Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences
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Buffalo milk: Influence of the first stage of lactation on plasmin system and free fatty acids of buffalo milk

Buffelmjölk: Påverkan av den första perioden av laktationen på plasminsystemet och fria fettsyror

Caroline Blänning
Sofie Sandelius

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Caroline Blänning
Sofie Sandelius

Supervisor: Monika Johansson, Swedish University of Agricultural Sciences, Department of Food Science

Examiner: Lena Dimberg, Swedish University of Agricultural Sciences, Department of Food Science

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Abstract

Buffalo milk is the second most produced milk after conductive bovine and mozzarella is one of the most important products of buffalo milk. The milk contains higher levels of nutrients, mainly protein and fat, which gives buffalo milk good physical and chemical properties. Milk from early lactation however, is difficult to use in mozzarella manufacturing. This study analyse the impact of plasmin (PL), plasminogen (PG) and free fatty acids (FFA) in buffalo milk from the first weeks of early lactation, since they are important factors in milk and can influence the quality in cheese. The aim was to investigate if these factors can be involved in the problematic of mozzarella manufacturing. Both individual buffalo milk and bulk milk were analysed.

The milk samples, collected from six buffalos twice a week during the first 6-7 weeks of lactation, were analysed in duplicates. PL and PG activity were determined by measuring the cleavage of chromogenic substrate and the formation of paranitroanilide (pNA) at 405 nm. The present results in the study should only be taken as an indication, since no statistical analyse has been performed. The average PL activity from individual buffalos indicates an increase in the second week of lactation. This activity dropped after four weeks. The average PG activity from the individual buffalos showed a decrease from week one to week three. After this period was the activity stabilized. Bulk milk showed similar results, where the activity decrease between week one and week eleven for PL and PG. FFA were determined through an extraction-titration method. The average FFA levels from the individuals increased from 0.12 mmol/100 g fat to 0.23 mmol/100 g fat between first to the sixth week of lactation. The bulk milk showed similar results. The fat concentration was around 7% in both the individual and bulk milk samples.

The quality of the first milk unsuitable for the mozzarella production is probably not influence by FFA and protolytic enzymes such as PL and PG.

Keywords: WATER BUFFALO, MILK, MOZZARELLA, PLASMIN, PLASMINOGEN, FREE FATTY ACIDS.

Sammanfattning

Buffelmjölk är världens andra mest producerade mjölk efter komjölken och mozzarella är en av de viktigaste produkterna som produceras av buffelmjölk. Mjölken innehåller höga nivåer av näringsämnen, främst protein och fett, som ger buffelmjölk bra fysiska och kemiska egenskaper. Mjölk från den tidiga laktationen går dock inte att använda till mozzarellaproduktion. Det här arbetet analyserar inverkan på plasmin (PL), plasminogen (PG) och fria fettsyror (FFA) i mjölken från de första veckorna av tidig laktation, eftersom dessa faktorer är viktiga i mjölk och kan påverka ostkvaliteten. Syftet var att utreda om dessa faktorer kan vara involverade i svårigheten att tillverka mozzarella. Både individuella buffelmjölkprover och tankmjölk analyserades.

Mjölkproverna insamlades från sex stycken bufflar två gånger i veckan under de första 6-7 veckorna av laktationen och analyserades i duplikat. PL- och PG-aktiviteten bestämdes genom att mäta när PL formade en fluorescerande produkt genom klyvning av det tillsatta substratet vid 405 nm. Resultaten i den här studien är endast en indikation, då ingen statistisk analys genomförts. Medelvärdet för PL från de individuella bufflarna ökade fram till andra veckan av laktationen. Därefter sjönk aktiviteten fram till vecka fyra följt av en stabilisering i ursprungsaktivitet i sista perioden av undersökningen. Medelvärdet för PG aktiviteten från de individuella bufflarna visade en minskning i aktivitet från vecka ett till tre. Efter vecka tre blev aktiviteten stabil. Tankmjölken visade på liknande resultat, där aktiviteten minskade mellan vecka ett och elva för både PL och PG. FFA bestämdes genom en extraktion-titrationsmetod. Medelvärdet för FFA från de individuella bufflarna ökade från 0.12 mmol/100 g fett till 0.23 mmol/100 g fett från första till sjätte veckan av laktationen. Tankmjölken visade på liknande resultat. Fett koncentrationen låg på ca 7 % för både individuella prover och tankmjölkens prover.

Kvalitéten på den första mjölken som inte passar för mozzarellatillverkning påverkas troligen inte av FFA eller proteolytiska enzym som PL och PG.

Nyckelord: VATTENBUFFEL, MJÖLK, MOZZARELLA, PLASMIN, PLASMINOGEN, FRIA FETTSYROR.

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Abbreviations

PG	Plasminogen
PL	Plasmin
PA	Plasminogen activators
TAG	Triacylglycerols
FFA	Free fatty acids
BDI	Bureau of dairy industry
LPL	Lipoprotein lipase
pNA	Paranitroanilide
CCP	Colloidal calcium phosphate
t-PA	Tissue type plasminogen activators
u-PA	Uroinase type plasminogen activators
PAI	Plasmin activator inhibitors
UHT	Ultra high temperature

1 Introduction

1.1 Background and purpose of the study

Mozzarella is one of the most important products made of water buffalo milk and is mainly used in cooking as topping on pizza (Walstra *et al*. 1999). It is a white spherical cheese belonging to the Pasta-filata family. Pasta filata is cheese that has been heated in water (75-85°C) and thereafter kneaded and stretched to get the desirable texture: smooth and elastic. It is preferable made of buffalo milk since buffalo milk in comparison with cow milk is richer in most of the constituents like fat and protein (Aspilcueta-Borquis *et al*. 2010). The milk from buffalos also has a whiter colour than cow milk, because cow milk contains a richer carotene content. Mozzarella can also be made of cow milk, but the process needs to be modified to give a good result (Jana & Mandal 2011).

Italy is the biggest producer of buffalo milk in Europe, where the milk is almost exclusively used for mozzarella production (Zicarelli 2004), and they consume most of the their mozzarella themselves. India is the country that produces the biggest volumes of buffalo milk in the world (Ménard *et al*. 2009) and like other developing countries; the buffalo milk is often used as drinking milk (Zicarelli 2004).

However, buffalo milk from the first period after calving is difficult to use for the production of mozzarella, since stretching of the cheese curd is impossible. The first milk, called colostrum, is not used in the dairy industry (Coroian *et al*. 2013) but there are also problems with the following milk for a couple of weeks after the colostrum period. This is not a well-known problem except in the industry and little research has been done on buffalo milk. A major reason for problem with this early milk for mozzarella production is probably the calcium concentration, which is higher in the first lactation period (but also in the late lactation period) (Fox & McSweeney 1998). Too much calcium makes the cheese curd impossible to stretch (Joshi, Muthukumarappan & Dave 2004).

Today it is not known if there are other factors that can contribute to the problem with the unsuitable milk for mozzarella manufacturing. It is a problem that can result in big economical losses, both for smaller and bigger producers.

This project is a part of a larger study investigating factors as pH, calcium, fat, protein content and the rheological properties of the buffalo milk in the first stage of lactation and how the buffalo milk affects the properties important for the mozzarella production. The purpose of this study was to analyse how the activity of plasmin (PL), plasminogen (PG) and concentration of free fatty acids (FFA) change during the first weeks of lactation, because they are also factors that affect

cheese quality. PL is an enzyme that hydrolyses casein, which give poor cheese quality and PG is its proenzyme (Aslam & Hurley 1998). FFA are more known for the sensorial and nutritional value (Coroian *et al*. 2013) in cheese, but the possible impact of the physical properties should not be excluded.

2 Literature review

2.1 Water buffalo (*Bubalus bubalis*)

Water buffalos are the world's second largest producer of milk behind the conductive cows. There are two main groups of water buffalos: river buffalo and swamp buffalo. The river buffalo represents the best characteristics for milk production. Majority of the buffalos are located in India followed by China (Han *et al*. 2012). In some developing countries like India, Pakistan and Egypt buffalo milk account for 50% of drinking, while in other countries as Italy and Brazil the buffalo milk is almost exclusively used for mozzarella production (Aspilcueta-Borquis *et al*. 2010).

