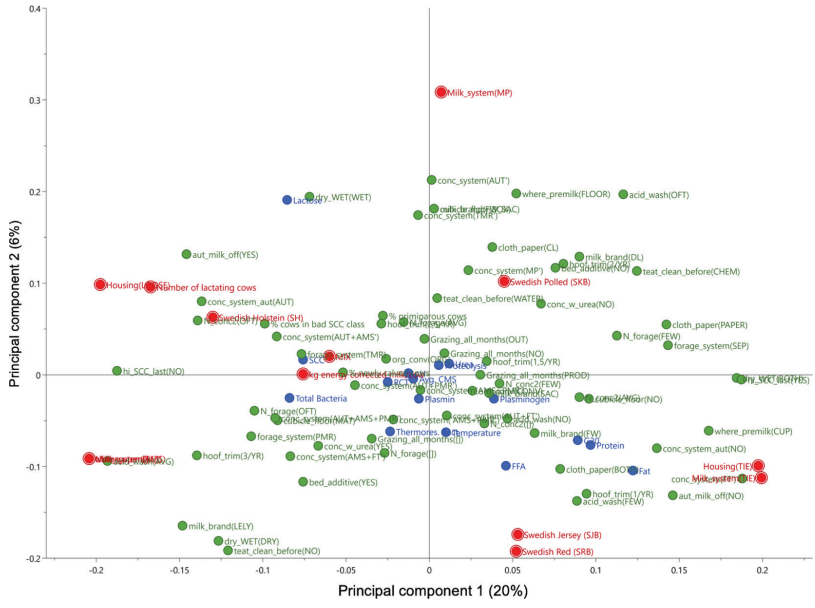

Figure 1. Principal component analysis (PCA) of on-farm (green labels) and milk quality (blue labels) variables used for selection of on-farm factors for further analysis; variables selected for further analysis are denoted with the red label. All data considered in this PCA are listed in Supplemental File S1 (https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/WKFDIJ).

from another farm, collected in April, was also deemed a serious outlier, as it was more than 4 standard deviations from the multivariate mean.

For the multivariate analysis, 15 variables reflecting milk quality attributes were assembled in a matrix comprising all monthly variables for the 41 remaining farms. The variables were preprocessed with mean centering, and each was set to unit variance by multiplication by its inverse standard deviation. Additionally, the following milk properties were logarithmically scaled (base 10) to adjust for skewness: total bacterial count, thermoresistant bacteria count, FFA, and SCC. We used PCA to compress the multidimensional data into a few principal components using the software Simca 16.0 (Sartorius Stedim Data Analytics AB). This model was restricted to variations associated with milk quality, with on-farm factors used only for interpretation of the results. The PCA score vectors were visualized in 2-dimensional scatter plots to assess similarities, trends, and groupings for the farms investigated. The PCA loading vectors were also used in 2-dimensional scatter plots to display similarities or differences between the milk quality attributes and to interpret the score scatter plots.

Univariate analysis was performed using NCSS 9 (NCSS Statistical Software) and repeated-measures ANOVA, and differences were considered significant at $P < 0.05$ using the Tukey-Kramer post hoc test. For univariate analysis, dependent variables (15 variables reflecting milk quality attributes) from all monthly values of all participating farms (n = 42) were evaluated according to the independent variables milking system and dominant breed, respectively. The model took the following form:

$$Y_{ijkl} = \mu + \alpha_{ij} + \beta_j + \gamma_k + (\beta\gamma)_{jk} + e_{ijkl},$$

where Y_{ijkl} is the response (dependent variable) for observation l in farm i (1–42), with milking system j (AMS, milking parlor, or tiestall) on months k (February 2016–February 2017); μ is the general mean; α_{ij} is the random effect of farm i within milking system or breed j; β_j is the effect of the milking system or breed j; γ_k is the effect of month k; $(\beta\gamma)_{jk}$ is the interaction between the milking system or breed j and month k; e is a random residual. The farm was used as an error term for testing the significance of the milking system or breed effects.

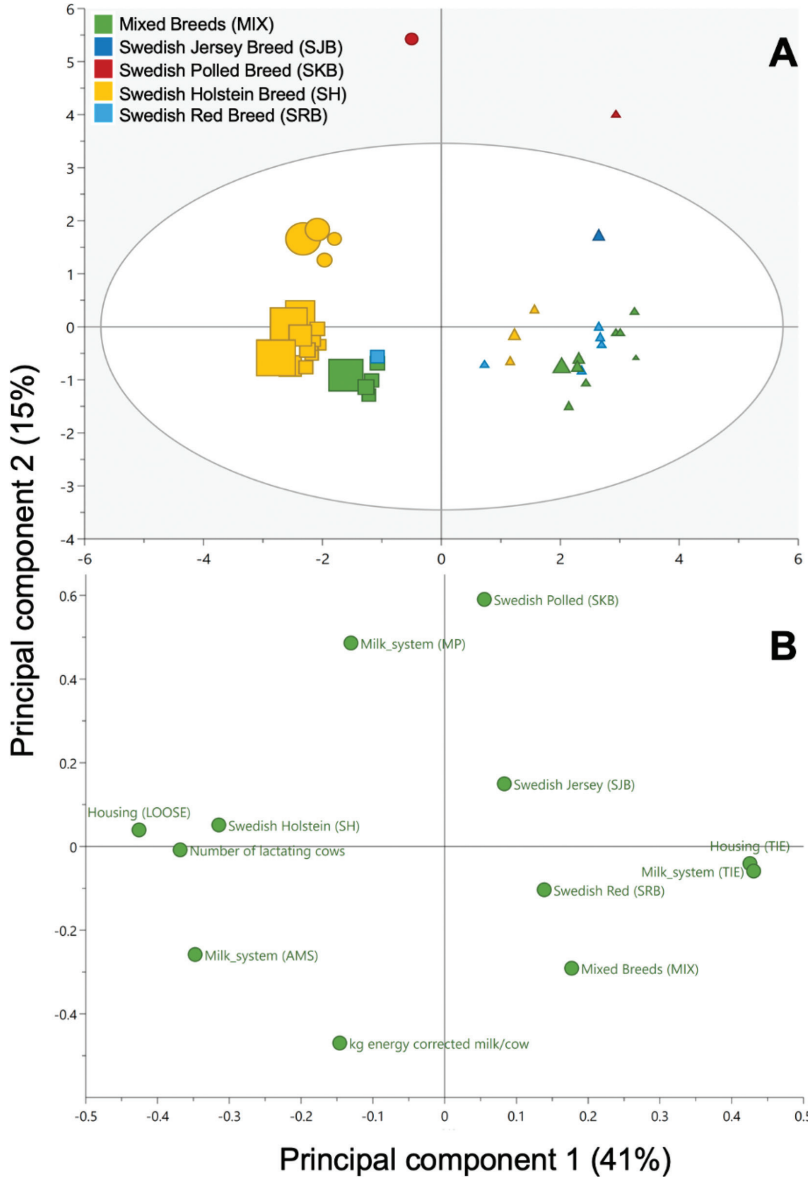

Figure 2. Principal component analysis (PCA) score plot (A) and loading plot (B) of selected on-farm factors documented on participating farms. In the score plot, each symbol represents an individual farm, with color indicating dominant breed, and shape indicating the milking system. Square = automatic milking system (AMS), circle = milking parlor (MP), triangle = tiestall milking system (TIE). Symbol size indicates the number of lactating cows in the herd.

RESULTS AND DISCUSSION

Variation in On-Farm Factors Between Participating Farms

The variation associated with on-farm factors was studied using data collected from the questionnaire, farm visits, and the Swedish cow-recording scheme (Kokontrollen, 2016). Data included in this study are listed in Supplemental File S1 (https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/WKFDIJ). From the preliminary multivariate screening of farm and milk data (Figure 1), the following farm factors were considered in the future models: (1) dominant breed (defined as the breed comprising >70% of the total herd), (2) milking system (AMS, tiestall, or milking parlor), (3) housing (tied or loose), (4) number of lactating cows, and (5) ECM yield (kg) per cow. Differences in milk quality attributes between organic (n = 5) and conventional (n = 37) farms were not observed in this study (Supplemental File S2, https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/BPJEC5), which is likely explained by similarities between the production systems (i.e., breeds and milking system).

The dominant breed on most farms was SH (19 farms), whereas Swedish Jersey (**SJB**) was only reported as dominant on 1 of the participating farms. These figures correspond well with data in the Swedish official cow-recording scheme (Växa Sverige, 2017) and with findings by Frössling et al. (2017), who reported SH to be the most common breed (50% of the dairy cow population in Sweden), followed by Swedish Red (**SRB**; 44%). In contrast, SJB and Swedish Polled (**SKB**) are rare dairy breeds in Sweden (Växa Sverige, 2017). Considering the low number of farms reporting SJB and SKB as the dominant breed in their herd, results on composition and properties of milk from these cows must be interpreted with caution.

The proportions of AMS (42% of farms) and tiestall milking (45% of farms) among participating farms reflect a situation where AMS is being increasingly installed on dairy farms. In 2018, the total proportion of farms in the region using AMS was 34%, in comparison with 28.7% on a national level (Växa Sverige, 2017). This suggests that the change from conventional milking to AMS has been more intense in the investigated region in comparison with the rest of Sweden. Daily milk yield (ECM) was 32.1, 29.6, and 29.2 kg in AMS, MP, and tiestall herds, respectively (Table 1). The average herd size (n = 66 lactating cow) and annual milk yield (9,806 kg of ECM per cow) in participating farms were also representative of northern Sweden (Växa Sverige, 2017).

Using PCA to assess the variation associated with the selected on-farm factors, 2 major groups of farms were identified based on principal component 1 (Figure 2). One cluster consisted of tiestall farms with various breeds in the herd, and the other cluster of farms with loose housing and milking parlor or AMS, with SH as the dominant breed (73.8% and 75.8% of SH in herds using milking parlor and AMS, respectively; Table 1). A higher number of lactating cows (i.e., larger farms) was strongly associated with SH as the dominant breed, loose housing, and AMS (~85 cows) and milking parlor (approximately 86 cows), whereas tiestall farms were smaller (~30 cows; Figure 2). In tiestall farms, SRB and SH percentages were 47.4 and 30.8, respectively (Table 1). The selected farm factors (milking system, housing system, number of lactating cows, ECM yield, and breed) were clearly confounded. This must be kept in mind when assessing the effect of individual on-farm factors on milk quality attributes (e.g., milking system or dominant breed in the herd).

Variation in Milk Quality Attributes Associated With Farm Type

The variation in the analyzed quality attributes of the milk samples is illustrated in the PCA plots in Figure 3. There was a clear effect of farm type on milk quality attributes, illustrated in Figure 3A by the color of the dots. Although there were exceptions, milk samples from farms with tiestall milking were mainly located to the left on principal component 1, milk samples from farms with AMS more to the right, and milk samples from farms with milking parlors in intermediate positions (Figure 3A). This distribution can be attributed to differences in milk composition, as visualized in the loading plot (Figure 3B). The PCA suggested that milk from farms with tiestall milking, where the dominant breed was SRB but also mixtures of breeds were common, was associated with a higher fat and protein content, but a lower lactose content, in contrast to milk from farms with AMS and MP, where the dominant breed was SH. This is illustrated in Supplemental File S3 (https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/YLFI4T) by coloring the score plot in Figure 3 according to the 2 dominant breeds (SRB and SH). These results agree with the previous finding by Wedholm et al. (2006), where fat and protein contents are higher in milk from SRB compared with SH cows, and also comply with the national data (Växa Sverige, 2017).

As mentioned, herd size, breed, housing system, and milking system were confounding factors in this case study. It is well known that cow breed is linked to several milk properties, such as fat and protein content.

Table 1. Herd data and quality attributes of farm tank milk samples collected monthly for 1 yr from the participating dairy farms (n = 42); averages of monthly data categorized according to farm type, based on milking system[1]

	AMS (n = 18)			Milking parlor (n = 5)			Tiestall milking system (n = 19)				
Item[2]	Mean	SE	N	Mean	SE	N	Mean	SE	N	Main effect *P*-value[3]	Interaction *P*-value
No. of lactating cows	84.8	3.29	180	86.1	5.98	60	29.5	1.10	210	*	*
Daily milk yield (kg of ECM/cow)	32.1	0.25	180	29.6	0.82	60	29.2	0.50	210	0.33	0.31
% SH	75.8	1.51	179	73.8	4.96	60	30.8	2.33	209	*	*
% SRB	17.3	1.53	179	3.9	0.75	60	47.4	2.45	209	*	*
% SH × SRB	6.2	0.55	179	2.2	0.40	60	5.8	0.73	209	0.66	0.56
% SJB	0.4	0.06	179	0.2	0.05	60	9.4	1.01	209	0.20	*
% SKB	0.0	0	179	18.0	4.68	60	5.7	1.48	209	0.28	*
% other breeds and crossbreds	0.3	0.06	180	2.0	0.41	60	0.8	0.22	210	0.43	*
Fat (g/100 g)	4.32	0.020	221	4.47	0.031	63	4.74	0.032	221	*	*
Protein (g/100 g)	3.47	0.009	221	3.54	0.015	63	3.63	0.014	221	*	*
Lactose (g/100 g)	4.74	0.004	214	4.79	0.006	62	4.67	0.008	218	*	*
Urea (mmol/L)	3.94	0.052	221	4.09	0.074	63	4.11	0.072	221	0.72	0.07
FFA (mmol/100 g of fat)	0.75[ab]	0.006	221	0.68[b]	0.009	63	0.83[a]	0.017	221	*	0.41
PL (units/mL)	3.08	0.093	153	3.16	0.188	42	2.85	0.087	151	0.41	0.93
PG (units/mL)	63.6	0.95	153	62.1	1.85	42	67.4	0.98	155	0.06	0.78
TP (m*M* Leu equivalents)	32.0	0.50	183	33.5	1.07	53	32.4	0.51	184	0.45	0.07
pH	6.72	0.004	199	6.72	0.010	59	6.71	0.004	203	0.37	0.16
SCC (10^3/mL)	195[a]	4.6	221	190[ab]	10.8	63	150[b]	5.1	221	*	0.29
TBC (10^3/mL)	14.2[a]	1.00	205	10.3[ab]	2.19	57	7.8[b]	0.62	208	*	0.25
TRBC (number/mL)	1,503	208.5	143	805	266.2	40	1,329	210.5	147	0.78	0.88
CMS (nm)	140	3.1	179	132	4.7	54	136	2.7	184	0.31	0.45
RCT (s)	455	6.6	129	446	11.7	38	446	7.4	130	0.96	*
G20 (Pa)	65.9[b]	1.17	128	70.7[ab]	2.33	38	79.1[a]	2.10	130	*	0.08

[a,b]Mean values with different superscripts within rows are indicated when only main effect is significantly different between farm types.

[1]AMS = automatic milking system; n = number of farms based on milking system; N = number of milk samples analyzed.

[2]% indicates average percentage of each breed in herds using a certain milking system; SH = Swedish Holstein; SRB = Swedish Red; SH × SRB = crosses of Swedish Holstein and Swedish Red; SJB = Swedish Jersey; SKB = Swedish Polled; FFA = free fatty acids; PL = plasmin activity; PG = plasminogen-derived activity; TP = total proteolysis measured as free amino terminals; TBC = total bacteria count; TRBC = thermoresistant bacteria count; CMS = casein micelle size; RCT = rennet coagulation time; G20 = gel strength at 20 min.

[3]Main effect and interaction effect with monthly repeated measurements are indicated with their *P*-values.

*$P < 0.05$.

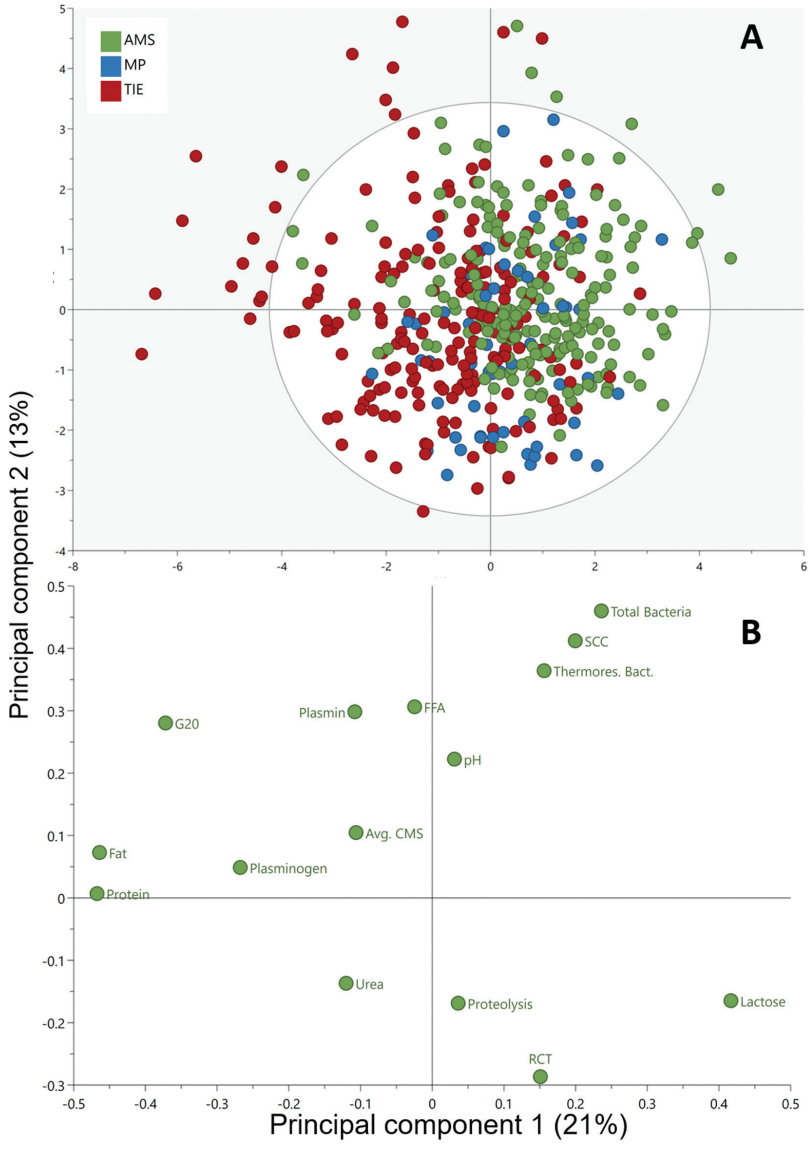

Figure 3. Principal component analysis score plot (A) and loading plot (B) of milk quality attributes (monthly data per farm). In the score plot, each dot represents a milk sample, and dot color indicates the milking system on the farm. AMS = automatic milking system; MP = milking parlor; TIE = tiestall milking. In Supplemental File S3 (https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/YLFI4T), score plots are colored according to the prevalence of the 2 major breeds (Swedish Red and Swedish Holstein).

Because the breed distribution on the participating farms was closely associated with housing and milking systems, these factors were expected to contribute to the observed differences to various extent; however, the effects of the individual factors cannot always be separated in our study. Descriptive data for the milk quality attributes in monthly samples, categorized according to the milking system and dominant breed, are shown in Tables 1 and 2, respectively.

As can be seen in Table 1, despite the significant interaction, there were differences in fat and protein content between milking systems, with higher values observed for tiestall compared with AMS or milking parlor farms. Milking frequency has previously been found to be negatively correlated with fat and protein content (e.g., Svennersten-Sjaunja et al., 2007; Løvendahl and Chagunda, 2011). In a comparison of tank milk from commercial Swedish dairy farms with conventional or robotic milking systems, Johansson et al. (2017) reported lower fat and protein content in milk from AMS than in milk from MP, which they attributed to the higher milking frequency in AMS. The daily milking frequency on AMS farms in this study was on average 2.7, and farms with tiestalls or milking parlors 2.0, according to questionnaire responses. Therefore, lower milking frequency in tiestall or milking parlor systems compared with AMS likely contributed to the observed difference in fat and protein contents. However, the compositional differences were most likely also linked to the higher occurrence of breeds that yield higher fat and protein content in milk from tiestall farms (Table 1, 2), and SH as the dominant breed on AMS farms (Figure 2). Despite the significant interactions, milk from herds with SRB cows showed numerically higher ($P = 0.07$) protein and fat percentage (3.66% and 4.85%, respectively) compared with milk from herds with SH as the dominant breed (3.55% and 4.33%, respectively; Table 2). Likewise, Bieber et al. (2019) and Wedholm et al. (2006) reported that milk from SRB contained higher fat and protein than milk from SH. The nonsignificant differences in the present study may be due to the fact that we used herd milk samples where one breed may be dominant, but not the only breed contributing to the farm milk sample. Milk from the farm with SJB as the dominant breed had the highest protein and fat content of all farms (4.06% and 6.21%, respectively).

Despite the significant interaction, lactose levels were lower in milk from SRB than in milk from SH in this study (Table 2). Likewise, Wedholm et al. (2006) found a significantly higher lactose content in milk from SH than in milk from SRB cows, whereas there was no difference when milk from SH and Danish Holstein cows were compared. Because results for SJB and SKB were based on only 1 and 2 herds, respectively, one should be cautious with too long-reaching interpretations. The lowest lactose content was in milk from the SJB farm. Considering that SJB cows produce two-thirds of the amount of milk compared with SH cows (Växa Sverige, 2017), the lower lactose content in milk from Jersey was expected. However, the highest lactose content was found in milk from the 2 farms with SKB cows. This is difficult to explain because SKB cows produce even less milk than SJB cows, averaging 54% of an SH cow (Växa Sverige, 2017). However, milk yield is not only governed by lactose concentration but also many other factors (e.g., genetics, feed, nutrition, metabolism, parity, and stage of lactation; McGuffey, 2017).

An elevated level of FFA in milk, resulting from enzymatic degradation of milk fat, is undesirable because it may confer a rancid taste to the final product (McSweeney et al., 1997). Irrespective of breed or milking system, FFA values in the present study were generally low (0.68–0.83 mmol/100 g of fat, Table 1) compared with values reported by Wiking et al. (2006), who investigated the effect of milking frequency on FFA levels in milk from SRB cows. The lowest FFA level in their study, 0.72 mEq/100 g of fat, was found when cows were milked twice per day and milk samples were analyzed immediately after milking. The FFA content in milk has been reported to be positively correlated with higher milking frequency (Klungel et al., 2000; Wiking et al., 2006). A suggested explanation for this is the disruption of the milk fat globule membrane as a result of mechanical stress in AMS and exposure of triglycerides to lipase (Hogenboom et al., 2019). Therefore, we expected higher FFA content to be associated with milk from AMS farms in our study. In fact, we found that milk from tiestall farms had higher numerical FFA content than milk from AMS farms (Table 1). It is difficult to suggest an explanation for this unexpected result. The results could also be related to factors associated with the status of the milking equipment on the farms (e.g., age and function) because higher FFA values were mostly reported from the oldest and smallest barns in our study, which all belonged to tiestalls farms. There are also studies reporting that no differences in FFA levels in milk from farms with AMS, MP, or tiestall milking (e.g., Johansson et al., 2017). Factors reported to contribute to variation in FFA levels include differences in mechanical treatment of the raw milk between farms with tiestall milking systems and AMS (e.g., pumping, foaming, and posthandling time; de Koning et al., 2003). The individual farm factor likely contributes to the fact that varying results on FFA in milk from different milking systems are reported.

