Sveriges lantbruksuniversitet
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Swedish University of Agricultural Sciences
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Proteolysis in UHT milk due to

Staphylococcus aureus

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Abstract

The aim of this project was to analyse the effect of mastitis pathogen *Staphylococcus (S.) aureus* on proteolysis in UHT milk. *S. aureus* is one of the main pathogens that causes mastitis and reduces milk yield and quality which both leads to large economical losses. UHT milk was inoculated with four strains of *S. aureus* and then incubated at 37°C. Milk was sampled at six different time points (0, 0.5, 1, 2, 4, and 6 h). Samples were stored at -20^{0}C until analysis for protein separation by capillary electrophoresis (CE). CE was run for two strains (333 and 485) and electropherograms were obtained. Due to the quality of electropherograms only strain 485 was further evaluated for protein quantification. The level of milk proteins (caseins and whey proteins) was estimated for each sample by quantification of peak area. Data were statistically analysed using SAS ANOVA procedures. The lowest pH value of inoculated milk was 6.2, while pH of non-inoculated milk remained constant and was 6.7. Bacterial growth in log phase reached levels of 8x10^{8} and 8.7x10^{8} colony forming units for strain 333 and 485, respectively. The overall casein content was reduced by up to 21% due to *S. aureus*.

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1. **Reference list**

1. Introduction

1.1. Milk proteins

There are four types of caseins in milk, α_{s1}, α_{s2}, β and κ casein and their approximate ratio is 4:1:4:1, respectively (de Jong et al., 1993). Caseins are present in milk in form of casein micelles, which additionally contain colloidal calcium phosphate (Walstra, Wouters and Geurts*,* 2006). Normal bovine milk protein content is 3.5% and caseins make 76-86% of that amount (Swaisgood, 2003). Milk proteins also include the proteins dissolved in the serum: α-lactalbumin, β-lactoglobulin, serum albumin, immunoglobulins and lactoferrin, which are usually referred to as the whey proteins (Walstra, Wouters and Geurts*,* 2006).

1.2. *Staphylococcus aureus*

Staphylococcus (S.) aureus is a gram positive bacterium, morphologically expressed in form of spheres which are usually further grouped in grapelike clusters (Madigan *et al.*,2010). It is non-sporulating, facultatively anaerobic, catalase and coagulase positive bacteria, with golden pigmented colonies and is frequently found to be the cause of mastitis in bovines.

According to its epidemiology *S. aureus* together with *Streptococcus agalactiae*, and *Mycoplasma spp.* belongs to contagious pathogens, whose main reservoir is an infected mammary gland. There are also environmental pathogens like *Escherichia coli*, *Klebsiella spp.*, *Coagulase negative staphylococci* (CNS), *Streptococcus* (other than *agalactiae*) and *Enterococcus sp*., mainly located in cow's surroundings like bedding or pasture (Contreras *et al*. 2011). Due to the infected quarters as a main pathogen source, *S. aureus* is most easily spread during the milking of the cows.

Wilson *et al*. (1995) have demonstrated the significance of pathogen transmission at milking time. Since teat cup milking inflation or liner is a main means for spreading of infection, positive tested cows were milked last or by separate milking units which reduced the prevalence 12.7%. Decrease in bulk tank milk (BTM) somatic cell count (SCC) from 600.000 to 345.000 was also evident. Control herds had prevalence reduced for only 2.3%, and BTMSCC from 698.000 to 484.000 (Wilson *et al*.,1995).

Improving of milking procedure like increasing hygiene, introduction of milking order and teat dipping is considered as important control measure in reducing infection incidence caused by contagious pathogens. Skin lesions including hock

skin were identified to be a significant source of *S. aureus* (Capurro *et al*. 2010). Even more, *S. aureus* was found to be more prevalent when animals were kept in tie-stalls in comparison to free-stall barns, especially to those not using mattresses or rubber mats (Olde Riekerink *et al.* 2010).

1.3. Mastitis

Mastitis or udder inflammation has two distinctive forms: -clinical (symptomatic) and subclinical (asymptomatic) form. Different pathogens can provoke intramammary infection and subsequent mastitis. Level of pathogenicity expressed by increased immune response of the host is commonly reflected in elevated somatic cell count, acute phase proteins (haptoglobin and serum amyloid A) and N-acetyl-β-D-glucosaminidase activity in milk. According to these parameters severity of pathogens was found to increase in the following order: *Coagulase negative staphylococci*, *Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli* (Pyörälä, et al. 2011).

Mastitis is considered to have a negative economic impact (Halasa *et al*. 2011). Critical aspects contributing to economic costs involve milk production losses, drugs, discarded milk, veterinary services, labour, product quality, materials and investments, diagnostics, other diseases, culling (Halasa *et al*. 2011).

S. aureus has been identified as the most prevalent mastitis pathogen in many countries (Reksen *et al. 2007,* Østerås *et al.*, 2006, Persson *et al.* 2011). With such a high prevalence *S. aureus* is an important contagious pathogen causing large economic losses, very much associated with a reduced milk yield and increased somatic cell count, both of which are substantial consequences of subclinical mastitis (Sørensen *et al.* 2010). Results from Norway showed seasonal prevalence of *S. aureus*, being greatest during the outdoor season (June and July) (Østerås *et al.*, 2006).

Comparison of milk yield of non-infected and infected multiparous cows (at least one udder quarter) showed reduction for 94 and 161 kg in 2nd and >3rd parity, respectively, in infected cows during the full lactation. Infection of two or more quarters further decreased milk yield by 303 and 390 kg in 2nd and >3rd parity, respectively. Primiparous cows with two or more culture-positive quarters produced 229 kg less than pathogen-free cows during the full lactation, while no difference was found when only one udder quarter tested positive (Whist *et al*., 2009). In the same study, *S. aureus* udder infection in cows showed very significant association with a risk of being treated for clinical mastitis. When causing a clinical mastitis *S. aureus* showed deleterious effects on milk yield. Milk production in primiparous cows was reduced for 8.4 kg/day during the first 2 weeks post-detection, while multiparous cows had the milk production decreased by up to 5.5 kg/day in the first week post-detection (Gröhn *et al.* 2004).

Milk composition was deteriorated by *S. aureus* infection and showed decreased levels of lactose, casein/protein ratio and increased level of somatic cell count (Coulon, *et al.*2002). In subclinical mastitis, with quarters chronically infected by *S. aureus*, SCC was elevated to 865000±727 (LSMeans±SE). Deterioration of technological properties of mastitic milk was shown in a clotting time increase of 428 s when compared to control milk samples. Curd firmness was more than three and two times lower measured 20 and 30 minutes after adding rennet when compared to control, respectively (Leitner *et al*., 2006). Cheese made from high SCC milk have had increased moisture levels as well as higher proteolysis during ripening process with potential adverse impact on sensory quality of the cheese (Mazal *et al*. 2007). Increased proteolysis of α_{s1}- and β-casein in UHT milk has also been associated with a high level of raw milk SCC (Fernandes *et al*. 2008). Similar effect of substantial decrease in α_{s1}- and β-casein levels was shown on raw milk samples high in SCC (Verdi et al. 1987).High SCC has a negative impact on yoghurt quality, mostly by increasing lipolysis and free fatty acid content (Fernandes *et al*. 2006)

1.4. Indigenous milk proteinases

1.4.1. Plasmin

Plasmin is an alkaline serine proteinase regulated through the plasmin system consisted of plasmin (active enzyme), plasminogen (zymogen), plasminogen activator (PA) and inhibitors of plasmin (PI) and plasminogen activator (PAI). Two types of plasminogen activators are present in bovine milk: t-PA or tissue type and u-PA or urokinase type. Plasmin, plasminogen and t-PA are casein bound, while u-PA is neutrophil bound by the u-PA receptor (u-PAR) attached to the cell membrane (Politis *et al.,* 2002). More exact, t-PA is bound to κ- and α_{s2}-casein, and not to α_{S1}- and β-casein (Heegaard, *et. al.* 1994). Inhibitors of plasmin and PA are found in milk serum. Plasminogen to plasmin conversion is achieved by action of u-PA or t-PA on Arg_{557}–Ile_{558} bond. Optimal conditions for plasmin activity are 37^{0}C and pH of 7.5 (Fox et al. 2006).

Human strains of *S. aureus* synthesise staphylokinase, a protein with an ability to bind and activate plasminogen into plasmin (Mölkänen et al., 2002). Unlike human strains, bovine strains possess no ability of staphylokinase expression and thereby no plasminogen converting activity could be achieved. Although, *S. aureus* presence enhanced u-PA production in epithelial and myoepithelial mammary cell culture media (Zavizion et al., 1997).

Concerning the heat treatment it was shown that both plasmin and plasminogen in milk persevere after pasteurisation. Even more plasminogen activation and plasmin increase since PA is significantly heat stable contrariwise to PAI. Unfolding of plasminogen in pasteurisation process results in its denaturation which makes it even more prone to PA (Ismail et al., 2010).

