When does the protein profile in milk normalize after antibiotic treatment against clinical mastitis?

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Master Thesis

Institutionen för Livsmedelsvetenskap Publikation nr 293

Swedish University of Agricultural Sciences Uppsala 2010
Department of Food Science

Master thesis, Agricultural programme, Food Science, Swedish University of Agricultural Sciences

Självständigt arbete 30 högskolepoäng, agronomprogrammet livsmedel, SLU

Title:
When does the protein profile in milk normalize after antibiotic treatment against clinical mastitis?

Titel:
När återställs mjölkens proteinprofil efter antibiotikabehandling mot klinisk mastit?

Key words:
mastitis, protein, casein

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Course code:
Ex0427

Type of student level:
30 ECTS Advanced D

Place of publication:
Uppsala

Year of publication:
2010

Cover picture: Hedvig Åkesson

Electronical publication: http://stud.epsilon.slu.se

Abstract

Concentration of protein in bovine milk is one of the most significant milk quality parameters, to a large extent determining the price for milk to the producer. Mastitis is a common disease among dairy cows, negatively affecting not only milk yield but also milk protein composition. Milk from mastitic cows tends to have lower cheese yield, negatively affected processability properties and sensory quality, due to changed protein quality and composition. Poorer milk protein quality would have an economically negative impact on dairy industry. Only few studies have investigated the short term effects of mastitis on milk protein composition. Considering the severe secretory damage on the epithelial gland cells caused my mastitis, the risk of altered milk protein concentrations immediate after withdrawal time is imminent.

The objective with this study was to follow the changes in milk protein composition from the time of mastitis treatment during the withdrawal period and until five days after the withdrawal time has expired. The proteins studied are α-lactalbumin and β-lactoglobulin, α_{S2}-casein, α_{S1}-casein, α_{S0}-casein, κ-casein and β-casein. The hypothesis was that protein composition is still affected after the elapse of the withdrawal time.

Sample collection and analysis were performed during summer and autumn 2009. The animals used were seven cows with mastitis, of which five Swedish Red (SRB) and two Holstein breed. Cows were housed at Jälla agricultural school and at Kungsängens Research Station, close to Uppsala, Sweden. Collection of udder composite milk samples started on the first milking after administration of the antibiotic treatment and proceeded during treatment and withdrawal periods until five days after end of withdrawal time. From each animal, approximately 30 samples were taken in connection with morning and afternoon milking over 15 days. The experiment period was divided into three parts: treatment period, withdrawal period and the five days after the end of the withdrawal period. Protein analysis was performed with capillary electrophoresis (CE). CE separates protein due to their physiochemical properties by an electric gradient making proteins migrate through a capillary in different velocities depending on their respective charge in a determined pH. Protein quantification was obtained by comparing electropherogram protein peak areas with those of Sigma standards. Concentrations of individual proteins during treatment period, withdrawal period and post withdrawal period respectively were compared with concentrations in reference bulk tank milk. Also, milk from cows infected by different pathogens was compared regarding protein composition.

Concentrations of whey proteins analysed, α-LA and β-LG (α-lactalbumin and β-lactoglobulin) did not change noticeable during the period of mastitis treatment and recovery. Concentrations of individual caseins analysed (α_{S2}-CN, α_{S1}-CN, α_{S0}-CN, κ-CN and β-CN) were found to be highest during treatment period, lower during the withdrawal period and lowest after the withdrawal period. All casein concentrations except that for κ-CN were lower during the first five days after the withdrawal time had expired than in normal bulk tank milk. The hypothesis that protein composition is still affected after the withdrawal time has expired cannot be rejected. The question when protein concentrations in milk normalize after treatment can still not be answered. For most animals and most proteins analyzed in this study, protein levels seemed to continue decreasing or increasing by the last sample taking. In future studies, the length of the experiment period must be extended to find the time when protein concentrations may normalize or stabilize. Concentrations of individual proteins showed large variation between animals. Various pathogens causing the mastitis may also affect milk protein concentrations differently. Therefore, to be able to draw more reliable conclusions from results in future studies, number of animals studied must be greater.

Sammanfattning

Proteinsammansättning är en av de viktigaste kvalitetsparametrarna hos mjölk. Samtidigt påverkar mastit, en av de vanligaste produktionssjukdomarna hos mjölkkor, både proteinhalten och proteinsammansättningen i mjölken negativt. Mjölk från kor med mastit har generellt sämre processegenskaper, sensorisk kvalitet och hållbarhet och ger färre kg ost per kg mjölk, vilket torde innebära stora ekonomiska förluster för mejerierna. Karenstiden, d.v.s. den tiden då mjölken inte får levereras till mejeriet, syftar till att restsubstanser av använda läkemedel i mjölken ska hinna minska till lägsta tillåtna gränsvärde. När karenstiden bestäms beaktas dock inte mjölkens sammansättning. Få studier har utrett hur proteinsammansättningen i mjölk från kor med klinisk mastit förändras under behandling och karenstid. Med bakgrund av de allvarliga skadorna mastit orsakar i den sekretoriska juvervävnaden är sannolikheten stor att mjölkens proteinsammansättning inte har återgått till det normala vid karenstidens slut.

Syftet med denna studie var att följa förändringar i mjölkens proteinsammansättning från behandlingens början till fem dagar efter karenstidens slut. De enskilda proteinerna vars koncentration analyserades var vassleproteinerna α-laktalbumin, β-laktoglobulin och kaseinerna α_{S2}-kasein, α_{S1}-kasein, α_{S0}-kasein, κ-kasein och β-kasein. Hypotesen var att proteinsammansättningen hos mjölken från en ko med mastit är påverkad av mastiten ännu efter karenstidens slut.

Studien utfördes under sommaren och hösten 2009. Korna som användes i försöket var av rasen SRB (Svensk Röd och vit Boskap) och två av rasen SRB (Svensk Låglandsboskap), samtliga med mastit. Djuren fanns på Kungsängens försöksgård och på Jälla Naturbruksgymnasium utanför Uppsala. Mjölkningspersonalen tog samlingsprover från juvrets alla juverdelar vid varje mjölkningstillfälle med start efter behandlingens början. Provtagningen pågick t.o.m. fem dagar efter karenstidens slut. Behandlingstiden och karenstiden var vanligen fem respektive sex dygn. Försöksperioden delades in i tre perioder: behandlingstid, karenstid och fem dygn efter karenstid. För analys av mjölkens proteinsammansättning användes kapillärelektrofores (CE). CE separerar proteiner m.a.p. fysiokemiska egenskaper med en elektrisk gradient som får proteiner att migrera olika snabbt genom en kapillär beroende på sin laddning i ett givet pH. Koncentrationer av de enskilda proteinerna under behandlingstid, karenstid och efter karenstiden jämfördes med koncentrationer i normal tankmjölk. Vidare jämfördes proteinsammansättningen i mjölk mellan kor hos vilka olika patogener orsakat mastiten.

Koncentrationer av vassleproteinerna (α-laktalbumin och β-laktoglobulin) förändrades inte nämnvärt under försöksperioden. Kaseinerna däremot (α_{S2}-kasein, α_{S1}-kasein, α_{S0}-kasein, κ-kasein och β-kasein) visade sig ha högst koncentration under behandlingstiden för att sedan sjunka under karenstiden och vara som lägst efter karenstidens utgång, då halterna var lägre än i normal tankmjölk. Hypotesen att proteinsammansättningen ännu är påverkad av mastiten efter karenstidens slut kunde inte förkastas. Hos flertalet djur verkade förändringen i koncentration för merparten proteiner fortsätta vid tidpunkten för den sista provtagningen. Tidpunkten då proteinkoncentrationen i mjölken eventuellt stabiliseras eller normaliseras torde vara senare än 15 dygn efter påbörjad behandling mot mastit. Variationen mellan individer m.a.p. koncentration av proteiner i mjölken var stor. Olika patogener orsakande mastiten antas också ha påverkat proteinsammansättningen på olika sätt. I fortsatta studier krävs ett större djurantal och en längre försöksperiod för att kunna dra säkra slutsatser av resultaten.

Abbreviations

Casein number:	Percentage of casein of total protein
Clinical mastitis:	Udder inflammation with visible symptoms in udder or/and in milk
Subclinical mastitis:	Udder inflammation without any visible symptoms
CN:	Casein
CE:	Capillary Electrophoresis
CZE:	Capillary Zone Electrophoresis
EOF:	Electro Osmotic Flow
HPLC:	High Pressure Liquid Chromatography
LIF:	Laser-Induced Fluorescence
PMN:	Polymorphonuclear leucocytes
Proteolysis index:	Concentration of γ-CN divided by concentration of β-CN
SCC:	Somatic Cell Count, somatic cells per ml of milk
UV:	Ultra Violet (high energy light, used e.g. in detectors)

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1. Introduction

1.1 Background

Concentration of protein in bovine milk is one of the most significant milk quality parameters, to a large extent determining the price for milk to the producer (Åkerstedt *et al.*, 2008). However, for cheese production, also protein quality and composition is crucial, since lower proportions of caseins generally result in lower cheese yield and impaired processing properties of milk, including heat stability, texture and sensory quality (Le Roux *et al.*, 1995; Auldist *et al.*, 2006; Leitner *et al.*, 2006; Wedholm *et al.*, 2006; Åkerstedt *et al.*, 2008). Hence, casein number would be an additional accurate quality parameter of milk protein quality (Åkerstedt *et al.*, 2008). Barbano *et al.*, (1991) states that for a better milk payment system reflecting true differences in milk functional value, a better qualitative index of overall milk quality is needed. Furthermore, an economical and accurate analysis of casein may be of great value for dairy industries when incorporated in the milk payment system. Milk protein composition, the relative concentrations of the different milk proteins, is the most economically important factor of bovine milk quality besides milk yield, protein and fat content. Milk technological properties and nutritional value is to a large extent determined by its protein composition (Heck *et al.*, 2008). However it is not included in the milk payment system. Udder health state influences milk composition and milk yield (Barbano *et al.*, 2005; Leitner *et al.*, 2006). Only with all udder quarters being healthy, the production of milk with a quality meeting consumer expectations and processor demands is guaranteed (Hamann, 2002). Bovine milk protein composition has been extensively studied due to its importance for dairy industry (Auldist *et al.*, 1995; Urech *et al.*, 1999; Ng-Kwai-Hang, 2002; Barbano *et al.*, 2005; Fernandes *et al.* 2008). Extensive research has been done to study changes of milk composition due to mastitis. Few studies though have investigated the short term effects of mastitis on milk protein composition. To our knowledge, there are currently no studies reporting on how long time after antibiotic treatment against clinical mastitis milk protein composition normalizes.

Mastitis is the most significant disease in bovine dairy herds (Viguier *et al.*, 2009) and accounts for almost 50% of veterinary treated diseases (Swedish Dairy Association, 2008). Mastitis leads to decreased synthesis of milk and milk components, elevated SSC and overall reduced milk quality with altered milk protein composition (Harmon, 1993; Barbano *et al.*, 2005). Abnormal protein composition has adverse economical effects for the dairy industry, e.g. shorter shelf life of dairy products and lower cheese yield (Lemieux & Simard, 1992; Barbano *et al.*, 2005; Fernandes *et al.* 2008). Mastitis has negative impact both on individual animal welfare and on farm and dairy industry economics (Hogarth *et al.*, 2004; Kietzmann & Bäumer, 2008; Viguier *et al.*, 2009). The intramammary infection may develop into an inflammation, leading to permanent injuries in the udder secretory tissue and reduced milk synthesis.