For certain types of long-ripening cheeses, plasmin plays an important role in protein degradation during

Table 2. Quality attributes of farm tank milk samples collected monthly for 1 yr from the participating dairy farms; averages of monthly data categorized according to the dominant breed[1] on the farm

	SH (n = 19)			SRB (n = 6)			SKB (n = 2)			SJB (n = 1)			MIX (n = 14)			Main effect	Interaction
Item[2]	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	*P*-value[3]	*P*-value
No. of lactating cows	86.1	3.47	200	27.4	1.47	57	32.1	2.71	24	49.7	1.75	12	41.2	2.15	157	*	*
Daily milk yield (kg of ECM/cow)	32.7	0.25	200	31.7	0.62	57	16.5	0.56	24	20.5	0.54	12	30.0	0.45	157	0.23	0.22
Fat (g/100 g)	4.33	0.017	227	4.85	0.038	72	4.46	0.026	25	6.21	0.078	11	4.54	0.028	170	*	*
Protein (g/100 g)	3.50	0.008	227	3.66	0.023	72	3.47	0.033	25	4.06	0.027	11	3.55	0.014	170	0.07	*
Lactose (g/100 g)	4.76	0.004	222	4.64	0.008	71	4.83	0.016	25	4.39	0.013	11	4.70	0.008	165	*	*
Urea (mmol/L)	3.98	0.050	227	4.35	0.099	72	3.17	0.174	25	3.58	0.164	11	4.12	0.079	170	0.24	0.12
FFA (mmol/100 g fat)	0.74	0.006	227	0.82	0.030	72	0.78	0.027	25	0.64	0.011	11	0.81	0.018	170	0.47	0.21
PL (units/mL)	2.98	0.090	157	3.00	0.188	47	2.97	0.300	17	3.43	0.352	8	2.97	0.097	117	0.99	0.25
PG (units/mL)	64.2	0.90	158	69.6	1.89	50	63.9	3.29	17	75.6	4.64	8	63.8	1.09	117	0.43	*
TP (m*M* Leu equivalents)	32.6	0.50	188	32.5	1.12	59	31.2	1.07	22	34.9	2.69	8	32.1	0.56	143	0.73	0.25
pH	6.72	0.004	205	6.71	0.008	63	6.74	0.015	25	6.73	0.020	10	6.72	0.005	158	0.35	*
SCC (10^3/mL)	185	4.5	227	134	7.8	72	208	22.2	25	183	18.1	11	172	6.3	170	0.39	*
TBC (10^3/mL)	11.5	0.95	206	7.4	0.77	69	10.5	2.30	25	7.5	1.39	11	12.0	1.14	159	0.77	0.92
TRBC (number/mL)	1,322	203.3	141	1,928	527.3	46	2,947	645.5	19	387	117.2	8	934	158.1	116	0.55	0.58
CMS (nm)	140	2.8	184	134	5.1	57	136	7.3	23	130	9.2	9	136	3.3	144	0.92	0.99
RCT (s)	460	5.8	130	457	13.7	38	416	25.7	16	418	26.7	6	443	8.3	107	0.19	0.99
G20 (Pa)	65.0[c]	1.17	130	78.4[b]	3.09	38	83.7[b]	3.29	16	139.0[a]	3.91	6	73.6[bc]	1.86	106	*	0.72

[a–c]Mean values with different superscripts within rows are indicated when only main effect is significantly different between breeds

[1]SRB = >70% of cows are Swedish Red; SH = >70% of cows are Swedish Holstein; SJB = >70% of cows are Swedish Jersey; SKB = >70% of cows are Swedish Polled; MIX = mixture of breeds with <70% of 1 breed, n = number of farms based on the dominant breed in average over the year, N = number of milk samples analyzed,

[2]FFA = free fatty acids; PL = plasmin activity; PG = plasminogen-derived activity; TP = total proteolysis measured as free amino terminals; TBC = total bacteria count; TRBC = thermoresistant bacteria count; CMS = casein micelle size; RCT = rennet coagulation time; G20 = gel strength at 20 min.

[3]Main effect and interaction effect with monthly repeated measurements are indicated with their *P*-values.

*$P < 0.05$.

cheese maturation (Ismail and Nielsen, 2010). This was the reason for including analysis of plasmin and plasminogen-derived activity in raw milk and investigating causes of its variation in this study. The average plasmin activity in tank milk was 3.08, 3.16, and 2.85 U/mL for farms with AMS, MP, and tiestall, and plasminogen activity was 63.6, 62.1, and 67.4 U/mL, respectively. In contrast to Johansson et al. (2017), we found no significant differences in plasmin activity when comparing milk from farms with different milking systems (Table 1), and no differences between farms with different dominant breeds (Table 2). Likewise, Bastian et al. (1991) found no differences in plasmin activity between breeds in milk samples collected monthly from Holstein and Jersey cows during 10 mo of lactation. Those authors concluded that lactation number (parity) had the greatest influence on plasmin activity, with higher plasmin activity in milk from cows in their fourth and later lactations. We did not observe any effect of the milking system (Table 1) or breed (Table 2) on plasminogen or total proteolysis, measured as free amino terminals, in milk in the present study.

The average SCC was low from an international perspective, but it was higher in milk from AMS farms than in milk from farms using a tiestall milking system in this study (Table 1). Likewise, although average total bacteria counts were very low in farm tank milk (7.8–14.2 10^3/mL), significantly higher levels were observed in milk from herds with AMS than in milk from herds with tiestall milking (Table 1). In a review, Svennersten-Sjaunja and Pettersson (2008) discussed the reasons for the observed increase in SCC and total bacteria numbers in milk after introducing AMS in Europe. Higher SCC was suggested to be linked to several factors, some related to AMS per se (e.g., variation in the length of milking interval), whereas other factors were related to herd management on AMS farms. Using data from the Swedish Official Milk Recording Scheme, Frössling et al. (2017) also showed that a higher incidence of elevated SCC was associated with milk from AMS farms. Persson Waller et al. (2009), reported that milk from SRB cows had lower SCC compared with SH milk due to better udder health, inherent mastitis resistance, and efficient immune defense. In the present study, a probable reason for not observing differences in SCC between breeds could be that this study used tank milk samples classified according to the dominant breed in the herd, not individual cow milk samples. The fact that breed and milking system were confounding factors, with SH commonly being the dominant breed on AMS and milking parlor farms (Figure 1), likely explained the higher numbers of SCC in milk from AMS herds compared with farms with tiestall milking, and that milk from milking parlor farms showed numbers between the other 2 systems.

The total number of bacteria in milk is known to be associated with microbial contamination from the exterior of the udder, cleaning and sanitizing practices, and raw milk storage temperature and time (Bramley and Mckinnon, 1990). Mastitis pathogens are also known to contribute to the total bulk milk bacteria count, and cows with mastitis shed more bacteria into the milk than healthy cows (Bramley and Mckinnon, 1990). Fenlon et al. (1995) suggested that a mammary infection caused by mastitis pathogens will give rise to an increase in SCC as well as in total bacteria count in the milk. In the literature, the SRB breed is associated with a lower incidence of veterinary-treated clinical cases of mastitis compared with SH cows (Nyman et al., 2007; Bieber et al., 2019). This may be in line with the numerically lower total bacteria counts in milk from SRB cows compared with milk from SH cows in this study. There was no significant effect of the milking system or breed on the number of thermoresistant bacteria. The low number found in SJB milk and the high number in SKB milk was probably a consequence of these breeds being represented by only 1 and 2 farms, respectively (Table 2).

In the present study, we observed no significant difference in casein micelle size when comparing milk from farms with different milking systems (Table 1) or breeds (Table 2); we only found differences in numerical values, with larger casein micelles in milk from farms with SH compared with SRB as a dominant breed (Table 2). This agrees with Glantz et al. (2010), who found that milk from SH cows had a larger average casein micelle size (200 nm) than milk from SRB cows (191 nm). The differences in numerical values between our study and the study by Glantz et al. (2010) are likely explained by the different measuring techniques applied [i.e., NTA in our study and dynamic light scattering in the study by Glantz et al. (2010)].

Milk coagulation properties, measured as G20, were influenced particularly by breed, where milk from the SJB farm had an average G20 of 139 Pa, in comparison with 65.0 Pa for milk from farms with SH as the dominant breed (Table 2). Likewise, Jensen et al. (2012) found that milk from the Jersey breed had superior coagulation properties compared with milk from Holstein-Friesian cows, likely explained by the higher protein content (4.49 g/100 g), and specifically a high total casein content (3.13 g/100 g), in Jersey milk. Frederiksen et al. (2011) showed that milk from Jersey cows exhibited superior coagulation properties to milk from Danish Red or Danish Holstein-Friesian, owing to higher protein content (3.81 g/100 g) in Jer-

sey milk compared with milk from the other 2 breeds (3.52 and 3.47 g/100 g, respectively). The effect of the breed was also significant when comparing G20 for the different milking systems in our study (Table 1). Milk from farms with tiestall milking systems, which was positively associated with breeds other than SH (Figure 1), had a significantly higher G20 value than milk from farms with AMS or MP. Moreover, the difference in G20 might also result from the lower protein and fat content associated with higher milking frequency in AMS as observed in the present study, in agreement with Løvendahl and Chagunda (2011). In contrast, the milking system or breed did not affect RCT. Rennet coagulation time and G20 are generally inversely related; milk that coagulates rapidly (low RCT) has higher gel strength (high G20). This is visualized in the PCA in Figure 3B, where RCT and G20 are located on opposite sides in the loading plot, previously also reported by Priyashantha et al. (2019). Hallén et al. (2007) investigated the rheological properties of individual milk samples from SRB and SH and observed that protein concentration was positively associated with G20, but not with RCT, also confirming our observations. Problems previously reported to be associated with milk from SRB cows [e.g., noncoagulating properties and significantly lower gel strength in milk from individual cows (Frederiksen et al., 2011; Nilsson et al., 2019) and also in farm milk (Gustavsson et al., 2014)] were not evident in our study.

CONCLUSIONS

Two types of dairy farming systems were distinguished in this study (i.e., larger farms with AMS or milking parlors with SH as the dominant breed, and smaller tiestall farms with other breeds than Holstein). The shift toward larger farms is an effect of the structural dairy intensification that has gone on for some time in the investigated region. The continuous change in dominant breed, housing, and milking system, and the increasing herd size has most likely influenced the raw milk properties, and thus the following dairy processing. The type of dairy farming system showed a significant effect on many of the investigated milk quality traits. Because the housing and milking systems and dominant breed in the herd were confounding factors, it was difficult to distinguish their individual effects on milk quality attributes in this case study. Overall, we found that milk produced in the region was of high quality, irrespective of the dominant breed or milking system. To gain deeper insights into causes for the variation in raw milk, the influence of individual on-farm factors would need to be investigated further. Studies on the effects of raw milk variation as influenced by on-farm factors on cheese ripening are ongoing.

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Composition and properties of bovine milk: A study from dairy farms in northern Sweden; Part II. Effect of monthly variation

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ABSTRACT

This study investigated the influence of monthly variation on the composition and properties of raw farm milk collected as part of a full-scale cheese-making trial in a region in northern Sweden. In our companion paper, the contribution of on-farm factors to the variation in milk quality attributes is described. In total, 42 dairy farms were recruited for the study, and farm milk samples were collected monthly over 1 yr and characterized for quality attributes of importance for cheese making. Principal component analysis suggested that milk samples collected during the outdoor period (June–September) were different from milk samples collected during the indoor period. Despite the interaction with the milking system, the results showed that fat and protein concentrations were lower in milk collected during May through August, and lactose concentration was higher in milk collected during April through July than for the other months. Concentrations of free fatty acids were generally low, with the highest value (0.86 mmol/100 g of fat) observed in February and the lowest (0.70 mmol/100 g of fat) observed in June. Plasmin and plasminogen-derived activities varied with sampling month without a clear seasonal pattern. The pH of farm tank milk ranged from 6.60 to 6.82, with the lowest and highest values in September and February, respectively. The highest somatic cell count was observed in August (201×10^3 cells/mL) and the lowest in April (143×10^3 cells/mL). The highest value of gel strength, was recorded in December (88 Pa) and the lowest in July (64 Pa). Rennet coagulation time and gel strength were inversely correlated, with the lowest rennet coagulation time value observed in December. Orthogonal projections to latent structures (OPLS) and discriminant analysis adaptation of OPLS identified casein micelle size and total proteolysis as the milk quality attributes with major responses to sampling month, with smaller casein micelle size and higher total proteolysis associated with the outdoor months. Using discriminant analysis adaptation of OPLS to further investigate causes behind the variation in milk traits revealed that there were factors in addition to feeding on pasture that differed between outdoor and indoor months. Because fresh grass was seldom the primary feed in the region during the outdoor period, grazing was not considered the sole reason for the observed difference between outdoor and indoor periods in raw milk quality attributes.

Key words: season, monthly variation, raw milk characteristic, total proteolysis, casein micelle size

INTRODUCTION

The characteristics of cheese are known to depend on the composition and properties of raw milk (Skeie, 2007; Guinee and O'Brien, 2010), which has been shown to vary with the season in regions with a seasonal calving pattern (Li et al., 2019). Seasonal variation in the composition and properties of raw milk has been investigated for different dairy farming systems in regions with predominantly seasonal calving [e.g., New Zealand (Auldist et al., 1998) and Ireland (Lin et al., 2017)] and in regions with year-round calving [e.g., the Netherlands (Heck et al., 2009) and Sweden (Larsen et al., 2010; Karlsson et al., 2017)]. Various factors may contribute to observed seasonal differences in milk characteristics, depending on regional climate conditions and differences associated with lactation stage, cow nutrition, and cow health (Williams, 2002; Heck et al., 2009; O'Brien and Guinee, 2016). Concomitant interactions between these factors will influence the characteristics of milk, resulting in seasonal variation in the quality attributes of raw milk. In countries

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with pronounced seasonality, compositional changes in milk are mainly induced by calving patterns (e.g., newly calved cows as a percentage of the milking herd, feeding regimen, and udder health status; O'Brien and Guinee, 2016).

In Sweden, there is generally no seasonality in calving pattern; therefore, any observed seasonality in milk composition must be associated with other factors. To comply with Swedish animal welfare regulations, cows must have access to outdoor pasture during summer (Jordbruksverket, 2019). According to regulations, cows in the south of Sweden should have access to pasture during 120 d in the period April through October, whereas for cows in northern Sweden, regulations state 60 d on pasture in the period May through September. From this, at least 30 d must be in the period of June 1 to August 31, with at least 6 h of daily access to the field. During the indoor period in the region of our study, Swedish dairy cows were fed forages (i.e., grass silage and hay), some type of concentrate, and sometimes cereals. During the summer, some or all of the forage provision was substituted with pasture. Nevertheless, on some farms, cows were outdoors mainly for the purpose of exercise with only a little grass to consume. On these farms, the diet did not differ much over the year as reported by the farmers.

Few previous studies have investigated factors behind the variation in raw milk composition in Sweden. Larsen et al. (2010) reported lower fat and lower protein content in milk during summer months, and Frössling et al. (2017) observed elevated SCC toward the end of the grazing season. Lindmark-Månsson (2012) investigated the composition of Swedish dairy milk, analyzing 140 components in dairy silo milk sampled every second month during 1 yr from 9 dairy plants located throughout Sweden. The study showed that most components varied significantly during the year, whereas a lower number of components showed geographical differences. Some components, of which most were associated with milk fat quality, showed a more pronounced seasonal variation due to outdoor grazing in summer. Larsen et al. (2010) compared milk produced in central and southern Sweden and showed that milk quality attributes were influenced by differences in climate, feeding regimen, and breeds between the 2 regions of Sweden. Karlsson et al. (2017), who investigated dairy silo milk intended for the production of UHT processed milk in northern Sweden, reported monthly variation in the milk quality characteristics investigated, but variation was not associated with the season. The present study, which focused on dairy production in a cheese-producing region (Västerbotten) in northern Sweden, aimed to investigate the influence of monthly variation on the composition and properties of raw farm milk intended for long-ripening hard cheese.

MATERIALS AND METHODS

Experimental Design and Sample Collection

This study was part of a full-scale commercial cheese-manufacturing trial, using farm tank milk samples collected once per month during the period February 2016 to February 2017. The geographical area (from 64°2′ to 65°0′ N and 19°3′ to 21°5′ E) defines a relatively small region in the north of Sweden. The diversity of the farms, in terms of the dominant breed (making up >70% of the total herd) and milking system, is shown in Figure 1. The average annual temperature in the region during the study year (2016) varied from 0 to 4°C (Figure 1), and the average temperature during the period when cows were most active grazing (June–August) was 14.5 ± 2°C (Swedish Meteorological and Hydrological Institute; SMHI, 2016). Mean precipitation during June to August 2016 was 75 to 95 mm in the region (SMHI, 2016).

A total of 42 commercial farms, of which 5 were organic, were recruited for this study. Detailed characteristics of milk composition and properties, and how they are influenced by on-farm factors, are presented in a companion paper (Priyashantha et al., 2021). Herd and individual animal data (i.e., breed and milk yield) were obtained from the Swedish cow-recording scheme (Kokontrollen, 2016). The farms were visited on 2 occasions during the sampling period, in February and March (indoor period) and July (outdoor period), to get an update on the feeding, milking, and cleaning routines. During the study, a few monthly data from 2 farms were excluded in connection to closing down their dairy production. In conjunction with regular milk collection, a milk sample for the raw milk quality control program run by the official milk testing laboratory (Eurofins Steins Laboratory, Jönköping, Sweden) was collected. During this 1-yr study, an additional 250 mL of bulk milk was sampled by the tanker driver from each farm once per month parallel to the control sample. This extra tank milk sample was sent for analysis of additional milk quality attributes at the Swedish University of Agricultural Sciences, Uppsala, Sweden.

Milk Characterization

Procedures for handling and analysis of the milk samples are described in full in the companion paper (Priyashantha et al., 2021). In brief, gross composition, SCC, total bacteria, and thermoresistant bacte-

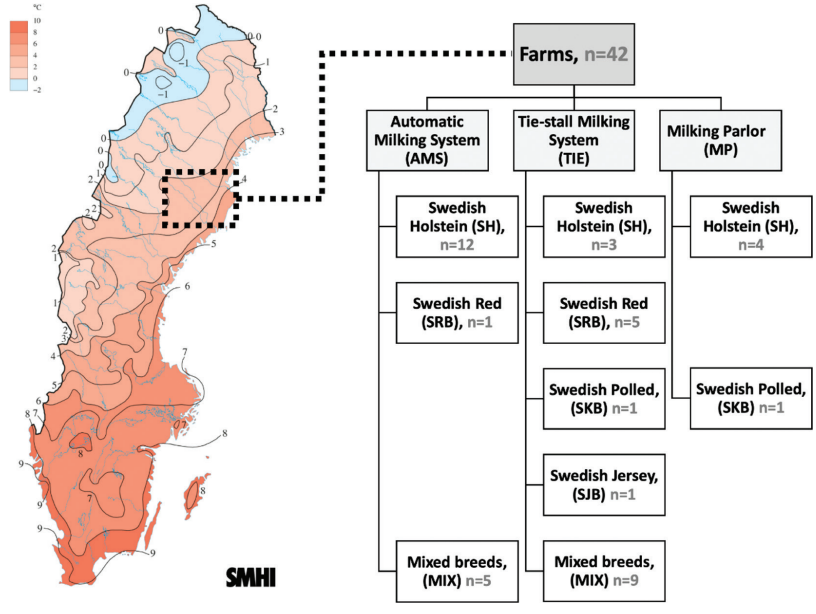

Figure 1. Participating farms in the study, categorized according to milking system and dominant breed in herd. n = number of farms; dominant breed = breed making up >70% of the total herd. Mixed breed = herd consisting of different dairy breeds or crosses (<70% of an individual breed). The map indicates mean annual temperature in 2016, sourced from the Swedish Meteorological and Hydrological Institute (SMHI, 2016), with the region where participating farms are located indicated by dotted lines in the map of Sweden.

ria counts were analyzed at a certified milk-grading laboratory (Eurofins Steins Laboratory, Jönköping, Sweden). Fat, protein, lactose, urea, and free fatty acids (**FFA**) were measured using Fourier transform infrared spectral analyses (CombiFoss 6000, Foss). The SCC and total bacteria count were analyzed using Fossomatic and BactoScan FC instrumentation, respectively (Foss). Thermoresistant bacteria were analyzed at Eurofins from May 2016 and onwards by culturing method (Wehr and Frank, 2004). The pH of milk samples was measured using a pH meter (Seven Compact S210) at room temperature. Average casein micelle size was determined by nanoparticle tracking analysis using NanoSight NS500 (Malvern Instruments) according to the method described by Priyashantha et al. (2019). Rennet-induced coagulation properties of skim milk were evaluated using a Bohlin CVOR-150–900 rheometer (Malvern Instruments Nordic AB) according to Johansson et al. (2015) using calf rennet (75/25 chymosin/bovine pepsin, 180 international milk clotting units, Kemikalia) at a concentration of 0.18 international milk clotting units per milliter. Rennet coagulation time (**RCT**, s), and gel strength after 20 min (**G20**, Pa) were recorded. Assessment of plasmin and plasminogen-derived activities were performed following the method by de Vries et al. (2016). Plasmin activity was measured in the resulting milk serum using a chromogenic substrate, and plasminogen-derived activity was calculated after activation with urokinase-type plasminogen activator. Total proteolysis was estimated by measuring free amino terminals (Leu equivalents) as described by Wiking et al. (2002) and modified by Johansson et al. (2017). Due to practical circumstances, on some sampling occasions, not all milk quality traits were evaluated.

Statistical Analysis

Principal component analysis (**PCA**; Wold et al., 1987) and orthogonal projections to latent structures (**OPLS**; Trygg and Wold, 2002) were used for multivariate analyses, using the software Simca 16.0 (Sartorius Stedim Data Analytics AB). The OPLS method is a progression from partial least squares regression (Wold et al., 2001); in OPLS, orthogonal variation to the response is removed from the independent variables

before fitting the model. One farm that reported Jersey as the dominant breed was excluded from the multivariate analyses because it was deemed an outlier, as all observations deviated by more than 2 standard deviations from the multivariate mean using the Hotelling T2 statistic (Jackson, 1991). One milk sample collected from another farm in April was also excluded as an outlier, as it deviated by more than 4 standard deviations from the multivariate mean.