In the UHT processing at a very high temperatures (135-150 ^{0}C, few seconds) β-lactoglobulin binds plasmin and plasminogen individually in an irreversible reaction involving thiol group. These complexes render both plasmin and plasminogen rather inert (Ismail et al., 2010).

Plasmin in milk expresses caseinolytic activity, especially on β- and α_{S2}-casein. It shows specificity for lysine (and arginine) part of carboxyl group in peptide bonds. Main products of β-casein proteolysis are following γ-caseins, γ1 (f29-209), γ^2 (106-209), γ^3 (108-209) and proteose peptones (PP), PP5 (f1-105/107), $PP8_{slow}$ (f29-105/107), $PP8_{fast}$ (f1-29) (Fox et al. 2006). Caseinolysis of α_{S1}-casein results in λ-casein formation, while κ-casein is not susceptible to proteolysis by plasmin (Kelly and McSweeney, 2003).

1.4.2. Cathepsin D

Cathepsin D is an intracellular lysosomal acid aspartic proteinase with an optimum activity at pH 4 and 37°C. There are four forms of cathepsin D: procathepsin, pseudocathepsin, single-chained and two-chained cathepsin D. The major form present in milk is procathepsin D (Hurley et al. 2000). It is partially inactivated by milk pasteurisation, and completely inactivated by heating for 10 min at 70°C. The gradual decrease of procathepsin D activity was registered after exposure to a range of increasing temperatures. Half of its original activity remained at low pasteurisation temperatures while it was significantly decreased at 99^{0}C (60 s) (Larsen et al. 2000). Both procathepsin and cathepsin D hydrolyse α_{s1}, α_{s2}, β, κ casein and α-lactalbumin, while no enzymatic activity was shown by using β-lactoglobulin as substrate. Enzymatic specificity was expressed preferentially for hydrophobic amino acid residues (Kelly and McSweeney, 2003). Proteolysis of α_{s1} and β caseins by cathepsin D is analogical to proteolysis by plasmin, while proteolysis of α_{s2} casein differs. Cathepsin D cleaves Phe_{23}- Phe_{24}, Phe_{24}-Val_{25}, Leu_{98}-Leu_{99}, Leu_{149}-Phe_{150} bonds in α_{s1}-casein and Leu_{99}-Tyr_{100}, Leu_{123}-Asn_{124}, Leu_{180}-Lys_{181}, Thr_{182}-Val_{183} in α_{s2}-casein. In κ-casein, Leu_{32}-Ser_{33}, Leu_{79}-Ser_{80}, Phe_{105}-Met_{106} bonds are cleaved, while Leu_{52}-Phe_{53} and Trp_{104}-Leu_{105} were identified as cleavage sites in α-lactalbumin. Cleavage sites in β-casein are Phe_{52}-Asn_{53}, Leu_{58}-Val_{59}, Pro_{81}-Val_{82}, Ser_{96}-Lys_{97}, Leu_{125}-Thr_{126}, Leu_{127}-Thr_{128}, Trp_{143}-Met_{144}, Phe_{157}-Pro_{158}, Ser_{161}-Val_{162}, Leu_{165}-Ser_{166}, Leu_{191}-Leu_{192}, Leu_{192}-Tyr_{193}, Phe_{205}-Pro_{206} (Larsen *et al*. 1996). It was shown that cathepsin D possesses the ability to initiate skim milk coagulation if present in concentration tenfold higher than its native milk concentration (Larsen *et al*. 1996). Whey is the main fraction where procathepsin D and cathepsin D are localised. Activity of cathepsin D protease positively correlates with somatic cell count in milk (O'Driscoll et al. 1999).

1.4.3. Cathepsin G

Cathepsin G is neutral serine lysosomal protease stored in monocytes and primary granules of polymorphonuclear neutrophils (Prin-Mathieu et al. 2002). Considine *et al*. (2002) found that Cathepsin G broadly hydrolysed α_{s1}- and β-caseins with the preference for the peptide bonds involving amino acids Glu, Ala, Phe, Arg and

Leu in α_{s1}-casein and Ser, Thr, Leu and Phe in β-casein. Cathepsin G and elastase have some cleavage sites of α_{s1} casein in common. The same is true for cathepsin G and cathepsin B (Considine *et al*. (2002).

1.4.4. Cathepsin B

Cathepsin B, lysosomal cysteine protease expresses its highest activity at pH 6, and undergoes inactivation at pH 7. In proteolysis of α_{s1} and β casein cathepsin B expressed its activity against a broad range of peptide bonds with an obvious affinity for hydrophobic amino acids Leu, Val, Gln, Pro and Ser (Considine *et al*. 2004). Protease cathepsin B is present in whey fraction and correlates positively with SCC (Larsen et al. 2004). Cathepsin D interacts with cathepsin B and has ability to initiate its proteolytic activity (Magbul *et al*., 2001).

1.4.5. Elastase

Elastase, a neutral serine lysosomal proteinase exhibited broad specificity in hydrolysing α_{s1}- and β-casein. Cleavage sites originating in hydrophobic regions f1-40 and f141-199 of α_{s1} casein were most frequently hydrolysed (Considine *et al*. 1999, 2000).

1.5. Exogenous proteinases from *S. aureus*

S. aureus possesses several extracellular proteases: aureolysin (aur), serine protease (SspA), two papain-like cysteine proteases staphopain A and staphopain B (ScpA and SspB).

1.5.1 Aureolysin

Aureolysin is a zinc dependent single-chain metalloprotease and belongs to thermolysin protease family. It is comprised of 301 amino-acids and binds one zinc and three calcium ions (Banbula, 1998). Aureolysin cleaves casein, expresses EDTA sensitivity and requires calcium for its activity (Arvidson, 1973). Concerning the substrate specificity aureolysin shows preference for N terminal side of large hydrophobic amino acids: alanine, valine, leucine, isoleucine, phenylalanine and tyrosine, with exclusion of glycine (Arvidson, 1973., Drapeau, 1978., Sabat, 2008.)

1.5.2. V8 protease

Serine protease SspA also known as V8 protease was found to hydrolyse caseins by cleaving peptide bonds on carboxyl-terminal side of glutamic or aspartic acid (Drapeau *et al*. 1972). When these two amino acids are compared, V8 protease

cleaves 3000 times faster peptide bonds involving glutamic acid (Sørensen et al. 1991). V8 protease also shows buffer depending activity. Buffers ammonium bicarbonate and acetate have an inhibitory effect on V8 protease activity when compared to sodium phosphate. These buffers do not express any influence on enzyme specificity (Sørensen et al. 1991). V8 protease activity is not influenced by EDTA presence (Drapeau *et al*. 1972). Active site of the protease is *Ser169.* Protease specificity for the negatively charged acidic amino acid residues is defined by its positively charged N-terminus (Prasad *et al.* 2003)

1.5.3. Staphopain A and B

Both enzymes, staphopain A (ScpA) and staphopain B (SspB) are cysteine, papain-like proteases with a molecular weight of ~20 kDa and nearly the same three-dimensional structure (Kantyka et al. 2011). ScpA and SspB express no proteolytic affinity towards caseins (Rice et al. 2001).

1.6. Analytical methods for protein analysis

Capillary electrophoresis is a technique used for protein separation based on difference in mobility of charged molecules exposed to applied electric field in unfused silica capillary with a background electrolyte (Weinberger, 2000). Protein separation is achieved by UV absorbance detection through a small window on a capillary. In a contact with an electrolyte solution silanol groups on the inner capillary wall achieve electronegativity which contributes to adhering of solution cations and forming of double layer. The layer next to negatively charged silanol groups is called compact or Stern layer (Corradini et al. 2003). Further from capillary walls in a diffuse layer where cations are water bound, applied voltage initiates cathode attracted flow of the whole electrolyte which is called electroosmotic flow (EOF) and can be altered by composition, pH and ionic strength of electrolyte solution. Ionic strength increase causes a reduction of a double layer, zeta potential and EOF (Corradini et al. 2003) At a high pH values ionisation of silanol groups is fully achieved, strong zeta potential is generated as well as dense double layer and consequently EOF is increased (Weinberger, 2000).

There are several techniques of capillary electrophoresis based on a different protein characteristics used for separation. For example differences in charge-to-mass ratio, isoelectric points and mobility in sieving matrices serve as protein separation parameters in capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIF), and sieving SDS capillary electrophoresis, respectively (Manabe, 1999). When capillary electrophoresis and high performance liquid chromatography (HPLC) methods for protein separation are compared, CE generally has higher resolution potential, requires toxin-free buffers, shorter

analysis time and smaller amounts of buffer and sample, and is a cheaper method since CE capillaries are less expensive than HPLC columns (Otte *et al*., 1997). Besides the basic protein analysis CE can be used for detection of protein polymorphisms, heat treatment and adulteration of dairy products (Recio, *et al.*1997).

1.7 Objective

This study was conducted in order to investigate the influence of several strains of mastitis pathogen *Staphylococcus aureus* on milk proteins. Considering the activity of the enzymes present in milk and their proteolytic activity, commercial UHT milk was used. In order to mimic the udder conditions incubation temperatures of 37^0C were applied.