The buffalos are reared in intensive systems where they are managed and fed. Females are kept loose and milked twice a day mechanically, while males get full breed and are kept on slatted floors and slaughtered around 15 months, when they have a weight of more than 400 kg. There are many products from buffalo. Milk provides known cheeses such as ricotta, provola scamorza and from the meat comes steaks, roast, ham and salami. But the most important product in Italy and on the international market is mozzarella (Borghese 2013).

Buffalo breeding in Europe reveals two different trends in which Italy increase their progress with high quality milk and meat products on the market. Italy shows good economic development with progress of breeding, genetics and technologies. In the rest of Europe, buffalo breeding is a declining trend. There are 370 000 buffalos in Italy, 50 000 of which are recorded each month during lactation and show a yield of 2220 kg over the lactations 270 days. Some buffalo champions could produce a milk yield of more than 5000 kg, which is a lot more compared to other Mediterranean countries where the maximum yield is 1900 kg (Borghese 2013).

2.2 Buffalo milk

Every year is 82 billion litres of buffalo milk produced in the world, which is around 13% of all global milk. The biggest producer is India with 91% of the buffalo milk (Table 1). Even though this big amounts of buffalo milk, cow milk is on top with 551 billion litres, which is 84% of all the milk produced (Ménard *et al*. 2009). Milk composition differs between water buffalos and cows (Table 2). Buffalo milk contains a higher concentration of all components, mainly protein and fat, but there are also differences in lactose, minerals and vitamin levels. The higher nutrition values bring good physical and chemical properties for use in the dairy industry and mainly for mozzarella production. The final product is largely com-

posed of fat and casein and these are the parameters that in many countries determine the price of milk. Buffalo milk shows, despite their high fat content, a lower cholesterol content than cow milk. That, along with studies showing higher content of polyunsaturated fatty acids compared to bovine and sheep milk, make the buffalo milk particularly interesting (Aspilcueta-Borquis *et al*. 2010; Han *et al*. 2012).

Table 1. *Whole fresh buffalo milk produced during the years 1990-2013 in Europe and Asia in tonnes. The percentage shows the total globally produced milk during the year*

	Europe	where of Italy	Asia	where of India
1990	64 142 (0.1%)	43 000	42 762 071 (97%)	29 057 000
2000	144 343 (0.2%)	135 100	64 336 899 (96.7%)	43 428 000
2005	222 314 (0.2%)	215 228	76 255 905 (96.8%)	52 070 000
2013	203 791 (0.3%)	194 893	77 290 169 (96.5%)	70 000 000

Data from FAO STAT.

Table 2. *Milk components in different buffalo breeds compared to Holstein cross (cow)*

	Mediterranean water buffalo[1]	Bangladeshi buffalo[2]	Swamp buffalo[2]	Holstein cross[3]
Protein (%)	4.65	3.50	3.97	2.76
Fat (%)	8.30	5.84	8.43	3.71
Lactose (%)	ND	4.75	4.80	4.60
Ca (%)	ND	14.8	ND	10.91

[1](Zicarelli 2004), [2](Islam *et al.* 2014), [3](Khan *et al.* 2007), ND = Not determined.

The composition of milk can differ but the biggest variation is between colostrum and the regular milk. The following milk after colostrum is the raw material used in the dairy industry. Colostral period is between 3-5 days. It is vital for development and normal growth of the calf and it is also of big importance for the immune system, since maternal antibodies cannot be transported across the placenta. Diet of the buffalo seems to have a major role in colostrum composition and calves that are born in the summer might have an advantage in more concentrated colostrum feeding. Factors as breed, age and lactation can also influence the composition of colostrum (Coroian *et al*. 2013).

2.3 Mozzarella cheese

Mozzarella is a small spherical cheese originally made in Italy from buffalo milk and acid whey starter (Romano *et al*. 2011). The taste is mild and should not be pronounced (Walstra *et al*. 1999). The FFA concentration is low, except for C_4, but a role for FFA in the characteristic flavour is not apparent (Woo & Lindsay 1983). The cheese is often used in cooking and especially suitable for pizza, due to its ability to become soft and smooth when heated (Walstra *et al*. 1999). It consists of 16.7 g/100g protein, 24.4 g/100g fat and 55.5 g/100g water (BDA food composition database 2008). Today there is an upcoming trend in the development countries for buffalo mozzarella as a delicacy cheese for special occasions.

Mozzarella is one of the most important food products in Italy, which produces 36 000 ton of this cheese each year with a value of 500 million Euros. Italy consumes 82% of the cheese and 18% is exported mainly to Germany, France, USA and UK (Borghese 2013).

Mozzarella di Bufala Campana is a traditionally made mozzarella cheese from certain regions in the south of Italy: Campana, Latium, Puglia and Molise (Romano *et al.* 2011). The ingredients must comply with the regulations in the council regulation (EC) no 1151/2012 and is approved since 1996 to be labelled with *Denomination Origin Protected* (DOP). From the 4th of January 2016 should all of the DOP products be labelled with EU:s logotype for DOP. In 1998 was *Guaranteed Traditionally Speciality* (GTS) registered for *Mozzarella*. It means that it is a product with a special composition, production or process that conforms to traditionally production for the product or that it is made of traditionally ingredients (Livsmedelsverket 2015; DOOR database).

2.3.1 Mozzarella manufacturing

The manufacturing of mozzarella is very specific and producers have their own touch. This is of course a company secret, but the cheese is basically made in the procedure that is shown in Figure 1 below (Walstra *et al.* 1999).

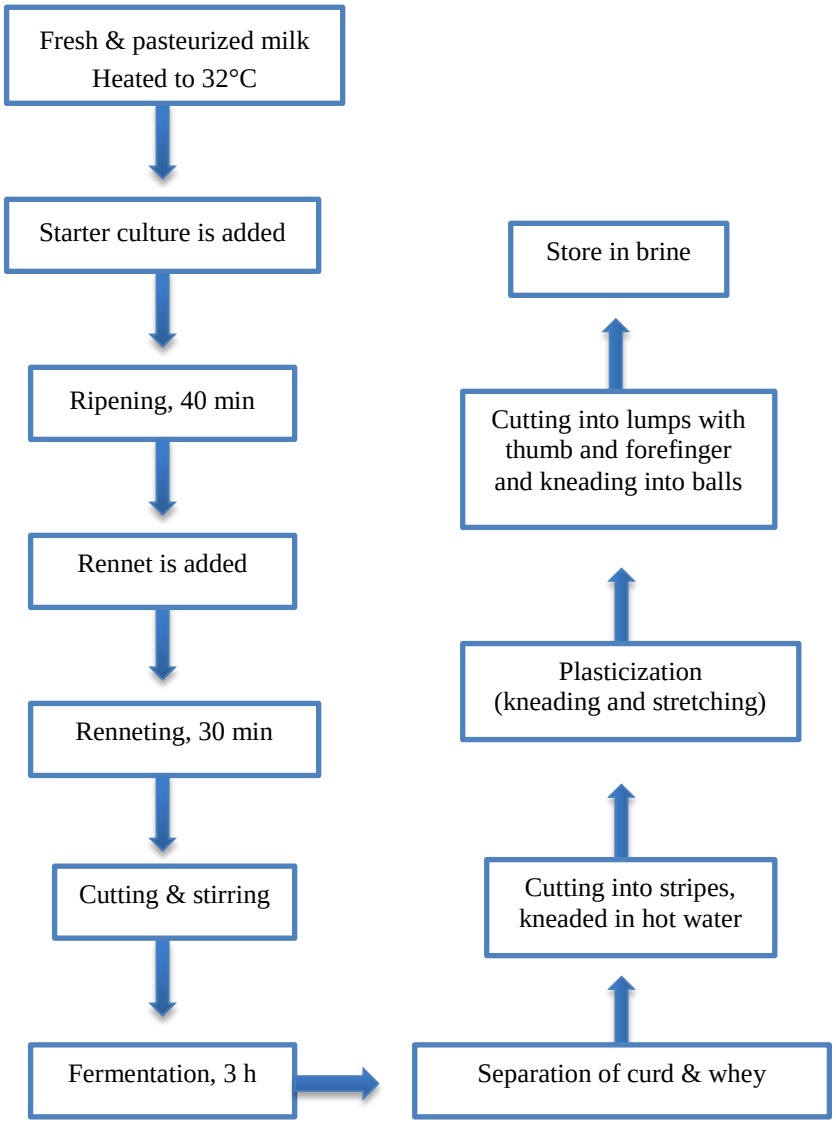

Figure 1. *Normal steps in mozzarella manufacturing. Picture based on Walstra et al. (1999).*

Fresh and pasteurized milk is heated to 32°C and starter culture is added. The starter culture is often added in form of whey from previous produced mozzarella and microflora is mainly composed of *Streptococcus thermophilus*, *Lactobacillus delbrückii ssp*. *bulgaricus*, and *Lactobacillus helveticus*. Lactobacilli produce lactic acid and decrease the pH from 6.7 to 5.2-.5.3. The pH is of big importance, since it makes stretching possible, due to solubilisation of micellar phosphate and increase colloidal calcium and para-casein hydration.