For the multivariate analysis, the 15 variables of milk quality attributes were assembled in a matrix, comprising all monthly variables for the 41 farms as observations. The variables were preprocessed with mean centering, and each was set to unit variance by multiplication of its inverse standard deviation. Additionally, the following milk properties were logarithmically scaled (base 10) to adjust for skewness before performing PCA: total bacterial count, thermoresistant bacteria count, FFA, and SCC. As this model was limited to variation associated with milk quality, sampling month was used only for interpretation. The PCA score vectors were visualized in 2-dimensional scatter plots for assessing similarities, trends, and groupings for the farms investigated. The PCA loading vectors were used in 2-dimensional scatter plots for displaying similarities or differences between the milk quality attributes and to interpret the score scatter plots.

We used OPLS to study milk properties in relation to a specific response (i.e., sampling month). Using Simca 16.0, the data matrix containing milk quality attributes was modeled with an OPLS batch procedure, with sampling month as the response variable, to identify factors associated with sampling month. For this, all observations from each farm collected throughout the year were treated as separate batches. Finally, orthogonal projections to latent structures discriminant analysis (**OPLS-DA**; Bylesjö et al., 2006) was used to study the effect of feeding regimen (e.g., grazing), with milk quality attributes modeled with different grazing practices. The OPLS-DA method is a version of OPLS especially aimed at the purpose of classification where the response matrix represents different classes using dummy variables of ones and zeros. In the special case of 2 classes, 1 dummy variable can be used, but for a higher number of classes, 1 dummy variable was added for every class. The different feeding regimens were set up as the responses, and the milk quality attributes served as independent variables in the OPLS-DA model. For the OPLS models, loading plots were inspected to identify significant factors in relation to the studied responses.

Univariate analysis was performed using NCSS 9 (NCSS Statistical Software). The effect of sampling month on milk quality attributes was analyzed by ANOVA with the Tukey post hoc test, and significance was considered at $P < 0.05$. Milk quality parameters (dependent variables) were studied according to monthly variation as well as the interaction with the milking system on the farm. The model took the following form:

$$Y_{ijkl} = \mu + \alpha_{ij} + \beta_j + \gamma_k + (\beta\gamma)_{jk} + e_{ijkl},$$

where Y_{ijkl} is the response (dependent variable) for observation l in farm i (1–42), with milking system j (automatic milking system, milking parlor, or tiestall) on months k (February 2016–February 2017); μ is the general mean; α_{ij} is the random effect of farm i within milking system or breed j; β_j is the effect of the milking system j; γ_k is the effect of month k; $(\beta\gamma)_{jk}$ is the interaction between the milking system j and month k; e is a random residual. The farm was used as an error term for testing the significance of the milking system.

RESULTS AND DISCUSSION

Variation in On-Farm Factors Associated With Sampling Month

Silage of grass and clover (mixed ley) was the dominant forage on the farms, mostly preserved in round bales, but also in the bunker or tower silos on some farms. There was a large variation in feed intake from pasture on the farms. On some farms, the cows had full indoor feeding even during summer months; however, on other farms, pasture provided a major part of the forage intake of the cows, at least during part of the summer. On average, the pasture was estimated to provide approximately 30% of the feed intake of the cows on the participating farms from mid-June until mid-August.

Under animal welfare regulations for the region, dairy cows should be outdoors for at least 2 mo during summer (Jordbruksverket, 2019). In 2016, the participating farmers reported that the grazing period was 2 to 2.5 mo on 16 of the farms, 2.5 to 3.5 mo on 11 farms, and longer than 3.5 mo on the remaining farms. The earliest date on which cows had outdoor access was May 2, and the latest date for bringing cows back indoors was October 26. In June through August, most cows had outdoor access, whereas in September there was wide variation in the number of farms that still had cows on pasture.

Variation in Milk Quality Attributes Associated With Sampling Month

A PCA model, explaining 21 and 13% of the variance in the first and second principal component,

respectively, was used to evaluate the monthly variation in different milk quality variables. The score plot suggested a tendency for milk collected during May through August to cluster in the lower-right quadrant, and milk samples collected during the other months grouped more to the upper-left quadrant of the score plot (Figure 2A). According to the loading plot (Figure 2B), the underlying reasons for this variation included differences in fat, protein, and lactose concentrations, in addition to the possible effects from plasmin activity, coagulation properties, casein micelle size, and total proteolysis (free amino terminals).

The variation in milk quality attributes with sampling month is shown in Table 1, which presents averages calculated from monthly data for the individual farm milk samples and their interactions with the milking system. As found previously by Lindmark-Månsson (2012) and Lindmark-Månsson et al. (2003) for Swedish dairy silo milk, milk gross composition was influenced by month. In our study, despite the interaction of monthly variation in fat content with the milking system, milk delivered during May through August had lower fat content compared with the rest of the year. Similarly, protein concentration in milk was higher in the indoor period (e.g., October–December) than in the outdoor period (e.g., May–August).

Similarly, Karlsson et al. (2017) observed higher protein content in November and December than during the rest of the year when studying unprocessed dairy silo milk produced in the same region. Heck et al. (2009) observed similar trends regarding variation in the contents of fat and protein in Dutch dairy milk. The authors suggest that the differences in feeding regimens between seasons, in particular the difference in the inclusion of concentrate in the diet, could influence milk production and thereby also the technological properties of the milk.

Lactose concentrations observed in the present study were in the same range as the average of 4.70 g/100 g of milk reported by Lindmark-Månsson, (2012) for Swedish dairy silo milk, but lower than 4.87 g/100 g of farm tank milk (Toledo et al., 2002). In our study, lactose concentrations ranged between 4.64 and 4.75 g/100 g of milk, with slightly higher values observed during April through July (4.74–4.75 g/100 g) compared with milk from November and December (4.64–4.65 g/100 g (Table 1), despite the interactions with milking system. Glantz et al. (2009) found lower lactose content in bulk milk during winter (4.51 g/100 g) than during summer (4.54 g/100 g), and also Heck et al. (2009) and Chen et al. (2014) observed variation in lactose content in milk over the year, but with no significant differences between months. Lactose, through its osmotic properties, regulates the water content of milk, and concentrations in milk from healthy cows are expected to be quite constant during the lactation (Fox et al., 2015). Evaluating data from the cow-recording scheme (Kokontrollen, 2016; data not shown), we found that the slightly higher lactose values observed during April through July were not associated with an increase in milk yield.

The average milk urea concentration in our study was 4.0 mmol/L, calculated for all months and using data from all farms, with values ranging from 3.8 mmol/L in January to 4.2 mmol/L in May. In agreement with Chen et al. (2014), who investigated variation in raw bulk milk from Holstein cows in the United Kingdom, we did not observe any seasonal variation in milk urea. Protein-rich diets are reported to result in higher milk urea (Nousiainen et al., 2004); therefore, seasonal differences are usually associated with differences in protein feeding during indoor and outdoor periods. In the present study, herds seemed to be provided with a balanced diet, likely explaining the lack of a seasonal pattern in milk urea concentration.

Throughout the year, average FFA concentrations in milk from the participating farms were below 1.0 mmol/100 g of fat (Table 1), which is considered the threshold for distinguishing a rancid off-flavor in raw milk (McSweeney et al., 1997). However, as seen from the maximum values, milk samples from individual farms occasionally had higher concentrations of FFA (data not shown). The highest average FFA concentration (0.86 mmol/100 g of fat) was observed in February 2017 and the lowest in June (0.71 mmol/100 g of fat). In a study of dairy silo milk from northern Sweden, Lu et al. (2018) observed higher FFA concentrations in milk sampled in the months of March and September, and they attributed this to a lower forage quality associated with these transition months. Furthermore, previous investigations on the causes of elevated FFA in milk in Sweden concluded that maintenance of milking equipment and the interval between milkings were important factors (Lindberg et al., 2004). Seasonal variation in FFA concentrations may also be due to stress factors, such as feed transitions, feed shortages, and temperature fluctuations. According to Anderson (1983), the formation of FFA typically occurs at the farm level, but further increases may occur within the dairy factory due to disruption of fat globules as a result of mechanical stress (e.g., pumping).

In this study, we observed a monthly variation associated with plasmin and plasminogen-derived activities in milk (Table 1). Plasmin activity in milk ranged from 2.16 units/mL in July to 3.43 units/mL in March, whereas plasminogen-derived activity varied from 59.93

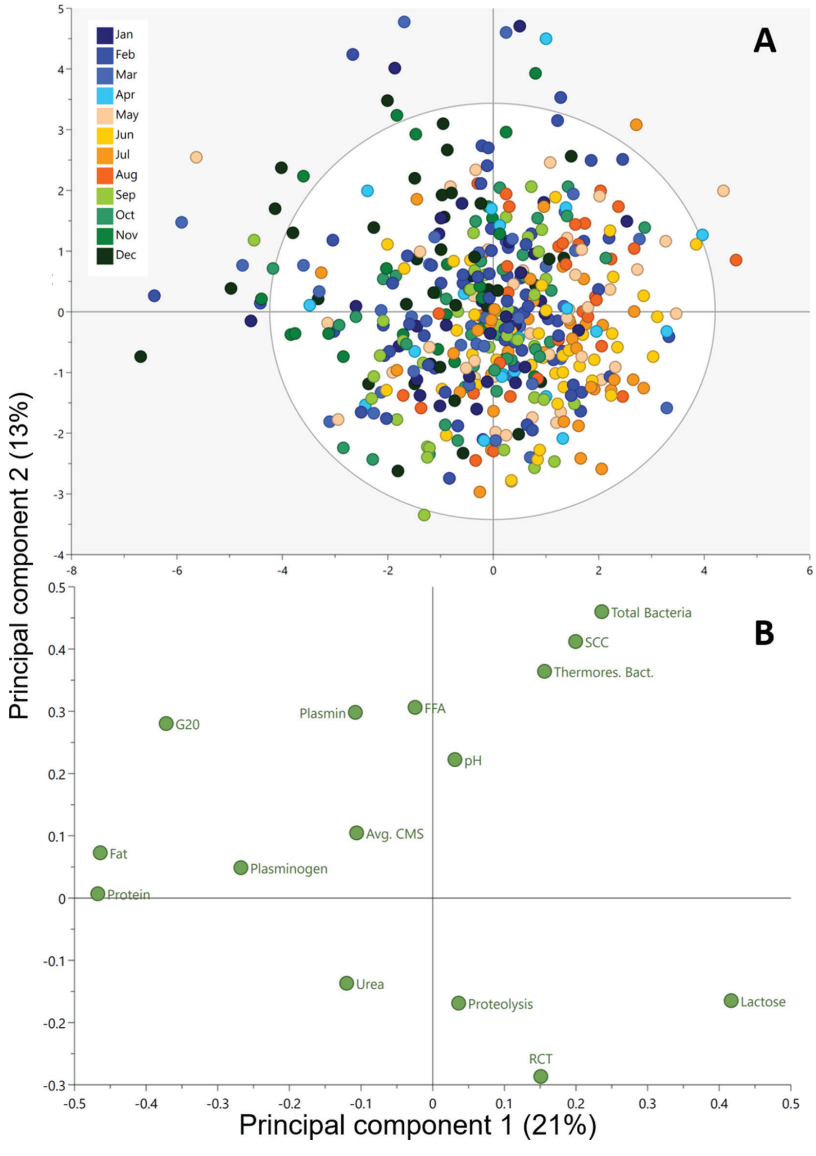

Figure 2. Principal component analysis score plot (A) and loading plot (B) of milk quality attributes as influenced by sampling month. Colors in the score plot indicate month. Monthly variation appeared to follow a diagonal trend from upper-left quadrant (indoor months) to lower-right quadrant (outdoor months). FFA = free fatty acids; RCT = rennet coagulation time; G20 = gel strength; Avg. CMS = average casein micelle size.

Table 1. Average milk composition [mean (SD)] based on the monthly analyses of bulk milk samples from the participating farms during 1 yr, February 2016 to February 2017; the number of farm milk samples analyzed varied between months for different reasons (e.g.,samples from individual farms occasionally missing)

Parameter[1]	Feb 2016	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 2017	Feb	P main[2] effect	P month[2] × MST
Fat (g/100 g of milk)	4.58	4.62	4.53	4.47	4.42	4.41	4.38	4.53	4.56	4.66	4.60	4.59	4.52	**	*
	(0.50)	(0.49)	(0.37)	(0.45)	(0.46)	(0.45)	(0.39)	(0.42)	(0.35)	(0.44)	(0.40)	(0.40)	(0.31)		
Protein (g/100 g of milk)	3.54	3.56	3.55	3.51	3.46	3.48	3.47	3.60	3.61	3.65	3.60	3.59	3.56	**	*
	(0.22)	(0.20)	(0.22)	(0.19)	(0.17)	(0.16)	(0.16)	(0.14)	(0.16)	(0.14)	(0.18)	(0.16)	(0.15)		
Lactose (g/100 g of milk)	4.73	4.71	4.74	4.75	4.75	4.74	4.72	4.71	4.71	4.65	4.64	4.72	4.72	**	*
	(0.10)	(0.11)	(0.09)	(0.10)	(0.10)	(0.10)	(0.11)	(0.09)	(0.10)	(0.11)	(0.12)	(0.10)	(0.07)		
Urea (mmol/L)	4.07	4.05	4.11	4.19	3.97	3.91	4.03	3.90	3.98	4.14	4.14	3.78	4.11	0.26	0.07
	(1.01)	(0.98)	(0.97)	(0.97)	(0.82)	(0.77)	(0.90)	(0.84)	(1.04)	(0.87)	(0.92)	(0.70)	(0.90)		
FFA (mmol/ 100 g of fat)	0.75^{ab}	0.83^{ab}	0.80^{ab}	0.79^{ab}	0.71^{b}	0.80^{ab}	0.76^{ab}	0.77^{ab}	0.75^{ab}	0.73^{ab}	0.73^{ab}	0.79^{ab}	0.86^{a}	*	0.40
	(0.13)	(0.34)	(0.35)	(0.16)	(0.09)	(0.09)	(0.10)	(0.12)	(0.10)	(0.16)	(0.13)	(0.12)	(0.19)		
PL (units/mL)	NA[3]	3.43^{a}	NA	3.24^{a}	2.99^{a}	2.16^{b}	3.08^{a}	NA	3.36^{a}	3.12^{a}	3.01^{a}	2.70^{ab}	2.97^{ab}	**	0.93
		(1.27)		(1.10)	(1.00)	(0.75)	(1.19)		(0.91)	(1.09)	(1.08)	(1.21)	(1.20)		
PG (units/mL)	NA	63.34^{b}	NA	65.32^{b}	60.17^{b}	60.73^{b}	61.51^{b}	NA	68.3^{ab}	77.06^{a}	68.01^{b}	64.12^{b}	59.93^{b}	**	0.78
		(17.00)		(8.07)	(10.18)	(8.00)	(10.39)		(10.45)	(12.36)	(10.00)	(9.77)	(13.05)		
TP (mM leucine equivalent)	NA	31.27^{cde}	32.79^{bcde}	39.06^{a}	36.57^{ab}	35.34^{abc}	33.94^{bcd}	33.31^{bcd}	33.94^{bcd}	29.89^{def}	27.87^{ef}	27.70^{ef}	26.51^{f}	**	0.72
		(5.63)	(6.88)	(6.72)	(5.11)	(4.92)	(4.43)	(8.17)	(6.51)	(4.74)	(3.97)	(6.69)	(6.63)		
pH	6.72^{cd}	6.71^{de}	6.73^{cd}	6.74^{c}	6.72^{cd}	6.68^{f}	6.75^{bc}	6.60^{g}	6.68^{ef}	6.77^{b}	6.70^{def}	6.72^{cd}	6.82^{a}	**	0.16
	(0.02)	(0.04)	(0.06)	(0.05)	(0.03)	(0.03)	(0.03)	(0.06)	(0.04)	(0.03)	(0.03)	(0.03)	(0.05)		
SCC (10^3/mL)	159^{ab}	154^{ab}	143^{b}	171^{ab}	171^{ab}	172^{ab}	201^{a}	181^{ab}	175^{ab}	185^{ab}	191^{ab}	191^{ab}	181^{ab}	*	0.29
	(70)	(66)	(62)	(83)	(76)	(76)	(88)	(86)	(81)	(85)	(76)	(83)	(60)		
TBC (10^3/mL)	7.73	13.46	9.78	13.24	11.03	13.70	12.59	7.74	10.06	11.54	9.20	10.58	11.95	0.06	0.24
	(5.51)	(18.89)	(9.12)	(11.60)	(9.12)	(20.24)	(12.60)	(6.05)	(7.11)	(15.91)	(9.08)	(15.60)	(18.49)		
TRBC (number/mL)	NA	NA	NA	$1{,}613^{ab}$	$1{,}300^{ab}$	$1{,}198^{ab}$	$3{,}032^{a}$	$1{,}154^{ab}$	$2{,}023^{ab}$	932^{ab}	530^{b}	843^{b}	$1{,}021^{ab}$	*	0.88
				(2,715)	(2,599)	(2,179)	(4,022)	(1,904)	(3,064)	(1,924)	(800)	(1,709)	(1,907)		
CMS (nm)	136^{de}	134^{de}	133^{de}	109^{f}	120^{ef}	NA	72^{g}	149^{cd}	107^{f}	144^{d}	165^{bc}	181^{ab}	184^{a}	**	0.45
	(22)	(24)	(18)	(14)	(17)		(13)	(35)	(23)	(24)	(27)	(23)	(21)		
G20 (Pa)	75^{ab}	76^{ab}	NA	69^{b}	68^{b}	64^{b}	74^{ab}	NA	72^{b}	NA	88^{a}	NA	65^{ab}	**	0.08
	(21)	(18)		(18)	(17)	(19)	(20)		(13)		(24)		(10)		
RCT (s)	46	444	NA	441	465	456	442	NA	483	NA	361	NA	529	**	*
	(46)	(59)		(95)	(40)	(52)	(68)		(40)		(123)		(67)		

[a–g]Mean values within rows with different superscripts are indicated when only main effect is significantly different at $P < 0.05$ or $P < 0.01$.

[1]FFA = free fatty acids; PL = plasmin; PG plasminogen; TP = total proteolysis based on free amino terminals; TBC = total bacterial count; TRBC = thermoresistant bacteria; CMS = casein micelle size; RCT = rennet coagulation time; G20 = gel strength at 20 min.

[2]MST = milking system; main effect and interaction effect with milking system are indicated with their P-values.

[3]NA = not analyzed.

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

units/mL in February 2017 to 77.06 units/mL in November. Despite a significant effect of the month on plasmin and plasminogen-derived activities, variation showed no clear seasonal pattern. Similarly, on studying the effect of season on plasmin-derived proteolytic activity in milk from pasture-fed dairy cows in New Zealand, Nicholas et al. (2002) found that time of the year did not influence plasmin activity. They observed an effect on plasminogen-derived activity, with higher values in late lactation. They concluded that plasmin and plasminogen-derived activities were not strongly influenced by milk yield or feed and that the effect of the lactation stage was greater than that of time of the year (Nicholas et al., 2002). Similarly, Karlsson et al. (2017) did not observe any influence of season on plasmin-derived activity in dairy silo milk in northern Sweden. Considering that year-round calving is generally applied in Sweden, the lack of a seasonal pattern in plasmin activity in our study was expected.

The average pH value in farm tank milk in our study varied between sampling months, with the lowest average pH value (6.60) recorded in September and the highest in February 2017 (6.82). This range of pH values was broader than that recorded for Swedish dairy silo milk (6.68 in March to 6.73 in May) by Lindmark-Månsson et al. (2003). The pH value of 6.82 measured in February was high; however, one must consider that the values represent averages of milk sampled from individual farms, not milk sampled from dairy silos. Chen et al. (2014) observed a seasonal variation in pH in UK farm tank milk, with lower values in June, July, and August than during the rest of the year.

The SCC was generally low in the present study (Table 1), with small but significant variation between months, and with the highest average SCC value observed in milk collected in August (201×10^3 cells/mL). The SCC values were in the same range ($140–230 \times 10^3$ cells/mL) as those in dairy silo milk from the same region reported by Karlsson et al. (2017). Frössling et al. (2017) reported an increase in SCC during the latter part of the pasture season (August and September) in milk samples from dairy herds enrolled in the Swedish official milk recording scheme. A similar trend was observed by Olde Riekerink et al. (2007), who reported elevated SCC in milk collected during late summer using data from the Dutch national milk recording system. Higher SCC during late summer may be associated with a higher incidence of clinical mastitis, explained by seasonal differences in the occurrence of mastitis pathogens (Olde Riekerink et al., 2007). Total bacteria count was not influenced by the sampling month in this study, indicating uniform and good hygienic conditions on the participating farms throughout the year. Low numbers of bacteria result from proper handling of the raw milk, as well as good hygiene and cleaning routines on-farm (Guerra et al., 2013). The trend for slightly higher numerical values during the outdoor period in this study was possibly related to higher contamination from the environment when the cows were out on pasture, which has also been reported by Doyle et al. (2016). During the outdoor period, cows were subjected to higher contamination with soil bacteria, enhancing the total bacterial count in milk. As can be seen in the PCA in Figure 2, SCC and total bacteria were correlated; however, both bacteria and SCC count were generally low in our study (Table 1), not suggesting udder health issues.

The highest average G20 value (88 Pa) was recorded in milk collected in December, and the lowest numerical value was associated with milk collected in July (64 Pa; Table 1). Despite the interaction effect, the shortest RCT was also recorded with milk from December (361 s), and the longest was 529 s for milk collected in February. The highest G20 was associated with the lowest RCT value, which is in agreement with results reported by Priyashantha et al. (2019). The observed differences in G20 could likely to be an effect of variation in protein content (e.g., higher protein resulted in stronger gel compared with lower protein content; Panthi et al., 2019). However, differences in coagulation properties between months may also be attributed to concomitant changes in pH values (Chen et al., 2014). In contrast, Lin et al. (2017) observed no seasonal influence on the coagulation properties of milk from a mixed herd of spring- and autumn-calving cows.

To further evaluate the effect of month on milk composition, we performed an OPLS analysis. In Figure 3, bars are based on the loadings of the OPLS predictive component, and the level of influence is correspondent with bar length (i.e., the higher the bar, the greater the influence). The results showed that casein micelle size and proteolysis (free amino terminals) represented the milk quality traits that were most influenced by the factor month, followed by lactose and protein content, pH, SCC, and thermoresistant bacteria count.