2. Materials and methods

2.1. Bacterial growth

Four different strains of *S. aureus* were investigated. One colony from the original agar plate was cultured overnight in 5 ml Nutrient Broth (10% horse serum) at 37^0C. 0.5 ml of the overnight culture was then incubated in the 9.5 ml of Nutrient Broth for one and a half hour at 37^0C in order to bring the bacteria to log phase. Commercial milk from a 2 dl package was used (Arla Dairy, Sweden). 3.2 ml of sterile 2.5 M MOPS (3-(4-Morpholino) propane sulfonic acid) was added to 80 ml of milk. The pH of the mixture was adjusted to the pH of UHT milk of 6.7 by adding 5 M NaOH. 30 ml and 28.5 ml of milk was added to two E-flasks marked 1 and 3, respectively. 30 and 28.5 ml of milk and MOPS mixture was added to E flasks marked 2 and 4, respectively.

2.2. Colony forming units

After 1.5 hour incubation time 1.5 ml of the bacterial culture was added to flasks 3 and 4, each. E-flasks were then put in incubator at 37^0C. E-flasks were sampled at the following time points (0, 0.5, 1, 2, 4 and 6h) by collecting approximately 2 ml of milk from each sample to the 4 different Eppendorf tubes. At each time point 0.5 ml of the milk was added to 4.5 ml of sterile 0.86% NaCl solution for further dilutions. 100 µl of 10^4 and 10^6 dilutions were spread on blood agar plates in order to estimate the number of colony forming units. The same procedure was done for sample 3 at time point 6. Samples 1 and 2 were checked for absence of bacteria by spreading 100 µl of milk onto the blood agar plates at time points 0 and 6.

2.3. pH measurements

Sample pH measurements were done by using Orion digital pH meter. Preceding the use the pH meter was standardized at 37^0C with buffers of pH 4.0 and 7.0. Immediately after collecting samples at each time point pH values were measured and Eppendorf tubes were then put into the freezer at -20^0C for further electrophoretic analysis.

Table 1. Content of milk samples

Sample	1	2	3	4
Milk	30 ml	28.84 ml	28.5 ml	27.4 ml
MOPS	-	1.16 ml	-	1.1 ml
Bacteria	-	-	1.5 ml	1.5 ml

Sample 1: sterile milk, Sample 2: milk with MOPS, Sample 3: inoculated milk, Sample 4: inoculated milk with MOPS

MOPS as a buffer was used to maintain constant pH in samples 2 and 4 in order to provide conditions similar to those *in vivo*.

2.4. Protein analysis

2.4.1 Capillary electrophoresis

2.4.1.1 Sample preparation

Two strains (333 and 485) were further investigated and analysed by using capillary electrophoresis. Samples were defrosted and 800 µl form each sample was centrifuged and then defatted by removing fat layer with cotton swabs. Procedure was repeated for removing remnants of fat. 300 µl were added to a new tube and put in a water bath at 42^0C for 2x15 min. 700 µl of sample buffer was added to each sample tube, and left for 1 hour. Each sample was filtered by using syringe with 45 µm filter. 30 µl of each sample was used for the protein analysis in the capillary electrophoresis instrument.

2.4.1.2 Sample and run buffers

Sample buffer was prepared by using 0.167 M hydroxymethyl-aminomethane (Triss), 0.067 M ethylene-diamine-tetraacetic acid disodium salt dihydrate EDTA, 0.042 M MOPS, 0.017 M d,l-dithiothreitol (DTT), 6M urea, 0.05% (w/v) methylhydroxyethylcellulose MHEC and ion exchange resin. Run buffer was prepared by using 0.02 M trisodium citrate (dehydrate), 0.19 M citric acid, 6M urea, 0.05% (w/v) MHEC and ion exchange resin. Prior to use run buffer was filtered through a 0.45 µm filter. Protein separation was performed on capillary

electrophoresis system Agilent Technologies Co. using unfused silica capillary with 50 µM diameter x 52 cm (40cm effective length). Data were collected with Chemstation software version A 10.02.

2.4.1.3 Statistical analysis

Capillary electrophoresis electropherogram base lines were adjusted and area percentage was calculated by using ChemStation - Agilent Technologies software. The data retrieved were then used for statistical analyses using SAS software 9.2. Analysis of variance (ANOVA) was used for comparing the changes in amount of milk proteins at different time points and determining the levels of significance among the samples.

2.5 Results

2.5.1. Colony forming units

After transfer of bacteria from nutrient broth to milk samples lag phase (binary fission occurred) and exponential (log) phase of bacterial growth occurred. Growth and division of bacteria ranged from initial $10x10^6$ cfu ml^{-1} and $17x10^6$ cfu ml^{-1} to the maximum value (at 6h) of $8x10^8$ cfu ml^{-1} and $8.7x10^8$ cfu ml^{-1} in sample 4 of strain 333 and 485, respectively. Sample 3 had a higher maximal value of $12x10^8$ cfu ml^{-1} and $10x10^8$ cfu ml^{-1} for strains 333 and 485, respectively, which might be explained by lower pH values being more supportive for bacterial growth due to absence of MOPS. Colony forming units for strains 333 and 485 are numerically presented in Tables 2. and 3., respectively.

Table 2. Colony forming units - strain 333

Time	00:00	00:30	01:00	02:00	04:00	06:00
Sample 3						1195
Sample 4	17	23	42	123	548	804

Colony forming units (10^6 cfu ml^{-1}) at time 0h, 0.5h, 1h, 2h, 4h, and 6h. Sample 3: inoculated milk, Sample 4: inoculated milk with MOPS

Table 3. Colony forming units - strain 485

Time	00:00	00:30	01:00	02:00	04:00	06:00
Sample 3						1003
Sample 4	10	33	34	81	354	870

(10^6 cfu ml^{-1}) at time 0h, 0.5h, 1h, 2h, 4h, and 6h. Sample 3: inoculated milk, Sample 4: inoculated milk with MOPS

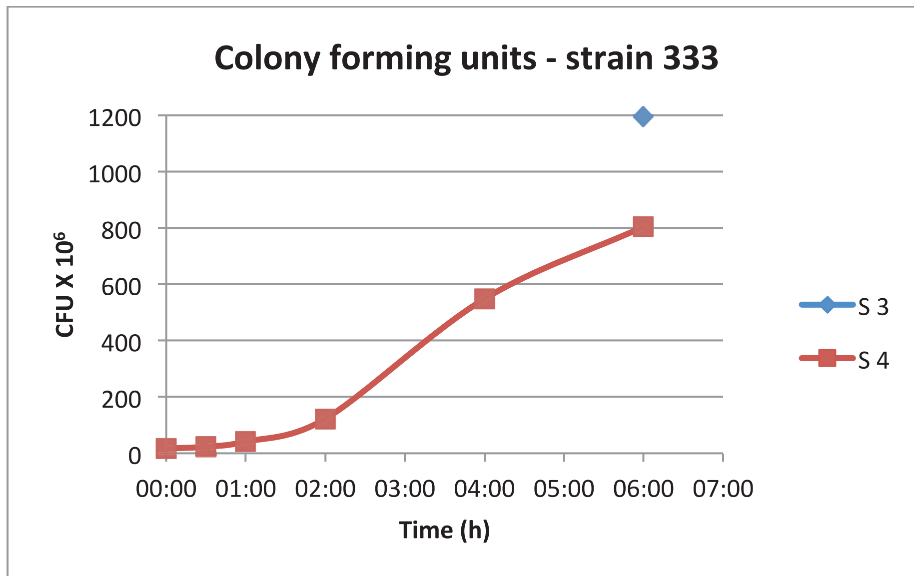

Figure 1. Colony forming units strain 333 (10^6 cfu ml^{-1}) at time 0h, 0.5h, 1h, 2h, 4h, and 6h. S3: inoculated milk, S4: inoculated milk with MOPS

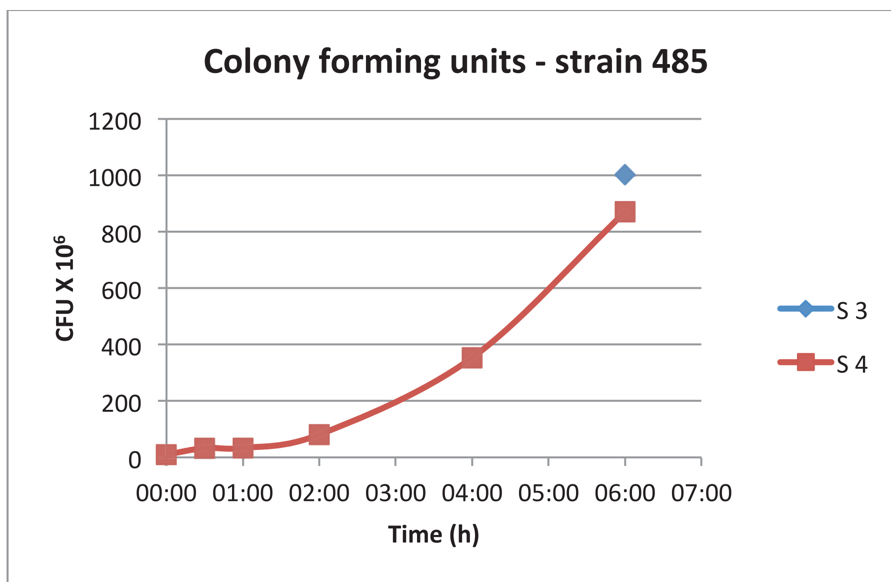

Figure 2. Colony forming units strain 485 (10^6 cfu ml^{-1}) at time 0h, 0.5h, 1h, 2h, 4h, and 6h. S3: inoculated milk, S4: inoculated milk with MOPS

2.5.2 Determination of pH

Measured pH values at each time point were almost constant (pH 6.7) for sample 1, 2 and 4 and slightly decreased for sample 3 (Figures 1 and 2). Drop of pH values of sample 3 from pH 6.7 to 6.2 indicates the lack of MOPS and consequently lower buffer capacity which led to slightly steeper pH decrease compared to sample 4. The lowest pH value of sample 4 was 6.5 (strain 485).

