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Total proteolysis in bovine raw bulk milk of Northern Sweden

Total protolys i tankmjölk från gårdar i norra Sverige

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Master´s thesis • 30 hp credits
Uppsala 2020

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Credits:	30 hp credits
Level:	Second cycle, A2E
Course title:	Degree project in Animal Science
Course code:	EX0872
Course coordinating department:	Department of Animal Breeding and Genetics
Place of publication:	Uppsala
Year of publication:	2020
Online publication:	https://stud.epsilon.slu.se
Keywords:	Total proteolysis, plasmin, total bacteria count, somatic cell count, SDS PAGE

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Abstract

Proteases are enzymes that catalyze protein breakdown. The major indigenous protease is plasmin, that together with proteases originating from somatic cells and bacteria, accounts for most of the proteolytic activity in bovine milk. In many dairy products, the key characteristics depends on the concentration and composition of the milk proteins. It has been seen in other studies that environmental and production parameters can influence the level and activity of proteases. The objective of this study was to measure the level of total proteolysis in raw bulk milk of Northern Sweden and investigate if there are correlations between the total proteolysis and any of the parameters: plasmin, somatic cell counts, total bacteria number, milk pH, total milk protein, breed, milking system, production system (tied or loose house), number of animals or different seasons. The total proteolysis, that was measured as the level of peptides in the milk samples, was significantly correlated to the milk protein content ($P<0.001$) and the milk pH value ($P<0.01$) in a Pearson correlation. The same connection was seen when one variable at a time was compared in a regression; there was a correlation between proteolysis and milk pH ($P<0.05$), as well as milk protein content ($P<0.001$). In addition, there were significant differences between the breeds, where Jersey cows had 25.7 % higher proteolytic activity compared to the other breeds. There was also a difference between the housing systems, where milk from tied cows had 10.6 % higher proteolysis, compared to milk from loose housed cows ($P=0.002$). However, when all variables were included in a multivariable regression model, no variable was significantly correlated to total proteolysis. Nevertheless, some interesting trends were observed. The β-value was negative for all the breeds (SRB, SLB, Mountain Breed and farms with both SRB and SLB), when they were compared to the Jersey breed. Also, a trend towards a correlation between plasmin and proteolysis, seen in the single regression, remained in the multivariable regression. The measurement of proteolysis was complemented with an SDS PAGE electrophoretic analysis of eight milk samples, in order to study the protein profile. The images fortified the speculation on plasmin being the major protease responsible for the proteolysis in this study, since β-CN (the major plasmin substrate) appeared to be more extensively degraded in the samples with high proteolytic activity. Because of the fact that the degree of explanation for the variance in total proteolysis was low in the multivariable regression, even in the best model (R-sq. adj. 12.16 %), it was concluded that there are probably other factors, e.g. individual differences – that was not included in this study, that highly affect the proteolytic activity in bovine milk. However, according to the results in this study, investigations with focus on genetical differences might be an interesting perspective for future investigations.

Keywords: Total proteolysis, plasmin, somatic cell counts, total bacteria count, SDS PAGE

Sammanfattning

Proteaser är enzymer som katalyserar nedbrytning av proteiner. I mjölk är den proteolytiska aktiviteten huvudsakligen orsakad av plasmin, vilket överförs från blodet, och proteaser som utsöndras från somatiska celler och bakterier. Många av de karaktäristiska kvalitetsegenskaper som önskas i mejeriprodukter påverkas av koncentrationen och sammansättningen av mjölkproteinerna. Tidigare studier har visat att såväl koncentrationen som aktiviteten av de olika proteaserna påverkas av miljö- och produktionsfaktorer. Syftet med den här studien var att mäta den totala proteolysen i tankmjölk från gårdar i norra Sverige, och undersöka om det finns statistiska samband mellan nivån av proteolys och faktorerna: plasmin, somatiska celltal, totalt bakterieantal, mjölkens pH-värde, mjölkens totala proteininnehåll, olika raser, mjölksystem, stallsystem (uppbundet eller lösdrift), antal djur eller olika säsonger. Den totala proteolysen, skattad som mängden peptider i mjölkproverna, var signifikant korrelerad med både det totala proteininnehållet ($P<0.001$) och pH-värdet ($P<0.01$) i mjölken, när detta undersöktes i en Pearson korrelation. Detta samband sågs även när de olika variablerna jämfördes i enkla regressioner; både proteinhalten och mjölkens pH-värde var positivt korrelerade till mängden proteolys ($P<0.001$ respektive $P<0.05$). Skillnader i medelvärden jämfördes för grupperade data. Detta visade på signifikanta skillnader mellan raserna från de medverkande gårdarna, där Jerseykor hade 25.7 % högre proteolytisk aktivitet jämfört med de andra raserna. Det var även 10.6 % högre total proteolys i mjölk från gårdar med uppbundna kor, jämfört med kor i lösdriftssystem ($P=0.002$). När alla variabler inkluderades i en multivariabel regressionsmodell var däremot ingen faktor signifikant korrelerad med nivån av proteolys. Dock fick samtliga raser (SRB, SLB, Fjällkor och gårdar med en blandning av SRB och SLB) ett negativt β-värde vid jämförelse med Jerseykorna. Resultatet visade även på en trend mot signifikant positiv korrelation mellan plasmin och total proteolys; denna trend syntes både vid enkel regressionsjämförelse och bestod i den multivariabla modellen. I detta arbete genomfördes även en elektroforetisk analys (SDS PAGE). Proteinprofilen i de analyserade proverna styrkte antagandet att plasmin var det mest aktiva enzymet i mjölkproverna då β-kasein, det främsta substratet för plasmin, bedömdes vara lägre i de prover där en hög proteolys uppmätts. Med tanke på att förklaringsgraden över variationen av den totala proteolysen var relativt låg (R-sq. Adj. 12.16 %), drogs slutsatsen att den totala proteolysen antagligen påverkas i stor utsträckning av andra faktorer än de som inkluderades i denna undersökning. Enligt resultatet i detta arbete, kan ett intressant utgångsläge för vidare studier vara fokus på genetiska skillnader.

Nyckelord: Total proteolys, plasmin, somatiska cellantal, totalt bakterieantal, SDS PAGE

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Abbreviations

AMS	Automatic Milking System
BME	Beta-Mercapto Ethanol
BSA	Bovine Serum Albumin
CFU	Colony Forming Unit
CMS	Conventional Milking System
HCl	Hydrochloric acid
Ig	Immunoglobulin
kDa	kilo Dalton
LDS	Lithium Dodecyl Sulphate
LF	Lactoferrin
MW	Molecular Weight
PA	Plasminogen Activator
PAGE	Polyacrylamide Gel Electrophoresis
PAI	Plasminogen Activator Inhibitor
PG	Plasminogen
PI	Plasmin Inhibitor
PL	Plasmin
PMN	Polymorphonuclear Neutrophils
PP	Proteose Peptone
PPPS	Precision Plus Protein™ Standards
RPM	Reference Protein Mixture
SCC	Somatic Cell Count
SCs	Somatic cells
SDS	Sodium Dodecyl Sulphate
SLU	Swedish University of Agriculture
t-PA	Tissue-type Plasminogen Activator
TBC	Total Bacteria Count
TCA	Trichloro acetic Acid

Tris	2-amino-2-(hydroxymethyl)1,3-propanediol
u-PA	Urokinase-type Plasminogen Activator
UHT	Ultra-High Treatment
α-LA	α-lactalbumin
α_{S1}-CN	α_{S1}-casein
α_{S2}-CN	α_{S2}-casein
β-CN	β-casein
β-LG	β-lactoglobulin
γ-CN	γ-casein
κ-CN	κ-casein

1 Introduction

Bovine milk is a high value nutritional fluid that contains all essential amino acids required by humans (Walstra *et al.,* 2006). In Sweden, milk is a traditional staple food, yet consumers preferences for specific dairy products has changed over the years (Nylander *et al.,* 2014). Since the 1960s, the direct consumption of milk has gradually decreased; however, the demand for other processed dairy products has increased (Jordbruksverket, 2019). Cheese consumption has raised by 37 % since 1980 (Jordbruksverket, 2018), and the request for other high protein products, such as quarg, has escalated during the past decennium (Gianuzzi, 2019).

Proteases are enzymes that catalyze protein breakdown (Walstra *et al.,* 2006). In milk, proteases of different origin are naturally present, and their activity may have an impact on the product quality. Uncontrolled proteolytic activity can lower the cheese yield (Law, 1979), or decrease shelf-life and cause bitterness of milk products (Ismail & Nielsen, 2010). On the other hand, during cheese ripening, development of desired flavor compounds is attributed to protein degradation products (Walstra *et al.,* 2006). Furthermore, the level of proteases has been observed to affect the ripening time (Bastian & Brown, 1996). The ripening process is expensive and energy demanding, since it requires maintenance and storage space (Walstra *et al.,* 2006). Dairy companies thrive for a reduced climate impact by optimizing their use of resources and energy consumption (Arla, 2020). Thus, the dairy industry has an interest in understanding and controlling these dynamic proteolytic systems in milk, that is driven by both the possibility to enhance product quality and prolong shelf-life, as well as economic and sustainability incentives with shortened ripening and optimum use of resources.

Pasteurization and storage conditions have an impact on the enzymatic activity during processing (Murphy *et al.,* 2016). However, raw milk of high quality is a prerequisite for production of premium dairy products. Environmental aspects and management routines influence the presence and activity of proteases, as do individual animal factors (Bastian & Brown, 1996). Thus, the basis for production of high-quality dairy products is initiated on the farm.

1.1 Objective

The objective of this thesis is to measure the level of total proteolysis in raw bulk milk samples, collected from eighteen farms in Northern Sweden, and investigate if any of the known production parameters are significantly correlated to the level of protein breakdown. This will be accomplished by a statistical analysis of variance in a linear multivariable regression model. Furthermore, eight selected samples will be analyzed in a protein separation method. This is performed with the intention to acquire complementary information on the milk protein profile in these individual samples, in relation to the total proteolytic activity.

2 Literature review

2.1 Protein composition of bovine milk

Milk is a complex liquid containing several structural elements in different physical stages (Walstra *et al.,* 2006). The fat fraction is structured as globules, which creates an oil-in-water emulsion. These globules are surrounded by a multi-layer membrane of polar lipids, such as phospholipids, and interspersed proteins, where some are enzymes. The average amount of protein in bovine milk plasma (the liquid surrounding the fat globules) is 3.3 % with a range between 2.3 - 4.4 % (Walstra *et al.,* 2006). Traditionally, the proteins are subdivided into two categories: caseins (CN) and whey proteins. Around 80 % of the proteins are caseins, which are defined as the proteins that precipitate when milk is acidified to a pH of 4.6, while the whey proteins remain in the solution (Jenness *et al.,* 1956). Based on their primary structure, there are four casein families: α_{S1}-, α_{S2}-, β- and κ-CNs (Farrell et al., 2004). In addition, there is a fifth category, called γ-CN. However, γ-CNs are degradation products, formed when β-CN is enzymatically degraded (Stelwagen, 2016), while the others belong to four separate genes (Fox & McSweeney, 2003).

The caseins tend to aggregate and form spherical structures, so called micelles (Fox & McSweeney, 2003). The exact micelle substructure is still unknown (De Kruif *et al.,* 2012), but apart from caseins and small amounts of other proteins, such as enzymes, the micelles consist of water and salts, mainly calcium phosphate, but also calcium and magnesium (Walstra *et al.,* 2006). The main idea is that thousands of casein molecules form subunits that are linked together by calcium phosphate (Fox & McSweeney, 2003). The micelles have an open structure and can therefore bind water; hence they have a large hydrodynamic size, meaning that the water volume per gram dry casein is 4 ml (Walstra *et al.,* 2006). While the core consists of α_{S1}-, α_{S2}- and β-CN, κ-CN is linked to the surface, with the negatively charged and hydrophilic end creating a surface layer, which provides colloidal stability.

The remaining 20 % of total proteins, the whey proteins, are also called serum proteins because they remain soluble while the caseins precipitate at pH 4.6 (Fox & McSweeney, 2003). The major whey proteins are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), lactoferrin (LF), Immunoglobulins (Ig) and proteose peptones (PP) (Farrell *et al.,* 2004). Proteose peptones are a heterogenous mixture of proteins where some are derived from proteolysis of caseins and other are indigenous to milk (Deeth & Bansal, 2019). Apart from the proteins mentioned, milk contains a substantial amount of minor proteins such as enzymes and globule fat membrane proteins. Approximate concentrations and particle sizes of the caseins are shown in Table 1.

Table 1. *Molecular weight and approximate composition of caseins in milk (Walstra et al., 2006; Stelwagen, 2016)*

Protein	MW (kDa)	Total protein (%)	Total casein (%)
α_{S1}-CN	23,60	32	38.1
α_{S2}-CN	25,20	8,4	10.2
β-CN	23,98	26	35.7
κ-CN	19,55	9,3	12.8
γ-CN	20,50	2,4	3.2

2.2 Milk protein profile

There are different variants of the major milk proteins, due to amino acid changes caused by genetic polymorphism (Farell *et al.,* 2004). The frequency of these genetic variants is different between breeds, and some genetic variants have been associated to yield and manufacture properties, such as coagulation and renneting time, or curd firmness during cheese ripening (Park & Haenlein, 2013).