The milk is left to ripen for 40 minutes and rennet is added. Renneting time is around 30 minutes and rennet makes the mixture to turn into a coagulum. The curd is stirred, cut and rested. This procedure makes the curd to lose whey and is repeated until the curd has got a desirable texture. Thereafter it is left for 3 hours and fermenting starts.

Next step is to separate the curd from the whey. The curd is cut into strips and kneaded in hot water. This makes the cheese to melt, but through kneading and stretching, a procedure called plasticization, it turns into a solid consistency, called *pasta filata*. The high temperature of the water and the low pH is conductive for the formation of para-casein fibers that gives a strong stretchability to the cheese when it is cooked on pizza. The pasta is cut into lumps with the thumb and forefinger, kneaded into balls and swallows in cold water. The last step is to store in brine before packaging (Walstra *et al*. 1999; Guinee *et al*. 2002).

2.4 Calcium and pH and their influence on casein micelles

Calcium content and pH are important since they influence the ability of curd to plasticize in hot water. When the pH is decreasing during fermentation, it promotes solubilisation of micellar calcium phosphate and increases the ratio of soluble to colloidal calcium and para-casein hydration. If the pH is above 5.4, the cheese mass is to firm and difficult to plasticize, and if the pH is below 5.1 it gets too crumbly (Guinee *et al*. 2002).

Concentration of calcium is important for controlling interactions of proteins in the matrix of mozzarella cheese. Majority of the total calcium in milk is present as a colloidal calcium phosphate (CCP), where it functions as a bridge to bind submicelles in the casein micelles (Joshi, Muthukumarappan & Dave 2004). Colloidal calcium bind to the casein trough phosphoserine groups. These bindings, together with the hydrophobic regions of casein that also form binding, give micelles a great stable networks (Coultate 2009). When pH decreases during cheesemaking, the CCP dissociates from the casein micelle, leaving calcium and phosphate at the terminals of casein. This decrease in calcium content is associated with decrease in numbers of binding sites for casein particles in cheese and makes the hard curd turn into a soft and stretchable structure. Casein associated calcium and phosphate

in too high amounts, results in tough curd that tears and fractures during stretching. Too little calcium and phosphate, however, result in complete loss of structure and stretch (Joshi, Muthukumarappan & Dave 2004).

2.5 Milk proteins

Despite the global spread of buffalo few studies are done on the protein composition of buffalo milk. The studies available are contradictory and studies on the buffalo milk's proteins are often with samples from few animals (Bonfatti *et al*. 2012). Bovine milk could be used as a calibration standard and consists mainly of six different proteins αs1-, αs2-, β-, and κ-casein, β-lactoglobulin and α-lactalbumin (Heck *et al*. 2009). The distributions of the proteins are 80% casein with the four subtypes αs1-, αs2-, β-, and κ-casein and 20% whey in the milk. The main fraction of milk protein is α-casein consisting of αs1- and αs2-casein (Park *et al*. 2014). The nutritional value and technological properties are largely determined of the protein composition, like a high concentration of casein increases the cheese yield. The casein composition even affects the cheese properties like coagulation time and curd firmness. The protein composition in milk varies depending on the season and some of the factors affecting the composition are stage of lactation, feeding and health status, but the main affecting reason are genetic factors (Heck *et al*. 2009).

2.5.1 Proteolysis of milk proteins

Proteolysis is the degradation of proteins to smaller polypeptides and free amino acids. Proteolysis is mainly caused by proteases. Proteases are enzymes, which act against the protein and promote the breakdown into smaller parts. There are many various types of proteases in milk, which can be either exogenous or endogenous. Exogenous proteases can come from microorganisms and endogenous can be transported from the blood of the cow to the milk (Aslam & Hurley 1998).

2.5.2 Plasmin/plasminogen system

PG is the inactive pro-enzyme converted to the active form - PL. It mainly comes from the liver but also from adrenal glands, kidney and brain (Lijnen 2001; Francis & Castellino 2005). PL plays an important role to cleave fibrin, to dissolve blood clots. This action is also referred to as fibrinolysis (Turner *et al*. 2002). The activation of the fibrinolytic system depends on the conversion of PG to PL, mediated by two plasminogen activators (PA). The tissue type (t-PA) is in the circula-

tion and involves the degradation of fibrin. The other one, urokinase type (u-PA), contributes to the activation of cell-bound PG. u-PA has the ability to bind to specific cellular receptors. The system inhibits either at levels of the PA by specific activator inhibitors (PAI), or at levels of PL. PL is important for tissue remodelling and bacterial invasion. PL is also involved in other functions in the body, e.g. the breakdown of protein barriers that promotes cell migration, wound healing and the growth and spread of tumours (Lijnen 2001; Francis & Castellino 2005).

2.5.3 Plasmin and the dairy industry

PL is the most prevalent protease in milk. PL can be transported over to mammary epithelial cells from the blood plasma (Di Luccia *et al*. 2009). PL proteolysis influences the degradation of milk proteins, especially casein. PL attacks mainly β-casein. The other types of caseins are known to be more resistance to PL. During the action of PL undesirable off-flavour and bitterness occurs in milk and milk products.

The activity of PL depends on the interaction between the proenzyme PG and its activators and inhibitors. PL, PG and the PG activators are connected to the casein micelles and the fat globule membranes present in the milk (Aslam & Hurley 1998). The largest amounts of PL are mainly found in the curd while inhibitors of PG activators generally are lost with the whey. Storage, heat treatment and pH are some of the factors that can influence the PL activity in dairy products. PL survives high heat treatment and is still active during cold storage and can influence the coagulation properties of milk (Di Luccia *et al*. 2009).

Protein degradation caused by PL can be either positive or negative, depend on the dairy product and provide many different effects on product quality. Cheese flavour and texture may be dependent on PL activity where the protein degradation may be advantageous during ripening and contribute to desired tastes and texture. In pasteurized milk, it may instead provide a negative effect, such as undesired gelation or precipitation. The decrease in pH during cheese manufacturing causes dissociation of the casein micelles and the PL and PG leak and are lost with the whey. The cheese ripening process can be slowed if the casein micelles drop PL, leading to increased production costs. Uncontrolled proteolysis can lead to losses of curd formation (Ismail & Nielsen 2010).

Studies on bovine milk shows that the concentration of PL and PG are lower during early lactation and higher in the later state (Korlycka *et al*. 1982; Aslam & Hurley 1998). This is due to the increased permeability of the blood-milk barrier during the late stage. Also older cows show higher activity and the activity seems to increase with lactation number. The enzyme activity may depend on many factors. The breed of the cow is one factor and another is if the cow has some disease condition, as mastitis increases the number of somatic cells, which leads to in-

creasing the number of PG activators. Even factors related to post-harvest handling and feeding can influence the PL activity in milk (Aslam & Hurley 1998).

