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Effekter av mjölkningssystem på plasmin- och plasminogenaktiviteten i tankmjölk från ko

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Abstract

Protein is an important component in various milk products and has a crucial role in the final quality. Excluding non-protein nitrogen, bovine milk contains about 3.3% protein whereas about 80% of this fraction consists of casein. There are four major members in the casein family: α_{s1}-casein, α_{s2}- casein, β-casein and κ-casein. Proteolytic enzymes are the cause of degradation of the economically important milk proteins. By that, proteases play a more significant role than any other group of enzymes in dairy technology. The proteolytic activity is mainly achieved through activation of the serum protease plasmin. Plasmin alters from its inactive proenzyme, plasminogen and causes a degradation of the protein, such as casein.

The aim of this study was to investigate if the type of milking system significantly affected the plasmin and plasminogen activity in bulk milk. Milk from automatic milking system (AMS) was compared with milk from conventional milking system (CMS). The proteolytic enzyme activity was examined by fluorescence measurement. Totally 108 bulk milk samples was included in the study, 52 and 56 from AMS and CMS farms, respectively. In addition to analysis regarding plasmin and plasminogen activity, casein content and milk features such as pH, fat, total protein and SCC were determined. Results showed significantly higher content of α_{s1}-casein in CM milk and α_{s2}- casein was significantly higher in AM milk. Fat and protein were significantly higher in CM milk, meanwhile results regarding pH and SCC showed higher amount in milk derived from AMS. Percentage of proteolytic enzyme activity was significant higher (PL: 17% and PG: 7%) in CMS milk in comparison to AMS milk, suggesting that quality of the protein fraction is better preserved in milk from AMS farms.

Sammanfattning

Protein är en betydelsefull komponent i olika mjölkprodukter och har en avgörande roll för dess kvalité. Undantaget icke proteinbundet kväve, innehåller komjölk ca 3.3% protein och ca 80% av den totala proteininnehållet består av kasein. Kaseinfraktionen består av α_{s1}-kasein, α_{s2}- kasein, β-kasein och κ-kasein. Proteolytiska enzymer är orsaken till nedbrytningen av det ekonomiskt viktiga proteinet. I och med detta spelar proteaserna en mer betydande roll än någon annan grupp av enzymer inom mejeriindustrin. Den proteolytiska aktiviteten uppnås främst genom aktivering av serumproteaset plasmin. Plasmin transformeras från dess inaktiva proenzyme, plasminogen, och orsakar därefter en nedbrytning av främst kasein.

Syftet med denna studie var att undersöka om olika typer av mjölkningssystem har någon signifikant inverkan på plasmin- och plasminogenaktiviteten i tankmjölk. Mjölk från automatiskt mjölkningssystem (AMS) jämfördes med mjölk från konventionellt mjölkningssystem (CMS). Den proteolytiska enzymaktiviteten undersöktes genom fluorescensmätning. Totalt 108 tankmjölksprover ingick i studien, 52 från AMS gårdar och 56 från gårdar med CMS. Utöver analys av plasmin- och plasminogenaktivitet, fastställdes andelen kaseininnehåll samt övriga mjölkkomponenter såsom pH, fett, protein och SCC. Resultat visade på en signifikant högre halt av αs_1-kasein i CM-mjölk medan andelen αs_2-kasein var signifikant högre i AM-mjölk. Fett och protein var signifikant högre i CM-mjölk, medan resultat gällande pH och SCC visade på en högre andel i mjölk som härrörde från AMS. Procentandel av proteolytisk enzymaktivitet (PL: 17% and PG: 7%) var signifikant högre i CM-mjölk i jämförelse med AMS mjölk, vilket tyder på att proteinets kvalitet bevaras bättre i mjölk från gårdar med AMS.

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Abbreviations

AM	Automatic milked
AMS	Automatic milking system
CE	Capillary electrophoresis
CM	Conventional milked
CMS	Conventional milking system
MS	Milking system
MF	Milking frequency
PAIs	Plasminogen activator inhibitors
PG	Plasminogen
PGA	Plasminogen activators
PL	Plasmin
PLIs	Plasmin inhibitors
SCC	Somatic cell count
α_{s1}-CN	α_{s1}-casein
α_{s2}-CN	α_{s2}-casein
β-CN	β-casein
κ-CN	κ-casein

1. Introduction

Dairy production has for decades been undergoing development and technical progress. Heads per herds are rising in numbers and milk tanks are becoming larger. The increasing herd sizes results in higher demands on technically advanced equipment and more automation of systems (Andersson, 2012). During the nineties the interest for the automatic milking system (AMS) aroused (de Koning, 2010) and over 8000 commercial farms in the world are using one or more AMS for their herds (de Koning, 2010). The AMS has gained widespread acceptance also in Sweden, with over 800 farms using this technique (Växa Sverige, 2013).

Although automatic milking uses the same milking principles as conventional milking, there are differences in milk quality and thus affecting the end products (Svennersten-Sjaunja and Pettersson, 2008). Milk quality includes both compositional and hygienic aspects (Svennersten-Sjaunja and Pettersson, 2008). There are many features influencing the variability in composition and hygienic quality of milk. Factors such as genetic, physiologic, pathologic and environmental factors are the main aspects affecting the composition and properties of milk. The breed, stage of lactation, age of the cow, estrous, gestation, mastitis, feed, climate but also milking method can all conduct to an increase in compositional variability (Walstra *et al*. 1999). In terms of SCC in the bulk milk with AMS, research has highlighted changes within milk quality at introduction of automatic management, especially with a significant fluctuation the first three months after transition (Rasmussen *et al*. 2002; Bennedsgaard *et al*. 2006). Elevated SCC has shown to contribute to an increase in proteolytic enzymes which in turn results in changed quality of the end product such as cheese (Bastain *et al.* 1991; Walstra *et al*. 1999). Also total bacterial count in bulk milk increases and milk quality failures almost doubles as the self-monitoring program included in the AMS is not always sufficient enough (Rasmussen *et al*. 2002; Svennersten-Sjaunja and Pettersson, 2008). The advantage with AMS is that the milking frequencies can be enhanced. Three milkings daily are estimated to increase milk yield by approximately 10-15% (Klei *et al*. 1997; Österman and Bertilsson, 2003; Stelwagen, 2002). The possibility with AMS to control frequency at different stages of lactation enables to a significant increase of total milk production, for example by enhancing milking frequency during early lactation (Hale *et al.* 2003; Dahl *et al.* 2004; Soberon *et al.* 2011). Depending on stage of lactation the increase of milking frequency alters not only yield but also milk components such as fat and protein (Soberon *et al*. 2011; Klungel *et al.* 2000). Percentage of fat and protein decreases with increasing milking frequency, but total yield enhances (Klungel *et al.* 2000).