Regarding the genetic protein variants and interactions between composition of milk proteins, the B allele for β-LG is associated to a higher CN content, and the B allele for κ-CN affects the κ-CN concentration positively (Hill, 1993; Wedholm *et al.,* 2006). Jersey is found to have a higher frequency of the B allele for κ-CN, than both SLB and SRB (Gustavsson *et al.,* 2014). Van Eenennaam & Madrano (1991) determined the frequency of β-LG alleles A and B to be 0.43 and 0.47 respectively for SLB, and 0.37 and 0.63 for Jersey.

Gustavsson *et al.* (2014) examined the differences between breeds, in terms of variations in protein concentrations. Jersey and SRB were found to have a higher κ-CN concentration than SLB. Furthermore, SLB had the highest concentrations of α-LA, β-LG and β-CN, while Jersey had the highest α_{S1}- and α_{S2}-CN concentrations.

However, since the separate CN genes are located close by on chromosome 6, it is also reasoned that effects of haplotypes should be estimated instead of just separate gene variants, when looking at milk protein composition and manufacture properties (Park & Haenlein, 2013).

2.3 Proteolytic activity

Activity from around 60 different enzymes has been detected in bovine milk (Fox & McSweeney, 2003). These are often categorized based on their origin, where indigenous enzymes are excreted by the mammary gland, and exogenous enzymes originate from microorganisms. The indigenous enzymes can either be synthesized in the mammary secretory cells or be transferred from blood to milk – either through leaky tight junctions between cells, or via transcytosis (Fox & McSweeney, 2003). Most enzymes in milk are associated to the globule fat membrane and enter through exocytosis by the secretory cells (Fox & McSweeney, 2003).

Proteases are enzymes that catalyze the hydrolytic cleavage of peptide bonds (Walstra *et al.,* 2006). Thus, proteolytic activity will yield peptides and fragments from larger proteins.

2.3.1 Indigenous proteases

There are two major systems of indigenous proteases in milk; the plasmin system which is blood-derived, and proteases originating from somatic cells that migrate to the mammary gland (Kelly & McSweeney, 2003).

Plasmin (PL) is an important enzyme in blood since it is part of the fibrinolysis process (i.e. removing of blood clots) (Sjaastad *et al.,* 2016). It is transferred to milk through the mammary cell wall lining, but at quite small amounts; the concentration is around 0.3 µg/mL compared to 200 µg/mL in blood (Halpaap *et al.,* 1977). The activity of plasmin is regulated in a complex system of different components that interact with each other: an inactive zymogen of plasmin called plasminogen (PG), activators of plasminogen (PA:s), PL, and inhibitors of both plasminogen activators (PAI:s) and plasmin (PI:s) (Ismail & Nielsen, 2011). Plasminogen is usually the predominant component compared to PL, with concentrations between 0.8 - 2.8 µg/mL (Ozen *et al.,* 2003).

There are at least two groups of activators: urokinase-type PA (u-PA) and tissue-type PA (t-PA) (Heegaard *et al.,* 1994). These activators convert PG to PL by cleavage of the peptide bond Arg_{557}-Ile_{558} on PG, that contain 786 amino acid residues (Heegard *et al.,* 1994; Kelly & McSweeney, 2003). Plasmin specifically cleaves proteins at peptide bonds containing Lys or Arg residues and is active on α_{S1}-, α_{S2}-

and β-CN in milk (Fox & Kelly, 2006). The principal substrate is β-CN, from which $\gamma_1-, \gamma_2-, \gamma_3-$caseins and PP are gradually formed (Eigel *et al.,* 1984). The rate of α_{S1}-CN breakdown is slower compared to the hydrolysis of α_{S2}- and β-CN, and it has not yet been established whether PL is active on κ-CN (Ismail & Nielsen, 2010). The enzymatic activity of PL is down regulated by a heterogenous group of inhibitors of both PL and PA:s (Politis, 1996). These are present in the whey fraction, while PL, PG and PA:s are bond to the casein micelles (Baer *et al.,* 1994). Some PA:s are also found to be associated to somatic cells (Heegard *et al.,* 1994). Plasmin, PG and PA:s are quite heat resistant, to the extent that they can ”survive” pasteurization (Lu & Nielsen, 1993), whereas PI:s and PAI:s are heat labile (Prado *et al.,* 2006). Still, optimum conditions for plasmin activity is 37 °C and pH 7.5 (Kelly & McSweeney, 2003).

In addition to the PL system, proteases are released by somatic cells (Li *et al.,* 2014). Somatic cells present in milk mainly consists of leucocytes, where the predominant types are macrophages, polymorphonuclear neutrophils (PMNs) and lymphocytes (Auldist, 2011). In healthy milk, the proportions between these three types are 74, 18 and 8 % respectively (Saad & Ostensson, 1990) whereas the PMNs rapidly increase during mastitis (Kelly & McSweeney, 2003) Thus, an influx of SCs indicates mastitis and somatic cell count (SCC) is commonly used to evaluate udder health, but the composition of somatic cells (SCs) will be different during infection compared to milk from a healthy udder. Of the different leucocytes, the PMNs are found to have the highest capacity for protein breakdown (Le Roux *et al.,* 2003). Apart from leucocytes, a small amount of the somatic cells are shred epithelial cells (Kelly & McSweeney, 2003).

When Larsen *et al.* (2004) induced mastitis by inoculating 10^7 CFU of *Streptococcus uberis* into one teat cistern each on four dairy cows, SCC increased from <250 000 cells/mL, to 2-10 000 000 cells/mL during the trial period of 40 h. Total proteolytic activity (measured as the level of free primary amines), in relation to SCC showed increased proteolysis when cell counts were >1 000 000 cells/mL. No effect on proteolysis in relation to SCC was seen in uninfected milk samples.

Larsen *et al.* (2004) also investigated effects of specific enzymes. The most studied somatic cell proteinase is cathepsin D, which like PL is part of a system of zymogens and activators (Kelly & McSweeney, 2003). Activity from cathepsin D was significantly higher in the infected milk, and there was an increase that followed log SCC (Larsen *et al.,* 2004).

Cathepsin D is mostly present at low concentrations (0.4 µg/mL) in the whey fraction, and optimum conditions for activity are around pH 3-4 and 37 °C (Magboul *et al.,* 2001; Larsen *et al.,* 1996; Kelly & McSweeney, 2003). Compared to PL, it has quite low heat stability (Hayes *et al.,* 2001), but it is active on all caseins, including κ-CN (McSweeney *et al.,* 1995).

Besides cathepsin D, several other lysosomal proteases, such as cathepsin B and elastase, are probably released in milk after lysis of SCs (Li *et al.,* 2014). Their activity has been identified through chromatographic or immunological methods, yet little else is known (Magboul *et al.,* 2001). Given the knowledge on how milk is secreted and how substances are transferred to milk, it is considered likely that a range of other indigenous enzymes are present in milk as well (Kelly & McSweeney, 2003). Most likely, the balance between these complex systems with interacting components, will control the total proteolytic activity.

Li *et al.* (2014) also argued that the effect on milk proteins caused by SCs is difficult to estimate. When looking at SCC, the composition of cells should also be taken into consideration. Many studies are based on manipulated mastitis or a mixture of milk to create different concentrations of SCC. This makes it difficult to interpret the results, according to Li *et al.* (2014).

2.3.2 Exogenous proteases

Milk is a high-quality growth medium for many microorganisms (Walstra *et al.,* 2006). Due to different optimum growth environments for individual bacteria spp., the microflora will alter depending on processing and storage conditions of the milk.

Psychrotrophic bacteria are very diverse and include several genera of both gram-negative and gram-positive spp. that can proliferate in low temperatures, below 7 °C (Champagne *et al.,* 1994). Thus, they dominate the microflora of cold stored raw milk, since they are naturally selected for in refrigerated bulk tanks (Sørhaug & Stepaniak, 1991). The most common psychrotrophs in raw milk belong to the genus *Pseudomonas* (Cousin, 1982; Skeie *et al.,* 2019), predominantly *P. fluorescens* (Sørhaug & Stepaniak, 1991). Many strains of *Pseudomonas* spp. (as well as other psychrotrophs) produce heat-stable enzymes that degrade all variants of caseins, often with a preference for κ-CN (Sørhaug & Stepaniak, 1991; Kohlmann *et al.,* 1991). The extracellular proteases are generally secreted at the end of the log growth phase, when the bacteria population reach 10^6 CFU/mL and the cell density is high (Sørhaug & Stepaniak, 1997). However, it is also reasoned that an exact critical number of bacteria to influence the milk quality is difficult to determine, since the specific characteristics such as a high production of enzymes, may be strain dependent (Murphy *et al.,* 2016).

Even though it has been declared that psychrotrophic *Pseudomonas* dominate in refrigerated stored milk (Sørhaug & Stepaniak, 1991; Skeie *et al.,* 2019), the microflora of raw milk is complex and not yet fully explored. When von Neubeck *et al.,* (2015) analyzed 2906 isolates from milk samples stored at 4-5 °C for 3-4 days, 18% of the total bacteria belonged to unknown species, thus there is a wide range of bacteria that might have an impact on the enzymatic activity in bulk milk.

In addition, mastitis pathogens such as spp. from the genera *Streptococcus* and *Staphylococcus* are commonly found in bulk tank raw milk (Skeie *et al.,* 2019). *Staphylococcus aureus,* one of the major pathogens responsible for mastitis infections, can effectively produce extracellular enzymes that degrade caseins (Johansson *et al.,* 2013). When pasteurized milk was inoculated with field strains of *S. aureus* – bacteria counts reached 9.13 log CFU/mL after 6 h incubation at 37 °C – and the total CN content was reduced by 21 %.

2.4 Processing and product quality aspects

Processing conditions have an impact on the proteolytic activity in milk (Sørhaug & Stepaniak, 1991). During pasteurization, most psychrotrophs are eliminated, apart from some thermoduric spp., yet the heat-stable enzymes produced by the psychrotrophs during cold storage will remain (Champagne *et al.,* 1994). Heating also inactivates PIs and PAIs, thus the plasmin system is no longer down-regulated (Prado *et al.,* 2006).

Even though enzymes often are present at low levels in milk, they can be of considerable importance and have both beneficial and undesirable effects (Ismail & Nielsen, 2010).

2.4.1 Positive aspects

In the cheese making process, clotting of casein is induced by the enzyme chymosin (naturally occurring in calf rennet) which cut off the end of κ-CN, thus the micelle stability is disrupted (Walstra *et al.,* 2006). This creates a gel, from where the whey is separated, and the remaining curd can be shaped and pressed. This is followed by ripening of the cheese, during which chemical changes occur due to indigenous and bacterial proteases that – together with rennet – modify the cheese sensory quality (Sørhaug & Stepaniak, 1991). Rennet and plasmin releases peptides that are further broken down to amino acids by lactic acid bacteria. Depending on the cheese variety, different proportions of break-down products may be desired, as they are aroma precursors (Sørhaug & Stepaniak, 1991). Increased plasminogen activation or addition of plasmin has been seen to improve cheese flavor and quality (Farkye & Landkammer, 1992; Bastian *et al.,* 1997), yet the importance of the effects possibly also depend on differences in the manufacture, where cheese variants cooked under high temperature and pH close to PL optimum are probably more favored by the influence of plasmin proteolysis, since the rennet will be inactivated during these conditions (Bastian & Brown, 1996). Furthermore, the ripening process is expensive

(Ismail & Nielsen, 2011); in trials where PL or *P. fluorescens* has been added to milk prior to cheese making, the ripening process was accelerated due to increased proteolysis (Tye *et al.,* 1988). Thus, for specific cheese types, a high proteolytic activity may be economically beneficial.

2.4.2 Negative aspects

On the other hand, uncontrolled proteolysis before coagulation has been seen to decrease cheese yield due to lower casein concentration (Law, 1979). Other, undesirable consequences due to casein break-down are flavor bitterness caused by proteases from *Pseudomonas* spp. (Sørhaug & Stepaniak, 1997). Negative effects on flavor in hard cheese has been seen when CFU in raw milk were 7.5-8.3 log/mL – differences are explained by strain variations – and no exact threshold level has been established (Sørhaug & Stepaniak, 1997).

Ultrahigh-temperature (UHT) treated milk is a way to prolong shelf-life by heating the milk to 140-145 °C for a few seconds (Walstra *et al.,* 2006). During the heating, complexes are formed between β-LG and κ-CN. Breakage of κ-CN during storage, can cause these complexes to dissociate from the micelles and aggregate, which leads to gel formation (Datta & Deeth, 2001). This happening is partly attributed to proteases, amongst other production- and storage factors. Results on whether PL can be a causative factor or not have been conflicting (Datta & Deeth, 2001). However, gelation due to heat-stable proteases produced by psychrotrophs in raw milk during storage, has been observed by several authors (e.g. Law *et al.,* 1977; Griffiths *et al.,* 1988). As with cheese, bitter flavors as a result of proteolysis can also cause storage defects in milk (Champagne *et al.,* 1994). Stoeckel *et al.* (2016) found that bitterness always occurred before gelation when three different *Pseudomonas* spp. were incubated to raw milk prior to UHT treatment.