Because the effect of sampling month was most pronounced for casein micelle size and total proteolysis, variation over the year was plotted for these 2 variables (Figure 4). The variation in average casein micelle size measured over a year by the nanoparticle tracking analysis method is shown in Figure 4A. The average casein micelle size ranged from 72 nm in August to 184 nm in February 2017 (Table 1). There was a trend for smaller casein micelles between May and October in comparison with other months, according to Figure 4A. However, there was an abrupt increase, and a very

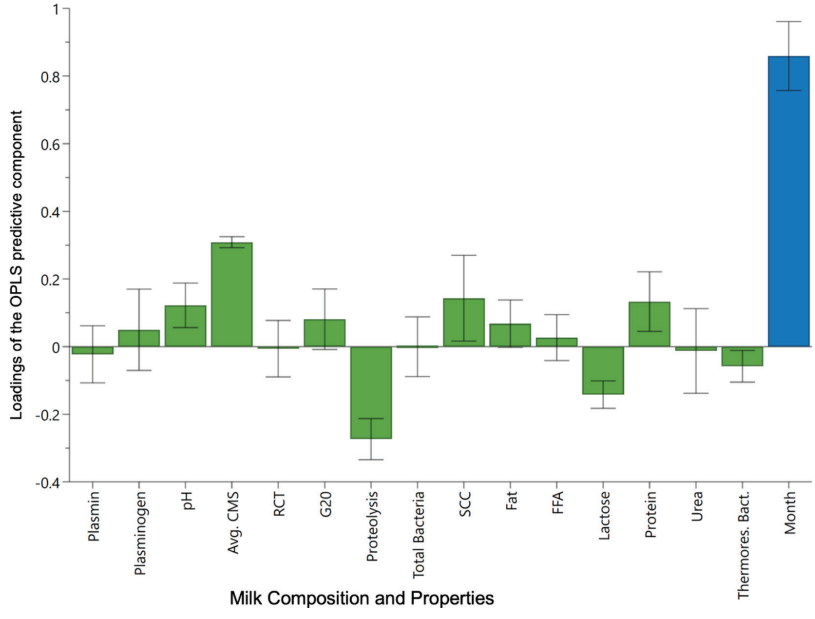

Figure 3. Orthogonal projections to latent structures (OPLS) analysis of milk quality attributes (green bars) in response to month (blue bar). Confidence intervals (95%) are given for each factor and for the response. Bars are based on the loadings of the OPLS predictive component, with bar length indicating level of influence (i.e., taller bars indicate stronger influence). FFA = free fatty acids; RCT = rennet coagulation time; G20 = gel strength; Avg. CMS = average casein micelle size.

large variation, in values for casein micelle size associated with milk samples collected in September. This is possibly explained by a large variation between farms with respect to cows being indoors or outdoors as well as the fact that it is the transition month for many of the cows from outdoor to indoor feeding. Holt and Muir (1978) first suggested that casein micelle size is affected by season, with significantly smaller micelles during summer compared with the rest of the year. A similar trend, although the values were not significant, was observed by Chen et al. (2014), with smaller micelles in milk sampled during June, July, and August; however, they were studying milk from farming systems where seasonal calving dominated.

Several factors have been reported to be important for the size of casein micelles, including κ-casein content, casein:protein ratio, genetic variants of caseins, pH, and calcium and citrate content (Devold et al., 2000; Glantz et al., 2010; Priyashantha et al., 2019). Studies by Bijl et al. (2014) and de Kruif and Huppertz (2012) concluded that casein micelle size in milk from individual cows is not influenced by the stage of lactation or protein content. In the study by Holt and Muir (1978), the size difference in casein micelles associated with season could partly be explained by differences in casein-bound calcium because micelle size was reported to show a negative correlation with casein-bound calcium and a positive correlation with colloidal phosphate. We observed similar relationships in a modeling study, where the increase of calcium concentration in milk resulted in reduced micelle size (Priyashantha et al., 2019). Total milk serum calcium concentration has been found to be higher in summer milk (June–August, 37%) compared with winter milk (28%) in seasonal calving systems (Lin et al., 2017). Moreover, Akkerman et al. (2019) found that ionic calcium content in milk is affected by pasture feeding, in addition to supplementary feeding and type of grass. Organic phosphate content has been reported to be lower in small casein micelles compared with large micelles (Bijl et al., 2014), and Lin et al. (2017) observed lower content of phosphorus in milk serum during summer compared with winter in spring-calving bulk milk. The addition of citrate to milk has been shown to increase casein micelle size

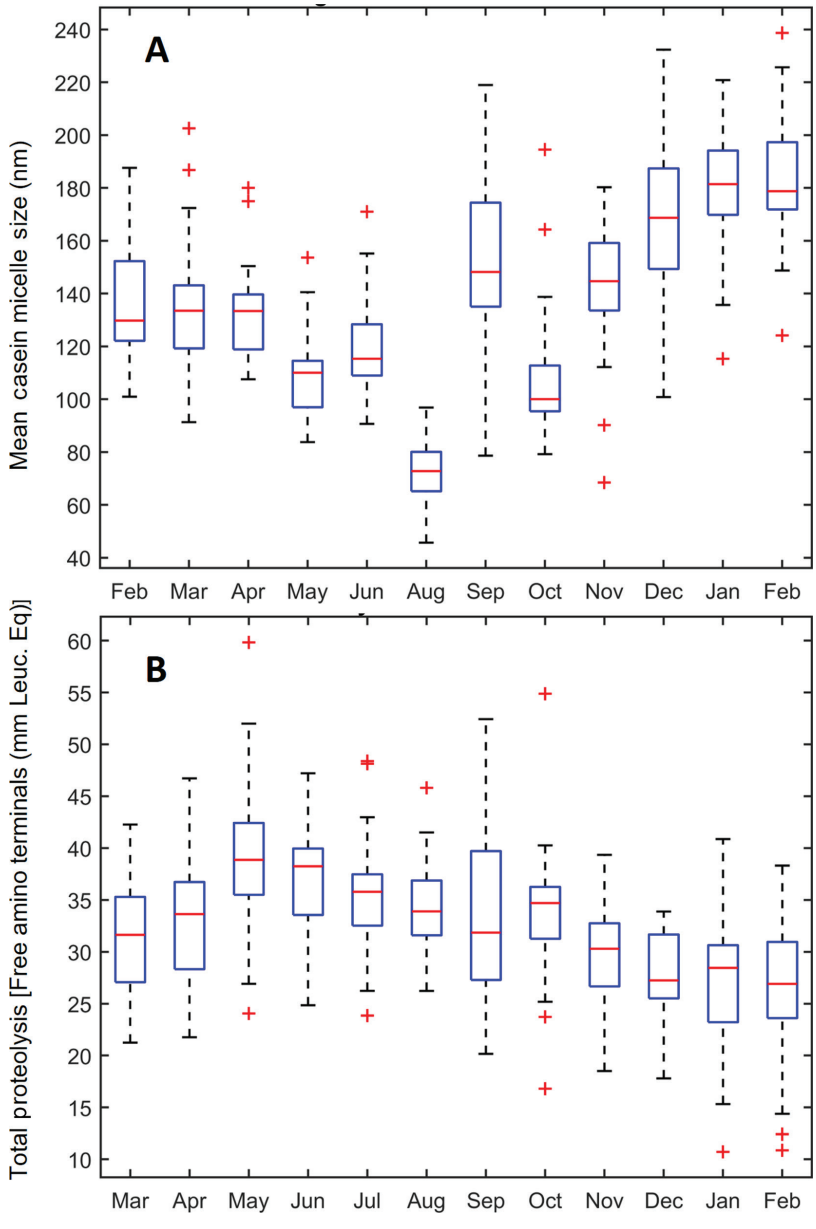

Figure 4. Box and whisker plot of the monthly variation in average casein micelle size (A) and total proteolysis, measured as content of free amino terminals (B), showing median values, interquartile range (height of the box), spread (upper and lower whiskers), and outlying values (red plus signs), for farm tank milk samples collected from participating farms (n = 41). Leuc. Eq. = leucine equivalent.

(Priyashantha et al., 2019). However, levels of citrate in bulk milk were not observed to vary with month or season (Chen et al., 2014; Karlsson et al., 2017; Akkerman et al., 2019). Garnsworthy et al. (2006) reported that citrate content varies with the stage of lactation and is related to de novo synthesis of fatty acids, but independent of diet and milk yield. Thus, increasing concentrations of calcium, decreasing concentrations of phosphorus, and relatively stable citrate content during summer months could be potential explanations for the differences in casein micelle size observed in this study, although these minerals were not analyzed. Higher protein content and larger micelles observed during indoor months also coincided with the highest gel strength value (G20) for milk sampled in December (Table 1). These observations are in agreement with results in our previous modeling study, where milk with larger micelles with higher protein concentration resulted in stronger gels (Priyashantha et al., 2019).

Milk proteolysis is influenced by several factors (e.g., environment, udder health, storage, and microbial count). Milk proteolysis indicates the potential of casein hydrolysis and it is often considered disadvantageous for yield in cheese making, whereas proteolytic activity in the raw milk can be advantageous in the production of long-ripening cheese (Kelly and Larsen, 2021). Total proteolysis in raw milk is a result of indigenous (e.g., plasmin and cellular proteases) and exogenous (e.g., microbial protease) proteolytic activities (Fox and Kelly, 2006; Zhang et al., 2019; Kelly and Larsen, 2021). It is not possible to assess the exact contribution from each of the proteolytic activities to the total proteolysis in this study. In certain months, total proteolysis seemed to follow the bacterial count, plasmin, and SCC, whereas on some occasions, a high variation was observed (Table 1). Variation in total proteolysis, as indicated by free amino terminals over 1 yr is shown in Figure 4B. Total proteolysis was higher during May through September compared with the rest of the year, with a weak pattern suggesting that the variation in proteolysis was inversely correlated with casein micelle size. Further research, however, is needed to confirm this observation. The values for total proteolysis showed the largest variation in milk from September, in parallel with the high variation in casein micelle size for that month. As previously discussed for casein micelle size, the higher variation in total proteolysis in September may be due to a greater variation in management of cows between farms. In September, some farms will still have their cows outdoors with access to pasture, whereas other farms will already have their cows indoors. The SCC was highest in milk from August. Slightly higher SCC in milk during the outdoor months may have contributed to the increase in total proteolysis because elevated SCC can contribute to proteolytic activity in milk (Senyk et al., 1985). In contrast, Karlsson et al. (2017) found no difference in total proteolytic activity in dairy silo milk between outdoor (June and July) and indoor periods in northern Sweden.

The monthly variation in milk quality attributes in our study was less likely to be explained by variation in on-farm factors because those observed to have an influence (e.g., dominant breed, housing and milking system; Priyashantha et al., 2021) were generally stable throughout the year, and calving incidence (percentage newly calved cows in the herd) was uniform throughout the year (data not shown). The outdoor temperature varied throughout the year, and although dairy barns are in general insulated in this region, we cannot exclude variation in the indoor climate during the study. Milk characteristics have been reported to be strongly influenced by heat stress (Bernabucci et al., 2010), but temperature and rainfall patterns were not extraordinary during the outdoor period and were not different from previous years (SMHI, 2016). Thus, we expected that the variation in milk composition and properties between months observed in this study was most likely explained by variation in feeding regimen between outdoor and indoor periods. Effects on milk composition and properties were therefore further examined using OPLS-DA to evaluate the effect of degree of grazing.

Figure 5 illustrates the influence of the degree of grazing on the variation in milk quality attributes for all months and milk samples with dots colored according to grazing practices (i.e., grazing, limited grazing, and no grazing) for the individual milk sample, using the data in Table 1. Indoor months are largely associated with the no grazing period, whereas limited grazing is mainly associated with outdoor months when cows are outdoor mainly for the purpose of exercise but still have silage as an important part of their forage intake. There was a tendency for milk samples from cows with access to grazing (both grazing group and limited grazing group) in the summer to be located to the left in the plot. In contrast, milk samples from cows that had no or restricted grazing, irrespective of the month of the year, were distributed more to the right. Grazing practice was confounded with the season, but in fact, when we compared only the milk samples that were collected during the summer months, we could not see a clear difference between the milk obtained from farms with grazing cows and milk from farms with cows that were nongrazing cows (data not shown). Thus, it is likely that grazing was not the only factor giving rise to the observed trend in Figure 5. Likewise, the widely-held hypothesis that a seasonal effect on milk quality

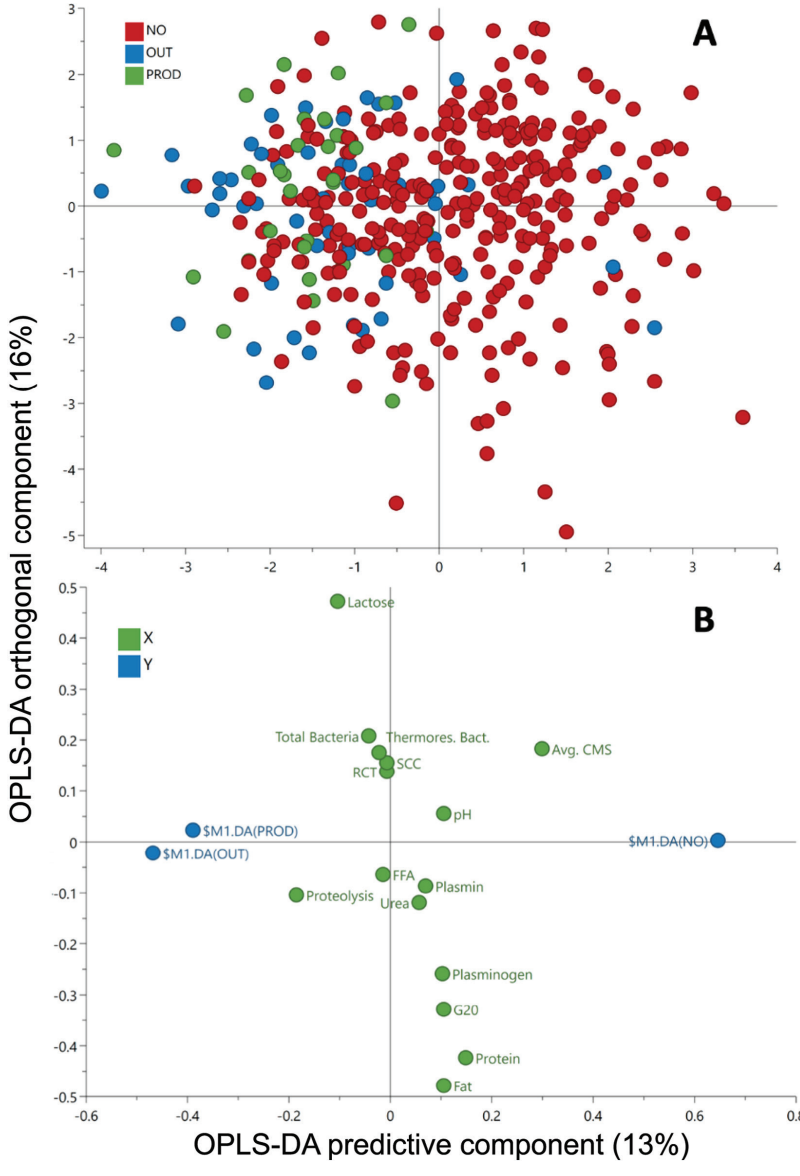

Figure 5. Orthogonal projection to latent structures discriminant analysis (OPLS-DA) of milk quality attributes from all months, as influenced by degree of grazing. NO = no grazing and no outdoor time; OUT = cows spent time outside with only limited grazing (mainly out for "exercise" on small area close to the barn); PROD = grazing actively, pasture contributing to the diet. (A) Cross-validated score values, with each dot representing 1 observation (milk from a farm). Predictive (related to response, i.e., grazing) scores on x-axis, orthogonal (not related to response) on y-axis. Colors indicate class. (B) OPLS-DA loadings, with each dot representing 1 milk quality variable. Predictive loadings on x-axis, orthogonal on y-axis. Blue dots represent response classes, green dots represent milk parameters. FFA = free fatty acids; RCT = rennet coagulation time; G20 = gel strength; Avg. CMS = average casein micelle size; Thermores Bact. = thermoresistant bacteria.

attributes is mainly attributable to pasture feeding would not be the sole reason for our observation. In this study, milk fatty acid profile, which is known to reflect the higher intake of UFA from the pasture (Rego et al., 2016), was not included. Nevertheless, our results suggest differences in milk characteristics between sampling months, indicating that additional factors that were not included in this study may have been involved. The major variables behind the seasonal variation in the farm tank milk can be seen from the OPLS-DA loadings (Figure 5B), such as variation in total proteolysis and casein micelle size. This corresponded well with the effect of season or production month shown in Figure 5, where lactose and proteolysis values were observed to be higher and casein micelle size was smaller, in milk samples sourced during the outdoor period compared with the rest of the year.

The novelty of this larger study in combination with the companion paper (Priyashantha et al., 2021) lies in the evaluation of dual effects on milk characteristics in milk sourced from farms in a cheese producing region in northern Sweden (i.e., variation in milk explained by on-farm factors and month). In combination, our work provides an overview of the nature of variation in raw milk and the influence of different factors. Multivariate techniques (PCA and OPLS) were needed to extract and elaborate upon relationships between the factors and responses investigated. Because the farms were only followed for 1 yr, the seasonal pattern observed in the study cannot be used to draw general conclusions; for this, the study would have needed to cover multiple years. The lack of large variation in milk properties over the year confirmed that raw milk sourced from farms in the region is suitable for cheese making on a year-round basis, at least from the perspective of the initial coagulation process. However, further investigations are currently underway to determine the effect of raw milk variation on the cheese ripening process.

CONCLUSIONS

We have investigated the composition and properties of raw farm milk aimed for the production of long-ripening cheese during 1 production year to determine the monthly variation. The use of univariate and multivariate statistical methods (PCA and OPLS) revealed that milk produced during the main outdoor period for the cows tended to deviate from milk samples collected during other months. The milk quality variables that showed the most pronounced monthly variation were casein micelle size (smaller during the outdoor period) and level of total proteolysis (higher during the outdoor period), as visualized by OPLS-DA. We expected a shift in feeding regimen, from pasture to indoor feed, to be the explanation for the observed seasonal differences, but concluded that additional factors not covered by this study must co-vary to give the results obtained. To confirm our findings and gain deeper insights into the causes of variation in raw farm milk, the study would need to span several years and consider multidimensional parameters that may be associated with the season. Finally, we concluded that the milk quality in the region was high all year, and the observed variation in the investigated quality attributes of raw farm milk was partly associated with season or month.

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Article

Variation in Dairy Milk Composition and Properties Has Little Impact on Cheese Ripening: Insights from a Traditional Swedish Long-Ripening Cheese

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Abstract: The monthly variation in raw dairy silo milk was investigated and related to the ripening time of the resulting cheese during an industrial cheese-making trial. Milk composition varied with month, fat and protein content being lowest in August (4.19 and 3.44 g/100 g, respectively). Casein micelle size was largest (192–200 nm) in December–February and smallest (80 nm) in August. In addition, SCC, total bacteria count, proteolytic activities, gel strength, and milk fatty acid composition were significantly varied with month. Overall sensory and texture scores of resulting cheese were mainly influenced by plasmin and plasminogen activity, indicating the importance of native proteolytic systems. Recently, concepts based on the differentiated use of milk in dairy products have been suggested. For the investigated cheese type, there might be little to gain from such an approach. The variation in the investigated quality characteristics of the dairy milk used for cheese making had little effect on cheese ripening in our study. In contrast to our hypothesis, we conclude that as long as the quality of the milk meets certain minimum criteria, there are only weak associations between cheese milk characteristics and the time required for the development of aroma and texture in the cheese. To find answers behind the observed variation in cheese ripening time, studies on the effects of process parameters are needed.

Keywords: cheese maturation; seasonality; raw milk quality; cheese making; fatty acids

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1. Introduction

The process of cheese making and the characteristics of the resulting cheese are associated with the composition and properties of the raw milk used. Hence, an in-depth understanding of the variation in raw milk quality and of factors contributing to this variation is important for assessing the suitability of raw milk for cheese making. The composition and properties of raw milk vary with numerous management and animal-related factors, e.g., feeding, milking practices, genetics, lactation, cow health status, and occasionally seasonality [1].

The concept of "seasonal variation" is well documented in the literature and has been shown to be associated with calving pattern, feed, and climate conditions [1–3]. Examples of milk characteristics reported to be associated with seasonal variation include protein and fat content [1,4], casein micelle size [5], pH [6], free fatty acid concentration [7], proportions of mono- and poly-unsaturated fatty acids [8], and somatic cell count [9]. The effect of seasons on milk quality attributes has been demonstrated by many researchers across the globe for different dairy farming systems, e.g., seasonal calving and pasture-based

systems [10,11] and year-round calving systems [1,5]. In recent work characterizing milk samples collected over one year from farms with continuous calving in northern Sweden, we observed variations in the characteristics of farm milk by sampling months [12]. Thus, it is apparent that milk composition and properties are subject to variation throughout the year, irrespective of the dairy farming system, potentially creating challenges or opportunities for dairy processors [6].

Despite the relatively high number of studies reporting variations in raw milk, the association with the maturation time of the resulting cheeses is not a well-researched area. The maturation of cheese to acquire characteristic organoleptic properties is important for consumer acceptability [13]; therefore, a release to the market of fully matured cheese is crucial for the cheese manufacturer. Traditionally, assessment of maturation is based on four key factors: appearance of the outer surface, appearance of the cut surface, consistency, and organoleptic properties (smell, taste, and aroma). In recent years, an increasing variation in the time required for maturation of a traditional long-ripening hard Swedish cheese has been observed by the cheese manufacturer. Using near-infrared (NIR)-hyperspectral image analysis, we demonstrated variation in the ripening process both within and between cheeses in that case [14]. In a parallel study, we evaluated monthly variations in the composition and properties of raw tank milk sourced from the farms that deliver milk to the cheese-making plant [12] and assessed whether the type of dairy farm contributed to the observed variation [15]. Dairy farms all over the world are undergoing rapid intensification [16], giving rise to variations in farm milk characteristics. An in-depth understanding of variations in dairy silo milk, i.e., a batch of pooled milk from individual farms, is equally important. However, it may be misleading to predict the composition and properties of dairy silo milk based only on the variation in milk from individual farms, since factors such as pooling of different volumes of milk from individual farms, mechanical stresses applied during pumping and transportation, and short-term storage at dairy silos could concomitantly impose influences not apparent on studying tank milk from individual farms.

Linking the variation in farm milk with variations in cheese maturation and ripening time is thus difficult. The aim of the present study was to investigate whether the observed variation in cheese ripening time is associated with variations in the composition and properties of the raw milk in the silo at the cheese-making plant. The hypothesis tested was that the variation in cheese ripening time is associated with variations in dairy silo milk quality.