Regarding other variables in milk composition, pH has proven to increase depending on which type of milking system (MS) is used. It has been shown that the pH was significantly higher in primiparous cows at farms with AMS then in farms with conventional milking system (CMS) (Abeni *et al.* 2008). The more or less voluntarily visits to the AMS results in variation in milking frequency and that milking interval per cow can vary with a range of 2.5 to over 3.0 milkings per day (de Koning, 2010). This irregularity in milking interval with AMS can be an explanation for the elevated pH due to a change in ionic equilibrium (Abeni *et al.* 2008).

However, there are just a few studies investigating if also proteolytic enzymes are significantly affected due to difference in milking system (MS) and milking frequency (MF) (Sorensen *et al.* 2001). Proteolytic enzymes are known as proteases and their activity is connected to the degradation of economically important milk proteins. By that, proteases play a more significant role than any other group of enzymes in dairy technology (Fox, 1981). The proteolytic activity is mainly achieved through activation of the serum protease plasmin. Plasmin alters from its inactive proenzyme, plasminogen and causes a degradation of milk protein, such as casein (Korycka-Dahl *et al*. 1983). Casein stands for the majority of the protein in bovine milk. The cheese yield is dependent on the casein quality (Walstra *et al*. 1999) whereas the plasmin concentration in the milk is linked to casein degradation with the reduction of cheese yield as a consequence.

Since there is an emerging demand on dairy products, the dairy industries' contribution becomes more important (IDF, 2013). The bovine milk production in 2013 was forecast to be 780 million tons worldwide (FAO, 2013) and FAO predicts that the demand for milk will grow over 1000 million tons in 2050 (IDF, 2013). In Sweden, 2013, 30% of all raw milk was used for cheese production and the yearly consumption of cream and processed cheese were 19.8 kg per capita (LRF, 2014). When 28% of the country's raw milk origin from farms with AMS, the influence on milk composition and quality due to type of MS, must be considered (Landin and Gyllenswärd, 2012).

1.1. Objective and hypothesis

The aim of this study was to investigate if the type of different milking systems significantly affected the plasmin and plasminogen activity in bulk milk. Milk collected from AMS was compared with milk collected from CMS and the plasmin and plasminogen activity in bulk milk was examined.The hypothesis is that:

- Plasmin and plasminogen activity will differ between milking systems.
- The casein content in bulk milk, primarily regarding β-casein, α_{s1}- and α_{s2}- casein will be lower in the milking system with higher proteolytic enzyme activity.
- Different milk components may be significant correlated with plasmin or plasminogen activity.

2. Literature review

2.1. Milk protein

Milk is a fluid consisting of an enormous quantity of components. The foremost constituents of milk are water, fat, protein, lactose, organic acids and minerals. Excluding non-protein nitrogen, bovine milk contains about 3.3% milk protein and about 80% of total protein content consists of casein. Chemically, casein is defined as milk protein precipitating at pH 4.6. The 20% of the remaining protein are referred as whey protein or milk serum protein and are still soluble at this pH (Walstra *et al*. 2006).

2.1.1. Caseins

There are four major proteins in the casein family: α_{s1}-casein, α_{s2}- casein, β-casein and κ-casein. α_{s2}- and β-casein constitutes approximately 34-38% of the total casein content, while the amount of α_{s1}-caseins and κ-casein is restricted to 10-15% (Fox *et al*. 2000). The characteristics of casein diverge from those of most proteins. Caseins are hydrophobic, with a fairly high negative charge, many prolines and limited cysteine residues (Ng-Kwai-Hang, 2011; Walstra *et al*. 2006). They form short lengths of α-helix and have small tertiary structure, which makes the caseins open and flexible. This facilitates the accessibility for degradation of the casein molecule by proteolytic enzymes. The α_{s1}-CN primary structure consists of 199 amino acid residues and eight phosphorylated serines. There are three hydrophobic regions, located at residues 1–44, 90–113, and 132–199. The polarity of residue sequence 41-80 is high due to seven phosphoserines, eight glutamates, and three aspartates. The α_{s2}-CN molecule consists of eight amino acids more than α_{s1}-CN. It has 10 prolines and is the casein with most phosphoserines and lysines. Among the casein, α_{s2}-CN is also shown to be the least hydrophobic, while β-CN is the most hydrophobic casein. The absences of cysteine and high proportions of prolines have a significant effect on the structure of β-CN. The N-terminal 21-recidue segment is strongly negatively charged at milk pH, while the remaining molecule is no net charged and is very hydrophobic. The amphipathic attribute, i.e. possession of both hydrophobic and hydrophilic properties, is the cause of its arrangement of micellar aggregates in the milk. In the casein family, κ-CN is the only protein that is glycosylated. The κ-CN molecules stabilize casein micelles against precipitation by calcium, due to its limited ability to bind Ca^{2+}. The amphipathic characteristics of κ-CN engenders to formation of micelles in the solution (Ng-Kwai-Hang, 2011).

2.1.2. Casein micelles

The heterogenic characteristics of caseins emerge from their interactions with each other, with other proteins and small ions. Due to calcium phosphate present in milk, the caseins exist as micelles (Ng-Kwai-Hang, 2011). The casein micelles are clusters of colloidal calcium phosphates and different casein proteins. The diameter varies between 50 to 500nm and the casein micelles are stable in aqueous solutions such as milk because of the hydrophilic surface layer consisting of κ-casein (Bomholt *et al*. 2011; Ng-Kwai-Hang, 2011). The distribution of κ-casein on the micellular surface has shown to be heterogeneous and this coverage enables a steric stabilization against larger particles such as other micelles. The

heterogeneity allows however access for molecules of smaller dimensions to integrate with the micelles (Dalgleish, 1998).