2.5 Factors influencing proteolytic activity in raw bulk milk

As described earlier, activity from plasmin, somatic cell-, bacterial proteases and possibly other, not yet characterized enzymes, are responsible for the total proteolytic activity in raw bovine milk. In turn, there are many different factors that have an impact on the presence and activity of these enzymes, and many of them are correlated to each other.

2.5.1 Plasmin – reasons for variations in level or activity

During mastitis, bacterial toxins can rupture the mammary epithelial tight junctions, thus leading to an increased leakage of plasmin to milk (Auldist, 2011). Saeman (1988) saw that PL activity remained at a higher level after full recovery of induced mastitis, which might be part of the explanation for why older cows have a higher PL activity compared to younger ones (Politis, 1989a), since it is more likely that older cows have suffered from mastitis (Fox & McSweeney, 2003).

Increased leakage is also assumed to be the reason for why milking frequency is negatively correlated to PL, since longer milking intervals lead to a higher pressure in the mammary gland (Stelwagen *et al.,* 1997). Stelwagen *et al.,* (1997) showed that the tight junctions shifted to a permeable state after 18 h, when eight cows in early lactation were examined. One of the major advantages with AMS is that more frequent milking is possible (Svennersten-Sjauna & Pettersson, 2008). Significantly lower plasmin levels have been measured in milk from farms with AMS, when compared to farms with CMS (Johansson *et al.,* 2017). Sorensen *et al.* (2001) compared plasmin levels in milk from cows where half the udder was milked twice a day, and the other half was milked three times a day. In consistency to Stelwagen (1997), it was seen that a more frequent milking kept a greater tight junction integrity with lower PL activity. However, Sorensen *et al.* (2001) also reasoned that part of the explanation for this effect, was due to the reduced time for proteolytic activity in the udder; since the conditions in the udder are close to optimum for PL – most protein breakdown may take place prior to milking.

Furthermore, during involution at the end of lactation, proteases presumably contribute to tissue breakdown (Dallas *et al.,* 2015), and the tight junctions are destroyed by apoptosis (Sjaastad *et al.,* 2016). Thus, the stage of lactation is yet another factor contributing to variations in PL levels due to a higher leakage from blood (Nicholas *et al.,* 2002; Politis *et al.,* 1989b).

Apart from an increased influx, PL levels can also increase due to an enhancement in PG activation. It has been observed that proteases from SCs can work as PAs; when a suspension of bovine milk somatic cells, equivalent to a concentration of 500 000 cells/mL, was mixed in a buffer solution with β-CN and PG, PL breakdown of β-CN increased from 5.9 to 28.4 % when incubated at 37 °C for 7 h (Verdi & Barbano, 1991).

Politis *et al.* (1989b) evaluated environmental effects on PL activity. Individual milk samples from 43 Holstein cows were collected monthly during an entire lactation. The result showed large variations in PL activity, which was attributed to the individual cow's stage of lactation and differences in SCC; PL activity more than doubled when SCC increased from 100 000 to 1 300 000 cells/mL. However, the samples were divided in sub-groups based on SCC, and there was no significant

effect on PL activity < 290 000 cells/mL. Apart from SCC – lactation number, stage of lactation and pH was also positively correlated to PL activity.

The same effect has been observed for microbial proteases – they can work as PAs. Plasmin activity gradually increased during 20 h in refrigerated milk, and PG was activated in a buffer solution, when *B. polymyxa* proteases were added (Larson *et al.,* 2006).

However, other studies have observed the opposite effect – PL activity can decrease in the presence of bacteria. Guinot-Thomas *et al.* (1995) examined changes in proteolytic activity, and interactions between PL and microbial proteases in raw milk, during storage in 4 °C for 6 days. Control milk was compared to milk treated in three different ways; with added u-PA (to activate PG), added bacteriocide (to kill off the bacteria) or both u-PA and bacteriocide. There was no change in total proteolysis no matter treatment, until after day 4, when the proteolytic activity attributed to bacterial proteases increased, at the same time as the PL activity decreased. The concentration of β-CN and κ-CN decreased after day 4, except in the ones where bacteriocide was added. The initial TBC was 2 x 10^4 CFU/mL, the growth log-phase lasted between day 2-4, and psychrotrophic count at day 4 was 10^6 - 10^7 CFU/mL in all samples apart from those where bacteriocides were added. Guinot-Thomas *et al.* (1995) concluded that bacterial proteases were more important than plasmin during storage at 4 °C, but only when bacteria counts reached 10^6-10^7 CFU/mL, which occured after 4 days.

When Fajardo-Lira *et al.* (2000) added *P. fluorescens* (M3/6) protease to milk, they found a decrease in PL levels in the CN fraction, and an increase in the whey fraction; thus, they concluded that bacteria proteases released PL into the whey by disrupting the micelles.

Summarizing these results, it appears as if proteases from bacteria, as well as SCs may have an impact on the PL system, even though the dynamic or required concentrations is not fully understood.

2.5.2 Plasmin and total proteolytic activity – effects of milk protein, breed and pH

Markus *et al.* (1993) found CNs to be effective substrates for accelerating the activity of PAs, where α-CNs were most effective. Furthermore, Baer *et al.* (1994) found that the level of bound PG was twice as high for α-CNs compared to β- and κ-CNs. Thus, it appears that interactions between caseins and the PL system is yet another factor that may affect the level of proteolysis. It has been discussed if this might be part of the explanation for why differences between breeds has been observed (Schaar, 1985). Schaar (1985) studied variations in both PL activity and PP content in fresh and cold stored milk from individual cows of different breeds. The PP

fraction was measured as the N-content, which was measured by the Kjeldahl method. Morning milk samples were collected from four different breeds: SLB, Jersey, SRB and SRB/SLB crosses. The cows were at different stages of lactation. Changes in PP content was measured by comparing fresh milk to milk stored at 5 °C for 72 h. Plasmin activity appeared to be higher for SLB compared to Jersey. However, when CN content was included in the analysis of variance, the effect was eliminated. Schaar (1985) came to the conclusion that this effect might be caused by competition between the casein and the synthetic substrate that is used to measure the PL activity; the more casein, the higher competition and a false low activity. Plasmin activity was measured by the use of a fluorescent synthetic peptide substrate, and the activity was expressed as compound units that are released over time. As with PL, there was no significant difference in content or changes of PP between the breeds, when the result was adjusted for the other variables. However, PP and changes in PP was still positively correlated to the CN content, after statistical adjustments. When looking at the protein genotypes, the highest PP and changes in PP occurred in milk from cows with the genotype BB for β-LG, and the lowest was found from β-LG genotype AA. There was no difference in PP content between the genotypes for different CNs. The variants of β-LG have been observed to affect the total protein composition, where the BB variant gives a lower concentration of β-LG, but a higher concentration in total CN (Hill, 1993).

Schaar (1985) also concluded that the stage of lactation had the highest impact on PL activity, and there were large variations between individual cows. Furthermore, there was a tendency (yet not significant) that the PP fraction was positively correlated to the pH value. The casein micellar structure is influenced by small variations in pH, since it affects the colloidal calcium phosphate (Sørhaug & Stepaniak, 1991). When Humbert *et al.* (1983) measured protease activity (expressed as changes in non-protein nitrogen) in morning milk from one herd of 120 Friesian cows, the correlation coefficient between proteolytic activity and milk pH was 0.46, and half of the variation in proteolytic activity was explained by the pH value. Abeni *et al.* (2008) evaluated the effects of AMS or CMS on milk enzymes and plasma metabolites. In this study, 6 pairs of Friesian twin cows were housed in the same stall and given the same feed. Individual milk samples were collected 6 times during the lactation period, thus the effect of stage of lactation was included in the statistical model. In average, pH was higher in milk from AMS compared to CMS, which was positively correlated to PL activity. Plasminogen levels and total PG and PL on the other hand, was significantly lower in AMS. Abeni *et al.* (2008) argued that irregular milking frequencies was the reason for the increased pH and since pH optimum for PAs is 7.5 (higher than average pH in milk), more PG may be converted to PL when closer to optimum conditions.

2.5.3 Somatic cells and total bacteria count – effects of milking system, management system, number of animals and seasonal variations

Given that psychrotrophic bacteria are represented in many different genera, they are present in all natural habitats, e.g. soil, dust, water, vegetation and faeces (Quigley *et al.,* 2013). Still, some species are more abundant in certain environments depending on their ecological niche (Walstra *et al.,* 2006). Thus, the composition of the microbial population in raw milk is complex and influenced by on-farm factors such as the feed, housing strategy, if the cows are kept on pasture or not, hygiene routines etc., as well as seasonal variations.

Porcellato *et al.* (2018) characterized the bacterial composition of raw silo milk from two dairies in Norway, and collected samples once every month, in order to study variations over the year. The total microbiota was investigated by gene sequencing, while total aerobic bacteria was determined by culturing techniques. The sample analyses took place one day after they were collected from the silos. Both cultured bacteria levels and compositions varied significantly between different months. *Pseudomonas* were most abundant during autumn and winter, while *Bacillus* were predominant during spring and summer.

Somatic cell count and total bacteria count (TBC) are in close relation to each other and they are both correlated to many production parameters. Johansson *et al.* (2017) investigated if there were differences in enzymatic activity in bulk milk from farms with AMS or CMS. Bulk milk was collected from 104 farms, where SLB or SRB were used for production. Plasmin activity was significantly lower in milk from AMS. However, there was a positive correlation between total proteolysis (i.e. not only PL activity) and both AMS and SCC, which implies that microbial or SC enzymes might be involved in the protein breakdown. Average somatic cell counts in AMS and CMS were 230 000 and 182 000 cells/mL respectively, whereas bacteria counts were unknown. According to Växa (2019), SCC >200 000 cells/mL is a clear indication of sub-clinical mastitis, and mastitis related pathogens are often present in individual fresh milk samples (Østerås *et al.,* 2006). Since udder milk is not sterile, infection status of the udder will influence the milk microbiota (Skeie *et al.,* 2019).

In a study conducted by Rasmussen *et al.* (2002), bulk milk quality on 98 farms in Denmark before and after installment of AMS was evaluated. Both SCC and TBC significantly increased when AMS was introduced. However, when the farmers participated in a monitoring program to detect cows with signs of clinical mastitis before they were milked with AMS, the effect on SCC almost diminished to the same levels as when CMS was used. The same effect was not seen on TBC; the levels were significantly higher even when the self-monitoring program was followed. Rasmussen *et al.* (2002) reasoned that this might be due to technical problems with

the automatic cleaning and cooling system. *Pseudomonas* contribute to biofilm formation by producing exopolysaccharides (Capodifoglio *et al.,* 2016). Biofilms support microbial growth on equipment and surfaces; thus, it is argued that preventive methods, with hygiene routines and minimized storage time, is the most reasonable strategy to control the risk of quality affecting bacteria, including psychrotrophs (Walstra *et al.,* 2006).

Nevertheless, according to statistics in Sweden 2010, environmental aspects still influence the milk quality. Both TBC and SCC are higher when AMS is used, compared to CMS (Svensk Mjölk, 2011). Somatic cell counts are higher in isolated loose housing, and there is a positive correlation between herd size and SCC.

2.6 Analytical method for detection of total proteolysis

In this thesis, the quantity of already produced peptides is used as a measurement for total proteolysis in raw milk samples.

2.6.1 Precipitation and separation of proteins

Since only the number of peptides is of interest in this assay, the milk proteins were separated and removed from the samples prior to further analysis. This is accomplished by trichloroacetic acid (TCA), a weak acid that is commonly used to precipitate proteins in aqueous solutions (Koontz, 2014), followed by centrifugation in order to separate the components. The negatively charged trichloro acetate ions disrupts the electrostatic interactions, that stabilizes the proteins secondary structure, hence they become denatured (Rajalingam *et al.,* 2009). In addition, the water solubility is decreased as a consequence of the exposure of non-polar surfaces when the proteins are unfolded, which implies that they can be recovered by centrifugation (Koontz, 2014). Thus, the TCA soluble peptides will remain in the supernatant.

2.6.2 Fluorescence assay

In this study, the fluorescamine method was applied in order to determine the quantity of peptides in the supernatant. The method, that was first described by Udenfriend (1972), is based on a reaction between fluorescamine and the primary amine at the terminal of each peptide. The formed complex is highly fluorescent, whereas the reagent (fluorescamine) does not possess any fluorescence by itself (Udenfriend, 1972). Hence, the fluorescence intensity is directly correlated to the quantity of free primary amines in the solution (BioTek, 2006) and the relative value can be expressed as equivalents when compared to a standard curve of known concentrations.

In this study, various concentrations of L-leucine were used to create a linear regression and the extent of proteolysis in the milk samples was expressed as mM Leucine equivalents (Leuc.Eq).