1.2 Objectives

The objective of this study was to follow the changes in milk protein composition during the time of mastitis treatment until the elapse of the withdrawal time, and to investigate if the protein composition is normal when milk is delivered to the dairy again. The proteins studied were the whey proteins α-lactalbumin and β-lactoglobulin, and the caseins α_{S2}-

casein, α_{S1}-casein, α_{S0}-casein, κ-casein and β-casein. The hypothesis was that the protein composition was still not normal after the elapse of the withdrawal time.

2. Literature review

2.1 Milk proteins

Milk protein is a heterogeneous group of compounds, serving important biological roles (Ng-Kwai-Hang, 2002). Milk nutritional value and technological properties are highly determined by its protein composition. In bovine milk, more than 200 proteins have been characterized and more are continuously getting discovered. The most important proteins in milk are caseins and whey proteins. Bovine milk also includes e.g. miscellaneous proteins and milk fat globule proteins.

2.1.1 Caseins

Caseins are a group of proteins specific for milk, constituting approximately 80% of total bovine milk protein. According to their net electrical charge, caseins are divided into groups: α_{S2}-casein (α_{S2}-CN), α_{S1}-casein (α_{S1}-CN), α_{S0}-casein (α_{S0}-CN), κ-casein (κ-CN) and β-casein (β-CN). Caseins have both acid and basic groups, thereby being ampholytes, with an average isoelectric point of 4.6. (Julien *et al.*, 1985). The low degree of secondary structure of caseins is due to their high content of the amino acid proline (Ng-Kwai-Hang, 2002). Caseins possess the unique property to bind calcium in high amounts, thereby remaining in solution at normal pH and in presence of calcium (Andrén, 2007). The main different forms of caseins are described shortly below:

In milk three types of **α-casein** are present: α_{S2}-CN, α_{S1}-CN and α_{S0}-CN. In contrast to κ-CN, α-CN is sensitive to calcium ions (Julien *et al.*, 1985). The most hydrophilic casein is α_{S2}-casein, due to its negative N-terminus and positive C-terminus (Ng-Kwai-Hang, 2002; Walstra *et al.*, 2006). α_{S2}-casein exists in multiple forms with different degrees of phosphorylation and forms dimers, appearing as multiple peaks in capillary electrophoresis analysis (Walstra *et al.*, 2006; Heck *et al.*, 2008). α_{S2}-casein contains two cysteine residues, resulting in a disulfide linkage. Among caseins, α_{S1}-CN has the highest net charge and phosphate content. It does not form dimers. α_{S1}-CN has two hydrophobic regions in each end of the polypeptide chain and in the middle a polar region in which almost all proline residues are found. α_{S2}-CN is more sensitive to calcium precipitation than α_{S1}-casein. The least studied of the α_S-caseins is α_{S0}-casein. It is degraded by plasmin activity to the same extent as α_{S2}- and α_{S1}-CN (García-Risco *et al.*, 2003). Among the caseins, **κ-casein** is widely the most studied (Ng-Kwai-Hang, 2002) and also of interest in mastitis research (Yang *et al.*, 2009). κ-CN accounts for about 10% of total bovine milk CN and is crucial in its role as casein micelle stabilizer. κ-CN is in contrast to other caseins water soluble as a result of its hydrophilic C-terminal (Walstra *et al.*, 2006). κ-CN is insensitive to high concentrations of calcium ions and contains low concentrations of phosphorus (Julien *et al.*, 1985). The properties of κ-CN are used in cheese manufacture. By adding rennet, e.g. chymosin, to milk the negatively charged C-terminal of κ-CN is cut, resulting in free glucomacropeptide (GMP) or caseinmacropeptide (CMP) and hydrophobic aggregation of the casein micelles (Walstra *et al.*, 2006; Andrén, 2007). The casein micelles make out the cheese curd, which is

further pressed and formed to cheese. Another property of κ-casein is its cysteine residues, resulting in disulfide bonds between κ-casein molecules. The disulfide bonds can be broken by heating the milk (Ng-Kwai-Hang, 2002; Andrén, 2007). The disrupted disulfide bonds result in exposed thiol ends, catalyzing disulfide interchange with whey proteins, especially β-lactoglobulin (Yang *et al.* 2009). In the production of many fermented dairy products, milk is heated, and primarily β-lactoglobulin aggregates on casein micelles. This gives the fermented milk product its characteristic texture and high water holding capacity (Andrén, 2007). Like α-caseins, **β-casein** is sensitive to calcium precipitation. It contains less phosphorus and more sulphur than α-caseins (Julien, *et al.*, 1985). Being the most hydrophobic casein, β-CN forms the major part of micelles, surrounded by an ampholytic layer of κ-CN (Ng-Kwai-Hang, 2002; Walstra *et al.*, 2006). Average isoelectric point for β-CN is 4.9. β-CN contains many proline residues at its hydrophobic region and a highly negatively charged N- terminal (Walstra *et al.*, 2006). When hydrophobic interactions are weakened due to low temperature, e.g. during cold storage, β-CN is released into the whey fraction and degraded by plasmin into proteose peptones and γ-CN.

In all mammalian milk, casein is mainly present as micelles in presence of calcium phosphate (Ng-Kwai-Hang, 2002; Yang *et al.* 2009). Casein micelles range between 30 and 300 nm in size (Julien, *et al.*, 1985). The loose structure of the casein micelle makes it easily degraded by proteolytic enzymes, serving a well-balanced source of amino acids for the young mammal (Ng-Kwai-Hang, 2002). The protein composition of casein micelles is generally: 1κ : 3β : 5α, the proportion of κ-CN negatively and β-CNs positively correlated with size of micelles (Julien, *et al.*, 1985). Acidification to a pH identical to the average isoelectric point of caseins neutralizes negative charges thus destabilizing the casein micelle. Coagulation of caseins may start as soon as at pH 5 at normal temperatures. Also, salts and ion strength influence stability of casein micelles. Increase of any negative charge (citrates or phosphates) increases micelle stability in most milks whereas increasing amount of calcium ions destabilizes micelles.

2.1.2 Whey proteins

Whey proteins are generally more heat sensitive and have more organized secondary structure than caseins (Julien *et al.*, 1985; Ng-Kwai-Hang, 2002). The two main whey proteins are β-lactoglobulin (constituting 80% of total whey protein) and α-lactalbumin. **β-lactoglobulin** is the most abundant bovine whey protein. β-lactoglobulin binds retinol, possibly serving as a carrier of vitamin A (Ng-Kwai-Hang, 2002; Hogarth *et al.*, 2004). **α-lactalbumin** binds calcium and is involved in the synthesis of lactose and thereby regulation of total milk secretion, since lactose amount regulates milk yield by osmotic mechanisms. α-lactalbumin constitutes approximately 13% of total whey protein. Immunoglobulins constitute a very heterogenic group of whey proteins, together with serum albumins protecting against infections.

2.1.3 Variation in protein composition

Bovine milk protein concentration and composition have been extensively studied due to its importance for dairy industry and human health. Protein composition undergoes great variations due to factors such as genetics and breed, age, lactation stage, season, health state and nutritious status of the animal (Ng-Kwai-Hang, 2002). Protein concentration is on average 3.4 % (Lindmark-Månsson *et al.*, 2005; Walstra *et al.*, 2006). Heck *et al.* (2008) showed a large variation among individual animals in relative concentrations of

the main milk proteins. One important measure of milk protein quality is casein number, defined as casein concentration divided by total protein concentration. Casein number decreases with cow age and incidence of mastitis (Ng-Kwai-Hang, 2002). Milk proteins change in concentrations and in total yields over the year and over the lactation period (Verdi *et al.*, 1987; Barbano *et al.*, 1991; Auldist *et al.,* 1995; Le Roux *et al.* 1995; Martin *et al.* 1997; Auldist *et al.* 1998). Auldist *et al.* (1995) found the change in milk constituents over lactation to follow the same pattern for mastitic as for healthy cows, being confirmed by a later study (1998) as well as by Martin *et al.* (1997). Protein quality decreases with lactation stage regardless of SCC (Le Roux *et al.*, 1995). Barbano *et al.* (1991) found a significant decrease in casein number with lactation stage. Auldist *et al.* (1995) found casein number being lowest in late lactation. In early lactation, casein yield is as highest (Barbano *et al.*, 1991; Ng-Kwai-Hang, 2002). Milk composition and somatic cell count (SCC) change also during the milking occasion, with SCC being higher and casein number being lower in the foremilk and strip milk than in bucket milk (Urech *et al.* 1999). A large genetic variability exists among all main milk proteins, (Andrén, 2007; Heck *et al.* 2007). Genetic variants of a protein include deletion, substitution or addition of amino acids (Andrén, 2007) changing its three dimensional structure and physicochemical properties like hydrophobicity and net electric charge, this affecting milk composition, processing properties of the milk and product quality (Ng-Kwai-Hang, 2002; Andrén, 2007). Due to differences in composition as well as the large genetic polymorphism among milk proteins, the processing properties such as clotting time are very hard to predict for a certain milk batch (Andrén, 2007).

2.2 Mastitis

Mastitis is the most extensive disease of dairy cows with enormous effect on farm economics (Viguier *et al.*, 2009). In Sweden, more than 17% of dairy cows are treated for diseases in the udder every year (Lägesrapport Djurhälsa, Swedish Dairy Association, 2009). The progression of mastitis is a complex scheme of events leading to increased permeability of the blood-milk barrier, raised somatic cell count, reduced synthesis of milk constituents and changed composition of the milk (Harmon, 1993; Auldist *et al.*, 1995; Ma *et al.*, 1999; Hogarth *et al.*, 2004; Barbano *et al.*, 2005; Leitner et al, 2006). Mastitis results in increased casein hydrolysis, changed leucocyte distribution and decreased lactose concentration (Leitner *et al.*, 2006). The complexity of mastitis is reflected in the variety of microorganisms and the variation in effect on physiological responses (Harmon, 1993; Leitner *et al.*, 2006). Mastitis is an inflammatory reaction in the udder as a response to mechanical damage or infection of pathogenic microorganisms, mainly bacteria (Harmon, 1993; Barbano *et al.*, 2005). The bacterial infection in the mammary gland and accumulation of bacteria induce the onset of the mammary defence system (Leitner *et al.*, 2006). The mammary gland is protected by a range of various defence mechanisms against pathogens (Yang *et al.* 2009). This inflammatory response being localized exclusively to the mammary gland not including systemic symptoms such as redness of the udder or oedema, the inflammation is classified as subclinical (Harmon, 1993; Leitner *et al.*, 2006). Subclinical mastitis is characterized by an increase in SCC, altered milk composition and reduced milk secretion but without any visible changes appearing in the milk or the udder (Harmon, 1993; Kietzmann & Bäumer, 2008). Individual cows may vary largely in SSC without showing any visible symptoms (Lindmark-Månsson *et al.* 2005). When the inflammatory response includes systemic symptoms such as elevated rectal temperature and swelling and redness of the udder the inflammation is defined as clinical (Harmon, 1993; Kietzmann & Bäumer, 2008).

Mastitis may also become chronic, resulting in permanent inflammation of the udder (Viguier *et al.*, 2009). The major mastitis causing pathogens are *Staphylococcus aureus*, *Streptococcus dysagalactiae* and *Streptococcus uberis*, the two latter mostly of environmental origin. Clinical mastitis and marked compositional changes of milk are infrequently associated with other than those major pathogens.