2.2. Milk proteolysis

By screening bovine mammary gland cDNA using northern blotting analysis, Berglund *et al.* (1995) detected that there was no expression of plasminogen (PG) in the mammary gland, but could observe an occurrence in bovine liver tissue. This in turn indicated that PG found in milk enters from the blood to the milk (Berglund *et al.* 1995). In agreement, analogy patterns between PG existing in blood and in milk were strongly confirmed. It has been shown that the PG sequence in milk is identical to the equivalent sequence of PG isolated from bovine blood (Benfeldt *et al*. 1995). Coagulation of blood is an important defence mechanism against loss of blood and reduces the risk of bacterial invasion (Bastian and Brown, 1996). Through an activation of the fibrinolytic system, blood cloths can be proteolytically dissolved. Plasmin (PL) plays an important role due to its capacity to cleave blood clotting protein, fibrin (Fox *et al.* 2000) and therefore also referred to as fibrinase or fibrinolysin (Bastian and Brown, 1996). Plasmin is converted from its inactive zymogen plasminogen. This alteration is modulated by plasminogen activators and indirectly by plasminogen activator inhibitors (Korycka-Dahl *et al*. 1983; Ismail and Neilsen, 2010). The plasminogen activators (PGA) are consisting of two types, tissue-type (t-PA) and urokinase-type (u-PA) (Bastian and Brown, 1996). The activation occurs when the Arg557-Ile558 bond in PG are cleaved when the milk is situated in the lumen of the mammary gland and also after milking, during storage (Schaar, 1985). The activity of PL and PA is regulated by plasmin inhibitors (PLIs) such as α_2-antiplasmin and plasminogen activator inhibitors (PAIs) (Grufferty and Fox, 1988; Ismail and Nielsen, 2010). In the absence of inhibitors, PG activator produces active PL, resulting in casein degradation (Bastian and Brown, 1996). All linked constituents in the fibrinolytic system are presented in Figure 1.

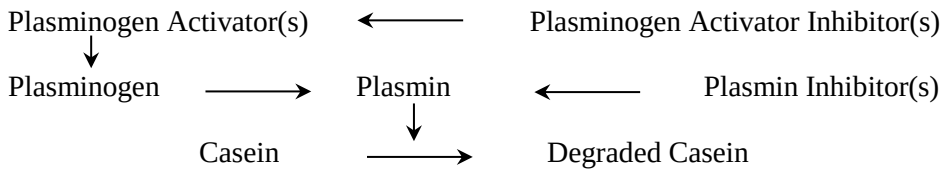

Figure 1. Schematic presentation of the plasmin system (Bastian and Brown, 1996).

Plasmin has an adversely effect on processing characteristics of bovine milk because the enzyme causes degradation of milk proteins. It has been proven to be a negative correlation between occurrence of plasmin and the quantity of α_s- and β-CN (Kelly, *et al*. 2006; Politis and Kwai Hang, 1989b). Primarily β-CN and α_{s2}-CN are degraded, but also α_{s1}-CN and κ-CN are hydrolyzed (Fox, 1981; Fox *et al.* 2000; Kohlmann *et al*. 1991; Richardson *et al*. 1981). Degradation of α_{s1}- and κ-CN are slower due to their higher resistance to plasmin hydrolysis (Fox *et al*. 2000). PL has affinity for lysine and arginine residues and preferentially cleaves Lys-X and Arg-X bonds in the caseins (Bastian and Brown, 1996). Cleavage of β-CN bonds occurs primarily at three sites and results in the formation of polypeptides, proteose peptone 5, proteose peptone 8-slow and proteose peptone 8-fast. Cleavage of α_{s2}-CN bonds by plasmin befalls at eight locations and products formed at this fission are thereby

approximately 14 peptides and three of them are potentially bitter. α_{s1}-CN are restricted degraded by PL resulting in a minor casein component, λ-CN. There are numerous sites for potential cleavage of κ-CN bonds, but κ-CN is said to be very resistant to PL and its products has not yet fully been identified (Fox *et al*. 2000).

2.3. Consequences of plasmin in milk

2.3.1. Negative effects

Manufacturing of cheese is fundamentally a dehydration process in which the fat and casein in milk is concentrated 6-10 fold depending on category of cheese. Traditionally the dehydration is achieved by an enzymatic or isoelectric coagulation of casein, or by a combination of acid and heat. Almost every ripened cheese is produced by rennet coagulation, i.e. by proteolysis and are said to be the most important biochemical part during the ripening of most cheeses varieties. Proteolysis also occurs in milk before coagulation and the cheese milk pre-manufacture are mainly caused by microbial and indigenous milk proteinases (Fox, 1989). High plasmin activity is linked to impaired coagulation features and degradation of caseins, resulting in reduced cheese yield and changes in functionality of milk protein (Srinivasan and Lucey, 2002). According to Okigbo *et al.* (1985) curd firmness is directly related to total casein in milk and it was suggested that PL might play a role in reducing the curd firmness of rennet-coagulated milk.

Moreover, in products such as pasteurized milk, ultra heat treated (UHT) milk and non-fat dry milk occurs also an undesirable sedimentation and gelation (Kohlmann *et al.* 1991). PL, PG and PGA have showed to be heat resistant and therefore survive pasteurization (Dongjin and Neilsen, 1993), while its inhibitors are heat labile (McSweeny, 2007). Kinetic data on heat inactivation of plasmin are shown not to be very consistent. The plasmin system in milk is very complex and the heat inactivation depends on the conditions applied as well as the performance of PG, PG- and PL- inhibitors. Heating time seems to be more important than temperature, especially heat treatment above 110°C. This heat resistance may be explained by either a high conformational stability or its molecular structure that readily unfolds; with the unfolded enzyme being resistant to subsequent degradative chemical reactions, which allows refolding of the protein back to an active enzyme when cooled down (Metwalli *et al*. 1998). When the cooking temperature elevates an inactivation of inhibitors occurs leading to an intensification of plasminogen and thereby an increase of plasminogen activity (Walstra *et al*. 2006). According to Andrews' (1983) measurements the breakdown of β-CN and α_{s1}-CN occurs faster in pasteurized milk in comparison to raw. In raw milk β-CN hydrolysis was marginally faster than the loss of α_{s1}-CN, while there was a more significant difference in pasteurized milk. Almost 90% of the β-CN was hydrolysed, whereas only 60% of the α_{s1}-CN were broken down, when stored at 37°C for 7 days (Andrews, 1983). A major limiting factor for the shelf life of milk that has been UHT treated is proteolysis of caseins. The reason may be due to residual heat-stable bacterial proteases, indigenous milk proteases or combinations of both (Datta and Deeth, 2003; Kelly and Foley, 1997). Such proteolysis can cause development of bitter off-flavours, precipitation and age gelation (Datta and Deeth, 2003; Walstra *et al*. 1999). PL has shown to be closely linked to age gelation of UHT (Kelly and Foley, 1997). The gel which occurs in milk that has been UHT treated is a three dimensional

protein matrix structured by whey proteins, chiefly β-lactoglobulin. The β-lactoglobulin denatures and interacts with casein, primarily with κ-CN. The proteinaceous linkages results in a β-lactoglobulin-κ-CN complex via disulphide bonds, due to the heat treatment. The formed complex aggregates and creates a gel (Datta and Deeth, 2001). There is a more susceptibility to gelation in UHT processed skim milk than in UHT whole milk. This phenomenon can be explained by the fact that the fat in whole milk impedes access of proteolytic enzymes to casein substrates. A suggestion is also that the higher proportion of denatured whey proteins not attached to the surface of the micelles in skim milk may be a cause for its reduced resistance to sedimentation (Chavan *et al*. 2011).