The products formed in the reaction are relatively stable for several hours (Lorenzen, 1993). However, the intensity may be affected by exposure to light (BioTek, 2006). Furthermore, the level of fluorescence differs between peptides and free amino acids depending on the pH, where maximum fluorescence for peptides is achieved near pH 9, whereas amino acids maximum appears around pH 7 (Udenfriend, 1972). One drawback with this method might be that it does not provide information on proteinase specificities and it is not considered to be that accurate, yet it is a fast method that can be used to measure moles of free primary amines, in order to compare levels of peptides in different samples (Le *et al.,* 2006).

2.7 Separation of proteins by SDS PAGE Electrophoresis

Proteins in complex samples can be separated and characterized by electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), is a commonly used electrophoretic method, that was first described by Laemmli (1970). In this procedure, the proteins are first heated in a buffer containing SDS, which dissociates noncovalent bonds, and a thiol reductant (often ß-mercaptoethanol, BME) that breaks covalent disulphide bonds (Strange *et al.,* 1992). Thus, the proteins lose their secondary and tertiary structure and the negatively charged SDS molecules can bind to all proteins in proportion to their weight, (approximately 1.4 g SDS/g protein; Reynolds & Tanford, 1970) no matter their native qualities in a folded structure. Because of this, the proteins can be separated by electrophoresis solely based on their size.

Once the samples have been treated with SDS, they are run through a gel placed in a Tris-buffer (Strange *et al.,* 1992). When attached to power, an electric field arises between the buffer ions which creates a moving boundary. The polyacrylamide gel is cross-linked at different levels (expressed in percentage), thus the pore size can be optimized for the proteins in the sample. Since the migration rate will depend on the molecular size, the proteins separate and the molecular weight (MW) can be determined by comparison to standard proteins of known MW, that are run on the gel together with the sample of interest. However, it has been described that the caseins behave abnormally in SDS PAGE, and only migrate to an expected MW around 30,000 (Basch *et al.,* 1985), when they indeed have a weight in the range between 19,000 – 25,000 (see table 1). This behaviour may be due to interactions between different caseins, leading to various levels of SDS-binding which in turn may cause an anomalous result (Cheeseman & Jeffcoat, 1970). Furthermore, by

looking at the MW of α_{s1}-CN and β-CN, β -CN is slightly larger and should therefore migrate shorter than α_{s1}-CN. Still, α_{s1}-CN has a larger hydrodynamic size when bound to SDS and tend to separate above β-CN (Strange *et al.,* 1992). To be able to interpret the electrophoretic patterns in the gel lanes, purified proteins can be run separately prior to analysis of the samples of interest. This makes it possible to add a mixture of these proteins on the gel together with the samples, and then compare the migrating patterns in order to identify which specific protein that correspond to each band. Thus, in each gel, there will be one lane with a standard mixture of proteins of known MW, and one lane with similar migrating patterns as the proteins in the sample.

SDS PAGE can be assessed visually based on characteristic patterns from the included proteins, but it can also provide quantitative information when the intensity of the bands is compared to a calibration curve (Bio-Rad, 2017).

3 Materials and Methods

All laboratory work was performed in BioCenter, at the Department of Molecular Science, Swedish University of Agriculture.

3.1 Milk samples

Raw milk samples from 18 different farms located in Northern Sweden were obtained at three different occasions; November 2017, February 2018 and September 2018. At each trial, three samples with one day in between were collected from each farm tank. However, some samples were missing from the trials. Thus, for some farms, only two samples from each trial were included. In total, 148 samples were analysed in this study.

Cooled to 4 °C, the samples were transported from Umeå to the Swedish University of Agriculture (SLU), Uppsala, where the milk was defatted and transferred to 2 mL micro tubes before it was stored at -80 °C as skim milk. During the time for this assay, the samples were temporary kept at -20 °C.

The participating farms differ in aspects of management such as milking- and housing system, number of dairy cows and breed used for milk production (presented in Table 2). Prior to this thesis, the milk samples have been analyzed by Eurofins Steins Laboratorium AB (Jönköping, Sweden) for protein content (%), somatic cell count (cells/mL) and total bacteria count (x 10^3 CFU/mL). In addition, pH has been measured at SLU, and the level of plasmin activity (Units/mL) has been examined for each sample respectively (Khaled, 2019).

Table 2. *Presentation of the 18 participating farms; their main breed and number of dairy cows used for milk production, as well as milking system and production system, i.e. tied or loose housing*

Farm nr	Nr of dairy cows	Breed	Milking system	Production system
1	67	SLB[1]	AMS[5]	Loose
2	177	SLB	CMS[6] (parlour)	Loose
3	120	SLB	AMS	Loose
4	73	SLB	AMS	Loose
5	60	SLB	CMS (parlour)	Loose
6	120	SLB	AMS	Loose
7	42	SLB	CMS	Tied
8	63	Jersey	CMS	Tied
9	18	MB[2]	CMS	Tied
10	32	SRB[3]	CMS	Tied
11	62	SLB	AMS	Loose
12	120	SLB	CMS	Tied
13	76	SLB	AMS	Loose
14	35	SRB	AMS	Loose
15	87	SRB/SLB[4]	CMS	Tied
16	70	SRB/SLB	AMS	Loose
17	29	SRB/SLB	CMS	Tied
18	41	MB	CMS (parlour)	Loose

[1] SLB, Swedish Friesian cattle (svensk låglandsboskap).
[2] MB, Mountain Breed, (fjällko).
[3] SRB, Swedish red and white cattle.
[4] SRB/SLB, ~ 50% of each breed is used for production.
[5] AMS, Automatic Milking System.
[6] CMS, Conventional Milking System.

3.2 Total proteolysis

The following procedure to determine the total proteolysis in samples of raw milk was carried out as described by Wiking (2002), modified according to Johansson et al. (2017).

3.2.1 Preparation of solutions

Three different solutions were needed in order to measure proteolytic activity in this assay; a TCA solution, a liquid with fluorescamine dissolved in acetone and a buffer of sodium tetraborate. The amount of chemical compounds used for each solution was calculated due to the equation:

$$m\,(g) = c\,(\mathrm{M}) \times V\,(L) \times M\,(\frac{g}{mole})$$

Fluorescamine- and TCA solutions were prepared in amounts to be sufficient for one week, thus the bottles were wrapped in tinfoil to avoid exposure to light. The tetraborate buffer was freshly prepared before each plate to be analyzed.

The table below briefly summarizes the preparations.

Table 3. *Specification of buffers and chemicals*

Chemical (supplier and batch nr)	Preparation of solution	Final concentration
TCA[1] (Sigma-Aldrich, T6399)	24 g TCA was weighed and transferred to a beaker. Milli Q was added up to 100 mL	24 % (w/v) TCA
Fluorescamine (Sigma-Aldrich, F9015) Acetone (MerckKGaA, 67-64-1)	10 mg fluorescamine was dissolved in 50 mL acetone	0.2 mg/mL fluorescamine
Sodium borate (Sigma-Aldrich, S-9640)	500.3 mg sodium borate was weighed and transferred to a beaker. Milli Q was added up to 23 mL. The liquid was placed on magnetic stirring until the borate was completely dissolved. Finally, pH was adjusted to pH 8 by adding approximately 2 mL 1M HCl.	0.1 M sodium borate buffer

[1] TCA, Trichloroacetic acid.

3.2.2 Leucine standard curve

A quantity of 132.2 mg L-leucine (Sigma-Aldrich, L8000-25 G) was accurately weighed and transferred to a beaker, where 1 mM HCl was added to a total volume of 10 mL. This gave a 0.1 M Leucine stock solution that was further diluted to create concentrations of 0.05, 0.3, 0.5, 0.75 and 1.0 mM Leucine by pipetting 5, 30, 50, 75 and 100 µL to new separate tubes and adding of 1 mM HCl up to 10 mL. These standards were included in each run of analyzed milk samples, in order to create a standard curve.

3.2.3 Analytical procedure

On the day before analysis, samples of skim milk were retrieved from the freezer (-20 °C) and placed in a 4 °C fridge to be thawed overnight. For each analyzed batch, a control sample of supernatant from defatted commercial milk (Arla, Sweden) was included. During the laboratory work, the samples were kept on ice.

3.2.3.1 Protein precipitation

From each sample to be analyzed, 0.5 mL of defatted milk was transferred to a 1.5 mL micro tube (Sarstedt, Nümbrecht, Germany) and mixed with an equal amount of TCA. The samples were vortexed and then placed on ice for 30 minutes to allow precipitation. Following that, the samples were centrifuged (20 min, 160 000 rpm, 4 °C, Himac CT 15RE, Hitachi Koki Co., Ltd.). After the centrifugation, 0.5 mL of the supernatant was carefully pipetted to an empty micro tube without disturbing the pellet. Before analyses, the samples were once again kept on ice.

3.2.3.2 Fluorescence assay

Tetraborate (600 µL) was pipetted to a 1.5 mL microtube, followed by the addition of supernatant (20 µL) from the protein precipitation step. Lastly, fluorescamine (200 µL) was added and the tube was rapidly mixed by vortexing. Each sample was analyzed in triplicate in a 96 micro well plate (Sarstedt, Nümbrecht, Germany) where 200 µL of the final solution was pipetted to each well. Furthermore, triplicates of blank samples (i.e. only buffer and fluorescamine), leucine standards and control milk samples were included in each micro plate. The fluorescence released was determined with a Luminescence spectrometer (Perkin Elmer LS55) after excitation at 390 nm and emission at 480 nm. Every plate was measured 25 minutes after addition of fluorescamine.

3.2.4 Pilot study

A pilot study was performed prior to the adequate investigation, in order to determine whether there were differences in proteolytic activity between whole milk and skim milk. Non homogenised, nor defatted milk from Arla (Sweden) was used in the study, where 13 samples of whole milk and 13 samples of skim milk were included. The latter was prepared to skim milk by centrifugation for 10 min at 400 rpm (Sorvall Super T21, Sorvall Products L.P., Newton, Connecticut, USA), followed by gentle removal of the cream with a cotton stick. Proteolytic activity was measured as previously described.

3.2.5 Statistical analysis and calculations

The fluorescence intensity data was corrected regarding the values from the blank samples; meaning that, the mean blank value was subtracted from the total fluorescence value in each well containing a milk sample. Furthermore, the results used for statistical calculations for each sample is a mean of three fluorescence readings,

since every sample was analyzed in triplicate in the micro well plate. If the standard deviation between the triplicates was >2 Leuc.eq., the sample was re-analyzed.

Total proteolysis is expressed as Leuc.eq. by reference to the standard curve, calculated according to:

$$Leuc.eq. = \frac{(Fluorescencce - blank) - m}{k \times df}$$

where m is the y-intercept, k is the curve slope and the total dilution factor (df) is 82. A high level of confidence (R-sq. > 0,95) was required for every obtained regression.

Statistical analysis was performed using MINITAB 18 (Minitab, Inc., USA) to determine effects of plasmin, SCC, pH, total protein, breed, number of animals, management system, milking system and season. Analysis of variance in a linear model (ANOVA or regression analysis) was examined for one variable at a time. Parameters with significant correlation or differences between means were included as explanatory variables in the following general linear model, as were plasmin and somatic cells, per definition.

$$T.PROTEOL_i = \beta_0 + \beta_1 PLAS_i + \beta_2 SCC_i + \beta_3 pH_i + \beta_4 T.PROT_i + \beta_5 BREED_i + \beta_6 X_i + \varepsilon_i \quad (1.)$$

Where T.PROTEOL is total proteolytic activity (Leuc. Eq.), PLAS is plasmin activity (U/mL), SCC is somatic cell count (cells/mL), pH is pH values, T.PROT is total protein (%), BREED is a dummy variable of five breeds (SLB, Jersey, Mountain Breed, SRB and a mixture of SRB/SLB where both pure breeds are used for production), X symbolises possible control variables and e_i is the random residual effect.

Additionally, the general model was tested by adding of remaining control variables, in order to examine any possible effects on the degree of explanation (R-sq. adj.):

$$T.PROTEOL_i = \beta_0 + \beta_1 PLAS_i + \beta_2 SCC_i + \beta_3 pH_i + \beta_4 T.PROT_i + \beta_5 BREED_i + \beta_6 NUMB_i + \varepsilon_i \quad (2.)$$

$$T.PROTEOL_i = \beta_0 + \beta_1 PLAS_i + \beta_2 SCC_i + \beta_3 pH_i + \beta_4 T.PROT_i + \beta_5 BREED_i + \beta_6 NUMB_i + \beta_7 MGMT_i + \varepsilon_i \quad (3.)$$

$$T.PROTEOL_i = \beta_0 + \beta_1 PLAS_i + \beta_2 SCC_i + \beta_3 pH_i + \beta_4 T.PROT_i + \beta_5 BREED_i + \beta_6 NUMB_i + \beta_7 MGMT_i + \beta_8 M.SYS_i + \varepsilon_i \quad (4.)$$

$$T.PROTEOL_i = \beta_0 + \beta_1 PLAS_i + \beta_2 SCC_i + \beta_3 pH_i + \beta_4 T.PROT_i + \beta_5 BREED_i + \beta_6 NUMB_i + \beta_7 MGMT_i + \beta_8 M.SYS_i + \beta_9 SEAS_i + \varepsilon_i \quad (5.)$$

Where NUMB is the number of animals used for production on each of the 18 farms, MGMT is a dummy variable for what housing system that is practiced, i.e. tied- or loose housing, M.SYS is yet another dummy variable for two different milking systems (AMS or CMS), as is SEAS which is referring to three different trials, where the sampling was repeated at different months (Nov-17, Feb-18 and Sep-18) during a period of ten months. For all dummy variables included, one group was excluded to avoid multicollinearity.