2. Materials and Methods

2.1. Experimental Design and Sample Collection

Dairy silo milk was sampled monthly (2 or 4 times per month) at a full-scale commercial cheese-making plant between February 2016 and February 2017. In total, 65 dairy milk silos containing milk delivered from approximately 80 dairy farms located between 64°2′–65°0′ N and 19°3′–21°5′ E in the region of Västerbotten in northern Sweden were sampled for this study. The quality characteristics of tank milk from 42 of these dairy farms, most of which deliver all their milk to the cheese-making plant, are described in a previous study [15]. During the trial period, on every sampling, approximately 100 mL of milk was sampled from a dairy silo and transported at 4 °C to the official milk-testing laboratory (Eurofins Steins Laboratory, Jönköping, Sweden) for routine raw milk quality analyses. Another sample of 250 mL of milk sampled from the same silo on the same sampling occasion was transported at 4 °C to the Department of Molecular Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, for further detailed analysis of milk composition and properties. Upon arrival, the pH of milk samples was measured using a pH meter (Seven Compact S210, Mettler Toledo, Switzerland). For practical reasons, not all milk quality parameters could be analyzed at every sampling point, resulting in different numbers of data points for the different milk quality parameters. Certain milk quality traits, e.g., casein micelle size and rennet-induced coagulation properties, were analyzed

using fresh skimmed milk. Due to logistical difficulties, on rare occasions, coagulation properties were analyzed in milk that had been stored frozen at −20 °C for a maximum of two weeks. The remaining milk sample was aliquoted (as whole milk) and stored at −80 °C until further analyses (i.e., milk fat composition, plasmin/plasminogen activity, and total proteolytic activity).

2.2. Raw Milk Gross Composition, SCC, and Bacteria

Raw milk gross composition, i.e., concentration of fat, protein, urea, and free fatty acids (FFA), was analyzed at the official milk testing laboratory using Fourier-transform infrared spectral analysis (CombiFoss 6000, Foss, Hillerød, Denmark). Somatic cell count (SCC) was determined using a Fossomatic electronic cell counter (Foss Electric, Hillerød, Denmark). Thermo-resistant bacteria were analyzed by a culture method [17]. Total bacteria and psychrotrophic bacteria counts were analyzed using colony-count techniques at 30 °C [18] and 21 °C [19], respectively, in the dairy laboratory of the cheese-making plant.

2.3. Casein Micelle Size

Casein micelle size was determined on freshly skimmed milk by nanoparticle tracking analysis (NTA), according to Priyashantha et al. [20], using a NanoSight NS500 device (Malvern Instruments, UK). Video clips were captured using a camera fixed at 90° angle and 658 nm wavelength for 2000-fold diluted sample flow through the laser beam. Recorded video clips were batch-processed using NanoSight 2.3 NTA to obtain the average casein micelle size.

2.4. Rennet-Induced Coagulation

Rennet-induced coagulation properties of skimmed milk were studied at 35 °C using calf rennet (75/25 chymosin/bovine pepsin, 180 international milk clotting units (IMCU), Kemikalia, Skurup, Sweden) at a concentration of 0.18 IMCU/mL according to the method described by Johansson et al. [21]. Rennet coagulation time (RCT, s) and gel strength (Pa) after 20 min of rennet addition (G20) were analyzed in duplicate, using a Bohlin CVOR-150–900 rheometer (Malvern Instruments Nordic AB, Uppsala, Sweden).

2.5. Plasmin Activity

Plasmin- and plasminogen (PL/PG)-derived activity were determined in duplicate using a spectrophotometric method, as described by de Vries et al. [22]. Plasmin activity was measured in ultracentrifuged milk serum using 2.5 mg/mL of a chromogenic substrate, pyro-GLU-Phe-Lys-p-nitroanilide hydroxy chloride (Aniara, West Chester, OH, USA). Plasminogen, i.e., the inactive precursor of the enzyme, was converted into plasmin after activation with urokinase (49.5 Plough units), and both plasmin activity and total activity (i.e., plasmin and plasminogen activity) were measured using a multimode microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at 37 °C. Plasminogen activity was finally calculated as the difference between total activity and plasmin activity.

2.6. Total Proteolysis

Total proteolysis was estimated in triplicate by a fluorescamine method, as described by Johansson et al. [23], using a PerkinElmer LS55 luminescence spectrometer (Waltham, MA, USA). In brief, milk mixed with an equal volume of 24% trichloroacetic acid was kept on ice for 30 min before centrifugation at 16,000× *g* for 20 min. The resulting supernatant was mixed with sodium tetraborate, and fluorescamine was added before loading into a 96-microwell plate to measure the fluorescence at excitation wavelength 390 nm and emission wavelength 480 nm. The extent of proteolysis, i.e., the content of primary amino groups of trichloroacetic acid-soluble peptides and free amino acids, was expressed as leucine equivalents (mM Leuc. Eq.).

2.7. Milk Fat Composition

Fat extraction was performed in duplicate according to Hara & Radin [24] using 2 mL of milk sample, 10 mL of HIP (hexane:isopropanol (3:2), *v*/*v*), and 4.5 mL of a Na_2SO_4 solution (6.67%). The fat content was determined gravimetrically by weighing the dissolved extract on a microbalance (Mettler, Toledo, Switzerland). Fatty acids were methylated with BF_3 according to the method described by Appelqvist [25]. The fatty acid composition was then analyzed by gas chromatography using a CP 3800 instrument (Varian AB, Stockholm, Sweden), as described in Blomqvist et al. [26]. Peaks were identified according to their retention time in comparison with that of the standard mixture GLC 68A (Nu-check Prep, Elysian, MN, USA) and other authentic standards. Peak areas were integrated using Galaxie chromatography data system software version 1.9 (Varian AB, Stockholm, Sweden).

2.8. Cheese Production

The cheese in this study was commercially produced at the local cheese-making plant of the collaborating dairy company. The cheese originated from the characterized milk from 65 dairy silos and was evaluated for sensory properties and maturity during the ripening process. Within the total number of cheese batches (n = 208), 34 batches were produced from a combination of milk from two silos, for logistics reasons in the cheese-making plant. In such cases, the milk composition of the cheese batch was calculated proportionately, using values from the individual silos. The cheeses were manufactured in full production scale according to Rehn et al. [27]. In brief, a precultured bulk starter was added to the pasteurized milk and rennet (180 IMCU, Kemikalia, Skurup, Sweden), containing 75:25 chymosin: bovine pepsin, was added at a concentration of 0.3 mL L^{-1} milk. Cheese was produced in 18 kg cylinders (~16 cm height), which were brine-salted up to 1.2% salt content. The cheese surfaces were waxed and the cheeses were transferred to the manufacturer's ripening facility, for ripening at constantly monitored and controlled moisture and temperature.

2.9. Cheese Final Sensory Evaluation and Ripening Time

The sensory properties of the cheese were evaluated by at least three trained sensory panelists from the dairy company, starting with cheese aged 14 months and repeated at two-month intervals until full maturity was achieved [14]. The cheeses were evaluated against an in-house standard protocol concerning outer appearance, smell and taste, and texture, to determine whether they met the criteria for release to market. Comments on quality for the different cheese batches were noted in a specific protocol, e.g., the appearance of the cut surface (number and distribution of eyes, color, mold, and smear formation), smell and taste (cheese flavor, acidic taste, saltiness, sweetness, rancidity, burned, and fruity) and texture (evaluated with a finger, i.e., hardness, toughness and evaluated with the mouth, i.e., chewing resistance, dryness, grainy, rubbery, etc.). On each sensory evaluation occasion, each of the cheese batches was given a smell and taste score by the sensory panelists. These scores essentially reflected the assessed market readiness of a particular cheese batch. The ripening time (days), defined as time from production to final sensory approval, was recorded for each cheese batch.

2.10. Statistical Design

Minitab 18.1 software (Minitab Inc., State College, PA, USA) and Simca 16.0 software (Sartorius Stedim Data Analytics AB, Umeå, Sweden) were used for univariate and multivariate analysis, respectively. The monthly variation in milk quality attributes was analyzed by ANOVA with the Tukey post hoc test, using Minitab. Two different methods of multivariate analysis were used, principal component analysis (PCA) [28] and orthogonal projections to latent structures (OPLS) [29]. In constructing the PCA plots, individual values deviating by more than four standard deviations from the mean were considered outliers and excluded from further analysis. All the milk quality attributes were UV-scaled,

auto-transformed, and displayed in PCA loading plots, while silo milk samples were visualized in the PCA score plot. The OPLS approach was used to investigate the relationship between milk quality attributes, ripening time, and sensory scores. For the OPLS model, a loading plot was inspected to identify significant influencers (e.g., milk quality attributes) associated with the studied responses (e.g., ripening time and sensory scores).

3. Results and Discussion

3.1. Variation in Raw Milk Composition and Properties

The monthly variation in the composition and properties of the milk used for commercial full-scale cheese production was investigated based on the PCA results. The PCA score plot showed a tendency for silo milk samples obtained during June–August to be located on the right side, while silo milk from the other months grouped towards the left side of the plot (Figure 1A). The PCA loading plot suggested that the differences were mainly related to the fat and protein content, casein micelle size, SCC, and proportions of saturated or unsaturated fatty acids in the milk (Figure 1B).

The monthly variation in the milk quality attributes investigated in this study is shown in Table 1, which presents the average values for the silo milk at the dairy plant during each month. The highest and lowest fat and protein content were seen in November–December and May, respectively. This is in agreement with findings in our previous study based on farm tank milk [12], where fat and protein concentrations were lower in May–August than in other months, and in other studies based on Swedish bulk milk samples [30,31]. Heck et al. [1] attributed lower fat and protein content during the outdoor period to changes in feeding regimen associated with the season, and suggested that the increase in readily fermentable carbohydrate content in the feed as a result of higher concentrate:forage ratio in winter could lead to higher production of propionic acid in the rumen. Since propionic acid is the major precursor of glucose, modification of hormonal signals with the glucogenic nutrient supply could stimulate milk protein synthesis. Heck et al. [1] also suggested that higher linolenic acid content in fresh grass compared with silage inhibits de novo fatty acid synthesis in the mammary gland, thereby reducing the milk fat content. Variations in protein and fat content are likely to have an influence on cheese yield, considering that 77% of fresh cheese yield derives from the fat and protein content [32]. However, in the present case cheese milk is standardized at the cheese-making plant with regard to fat content and protein:fat ratio, and therefore this variation would have less impact on the production process.

The average urea concentration in dairy silo milk did not vary with month, which is in agreement with our previous results for urea concentrations in farm tank milk [12]. By contrast, Karlsson et al. [33] observed seasonal variation in urea content in dairy silo milk sampled from the same dairy company, with the lowest urea content in summer months. In the present study, FFA levels did not vary by month, contradicting findings in our previous study on FFA in farm tank milk [12]. A probable explanation for this is that the dairy silos contained farm milk from other herds, in addition to those described in our previous study. Another explanation could be bulking of milk from farms delivering differing volumes of milk. The average FFA level in silo milk over the year was always below 1.0 mmol/100 g (Table 1), which is considered the threshold value for no distinguishable off-flavor associated with the raw milk or final product [34]. Elevated levels of FFAs may cause a rancid flavor in cheese [35]. The pH value of silo milk showed monthly variation, with the lowest value in September (6.48) and the highest in February 2017 (6.81). Likewise, we observed the lowest pH (6.60) in farm milk in September [12]. However, Lindmark-Månsson [31] reported no seasonal variation in pH in another similar study. Variation in pH affects cheese making through modification of protein interactions and their functionality, with lower pH resulting in a higher content of soluble calcium and contraction of the protein matrix in the resulting cheese [36].

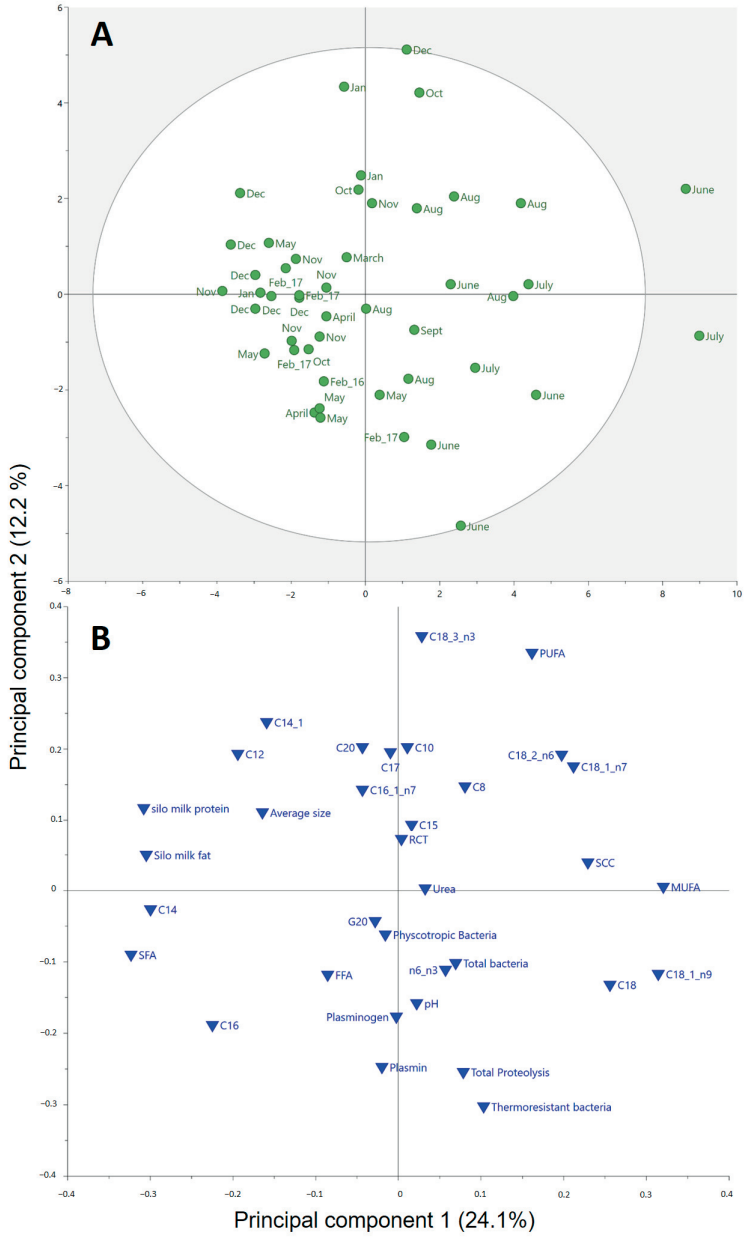

Figure 1. Results of principal component analysis (PCA) of milk quality variables in silo milk (n = 65) collected over one year. (**A**) PCA score plot, with each dot representing an individual silo milk sample and (**B**) PCA loading plot. RCT: rennet coagulation time, G20: gel strength at 20 min, SCC: somatic cell count, FFA: free fatty acids, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, and n6_n3: linoleic acid/linolenic acid.

Table 1. Quality attributes of dairy silo milk samples ($n = 65$) collected monthly during one year at the participating cheese-making plant.

Parameter		p	Sampling Month											
			February-16	March	April	May	June	July	August	September	October	November	December	January	17-February
			$n = 2$	$n = 4$	$n = 5$	$n = 7$	$n = 5$	$n = 7$	$n = 7$	$n = 1$	$n = 5$	$n = 6$	$n = 7$	$n = 4$	$n = 5$
Fat	Mean	**	4.39 ^{abc}	4.43 ^{abc}	4.36 ^{abc}	4.43 ^{abc}	4.17 ^{bc}	4.28 ^{abc}	4.19 ^{c}	4.40 ^{abc}	NA	4.42 ^{a}	4.45 ^{a}	4.39 ^{ab}	4.36 ^{ab}
(g/100 g)	(SD)		(0.00)	(0.00)	(0.09)	(0.09)	(0.07)	(0.22)	(0.05)	(0.00)	NA	(0.09)	(0.04)	(0.04)	(0.10)
Protein	Mean	**	3.50 ^{abc}	3.47 ^{abc}	3.52 ^{abc}	3.45 ^{bc}	3.43 ^{c}	3.46 ^{abc}	3.44 ^{c}	3.53 ^{abc}	NA	3.59 ^{a}	3.59 ^{a}	3.57 ^{ab}	3.48 ^{abc}
(g/100 g)	(SD)		(0.04)	(0.00)	(0.01)	(0.02)	(0.01)	(0.00)	(0.02)	(0.00)	NA	(0.06)	(0.04)	(0.03)	(0.23)
Urea	Mean	0.55	4.35	4.20	4.05	4.00	4.60	4.00	4.24	4.10	NA	4.40	4.05	3.97	4.16
(mmol/L)	(SD)		(0.35)	(0.00)	(0.35)	(0.28)	(0.85)	(0.14)	(0.21)	(0.00)	NA	(0.28)	(0.37)	(0.28)	(0.22)
FFA	Mean	0.99	0.82	0.81	0.74	0.88	0.83	0.72	0.76	0.81	NA	0.80	0.87	0.74	0.83
(mmol/100 g fat)	(SD)		(0.16)	(0.00)	(0.00)	(0.15)	(0.47)	(0.00)	(0.14)	(0.00)	NA	(0.05)	(0.26)	(0.02)	(0.09)
pH	Mean	**	6.73 ^{abc}	6.70 ^{abc}	6.72 ^{ab}	6.74 ^{ab}	6.80 ^{ab}	6.73 ^{ab}	6.76 ^{ab}	6.48 ^{c}	6.67 ^{bc}	6.78 ^{ab}	6.70 ^{abc}	6.70 ^{abc}	6.81 ^{a}
	(SD)		(0.02)	(0.02)	(0.03)	(0.03)	(0.10)	(0.00)	(0.06)	(0.00)	(0.02)	(0.02)	(0.03)	(0.00)	(0.00)
PL	Mean	*	NA	2.91 ^{a}	NA	2.70 ^{a}	2.81 ^{a}	NA	1.56 ^{c}	NA	2.67 ^{ab}	NA	1.80 ^{bc}	NA	2.39 ^{abc}
(units/mL)	(SD)		NA	(0.76)	NA	(0.73)	(0.32)	NA	(0.54)	NA	(0.73)	NA	(1.03)	NA	(0.94)
PG	Mean	**	NA	70.83 ^{a}	NA	66.59 ^{a}	64.44 ^{ab}	NA	43.51 ^{c}	NA	58.37 ^{abc}	NA	48.89 ^{bc}	NA	48.72 ^{bc}
(units/mL)	(SD)		NA	(4.88)	NA	(9.91)	(7.63)	NA	(9.74)	NA	(8.63)	NA	(6.69)	NA	(9.28)
TP	Mean	**	NA	32.06 ^{ab}	NA	33.82 ^{ab}	29.23 ^{bc}	40.53 ^{a}	NA	NA	NA	NA	20.73 ^{c}	NA	NA
(mM Leuc. Eq.)	(SD)		NA	(1.17)	NA	(2.74)	(4.33)	(8.88)	NA	NA	NA	NA	(4.78)	NA	NA
SCC	Mean	**	174 ^{abc}	180 ^{abc}	133.5 ^{c}	182.5 ^{abc}	221 ^{ab}	197 ^{abc}	233.8 ^{a}	231 ^{abc}	NA	174.8 ^{bc}	191.3 ^{abc}	174.2 ^{bc}	197.2 ^{abc}
(10^3/mL)	(SD)		(9.90)	(5)	(7.78)	(30.4)	(20.7)	(21)	(41.1)	(0)	NA	(6.5)	(25.9)	(18.8)	(18.4)
TBC	Mean	**	27 ^{b}	32 ^{b}	15.2 ^{b}	50.3 ^{b}	58.2 ^{ab}	60.4 ^{ab}	109 ^{a}	53 ^{ab}	14.5 ^{b}	64.0 ^{ab}	65 ^{b}	21.2 ^{b}	36.6 ^{b}
(CFU 10^3/mL)	(SD)		(8.7)	(12.19)	(10.8)	(29.6)	(12.15)	(36.0)	(58.4)	(0)	(5.0)	(59)	(113)	(9.3)	(25.3)
PBC	Mean	0.87	1.6	12.7	1.4	4.6	7.5	12.1	11.02	1.60	7.1	6.5	11.2	5.3	4.5
(CFU 10^3/mL)	(SD)		(0.7)	(13.7)	(1.3)	(2.9)	(5.9)	(15.9)	(12.4)	(0)	(9.8)	(7.8)	(17.5)	(4.1)	(3.4)
TRBC	Mean	0.06	7.5	2.0	6.7	6.7	9.9	6.5	3.5	1.0	NA	1.9	0.7	1.1	4.3
(CFU 10^3/mL)	(SD)		(2.1)	(0)	(4.6)	(4.5)	(0)	(5.5)	(2.4)	(0)	NA	(1.1)	(0.3)	(0.5)	(2.9)
CMS	Mean	**	170 ^{ab}	153 ^{abc}	138 ^{abc}	129 ^{bc}	130 ^{bc}	NA	80 ^{c}	117 ^{abc}	127 ^{bc}	NA	192 ^{a}	200 ^{a}	197 ^{a}
(nm)	(SD)		(1)	(16)	(24)	(39)	(10)	NA	(2)	(0)	(29)	NA	(38)	(28)	(9)
G20	Mean	*	60 ^{ab}	66 ^{ab}	66 ^{ab}	67 ^{a}	60 ^{ab}	52 ^{ab}	58 ^{ab}	NA	65 ^{ab}	NA	57 ^{ab}	NA	42 ^{b}
(Pa)	(SD)		(5)	(3)	(2)	(8)	(3)	(10)	(21)	NA	(14)	NA	(5)	NA	(14)
RCT	Mean	0.87	504	457	467	450	471	494	461	NA	564	NA	462	NA	581
(s)	(SD)		(40)	(11)	(11)	(39)	(11)	(59)	(80)	NA	(174)	NA	(128)	NA	(50)

SD: standard deviation. NA = not analyzed. Due to practical circumstances, on some sampling occasions not all milk quality traits were evaluated. FFA: free fatty acids. PL: plasmin activity. PG: plasminogen-derived activity. TP: total proteolysis (measured as free amino terminals). SCC: somatic cell count. TBC: total bacterial count. PBC: psychrotrophic bacterial count. TRBC: thermo-resistant bacterial count. CFU: colony-forming units. CMS: casein micelle size. RCT: rennet coagulation time. G20: gel strength at 20 min. Mean values within rows with different superscripts are significantly different at $p < 0.05$ * or $p < 0.01$ **.

The PL- and PG-derived activity varied significantly with month but with no clear seasonal pattern (Table 1). Likewise, in our previous characterization of tank milk from individual farms, we observed significant monthly variation in PL and PG activity with no seasonal pattern [12]. The PL and PG activity is critically important for the maturation of many types of long-ripening cheeses, as it reflects an important source of proteolytic enzyme that facilitates development of texture and flavor of cheese through a series of reactions mediating the degradation of protein [35–37].