Samples with no bacteria showed almost completely constant pH values of 6.7 irrespective of MOPS presence.

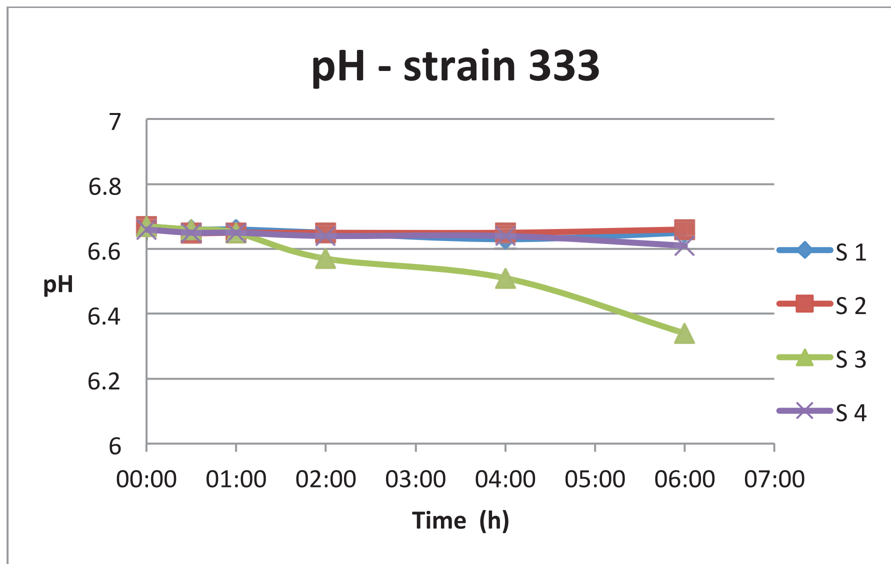

Figure 3. Average pH levels (strain 333) of milk samples at time 0h, 0.5h, 1h, 2h, 4h, and 6h. S1: sterile milk, S2: milk with MOPS, S3: inoculated milk, S4: inoculated milk with MOPS

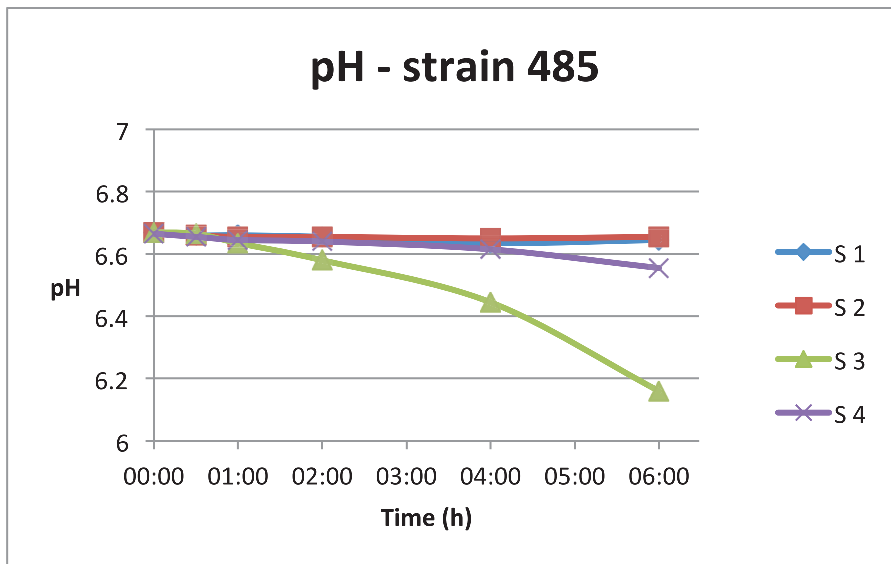

Figure 4. Average pH levels (strain 485) of milk samples at time 0h, 0.5h, 1h, 2h, 4h, and 6h. S1: sterile milk, S2: milk with MOPS, S3: inoculated milk, S4: inoculated milk with MOPS

2.5.3. Statistical analyses of data

Three time points were compared (0, 0.5 and 6 h) for all four samples. At time 0h no statistical difference was found between the samples in any of the milk protein components. At time 6h samples did differ significantly in β-lactoglobulin due to accretion in peak area percentage, and α_{S1}-casein, α_{S0}-casein and κ-casein due to reduction in peak area. Comparison done for each sample separately (1, 2, 3, or 4)

revealed no significant change in proteins between the time points of samples 1 and 2. Sample 3 time point 6h proved different in all proteins except α_{S0}-casein. Further analysis showed that difference in β-lactoglobulin (increased level) and κ-casein (decreased level) was very significant with $p<0.0001$, while for β-casein A2 (decreased) p was 0.0001 (Figures 3, 4 and 6). Sample 4 time point 6h was different in β-lactoglobulin (increased level), κ-casein, and β-casein A1 and A2 (decreased levels).
CE-electropherograms of samples 3 and 4 at time 6h show the occurrence of double peak at β-lactoglobulin migration time (Figure 8 and 9), while peak area percentage was increased 3 and 3.5 times (Figure 3) respectively. At the same time peak area percentage of κ-casein was decreased approximately by half (for 61 and 55% for samples 3 and 4 at time 6h respectively) (Figure 4).

Overall level of caseins was decreased by 18% and 21% for samples 3 and 4, respectively.Only strain 485 was submitted to statistical analysis since electropherograms obtained for strain 333 could not be properly evaluated.

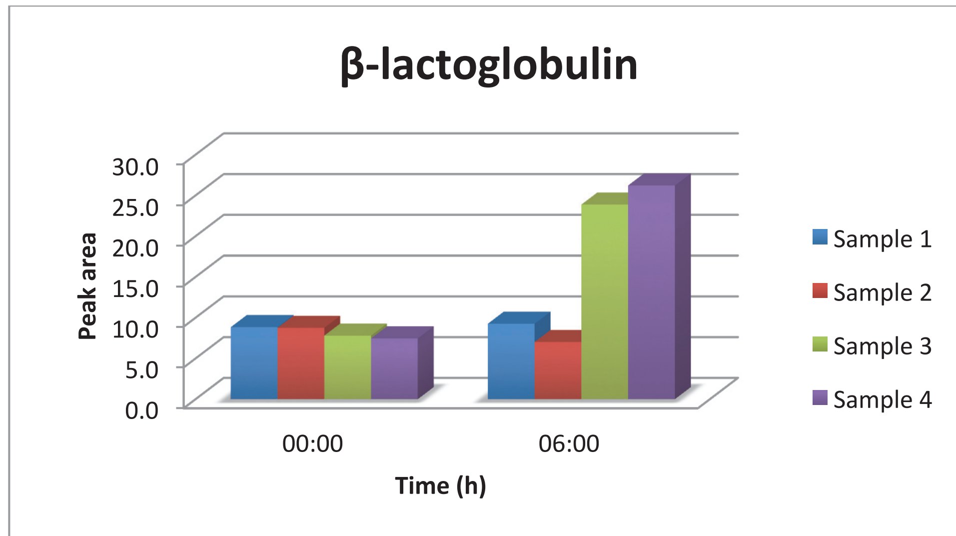

Figure 5. Level of β-lactoglobulin at time 0h and 6h (strain 485), Sample 1: sterile milk, Sample 2: milk with MOPS, Sample 3: inoculated milk, Sample 4: inoculated milk with MOPS