Total bacteria count was not included in any model, since not all samples were analysed for TBC. Thus, the effect of TBC was only examined by linear correlation with total proteolysis (n = 104).

Statistical significance was declared as *= p<0,05; **= p<0,01 and ***= p<0,001.

3.3 SDS PAGE Electrophoresis

In this thesis, eight selected milk samples were analyzed by SDS PAGE in a gradient gel that optimizes separation of a broad range of proteins at different MW.

In average, Jersey had the highest measured proteolysis, while SRB had the lowest proteolysis (measured by the fluorescamine method). Two samples from each of the two breeds were analyzed by SDS PAGE, the highest and the lowest within the breed. In addition, four samples that were highest or lowest in total proteolytic activity, when all 148 milk samples were taken into consideration, were analyzed to investigate if there were differences in the breakdown pattern that could be visualized by SDS PAGE.

The milk samples were diluted to an equal protein concentration prior to SDS treatment; hence the same amount of protein was loaded from each sample and the relationship between the caseins in particular, may be compared between the samples. A stain-free gel was used, which means that trihalo compounds (2,2,2-Trichloroethanol) are included in the gel (Ladner *et al.,* 2004). These form fluorescent complexes with tryptophan residues which can be visualized in an imaging system.

3.3.1 Buffer and sample preparations

A sample buffer was prepared by 270 µL of 4 x Laemmli Sample Buffer (250 mM Tris-HCl, 4 % LDS, 40 % (w/v) glycerol, 0.02 % bromophenol blue, ready made by Bio-Rad, Hercules, CA, USA) with the addition of 30 µL BME (Merck Schuchardt OHG, Hohenbrunn, Germany, 60-24-2).

The skim milk samples were diluted to an equal protein concentration of 3.3 % – dilution series were based on the individual protein content in each sample. All milk samples were further diluted to a relationship of 1:40 by mixing 2.5 µL milk with 97.5 µL Milli Q. Lastly, 20 µL of each milk was mixed with an equal volume of sample buffer.

Purified standard solutions of single milk proteins α-LA (1µg/µL), β-LG (2 µg/µL), α-CN (3 µg/µL),
β-CN (3 µg/µL), κ-CN (3 µg/µL), LF (2 µg/µL) and BSA (2 µg/µL) were used to create a standard reference mixture; 2 µL of each stock was mixed together in a 1.5 µL microtube with 28 µL of sample buffer.

Finally, all tubes were rapidly vortexed, followed by incubation in a 95 °C heat element for 5 minutes.

3.3.2 Electrophoresis and gel scanning

The electrophoresis was performed in a Bio-Rad Mini-PROTEAN Tetra Cell. The gel cassette (Mini-PROTEAN TGX Stain-Free Precast 4-20 % gel, 456-8093, Bio-Rad) was attached against the gasket of the electrode assembly, creating the inner chamber. The chamber was lowered into the electrophoresis tank and Tris/Glycine/SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1 % (w/v) SDS ready made by Bio-Rad, 161-0772) was poured to cover the wells of the gel in the inner chamber and above the platinum wire in the outer tank. The wells were loaded with 10 µL of each sample by the use of a micropipette. One lane was loaded with 10 µL molecular weight standard (PPPS, Precision Plus Protein™ Standards, Unstained, 161-0363, Bio-Rad) and one lane was loaded with 10 µL purified milk protein standards, as a reference. The tank was connected to power and gel electrophoresis was run at 200 V, 3 A, 60 W for approximately 40 minutes (until the blue dye front had reached the bottom of the gel).

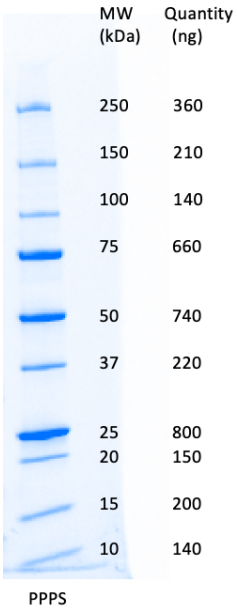

Figure 1. Protein amount in nanogram (ng), when 10 µl Precision Plus Protein Standard (PPPS) was loaded on the gel.

After completed electrophoresis, the gel was gently placed on a tray and image analysis was performed during 5 minutes in a Gel Doc™ EZ Imager (Bio-Rad) with the software Image Lab™ (Version 6.0.0, Bio-Rad). The amount of each protein in the standard solution was provided from Bio-Rad (Figure 1). By knowing the quantity in the PPPS lane, protein quantification of the milk samples can be calculated by use of the software tool, where the estimated amount of milk protein in each band is based on the calibration curve received from the intensity of the bands in the PPPS lane (Figure 2).

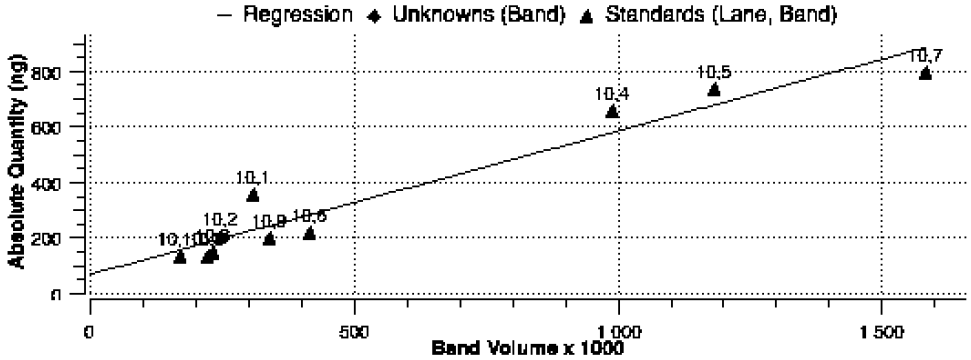

Figure 2. Linearity and intensity of Precision Plus Protein Standard (PPPS), for which sample intensity may be compared to, by the software tool.

4 Results

4.1 Pilot study

There was no significant difference (P = 0.159) between means of whole milk, 24.97 mM Leuc.eq. and skim milk, 26.57 mM Leuc.eq (Figure 3).

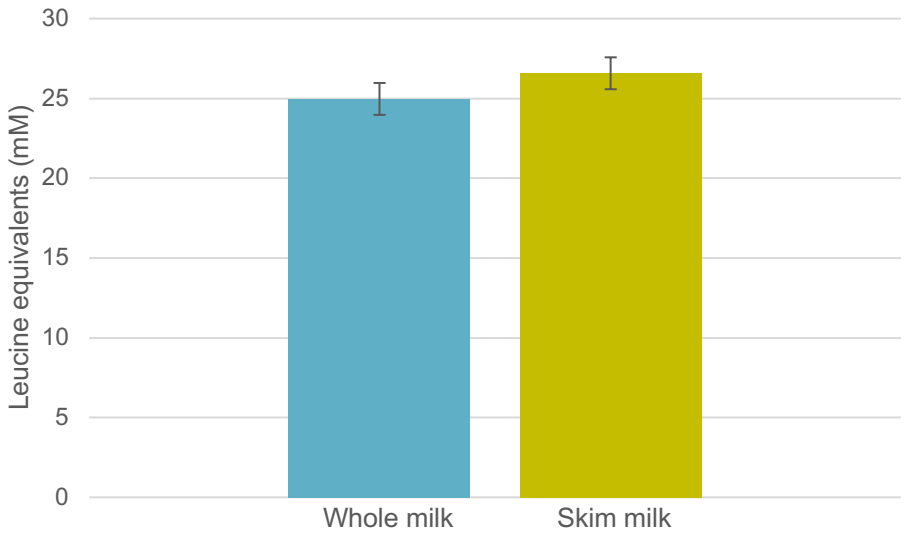

Figure 3. Mean values of proteolytic activity (mM Leuc.Eq.) of whole milk (n = 13) and skim milk (n = 13). Standard deviation is indicated.

4.2 Total proteolysis

Descriptive statistics of total proteolysis analyzed in this assay, together with data received from Eurofins Stein Laboratory and Kahled (2019) are presented in Table 4. One observation of plasmin activity was an assumed error, with a value of 83.67 U/mL compared to the second highest of 8.33 U/mL, thus it was excluded.

Table 4. *Descriptive statistics of analysed milk quality traits with mean value ($\bar{x}$), standard deviation (SD), minimum and maximum value*

Variable	$\bar{x}$	SD	min	max
T. PROTEOL[1]	25.3	4.81	14.0	45.3
PLAS[2]	4.07	1.26	1.67	8.33
T. PROT[3]	3.63	0.264	3.25	4.64
pH[4]	6.67	0.0473	6.54	6.77
SCC[5]	184	85.5	46.0	510
TBC[6]	8.40	6.61	3.00	34.0

[1] T.PROTEOL, Total proteolysis (mM Leuc. Eq.), n = 148.
[2] PLAS, Plasmin (U/mL), analysed by Khaled (2019), n = 147.
[3] T.PROT, Total protein (%), analysed by Eurofins Stein Laboratory AB, n = 148.
4 pH, pH values, analysed at SLU.
[5] SCC, Somatic cell count (x 10^3 cells/mL), analysed by Eurofins Stein Laboratory AB, n = 148.
[6] TBC, Total bacteria count (x 10^3 CFU/mL), analysed by Eurofins Stein Laboratory AB, n = 104.

Table 5 presents Pearson correlation coefficients, where total proteolysis was significantly correlated to both total protein content and pH, with coefficients of 0.3 and 0.167 respectively.

Table 5. *Pearson correlation coefficients for milk quality traits on all observations (n = 147)*

	T.PROTEOL[1]	T. PROT[2]	SCC[3]	pH[4]
T.PROT.	0.300***			
SCC	0.094	0.247**		
pH	0.167**	0.179*	0.125	
PLAS[5]	0.146	0.009	0.010	0.186*

[1] T.PROTEOL, Total proteolysis (mM Leuc. Eq.).
[2] T.PROT, Total protein (%), analysed by Eurofins Stein Laboratory AB.
[3] SCC, somatic cell count (x 10^3 cells/mL), analysed by Eurofins Stein Laboratory AB.
[4] pH, pH values, analysed at SLU.
[5] PLAS, Plasmin (U/mL), analyzed by Khaled (2019).
Significance levels are: *p <0.05, **p <0.01, ***p <0.001.

Neither plasmin (Figure 4) nor SCC (Figure 5) were significantly correlated to total proteolysis when analysed in a regression; p-values were 0.152 and 0.258 respectively.

The highest SCC (cells/mL) was 510 000, yet 88.5 % were below 300 000 and the average value was 184 000. Average SCC (cells/mL) within the breeds were 176 000 for SLB, 269 000 for Jersey, 165 000 for MB, 176 000 for SRB and 198 000 for the group with a mixture of the breeds SRB and SLB.

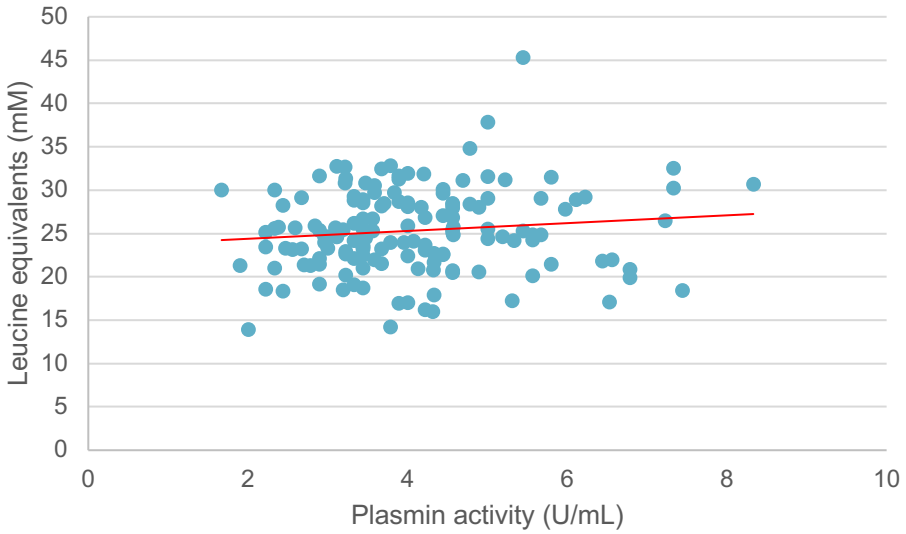

Figure 4. Regression analysis of correlation between total proteolysis and plasmin (PL) (n = 147).