2.2.1 Mastitis control measures, diagnosis and therapy

Mastitis control implies mainly proper milking hygiene, actions to reduce exposure to the pathogens causing mastitis, e.g. clean environment, and therapy, usually relying on antibiotics (Eckersall *et al.*, 2001; Kietzmann & Bäumer, 2008; Pyörälä, 2008). Since the risk for mastitis is greatest at parturation, udder health should be assessed as quick as possible after calving. During the first days after calving, diagnosis of mastitis must be adjusted for the raised SCC in connection with parturition (Pyörälä, 2008). Early detection and treatment of mastitis is crucial for minimization of economic losses (Yang *et al.*, 2009; Viguier *et al.*, 2009). Mastitis diagnosis on the farm relies e.g. on palpation of the udder and observation of the foremilk appearance. Since some cows show symptoms only in secretions whereas others also in the teats or the udder, regular examination by trained dairy personnel is required for diagnosis of mastitis (Eckersall *et al.*, 2001). Diagnosis of mastitis is based on confirming the presence of bacteria in combination with high somatic cell count. Mastitic milk is characterized by increased somatic cell count (SCC) (Ma et al 1999; Larsen *et al.*, 2004) and SCC is the most commonly used standard for udder health evaluation at dairy and farm level (Leitner *et al.*, 2006). Somatic cell count is an indicator of the intensity of the cellular immune defence, reflecting the integrity of the blood-udder barrier (Hamann, 2002) and seen as a good indicator also for bio-chemical changes in the composition of milk (Le Roux *et al.* 2002). Average bulk tank somatic cell count in Swedish dairy herds is 207 000 cells/ml (Swedish Dairy Association, 2009). In the case of mastitis, SCC may elevate up to millions of cells per ml milk (Larsen *et al.*, 2004) due to the migration of immune defence cells into the mammary tissue. Treatment of bovine mastitis usually relies on antibiotics and mastitis therapy is the most common use of antibiotics in dairy cows worldwide (Zecconi, A., 2007; Kietzmann & Bäumer, 2008). In Sweden, antibiotic treatment is commonly applied by a veterinarian after diagnosis (Swedish Dairy Association, 2007e). Antibiotic residues in milk is a consumer ethical problem as well as an obstacle in milk fermentation. Extensive and regular use of antibiotics may result in resistant bacteria. Zecconi (2007) concludes that the four last decades of antimicrobial drug use in the treatment of mastitis has not led to an observable progression of resistance among the disease causing bacteria, however pointing out the need of monitoring the potential risk of resistant mastitis causing bacterial strains.

2.2.2 Costs of mastitis

With treatment and veterinary costs, losses of income from withdrawal of milk during and after treatment period and lower payment for high SCC milk, mastitis has huge impact on farm economics (Viguier *et al.*, 2009). Within European Union, milk from cows with clinical mastitis is not to be delivered to the dairy (Swedish Dairy Association, 2007c). Milk from animals treated with drugs may not to be delivered before the elapse of the withdrawal time (Swedish Dairy Association, 2007d) and it must be ensured that milk to be delivered to the dairy plant is free from antibiotic traces.

Beside the acute cost of withdrawal of milk during treatment and withdrawal time, mastitis inflammation may permanently reduce milk component synthesis by irreversible damage to mammary secretory tissue. Subclinical mastitis causes overall greater costs than clinical mastitis (Viguier *et al.*, 2009). An important cost of mastitis on the farm level is slaughter of animals with chronic mastitis (Hogarth *et al.*, 2004; Kietzmann & Bäumer, 2008). Abnormal milk composition has negative economical effects on dairy economy, leading to reduced product quality, reduced shelf life and sensory quality and reduced cheese yield (Lemieus & Simard, 1992; Fernandes *et al.*, 2008). Mastitis is not only affecting farm and dairy industry economy but also welfare of individual animals (Hogarth et al, 2004; Kietzmann & Bäumer, 2008).

2.3 Variation in milk composition due to mastitis

2.3.1 General effects of mastitis on milk composition

Mastitis and the elevation of SCC are accomplished by noticeable changes in milk composition (Auldist *et al.* 1998b; Le Roux *et al.* 2002; Hamann, 2002; Leitner *et al.*, 2006; Forsbäck *et al.*, 2009). Both clinical and subclinical mastitis may affect milk composition and manufacturing properties by altering protein composition, concentration of salts and lactose (Harmon, 1993; Auldist & Hubble, 1998; Forsbäck *et al.*, 2009). The extent of change of milk composition is dependent on infecting agent and severity of the inflammation (Pyörälä, 2003). The mechanisms behind altered milk composition are reduced synthesis of milk components e.g. caseins, increased proteolytic activity and permeability of the blood-milk barrier due to damage on the mammary gland alveolar cells (Le Roux *et al.*, 2002). Mastitis reduces synthesis of lactose, associated with the decrease in milk yield (Auldist *et al.*, 1995; Hamann *et al.*, 2002). Lactose concentration is lower in high SSC milk than in low SCC milk (Leitner *et al.*, 2006; Wickström *et al.*, 2009). Leitner *et al.* (2006) did not find any significant effects of mastitis on fat or total protein concentration. This is in contrast with other studies, finding total protein concentration to be higher in mastitic milk (Auldist *et al.*, 1995; Urech *et al.*, 1999), suggesting the reason to be influx of plasma proteins exceeding the breakdown and reduced synthesis of milk proteins. However, total milk protein concentration is usually not found to increase during intramammary infection (Hogarth *et al.*, 2004). Åkerstedt *et al.* (2008) found lower protein concentrations in high SCC milk than in low SCC milk. In a review on mastitis and effects on milk composition Pyörälä (2003) reports protein concentration being lower in mastitic milk, which is visualized in *Table 1* below. A recent Chinese study by Li *et al.* (2008) is one of few investigating change in milk composition during antibiotic mastitis therapy. Li *et al.* found “little” but no significant changes in concentrations of fat, protein, casein, and total solids during the time of mastitis therapy; however lactose significantly decreased with treatment time.

Table 1. *Main changes in the relative concentrations of milk constituents caused by mastitis. From Pyöräla, 2003.*

Decrease	Degree of change	Increase	Degree of change
Quarter milk yield	- (- -)	Somatic cell count	+ + +
Dry matter	-	Whey proteins	+ + +
Lactose	-	Bovine serum albumin	+
Fat	-	Immunoglobulins	+ + +
Long-chained fatty acids	-	κ-casein	+ (+)
Total casein	- -	Proteose peptones	+ +
A_{S1}-casein	- -	Free fatty acids	+ +
B-casein	- - -	Short-chained fatty acids	+
A-lactalbumin	-	Sodium	+ +
B-lactoglobulin	- - -	Chloride	+ +
Calcium	- - -	Lactate	+ + +
Magnesium	- - -	Enzyme activity	
Phosphorus	- - -	Lipase	+ +
Zinc	-	Lysozyme	+ + +
Potassium	-	NAGase	+ + +
		B- glucuronidase	+ + +
		Plasmin	+ + +

Since mastitis not usually occurs in all udder quarters simultaneously, SCC and milk composition may vary significantly between udder quarters (Le Roux *et al.,* 1995; Urech *et al.*, 1999; Hamann, 2002; Lindmark-Månsson, 2005; Leitner *et al.*, 2006; Forsbäck *et al.*, 2009). Leitner *et al.* (2006) found significantly increased whey protein concentration and lower lactose concentration in milk from infected udder quarters compared to uninfected. Lindmark-Månsson (2005) stated that the possible difference in milk composition between udder quarters may motivate automated milking systems excluding abnormal milk from individual udder quarters. However, in spite of the anatomical structure of the mammary gland with four separate glands, some reports show interactions between udder quarters. Merle *et al.* (2007) found infection of one udder quarter significantly affecting SCC in neighbouring udder quarters. Larsen *et al.* (2004) found the infection in one gland affecting milk protein degradation also in other udder quarters, therefore suggesting that separation of high SCC milk at quarter level by automated milking systems may not be to recommend, while the milk composition in the healthy glands although with normal SCC may be influenced by the inflammation in the infected gland. In contrast to the findings of Merle *et al.* (2002) and Larsen *et al.* (2004), Lindmark-Månsson (2005) found differences in lactose content and protein content in relation to SCC between different udder quarters being negligible. Forsbäck *et al.* (2009) found that an inflammation in infected udder quarters affected the SCC to a much greater extent than the milk composition. In a review by Le Roux *et al.* (2002) a wide variability

in milk composition is reported for milk with SCC in the range of 400 000 – 600 000 cells/ml, but normally no major changes in milk composition at SCC below 200 000 cells/ml. Hamann *et al.* (2002) found concentrations of each milk component changing distinctly being no longer within its normal physiological range by SCC reaching 100 000 cells/ml, which was also found by Pyörälää (2003), suggesting the physiological threshold of quarter SCC to be 100 000 cells/ml. The altered milk composition at SCC above 100 000 cells/ml resulted in a disturbance of the processing and technological properties of milk (Hamann, 2002) with changes in milk composition occurring already at SCC as low as 10 000 cells/ml. According to Hamann *et al.* (2002), the official standards for optimal milk quality aim to eliminate possible consumer health risks and have weak relations to the criteria measuring udder health, thus resulting in an inconsistency between the maintaining of udder health and the economical signals directing milk quality to meet consumer and processor demands. The requirements of the Pasteurized Milk Ordinance (PMO) for maximal bacteria and SSC are not intended as dairy industry quality standards for raw product quality but aim to ensure public health (Barbano *et al.*, 2005). In Sweden, except for the SCC, the milk must be analysed for visible appearance (24 times a year), avoiding clotted and blood mixed milk being delivered to the dairy plant. Bulk farm milk must also be assessed for freezing point (6 times a year), aiming to discover abnormal milk composition resulting from e.g. mastitis. Dairy companies are responsible for the control. Additionally, occurrence of inhibitory substances (e.g. antibiotic residues) is to be controlled 12 times yearly and at least 3 times per month in the bulk milk tanker (Swedish Dairy Association, 2007b).

2.3.2 Effect of mastitis on protein profile

Mastitic cows tend to yield milk with lower casein number (Wickström *et al.*, 2009; Ng-Kwai-Hang, 2002) and casein concentration throughout lactation (Auldist *et al.*1995). The effect of mastitis on casein concentration can be explained by reduced synthesis of caseins in combination with breakdown of caseins (Wickström *et al.*, 2009).