2.3.2. Positive effects

Depending on the extent of hydrolysis, the plasmin activity has been shown to improve the flavour and overall quality on some cheeses (Tieleman and Warthesen, 1991; Bastian *et al*. 1997), for instance Swiss and Cheddar cheese. The flavour compounds and texture modification are due to the proteolysis (Bastian *et al*. 1997) because of added starter enzymes, such as PL. In Swiss cheese, rennet has little or no contribution to proteolysis due to chymosin and most other coagulant enzymes are inactive at the high cooking temperature used during manufacturing (Bastian *et al*. 1997). This leaves bacterial proteinase-peptidase system in combination with PL to hydrolyze casein in the cheese (Garnot and Molle, 1987). After completed coagulation and pH corrected to desirable level, cheeses are brined and stored according to traditional processes (Bastian *et al.* 1991).

2.4. Factors influencing the activity of plasmin in milk

2.4.1. Milking systems

The definition of the term automatic milking system are defined as a system that automates functions of the milking process and management of the cows undertaken in CMS, by a combination of manual and robotic systems. Automatic milking systems emphasis on self-service manner and the cow's motivation to be milked (de Koning and Rodenburg, 2004). In contrast to CMS, where cows are retrieved usually twice a day to be milked, the AMS allows an increase in milking frequency and an adaption to the stage of lactation (Svennersten-Sjaunja and Pettersson, 2008). The interval between milking can be as short as 6 h (Hogeveen *et al.* 2001; Bruckmaier and Wellnitz, 2008). In an evaluation conducted by Abeni *et al.* (2008) effects as alterations in milk enzymes in different milking systems such as automatic milking and twice-daily conventional milking, were assessed. The study confirmed that both PL and total activity (PL + PG) were higher in CM than in AM milk. There was a reduced PL/PG ratio at four weeks of lactation in AM than CM milk, due to a reduced quantity of PL and greater amount of PG in the AM milk (Abeni *et al*. 2008; Stelwagen *et al.* 1994a; Stelwagen *et al.* 1994b). In agreement, Svennersten-Sjaunja *et al*. (2007) and Le Roux *et al.* (2003) concluded a decreased PL and PG-derived activity increased with milking frequency (MF), which was believed to be explained by an improved integrity of tight junctions. In addition, with an increased MF reduces also the time available for PG to be converted to PL (Sorensen *et al.* 2001).

2.4.2. Lactation number

According to Bastian *et al* (1991) the most important factor that influences the plasmin activity and percentage of plasmin is the lactation number. High activity of plasmin in milk is a result of an increased activation of plasminogen due to the age of the cow. Thus, elder cows have a higher level of plasminogen activation (Bastian *et al*. 1991). In a least squares analysis conducted by Politis *et al.* (1989b), the effect of lactation number on PL activity was examined. Lactation numbers were divided into five categories: 1, 2, 3, 4, ≥ 5. During the first three lactations the activity of PL was relatively low; 90 x 10^{-6}units/ml. During the fourth and beyond fifth lactation the PL activity concentration increased to an average of 110 and 138 x 10^{-6}units/ml. Even after statistically adjustment for stage of lactation, season and SCC, a significant association between PL activity in milk and lactation number could be confirmed (Politis and Kwai Hang, 1989b; Bastian *et al.* 1991).

2.4.3. Stage of lactation

Stage of lactation might affect the concentration of plasmin (Korycka-Dahl *et al*. 1983; Shaar, 1985). It has been shown that both plasmin and plasminogen increase as lactation progress and the levels are highest during latter part of lactation (Politis *et al*. 1989b, Politis *et al*. 1992). According to a study conducted by Baldi *et al.* (1995) there is a significant increase ($P < 0.05$) in PL activity during the fifth and seventh month of lactation in comparison with early lactation (Table 1). The ratio of PG and PL was decreasing until the fifth month, implying accelerated conversion of PG to PL. The reduction in PG/PL ratio with advancing lactation corresponds with the increase in activity of PG activators. Two mechanisms could elucidate the elevated levels of PL during the fifth month of lactation. One is increased concentration of PG, while the PG/PL ratio remained unaltered, due to PG is located in all body fluids, but not synthesized by epithelial cells in the mammary glands; thus would be an indication on increased epithelial permeability of the mammary glands. The second explanation is an enhanced conversion of PG to PL by the impact of PG activators. According to Baldi *et al*. (1995) are both of these mechanisms involved in the fluctuation of PL activity associated with advancing lactation, with a predominance of the second mechanism in the fifth and seventh months of lactation.

Table 1. *Alterations in activity of plasmin, plasminogen and plasminogen activator with advancing lactation (Baldi, et al. 1995)*

Changes in plasmin, plasminogen and plasminogen activator with advancing lactation					
Activity (units/ml)	*Month*				*SE*
	1	*3*	*5*	*7*	
Plasmin	5.46b	5.49b	17.04a	13.39a	2.55
Plasminogen	32.95b	30.66b	40.56a	32.28b	1.91
Plasminogen activator	206.95b	296.92b	445.92a	441.87a	46.37
PG/PL	6.03a	5.58a	2.38b	2.41b	0.75

Means followed by same symbol within the same row were not significantly different from each other (P <0.05)

Pursuant with Baldi *et al*. (1995), Bastian *et al.* (1991) also reports that stage of lactation has an influence on PL- and total enzyme activity. Primary an increase in percentage of PL can be observed during the last two months of lactation (Bastian *et al.* 1991), which is consistent with PG activation noticed during late lactation (Politis *et al*. 1989a; Politis and Kwai Hang, 1989b; Baldi *et al.* 1995).

2.4.4. Breed of cow

The activity of PL in milk has been observed in limited number of Holstein-Friesian cows in comparison to Jersey cows. The Holstein-Friesian and Jersey cows were subdivided into early and late lactation. Throughout the lactation the PL activity was consistently higher for the Holstein-Friesian cows (0.27-0.53mg/l) than for Jersey cows (0.15-0.37mg/l) (Richardson, 1983). In agreement, Schaar (1985) also concluded that PL activity differed between breeds, with highest activity in milk from Swedish Friesian and crosses (Swedish Friesian x Swedish Red and White). Lowest activity was measured in milk from Swedish Jersey. However, when the effect of the casein content was considered, the breed effect was eliminated. Consequently the breed differences were thus mainly due to difference in milk casein content, with the PL activity decreasing with increasing casein content (Schaar, 1985).