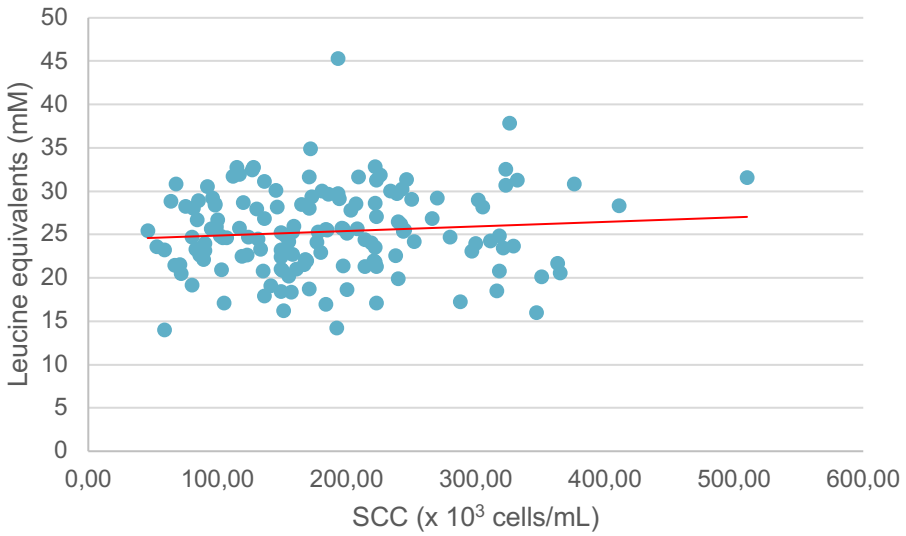

Figure 5. Regression analysis of correlation between total proteolysis and somatic cell count (SCC) (n = 148).

Figure 6 and 7 illustrate the regressions for pH and protein content, in relation to proteolytic activity. Both parameters were positively correlated to total proteolysis; milk pH with P<0.05 and milk protein content with a t-value of 3.8 (P <0.001), R-sq of 9 % and a β-value of 5.46. Mean protein values within the breeds were 3.55 % for SLB, 4.55 % for Jersey, 3.58 % for MB, 3.63 % for SRB and 3.61 % for SRB/SLB, thus it can be concluded that the upper right cluster belong to milk samples retrieved from Jersey cows.

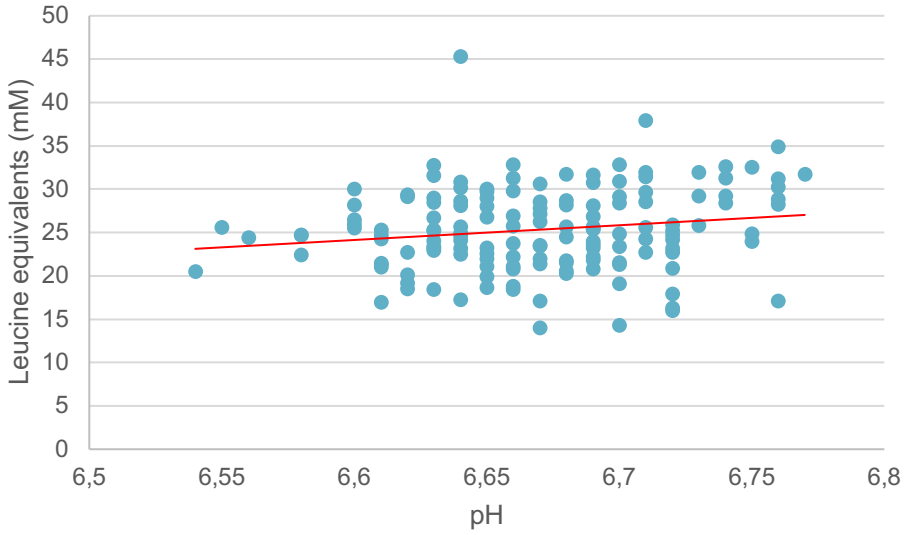

Figure 6. Regression analysis of correlation between total proteolysis and pH (n = 148).

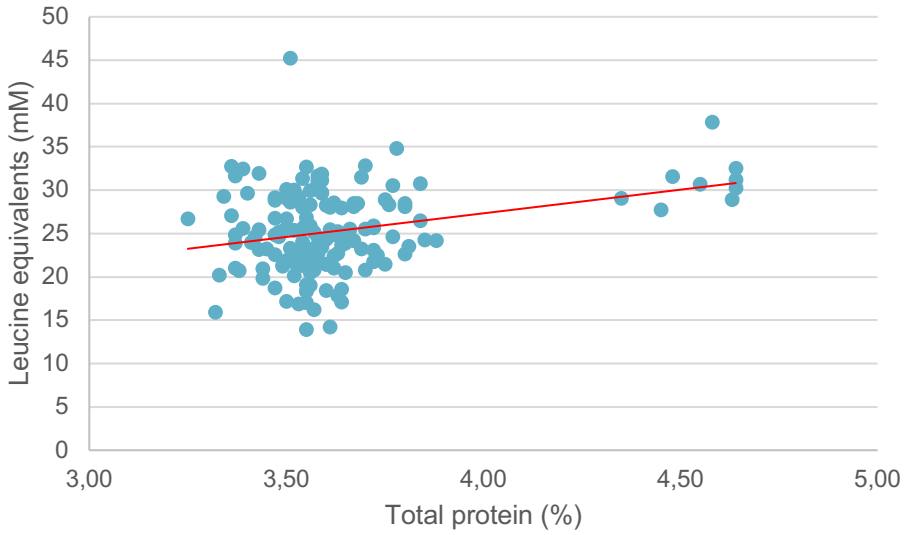

Figure 7. Regression analysis of correlation between total proteolysis and total protein (n = 148).

There were differences between the breeds (P = 0.003), which is illustrated in Figure 8. Milk samples from Jersey (breed nr 2) were significantly higher in proteolytic activity compared to the other breeds. Mean values for SLB, Jersey, MB, SRB and SRB/SLB were 25, 31, 24, 24 and 26 Leuc.Eq. respectively. Thus, Jersey cows had 25.7 % higher proteolytic activity compared to the average of 24.8 Leuc.eq. for the other breeds. Number of animals on the farm had no significant effect on proteolysis (P = 0.154, Figure 9).

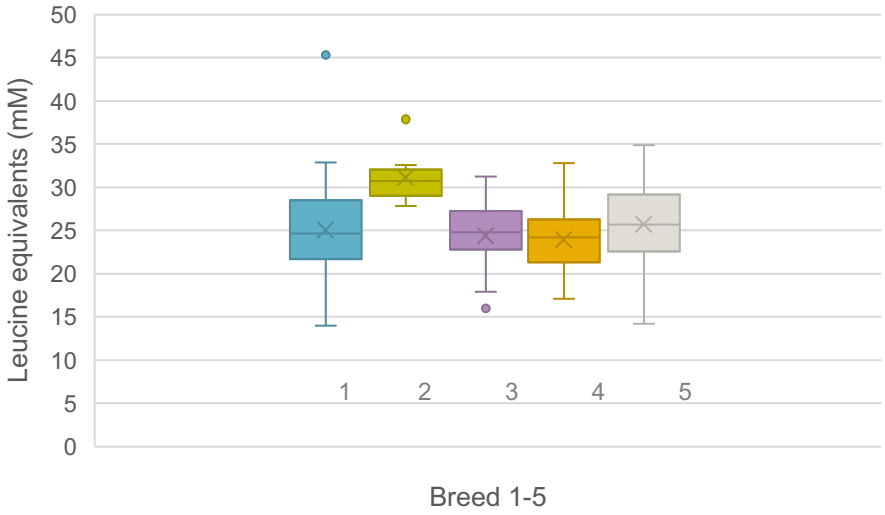

Figure 8. Analysis of difference between breed means, where breed nr 1 is SLB (n = 81), nr 2 is Jersey (n = 9), nr 3 is Mountain Breed (n = 18), nr 4 is SRB (n = 13) and nr 5 is SRB/SLB (n = 27).

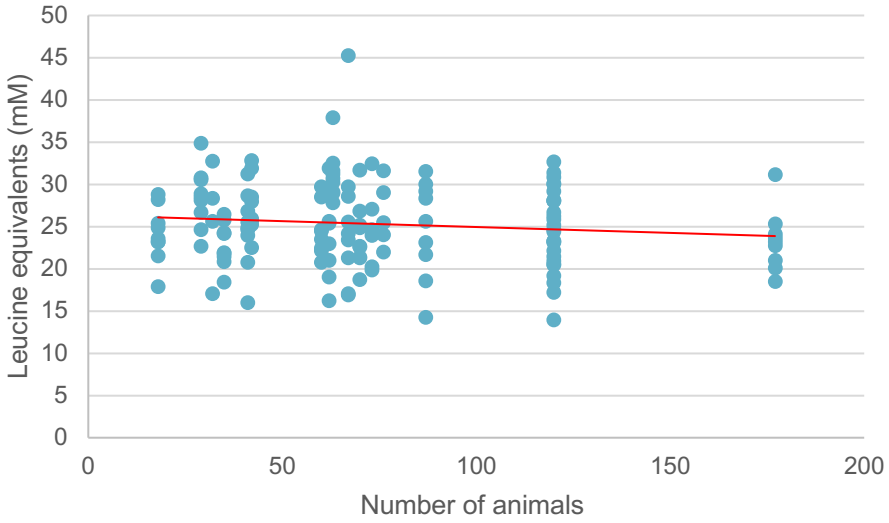

Figure 9. Regression analysis of correlation between total proteolysis and number of animals on the farm (n = 148).

Figure 10 and 11 illustrates the differences between means of tied cows compared to loose housed cows, as well as AMS compared to CMS. Milk from tied cows had 10.6 % higher proteolytic activity compared to loose housed cows (P = 0.002). However, there was no difference between the groups of different milking systems (P = 0.209).

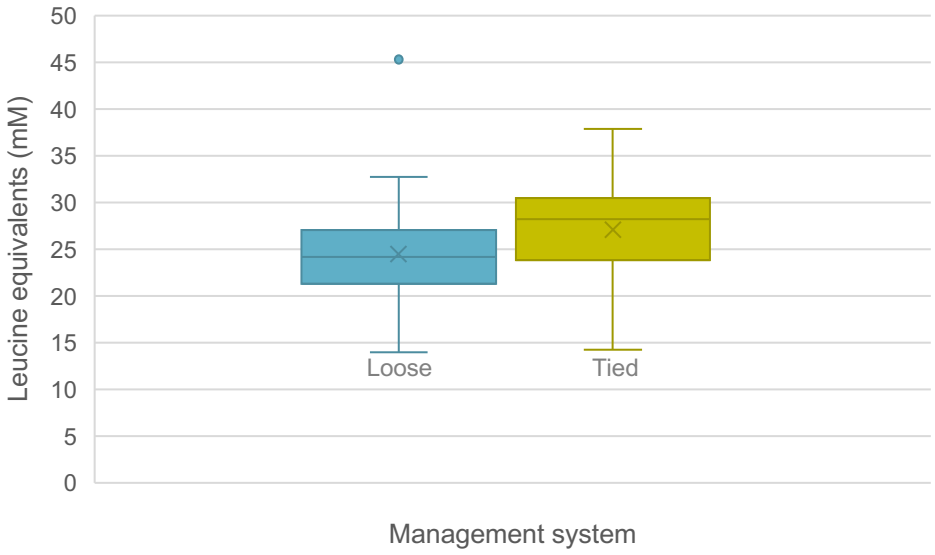

Figure 10. Analysis of difference between means, due to tied (n = 48) or loose housing (n = 100).

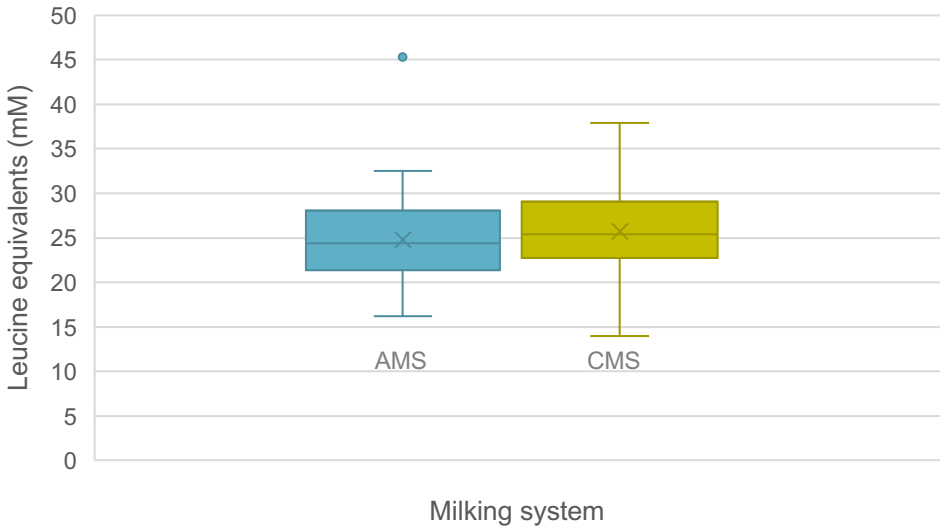

Figure 11. Analysis of difference between means when comparing Automatic milking system, AMS (n = 65) and Conventional milking system, CMS (n = 83).