Altering casein concentration in milk would change its physic-chemical properties (Yang *et al.*, 2009). Auldist *et al.* (1996) found high SCC milk having significantly lower proportions of κ- and β-CN and higher proportions of γ-CN, which was thought to be due to the elevated plasmin activity in high SCC milk. As a result of mastitis, the ability of producing casein in the mammary gland is often severely reduced by the damage on the mammary epithelial cells (Auldist *et al.*, 1995; Hogarth *et al.*, 2004). Fernandes *et al.* (2008) found a reduction in all casein fractions except for α_{S2}-CN in high SSC milk during storage. Hogarth *et al.* (2004) followed concentrations of individual whey proteins during mastitis infection, finding increased concentrations of proteins of serum origin including albumin and serotransferrin. Auldist *et al.* (1995) found a significant positive correlation between SCC and concentration of whey proteins, suggesting the influx of plasma proteins into the mammary gland to be the reason for the increased concentrations of whey proteins, which was also suggested by Urech *et al.* (1999) and Harmon *et al.* (1994). Lindmark-Månsson *et al.* (2005) found no major differences in β-LG and α-LA concentration in udder quarters with high SSC compared to glands with low SCC. Åkerstedt *et al.* (2008) found reduced concentrations of both whey protein and caseins in high SCC milk, suggesting that also synthesis of whey protein was reduced in the high SCC milk. The major whey proteins β-LG and α-LA where found to decrease in response to mastitis infection, in agreement with Hogarth *et al.* (2004), who suggested reduced synthesis of proteins as a response to the infection or as a result of damage on the mammary epithelium by bacterial toxins. The proteolysis of whey proteins as well as of caseins is thought to be an action of proteinases derived from blood, bacteria or from

leucocytes. Major whey proteins though are quite resistant to proteolysis. Serum derived whey proteins such as serum albumins were found to increase as a result of mastitis, which was associated with increased permeability of the blood-milk barrier (Hogarth *et al.* 2004).

2.4 Capillary electrophoresis for analysis of milk proteins

Proteins can be separated and identified using a range of different methods. Separation of proteins depends on physiochemical properties e.g. charge, solubility in different agents and pH. Milk proteins are heterogeneous a group of compounds and that is why they are difficult to fractionate and isolate. Many methods separating caseins use their different isoelectric precipitation (Ng-Kwai-Hang, 2002). A range of electrophoretic and chromatographic methods fractionate and isolate proteins by ionic strength, pH and temperature. The method used in this study is Capillary Electrophoresis (CE).

Conventional capillary electrophoresis (CE) was introduced in late 1960s, with capillary-based instrumentation and emergence of fused-silica in mid-1980s. Since then, large progress has been made and a large number of electrophoretic methods using thin capillaries have been developed (Janson and Rydén, 1998). Commercially available reagents, capillaries, instruments and complete CE kits provide a useful alternative to other liquid phase separation techniques, HPLC and gel electrophoresis, especially for protein separation and analysis. CE methods should be considered as attractive alternatives or complements to other capillary separation methods, such as liquid chromatography and gas chromatography. CE analyses are driven by an electrical field (Taliha, 2008) and advantages are easy automation, high sensitivity, small sample size and volumes of chemicals used (Janson and Rydén, 1998; Taliha, 2008). The diameter of CE capillaries ranges from 10 to 100 μm. CE may in fact be applied for characterization, determination and separation of a large range of particles, from big particles such as living cells to small mole mass molecules of industrial, pharmacological, nutritional and environmental interest. Unlike chromatographic methods, CE has shown to be able to separate also very small molecules, such as metal ions and amino acids (Janson and Rydén, 1998).

Different CE techniques exist, of which capillary zone electrophoresis (CZE) is often used in protein analysis. Proteins are made up by a large number of amino acids, containing ionizied groups exhibiting negative, positive or no charges depending on pH (Janson and Rydén, 1998). Using CZE, based on the variation in isoelectric points and molecular mass, proteins can be separated obtaining high resolution and sensitivity (Ng-Kwai-Hang, 2002; Heck *et al.*, 2007). Capillary electrophoresis is used for a growing number of applications, of which the analysis of proteins in food is an important application area. Foodstuffs most commonly analyzed for protein by CE are milk, fish, egg and meat (Hutterer and Dolník, 2003). Several researchers have used CE for the determination of milk protein composition and degradation, (Recio *et al.*, 1997; Molina *et al.*, 1999; Cartoni *et al.*, 1999; Miralles *et al.*, 2001; Strickland *et al.*, 2001; Civardi *et al.*, 2002; Hutterer and Dolník, 2003; Heck *et al.*, 2008). Milk proteins of other origin than bovine, e.g. goat and donkey, have also been analysed by CE (Recio *et al.*, 1997; Cartoni *et al.*, 1999; Molina *et al.*, 1999; Civardi *et al.*, 2002). Both qualitative and quantitative determination of milk proteins have been carried out by CE (Recio et al, 1997; Otte *et al.*, 1997; Strickland *et al.*, 2001; Miralles, 2001; Heck *et al.*, 2008). CE is suitable for rapid

separation and determination of relative amounts of the major milk proteins (α-LA, β-LG, α_{S2}-CN, α_{S1}-CN, β-CN, κ-CN). CE is also capable of separating proteins with different degrees of phosphorylation. CE possesses good possibilities for protein quantification. Heck *et al.* (2007) succeeded to distinguish α_{S2}-CN from α_{S1}-CN and identify different phosphorylation states of α_{S2}-CN. Analysis of peptides and proteins are often challenged by their low concentrations (Hutterer and Dolník, 2003).

CE techniques with various modes of operation have been developed. Conventional CE, in which a current is applied parallel to the axis of the capillary, includes capillary isotachophoresis (CITP), capillary zone electrophoresis (CZE) and capillary isoelectric focusing (CIEF) among others (Janson and Rydén, 1998). In CZE, a continuous buffer and the sample is the only discontinuity present, separated by differences in net mobility. The basic arrangement of CE is shown in Figure 1. The capillary connects two electrode compartments, with the sample being injected into the source end of the capillary and being detected at the destination end. The length of capillary is usually 1 – 120 cm. (Janson and Rydén, 1998; Taliha, 2008). Buffer and sample are injected with vacuum and/or a pump and voltage is applied along the capillary. The apparatus is controlled by a computer, monitoring injection, temperature, currency, fraction detection and storing information.

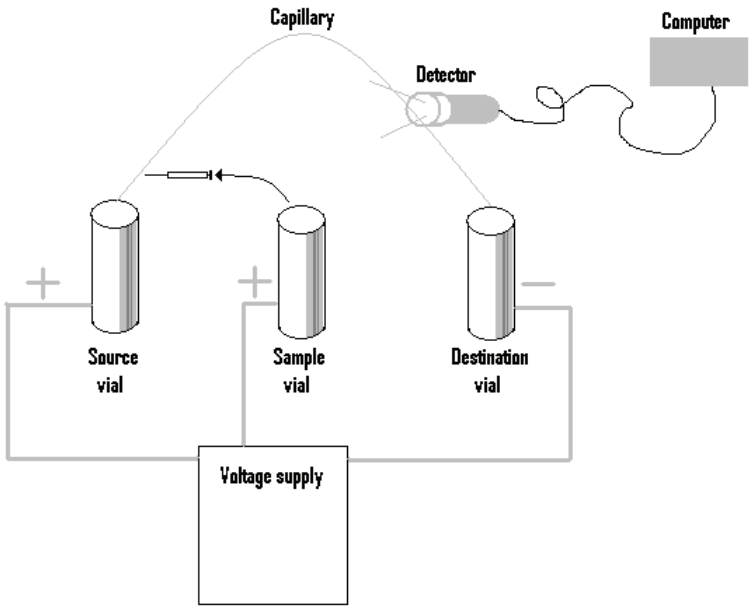

Figure 1. Set up of the basic units in capillary electrophoresis.

In CE, the applied electric field induces a bulk movement of entire liquid through the capillary, termed Electro Osmotic Flow (EOF), as a result of the charged (ionized) inner wall of the capillary (Janson and Rydén, 1998, Taliha, 2008). With the capillary inner wall being negatively charged (e.g. in fused silica capillaries), an inner layer of positive ions is built up on the wall surface, resulting in a potential difference known as the Zeta potential (Taliha, 2008). As voltage is applied along the capillary, positively charged ions on the inner capillary wall are attracted towards the negative end of the capillary, directing the EOF towards the cathode (negative end). The movement of positive ions towards the negative capillary end drags other particles with it, creating a bulk

movement. Velocity of each particle is dependent on its charge density, i.e. its size and net charge. A smaller particle has less friction forces as it moves through the capillary than a larger particle. A more charged particle moves faster as it binds harder to ions with opposite charge. Magnitude of EOF is usually larger than the movement of single particles respectively, although every particle finally is attracted to the destination capillary end even if attracted to the injection end, as a result of the bulk flow (Taliha, 2008). The zeta potential and resulting EOF are dependent on the charge of the capillary wall, which is large at high pH and low at low pH. Other parameters to control EOF are currency applied being proportional in change to EOF, ionic strength of run buffer (decreasing zeta potential when decreased), temperature (changing viscosity) or neutral hydrophilic polymer (absorbing to the capillary wall shielding surface charge thereby reducing EOF). Flowing through the capillary, proteins and other analytic compounds may interact with or/and adsorb to the capillary wall. Hydrophobic and hydrophilic based interactions as well as interactions between highly charged proteins and capillary (mainly positively charged proteins in a fused silica capillary with negatively charged walls) alter EOF and may cause peak broadening, peak asymmetry or preventing proteins from being separated (Hutterer and Dolník, 2003; Janson and Rydén, 1998). In this context, one must optimize CE conditions to obtain efficient separation of sample, considering electrophoretic buffer, capillary size and its material and inner surface. Prior to analysis by e.g. capillary electrophoresis, proteins must be denaturized to prevent them from interacting with each other, which would interfere with separation (Ng-Kwai-Hang, 2002). The most common measure to reduce wall absorption is usage of static or dynamic capillary wall coatings (Hutterer and Dolník, 2003). Dynamic wall coating implies the addition to the buffer of a detergent, polymer or another molecule interfering with the interaction between capillary wall and analytes by serving as a bilayer on the capillary wall. Static coatings are more stable and usually more effective than dynamic coatings but more difficult and expensive to regenerate and to produce (Janson and Rydén, 1998; Hutterer and Dolník, 2003).

Injection time (seconds) and injection pressure (mbar) determines the volume and velocity of injected sample following: *Injection time (s)* • *Injection pressure (mb)* = *Injection volume (mbs)*. Either the injection time can be modified or the injection pressure. The sample should not be injected into the column with too high pressure, while the velocity of sample must be slower than the present EOF, which will otherwise not influence the protein migration time. With too high injection pressure, the first fractions may not get separated or detected while passing too quickly. The injection time should also not be too short, since the time limits the volume of sample injected and thereby limits resolution. If the injection time is too short, small concentrations of protein will not be detected because of too small sample volume. To determine protein composition by CE, peak area has to increase linearly with injection volume (Heck *et al.*, 2008). For protein analysis by CE, many detection modes developed for HPLC may be used, of which the most common detection modes used are spectroscopic techniques such as UV absorbance and fluorescence, UV offering the simplest method however the least sensitive (Janson and Rydén, 1998; Hutterer and Dolník, 2003). Usually, the wavelength used by UV detection is between 200 and 220 nm, here absorption being proportional to the number of peptide bonds in the sample, which is proportional to gram of protein for most proteins (Hutterer and Dolník, 2003; Heck et al, 2007). Aromatic residues however have an influence on absorption in those wavelengths. Also degree of phosphorylation is an important factor with direct impact on the migration time of the protein. Every phosphorylation results in an additional negative charge on the protein surface resulting

in longer migration time (Recio *et al.* 1997b). The charge of a protein dependent on e.g. its phosphorylation state correlates linearly with its migration time, which is also dependent on size and shape of the protein. To enhance separation of the proteins, reducing and denaturising agents are used (Ng-Kwai-Hang, 2002).