Proteolysis plays a crucial role in texture and flavor development in aged cheeses [38]. During the ripening process, protein hydrolysis takes place due to the action of coagulants, plasmin, and microbial and somatic cell proteinases [39]. The resulting intermediate peptides are further degraded by the activity of starter and nonstarter lactic acid bacteria (LAB), which produce proteinases and peptidases, catalyzing the hydrolysis of proteins and peptides during the aging of cheese. Nonstarter LAB, which dominate towards the end of cheese ripening, continue the proteolytic activity and are important for the characteristic cheese flavor of many types of cheeses [40]. In the present study, total proteolysis, measured as free amino terminals, varied with sampling month, with the lowest value in December (20.73 mM Leuc. Eq.) and the highest in July (40.53 mM Leuc. Eq.). We observed a similar tendency in the individual farm tank milk samples, with an increase in total proteolytic activity during the summer/outdoor months (May–July) [12]. However, a recent study by Glantz et al. [41], conducted in three geographical regions of Sweden, found that protease activity in dairy silo milk did not differ between the outdoor (May–September) and indoor (October–March) periods. This discrepancy may depend on differences in the technique used for measuring proteolytic activity. In the present study, we used fluorescamine (at 480 nm) as a substrate, while Glantz et al. [41] used azocasein (at 345 nm). It could also be associated with the differences in SCC we found between outdoor and indoor seasons, whereas Glantz et al. [41] observed no such differences. Higher SCC could have acted as an additional source of proteolytic vector during the outdoor months, since SCC is often correlated with higher plasmin activity in milk [42].

In the present study, SCC and TBC also varied by month, with higher counts during the main outdoor months and the highest values observed in August (233×10^3/mL and 109×10^3/mL, respectively). Higher SCC and TBC could be a contributor to the observed increase in total proteolysis seen during July, since elevated SCC and TBC can contribute to the proteolytic activity [43,44]. Bobbo et al. [45] reported higher SCC associated with higher milk pH and resulting negative effects on cheese-making properties. In general, stress caused by changing environmental conditions, in addition to poor hygiene practices at milking and cow nutritional status, can contribute to increased SCC in dairy milk [46,47]. As an example, Frössling et al. [9] found that SCC was higher in Swedish bulk milk during the latter part of the pasture season (August–September). In general, increased SCC (above 200×10^3/mL) results in greater protein losses in whey, thereby reducing dry matter yield of cheese [48]. Further, higher SCC has been shown to have a negative influence on the suitability of milk for cheese making, with e.g., a decrease in the proportion of casein and breakdown of β-casein, while releasing γ-casein [49]. However, it is difficult to assess the effect of SCC on potential quality defects in the final product, as specific threshold levels and effects of different types of somatic cells are not known for this particular type of cheese. According to Auldist [49], a negative effect can be detected from 100×10^3/mL onwards in some dairy products but not until 500×10^3/mL in others.

The higher TBC in the outdoor period (mainly August) may be related to contamination of the animals by the environment (soil, pasture, and water), compared with conditions during the indoor period [50]. However, unlike in this study investigating dairy silo milk, in our previous study on farm tank milk [12], we did not observe a significant effect of sampling month on TBC. Nevertheless, the TBC in the present study ranged between 14 and 109×10^3 colony-forming units (CFU)/mL (Table 1), which is higher than observed in the farm milk samples (range 8–14×10^3 CFU/mL) [12]. This could be an effect of longer cold storage of dairy silo milk compared with farm tank milk, i.e., the increment

could be associated with growth of psychrotrophs over time. In most dairy marketing systems, analysis of SCC and TBC is compulsory, and milk values exceeding the cut-off level have a severe effect on the price paid to the dairy farmer [51]. In this regard, the milk used in cheese production in this study met the highest quality standards stipulated by the cheese manufacturer.

In this study, psychrotrophic bacterial counts in dairy silo milk did not vary significantly, and there was no seasonal pattern. In general, the presence of high numbers of psychrotrophic bacteria in raw milk poses challenges for the quality of milk, and the resulting cheeses. Psychrotrophs are capable of growth at temperature $\leq$7 °C [52]. Increasing numbers of psychrotrophic bacteria have therefore been associated with prolonged cold storage and longer time between milk collections at farms, whereas freshly drawn milk from the udder does not contain psychrotrophic bacteria [53]. These bacteria are eliminated by pasteurization, but their extracellular, heat-resistant enzymes can remain active in pasteurized milk and lead to a slight reduction in cheese yield, as a result of degradation of casein into soluble end-products that may be lost in the whey fraction [54]. Another negative effect associated with psychrotrophic bacteria is flavor defects, which are often associated with characteristics such as rancidity and bitterness.

The number of thermo-resistant bacteria (mainly *Enterococcus* and *Streptococcus*) in raw milk can be used as an indicator of the efficacy of cleaning of milking equipment at farm level [55]. It is also an important consideration for the suitability of milk for cheese making, since thermo-resistant bacteria can survive heating at 60 °C for 30 min and can thus be present in the pasteurized milk and in cheeses [56]. In the present study, the number of thermo-resistant bacteria did not vary significantly with sampling month. In a Danish study [57], nine different nonstarter *Lb. paracasei* strains demonstrated thermo-resistant properties in association with thermization (60 °C, 5 min), and pasteurization (73 °C, 15 s). This suggests that the thermo-resistant bacteria observed in the present study might be important in cheese production, as they are likely to survive pasteurization and may be active in the cheese during the ripening process.

Casein micelles are important structural components in milk and crucial functional units in cheese making. The size of casein micelles is known to influence the cheese-making process, with larger micelles reported to result in stronger gels [20] and smaller micelles in weaker gels [58]. In this study, we observed a seasonal variation, with casein micelles being smaller in the main outdoor period than in the indoor period. The smallest average casein micelle size was observed in August (80 nm) and the largest in December (192 nm), January (200 nm), and February 2017 (197 nm). This is in agreement with findings in our previous study on farm tank milk samples [12], where casein micelle size varied from 72 nm in August to 184 nm in February. In a study by Holt and Muir [5] on milk samples originating from seasonal calving dairy farming systems in Scotland, casein micelle size was significantly smaller in summer milk than in milk from other months of the year.

The dairy farms in the present study followed a year-round calving system; therefore, observations from dairy farming systems with seasonal calving may not be directly applicable. The similarity of the observations, irrespective of calving system, could be because casein micelle size has been shown to be unaffected by lactation stage of the cow or fat or protein content of the milk [59]. The seasonal difference in casein micelle size observed in our study could instead be attributable to differences in minerals in the feed during the summer months (e.g., higher Ca and lower P), as discussed in our previous study [12].

There was a significant effect of month on gel strength (described as G20), with the highest G20 values observed in May (67 Pa) and the lowest in February (42 Pa) and July (52 Pa). The RCT ranged from 581 s in February 2017 to 450 s in May, indicating the inverse correlation between RCT and G20 [20], as also observed in our previous study in the region [12]. One potential reason for the low G20 quality in February might be the higher pH in milk sampled during that month (Table 1), since higher pH decreases the soluble calcium content [36] and free calcium is critical in creating a firm gel [60]. Gel firmness depends on milk protein concentration to the power of three for milk from

Jersey cows [61]. However, multiple factors in addition to the protein content co-vary to influence gel strength, e.g., genetic milk protein variants are decisive for milk coagulation properties [62]. However, this was not covered during the present study when analyzing the pooled dairy silo milk.

The variation in fatty acid composition of the milk at the dairy plant is shown in Table 2. In general, the fatty acid composition of milk determines the fatty acid composition in cheeses, since processing has a negligible influence on fatty acids, highlighting the importance of studying the variation in the raw material [63]. We observed lower concentrations of saturated fatty acids (SFA) and higher concentrations of monounsaturated fatty acids (MUFA) in the main outdoor period (June–August) compared with the rest of the year (Figure 1). In an earlier study on the composition of dairy silo milk in Sweden, Lindmark-Månsson [31] also observed significant variation in MUFA over the year, with the highest levels in July and the lowest in January. However, Lindmark-Månsson [31] did not observe any significant variation in the polyunsaturated fatty acid (PUFA) content of the milk between seasons or regions. Similarly, in the present study, PUFA content seemed to be less affected by seasonality, as illustrated in Figure 1. In another study characterizing dairy milk destined for ultrahigh temperature treatment at a dairy plant in the same region as in this study (Luleå, Sweden), it was found that the milk contained a higher content of PUFA in July than in other months [64]. Similar results were found in a Swiss study comparing the fatty acid composition in milk delivered to dairies in the mountain regions of Switzerland in summer and winter months [8]. A French study concluded that milk fatty acid composition is closely associated with feeding and husbandry practices [65]. The participating dairy farms in our previous study in northern Sweden applied a wide range of feeding practices during the outdoor period [12]. Some farms had full indoor feeding even during summer, with the time cows spent on pasture mainly serving as exercise, whereas other farms had pasture providing up to 95% of the animals feed intake. On average, pasture provided about 30% of the feed intake from mid-June until mid-August on those farms [12]. Despite the variation in feeding strategies during outdoor months on participating farms, we observed a significant effect of month on the FA composition of milk at the dairy plant [12]. In the present study, we observed lower dairy silo milk fat content during the summer (main grazing period) than in winter (commonly fed silage). Since the high C18:3 (linolenic acid) content in fresh grass is associated with ruminal production of *trans*-10- and *cis*-12-conjugated linoleic acids, inhibiting de novo fatty acid synthesis in the mammary gland [66], this could contribute to the lower milk fat content observed during outdoor months.

Medium-chain fatty acids (C12:0, C14:0 and C16:0) were more associated with indoor months, whereas long-chain fatty acids (C18) were located on the right side of the PCA, indicating that they were more associated with outdoor months (Figure 1). July (the main outdoor month in this study) had the lowest concentrations of C10:0 (capric acid), C12:0 (lauric acid), and C14:0 (myristoleic acid), and the highest concentrations of C17:0 (margaric acid), C18:0 (stearic acid), and C18:1 (oleic acid) (Table 2). The fatty acids C8:0 and C10:0 are not naturally present in feedstuffs and are synthesized de novo in the mammary gland, whereas C16:0 and long-chain fatty acids (C18) are taken up from the blood in the udder. The differences in FA profiles can be attributed to differences in feeding strategy during indoor and outdoor months. In a study in central and southern Sweden, Larsen et al. [4] also observed lower fat content and higher amounts of C18:1 cis-9, conjugated linoleic acid cis-9 and trans-11, and lower amounts of C4-C16 fatty acids, in bulk milk in summer compared with winter.

Table 2. Fatty acid composition of milk samples collected monthly during one year at the participating cheese-making plant.

Fatty Acids		*p*	Sampling Month												
			February-16	March	April	May	June	July	August	September	October	November	December	January	17-February
			n = 2	*n* = 2	*n* = 2	*n* = 5	*n* = 4	*n* = 4	*n* = 6	*n* = 2	*n* = 3	*n* = 6	*n* = 8	*n* = 4	*n* = 3
C8:0 (Caprylic acid)	Mean	0.72	0.16	0.13	0.00	0.04	0.05	0.13	0.06	0.00	0.02	0.00	0.07	0.13	0.18
	(SD)		(0.22)	(0.18)	(0.00)	(0.09)	(0.05)	(0.26)	(0.15)	(0.00)	(0.02)	(0.00)	(0.11)	(0.15)	(0.16)
C10:0 (Capric acid)	Mean	**	0.36 ^{ab}	0.62 ^{ab}	0.07 ^{ab}	0.34 ^{ab}	0.50 ^{ab}	0.24 ^{b}	1.01 ^{a}	0.69 ^{ab}	1.00	0.90 ^{ab}	0.62 ^{ab}	0.39 ^{ab}	0.20 ^{ab}
	(SD)		(0.29)	(0.49)	(0.10)	(0.25)	(0.44)	(0.27)	(0.31)	(0.68)	(0.57)	(0.43)	(0.16)	(0.04)	(0.18)
C12:0 (Lauric acid)	Mean	**	3.12 ^{abc}	3.01 ^{abc}	2.59 ^{abc}	2.98 ^{abc}	2.57 ^{bc}	2.41 ^{c}	3.30 ^{ab}	2.52 ^{abc}	3.38 ^{abc}	3.61 ^{a}	3.45 ^{a}	3.27 ^{abc}	2.66 ^{abc}
	(SD)		(0.14)	(0.23)	(0.27)	(0.25)	(0.55)	(0.39)	(0.43)	(0.49)	(0.35)	(0.21)	(0.29)	(0.32)	(0.81)
C14:0 (Myristoleic acid)	Mean	**	13.85 ^{ab}	13.64 ^{abc}	13.66 ^{abc}	13.57 ^{ab}	12.74 ^{bc}	12.54 ^{c}	13.16 ^{abc}	12.69 ^{abc}	13.27 ^{abc}	13.79 ^{a}	13.79 ^{a}	13.69 ^{a}	13.80 ^{a}
	(SD)		(0.17)	(0.43)	(0.08)	(0.07)	(0.39)	(0.82)	(0.47)	(0.00)	(0.16)	(0.30)	(0.20)	(0.48)	(0.45)
C15:0 (Pentadecylic acid)	Mean	0.20	1.17	1.43	1.21	1.17	1.14	1.26	1.25	1.14	1.07	1.11	1.31	1.34	1.19
	(SD)		(0.03)	(0.03)	(0.04)	(0.07)	(0.04)	(0.07)	(0.16)	(0.01)	(0.33)	(0.25)	(0.16)	(0.15	(0.03)
C16:0 (Palmitoleic acid)	Mean	*	38.59 ^{abc}	37.73 ^{abc}	39.09 ^{abc}	39.01 ^{a}	36.99 ^{abc}	36.13 ^{bc}	36.46 ^{c}	38.08 ^{abc}	37.13 ^{abc}	37.47 ^{abc}	38.05 ^{abc}	37.48 ^{abc}	38.20 ^{abc}
	(SD)		(0.01)	(0.53)	(1.16)	(0.27)	(1.68)	(0.89)	(0.86)	(1.00)	(2.02)	(1.52)	(0.83)	(1.16)	(0.50)
C17:0 (Margaric acid)	Mean	*	0.55 ^{ab}	0.55 ^{ab}	0.62 ^{ab}	0.53 ^{b}	0.58 ^{ab}	0.68 ^{a}	0.56 ^{ab}	0.52 ^{ab}	0.55 ^{ab}	0.55 ^{ab}	0.56 ^{ab}	0.66 ^{ab}	0.57 ^{ab}
	(SD)		(0.00)	(0.04)	(0.09)	(0.02)	(0.04)	(0.13)	(0.03)	(0.02)	(0.04)	(0.04)	(0.03)	(0.14)	(0.03)
C18:0 (Stearic acid)	Mean	**	12.28 ^{abc}	12.06 ^{abc}	12.10 ^{abc}	12.16 ^{bc}	13.22 ^{ab}	13.53 ^{a}	12.58 ^{abc}	12.97 ^{abc}	12.18 ^{abc}	12.39 ^{abc}	11.96 ^{c}	12.19 ^{bc}	12.52 ^{abc}
	(SD)		(0.60)	(0.14)	(0.09)	(0.22)	(0.63)	(0.37)	(0.70)	(0.06)	(0.53)	(0.64)	(0.45)	(0.21)	(0.85)
C20:0 (Arachidic acid)	Mean	0.35	0.20	0.18	0.23	0.23	0.19	0.20	0.31	0.20	0.27	0.20	0.21	0.39	0.24
	(SD)		(0.01)	(0.01)	(0.04)	(0.05)	(0.09)	(0.05)	(0.18)	(0.02)	(0.07)	(0.05)	(0.05)	(0.22)	(0.21)
C14:1 (Myristoleic acid)	Mean	**	1.13 ^{abcd}	1.29 ^{abcd}	1.11 ^{abcd}	1.23 ^{abcd}	1.05 ^{cd}	1.05 ^{bd}	1.14 ^{abcd}	1.05 ^{abcd}	1.32 ^{ab}	1.20 ^{abcd}	1.27 ^{a}	1.19 ^{abcd}	1.14 ^{abcd}
	(SD)		(0.04)	(0.06)	(0.03)	(0.15)	(0.05)	(0.09)	(0.15)	(0.01)	(0.09)	(0.05)	(0.13)	(0.02)	(0.04)
C16:1 (Palmitoleic acid)	Mean	0.15	1.39	1.91	2.02	1.55	1.57	1.72	1.68	1.71	1.80	1.60	1.81	1.92	1.80
	(SD)		(0.60)	(0.16)	(0.34)	(0.23)	(0.13)	(0.14)	(0.22)	(0.03)	(0.11)	(0.24)	(0.24)	(0.38)	(0.14)
C18:1 (Oleic acid)	Mean	**	23.84 ^{bcd}	23.34 ^{cd}	23.70 ^{cd}	23.59 ^{cd}	25.54 ^{ab}	26.33 ^{a}	24.51 ^{bc}	25.16 ^{abc}	24.12 ^{bcd}	23.75 ^{cd}	23.30 ^{d}	23.42 ^{cd}	23.81 ^{cd}
	(SD)		(0.08)	(0.48)	(0.25)	(0.56)	(0.59)	(0.86)	(0.86)	(0.06)	(0.31)	(0.62)	(0.45)	(0.28)	(0.73)
C18:2 (Linoleic acid)	Mean	0.73	1.69	1.76	1.75	1.71	1.96	1.88	1.85	1.66	1.99	1.74	1.81	1.89	1.74
	(SD)		(0.00)	(0.19)	(0.15)	(0.15)	(0.33)	(0.20)	(0.21)	(0.01)	(0.39)	(0.07)	(0.33)	(0.17)	(0.07)
C18:3 (Linolenic acid)	Mean	0.78	0.58	0.55	0.55	0.59	0.63	0.58	0.62	0.52	0.66	0.61	0.59	0.69	0.70
	(SD)		(0.03)	(0.02)	(0.00)	(0.13)	(0.08)	(0.05)	(0.11)	(0.00)	(0.10)	(0.08)	(0.09)	(0.18)	(0.23)

SD: standard deviation. *n* = number of samples analyzed. Mean values within rows with different superscripts are significantly different at $p < 0.05$ * or $p < 0.01$ **.

The amount and composition of milk fat are known to influence the physicochemical and organoleptic properties of cheeses (e.g., flavor, aroma, mouthfeel, hardness, melting properties, etc.). Short-chain volatile fatty acids in particular play a crucial role in the development of cheese aroma, in addition to being precursors of strong volatiles (not covered in the present study). The composition of fatty acids in milk has a strong, yet complex and indirect, relationship with the composition of the animal's diet, as rumen microbial activity largely converts unsaturated fatty acids into saturated fatty acids [67,68].

3.2. Cheese Ripening Time

The overall aim of our research is to determine how different factors contribute to the observed variation in ripening time of a traditional Swedish long-ripening cheese produced by our dairy collaborative partner. In this study, cheese-ripening time varied between 485–721 days, with no clear seasonal pattern (Figure 2). However, the average ripening time showed greater variation for cheeses produced during the period June–October, which includes the transition to and from pasture feeding. Visualizations of the variation in cheese maturation using NIR-hyperspectral images of cheese resulting from the same project [14] showed that cheeses within the same batch and cheese from different batches matured at different rates. Owing to the cost associated with an extended cheese ripening period, there is great commercial interest in controlling and predicting the cheese ripening process [69].

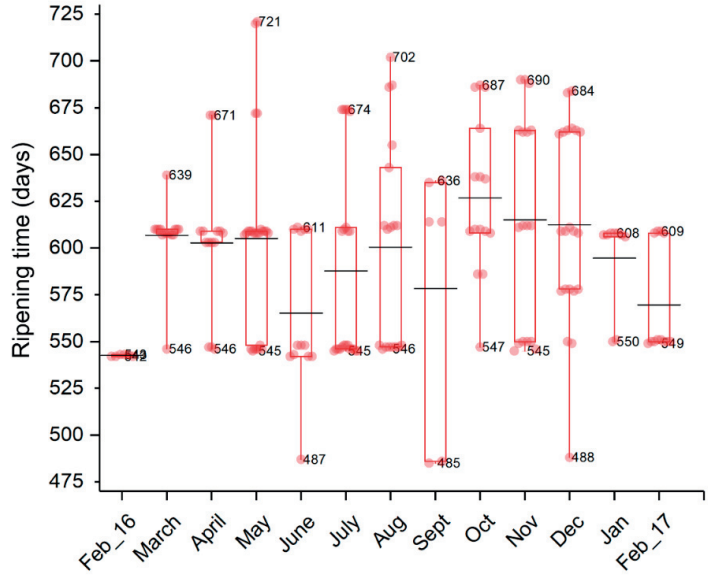

Figure 2. Box and whisker plot illustrating the variation in ripening time (days) of long-ripening cheeses, arranged according to production month (*X*-axis), showing median values (black horizontal line), 25–75th percentile interquartile range (height of the box), and spread (red dots and whiskers). In total, 208 cheese batches are represented.

3.3. Ripening Time and Milk Quality

Based on the variation observed in milk quality attributes and ripening time of the resulting cheese over one year, we evaluated the relationship between milk quality and cheese ripening time. For this, we performed an OPLS analysis to explore the influence of milk quality parameters on the ripening time of the resulting cheese (Figure 3). We found that none of the milk quality parameters studied had a strong effect on cheese-

ripening time. Some fatty acids had a weaker influence, e.g., longer ripening time was associated with C14:1, and shorter ripening time was associated with C18:0 and C18:1. This was probably because the concentration of C18:1 was highest in July (Table 2), when the ripening time was comparatively short. The C18:1 and C18:0 acids are closely associated with pasture feeding, and it has been shown that cheeses made from milk from pasture-fed cows have higher lipolytic activity, with a more elastic and creamier texture, and a yellower tone, than cheeses made from milk from silage-fed cows [70]. However, identifying a link between cheese ripening and milk quality aspects is challenging, since in industrial cheese production, there is greater scope for variation in cheese quality, e.g., between samples of cheeses, between cheeses produced from a vat, between vats produced within a day, and between different production days. Factors not covered in this study, e.g., the microbiota in the raw milk and cheese and minor variations in process parameters, also contribute to variation in ripening time of the cheeses. For these reasons, direct and obvious relationships between milk quality parameters were not found in this study.