Mean values of Leuc.eq. for samples collected during different times of the year were 25.8 for season 1 (Nov-17), 25.0 for season 2 (Feb-18) and 25.2 for season 3 (Sep-18). There was no significant difference between the trial periods (P = 0.71), as illustrated in Figure 12.

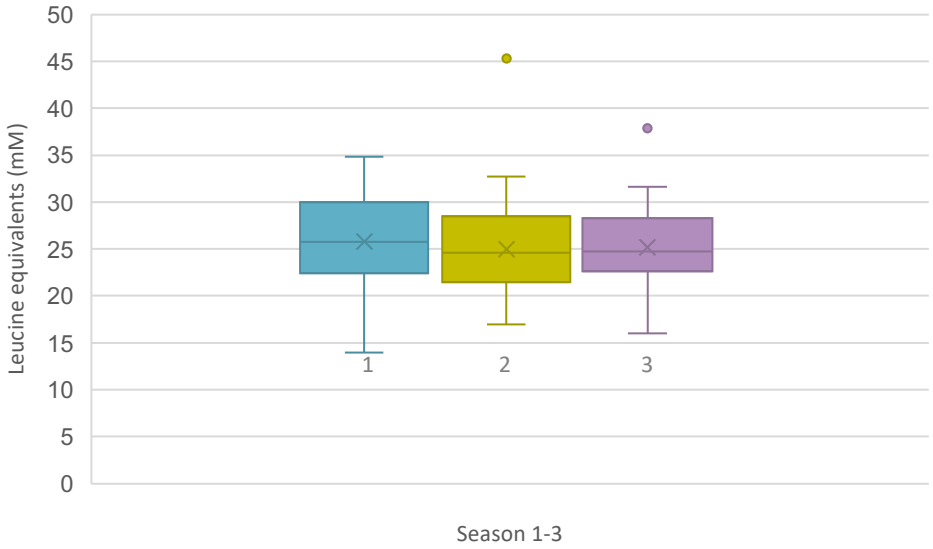

Figure 12. Analysis of differences between trial means, where season nr 1 is Nov-17 (n = 49), nr 2 is Feb-18 (n = 47) and nr 3 is Sep-18 (n = 52).

A regression analysis for correlation between TBC and proteolysis was performed for the 104 samples that had been analysed for bacteria counts (Figure 13). There was no significant correlation (P = 0.46) between the two parameters. The highest bacteria count was 34 000 CFU/mL, yet average value was 8.40 and 78 % of the milk samples were ≤10 000 CFU/mL.

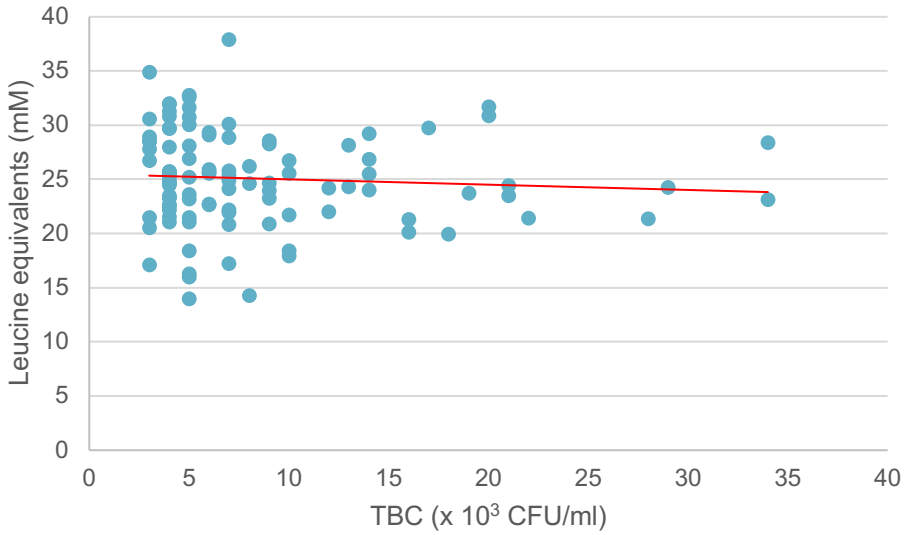

Figure 13. Regression analysis of correlation between total proteolysis and total bacteria count (TBC) (n=104).

Table 6 summaries the multivariable regression analysis for the general model (1.) as well as models 2-5, where all remaining control variables are tested. Model 3 gave the highest degree of explanation of variance, with an R-sq. (adj.) of 12.16 %. No variable had a significant effect on proteolysis in model 3, nor in any other model.

Since breed 2 (Jersey) differed significantly in relation to the other breeds in the analysis of difference between the means (Figure 8), Jersey was chosen as the excluded dummy variable. In all models, the β-value was negative for all breeds, in comparison to Jersey, with p-values between $0.141<0.568$.

The t-value for plasmin in model 3 was 1.33, for SCC 0.67 and for protein 0.23. Management, pH and number of animals all had t-values closer to the significant confidence level (± 0.96), compared to the previous mentioned variables, with values of -1.53, 1.57 and -1.5 respectively.

Table 6. *Summary of multivariable regression analysis with all control variables tested*

	Model 1				Model 2				Model 3				Model 4				Model 5			
Variable	***β***	**SE**	**t-val.**	**p-val.**	***β***	**SE**	**t-val.**	**p-val.**	***β***	**SE**	**t-val.**	**p-val.**	***β***	**SE**	**t-val.**	**p-val.**	***β***	**SE**	**t-val.**	**p-val**
PLAS[1]	0.08	0.06	1.42	0.16	0.07	0.06	1.28	0.20	0.08	0.06	1.33	0.19	0.08	0.06	1.32	0.19	0.08	0.06	1.30	0.19
SCC[2]	-0.00	0.01	-0.09	0.93	0.00	0.01	0.39	0.70	0.00	0.01	0.67	0.51	0.00	0.01	0.67	0.50	0.00	0.01	0.67	0.50
pH	13.61	9.05	1.50	0.14	13.75	8.95	1.54	0.13	13.94	8.91	1.57	0.12	14.01	9.00	1.56	0.12	13.40	10.30	1.30	0.20
T.PROT[3]	3.19	3.24	0.98	0.33	2.19	3.25	0.68	0.50	0.79	3.36	0.23	0.82	0.76	3.40	0.22	0.82	0.69	3.49	0.20	0.84
BREED[4] **1**	-2.14	3.68	-0.58	0.56	-2.18	3.64	-0.60	0.55	-2.07	3.63	-0.57	0.57	-2.09	3.65	-0.57	0.57	-2.16	3.77	-0.57	0.57
BREED[4] **3**	-3.52	3.69	-0.95	0.34	-5.10	3.74	-1.36	0.18	-5.28	3.72	-1.42	0.16	-5.35	3.85	-1.39	0.17	-5.40	3.93	-1.37	0.17
BREED[4] **4**	-4.12	3.60	-1.14	0.26	-5.54	3.63	-1.52	0.13	-5.36	3.62	-1.48	0.14	-5.37	3.64	-1.48	0.14	-5.45	3.76	-1.45	0.15
BREED[4] **5**	-1.92	3.53	-0.54	0.59	-2.73	3.52	-0.78	0.44	-3.36	3.53	-0.95	0.34	-3.38	3.55	-0.95	0.34	-3.44	3.65	-0.94	0.35
NUMB[5]					-0.02	0.01	-2.00	0.05	-0.02	0.01	-1.50	0.14	-0.02	0.01	-1.41	0.16	-0.02	0.01	-1.40	0.16
MGMT[6] **1**									-1.72	1.12	-1.53	0.13	-1.66	1.39	-1.19	0.24	-1.66	1.40	-1.19	0.24
M.SYS[7] **1**													-0.08	1.12	-0.07	0.95	-0.08	1.13	-0.07	0.95
SEAS[8] **1**																	0.15	1.04	0.14	0.89
SEAS[8] **2**																	0.04	0.95	0.05	0.96
R-sq		14.31%				16.73%				18.14%				18.14%				18.15%		
R-sq(adj)		9.37%				11.30%				12.16%				11.52%				10.21%		

[1] PLAS, Plasmin; [2] SCC, somatic cell count; [3] T.PROT, Total protein; [4] BREED 1 = SLB, 2 = Jersey, 3 = Mountain Breed, 4 = SRB, 5 = SRB/SLB; [5] NUMB, Number of animals; [6] MGMT, Management 1 = loose, 2 = tied; [7] M.SYS, Milking system 1 = AMS, 2 = CMS; [8] SEAS, Season 1 = 17 nov, 2 = 18 feb, 3 = 18 sep, SE = Standard Error.

4.3 SDS PAGE Electrophoresis

Eight samples were analyzed by SDS PAGE in an attempt to compare the protein profiles and quantify the caseins.

Samples 1 and 2 were from Jersey and samples 3 and 4 were from SRB (Figure 14). Samples 5 and 6 were highest in total proteolysis, of all 148 samples, and originated from SRB/SLB and SLB (sample 5 is the third highest value, since one of the Jersey samples was the second highest of all samples), while 7 and 8 were lowest in total protein breakdown, measured as Leuc.eq. Samples 7 and 8 originated from SRB/SLB and MB respectively.

The protein profile appeared to be slightly different between the breeds, as can be seen in Figure 14. It may not be possible to determine which specific casein each band represents, yet the written CN variants arranged in the left side of the gel as reference protein mixture (RPM) shows the expected separation pattern, based on the CNs MW. Furthermore, when comparing samples 5-8, the casein bands for assumed α_{S2}- and β-CN in samples 7 and 8 were more heavily stained compared to samples 5 and 6, indicating a higher concentration of these proteins.

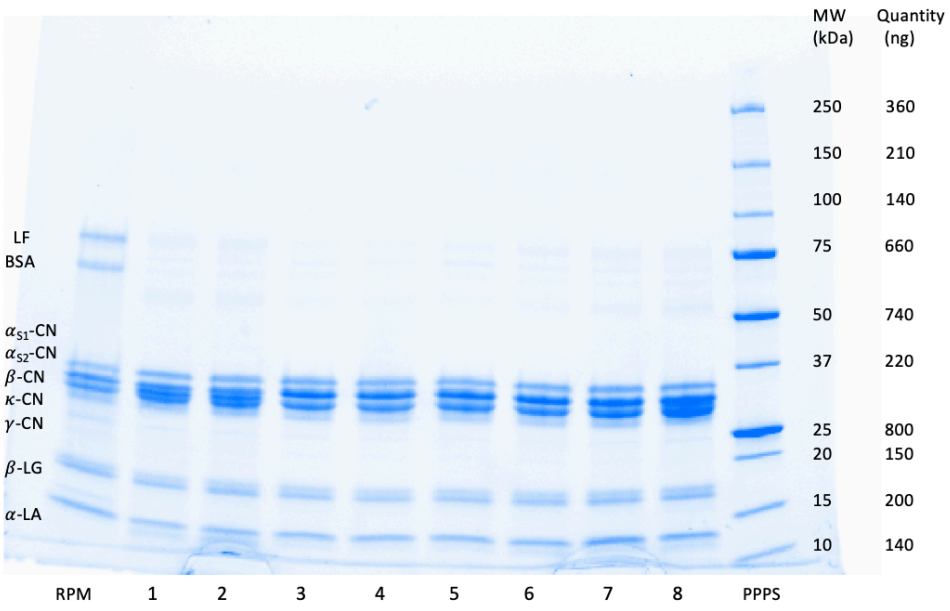

Figure 14. Protein patterns of SDS PAGE on eight samples; 1, Jersey highest; 2, Jersey lowest; 3, SRB highest; 4, SRB lowest; 5, third highest of all samples; 6, highest of all samples; 7, lowest of all samples; 8, second lowest of all samples. RPM, Reference Protein Mixture; PPPS, Precision Plus Protein Standard.

The software tool in Image Lab™ was used in an attempt to quantify the single caseins (α_{S1}-, α_{S2}-, β- and κ-CNs) (Table 7). However, the bands were not separated enough for the tool to make an accurate estimation; only three bands were quantified in lane 2 and 8, although when studying the gel image, it was obvious that there were at least four bands in the area of expected caseins.

Table 7. *Received values of casein (CN) quantity (ng) by use of the software tool in Image Lab™*

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
T.PROTEOL[1]	37.9	29.0	32.8	17.1	34.9	45.3	14.2	16.0
α_{S1}-CN	195.4	183.9	189.8	181.0	181.3	194.0	208.5	222.5
α_{S2}-CN	129.8	115.3	333.6	332.9	334.3	324.0	308.5	311.3
β -CN	138.9	146.1	238.0	249.6	259.5	236.2	237.4	225.1
κ -CN	70.1	-	84.4	83.3	84.0	80.9	85.3	-
Total CN	534.2	445.3	845.8	846.8	859.1	835.1	839.7	758.9

[1] T.PROTEOL, Total proteolysis (mM Leuc.Eq.)