2.5 Conclusion of literature

Milk protein concentration and composition is one of the most economically important properties of bovine milk, influencing its processability and economical value. Concentration and composition of milk proteins is significantly influenced by mastitis, normally decreasing total protein content and quality. Mastitis is normally caused by a bacterial infection in the udder and causes great suffering in affected cows. Mastitic milk is not to be delivered to the dairy and antibiotic treatment is often undertaken. Mastitis influences farm economics by lowering protein and milk yield on top of costs for treatment and withdrawal of the milk. For the dairy industry, lower protein quality and protein content result in lower cheese yield (kg cheese produced from kg of milk) and less favourable processability properties.

For analysis of milk proteins, a great number of various methods are available. Since the 1980'ies, capillary electrophoresis (CE) is widely used. CE separates proteins after their molecular masses and net charges at a selected pH. Sample is injected in the capillary source end, forced through the capillary by an electrical field causing an electro osmotic flow (EOF). Particles with higher charge density (smaller or/and with more charges) move faster than particles with lower charge density. At the capillary destination end, proteins are detected, usually by UV-detection.

3. Material and Methods

3.1 Animal materials and collection of milk samples

Seven cows, five Swedish Red (SRB) and two of Holstein breed, diagnosed with an intramammary infection, mastitis, were selected for this study during summer and autumn 2009. Cows were housed at Jälla agriculture school and on Kungsängens dairy research station, Uppsala, Sweden. The milking personnel were instructed to collect samples in connection with every milking occasion, mornings and afternoons/evenings. At Kungsängens dairy research station (K), milking was performed at 7 am and 4 pm, at Jälla agriculture school (J) milking was at 5 am and 5:30 pm. Sampling began on the first milking occasion after start of mastitis treatment and proceeded during treatment and withdrawal periods and until five days after the end of the withdrawal time. Typical lengths of treatment and withdrawal periods were 5 and 6 days, respectively, see Table 1. From each animal, in total approximately 30 samples were taken.

Table 2. *Animal data including length of treatment and withdrawal period. K = Kungsängen, J = Jälla. SRB (Svensk rödbrokig boskap) = Swedish Red. SLB (Svensk Låglandsboskap) = Swedish Lowland.*

Animal ID	**1291**	**1389**	**1136**	**1294**	**1332**	**9355**	**1438**
Farm	K	J	K	J	K	J	J
Breed	SRB	SLB	SRB	SLB	SRB	SRB	SRB
N:o of previous lactations	4	1	6	4	4	1	1
Previous parturition	2009 10.03	2008 12.11	2009 07.30	2009 07.07	2009 08.02	2009 09.18	2009 09.02
Pathogen	*Staph. aureus*	Coagulase neg. *Staph.*	*Strept. dysgal.*	*Strept. uberis*	*Staph. aureus*	*Strept. dysgal.*	*Strept. dysgal.*
Medicinal preparation (ml/day)	Penovet 45	Penovet 65	Metacam 17.5 Penovet 250	Ethacilin 50	Metacam 17.5 Penovet 250	Penovet	Ethacilin Carepen Metacam
Treatment time (days)	5	5	5	5	5	5	5
With-drawal time (days)	6	8	6	6	5	5	5

3.2 Sample handling

Samples were stored at -20ºC for not more than 2 months before protein analyses. Time during which samples are subjected to room temperature is critical for deterioration of milk proteins by enzymes in the milk, while plasmin degradation occurs at room temperature. For this reason, preparation of samples was performed within as short time in room temperature as possible and number of samples run in each batch in CE was limited to 10.

3.3 Analytical methods

The instrument used for protein analyses was Agilent CE with CE silica standard capillary, 50 µm inner diameter, 40 cm active length.

3.3.1 Sample preparation

Milk samples were defatted by centrifugation at 4°C for 10 minutes at 3500 rpm. 300 µl skim milk fractions were incubated with 700 µl sample buffer at room temperature for 1h. The buffer sample mixture was filtrated through a 0.45 µm, nylon membrane filter. Buffer sample mixtures and running buffer were stored at -20ºC prior to analysis.

3.3.2 Preparation of buffer solutions

Sample buffer and running buffer were prepared for CE protein analyses: 2 g of ion exchange resin per 100 ml were used (AG® 501-X8 and Bio-Rex® MSZ 501(D) Mixed Bed Resin, Bio-Rad Laboratories, Inc.). The ion exchange bed resin decreases the ion strength of the buffers and the sample. Mixed bed resins are usually used for deionizing non-ionic substances like urea etc. as well as water (Bio-Rad Laboratories, 2000). The ion exchange resin captures ions, thereby neutralising ions yielding water (Bio-Rad Laboratories, 2000): Resin-H + Resin-OH + NaCl → Resin-Na + Resin-Cl + H_2O. The pH of the running buffer influences the charge of the proteins. Urea has the function of denaturising or solubilising proteins and modifying EOF whereas trisodium citrate dehydrate stabilizes pH (Landers, 1997). MOPS and Tris are added as buffering agents to stabilize pH. The function of monohydrate citric acid is to adjust pH. Methylhydroxyethylcellulose serves to control or suppress electro osmotic flow in the capillary (Landers, 1997). DTT is a reducing agent normally added to convert dimers of α_{S2}-CN to its monomer form (Heck *et al.*, 2008). EDTA has the function of capturing divalent ions. The sample buffer and the run buffer were prepared according to Heck *et al.* (2007) with minor adjustments. For all buffers, urea solution was made by dissolving 6.00 M urea (Sigma) and 7 M of ion exchange resin in water until conductivity reached below 2 µS. For running buffer, 0.19 M monohydrate citric acid (Sigma), 0.02 M trisodium citrate dehydrate (Sigma) and w/w 0,05% methylhydroxyethylcellulose 3000 (MHEC, Serva) were dissolved in the urea solution. For sample buffer, 0.167 M (tris)hydroxymethylaminomethane (Tris, Sigma), 0.067 M ethylene-diamine-tetraacetic acid disodium salt dihydrate (EDTA, Merck), 0.042 M 3-morpholino-propanesulphonic acid (MOPS, Sigma) and refrigerator stored 0.017 M D,L-dithiothreitol (DTT, Sigma) were dissolved in the urea solution. The sample and running buffer were filtered with 0.45 µm filter papers (Durapore® membrane filters, Millipore) and portioned for use prior to freeze storage.

3.3.3 Effect of bed resin on running buffer and sample buffer

Two different buffer solutions were prepared for running buffer and sample buffer, one with ion exchange resin (AG® 501-X8 and Bio-Rex® MSZ 501(D) Mixed Bed Resin, Bio-Rad Laboratories, Inc.) and one without ion exchange resin. The aim with testing buffer with and without resin was to investigate the effect of resins on the sample separation. Reference sample used in selection of optimal buffer solution was hydroxy acetone (10 M, Agilent Technologies).

3.3.4 Selection of optimal sample injection and pressure

The injection time was set to 7 seconds with the injection pressure of 50 mbar. The parameters and method was optimized for analysis of milk from a healthy udder though showed to be successfully also in analysis of mastitic milk. Optimization of injection volume was made by analyzing different concentrations of 4-Hydroxy acetophenone (0.1 mM, 0.5 mM, 1 mM and 5 mM, Agilent Technologies) in buffer pH 9.3 for HPCE (20 mM borate, Agilent Technologies). 4 different injection pressures were tested for best resolution: 10.7 mbar (74.9 mbs), 21.4 mb (149.8 mbs), 35.7 mbar (249.9 mbs) and 50 mbar (350 mbs). The amount of sample injected should respond to pressure multiplied with injection time and therefore be greatest at 50 mbar. The injection pressure of 50 mbar resulted in the highest resolution of protein fractions. In samples injected with pressures of 10.7, 21.4 and 35.7 mbar, peaks for single proteins were under detection limits. Therefore, injection pressure of 50 mbar during 7 seconds, resulting in 350 mbs was chosen for this trial.

3.3.5 Reproducibility test

A reproducibility test was made by injecting acetone (4-Hydroxy acetophenone, 1.0 mM, Agilent Technologies) approximately 50 times, using buffer pH 9.3 for HPCE (20 mM borate, Agilent Technologies). Before each separation, capillary was flushed with buffer solution (20 mM borate) in reverse direction for 2 minutes. Acetone was injected at 40 mbar for 5 seconds. Separation was performed at 20 ºC and a linear voltage of 30 kV. Absorbance was measured at 325 nm.

3.3.6 Procedure

Before starting a separation, capillary was flushed in reverse direction 3 minutes with water and 5 minutes with run buffer. If needed, capillary was flushed with 1.0 M alternatively 0.1 M NaOH for 15 minutes, then with water and run buffer solution for 15 minutes respectively. Samples were divided into series where every series consisted of 8 samples and one external control sample (bulk tank milk from Kungsängens research station). The order of samples analysed was: C (control), 1, 2, 3, 4, 5, 6, 7, 8, 1, C. The first sample in the batch, which was included in the standard error, served as control of the repeatability within each batch. The reliability of the analysis method was determined by comparing internal and external standards (Sigma) within and between series of 10 samples analyzed in a row. Separation was performed at 45ºC and a linear voltage of 25 kV and 75μA for 30 minutes. Sample was injected during 7 seconds at pressure of 50 mb. Detection was measured at 214 nm during 25 minutes.

Identification of peaks

Identification of peaks and assigning peaks to known proteins was made by comparing electropherograms with Sigma standards and literature electropherograms (Heck *et al.*, 2007). Multiple peaks that arose around the main peak of α_{S2}-CN were assigned to α_{S2}-CN according to Heck *et al.* (2007). In many samples, the peak addressed to κ-CN showed a remarked shoulder, which in many samples appeared as a separate peak. When appearing as a separate minor peak, it was not included in the estimation of κ-CN concentration.

Protein quantification and data evaluation

Protein quantification was based on standard curves where purified CN samples (Sigma) with known protein concentration were used. The protein concentration of the milk samples was related to peak area in the electropherogram. Concentrations of individual

proteins during treatment period, withdrawal period and post withdrawal period respectively were used to calculate an average concentration for the period. Those values were compared with concentrations of the reference bulk tank milk.

4. Results and Discussion

Proteins were separated with retention times between 11 and 24 minutes, shown in Fig 2.

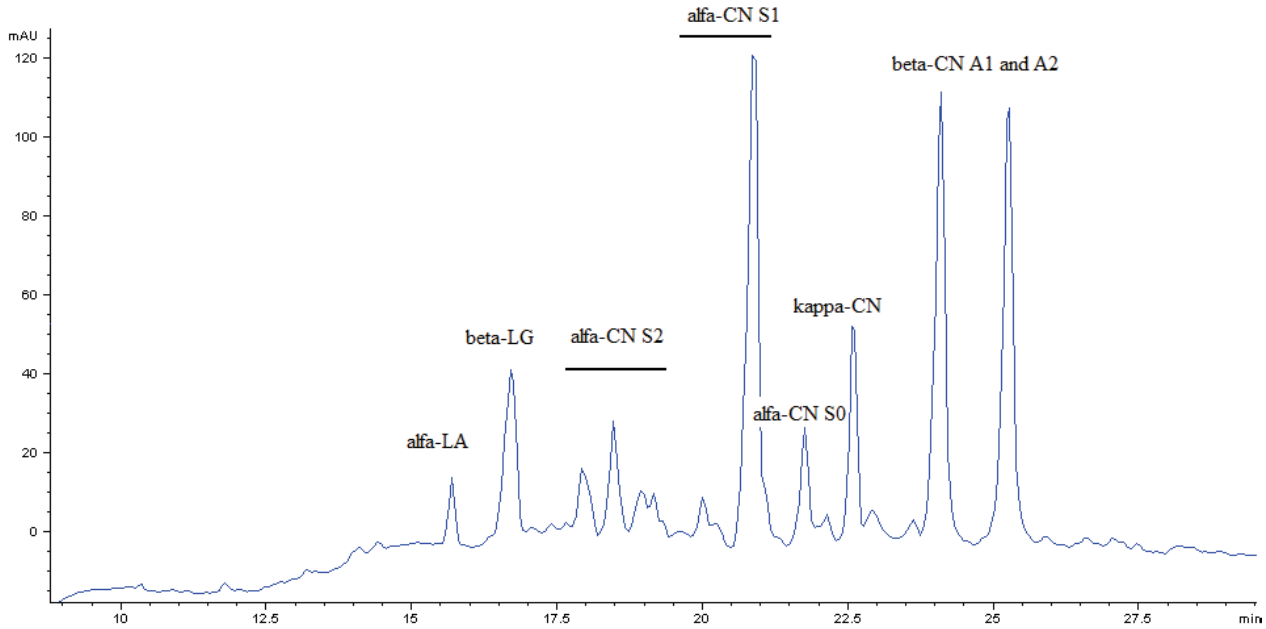

Figure 2. Typical electropherogram from CE with UV absorption peaks addressed to milk proteins.