2.4.5. Season

The concentration of PL and PG has a seasonal fluctuation. During summer and fall plasmin is at its highest level and plasminogen is highest during fall and winter (Bastian *et al*. 1991). However, there was a strong influence of season on activities and yields of plasminogen and total enzyme, with highest activities in spring and summer (Nicholas *et al.* 2002). The opposite finding that was observed by Bastian *et al*. (1991) is explained by that they did not include cow management details in the calculations. Level of feeding, such as quantity and quality, can also influence the proteolytic activity and this can varies between seasons (Nicholas *et al*. 2002). According to Politis *et al.* (1989) could no independent relationship between PL and season be confirmed after statistical adjustments for milk yield, SCC, stage of lactation and lactation number.

2.4.6. Milk composition

It has been shown in the study conducted by Bastian *et al*. (1991) that neither pH, protein- nor fat content influences PL or PG activity. In this study the correlation coefficient between pH and PL activity was r = 0.22. However, both pH and whey proteins can instead have an impact on the kinetics of the PG-induced hydrolysis and additionally, thermal processing, mineral content and storage temperature has also an effect (Ismail and Nielsen, 2010). In agreement with Ismail and Nielsen (2010), Politis *et al*. (1989b) found a significant and positive correlation between PL activity and pH. There was also a significant correlation after adjustment for SCC, which indicates an independent relationship between PL and pH in bovine milk. In the study of Politis *et al.* (1989b) a negative correlation between PL and amount of α_s – and β-CN was found.

2.4.5. Mastitis

It has been reported that there is a correlation between SCC (> 300.000) and plasmin activity (Walstra *et al*. 1999). But according to studies conducted by Bastian *et al*. (1991) could no correlation between SCC and plasmin be observed. The correlation coefficient was of 0.42, and thus no relationship between SCC and increased plasmin activity. The reason for this was explained by the lacking availability to enough milk samples with high SCC (> 300.000). So there was no linear relationship between plasmin and SCC in the range of 100.000 to 300.000 somatic cells (Bastian *et al*. 1991). Whereas Politis *et al*. (1989b) reported a high correlation,

r = 0.62, with a more normal distribution of SCC. At higher cell numbers, the concentration of the active enzyme PL increases, due to somatic cells, particularly polymorphonuclear leucocytes contains PG activators (Considine *et al*. 2002; Lindmark, Måsson. 2002). In other words, health status of the udder has showed to have an effect on PG activity within milk somatic cells (Zachos *et al.* 1992). According to Politis *et al.* (1989a) an increase of SCC from less than 250.000 to more than 1.000.000 results in an intensification of PL and PG concentration from 0.18 to 0.37mg/l and from 0.85 to 1.48mg/l, respectively. The ratio of PG and PL decreased from 4.7 to 4.0, as SCC increased. In a subsequent work, where SCC increased from 0.29-1.3 million, plasmin activity increased from 107 to 230 10^{-6}U/ml (Politis *et al.*, 1989b). A study conducted by Saeman *et al*. (1988) showed that mastitis increases proteolytic activity in milk. This was confirmed by induction of mastitis to six Holstein cows and the plasmin activity was measured during pre-infection, infection and post-infection. Levels of PL were significantly higher during the infection and after treated mastitis the SCC had decreased, the proteolytic activity declined but not to its pre-infection values. Thus, detrimental effects on milk quality may continue even after elimination of intra-mammary infection and normal levels of SCC has been obtained (Seaman *et al.* 1988). This can be a cause why elderly cows have higher PL activity in their milk then young cows (Bastian and Brown, 1996). This was also observed by Politis *et al*. (1989b) who emphasized a correlation between elevated levels of SCC and increased activity of plasmin, due to the higher permeability of the epithelial layer of the alveoli in the mammary gland tissue.

3. Materials and methods

All analysis of milk samples was executed at the laboratory at the Uppsala BioCenter at the Swedish University of Agricultural Sciences, Department of Food Science.

3.1. Origin of milk samples

Samples of bulk milk were delivered by Eurofins Steins Laboratorium AB and each sample was obtained during late fall from different Arla and Grådö suppliers in the Mälardalen region. The selection of participators was done by Växa Sverige. The samples were divided in to two categories, bulk milk from farms with robotic milking system, AMS, and bulk milk from farms with CM cows. The number of samples that was delivered from Eurofins was 109 in total, 53 of these from farms with AMS and 56 from farms with CMS. However, only 108 samples could be analysed.

3.2. Milking procedure and milking equipment

Bulk milk samples received from dairy farms with AMS were using robotic system provided by DeLaval International AB, Tumba, Sweden or Lely International, Maassluis, Netherlands. In the AMS cows were milked > 2 times a day. Milk samples from farms with CMS were equipped with traditional parlour and the cows were milked twice a day.

3.3. Milk plasmin and plasminogen analysis

Each milk sample from AMS and CMS were analysed for both plasmin and plasminogen activity. Plasmin and plasminogen activity was measured by modified method from

(Korycha-Dhal *et al.* 1983). The result of PL and PG activity was established by fluorescence measurement.

3.3.1. Buffer preparation

Chemicals were obtained from Sigma-Aldrich Stockholm, Sweden, except where otherwise stated. Preparation of plasmin buffer was made according to given instructions. Calculation ($m = M_w \times c \times V$) of the amount of compounds needed for the buffer were made and weighed; 2.6g ε-amino-n-caproic acid, EACA (20mM), 8.4g Trizma buffer (53mM), 6.8g NaCl (117mM). The buffer components were added to half of the needed purified water (1000ml final volume) and thereafter pH (desirable pH; 7.4) was regulated by 2.5M NaOH. The enquired volume of plasmin buffer was to be sufficient to all samples.

3.3.2. Sample preparation

A manageable amount of milk samples (6-18) were defrosted overnight in a 4°C fridge and were put in a water bath at 45°C for 10 minutes to dissolve potential clusters of varied milk components. Each sample was convulsed in a vortex mixture (Vortex-Genie 2, Scientific industries, inc., U.S.) and 2ml of aliquot of milk samples in eppendorf safe lock tubes (Eppendorf, Germany) were placed once more in water bath for 15 minutes at the same temperature. After the samples had been cooled down the pH was checked two times with a pH/ION meter (ProLab 3000, SI Analytics, Xylem Inc.). Skim milk was prepared by centrifugation (Himac CT15RE, Hitachi Koki Co., Ltd.) at 15 000 revolutions per minute (rpm) at 4°C for 10 minutes and the cream was discarded. After the defatting, 0.5ml skim milk was incubated with 7.5ml buffer at room temperature for 2h to dissociate plasmin and plasminogen from the casein micelles and to enable their transfer to the buffer medium. The weight of the centrifugation tubes were checked so the weight of tube one was equally with tube two, tube three equal with tube four, tube five equal with tube six. After incubation the samples were ultra-centrifuged (LKB Ultrospin) with a RP55T angle rotor (12ml x 12) at 100 000 x g at 4°C for 1h to obtain the supernatant milk serum fraction. The supernatants (1.5ml) were stored in freezer at -20°C for later analysis of plasmin and plasminogen.