2.6 Milk fat

Lipids have an essential role in metabolism. It provides the cell with energy and the cell membranes is mainly built up of lipids. It is also a growth factor and is involved in the immune system. There are few studies made on fatty acids from buffalo milk, but it is known that there are more total lipids in buffalo milk than in cow milk (Coroian *et al*. 2013). Mean value of fat in buffalo milk varies between 6.87% and 8.59% (Aspilcueta-Borquis *et al*. 2010), which is considerably higher than cow milk with around 4,2% fat (Månsson 2008). The high amount of fat is interesting since the cholesterol content is surprisingly low in comparison with cow milk (Aspilcueta-Borquis *et al*. 2010). As mentioned before, the season and breed can influence the composition of milk and thereby the amount of lipids. Coroian *et al* (2013) investigated the seasonal changes of buffalo colostrum milk and although the results were quite similar, there were differences in fat content in the milk depending on *postpartum* day and season. Colostrum from the summer season showed higher concentration of all sixteen studied fatty acids in relation to winter colostrum. In the summer season, mean values of fat were 11.31% on the first day and 7.56% in the seventh day. In the winter season the mean fat content were 11.22% and 7.51%, on the first and seventh day respectively. On the fifth day, the amount of total fat had reached the normal amounts of fat in buffalo milk. The slightly higher values were possibly due to pasture in the summer season (Coroian *et al* 2013).

Milk fat is dispersed in form of spherical droplets called milk fat globules. They are mainly composed of triacylglycerols (TAG), which represents 98% of the milk fat. The milk fat globules are bigger in buffalo milk than cow milk and the major fatty acids are palmiatic acid (C16:0), oleic acid (C18:1), myristic acid (C14:0) and stearic acid (C18:0) (Ménard *et al*. 2009). Even though milk from buffalo contains more fat than cow milk, the proportions of mono- di- and triacylglycerols are similar to cow milk (Park & Haenlein 2006).

2.6.1 Lipolysis

Lipolysis is the hydrolysis of TAG, performed by an enzyme called lipase. It results in free, unesterified fatty acids known as FFA and mono- or diglycerides or glycerol.

Lipolysis increases the FFA concentration in the milk. The main lipolytic enzyme lipoprotein lipase (LPL) liberates fatty acids from TAG lipoproteins and

chylomicrons in blood. The enzyme has a temperature optimum about 33 °C and pH optimum about 8.5 (Walstra *et al*. 1999). The lipolytic activity of LPL in milk can depend on different factors: time elapsed between milking and heat treatment, temperature of the raw milk before processing and handling of the milk. Rough handling can cause breaking of the fat micelles, which expose the lipids for the lipolytic enzymes. LPL is inactivated during heating processes. Even if LPL is causing most of the lipolysis, it can also be derived from microbial milk lipase. The microbial lipases are produced by psychrotrophic bacteria, and can grow in milk in temperatures around 4 °C. Unlikely LPL, microbial lipases are thermoresistant at the temperatures of pasteurisation and ultra high temperature (UHT). Determination of FFA quantity can therefore be useful information of the lipase activity and milk quality (Antonelli *et al*. 2002).

2.6.2 Free fatty acids

Lipids contribute to sensorial and nutritional quality of different cheeses and provide many benefits to human health (Coroian *et al*. 2013). The most important flavour compounds in cheeses come from the FFA formed by lipolysis but the FFA are also correlated to a soapy-rancid flavour, which is not favourable (Walstra *et al*. 1999). It is especially the FFA with C_4-C_{12} that contribute to the flavours, but they also act as precursors for further reactions that produce volatile compounds important for aroma. Long chain saturated and monounsaturated FFA (C_{14}-C_{18}) are not associated with rancidity (Duncan, Christen & Penfield 1991; Vélez *et al*. 2010).

FFA occurs in a low amount in milk but they have a big impact on quality. The presence in milk is due to three different sources; blood, intracellular or bound to serum albumin; by the passive loss of unesterified fatty acid of the secretory cells of the mammary gland; or by hydrolysis of TAG. The occurrence in fresh milk is mainly depending on unsynthesized fatty acids, while in stored milk it is a result of lipolytic activity (Cardak, Yetismeyen & Brückner 2003).

3 Material and Methods

3.1 Milk sampling

The milk from six Mediterranean buffalos from Ängsholmen farm was collected directly from individuals after milking and from bulk milk *post partum,* twice a week. Samples have been collected from the individuals during seven weeks and eleven weeks from the bulk milk and were already collected when used in the present study. The milk samples were preserved by bronopol, in order to inhibit the microbial activity. The samples were stored at -20°C prior analysis. The bulk milk was gradually admixed from all six buffalos after which they calved.

3.2 Plasmin/plasminogen analysis

The method was based on Korycka-dahl *et al* (1982) with the exception that the absorbance was measured with a 96-plate spectrophotometer from Ohmega, instead of a cuvette spectrophotometer.

3.2.1 Plasmin extraction

The frozen milk samples were thawed one day in advanced at 4°C and were placed in 45°C water bath for 15 minutes before use. Two ml of milk was defatted by centrifugation at 1500 rpm 4°C for 10 minutes, the fat layer was removed. 0.5 ml of defatted milk was mixed with 7.5 ml buffer containing e-amino-n-caproic acid (EACA) (20 mM), Trizma buffer (53 mM), NaCl (117 mM), pH 7.4. The samples were incubated at room temperature for 2 h in order to dissolve the PL and PG from casein micelles. The milk serum was separated from casein micelles by ultracentrifugation at 100 000 g at 4°C for 1 h. The weight of centrifugation tubes with the samples was adjusted before the centrifugation. Two ml of the serum was collected in Eppendorf tubes and stored in -20°C for further analysis of the PL/PG activity.

3.2.2 Plasmin and plasminogen activity analysis

PL and PG activity were determined by measuring the cleavage of substrate S2251 and the formation of paranitroanilide (pNA) at 405 nm at pH 7.4 and 37°C. The change in absorbance was measured with 3 minutes interval for 2 h, 41 cycles. The assay was performed with a 96-wells plate placed on the ice before measurements, to reduce the enzymatic activity during preparation. For the PL

activity 150 µl of serum and 40 µl of substrate were used. PG activation to PL was both in the presence of substrate and 4.5 µl urokinase. The activities were measured in duplicates. Two hundred µl of buffer was used as a blank.

3.2.3 Definition of plasmin

PL and urokinase activated PG activity were converted into the same unit, where one unit is defined as the amount of enzyme producing a Δ A1 of 0.001 for 1 min at 405 nm, pH 7.4 and 37°C during formation of pNA from the substrate. The amount was calculated with the following equation:

$$\frac{(A2 - A1)/(T2 - T1)/0.001}{V * 1000}$$

Where A1 = absorbance start, A2 = absorbance end, T1 = time start (min), T2 = time end (min), 0.001 = factor for the amount of enzyme produced, V = serum volume (µl) and 1000 = conversion factor.

3.3 Free fatty acids analysis

The FFA method is based on the method described by Evers (2003). The samples were thawed in a refrigerator the day before. On analyse day, samples were prewormed in a 45 °C water bath for 15 minutes and vortexed. Two ml of milk sample was pipetted into teflon tubes (40 ml). Ten ml of diethyl eter:hexane (80:20 v/v), 2.5 ml of NaCl (26% w/v) and 14.4 ml methyl orange indicator (0.25% in water) was added and the tubes were shaken for one minute. Diethyl eter:heaxane is used as the organic solvent to solve the fatty acids and NaCl to make the two phases more separated. H_2SO_4 was added until the pH reached 2-3, which could be observed as the water phase got a pink colour. The tubes were shaken for another one minute and centrifuged at 3000 rpm and 21°C for five minutes to separate the organic and unorganic layers. The tubes were carefully taken out from the centrifuge. Blanks were made in the same way, except for addition of milk; water was added instead. Four ml of supernatant containing the organic layer was analysed in replicates. Ten µl of α-naphtolholphtalein indicator and ten µl of phenolphthalein indicator were added to the supernatant right before the titration with KOH (0.01 M) started. The replicates were stirred with a magnet during titration for better distribution of KOH. KOH was added until the solution turned from white transparent to purple. The volume of added KOH was thereafter used for calculation of the FFA amount according to the equation:

$$\frac{\left(\frac{(Vp-VB)CKOH*100}{m}\right)}{V}$$

Where V_p = volume KOH of sample (ml), V_B = volume blank (ml), C_{KOH} = concentration of KOH (0.01 mol/l), 100 = factor for 100 g FFA, m = total fat in the sample (%), V = volume of supernatant (ml).