Multiple peaks

Milk from two animals (1291 and 9355) showed double peaks at the migration time of β-lactoglobulin (beta-LG) in electropherograms. Both were considered to arise from the presence of β-lactoglobulin. Some milk samples from animal 9355 also showed double peaks of α-lactalbumin (alfa-LA). The multiple peaks of **α_{S2}-CN** appearing in electropherograms are assumed to arise from different phosphorylation steps of α_{S2}-CN and degradation products of α_{S2}-CN according to Heck *et al.*, 2008. The total α_{S2}-CN and α_{S1}-CN concentrations were calculated by adding all assigned peaks respectively, α_{S1}-CN-8P, α_{S1}-CN-9P together with main α_{S1}-CN peak for α_{S1}-CN, and peaks for α_{S2}-CN-10P, α_{S2}-CN-11P, α_{S2}-CN-12P together with the main α_{S2}-CN peak for α_{S2}-CN, as shown in Figure 2. **κ-CN** sometimes appeared as multiple peaks beside one major, as was also shown in previous studies (Miralles *et al.* 1999; Heck *et al.*, 2007). Different variants of κ-CN have different migration times, appearing as multiple peaks, of which some with the same migration time as β-CN A1 and A2 and thereby not visible in presence of the latter (Heck *et al.*, 2008). The major peak of κ-CN, its mono-phosphorylated form, should comprise ±50% of total κ-CN (Heck *et al.* 2007). The minor peaks, migrating at the same time as β-CN A1 and A2 are thought to comprise glycosylated and/or phosphorylated forms (Heck *et al.*, 2008). In electropherograms of some samples in this trial the peak of κ-CN rather appeared being double than being split in the top, leaving the nature of the minor peak unclear. The difference between the genetic variants A and E is the

substitution of a glycine residue by a serine residue on position 155 of the protein (Heck *et al.*, 2008) which would not influence polarity and migration times at the present pH of either this analysis or the analysis of Heck *et al.* (2008). Taking this into consideration, the minor peak beside the main peak observed in this study is not likely to show this genetic variant of κ-CN. The nature of the minor peak may be degradation products of proteins, however this remains unclear. Only the area of the main peak was measured.

Milk protein concentrations

Concentrations of milk proteins changed to a various extent during the time between start of treatment and 5 days after end of withdrawal time is shown in Figure 3.

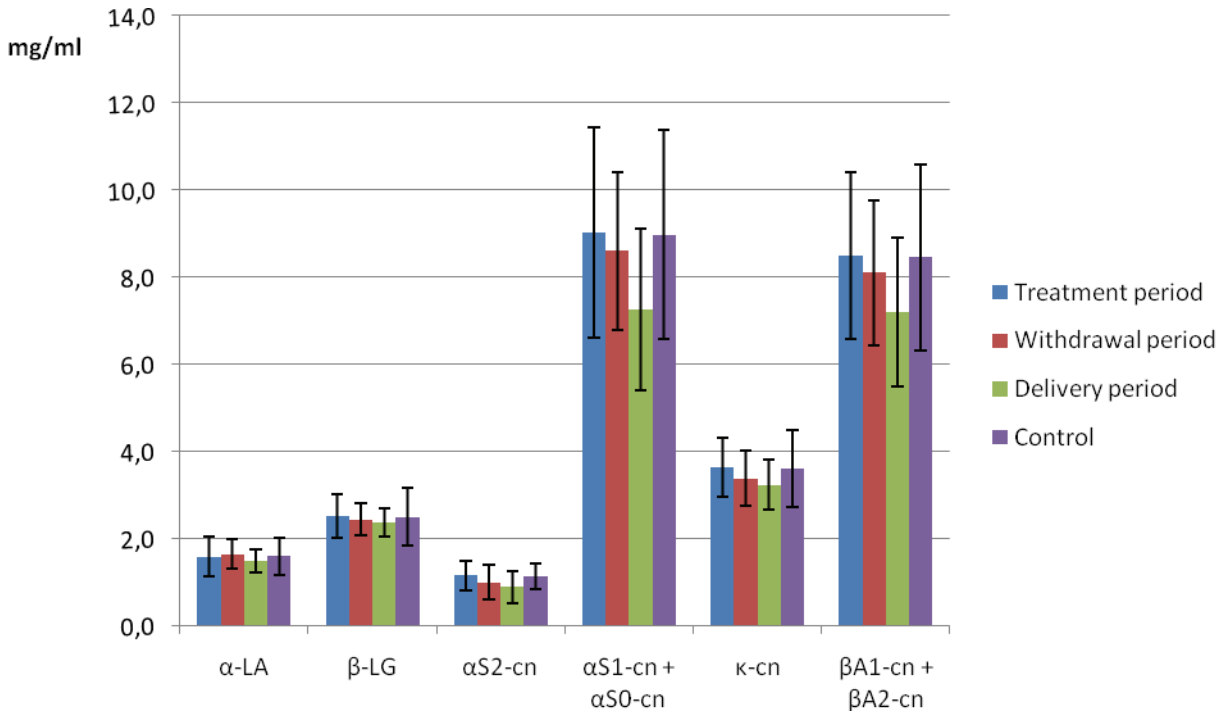

Figure 3. Concentrations of the individual milk proteins analyzed during treatment period, withdrawal period and delivery period. Error bars denote standard deviation within results of each protein and time period.

Concentrations of individual caseins analysed (α_{S2}-CN, α_{S1}-CN, α_{S0}-CN, κ-CN and β-CN) were found to be highest in treatment period, lower during the withdrawal periods and lowest in the five day period after withdrawal time expired. All caseins concentrations are lower during the first five days after withdrawal time has expired than in normal bulk tank milk from healthy cows. It should be noted that all animals in this study were at the beginning of lactation, were changes in milk composition especially protein concentration are dramatic. The decrease of concentrations of proteins found in this study may be an effect of changes taking place in early lactation.

A question arising from these results is why casein concentrations do not decrease already during the time of treatment and recovery instead of decreasing after treatment to continue decrease beyond end of withdrawal time. Mastitis is reported to cause casein degradation and decreased synthesis of caseins, followed by an increase in casein as a result of recovery from the inflammation. Results of this study indicate that the expected

increase in casein does not occur within the time period of this study. Results are in line with those of Pyörälä (2003) and Åkerstedt *et al.* (2008), finding the total casein concentration being lower in mastitic milk. Pyörälä reviewed research presenting decreased concentrations of α_{S1}-CN and β-CN, which is also found by this study. However, Pyörälä presents no data regarding when during the cease of mastitis, that protein concentration is analysed, wherefore comparison with results of this study may be inappropriate. It seems like the effect of mastitis on casein concentrations is delayed, since average concentrations of all caseins are in the range of those of control milk during treatment time, to later decrease during withdrawal period and after withdrawal time has expired. Those results indicate that synthesis and degradation of caseins are still not influenced by mastitis infection at the time of treatment. It is difficult to say whether casein concentrations normalize from abnormally high levels to normal or decrease from a normal concentration down to low. Comparison with bulk tank milk though indicates the latter – protein concentrations during treatment period correspond to those of normal milk. One possible explanation for the decreasing casein concentrations during treatment and withdrawal periods is increased milk volume, diluting the proteins, however, in this study, none of animals showed a marked increase in milk volume during treatment and withdrawal time. Especially for α_{S1}-CN, α_{S0}-CN and β-CN, concentrations tend to sink markedly from treatment period until five days after the end of withdrawal time. α_{S1}-CN, α_{S0}-CN and β-CN are known to be susceptible for enzymatic degradation. Since samples are not stored in room temperature, enzymatic degradation should only have taken place in the udder and by plasmin. Plasmin does not act on α_{S1}-CN and α_{S0}-CN. Presumably, decreased synthesis of α_{S1}-CN and α_{S0}-CN should have more influence on reduced casein levels than would enzymatic degradation.

Concentrations of whey proteins analysed (α-LA and β-LG) did not change noticeable between periods of treatment, withdrawal time or five days post withdrawal time. Total protein concentration has been found to be higher in mastitic milk (Auldist *et al.*, 1995; Urech *et al.*, 1999) due to influx of plasma proteins exceeding the proteolysis and decreased synthesis of caseins. Usually, though, intramammary infections are not considered to considerably increase protein concentration in milk (Hogarth *et al.*, 2004; Leitner *et al.*, 2006, Forsbäck *et al.*, 2009). Several authors state lower whey protein concentrations in mastitic milk than in milk from healthy cows (Pyörälä, 2003; Åkerstedt *et al.*, 2008). In contrast, Forsbäck *et al.* (2009) found whey protein concentration being higher in mastitic milk than in milk from healthy cows, especially for α-LA.

Variation among animals

In this study, protein concentrations in milk from each individual could be compared with concentrations in milk from the same cow in a healthy state adjusting for other factors influencing milk protein composition. Milk protein composition varies considerably throughout the cease of lactation, lactation number, season etc. Protein concentrations in milk from a certain animal at a certain time point is only to be compared with bulk tank milk from a great number of healthy individuals. Bulk tank milk is better suited for comparison of average milk protein concentrations since it consists of pooled milk from all animals. One source for the variation in protein composition between animals may be the pathogen causing the mastitis inflammation. Three out of seven animals were infected by *Streptococcus dysgalactiae ssp*. three by *Staphylococcus ssp.*, and one cow with *Streptococcus uberis*. However, no reliable conclusions from comparisons between those few individuals could be drawn regarding effect of pathogen.