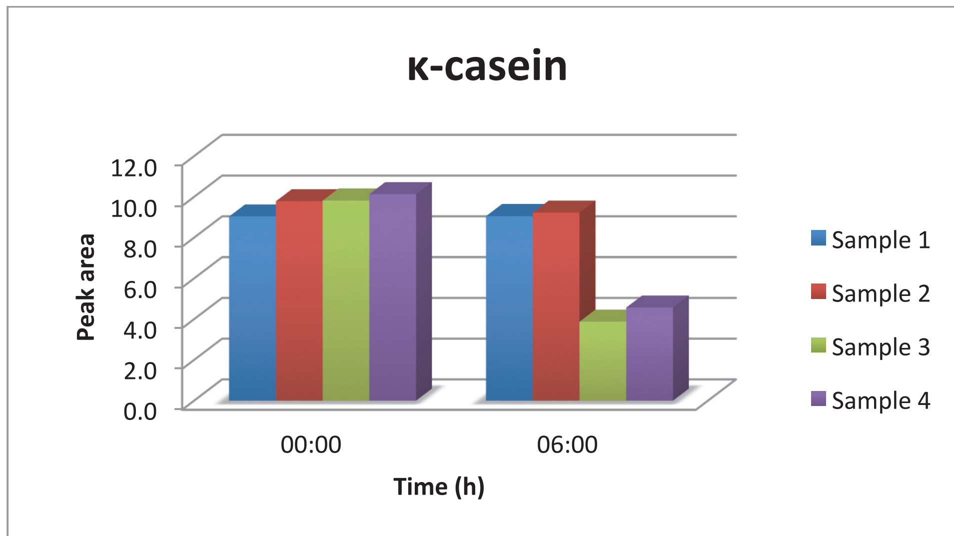

Figure 6. Level of κ-casein at time 0h and 6h (strain 485), Sample 1: sterile milk, Sample 2: milk with MOPS, Sample 3: inoculated milk, Sample 4: inoculated milk with MOPS

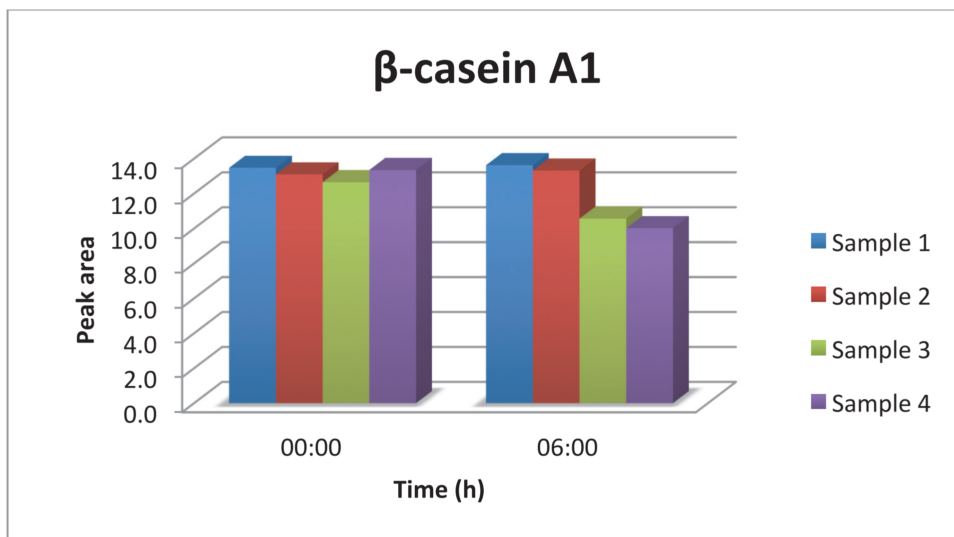

Figure 7. Level of β-casein A1 at time 0h and 6h (strain 485), Sample 1: sterile milk, Sample 2: milk with MOPS, Sample 3: inoculated milk, Sample 4: inoculated milk with MOPS

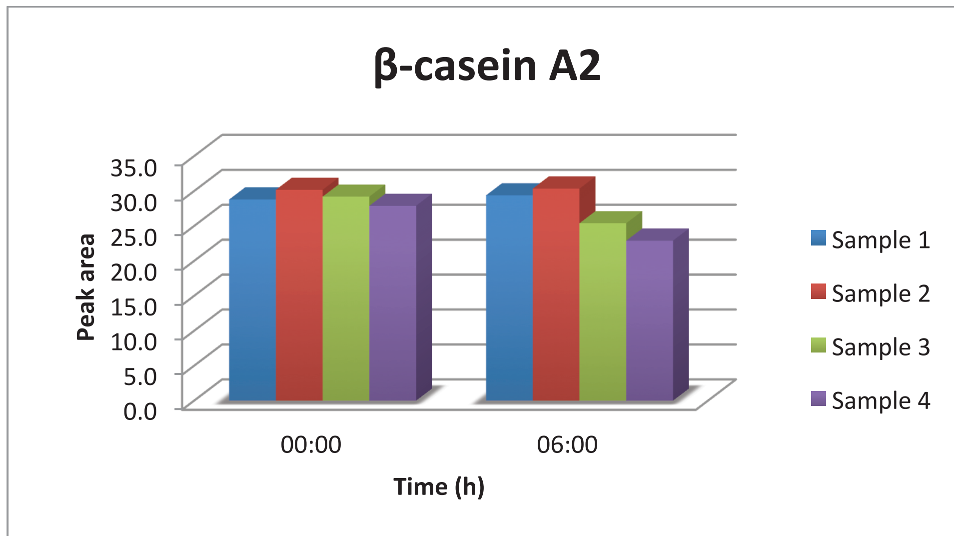

Figure 8. Level of β-casein A2 at time 0h and 6h (strain 485), Sample 1: sterile milk, Sample 2: milk with MOPS, Sample 3: inoculated milk, Sample 4: inoculated milk with MOPS

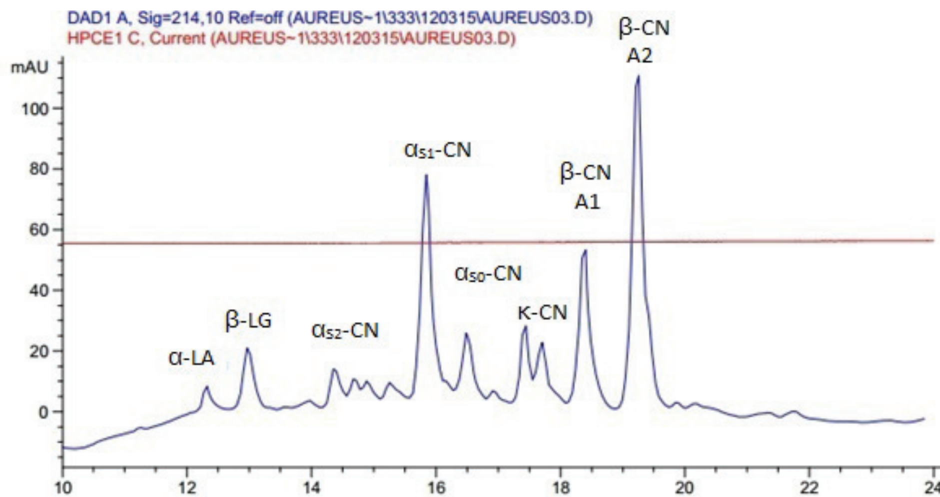

Figure 9. Electropherogram of sterile milk with no buffer (MOPS) added (Sample 1), time 0h, *X*-axis: time (min), *Y*-axis: mAU, milli-absorbance units, α-LA: α-lactalbumin, β-LG: β-lactoglobulin , α_{S2}-CN: α_{S2}-casein, α_{S1}-CN: α_{S1}-casein, α_{S0}-CN: α_{S0}-casein, κ-CN: κ-casein, β-CN A1: β-casein A1, β-CN A2: β-casein A2

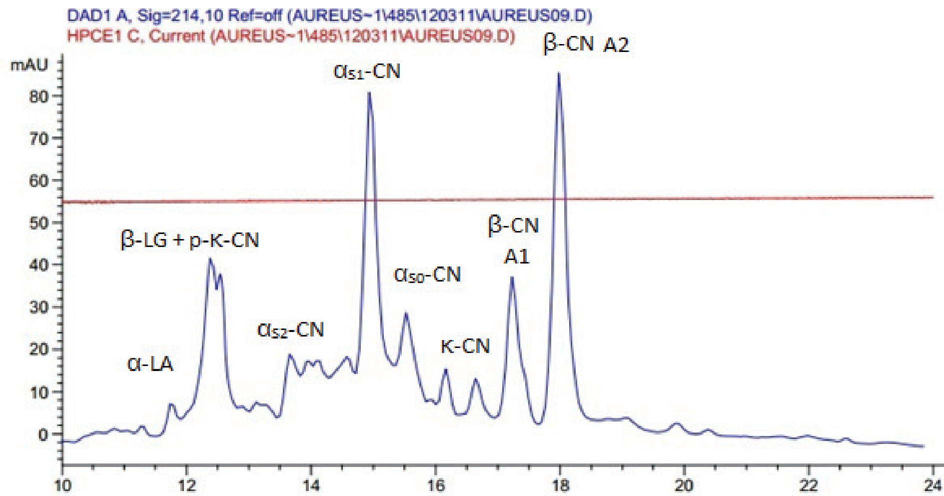

Figure 10. Electropherogram of inoculated milk with no buffer (MOPS) added (sample 3) at time 6h. Co-migration of β-lactoglobulin and para-κ-casein like peptide visible in a double peak form, *X*-axis: time (min), *Y*-axis: mAU, milli-absorbance units; α-LA: α-lactalbumin, β-LG: β-lactoglobulin , p-κ-CN: para-κ-casein like peptide, α_{S2}-CN: α_{S2}-casein, α_{S1}-CN: α_{S1}-casein, α_{S0}-CN: α_{S0}-casein, κ-CN: κ-casein, β-CN A1: β-casein A1, β-CN A2: β-casein A2