To be able to calculate FFA, the total fat concentration in every sample was needed. The results of the fat concentrations are presented in this study, but the measurements were performed earlier and not by the authors.

4 Results

4.1 Plasmin and plasminogen

PL and PG activity were measured for each buffalo, the first measurement a few days after calving continuing one month ahead. The results can only be seen as an indication as no statistical analysis has been performed.

Table 3. *Average plasmin activity for individual buffalos given in units/ml*

Buffalo Id	Week						
	1	2	3	4	5	6	7
14	5.43[1]	17.44[1]	14.60 ±0.92	6.05 ±0.69	6.61 ±0.76	7.95 ±2.79	7.33[1]
69	ND	4.88 ±2.18	4.96 ±0.50	5.16 ±2.13	6.36 ±0.87	3.55 ±1.66	ND
70	2.14[1]	3.72 ±0.35	4.90 ±3.93	1.56[1]	1.27[1]	ND	ND
74	13.61[1]	13.80 ±0.43	4.78 ±0.92	3.77 ±0.96	3.52 ±3.41	ND	ND
76	4.44[1]	7.84 ±2.83	7.80 ±3.63	7.20 ±1.41	10.96 ±2.48	8.11 ±0.65	ND
80	1.37[1]	3.80 ±0.66	2.74 ±0.06	3.95 ±1.55	2.99[2] ±0.82	ND	ND

[1] *One measurement,* [2] *four measurements, ND not determined.*

The PL activity showed varied results between the buffalos. All the buffalos showed at first an increase of the PL activity to week two. Buffalo 69, 70 and 74 showed from the first to the last measurement a decrease in PL activity while buffalo 14, 76 and 80 showed an increase, although it is difficult to see any clear pattern (Table 3). The average of the PL activity can be seen in Figure 2.

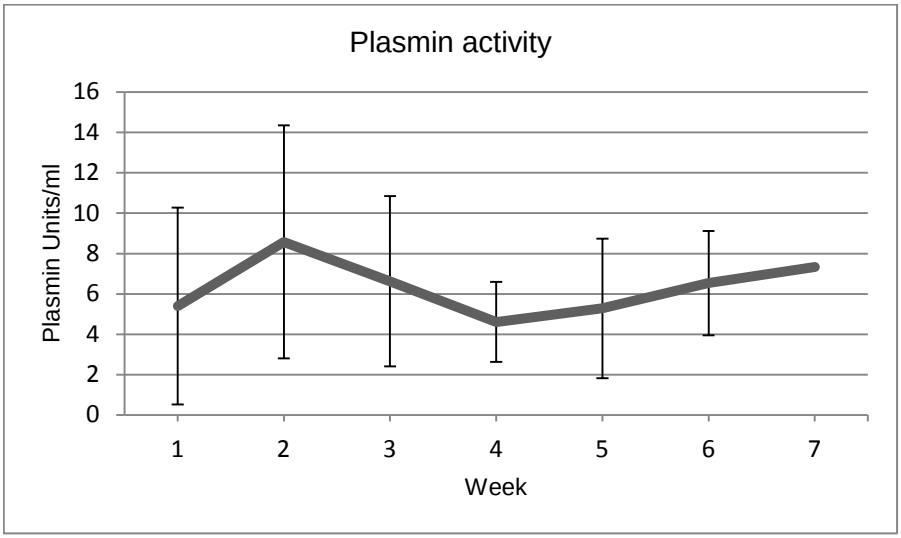

Figure 2. *Average of plasmin activity from all individual buffalos analysed. Total samples analysed n = 59.*

Table 4. *Average plasminogen activity for individual buffalos given in units/ml*

Buffalo Id	Week						
	1	2	3	4	5	6	7
14	37.43[1]	22.74[1]	16.61 ±0.61	18.29 ±2.61	19.18 ±4.95	15.31 ±3.16	17.11[1]
69	ND	32.90 ±18.12	18.77 ±1.96	13.41 ±7.74	12.06 ±4.46	17.80 ±6.03	ND
70	31.36[1]	32.34 ±11.54	25.17 ±5.21	17.33[1]	16.88[1]	ND	ND
74	72.10[1]	32.95 ±30.17	16.01 ±3.79	13.22 ±2.08	18.25 ±2.09	ND	ND
76	36.35[1]	23.91 ±0.59	16.40 ±1.42	15.18 ±0.73	14.28 ±3.60	20.87 ±0.16	ND
80	54.98[1]	20.20 ±11.61	15.32 ±1.68	17.48 ±1.15	16.12[2] ±2.15	ND	ND

[1]*One measurement,* [2]*four measurements, ND not determined.*

PG activity was high the first days after calving for all of the buffalos except for buffalo 69 where samples from the first week were missing (Table 4). Reduction in activity was observed around week three-four followed by stabilisation in the last weeks of the investigation. PG showed a higher activity compared to PL activ-

ity and showed a more decreasing trend (Table 3 and 4). The average of PG activity can be seen in Figure 3.

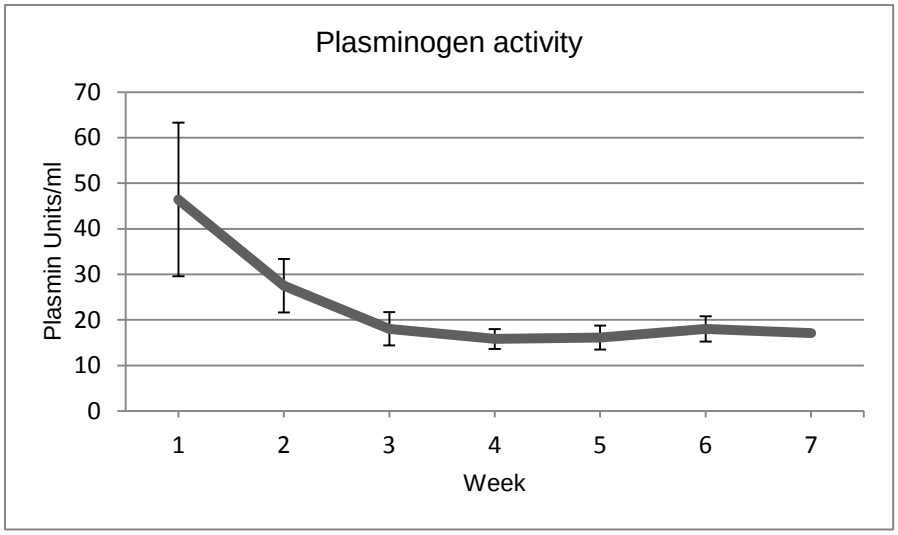

Figure 3. *Average of plasminogen activity in milk from all individual buffalos analysed, n = 59.*

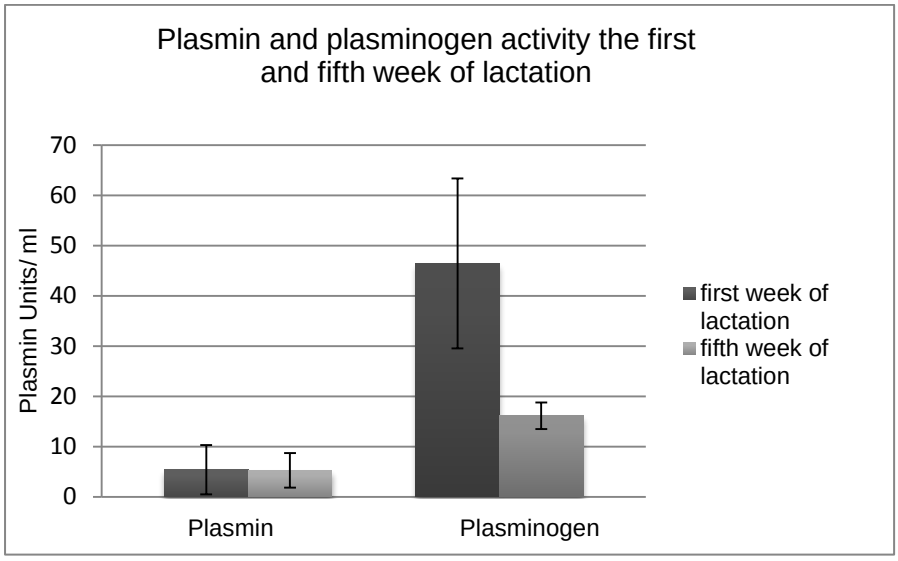

Figure 4. *Average of plasmin and plasminogen activity in milk the first and fifth week of lactation, n = 18.*

On average the PL activity indicated a 2 % decrease between first to fifth week of lactation while PG activity indicated a 65 % decrease (Figure 4). Week five was studied in the absence of samples during week six and seven.