The cow infected by *Streptococcus uberis*, id 1294, showed markedly lower concentrations of α_{S1}-CN and α_{S0}-CN during treatment and after the cease of the withdrawal period, this to a greater extent than in most other animals. This is suspected to be correlated with the severity of a *Streptococcus uberis* infection in comparison with other pathogens (Larsen *et al.*, 2004). In milk from the *Streptococcus uberis* infected animal, whey proteins, α_{S1}-CN, α_{S0}-CN and β-CN concentrations were slightly decreased during treatment, which was not seen in milk from other animals. It may be suggested that protein concentration decreased later in milk from this animal than in milk from other individuals resulting in a decrease of at least casein concentrations from treatment period to post withdrawal time. See Appendixes, Figure 7. In milk from animals 1332 and 1291 (both infected with *Staphylococcus aureus*) α_{S2}-CN concentrations did not decrease, as in other animals. See Appendixes, Figure 8 and 10. In one of the animals infected by *Streptococcus ssp.,* id 1136, κ-CN concentration decreased more rapidly and α_{S1}-CN and α_{S0}-CN slower than in cows infected with *Staphylococcus ssp.*

Table 3. *Overview of changes in protein concentrations in each animal from start of treatment to five days past withdrawal time. It can be seen that all caseins decreased on average, whereas whey proteins showed a slight or none decrease on average. This overview also visualises the great individual variation between animals.*
→ = no or little change
↓ = distinctive decrease
(↓) = slight decrease
↓ ↑ = initial decrease between treatment period and withdrawal period followed by increase between withdrawal period and post withdrawal period.

	α-LA	β-LA	α_{S2}-CN	α_{S1}-CN α_{S0}-CN	κ-CN	β-CN
Average (all animals)	→	*(↓)*	↓	↓	↓	↓
Animal ID 1438	*(↑)*	↓	↓	↓	↓	↓
9355	→	*(↑)*	*(↓)*	↓	*(↓)*	↓
1136	↓	↓	*(↓)*	*(↑)*	↓	↓
1294	↑ ↓	↑ ↓	↓	↑ ↓	*(↓)*	↑ ↓
1332	↓	↓	↓	↓	↑	↓
1389	→	→	→	↓	↓	*(↑)*
1291	→	→	↑ ↓	↓	↓	↓

Difficulties analysing clotted milk

In mastitic milk, proteins often clot, forming a non soluble precipitate in the milk. This was observed in some samples used in this study. Analysing highly clotted (mastitic) milk by CE may be accomplished with some difficulties with clotted proteins not able to go through the filtering of sample prior analysis, or later plugging the capillary. The question is whether a milk sample in which proteins are clotted gives a representative result in CE analysis. Using highly clotted milk samples, the electropherograms appeared remarkably different from electropherograms from not clotted milk. Peaks had different migration times and eluted a very short time after each other compared to peaks resulting from proteins in not clotted milk. However, analysis of samples in which proteins were

highly clotted as a pellet and therefore did not pass the filter prior analysis, resulted in almost equal electropherograms compared to samples in which proteins were eluted in the supernatant and therefore came through the filtering prior the analysis. This indicates that the supernatant may be a representative part of milk sample, as if predominately pellet is constituted of somatic cells rather than of aggregated caseins. Sample pH varied between 6.5 and 6.8, at which caseins would not precipitate (Julien *et al.*, 1985).The reason for clotting of caseins in some samples is unclear. For solubilisation of clotted proteins, pH may be adjusted and samples vortexed. Adding milk to buffer with optimal pH would adjust all samples to at least close to optimal pH. However, most samples from animals included in this trial have been analysed without those problems.

Udder quarter samples or commingled milk samples

According to previous research on correlations between udder health, SCC and milk composition (Le Roux *et al.* 1995), separate analysis of milk from of each udder quarter would be a more sensitive examination of milk composition change and maximize the variability of parameters measured than would milk composite samples. In addition, possible interactions between milk constituents in samples from different udder quarters can be eliminated by analyzing udder quarter samples separately (Le Roux *et al.*, 1995). As mentioned in the literature review, milk composition may vary widely within the same animal between healthy and mastitic udder quarters (Le Roux *et al.*, 1995; Leitner *et al.*, 2006; Lindmark-Månsson *et al.*, 2005). For this reason, the magnitude of change in milk composition in infected udder quarters will not fully show in analysis of udder composite samples. Verdi *et al.* (1987) found commingling of milk from udder quarters with different SCC resulting in elevated enzymatic activity in the low SCC milk. This would motivate separation of milk with different SCC in production as well as in sample taking for mastitis research.

Future research

In future studies, length of trial must be extended in seeking the time point when protein concentrations normalize or stabilize. For most animals and most proteins analyzed in this study, protein levels seemed to continue decreasing (or increasing) at the time for the last sampling. For the future, preparation of samples may be optimized for mastitis milk samples. It has to be investigated whether the clotting itself influences protein composition in filtered samples or not. To draw conclusions of results and formulate reasons for questionable results, results from a greater number of individuals must be compared together. Only by this, single aberrant results can be addressed to single sample batches (insufficient washing), level of capillary ageing, pH, micelle clotting and time of sampling (protein levels and protein degradation). To draw more sophisticated and reliable conclusions, number of animals used for sample collection must be greater.

5. Conclusions

The hypothesis that protein composition is still abnormal after withdrawal time has expired cannot be rejected.

The time point for stabilisation of protein concentrations after antibiotic treatment against mastitis is not yet found, though is suggested being not earlier than 15 days after start of treatment. The possibility that declines observed in milk protein concentrations may be due to the progress of lactation must also be considered. In this study, all cows were in the beginning of lactation.

Concentrations of individual proteins undergo great variation between animals. Type of pathogens causing the mastitis may also affect milk protein concentrations differently.

Concentrations of caseins as a group change to a larger extent than do concentrations of whey proteins during acute mastitis treatment and recovery.

6. Acknowledgements

I want to thank to all of those who have contributed with their support and knowledge in my work with this thesis. Especially thanks to Monika Johansson, Lotta Wall and Anders Andrén for their engagement and competence. To you who have encouraged me during this time: you know who you are and how important you are to me.

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8. Appendixes

Diagrams presenting individual protein concentrations for each animal during the different time periods are shown below.

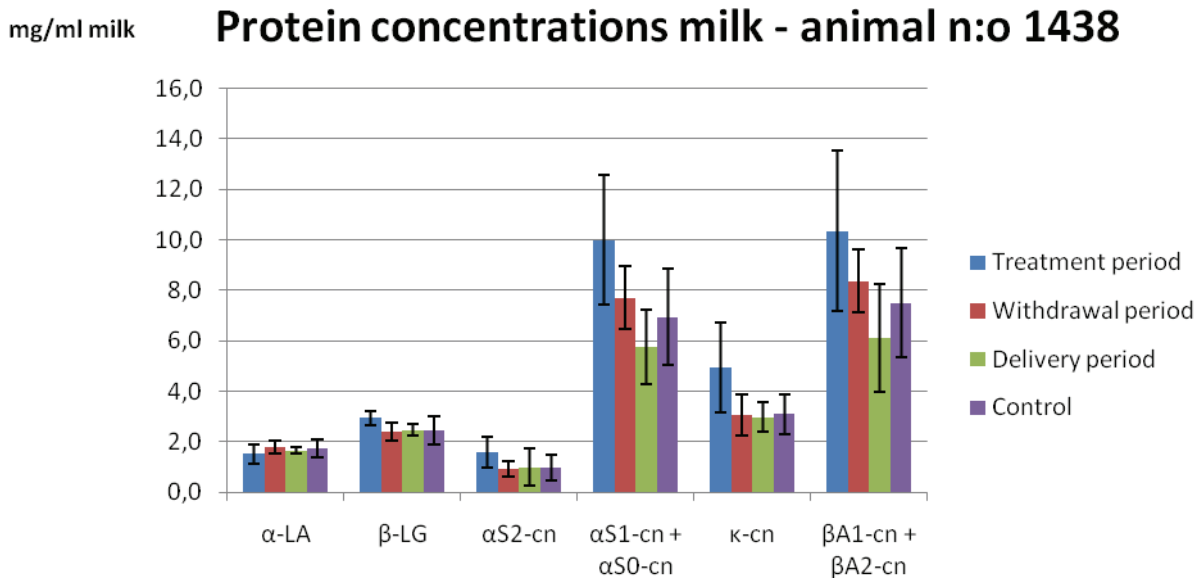

Figure 4. Concentrations of milk proteins analyzed for each time period respectively in milk from animal id: 1438. Each group of piles corresponds to one protein. The first pile in each group shows protein concentration in mg/ml milk during treatment period. The second pile shows protein concentration during withdrawal time and the third pile during the first five days after end of withdrawal time. Control milk protein concentrations is shown by the fourth pile in each group. Error bars denote standard deviation within results of each protein and time period.

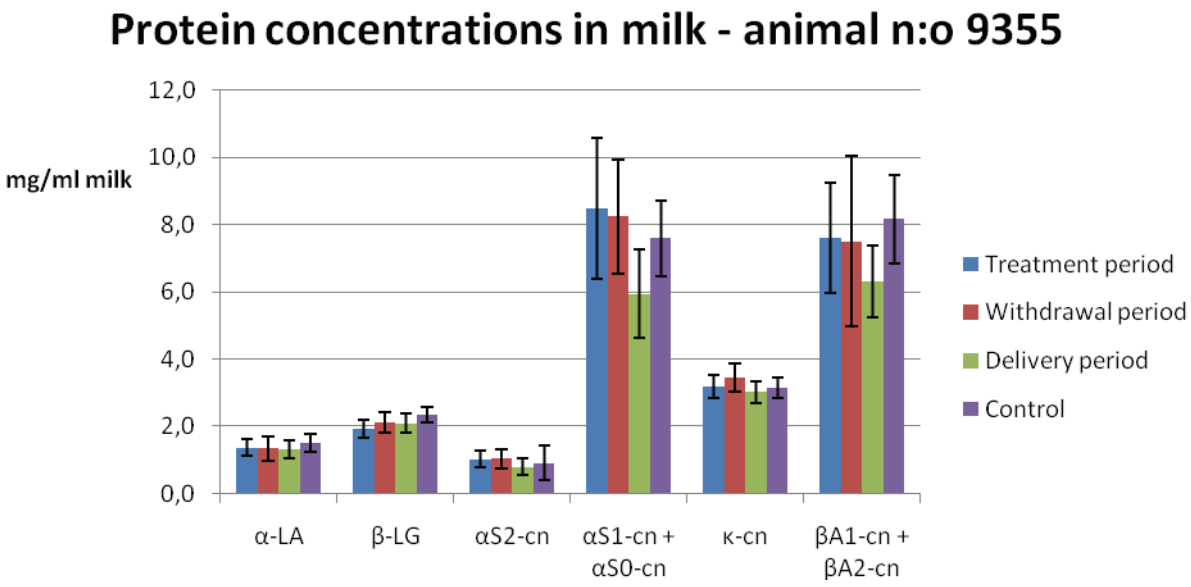

Figure 5. Concentrations of milk proteins analyzed for each time period respectively in milk from animal 9355.