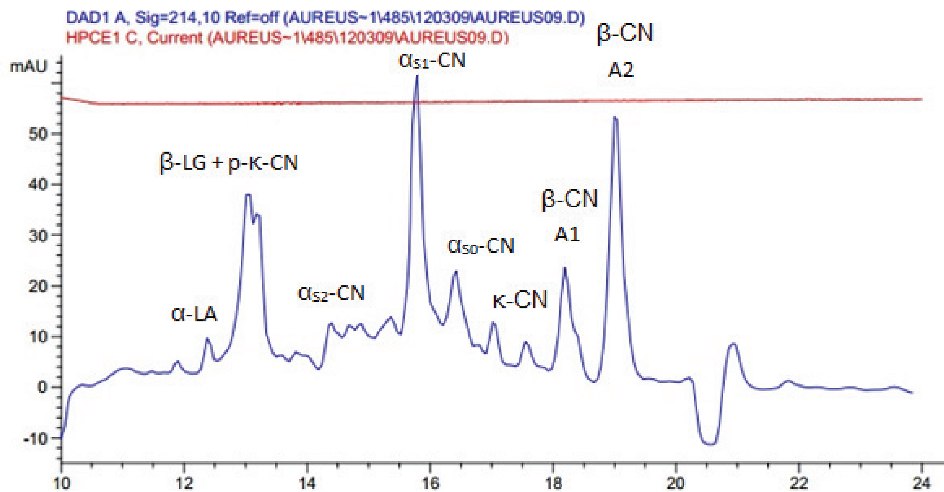

Figure 11. Electropherogram of inoculated milk with buffer (MOPS) added (sample 4) at time 6h. Co-migration of β-lactoglobulin and para-κ-casein like peptide visible in a double peak form, *X*-axis: time (min), *Y*-axis: mAU, milli-absorbance units; α-LA: α-lactalbumin, β-LG: β-lactoglobulin , p-κ-CN: para-κ-casein like peptide, α_{S2}-CN: α_{S2}-casein, α_{S1}-CN: α_{S1}-casein, α_{S0}-CN: α_{S0}-casein, κ-CN: κ-casein, β-CN A1: β-casein A1, β-CN A2: β-casein A2

3.Discussion

The results from this experiment show significant degradation of caseins. Peak area in α_{s1}-casein was reduced by 13% and 20% in bacteria containing samples 3 and 4, respectively. Significant proteolysis of both β-casein variants was evident from 16% and 24% decrease of β-casein A1 and 13% and 18% decrease of β-casein A2 in samples 3 and 4, respectively. These results also show increased hydrolysis in inoculated samples with MOPS buffer (sample 4) compared to samples containing milk and bacteria only (sample 3) probably due to lower pH drop in former ones. V8 protease exhibits maximum activity towards caseins at pH 7.8 (Drapeau *et al.*, 1972). *S. aureus* V8 proteolytic activity towards β-casein was shown in study done by Livney *et al.* (2004.b). When β-casein was digested by V8 protease the following peptides were obtained: f1-5, f1-11, f32-42, f32-43, f48-91, f109-117, f109-121, f109-129, f109-131, f109-121 and f109-31. Peptide fragments were identified by use of matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (Livney *et al.* 2004.b). By use of strong-cation-exchange chromatography it was found that V8 protease digests β-casein to the following peptide fragments: f6-14, f22-31, f45-56, f45-79, f45-91, f92-100, f101-108, f109-121, f132-141, f185-209 (Crimminis *et al.,* 1989). Identical cleavage sites of β-casein found in both of these two studies are Glu_5-Leu_6, Glu_{31}-Lys_{32}, Glu_{91}-Val_{92}, Glu_{108}-Met_{109}, Glu_{121}-Ser_{122}, and Glu_{131}-Asn_{132}. Peptide fragment f109-121 was the only fragment found in both studies.

The most extensively hydrolysed was κ-casein whose peak area was reduced by 60% ($p<0.0001$) and 55% in bacteria-containing samples 3 and 4, respectively. This might be explained by the structure of casein micelle since κ-casein is located on the surface of the casein micelle. V8 protease affinity towards κ-casein was shown when used for proteolysis of peptide complex obtained by heating of κ-casein/β-lactoglobulin solution mixture (Livney *et al*. 2004.a). β-lactoglobulin is a globular protein which becomes denaturated at 90^0C and alkaline pH above 7.5 (Bertrand-Harb *et al.* 2002.) Due to conformational changes and subsequent interactions thiol-disulfide bonds are formed between β-lactoglobulin and κ-casein. In the formed complexes V8 protease hydrolysed Glu_{12}-Lys_{13}, Glu_6-Gln_7, Asp_{14}-Glu_{15}, Glu_2-Gln_3, Glu_{15}-Arg_{16} and Asp_{115}-Lys_{116} bonds of κ-casein and Glu_{55}-Ile_{56}, Glu_{62}-Asn_{63}, Glu_{65}-Cys_{66}, Glu_{74}-Lys_{75}, Asp_{85}-Ala_{86}, Glu_{89}-Asp_{90}, Asp_{96}-Thr_{97} Asp_{98}-Tyr_{99}, Glu_{108}-Asn_{109}, Glu_{112}-Pro_{113}, Glu_{114}-Gln_{115}, Glu_{115}-Gly_{116}, Glu_{127}-Val_{128}, Asp_{129}-Asp_{130}, Asp_{137}-Lys_{138} bonds of β-lactoglobulin. The effect of V8 protease proteolytic activity on β-lactoglobulin was also analysed by using matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) and surface-enhanced-laser-desorption-ionization-time-of-flight-mass-spectrometry (SELDI-TOF-MS) in study done by Kosters *et al.* (2010). Proteolysis was evident from resulting 16 peptides identified as following fragments: A f115-127, B f46-65, B f63-89, B f109-131, AB f1-33, AB f1-45, AB f12-45, AB f29-45, AB f34-45, AB f56-62, AB f66-74, AB f75-89, AB f90-108, AB f135-158, AB f135-162, AB f138-158. Cleavage sites Glu_{65}-Cys_{66}, Glu_{74}-Lys_{75}, Glu_{108}-Asn_{109}, Glu_{127}-Val_{128}, Glu_{55}-Ile_{56}, Glu_{89}-Asp_{90}, Asp_{137}-Lys_{138}, Glu_{114}-Gln_{115}, Glu_{62}-Asn_{63} are also found in study done by

Livney *et al.*(2004.a). Cleavage of Glu_{15}-Arg_{16} and Asp_{115}-Lys_{116} bond of κ-casein resulted in para-κ-casein like peptide f (16-115) which was identified in supernatant of V8 digest. RP-HPLC was used for analysis and MALDI-TOF-MS for peptide identification (Livney *et al*. 2004.a).

Caseinomacropeptide (CMP) f(106-169), obtained by chymosin digestion of κ-casein was also cleaved by *S. aureus* V8 protease (Minkiewicz *et al.,* 1996) at 4 or 5 sites depending on glycosylation. Four bonds were hydrolysed in nonglycosylated CMP A: Glu_{137}-Ala_{138}, Glu_{140}-Ser_{141}, Glu_{147}-Asp_{148} and Glu_{154}-Ser_{155}, while one additional bond Glu_{151}-Val_{152} was hydrolysed in glycosylated CMP A. Fast atom bombardment mass spectroscopy (FAB-MS) and electrospray ionization mass spectrometry (ESI-MS) were used for peptide identification. In a study done by Talbo *et al.* (2001) nonglycosylated caseinomacropeptide A and B were also digested with V8 protease and similar results were obtained. Only four out of seven potential cleavage sites were actually obtained. Method used for peptide identification was MALDI mass spectrometry and the cleaved bonds were Glu_{137}-Ala_{138}, Glu_{140}-Ser_{141}, Glu_{147}-Asp_{148} and Glu_{151}-Val_{152}. No peptides were obtained by hydrolysis of any of the following three possible cleavage sites: Glu_{118}-Ile_{119}, Glu_{154}-Ser_{155} and Glu_{158}-Ile_{159}. The difference between the results of these two studies is in Glu_{154}-Ser_{155} bond which was not identified in a study done by Talbo *et al.* V8 protease was also used for digestion of a large C-terminal peptide retrieved after tryptic hydrolysis of κ-casein. Cleavage of Glu_{137}-Ala_{138} and Glu_{140}-Ser_{141} bonds in both A and B variants of κ-casein was determined by MALDI mass spectrometry (Holland *et al.* 2004).