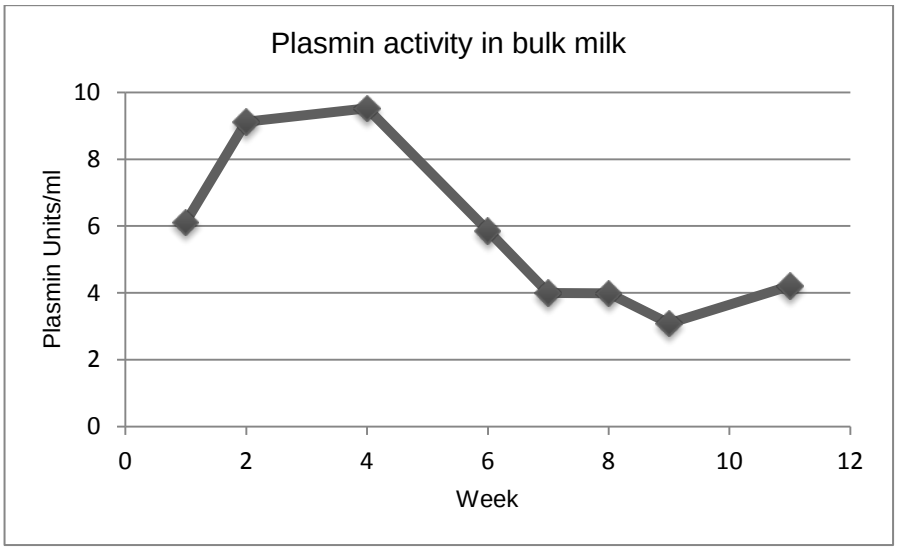

Figure 5. *Plasmin activity in the bulk milk, n = 13.*

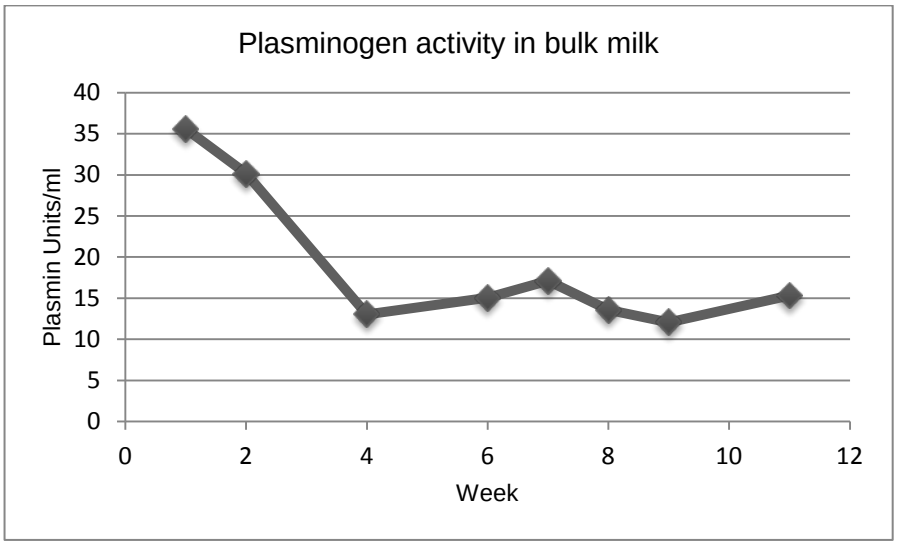

Figure 6. *Plasminogen activity in the bulk milk, n = 13.*

The bulk milk showed similar trends as the average of the six buffalo milk samples. PL activity was 31 % lower in the end of the trial period (Figure 5). PG activ-

ity decreased after four weeks with 63 % and remains more or less stabilised in the remaining weeks of investigation (Figure 6).

4.2 Free fatty acids

The FFA concentration in the milk samples showed an overall increase with 92 % from week one to six (Table 5). There was only one sample from week seven that could be analysed, why only week one to six is presented. For all of the buffalos, the concentration increased except for buffalo 14, where the FFA decreased. The amount varied within the individuals; there was not a straight increase or decrease of the FFA that could be observed. But in average, the increase was almost a straight line, which can be seen in Figure 7.

The first sample from buffalo 69 was taken from day nine and the others were taken from the end of the first week of lactation. The contributing samples to each week were mostly two, but for some weeks there were only one and sometime three samples. In total 57 samples were analysed for the FFA. The results can only be seen as an indication as no statistical analysis has been performed.

Table 5. *Average values for free fatty acid concentrations for individual buffalos during the first six weeks of lactation given in mmol/100 g fat*

Buffalo Id	Week					
	1	2	3	4	5	6
14	0.18[1]	0.14[1]	0.39 ±0.01	0.11 ±0.01	0.13 ±0.02	0.12 ±0.01
69	ND	0.12 ±0.00	0.16 ±0.00	0.18 ±0.05	0.21 ±0.09	0.27 ±0.01
70	0.13[1]	0.15 ±0.03	0.14 ±0.04	0.13[1]	ND	0.17[1]
74	0.10[1]	0.11 ±0.01	0.16 ±0.06	0.19 ±0.01	0.21 ±0.04	ND
76	0.10[1]	0.12 ±0.02	0.15 ±0.00	0.15 ±0.01	0.14 ±0.02	0.19 ±0.02
80	0.10[1]	0.17[2] ±0.04	0.21[1]	0.24 ±0.03	0.48[1]	0.43[1]

[1]One measurement, [2]Three measurements, ND = Not determined.

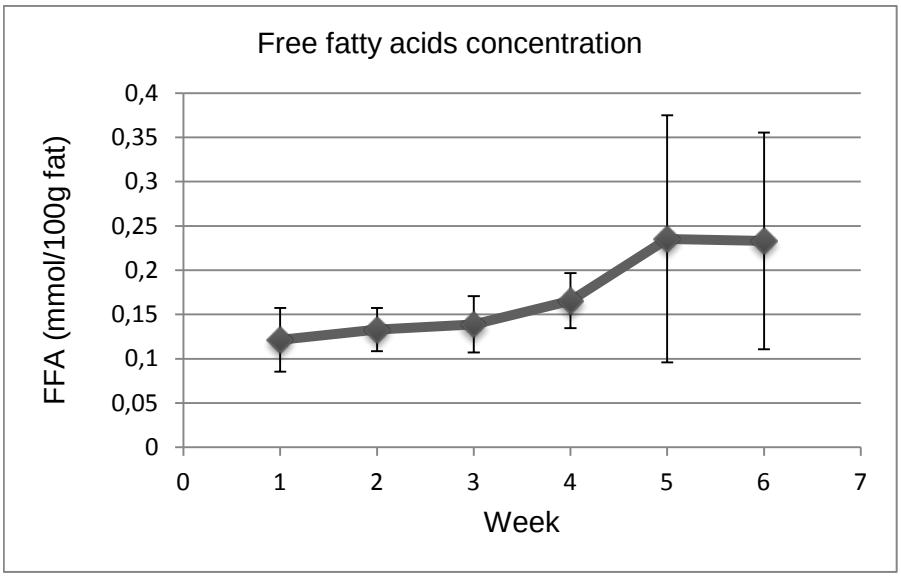

Figure 7. *Average concentration of free fatty acids, in the milk from all the individual buffalos analysed, n = 56.*

The average FFA levels from the individuals increased from 0.12 mmol/100 g fat to 0.23 mmol/100 g fat between first to the sixth week of lactation. The increase of FFA can easily be seen in Figure 7 and 8, where Figure 8 shows the differences between first and sixth week of measurement.