3.3.3. Fluorescence measurement

PL activity was measured in solution containing 150µl serum and 40µl chromogenic substrate CS-41(03) (pyroGlu-Phe-Lys-p-nitranilide hydroxychloride, Hypen BioMed, catalog nr. 229041) in Sarstedt 96 well plates. PG samples were complimented with 4.5µl urokinase (49.5 Plough U). Absorbance of PL and total activity was measured every third minute in 120 minutes continuously by a multi-mode microplate reader (FLUOstar Omega, BMG LABTECH, Germany). The change in absorbance ($\Delta A405/\Delta t$) was taken for the measurement of PL activity. PG activity was calculated as the difference between the total activity and PL activity. PL and activated-with-urokinase PG activities were expressed in the same units, with one unit being defined as the amount of enzymes that produces a ΔA_{405}^{1cm} of 0.001 in 1 minute at pH 7.4 and 37°C when p-nitroanilide (pNA) produced from CS-41(03) substrate were measured in the defined reaction mixture.

3.4. Milk protein analysis

Selected samples were analysed for protein content. The selection was based on PL activity. Five samples with high- respectively low PL activity were collected from each type of MS.

3.4.1. Buffer preparation

The buffer components were calculated according to given equation ($m = M_w \times c \times V$). Preparation of three different solutions was required: urea stock, run buffer and sample buffer. Urea buffer was prepared by mixing 126.1g Urea (6.0M), 0.0175g methylhydroxyethylcellulose, (MHEC, 0.05%), and 6.3g ion exchange resin (Bio-Rad, California, USA). Purified water was added to obtain 0.35l of urea stock. The compound was then mixed for > 3h to dissolve components and to enable ion exchange resin to lower the conductivity. The mixture was then filtrated. Run buffer was prepared by mixing 0.59g trisodium citrate dehydrate (0.02M), 4g citric acid (0.19M), 126.1g urea stock (6.0M) and 0.175g MHEC (0.05%). Purified water was added to obtain 100ml of run buffer solution. Sample buffer was prepared by mixing 4.05g hydroxymethyl-aminomethane (Triss, 0.167M), 5g ethylene-diamine-tetraacetic acid disodium salt dihydrate (EDTA, 0.067M), 1.8g 3-(N-morpholino) propanesulfonic acid (MOPS, 0.042M), 126.1g urea stock (6.0M), 0.175g MHEC (0.05%). DL-dithiothreitol (DTT, 0.017M) was added to sample buffer just before use, 0.039g DTT/15ml sample buffer. Purified water was added to obtain 200ml of sample buffer solution. Both sample and run buffer were stored in -20°C for prior use.

3.4.2. Sample preparation

Samples were defrosted overnight in a 4°C fridge and warmed up in a water bath at 45°C for 15 minutes. Each sample was convulsed in a vortex mixer (Vortex-Genie 2, Scientific industries, inc., U.S.) and was warmed in water bath ones more for 15 minutes at the same temperature. Thereafter, 150µl of each milk sample was pipetted into respective eppendorf safe lock tubes (Eppendorf, Germany). 350µl of sample buffer with supplementary 0.039g/15ml of DTT was added to the samples. Each sample was ones more convulsed in a vortexer and incubated for 1h in room temperature. After incubation the samples were defatted by centrifugation (Himac CT15RE, Hitachi Koki Co., Ltd.) at 10 000 rpm, at 4°C for 10 minutes and the cream was removed by cotton swabs. Each sample was filtered by using a syringe with a 45µm nylon membrane filter and 30µl was pipetted into conical vials (Agilent, Kista Sweden) for protein analysis by the capillary electrophoresis instrument.

3.4.3. Capillary electrophoresis

Protein separation was performed with 7100 capillary electrophoresis (CE) system (Agilent Technologies Co. America) as described by Johansson *et al.* (2013). Separations were performed using unfused silica standard capillary, 50µm inner diameter, 40cm active length (Chrom Tech, Märsta, Sweden). The calculation of relative concentrations of the individual proteins was based on the peak area and expressed as a percentage of the total areas recorded for all peaks in the electropherogram. Method reproducibility was determined by calculating the coefficient of variation (CV) of relative peak area, for all individual proteins. Result of the protein separation was displayed with Chemstation software version A 10.02 in a CE electropherograms.

3.5. Milk composition data

Data over bulk milk components for respective MS were obtained from Eurofins Steins Laboratorium AB, Sweden. Milk components included in the investigation were: pH, fat (%), protein (%) and SCC (cells/ml). SCC was analysed with flow cytometry and fluorescence techniques using Fossomtic equipment from Foss. Total fat and protein content was measured with Fourier Transform Infrared (FTIR) analysis, using CombiFoss 6000 equipment from Foss. Control system of received values was based on known reference values, both national and international obtained.

3.6. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (SPSS, inc., 2013) to calculate the differences between milking systems and level of significance. Milk compositional parameters and plasmin/plasminogen activities were tested for normality. Means of both milking systems were compared with an independent samples t-test. If the two-tailed p-value was <0.05, means were considered to be significantly different. Pearson correlations and their significance levels were calculated with the bivariate procedure in SPSS, both on the complete sample set as well as on subsets with either the AMS or CMS samples. Statistical analyses of SCC were done using its $\log_{10}$-value due to non-normal distribution of SCC values.

4. Results

4.1. Plasmin and Plasminogen activity in milk

Both PL and PG activity from bulk milk from CMS and AMS have been analysed. An overview on average PL and PG activity is given in Table 2. For bulk milk derived from AMS the mean value of PL activity was 3.61 units/ml and mean value of PG activity was 88.73 units/ml. For CMS bulk milk, PL and PG activity mean value was 4.33 units/ml and 94.93 units/ml respectively. Hence significantly higher PL and PG activity was observed in bulk milk from CMS. The percentage deviation between PL activities in the two different milking systems was 17% higher for CMS and for PG activity 7%.