From our electropherograms of inoculated milk (time 6h) it is possible to observe an occurrence of either double peak or peak shoulder at β-lactoglobulin migration time (Figures 10. and 11.). Due to this occurrence β-lactoglobulin peak area percentage increased at least three times its initial value ($p<0.0001$) in bacteria containing samples. In a study done by Miralles *et al*. (2001) it was shown that under applied electrophoretic conditions para-κ-casein co-migrates with β-lactoglobulin. This co-migration appeared in electropherograms in form of double peak or peak shoulder. Co-migration of para-κ-casein and β-lactoglobulin renders their quantification impossible. In order to achieve better resolution urea concentration and pH of run buffer were decreased which allowed separation and quantification of para-κ-casein and β-lactoglobulin (Miralles *et al*. 2001). Due to this co-migration it was not possible to determine the exact level of β-lactoglobulin alone at time 6h.

Considering the specificity of V8 protease and its capability of cleaving Glu_{15}-Arg_{16} and Asp_{115}-Lys_{116} bonds in κ-casein (Livney *et al*. 2004.a) it is possible that the co-migrated peak is para-κ-casein like peptide f (16-115) with a migration time very similar to para-κ-casein.

Besides V8 protease aureolysin is proven to have hydrolysing effect on caseins, preferably by cleaving N terminal side of hydrophobic amino acids: alanine, valine, leucine, isoleucine, phenylalanine and tyrosine (Arvidson, 1973a, 1973b). Similar

results of aureolysin proteolytic activity on β-casein were obtained in study done by Sabat *et al.* (2008). The same amino acid preference was determined with the exception of valine and isoleucine, while no preference for glycine was found.

If caseinolytic activity of *S. aureus* proteases is compared to caseinolysis resulting from *S. aureus* antigen-provoked immunological response, results differ substantially. In a study done by Larsen *et al.* (2010) intramammary infusion with *S. aureus* lipotechoic acid (LTA) induced rise in SCC (peaked at 6h p.i.) and plasmin (peaked 12/48h p.i). Delayed plasmin peak (12/48h p.i) is probably a result of u-PA effect on plasminogen. Proteomic and peptidomic study revealed complete hydrolysis of β-CN and α_{s1-}CN in milk sampled 6h p.i., while no α_{s2-}CN was affected. Retrieved peptides were attributed to the activity of plasmin, cathepsin B and D, elastase and potentially cathepsin G. In milk samples from non-infected quarters dynamics of proteolysis was very slow. Identified fragments of hydrolysed β-CN are f163-190, f163-191, f190-208, f191-207, f193-206, f193-207, f193-208, f193-209, f194-207, while α_{s1}-CN fragments are f1-23, f2-22, f2-23, f24-37, and f25-37. The retrieved peptides substantially differ from peptides produced by proteolytic activity of V8 protease on β-casein. None of the cleaved peptide bonds include Glu or Asp for which V8 protease is highly specific.

4. Conclusion

Mastitis pathogen *S. aureus* caused proteolysis in UHT milk incubated for 6 hours at 37^0C. The overall decrease in casein content reached 21%. Increase in β-lactoglobulin manifested in electropherogram as a double peak might be explained by co-migration of para-κ-casein like peptide f (16-115) which is formed by V8 enzymatic cleavage of κ-casein. Moreover increase in β-lactoglobulin and decrease in κ-casein shared the same level of significance $p<0.0001$. Further investigations including detailed peptide identification are necessary in order to determine the exact proteolytic effect of *S. aureus* proteases on milk proteins.

Appendix

1. Reference list

Arvidson, S. (1973a). Hydrolysis of casein by three extracellular proteolytic enzymes from Staphylococcus aureus, strain V8. Acta pathologica et microbiologica Scandinavica Section B Microbiology and immunology, 81(5), 538-544.

Arvidson, S. (1973b). The role of calcium for stability and activity of an extracellular proteolytic enzyme from Staphylococcus aureus. Acta pathologica et microbiologica Scandinavica Section B Microbiology and immunology, 81(5), 545-551.

Banbula, A, Potempa, J., Travis, J., Fernandez-Catalán, C., Mann, K., Huber, R., Bode, W., et al. (1998). Amino-acid sequence and three-dimensional structure of the Staphylococcus aureus metalloproteinase at 1.72 A resolution. Structure (London, England : 1993), 6(9), 1185-93.

Madigan, T. M., Martinko, M. J., Stahl, D., & Clark, P. D. (2010).Brock Biology of Microorganisms, 13th ed, Benjamin Cummings

Considine, T., Healy, A., Kelly, A. L., & Mcsweeney, P. L. H. (1999). Proteolytic specificity of elastase on bovine b-casein. Food Chemistry, 66(4), 463-470.

Considine, T., Healy, A., Kelly, A. L., & Mcsweeney, P. L. H. (2000). Proteolytic specificity of elastase on bovine α_{s1}-casein. Food chemistry, 69(1), 19-26. Elsevier.

Considine, T., Geary, S., Kelly, A. L., & Mcsweeney, P. L. H. (2002). Proteolytic specificity of cathepsin G on bovine α_{s1}- and β-caseins/Spécificité protéolytique de la cathepsine G sur les caséines α_{S1} et β bovines. Food chemistry, 76(1), 59-67. Elsevier.

Considine, T., Healy, Á., Kelly, A. L., & McSweeney, P. L. H. (2004). Hydrolysis of bovine caseins by cathepsin B, a cysteine proteinase indigenous to milk. International Dairy Journal, 14(2), 117-124.

Corradini, D., Sprecacenere, L., (2003). Dependence of the Electroosmotic Flow in Bare Fused-Silica Capillaries from pH, Ionic Strength and Composition of Electrolyte Solutions Tailored for Protein Capillary Zone Electrophoresis. Chromatographia, 58, 587-596

Coulon, J.-B., Gasqui, P., Barnouin, J., Ollier, A., Pradel, P., & Pomies, D. (2002). Effect of mastitis and related-germ on milk yield and composition during naturally-occurring udder infections in dairy cows. Animal research (Print), 51(5), 383-393. EDP sciences.

Crimmins, D. L., Thoma, R. S., McCourt, D. W., & Schwartz, B. D. (1989). Strong-cation-exchange sulfoethyl aspartamide chromatography for peptide

mapping of Staphylococcus aureus V8 protein digests. Analytical Biochemistry, 176(2), 255–260.

de Jong, N., Visser, S., & Olieman, C. (1993). Determination of milk proteins by capillary electrophoresis. Journal of chromatography. A, 652(1), 207-13. Elsevier.

Drapeau, G. R., Boily, Y., & Houmard, J. (1972). Purification and properties of an extracellular protease of Staphylococcus aureus. The Journal of Biological Chemistry, 247(20), 6720-6726. Elsevier Ltd.

Fernandes, A., Oliveira, C., & Lima, C. (2007). Effects of somatic cell counts in milk on physical and chemical characteristics of yoghurt. International Dairy Journal, 17(2), 111-115.

Fox, P. F., & Kelly, A. L. (2006). Indigenous enzymes in milk: Overview and historical aspects—Part 1. International Dairy Journal, 16(6), 500–516.

Fox P. F. and McSweeney P. L. H., (1998) Dairy Chemistry and Biochemistry (1st ed), Thomson Science

Gröhn, Y. T., Wilson, D. J., González, R. N., Hertl, J. A., Schulte, H., Bennett, G., & Schukken, Y. H. (2004). Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. Journal of Dairy Science, 87(10), 3358-3374.

Halasa, T., Huijps, K., Østerås, O., & Hogeveen, H. (2007). Economic effects of bovine mastitis and mastitis management: a review. The Veterinary quarterly, 29(1), 18-31.

Heegaard, C. W., Rasmussen, L. K., & Andreasen, P. A. (1994). The plasminogen activation system in bovine milk: differential localization of tissue-type plasminogen activator and urokinase in milk fractions is caused by binding to casein and urokinase receptor. Biochimica et Biophysica Acta, 1222(1), 45-55.

Holland, J. W., Deeth, H. C., & Alewood, P. F. (2004). Proteomic analysis of k-casein micro-heterogeneity. Proteomics, 4(3), 743–752.

Hurley, M. ., Larsen, L. ., Kelly, A. ., & McSweeney, P. L. . (2000). The milk acid proteinase cathepsin D: a review. International Dairy Journal, 10(10), 673–681.

Ismail, B., & Nielsen, S. S. (2010). Invited review: Plasmin protease in milk: Current knowledge and relevance to dairy industry. Journal of dairy science, 93(11), 4999–5009.