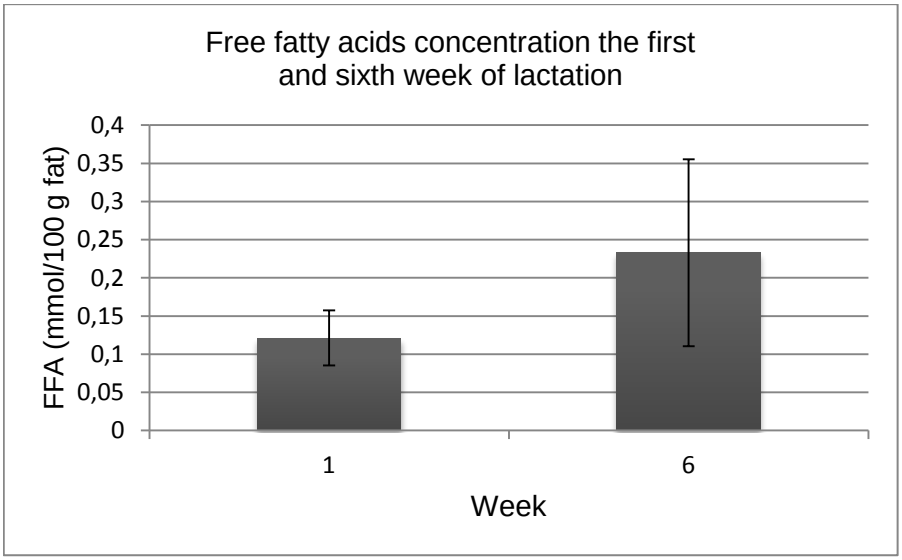

Figure 8. *Average free fatty acid concentrations in milk from first and sixth week of lactation, n = 13.*

Table 6. *Average fat concentration for individual buffalos during the first weeks of lactation given in %*

Buffalo Id	Week					
	1	2	3	4	5	6
14	6.90[1]	7.21[1]	6.77 ±0.06	6.70 ±0.47	6.58 ±0.15	6.56 ±0.05
69	ND	7.47 ±0.54	6.25 ±0.11	6.55 ±0.09	6.57 ±0.01	7.05 ±0.14
70	6.32[1]	4.99 ±0.10	8.24 ±0.64	7.79[1]	ND	7.20[1]
74	7.60[1]	7.19 ±0.25	6.95 ±0.06	7.24 ±0.05	7.48 ±0.24	ND
76	8.50[1]	6.94 ±0.15	7.05 ±0.59	7.25 ±0.26	7.35 ±0.04	7.77 ±0.33
80	7.90[1]	6.98[2] ±0.70	7.01[1]	6,50 ±0.11	6.57 ±0.37	6.68[1]

[1]*One measurement,* [2]*Three measurements, ND = Not determined.*

Table 6 shows the values of the total fat concentration during week one to six, with a fat concentration varying from 6.32% to 8.50 the first week. A small decrease with an average of 5% occurred mainly during the first two weeks, depending on some deviant values week one and two (Figure 9).

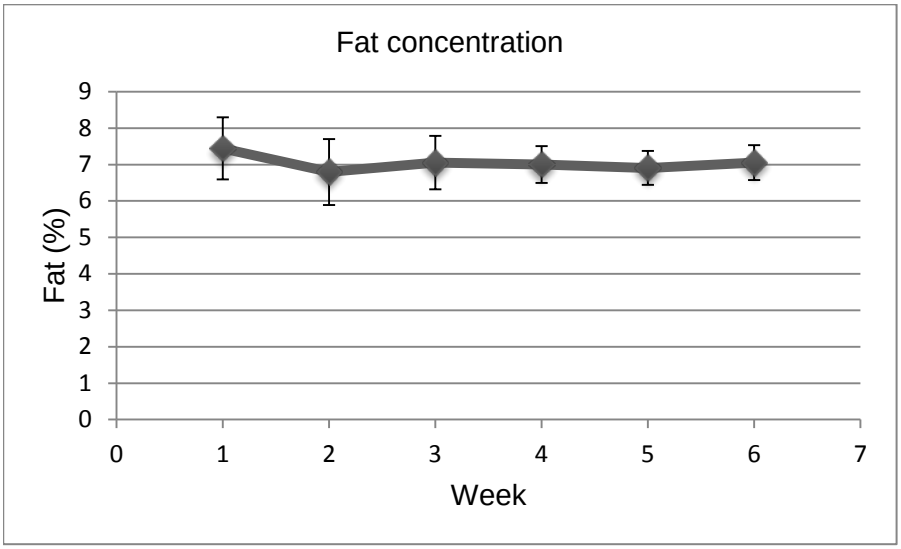

Figure 9. *Average total fat concentration in milk from all individual buffalos analysed, n = 57.*

Figure 10. *Fat concentration in milk the first and sixth week of lactation, n = 13.*

Figure 10 shows the fat content from week one and six and gives a picture of that the fat concentration was almost the same during these weeks with only a small decrease.

Only eight samples contributed to the evaluation of FFA concentration in the bulk milk, but it can be seen that FFA increased with time by 69% (Figure 11). The samples between week one and six could not be determined, since the fat concentration had not been measured for those samples.

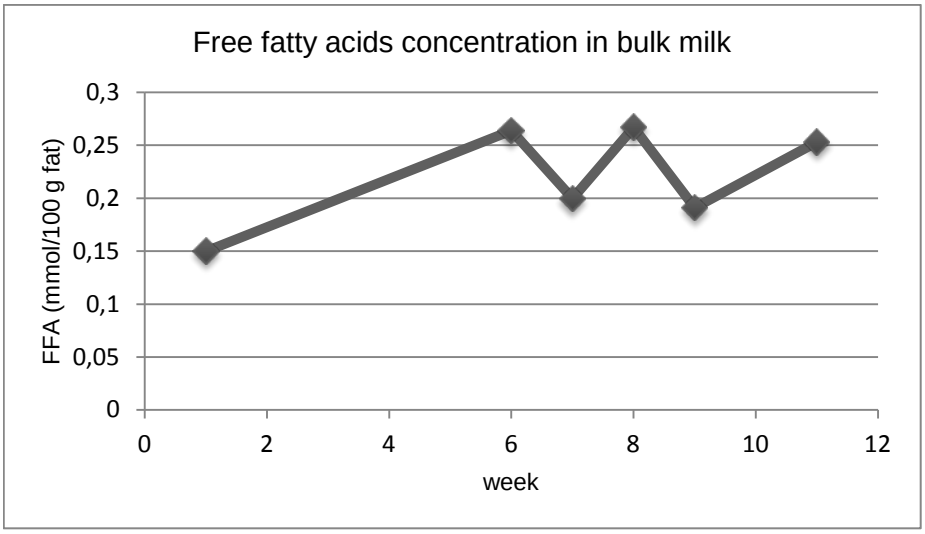

Figure 11. *Free fatty acids concentration in the bulk milk, n = 8.*

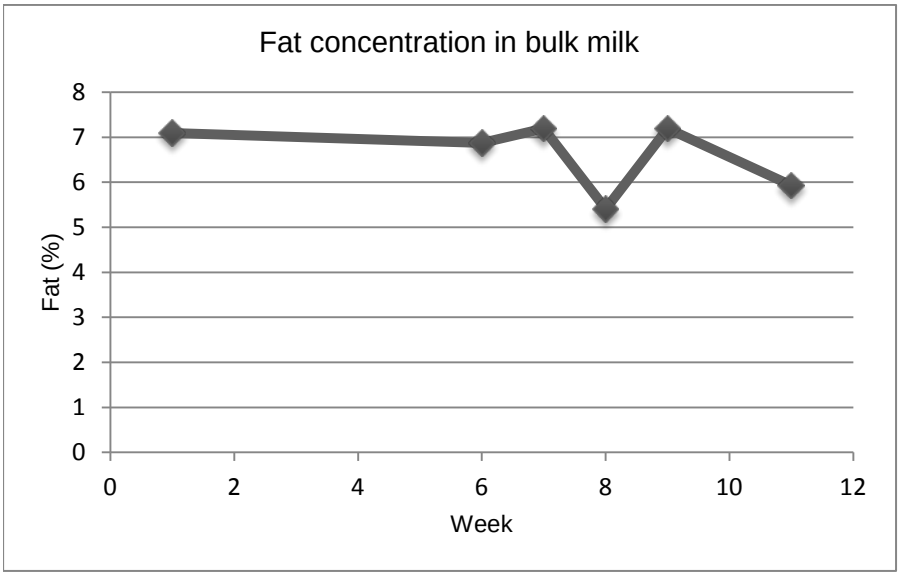

Figure 12. *Total fat concentration in the bulk milk, n = 8.*

The total fat concentration showed values between 5-7% (Figure 12), which was a wider range than for the individual samples (Figure 9). Like the samples for the individuals, however, a decrease in fat concentration could be observed, but here with 16 %.