5 Discussion

5.1 Pilot study

Considering the fact that most enzymes in milk enter through exocytosis and are associated to the global fat membrane (Fox & McSweeney, 2003), it is not unlikely that some proteases are linked to the encircling fat membranes. Because of this, total proteolytic activity was compared in whole milk and skim milk. Still, plasmin, the major indigenous protease, is transferred from the blood and through tight junctions before it associates to the milk caseins (Baer *et al.,* 1994). Furthermore, somatic cell proteases are mainly found in the whey (Kelly & McSweeney, 2003). This might be the reason for why there was no significant difference between the milks in this study, even though it is possible that some proteases are attached to the fat globules.

5.2 Total proteolysis in relation to included variables

Plasmin activity in raw milk is affected by many factors. Several studies have shown that the highest impact on level and activity of PL are individual aspects, such as health status, age and stage of lactation (Politis, 1989a; Nicholas *et al.,* 2002; Schaar, 1985). In this assay, bulk milk was analysed and no individual information was taken into consideration. However, SCC is an indicator of udder health status, that can have a direct impact on the PL activity by working as PAs (Verdi & Barbano, 1991; Politis, 1989b). Politis *et al.* (1989b) found no effect on PL <290 000 cells/mL. Average SCC in the analysed milk samples was 184 000 cells/mL, thus it is likely that somatic cell numbers were below the limit for where an influence on PL can be expected, and the majority of the PL fluctuations may be explained by individual variation. Nevertheless, if PL was accountable for most of the proteolysis, and the methods of analyses were specific and accurately executed, total

proteolysis and PL should correlate. In this study, the correlation between plasmin and total proteolysis was not significant when this was analysed in a regression analysis (P=0.152), which indicates that other proteases are involved in the protein break down as well.

Somatic cells can also be accountable for release of several proteases and SCC has been positively correlated to total proteolysis at an average level of 230 000 cells/mL (Johansson *et al.,* 2017). That correlation was not seen in this assay; the p-value was 0.258. The highest SCC of all samples was indeed 510 000 cells/mL – yet, like mentioned, average SCC was 184 000 cells/mL. This clearly indicates a good udder health status among the cows in general. When Larsen *et al.* (2004) studied proteolytic activity in relation to somatic cells, the increased cell counts was an effect of induced mastitis. It has been pointed out that different leucocytes possess different enzymatic activity due to their immunological mechanisms, where PMNs (leucocytes that increase most during clinical mastitis) have the highest capacity to break down proteins (Le Roux *et al.,* 2003). Larsen *et al.* (2004) found no effect on proteolysis in milk from uninfected cows. The milk samples from Jersey cows in this study had an average of 269 000 cells/mL. Considering that >200 000 cells/mL is an indication of sub-clinical mastitis (Växa, 2019) it is not unlikely that the composition of leucocytes in these samples are somewhat towards an infection status. Still, no correlation between total proteolysis and SCC was seen in this study.

Both total proteolysis and plasmin activity has been positively correlated to pH (Humbert *et al.,* 1983; Abeni *et al.,* 2008). In this study, a connection between pH and total proteolysis was seen both with a Pearson correlation coefficient of 0.167 (P<0.01) and a regression analysis (P<0.05). Based on the fact that the stability of the casein micelle is affected by small fluctuations in pH, it is not an unreasonable speculation that susceptibility to enzyme activity might also vary, depending on the milk pH value. The protein content was also significantly correlated with total proteolysis (P<0.001), both in the Pearson correlation and the regression analysis. Jersey cows have a higher milk protein content compared to other breeds; in this study the average was 4.55 %, while average for the other breeds were between 3.55 - 3.63 %. As seen in Figure 7, there is a cluster in the upper right part of the figure that stands out with highest values in both protein and proteolysis. There were only 9 samples in this study that originated from Jerseys, thus it is obvious that these samples are responsible for this cluster. The Jersey cows also differed from the other breeds with 25.7 % higher total proteolysis (P=0.003). This raises the question whether it is the total protein content or other factors within the breed that affects the milk proteolytic activity. Schaar (1985) found the level of PP to be positively correlated to the CN content, but there was no difference between the breeds (SLB, SRB, Jersey and SRB/SLB crosses). However, cows with genotype BB for β-LG had a higher PP content compared to cows with genotype AA. The B allele for β-

LG is associated to a higher CN content (Hill, 1993; Wedholm *et al.,* 2006). Neither the concentration of total or specific caseins nor the genotypes are known in this study, but Van Eenennaam & Madrano (1991) found the B allele for β-LG to be more frequent for Jerseys, when compared to SLB.

Automatic milking systems are associated to higher milk bacteria counts (Svensk Mjölk, 2011). When milking systems were compared in this study, there was no difference between AMS or CMS (P=0.209). The total bacteria counts were low in the analysed milk samples, with an average of 8400 CFU/mL. This clearly suggests healthy cows and good praxis of hygiene routines on the farms. Since the milk samples have been stored at 4 °C for 48-72 h, it can be assumed that there is an ongoing shift towards a psychrotrophic flora. In general, psychrotrophs secrete extracellular proteases when the cell density reaches levels around 10^6 CFU/mL, thus it is not very surprising that neither TBC nor AMS was positively correlated to total proteolysis in the analysed samples. In theory, there might still be differences over the year, since the composition of the flora alters, and different species possess different proteolytic qualities. However, when bacteria counts are this low, the composition of species might not make a difference. Indeed, there was no difference between the seasonal trials in this study.

By contrast, tied or loose kept animals differed in relation to total proteolysis, where tied animals had 10.6 % higher activity (P=0.002). Of the 18 farms, 7 have tied stall systems. One of them is the farm with Jersey cows, thus it can be suspected that this might be the reason for the higher total proteolysis in tied stall systems, rather than the fact that the housing system makes a difference in this case.

5.3 Multivariable regression

In a multivariable regression, the fact that there is almost never a simple explanation for a correlation between two variables is taken into consideration. When several models were tested, model nr 3 gave the highest degree of explanation with an R-sq (adj.) of 12.16 % (presented in Table 6). It must be mentioned that this is still a low degree of explanation for the variation of total proteolysis in the milk samples. However, some results were interesting. As expected, the effect of tied stall system was no longer significant; most likely, the difference in the single comparison was attributed to the Jerseys in that group. In addition, the effect of total protein content was completely diminished (P=0.82), thus the β-value was indeed false when only protein content and proteolysis were compared. No variable was significantly correlated to total proteolysis, as is seen in the table, yet the β-value for all breeds remained negative compared to Jersey, no matter the model or which parameters that were included. This implies that something within the breed have an impact on

the proteolytic activity. Even though it is quite possible that different casein variants, casein content or other differences between the breeds due to genetic factors, affects the level of proteolysis, the nine samples of Jerseys in this assay all originate from one farm. It is not possible to come to any conclusions based on so few observations from only one farm when environmental aspects are of such high importance, yet the result may arouse interest for further investigation.

Somatic cell counts were even less correlated in the multivariable regression, β=0.00, yet the p-value for PL did not change as much (0.15 compared to 0.19 in model 3). This was also the case for pH and number of animals; there was no significant correlation, yet the trend remained in the multivariable comparison. Taken all information in consideration, it appears most likely that the number of somatic cells were below levels that noticeably affect proteolysis; thus, PL should be accountable for most of the protein break down in the milk. The fact that PL and total proteolysis were not significantly correlated opposes this theory. However, there will always be some uncertainty in all analytical measurements and many analysed samples are required to counteract that effect. From a statistical point of view, there are not many observations included in this assay. Furthermore, PL activity and total proteolysis are analysed by two different methods, thus this must be taken into account when the results are compared.

Unfortunately, TBC could not be included in this analysis, since bacteria counts were not known for all samples. Yet like mentioned before, in the milk samples that indeed were analysed, TBC was low compared to when an effect on proteolysis has been observed in other studies. Still, it cannot be declared for sure that TBC did not have any effect on proteolysis, because it was not included in the multivariable regression.

5.4 SDS PAGE Electrophoresis

The attempt to quantify the caseins by the use of the software tool is admitted being a failure in this analysis. Considering that all caseins are in the range between approximately 19-25 kDa, it is not surprising that they were separated very close to each other in this gel. The software program was not able to detect and estimate the intensity of all bands. Thus, this method might not be suitable for CN quantification. When it comes to the MW, it is generally agreed that the caseins do not migrate as expected in relation to their MW in SDS PAGE (Basch *et al.,* 1985). This was also the case in this assay; as seen in Figure 14, the caseins are all above 25 kDa, while they should migrate a bit further. It has also been determined that α_{S1}- and α_{S2}-CNs separate the other way around, compared to what would be expected when looking

at their MW – α_{S2}-CN is slightly heavier than α_{S1}-CN, thus it should migrate shorter than α_{S1}-CN, but that is generally not the case (Strange *et al.,* 1992). It has been speculated that the reason for this is due to differences in hydrodynamic size. Interestingly, it appears as this was not the case in this study. In general, α_{S1}- and β-CN are close to the same concentration around 35-40 % of the caseins in milk, while α_{S2}- and κ-CN are around 10-15 % of the caseins. This makes it difficult to determine with safety which band that correspond to which casein in the gel image, since the middle casein bands in all the lanes appeared to be more concentrated. Based on theory about the general casein concentrations, the two bands in the middle should then correspond to α_{S2}- and β-CN. If this is the case, then the α_{S1}- and α_{S2}-CNs did not separate as expected – they were indeed separated based on their MW.

Nevertheless, even if there is some confusion on the concentrations and casein identification, the image still provides information that complement the other analysis. When visually comparing the samples in lanes nr 5 and 6 to nr 7 and 8, it certainly looks like the caseins have been more extensively hydrolysed in the samples 5 and 6, which basically fortifies the confidence in the results given from the fluorescamine method. Judging by the visual comparison of the intensity, β-CN concentration seems to be lower in samples 5 and 6. This is a possible indication of PL to be the most active enzyme, since β-CN is the main substrate for PL.

Comparing the samples in lanes 1-4, where nr 1 and 2 are from Jerseys and 3 and 4 are from SRB, it is interesting that the separation pattern clearly differs between the breeds. Like mentioned, the specific casein concentrations are unknown in this study, yet the casein bands in the milk from Jerseys seem to be more evenly distributed, compared to all the other samples. It is hardly possible to speculate on whether this is connected to the proteolytic activity; however, it has previously been determined that Jerseys have a higher concentration of α_{S1}- and α_{S2}-CNs than SRB, in general (Gustavsson et al., 2014).

5.5 Conclusion

The objective of this study was to measure the total proteolysis in milk samples from 18 farms and investigate if the level of proteolysis was correlated to any environmental-, on farm or gross composition parameters. Based on results from other studies, the levels of bacteria and SCC in the analysed milk samples were probably below the limit for when a noticeable effect on total proteolysis can be noticed. The results of the statistics in this assay was in agreement with this assumption, since no correlation was seen between total proteolysis and TBC or SCC. There was a trend, yet not statistically significant, towards a correlation between total proteolysis and PL activity. The break down pattern in the SDS PAGE analysis possibly showed

primary breakdown of β-CN, which would support this theory. In addition, Jersey cows differed compared to the other breeds in all statistical models tested. Since there are different caseins variants due to genetic polymorphism and this has been seen to affect the processing milk qualities, the fact that the breeds differed in this study might arouse interest for future investigations. Still, only one of the 18 farms participating in this project had Jersey cows, thus it would be essential with more observations from other farms with this breed.

It must be mentioned that the degree of explanation was low in all statistical models – thus there was a high level of variance in total proteolysis that was not explained in this thesis.

Furthermore, it should also be pointed out that the analyses performed in this assay gives no information on qualities for further processing, as the storage conditions and pasteurization will affect the milk enzymes and microflora. In this assay, the level of proteolysis was measured momentarily in the range of 48-72 h, and no changes over time was taken into consideration. It cannot be speculated on whether a high or a low level of proteolysis in these samples would have a positive or negative impact on processing, since that has not been investigated in this study. However, understanding of how the proteolytic systems are affected by on farm factors may contribute to increased knowledge on how to control and optimize the raw milk quality, which is the first link towards high quality dairy products.

Acknowledgements

I wish to express my deepest gratitude to my supervisor Monika Johansson. Thank you for making me feel so welcome at the institution, guiding me in the laboratory and being incredibly supportive all the way. I have really enjoyed working with this thesis! Thank you Hasitha Priyashantha for interesting discussions and your time when helping me with the results. Good luck with your PhD! I would also like to thank my friend Stephanie for all the long dog walks and coffee talk, I am so glad to have shared this time with you! To my brother Richard - there are no words for my gratitude towards your assistance with the statistics. Finally, to my family - thank you for your patience all those hours I have been sitting at the kitchen table absentminded. You are my everything.

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