Table 2. *Plasmin and plasminogen activity (units/ml) in bulk milk from automatic and conventional milking systems*

	AMS (n=52)	CMS (n=56)	
	Mean ± SD	Mean ± SD	*P*-value
Plasmin (units/ml)	3.61 ± 1.27	4.33 ± 1.18	0.01
Plasminogen (units/ml)	88.73 ± 9.54	94.93 ± 9.53	0.001

4.2. Casein content

There was no significant difference between MS regarding β-CN content, however a significant ($P < 0.01$) higher amount of α_{s1}-CN in milk from CMS was obtained. The concentration of α_{s2}-CN was significantly ($P < 0.5$) higher in milk from AMS and the quantity of breakdown products had no significant difference between MS (Table 3). Representative electropherogram of the protein separation is shown in Figure 2.

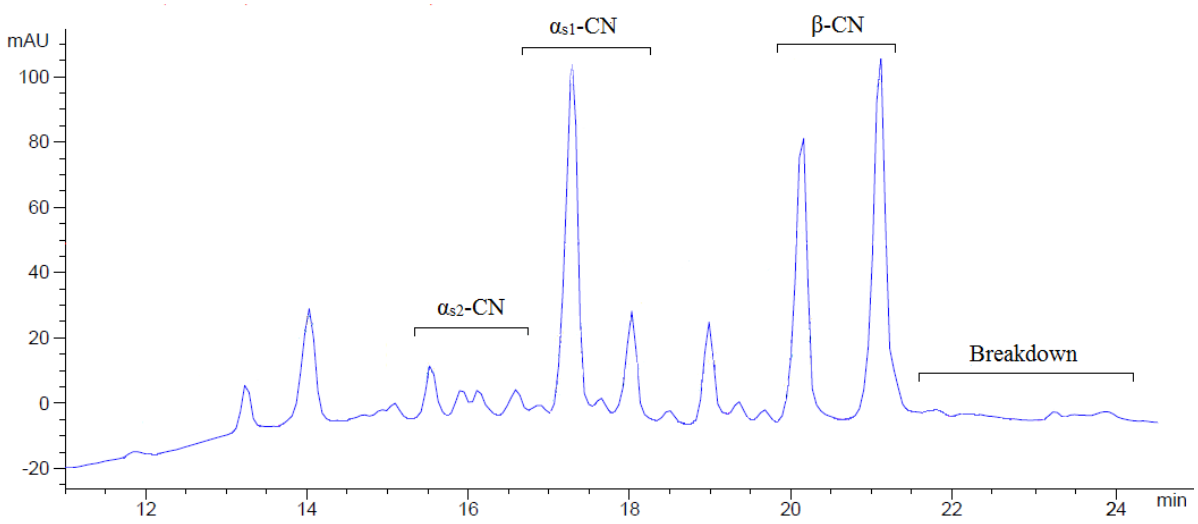

Figure 2. Representative elution profile of bulk milk protein obtained by capillary electrophoresis.

The electropherogram shows the retention time (minutes) from injection on the x axis and milli-absorbance units (mAU) on the y axis. α_{s2}-CN: α_{s2}-casein, α_{s1}-CN: α_{s1}-casein, β-CN: β-casein and breakdown products are indicated.

Table 3. *Plasmin sensitive caseins and breakdown products, in bulk milk from automatic and conventional milking systems. Mean values, significance level and coefficient of variations are indicated*

	AMS (n=10)		CMS (n=10)		
	Mean ± SD	CV	Mean ± SD	CV	P-value
β-CN	38.61 ± 1.08	2.7	38.80 ± 0.65	1.7	NS
α_{s1}-CN	29.15 ± 0.83	2.9	30.43 ± 0.83	2.7	0.01
α_{s2}-CN	7.83 ± 0.92	12.7	7.02 ± 0.57	8.1	0.05
Breakdown	2.54 ± 0.69	27.2	2.54 ± 0.25	9.2	NS

NS: Not significant

4.3 Bulk milk composition

There was a significant ($P < 0.05$) difference between MS regarding pH mean value. AMS had a slightly higher milk pH value in comparison to bulk milk from CMS. Fat and protein mean percentage was significantly ($P < 0.001$; $P < 0.01$) higher in CMS then in AMS. However, the SCC mean value was significantly ($P < 0.001$) higher in AMS (Table 4).

Table 4. *Overview of different parameters in bulk milk from automatic and conventional milking systems*

	AMS		CMS		
	Mean ± SD	N	Mean ± SD	N	*P*-value
pH	6.71 ± 0.03	52	6.70 ± 0.03	56	0.05
Fat (%)	4.37 ± 0.23	51	4.58 ± 0.26	55	0.001
Protein (%)	3.55 ± 0.16	51	3.63 ± 0.15	55	0.01
SCC (cells/ml)[1]	235 000 ± 77.81	51	185 000 ± 73.66	55	0.001

[1]SCC value are presented in its antilogarithmic value

4.4 Milk composition correlations

Correlations between the analysed variables in the received bulk milk samples from 53 farms with AMS and 56 with CMS are shown in Table 5. Total fat and total protein content was both positive correlated with PG at the level of significance P<0.01. The quantity of measurement values varied between 106 -108.

Table 5. *Correlations between different milk parameters in bulk milk from automatic and conventional milking systems*

	Plasmin	Plasminogen	pH	Fat	Protein	SCC
Plasmin	1	0.02	0.07	0.10	0.13	-0.13
Plasminogen	0.02	1	-0.16	**0.38****	**0.48****	-0.07
pH	0.07	-0.16	1	-0.07	-0.11	0.02
Fat	0.10	**0.38****	-0.07	1	**0.59****	-0.17
Protein	0.13	**0.48****	-0.11	**0.59****	1	-0.15
SCC	-0.13	-0.07	0.02	-0.17	-0.15	1

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

5. Discussion

The results from this study show that there was a significant difference in PL activity between MS. According to these results there was a higher PL activity in bulk milk derived from farms with CMS in comparison to milk from AMS. In percentage, the PL activity in CMS was 17% higher than the measurement for PL activity in AMS. These results are in agreement with research conducted by Abeni *et al*. (2008) and Sorensen *et al.* (2001). According to Abeni *et al*. (2008) both PL and total activity (PL + PG) were significantly higher in CM than in AM milk. In accordance, results obtained by Sorensen *et al.* (2001), also showed a reduction in PL activity by 17% when changing milking frequency from two to three times a day. In their study, the cause of decreasing PL concentration with increasing MF, were suggested to be induced by reduction of influx through tight junctions. An increase of influx would instead lead to a rise in blood components to the milk, resulting in a positive effect on PL activity, due to an increase of PG concentration. The epithelial barrier permeability could lead to arrival of PGA such as t-PA, urokinase or u-PA type and conduct to an increased conversion of PG to PL (Bastian and Brown, 1996; Le Roux *et al.* 2003). This in turn highlights the important impact milking frequency has on proteolytic enzymes. The high concentration of PL in CM milk in this study may well be due to an impaired integrity of tight junctions, but also to a prolonged time for PG to be converted to PL due to a lower milking frequency as suggested by Sorensen *et al.* (2001).