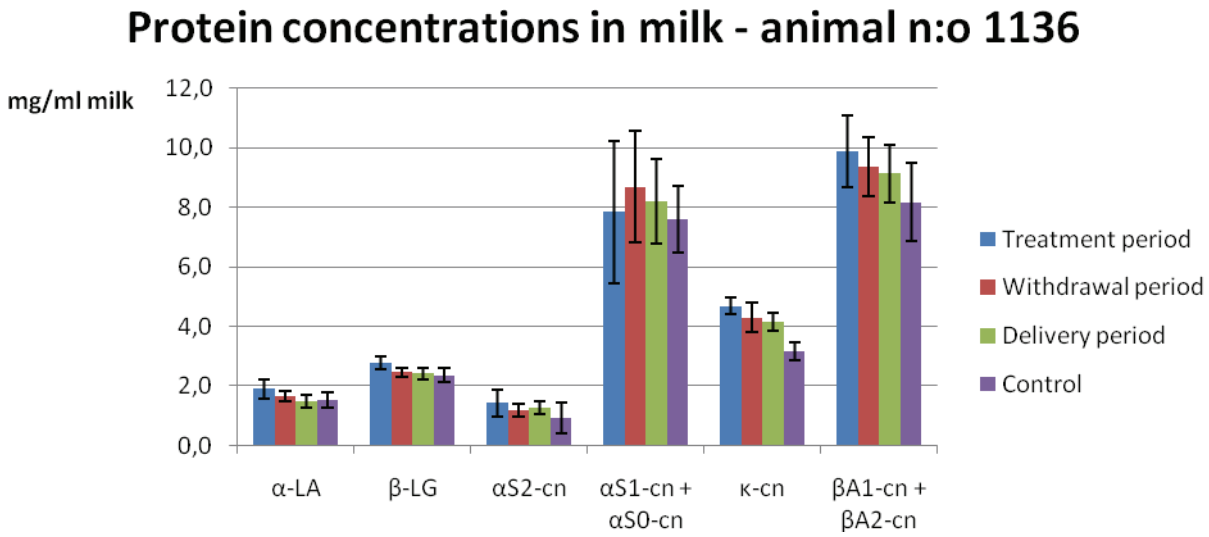

Figure 6. Concentrations of milk proteins analyzed for each time period respectively in milk from animal 1136.

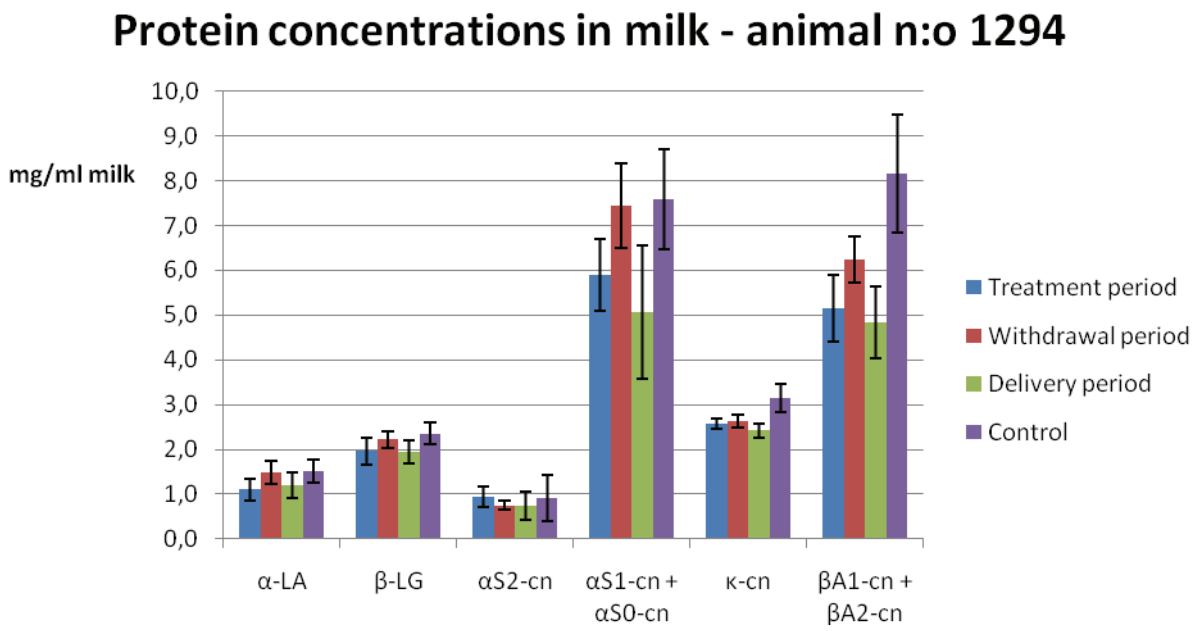

Figure 7. Concentrations of milk proteins analyzed for each time period respectively in milk from animal 1294.

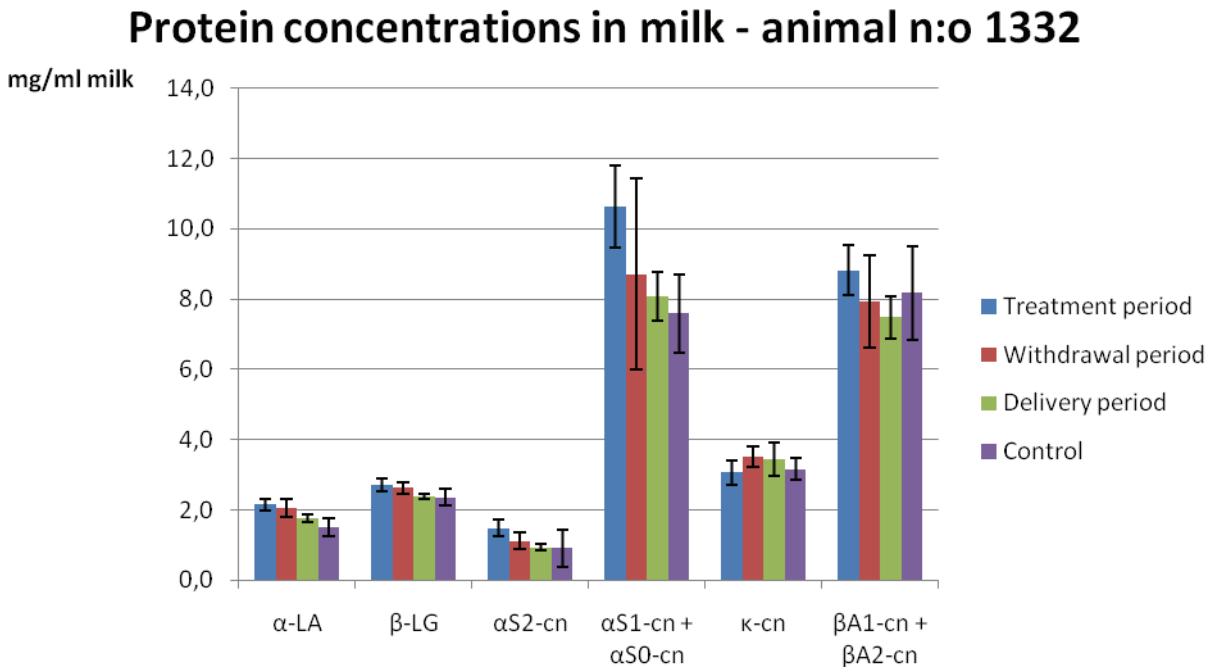

Figure 8. Concentrations of milk proteins analyzed for each time period respectively in milk from animal 1332.

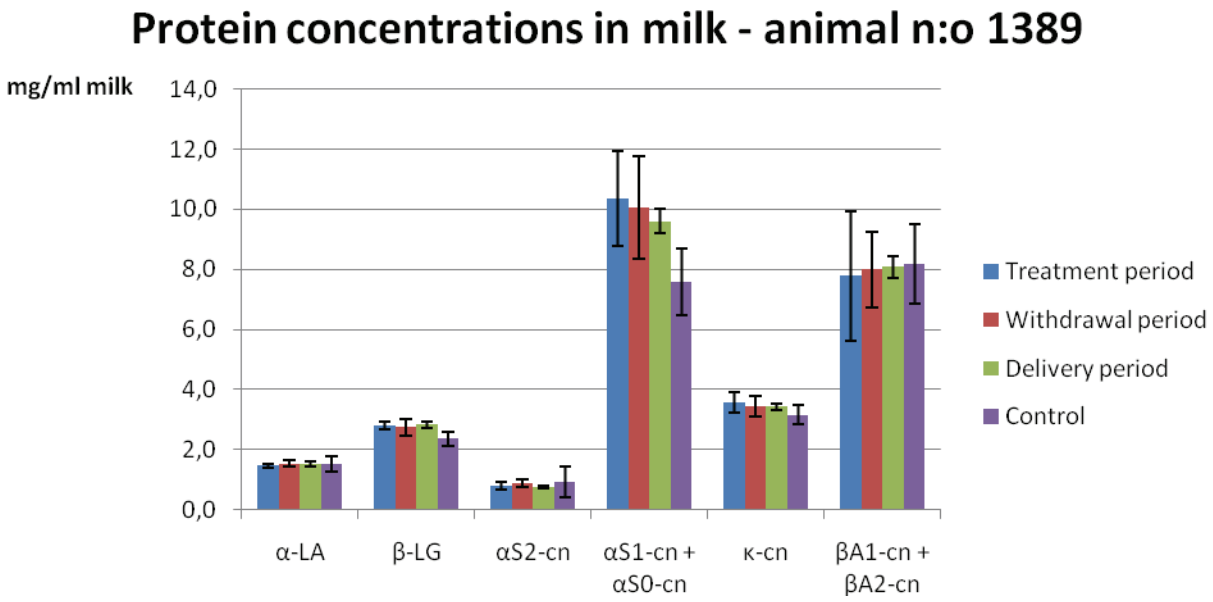

Figure 9. Concentrations of milk proteins analyzed for each time period respectively in milk from animal 1389.

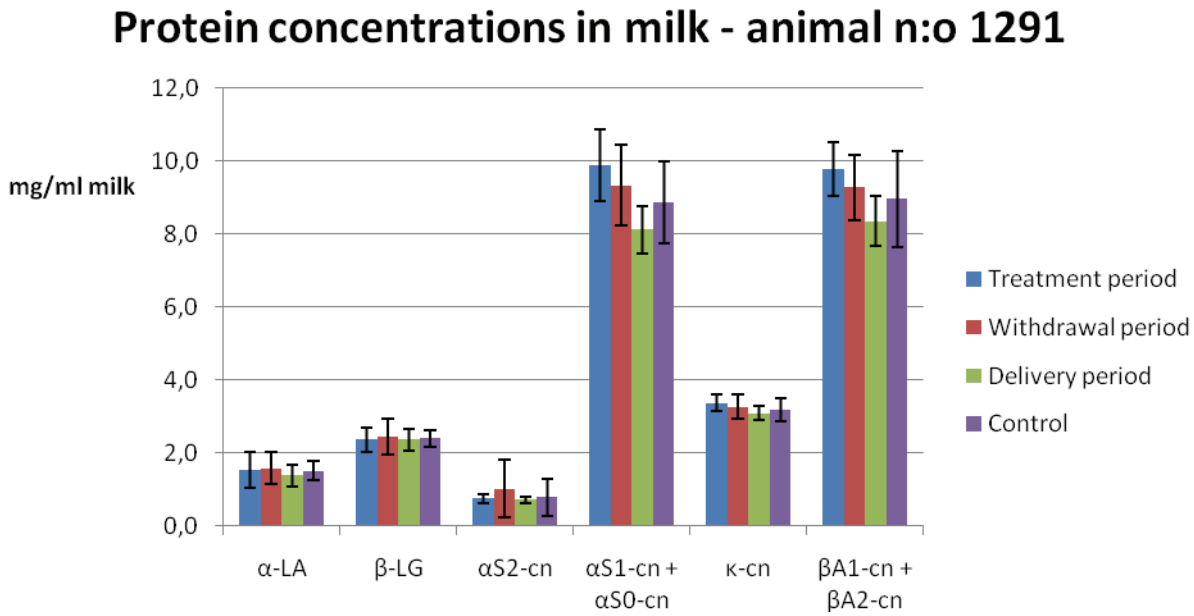

Figure 10. Concentrations of milk proteins analyzed for each time period respectively in milk from animal 1291.

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