Kantyka, T., Shaw, L. N., Potempa J. (2011). Papain-like proteases of *Staphylococcus aureus, (*p. 1-14) In: Robson, M., Dalton, J. (2011) Cysteine Proteases of Pathogenic Organisms, Springer

Kelly A. L. And McSweeney P. L. H. (2003), Indigenous proteinases in milk, in Fox, P. F. and McSweeney P. L. H. (Eds.), Advanced Dairy Chemistry, Proteins, Volume 1, part A, (3rd ed) Kluwer Academic/Plenum Publishers

Kosters, H. A., Wierenga, P. A., & Gruppen, H. (2010). SELDI-TOF-MS as a rapid tool to study food related protein–peptide interactions. Food Hydrocolloids, 24(6–7), 667–673. doi:10.1016/j.foodhyd.2010.03.011

Larsen, L. B., Benfeldt, C., Rasmussen, L. K., & Petersen, T. E. (1996). Bovine milk procathepsin D and cathepsin D: coagulation and milk protein degradation. The Journal of dairy research, 63(1), 119-130.

Larsen, L. B., Hinz, K., Jørgensen, A. L. W., Møller, H. S., Wellnitz, O., Bruckmaier, R. M., & Kelly, A. L. (2010). Proteomic and peptidomic study of proteolysis in quarter milk after infusion with lipoteichoic acid from Staphylococcus aureus. Journal of dairy science, 93(12), 5613-5626. Elsevier.

Larsen, L. B., Rasmussen, M. D., Bjerring, M., & Nielsen, J. H. (2004). Proteases and protein degradation in milk from cows infected with Streptococcus uberis. International Dairy Journal, 14(10), 899–907.

Larsen, L. B., Wium, H., Benfeldt, C., Heegaard, C. W., Ardö, Y., Qvist, K. B., & Petersen, T. E. (2000). Bovine milk procathepsin D: Presence and activity in heated milk and in extracts of rennet-free UF-Feta cheese. International Dairy Journal, 10(1-2), 67–73

Leitner, G., Krifucks, O., Merin, U., Lavi, Y., & Silanikove, N. (2006). Interactions between bacteria type, proteolysis of casein and physico-chemical properties of bovine milk. International Dairy Journal, 16(6), 648-654

Livney, Y. D., & Dalgleish, D. G. (2004.a). Specificity of disulfide bond formation during thermal aggregation in solutions of beta-lactoglobulin B and kappa-casein A. Journal of agricultural and food chemistry, 52(17), 5527–32.

Livney, Y. D., Schwan, A. L., & Dalgleish, D. G. (2004.b). A study of beta-casein tertiary structure by intramolecular crosslinking and mass spectrometry. Journal of Dairy Science, 87(11), 3638-3647.

Manabe, T. (1999). Capillary electrophoresis of proteins for proteomic studies. Electrophoresis, 20(15-16), 3116–21.

Malkoski, M., Dashper, S. G., O'Brien-Simpson, N. M., Talbo, G. H., Macris, M., Cross, K. J., & Reynolds, E. C. (2001). Kappacin, a novel antibacterial peptide from bovine milk. Antimicrobial agents and chemotherapy, 45(8), 2309–15.

Mazal, G., Vianna, P. C. B., Santos, M. V., & Gigante, M. L. (2007). Effect of somatic cell count on Prato cheese composition. Journal of dairy science, 90(2), 630-6. American Dairy Science Association.

Minkiewicz, P., Slangen, C. J., Lagerwerf, F. M., Haverkamp, J., Rollema, H. S., & Visser, S. (1996). Reversed-phase high-performance liquid chromatographic

separation of bovine kappa-casein macropeptide and characterization of isolated fractions. Journal of Chromatography A, 743(1), 123-135.

Miralles, B., Amigo, L., Ramos, M., & Recio, I. (n.d.). Analysing para-κ-casein and related peptides as indicators of milk proteolysis. Milchwissenschaft, 58(7-8), 412–415.

Miralles, B., Rothbauer, V., Manso, M. A., Amigo, L., Krause, I., & Ramos, M. (2001). Improved method for the simultaneous determination of whey proteins, caseins and para-kappa-casein in milk and dairy products by capillary electrophoresis. Journal of Chromatography A, 915(1-2), 225-230.

Mölkänen, T., Tyynelä, J., Helin, J., Kalkkinen, N., & Kuusela, P. (2002). Enhanced activation of bound plasminogen on Staphylococcus aureus by staphylokinase. FEBS letters, 517(1-3), 72–8.

O'Driscoll, B. M., Rattray, F. P., McSweeney, P. L. H., & Kelly, A. L. (1999). Protease Activities in Raw Milk Determined Using a Synthetic Heptapeptide Substrate. Journal of Food Science, 64(4), 606–611.

Olde Riekerink, R. G. M., Barkema, H. W., Scholl, D. T., Poole, D. E., & Kelton, D. F. (2010). Management practices associated with the bulk-milk prevalence of Staphylococcus aureus in Canadian dairy farms. Preventive Veterinary Medicine, 97(1), 20-28. Elsevier B.V.

Otte, J., Zakora, M., Kristiansen, K. R., & Qvist, K. B. (1997). Analysis of bovine caseins and primary hydrolysis products in cheese by capillary zone electrophoresis. Lait, 77(2), 241-257.

Østerås, O., Sølverød, L., & Reksen, O. (2006). Milk culture results in a large Norwegian survey--effects of season, parity, days in milk, resistance, and clustering. Journal of dairy science, 89(3), 1010-23. American Dairy Science Association.

Politis, I., Zavizion, B., Cheli, F., & Baldi, A. (2002). Expression of urokinase plasminogen activator receptor in resting and activated bovine neutrophils. The Journal of dairy research, 69(2), 195-204.

Prasad, L., Leduc, Y., Hayakawa, K., & Delbaere, L. T. J. (2004). The structure of a universally employed enzyme: V8 protease from Staphylococcus aureus. Acta Crystallographica Section D Biological Crystallography, 60(Pt 2), 256-259.

Prin-Mathieu, C., Le Roux, Y., Faure, G. C., Laurent, F., Béné, M. C., & Moussaoui, F. (2002). Enzymatic activities of bovine peripheral blood leukocytes and milk polymorphonuclear neutrophils during intramammary inflammation caused by lipopolysaccharide. Clinical and Diagnostic Laboratory Immunology, 9(4), 812–817.

Pyörälä, S., Hovinen, M., Simojoki, H., Fitzpatrick, J., Eckersall, P. D., & Orro, T. (2011). Acute phase proteins in milk in naturally acquired bovine mastitis caused by different pathogens. The Veterinary record, 168(20), 535.

Recio, I., Amigo, L., & López-Fandiño, R. (1997). Assessment of the quality of dairy products by capillary electrophoresis of milk proteins. Journal of chromatography B Biomedical sciences and applications, 697(1-2), 231-242.

Reksen, O., Sølverød, L., & Østerås, O. (2008). Relationships between milk culture results and composite milk somatic cell counts in Norwegian dairy cattle. Journal of Dairy Science, 91(8), 3102-3113.

Rice, K., Peralta, R., Bast, D., De Azavedo, J., & McGavin, M. J. (2001). Description of Staphylococcus Serine Protease (ssp) Operon in Staphylococcus aureus and Nonpolar Inactivation of sspA-Encoded Serine Protease. (E. I. Tuomanen, Ed.)Infection and Immunity, 69(1), 159–169.

Sabat, A. J., Wladyka, B., Kosowska-Shick, K., Grundmann, H., van Dijl, J. M., Kowal, J., Appelbaum, P. C., et al. (2008). Polymorphism, genetic exchange and intragenic recombination of the aureolysin gene among Staphylococcus aureus strains. BMC microbiology, 8, 129.

Sørensen, L. P., Mark, T., Sørensen, M. K., & Ostergaard, S. (2010). Economic values and expected effect of selection index for pathogen-specific mastitis under Danish conditions. Journal of Dairy Science, 93(1), 358-369.

Sørensen, S. B., Sørensen, T. L., & Breddam, K. (1991). Fragmentation of proteins by S. aureus strain V8 protease. Ammonium bicarbonate strongly inhibits the enzyme but does not improve the selectivity for glutamic acid. FEBS Letters, 294(3), 195–197.

Swaisgood, H. E. (2003), Chemistry of the caseins, in Fox, P. F. and McSweeney P. L. H. (Eds.), Advanced Dairy Chemistry, Proteins, Volume 1, part A, (3rd ed) (p. 140), Kluwer Academic/Plenum Publishers

Talbo, G. H., Suckau, D., Malkoski, M., & Reynolds, E. C. (2001). MALDI-PSD-MS analysis of the phosphorylation sites of caseinomacropeptide. Peptides, 22(7), 1093-1098.

Walstra P., Wouters, J. T. M., Geurts, T. J (2006), Dairy Science and Technology, 2nd ed., CRC Press

Weinberger R., (2000). Practical Capillary Electrophoresis, 2nd ed., Academic Press

Whist, A. C., Osterås, O., & Sølverød, L. (2009). Association between isolation of Staphylococcus aureus one week after calving and milk yield, somatic cell count, clinical mastitis, and culling through the remaining lactation. The Journal of dairy research, 76(1), 24-35.

Zavizion, B., White, J. H., & Bramley, A. J. (1997). Staphylococcus aureus stimulates urokinase-type plasminogen activator expression by bovine mammary cells. The Journal of infectious diseases, 176(6), 1637–40.

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