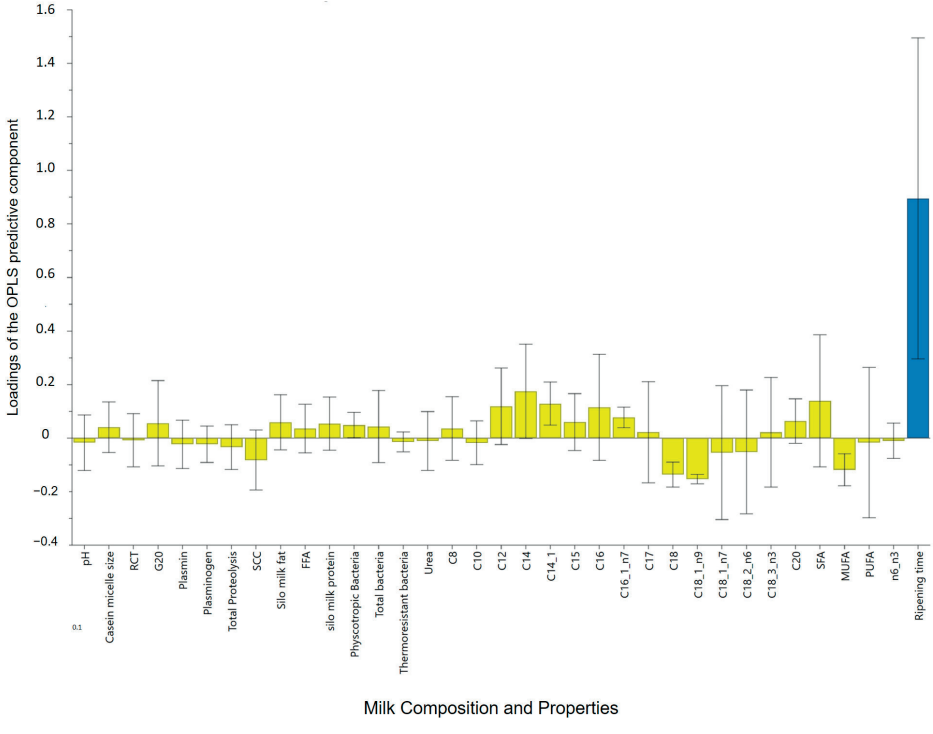

Figure 3. Orthogonal projections to latent structures (OPLS) analysis of milk quality attributes (yellow bars) in response to ripening time of the resulting cheese (blue bar). Confidence intervals (95%) are given for each factor and the response. Bars are based on loadings of the OPLS predictive component, with bar length indicating level of influence (i.e., taller bars indicate a stronger influence).

3.4. Effect of Raw Milk Quality Attributes on "Smell and Taste" Sensory Score Values during Ripening

Figure 4 shows the relationship between the sensory score values given to the cheeses and the quality attributes of the corresponding dairy silo milk for each of the occasions when sensory evaluation of the cheese was conducted. Interestingly, the influence of

milk components on sensory score values varied between different occasions during the ripening process. In the cheeses at 14, 18, and 20 months of ripening, the PL/PG and total proteolysis values contributed positively to the sensory score values, i.e., higher sensory score values were obtained for cheese produced from milk with high proteolytic potential. One likely explanation is that in cheese with high proteolytic activity, protein is degraded into flavor and odor active components, resulting in higher sensory score values. Fatty acids had a varying influence on sensory score values on different occasions during ripening, illustrating the complexity of the cheese-ripening process.

3.5. Effect of Milk Quality Attributes on Texture Scores of Cheeses

Since the texture of cheese plays a crucial role in determining the total ripening time, we also evaluated the effect of milk composition and properties on the texture score of the resulting cheeses at 14 months of ripening, using OPLS analysis (Figure 5). The texture score was positively influenced by gel strength (G20). Firmer gels are likely to retain more protein and fat than less firm gels [20], resulting in better gel properties and higher texture scores of the cheeses. We observed a positive influence of PL/PG on the texture score, probably due to a favorable proteolytic effect on the cheese matrix. However, the other two potent contributors to total proteolytic activity (i.e., SCC and TBC) had a negative influence on texture score, with higher values resulting in lower texture score values for the cheeses. This most likely indicates that indigenous enzymatic activity in milk results in favorable effects, whereas endogenous proteolytic activity results in weaker texture in sensory panel evaluations. However, elevated SCC and TBC may also be associated with overall milk compositional changes and catabolic activities with negative impacts on cheese texture. The causes of the observed differences need to be identified in a wider sensory evaluation.

Specific fatty acids also influenced the texture scores of the cheeses, with C16:0 and C18:0, and to some extent also C18:1, having a positive effect, while C12:0, C18:3, and C20:0 had a negative effect. Among the fatty acids, C16:0 and C18:1 are of major interest for cheese texture, since they are the major saturated and unsaturated fatty acid, with high and low melting points, respectively. A higher proportion of C16:0 results in a firmer cheese, while a higher proportion of C18:1 results in a creamier texture [71,72]. Since those aspects (overall textural quality assessment) were rated as favorable traits in the sensory evaluation, such cheeses received higher texture scores than cheeses with lower levels of C16 and C18:1 (Figure 5).

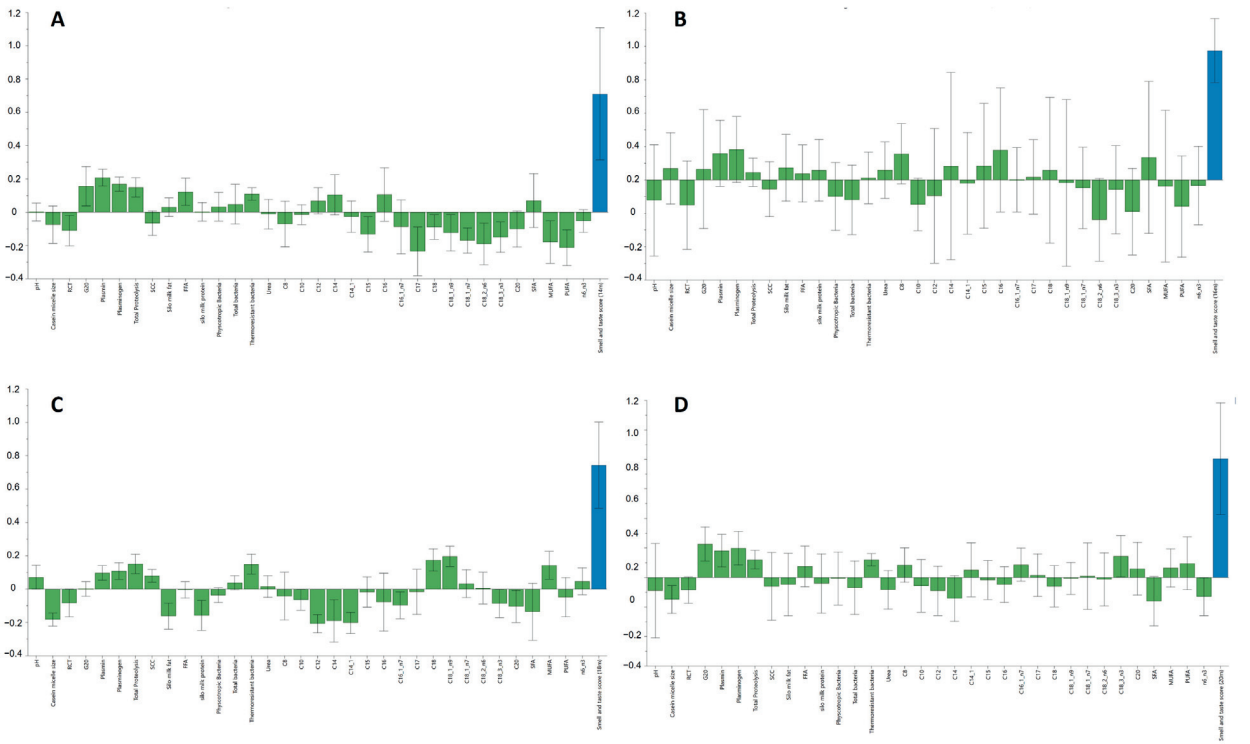

Figure 4. Orthogonal projections to latent structures (OPLS) analysis of quality attributes of the dairy silo milk (green bars) in response to smell and taste score values assigned by the sensory panel at (**A**) 14, (**B**) 16, (**C**) 18, and (**D**) 20 months of ripening of the resulting cheese (blue bar). Confidence intervals (95%) are given for each factor and the response. Bars are based on the loadings of the OPLS predictive component, with bar length indicating level of influence (i.e., taller bars indicate stronger influence). In total, 208 cheeses batches are represented.

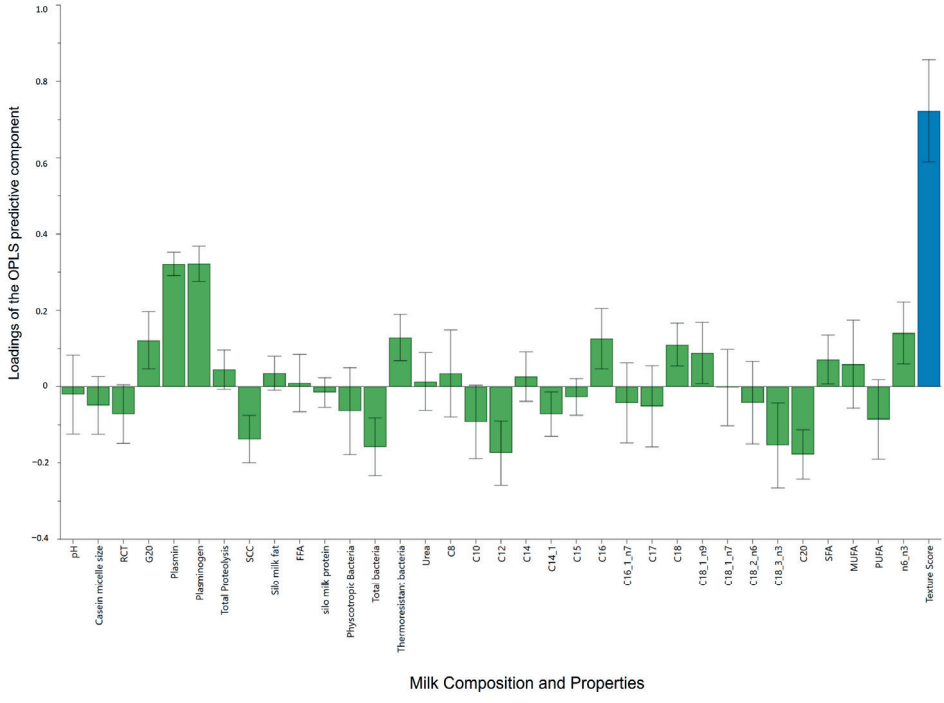

Figure 5. Orthogonal projections to latent structures (OPLS) analysis of milk and cheese quality attributes (green bars) in response to texture score (blue bar). Confidence intervals (95%) are given for each factor and the response. Bars are based on the loadings of the OPLS predictive component, with bar length indicating level of influence (i.e., taller bars indicate stronger influence). In total, 208 cheeses batches are represented.

4. Conclusions

This study demonstrated that the composition and properties of dairy silo milk are subject to monthly variation, with some quality attributes showing significant differences between the main indoor and outdoor periods of the year. However, investigated milk quality parameters showed weak relationships with the ripening time of the resulting long-ripening cheeses. This shows even though milk quality characteristics vary on monthly basis, the time required for the development of aroma and texture in resulting cheese is not associated with raw milk being used in cheese making, especially when the milk quality is of high standards. Hence, differential use of high-quality raw milk for cheese production is discouraged only based on raw milk quality characteristics. The sensory and texture scores of the cheeses were influenced by plasmin and plasminogen in the silo milk, highlighting the important role of the native proteolytic system during ripening for this Swedish cheese type. These findings indicate a need for more in-depth, holistic studies to identify the reasons for the variation in the time needed to reach the full maturity of the cheese.

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M.L. and M.H.; project administration, Å.L.; funding acquisition, Å.L. and A.H. All authors have read and agreed to the published version of the manuscript.

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Use of near-infrared hyperspectral (NIR-HS) imaging to visualize and model the maturity of long-ripening hard cheeses

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ABSTRACT

Spectroscopic measurements and imaging have great potential in rapid prediction of cheese maturity, replacing existing subjective evaluation techniques. In this study, 209 long-ripening hard cheeses were evaluated using a hyperspectral camera and also sensory evaluated by a tasting panel. A total of 425 NIR hyperspectral (NIR-HS) images were obtained during ripening at 14, 16, 18, and 20 months, until final sensorial approval of the cheese. The spectral data were interpreted as possible compositional changes between scanning occasions. Regression modelling by partial least squares (PLS) was used to explain the relationship between average spectra and cheese maturity. The PLS model was evaluated with whole cheeses (average spectrum), but also pixelwise, producing prediction images. Analysis of the images showed an increasing homogeneity of the cheese over the time of storage and ripening. It also suggested that maturation begins at the center and spreads to the outer periphery of the cheese.

1. Introduction

Long-ripening cheeses are important premium products for the dairy sector (Ardö, 1993), but cheese maturation is a costly and not fully controllable or predictable process (Fox et al., 1996). During maturation, the curd turns into a characteristic cheese with a particular flavour and texture depending on the microflora, milk quality, industrial processing steps, and storage conditions (e.g., Fox et al., 1996; Robinson and Wilbey, 1998; Rehn et al., 2010). The characteristic flavour and texture of different long-ripening cheeses are associated with the end-products of lipolysis and proteolysis in the matured cheese (Molina et al., 1999; Collins et al., 2003; Verdini and Rubiolo, 2002).

At present, maturation of long-ripening cheeses is mainly monitored by conventional methods based on chemical characterization and subjective evaluation of organoleptic properties (O'Shea et al., 1996; Coker et al., 2005). Destructive sensory evaluation at regular intervals is used to determine ripeness and readiness for the market. This approach is time-consuming and wastes material, and is therefore expensive for the producer. Thus, rapid non-destructive technologies for monitoring the maturation process in long-ripening cheeses are required. There is great interest in using non-destructive spectroscopic techniques to monitor cheese maturation and quality (Mazerolles et al., 2001; Downey et al., 2005; Currò et al., 2017; Lei and Sun, 2019). Cheese ripening has been studied with various novel techniques, including ultrasound (Benedito et al., 2001), X-ray computed tomography (Huc et al., 2014), confocal microscopic imaging (Soodam et al., 2014), and magnetic resonance imaging (Huc et al., 2014). During the past decade, near-infrared hyperspectral (NIR-HS) imaging applications have been developed for use as non-destructive quality and safety inspection tools in the food industry (Gowen, O'Donnell, Cullen, Downey and Frias, 2007; Liu et al., 2014). It has been shown that it is possible to use NIR-HS imaging to monitor the ripening of semi-hard cheese packed in transparent vacuum packages (Darnay et al., 2017).

A NIR-HS image is a parallelepiped, three-dimensional data array, sometimes called a hypercube. Two of the dimensions are pixel indices and the third dimension is a wavelength index. Each pixel in the hypercube is a complete spectrum, e.g. 256 wavelength bands from 900 to 2500 nm (Gowen et al., 2007). Identifying the key wavelengths with multivariate methods can improve the predictive capability and accuracy of a model (Burger and Gowen, 2011). Pre-processing of the images to improve the spectral information and to prepare data for further processing is therefore an important step in model development

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(Gowen et al., 2007). Due to the nature of the technique, both qualitative and quantitative analyses are possible with NIR-HS imaging, using spectroscopic and multivariate calibration techniques (Burger and Geladi, 2005). Advances in the hyperspectral imaging technique, integrating NIR imaging and spectroscopy, provide novel possibilities to analyse and characterize the spatial and spectral information on the sample of interest (Gowen et al., 2007). Each pixel in the NIR-HS image provides information based on the spectrum of that unique position, and thus allows visualization of the biochemical constituents and their distribution in the sample.

The objective of this study was to develop and evaluate predictive models based on the NIR-HS imaging technique for monitoring ripening of long-ripening cheeses. The hypothesis tested was that NIR-HS imaging, coupled with chemometric techniques, can predict the maturity and ripening process in paraffin wax-covered long-ripening hard cheeses in a commercial cheese manufacturing setting.

2. Material and methods

2.1. Cheese material and experimental design

This study was conducted in a full-scale commercial cheese manufacturing process at Norrmejerier, Sweden and was part of a larger project, in which raw milk used for cheese production was sampled and characterized twice a month during one year. The cheese resulting from the analyzed milk was subsequently used for this study. During the study, 209 cheeses were scanned (NIR-HS imaging) from February 2016 to February 2017, resulting in a set of 425 NIR-HS images. Each cheese comprised an 18 kg cylinder (40 cm diameter, 14 cm height) that was brine-salted to a content of around 1.2% NaCl, coated with paraffin wax, and ripened for at least 14 months under conditions previously described by Rehn et al. (2010). After 14 months of ripening, the cheeses were evaluated for maturity and organoleptic quality by a sensory panel, and this was repeated every two months until 20 months after production. At least three trained sensory panellists from the dairy company evaluated each cheese against a standard protocol considering outer appearance, flavour, smell and texture. Thereby, the three sensory panellists collectively determined whether the cheese could be considered sensorially approved (mature) or not. When a cheese was considered sufficiently mature, it was removed from the study and sent to the market for sale. If a cheese was considered to be still immature, it was sent back to the cheese-ripening facility and re-evaluated after another two months of storage. The NIR-HS images were captured in parallel with the sensorial evaluation of the cheeses, irrespective of the sensory approval. As a consequence of the analytical procedure, faster-maturing cheeses were sampled, evaluated by the sensory panel, and scanned by the NIR-HS camera on fewer occasions than slower-maturing cheeses. Not all cheeses were studied at every scanning occasion, and therefore different numbers of NIR-HS images were produced at each scanning occasion. The procedure resulted in 425 images obtained from 209 individual cheeses varying in age and maturity (Fig. 1). Out of these 425 HS images, 81 were acquired from sensorially approved cheeses that at this point exited the study, while 344 images were obtained from cheeses that were not yet approved. The chronological age (days) of each cheese was calculated as the difference between production date and imaging date.

2.2. Hyperspectral imaging system

To acquire NIR-HS images, an Umbio Inspector (Umbio AB, Umeå, Sweden) line-scan pushbroom system equipped with a moving belt was used as described in the literature (Geladi et al., 2007). The HS imaging system was set up as described by Hetta et al. (2017). In brief, a line-scan pushbroom system was used with a line-scan camera with a 22.5 mm sisuChema SWIR (short-wave infrared) objective (Specim, Spectral Imaging Ltd., Oulu, Finland) and equipped with a HgCdTe 2-D array detector. The spectral range recorded was 937–2542 nm at increments of 6 nm, resulting in a NIR-HS image (variable length x 320 pixels width) in 256 wavelength channels. Scanning speed was set to acquire square pixels.

2.3. Hyperspectral image acquisition

The cheeses were covered with a 1-mm layer of paraffin wax. They were carefully placed on the conveyor belt on the defined scanning occasions (14, 16, 18, and 20 months) to acquire the NIR-HS images. The cheeses were, however, not oriented in exactly the same way on the different scanning occasions. Illumination was supplied by quartz-halogen lamps at a 45-degree angle as a radiation source. For dark and white references, a shutter and a white spectralon surface, respectively, were used, and pseudo-absorbance was calculated. Reflectance standards are essential for image calibration, to correct pixel-to-pixel variations arising due to inconsistencies in capture and illumination of samples (Burger and Geladi, 2005). Each image had approximately 350 000 pixels, of which approximately 63% were cheese pixels and 37% represented background pixels. The average spectra of the images (cheese pixels) were calculated and modelled using Breeze and Evince software (Prediktera AB, Umeå, Sweden). The key steps in the imaging and data analysis procedure are illustrated in Fig. 2.

2.4. Image transformation and cleaning

Reflectance images (I_{raw}) were recorded using the dark (I_{dark}) and white (I_{white}) reference data and the reflectance was transformed into absorbance (A) using the equation: $A = -\log10\ [(I_{raw}-I_{dark})/(I_{white}-I_{dark})]$, according to Grahn et al. (2016). NIR-HS imaging captures a square-shaped image and the area surrounding the circular cheese was thus background information, giving rise to a noisy spectrum that needed to be eliminated before further processing. The background information (pixels representing the bare belt) was eliminated by removing absorbances over 1.5 at band 55 (1279 nm), to provide the best possible difference between the sample and the background. Objects in the images smaller than 1000 square pixels in total area were also removed.

2.5. Hyperspectral image analysis

The NIR-HS image analysis was conducted using the Evince software. In section 3.1, analysis of a single cheese is described in order to illustrate the analytical method. In section 3.2, the analytical method is also demonstrated using an individual cheese at four maturity levels, forming a composite image. The tools in the Evince software were used to create informative diagrams explaining the spectral and maturity differences in the cheeses.

2.6. Partial least squares discriminant analysis

Because the maturity criteria were only available for whole cheeses, the cheese NIR-HS images were replaced by average spectra after background removal. Noisy wavelengths were further removed by excluding wavelengths below 1000 nm and above 2400 nm. The spectra were used in the standard normal variate (SNV)-corrected and mean-centered form. A partial least squares (PLS) discriminant model was applied to a training dataset (n = 100 NIR-HS images). The maturity of the cheeses, expressed in days, was used to make the PLS calibration models. The diagnostics used for the selected PLS model were coefficient of determination for calibration (R^2) and root mean squared error of calibration (RMSEC).

2.7. Image visualization and distribution map

The maturity attributes for all pixels were predicted using the

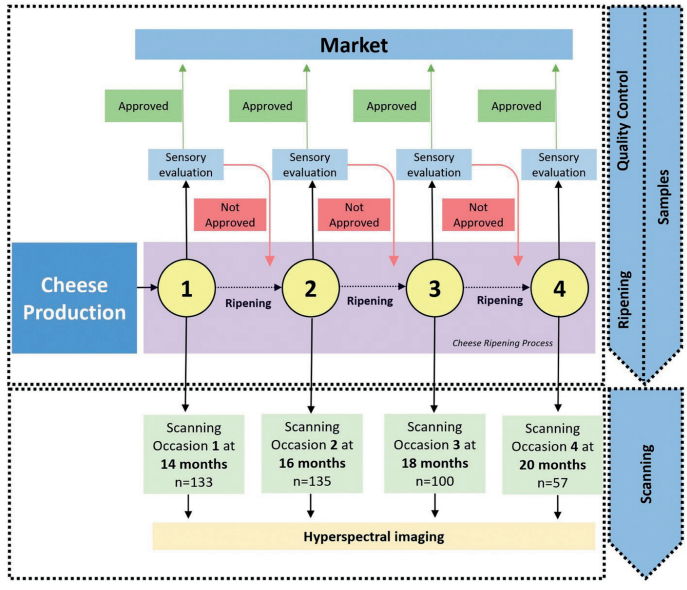

Fig. 1. Flow chart of sample handling and study design. Note: Not all cheeses remained throughout the study and, for practical reasons, not all cheeses were scanned on every occasion. This resulted in different numbers (n) of NIR-hyperspectral images on each scanning occasion.

calibration model developed from the training dataset. Predicted maturity values were applied to the region of interest (ROI) in the test dataset and distribution maps were developed for each cheese image. A high level of smoothing by merging 15 × 15 pixels was applied to pixels within the ROI, to improve the clarity of the pixels in the larger image.