The PG activity in this study was also significantly greater in CM milk. The concentration of PG was 7% higher than in milk derived from AMS. Correspondingly results given by Stelwagen *et al.* (1994a) and Stelwagen *et al.* (1994b) showed an increase of activity of PG in the milk as a result of reduced milking frequency. The reason of why PG enhances in milk due to lower milking frequency, hence milking system, corresponds to the reasons of the obtained results for PL. A greater leakage of PG from serum into milk, are due to amplified epithelial barrier permeability (Bastian and Brown, 1996). Hence, the result of both PL and PG activity thereby indicates that increased milking frequency may have a positive influence on protein quality.

The findings regarding casein content in the milk from the different MSs, displayed no difference in β-CN content. According to literature, β-CN along with α_{s2}-CN are the caseins primarily degraded by PL (Fox, 1981; Fox *et al.* 2000; Kohlmann *et al*. 199; Richardson *et al*. 1981). However, there was no significant result for β-CN concentration in this study and the hypothesis about impaired β-CN content in CM milk due to enhanced PL activity could not be verified. Nevertheless, the measurements for α_{s2}-CN had a significant inequality between AMS and CMS, with a greater amount of α_{s2}-CN in milk obtained from AMS. Hence, low α_{s2}-CN content could be an indicator of high PL activity. According to Fox, *et al.* (2000) degradation of α_{s1}-CN occurs slower in comparison to β-CN and α_{s2}-CN due to higher resistance to PL hydrolysis. Results regarding α_{s1}-CN content showed a significant higher peak area percentage of α_{s1}-CN in CMS. The explanation to the enhanced peak area in CM milk might thereby be due to this slightly higher resistance to degradation by PL. The hypothesis concerning an increased amount of breakdown residues due to degradation in CM

milk could not be confirmed. The quantity of these residues of degraded caseins should hypothetically be higher in CM milk as a result of greater PL activity, but no significance could be verified. All results regarding casein content are verified by coefficient of variation values, which shows the reliance to the performance of the method. The reason to the CV values > 10% might in this case be due to the limited numbers of casein analysed milk samples.

The total protein content was significantly higher in bulk milk from CMS in comparison to AMS. According to results obtained by Erdman & Varner (1995), O´Brien *et al.* (2001), Sapru *et al*. (1997) and Klei *et al*. (1997) milk protein content is negatively correlated with increasing milking frequency. This implies on higher total protein content in CM milk due to the extended milking interval and is thereby correspondingly to results in this thesis. Klungel *et al*. (2000) performed a comparative study between AMS and CMS, where milking frequency increased from two to three times per day. Findings showed that the increasing milking frequency in both MS led to a reduction of the milk protein concentration, which also strengthens the current result. The elevated quantity of protein in CM milk might be the underlying reason to the absence of significant difference in β-CN between MS. A significant difference between MS regarding β-CN might be obtained if adjustments for milk yield and percentage of protein are made.

Regarding the relationship between protein content and enzyme activity according to literature, protein percentage has no impact on either PL or PG concentration in the milk (Bastian *et al.* 1991). However, whey protein has shown to influence the PL system by alter the kinetics of the PL-induced hydrolysis of caseins. The kinetics is associated with the properties of PL and PGA, comprising the efficiency and rapidity of their activity during the chemical reaction. The hydrolysis of caseins is thereby impaired by the decreased action of PL or conversion of PG to PL by action of PGA (Ismail and Neilsen, 2010). Results concerning milk composition correlations in the current study presented a positive and significant correlation (0.48) between protein and PG. Correlations are however, not sufficient enough to draw any conclusions about interactions between, in this case, protein content and PG activity.

In addition to negative correlation between protein and MF, the literature has also described a relationship between fat and MF. According to Erdman & Varner (1995), O´Brien *et al.* (2001), Sapru *et al*. (1997) and Klei *et al*. (1997), there is a negative correlation between fat and increased MF. Hence, a higher fat quantity in CM milk then AM milk. At a significance level $P < 0.001$, results from current thesis showed a greater percentage of fat in CM milk. Result also shows a positive correlation (0.38) between total fat content and PG at a significance level 0.01. Presumably, this tendency to interaction concerning fat and PG are however rather due to MS then enzyme activity.

Concerning pH in the present study, the pH value was significantly higher in bulk milk from AMS. Politis *et al*. (1989b) found a significant and positive correlation between PL activity and pH, hence an increase in PL results in an increase of pH. The present results are thereby

contradictory to the findings of Politis *et al*. (1989b). However, according to Bastian *et al*. (1991), pH had no influences on either PL or PG activity. No correlation between proteolytic activity and pH could be definite in the present study and thus also no association between MS and pH was possible to confirm.

The SCC in the AM milk was significantly higher than the SCC in bulk milk derived from CMS farms. Changes in SCC levels due to MS are not unusual, with higher levels in AM milk (Rasmussen *et al*. 2002; Bennedsgaard *et al*. 2006). Elevated SCC has shown to contribute to an increase in proteolytic enzymes (Bastain *et al.* 1991; Walstra *et al*. 1999) due to impaired integrity of the tight junctions (Le Roux *et al.* 2003; Svennersten-Sjaunja *et al*. 2007). Hence, higher permeability of the epithelial layer of the alveoli in the mammary gland tissue leads to an increase in PL and PG activity, but then again the integrity can be sustained by an increased MF. However, no correlation between SCC and enzyme activity could be confirmed in this study. Nonetheless, it has been confirmed by consistent observations that there is a linear relationship concerning PL and SCC (Considine *et al*. 2002; Kroeker *et al*. 1985; Lindmark, Måsson, 2002; Politis *et al*. 1989b; Saeman *et al*. 1988; Zachos *et al.* 1992). Kroeker *et al*. (1985) reported a significant correlation between PL and SCC based on findings on impaired β-CN content with elevated SCC, due to proteolytic breakdown of β-CN by PL. Unfortunately, cannot the current result regarding enzyme concentration in milk with higher SCC levels, be verified by either decreased β-CN percentage or correlations.

6. Conclusion

Preliminary results on PL and PG activity from laboratory tests on bulk milk samples indicated that type of MS significantly influences the total content of proteolytic enzymes. The differences in enzyme activity between MSs, indicates that quality of the protein fraction are preserved better in milk from AMS than milk from CMS. With factors that may negatively affect the quality of food products relying on protein as a functional ingredient taken into consideration, AM milk seems more suitable for cheese manufacturing and UHT processes than CM milk.

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