3. Results and discussion

3.1. Hyperspectral analysis of an individual cheese

In Fig. 3A, a 14-month-old cheese is shown as a principal component one (PC1) image, coloured according to PC1 values (in the order

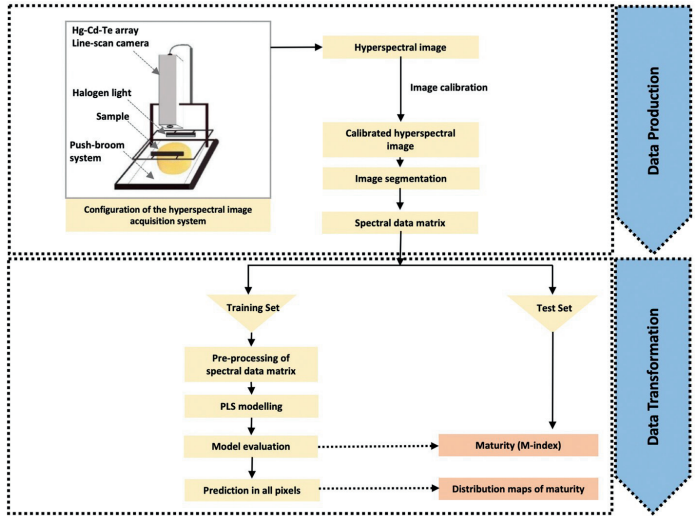

Fig. 2. Flow chart of the hyperspectral imaging, pre-processing, and partial linear squares (PLS) modelling procedure used for quantifying and predicting the maturity of long-ripening hard cheeses.

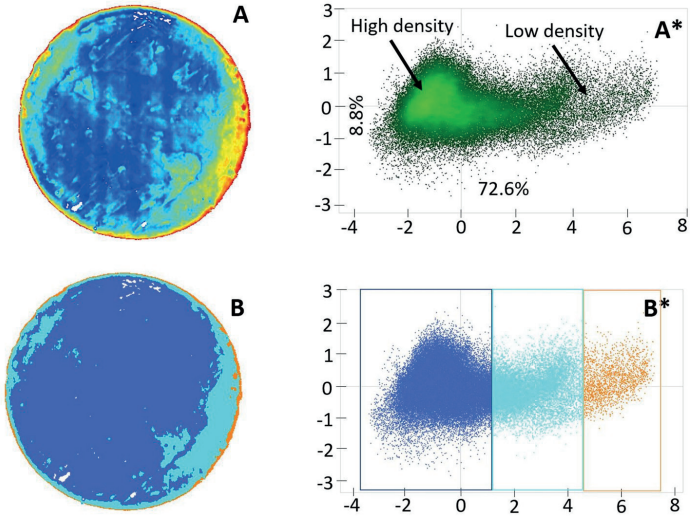

Fig. 3. A) The first principal component (PC1) of a 14-month-old cheese, colour-coded by PC1 values. A*) PC1 vs. PC2 score scatter plot of pixels of the same cheese, indicating different pixel intensity areas in PC1. B*) Preliminary selection of class regions (rectangular) in the score plot. B) Image coloured according to the classes selected in B* and projected onto the whole cheese using identical colours. The diagram should be read in the order A→A*→B*→B. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

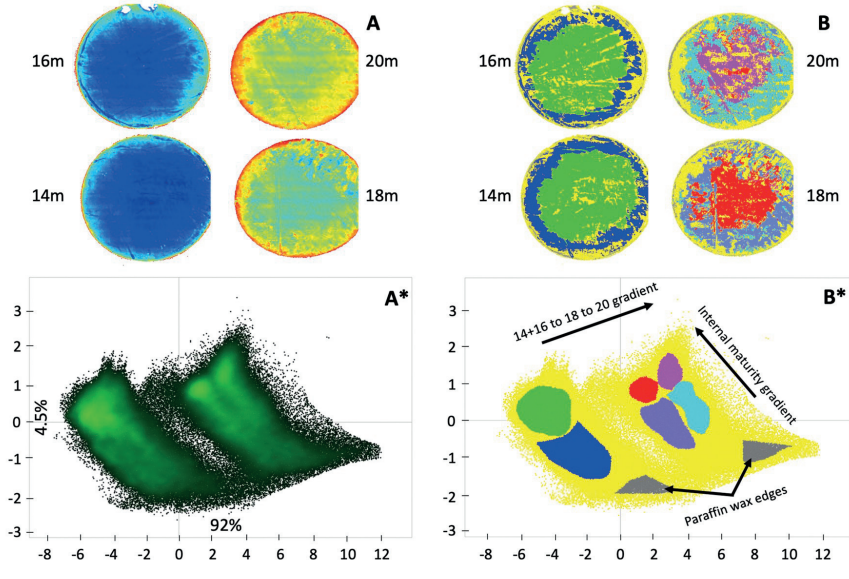

Fig. 4. A) Composite of the cheese at different maturity stages (14, 16,18, 20 months), showing the first principal component (PC1) score. A*) PC1 vs. PC2 score scatter plot for the composite image in A. B*) Manually selected regions of interest in the score scatter plot in A*. B) Projection of the regions selected in B* on the composite image. Colour codings are identical for B and B*. The diagram should be read in the order A→A*→B*→B. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

blue (lowest), cyan, green, yellow, orange, red (highest)). In order to interpret these images, a score scatter plot was created, as shown in Fig. 3A*. Three major regions with different score values and different densities, obtained after making a preliminary selection of three rectangular ROI (classes), are shown as coloured regions along PC1 in Fig. 3B*. The classes can also be seen in the coloured score image in Fig. 3B, produced using identical colours as in Fig. 3B*. Different regions in the cheese were revealed, with the edge of the cheese (red, yellow) mainly consisting of paraffin wax. Inside the cheese, two main regions were observed based on pixel intensities and PC1 (cyan and blue, respectively).

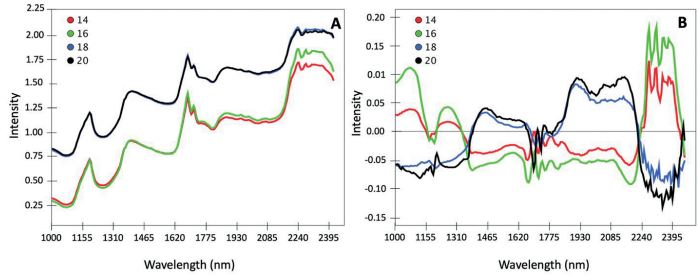

Fig. 5. A) Non-transformed and b) standard normal variate (SNV)-transformed spectral data for one randomly selected cheese scanned at 14, 16, 18, and 20 months after production.

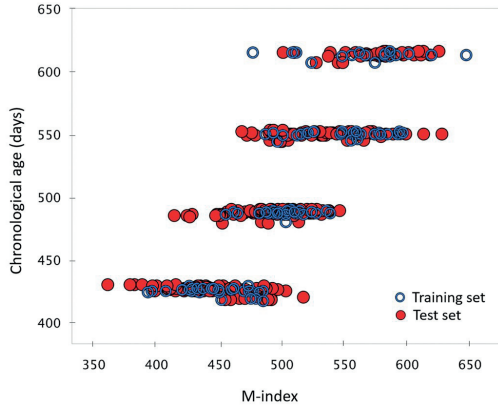

Fig. 6. Performance of the best prediction partial least squares (PLS) model for quantifying cheese maturity. Chronological age of the cheeses (423, 487, 550, and 614 days, corresponding to 14, 16, 18 and 20 months, respectively) was taken as the difference (days) between production data and imaging date. M-index: maturity index developed using NIR-hyperspectral image analysis.

3.2. Hyperspectral analysis of a composite image of a cheese at four maturity levels

The procedure described in section 3.1 for one cheese was repeated for the same cheese at different ages (14, 16, 18, and 20 months). The four images were made into a composite (Fig. 4A). After PCA and mean-centering of the composite image, a score scatter plot was obtained (Fig. 4A*). It revealed two large pixel clusters, one with a sub-set of pixels consisting of two separable clusters, forming three different main pixel clusters, i.e., 14 and 16 months maturity together and a sub-set of 18 and 20 months maturity levels (Fig. 4A*). In one of the large clusters, there was a region of high pixel density and a gradient away from it, i.e., a combined high pixel density area for the cluster of the cheese at 14 and 16-months of age. In the other large cluster, there were two high pixel density areas and their gradients within the sub-set of the cheese at 18 and 20-months of age. Pixel clusters and intensity gradients may relate to the maturity of the cheese. The three major high pixel density areas observed in Fig. 4A* were manually selected as shown in Fig. 4B* (green, red, and magenta). There were also gradients connected to each of the three clusters in Fig. 4A* and these were manually selected as shown in Fig. 4B* (blue, purple, and cyan). The selected regions of Fig. 4B* are shown in Fig. 4B using identical colours. Furthermore, some small clusters were identified in Fig. 4A* and coloured grey (Fig. 4B*). The smaller cluster in the lower part of Fig. 4A* turned out to represent the paraffin wax cover on the cheese. The yellow colour represents uncategorized pixels (Fig. 4B). The changes in colour illustrated in Fig. 4 indicates the occurrence of chemical changes, observed as spectral changes, that relate to age in months, and differences and gradients in composition within the cheese. A detailed discussion of possible explanations for this variation in composition is provided in section 3.3.

3.3. Wavelengths and their transformations

It is not meaningful to show many spectra at the same time, since the image becomes confusing. We therefore selected a single cheese for which spectra associated with the different months were representative for the whole dataset. For reasons of clarity, only the four average spectra (14, 16, 18, and 20 months) for this individual cheese presented

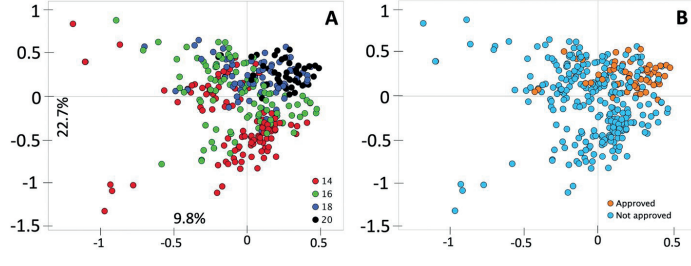

Fig. 7. A) Partial least squares (PLS) score scatter plot (component 2 vs. 3) of cheeses imaged at 14, 16, 18, and 20 months after production. B) PLS score scatter plot of the values in plot A, coloured according to approved or not approved for market by the sensory panel.

Table 1
Comparison of age and predicted maturity index (M-index), both in days, for six cheeses scanned at 14, 16, 18, and 20 months after production.

Cheese ID	1st scan, 14 months			2nd scan, 16 months			3rd scan, 18 months			4th scan, 20 months		
	Age	M-index	Dif	Age	M-index	Dif	Age	M-index	Dif	Age	M-index	Dif
C1	419	436	17	488	473	−15	551	538	−13	614	594	−20
C2	419	445	26	488	483	−5	551	554	3	614	613	−1
C3	419	466	47	488	498	10	551	580	29	614	626	12
C4	418	454	36	487	488	1	550	557	7	613	612	−1
C5	425	473	48	487	529	42	550	534	−16	613	571	−42
C6	425	477	52	487	532	45	550	568	18	613	597	−16

Calculations performed using the PLS model as described in section 3.5. Dif = difference between M-index and age of the cheese in days.

in Fig. 4 is shown in Fig. 5. Higher absorbance intensities were observed for the cheese scanned at 18 and 20 months compared with the same cheese scanned at 14 and 16 months (Fig. 5A). The reasons for this could be higher density (Darnay et al., 2017) and loss of gases and water during ripening (Hickey et al., 2013).

As shown in Fig. 5B, there were differences between the cheese at younger (14 and 16 months) and older (18 and 20 months) age in terms of SNV-corrected absorbance intensity at a given wavelength. These differences reflect the chemical changes that occur as a function of ripening. To relate the chemical components responsible for the differences in spectra, we compared the results against typical wavelengths reported for milk by Šašić and Ozaki (2000), who assigned differences observed in the 1208 nm band to milk fat (CH, CH_2, and CH_3 bonds). According to Fig. 5B, the intensity in this band was higher for the cheese at 14 and 16 months than for the same cheese at 18 and 20 months of age. Due to lipolysis of fat during ripening, less intact milk fat is available in older cheeses compared with young cheeses (McSweeney, 2004). Šašić and Ozaki (2000) assigned band 2056 and 2160 nm to amides. In our case, the intensities in the 2056 and 2160 bands were higher in the more mature cheese than in the younger cheese, indicating build-up of amides during ripening (McSweeney, 2004). The bands at 2316, 2340, and 2368 nm arise from combinations of CH_2 stretching and bending modes of protein side-chain groups (Šašić and Ozaki, 2000). In the present case, the intensities were higher for the younger compared with the older cheese, probably due to higher protein content (McSweeney, 2004). Our observations support findings by Hickey et al. (2013) that proteolysis increases during ripening.

3.4. PLS model between average spectra and age

The hyperspectral analyses (Figs. 3 and 4) reveal much about the cheese and variations in its maturity. The effect of chronological aging and changes due to maturity and differences between internal regions of the cheese are apparent. However, presenting corresponding data for hundreds of cheeses in a similar way would be tedious and nearly impossible to handle. Fortuitously, it was found that the average spectra (Fig. 5) could also be used to show differences in ripening. An attempt was therefore made to build a multivariate regression model of average spectra of all the scanned cheeses and their respective age. A PLS model was developed using average spectra calculated from the images for each cheese and their corresponding age in days. The dataset was split into a training set (n = 100 NIR-HS images) and a test set (n = 325 NIR-HS images), with the same distribution of scanning occasions.

The performance of the best PLS prediction model in assessing the maturity of the cheeses is shown in Fig. 6. The performance of the model was evaluated using the R^2 and RMSEC values; the higher the R^2 value and the lower the RMSEC value, the more powerful the model as a prediction tool (Vigneau et al., 2011). Five PLS components were found to be sufficient for the model. For the proposed model, R^2 was 0.76 and RMSEC was 36 days of age. The model was calibrated using cross-validation, resulting in root mean square error for the cross-validation of 34 days. The validated model was then used with the test set and its performance was evaluated with root mean square error for prediction, which was found to be 36 days.

The maturity (M) index and cheese age show a linear relationship until 18 months (~550 days), at which point a shift is observed. This indicates that cheeses older than 18 months needed a longer time to reach maturity and seem to follow a different pattern. There is a large variation in M-index for the cheeses on each particular scanning occasion (chronological age) (Fig. 6).

3.5. Scores of the PLS model

By using partial least squares regression (PLSR), quantitative estimates of particular relationships between the target variables and the spectral response were obtained. These were used to predict the concentration of different components in each pixel and to visualize their spatial distribution in the sample (see Vigneau et al., 2011). The PLS score scatter plot of components 2 and 3, describing the most meaningful variation in cheese maturity, showed that there was more variation between cheeses on the earlier scanning occasions (younger cheeses) than on the later scanning occasions (older cheeses), when they clustered together (Fig. 7A). Component 1 was not important and is possibly influenced by the paraffin wax layer, and was therefore not considered. This is in agreement with the diagram showing the same score scatter plot, but coloured in relation to approval by the sensory panel (Fig. 7B). To be approved by the sensory panel, a cheese has to have achieved certain characteristic properties and most of the approved cheeses were likely to be among the older ones. However, this could be partly due to the design of the study, as older approved cheeses were not scanned further and instead sent to market. The variation was greatest among the cheeses that were not approved by the pannel and is likely to derive from the young cheeses scanned on the earlier scanning occasions. Cheeses that were approved for the market, most of them originating from the scanning occasions at 18 and 20 months after production, showed less variation in the PLS score scatter plot (Fig. 7).

Table 1 shows the predicted maturity and the actual age for a selected set of six cheeses (C1-C6). On the earlier scanning occasions, the predicted maturity, i.e., M-index, tended to be higher than the age of the cheese. In contrast, towards the end of ripening, i.e., on the later scanning occasions, the predicted M-index mostly tended to be lower than the actual age of the cheese. This observation supports what was mentioned previously; that the more slowly maturing cheeses are kept in the cycle and faster maturing cheeses leave the study for the market.

3.6. Image visualization and distribution map

The PLS regression model for prediction of cheese maturity was applied to hypercubes of selected cheese images from different scanning occasions to visualize the maturity distribution of individual cheeses. A high level of image smoothing was applied to obtain visually comparable maturity distribution maps (non or intermediate levels of smoothing were found to give noisy results in our preliminary studies).

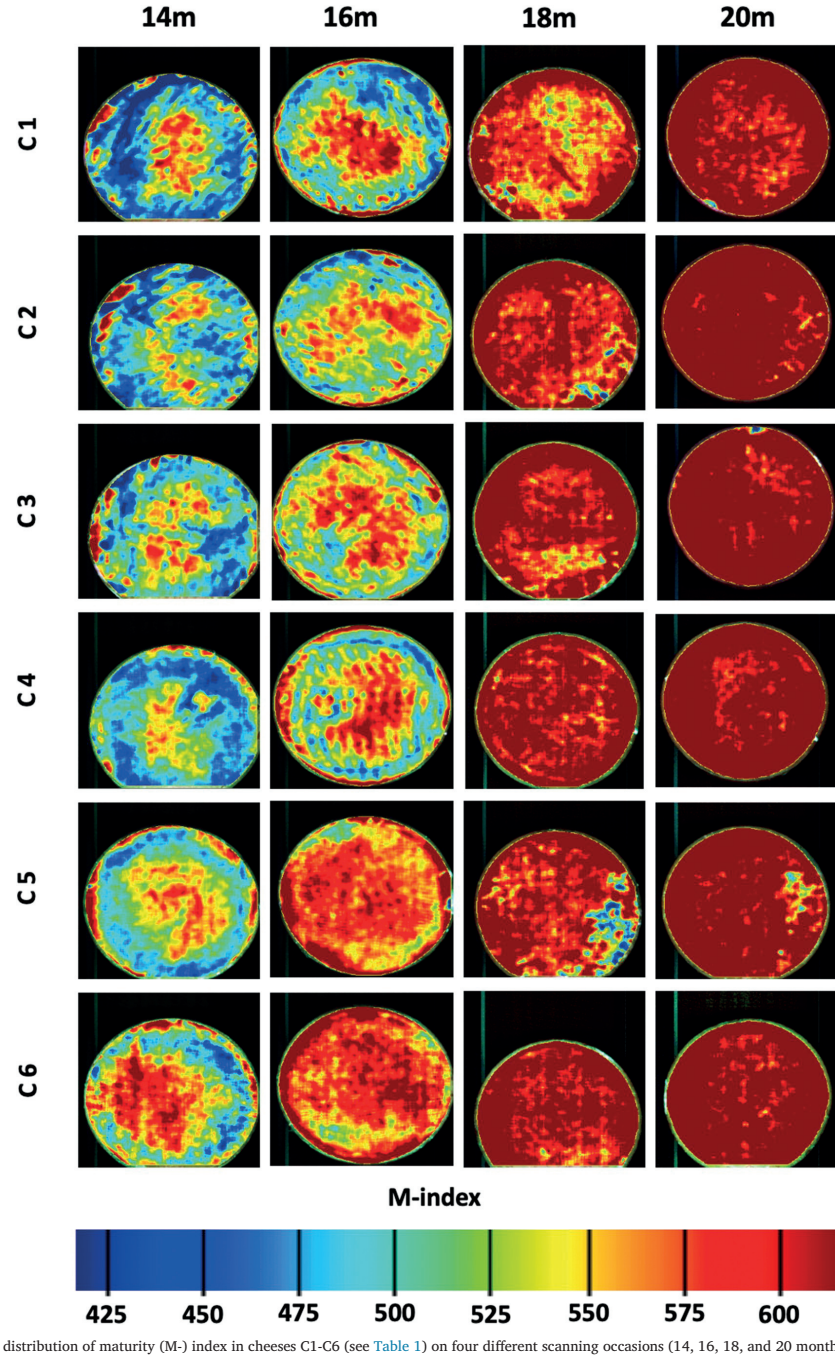

Fig. 8. Spatial distribution of maturity (M-) index in cheeses C1-C6 (see Table 1) on four different scanning occasions (14, 16, 18, and 20 months, corresponding to 423, 487, 550, and 614 days of age). M-index was developed using the PLS model, as described in section 3.4.

Fig. 8 shows the distribution of maturity in the six selected cheeses (C1-C6 in Table 1) on the four scanning occasions.

The images in Fig. 8 suggest that cheese maturation is non-homogeneous, i.e., within each cheese some parts are more mature than others. Moreover, the images indicate that ripening starts from the center of the cheese and moves to the outer periphery of the cheese rind. Similarly, as found in the PCA analysis (Fig. 4B), the images reveal that there are variations within an individual cheese and also within maturity level. For the 14- and 16-month cheeses in Fig. 8, green indicates the more mature center and blue the less mature edge. For the 18-month cheeses, red indicates the more mature center and purple the less mature edge. For the 20-month cheeses, the more mature center is coloured as magenta and the less mature edge is cyan.

Cheeses scanned on a particular occasion after production may have reached different degrees of maturity, thus showing different distributions of ripening of the curd (Fig. 8). This indicates that cheeses ripen internally in an uneven way and that variation occurs both within and between cheeses.

4. Conclusions

NIR-HS imaging is a powerful non-destructive method that provides the advantage of exploring simultaneous spatialized spectral information in each pixel. In the present study, NIR-HS imaging made it possible to generate meaningful composition classes based on individual images of a cheese and on a composite image representing a cheese at four maturity levels. Using chemometric and exploratory visualization techniques, the data were processed into meaningful and comprehensible information. The NIR-HS images provide indications on the chemical composition and on changes taking place during cheese ripening, potentially allowing prediction of cheese maturity. Considering that the model developed in our study achieved 76% accuracy in prediction of maturity (M-index), we conclude that the technique can become an important tool in cheese production for optimizing logistics and ensuring efficient use of costly cheese-ripening facilities.

Declaration of interest form

None.

Ethical statement

Conflicts of interest

The authors declare no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

Written informed consent was obtained from all study participants.

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Dairy farms in Sweden are undergoing structural changes that are reflected in the quality of the raw milk. In a full-scale cheese making trial, this thesis investigated how on-farm factors and season contribute to the variation in raw milk and whether this variation influences ripening time of a Swedish long-ripening cheese. The impact of selected milk quality parameters on cheese making was assessed in experimentally designed study, and NIR-hyperspectral image analysis was used to visualise and predict cheese maturation.

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