5 Discussion

This was a small study with milk samples from only six individual buffalos. Some of the samples resulted in deviant values in the analyses, which with few individuals had a big impact on the results. The analysis in this study give however an indication of the investigated parameters. A bigger study with more animals, more samples per week and statistical analysis would be an improvement of the present project.

5.1 Plasmin and plasminogen analysis

The enzyme assays on the buffalo milk were all measured on skim milk, as the major parts of PL and PG are found in the casein micelles, compared to the fat globule membrane, which only contain small amounts. The enzyme activity was determined through the milk serum. The milk samples were first pre incubated together with EACA buffer. EACA dissociates PL from the casein micelle. Compared to untreated milk the casein micelles are intact and the enzyme remains associated (Korycka-Dahl *et al*. 1983).

PL in milk is well studied and there are different methods of how to determined PL activity in milk. In the various methods different substrates and units are used which can make it difficult to summarize and get an overview picture of the factors affecting the PL activity (Kelly, O`Flaherty & Fox 2005). Synthetic chromogenic substrate is used in many cases due to its sensitivity and advantageous with just a small sample preparation needed (Bastian *et al*. 1995). The sample preparation differs between the methods, which may have impact on the final result, when PL displays optimal activity at 37 °C and pH 7.5 (Somers, Guinee & Kelly 2002; Bastian *et al*. 1995).

The different measurements of PL in milk after isolation comprises in a buffer, a milk-like environment or measuring the products of enzyme action. The measurements of PL activity after different isolations are much discussed. Concern between these methods may be, although the samples contain the same amount of PL. One sample may e.g. contain high concentration of PL inhibitors, which may affect the activity (Kelly, O`Flaherty & Fox 2005).

The method where the ultracentrifugation is used and resuspension occur in buffer, to separate the serum phase containing PL inhibitors, should provide a more equal result between samples. In the method where the trisodium citrate dissolves the casein to release PL just before the analyse, there should be a difference in the expositor of the inhibitors in both samples that influence the activity (Kelly, O`Flaherty & Fox 2005).

Inhibitors and activators play an important role and have an impact on the PL activity. Despite a good method it may be difficult to analyse PL, with the fact that the storage of the milk can complicate the measurement of enzyme activity. Further thing have come up with the well-known substrates Spectorzyme-PL and S-2251, used in this assay. They have been found to enhance PG activators. Processes in the milk may decrease or increase depending on many factors, which is important to take into account. Discussed situations above must be taken into consideration when measuring the PL activity in milk (Kelly, O`Flaherty & Fox 2005).

Many studies show that PL and PG activity increases at the end of lactation. Bastian and Brown (1995) concluded that the enzyme activity increases with the stage of lactation, severity mastitic infection and lactation number of milk. Even that older cows showing higher PL activity in the milk, PG content seem unchanged. Baldi *et al* (1996) made a study on cows that revealed no significant differences for either PL or PG content during the first three months of lactation, while they both have a peak during the fifth months. The study shows increased PL levels with advancing lactation. In this study it was also possible to see major differences between different herds, due to the PL system. The highest activity was measured in the herd were the cows were fed with the lowest level of hay. Difference between herds was probably due to differences in fed and managed. More studies are needed to make any conclusions but the feeding is an important observation.

According to the analysis of PL and PG on the water buffalos from Ängsholmens farm no increase of the enzyme activity could be seen. PG showed a reduction before the twelve days while the analysis of plasmin activity showed a fairly constant level (Figure 2 and 3). The PG result contradicts the other studies that show a constant level in the first period of lactation and an increase at the end. Difference may be due to the lost of inhibitors in the first period of milk after calving. The inhibitors seem to increase during the lactation with the decreasing activity but this factor was not investigated in this study. The constant level of PL consistent with Bastian and Brown (1995) and Baldi *et al* (1996). Since the measurement was made only in the first month it was not possible to see the expected increase at the end of the lactation. The bulk milk showed similar results as for the individuals but dilutive effect could be observed since the mixed milk was in different stages after calving (Figure 5 and 6). According to the results, PL is probably not the reason that the first milk cannot be used for mozzarella production as no major changes seem to occur during the first month. The change that can be seen is the sharp reduction of PG but how and whether it affects the milk for mozzarella production needs to be further investigated.

5.2 Free fatty acids analysis

The FFA concentration seemed to increase during the first stage of lactation (Figure 7 and 8). It varied a lot during the days but there was an overall increase and the total fat content does not seem to be correlated to the amount of FFA. The increased values of FFA during lactation confirm the results found by Cardak, Yetismeyen and Brückner (2003). The variation of milk fat observed in the beginning of lactation (Table 6 and Figure 9) was due to a few samples that contained deviant fat concentrations.

There were some samples with a really high FFA content. Buffalo 80 had a conspicuously high amount of FFA in comparison to the others (Table 5). It could be due to higher lipases activity in the milk from this individual.

The higher amount of FFA in the bulk milk can depend on how long the samples were in the tank before the samples were taken. The increase in FFA agrees with Antonelli *et al* (2002), which showed that the FFA (in bovine milk) increased in raw milk samples taken from a tank. The amount of FFA in the study did also varied between samples, but it was concluded that it was due to the different farms. Paseurized milk was also tested in the study, resulting in more constant values of FFA (Antonelli *et al* 2002).

When comparing the results for the individuals and bulk milk, there was a bigger variation in fat concentration in the bulk milk. That is interesting sine the individual samples gave almost equal and mostly higher results. Explanations could be that the tank contained different amounts of milk each sampling day if the samples were not taken from the same level in the tank or how long time the milk was stored before the samples were taken. Fat can easily stratify in the tank and it is important to take a sample that is presentable and equal for the tank each time.

Cardak, Yetismeyen and Brückner (2003) compared the FFA in goat, camel and cow and got a total FFA value of 13.56 μmol/10 ml for cow bulk milk, which is a result close to the present study. (The total FFA for goat and camel was 22.56 and 13.64 μmol/10 ml, respectively). This gives a clue that the content of FFA is probably not a contributing factor to the impossibility to make mozzarella from the early milk. The low values seem more likely to be favourable amounts, independent of stage of lactation. The most important thing seems to be to use freshly pasteurized milk in mozzarella production with respect to FFA.

Woo and Linsay (1983) investigated concentrations of FFA and flavour development in four Italian cheeses, where one of them was mozzarella. The FFA values were much lower in comparison to the other cheeses (Provolone, Parmesan and Romano). As an example, the amount of C_4 FFA for mozzarella was 48 ppm and for the others 376, 140 and 1756 ppm, respectively. It was similar for the other FFA except that there were hardly any of C_6 or C_8 FFA in the mozzarella. This is also a result that agrees with the idea that the low FFA content is not a factor that

makes the mozzarella manufacturing difficult. Instead, the low content is wanted because of the unpronounced flavour of mozzarella, and since mozzarella is made of fresh milk, the FFA concentration should be low. If the present results had shown high amounts, it would probably contribute to bad taste rather than physical or chemical problems of production of mozzarella.

There are many methods to measure FFA levels in dairy products and they all have advantages and disadvantages (Evers (2003). A major difficulty associated with FFA determination in dairy products is the incomplete extraction of especially the short chain fatty acids, which are important since they have a big role in producing off flavours (Salih, Anderson & Tuckley 1976).

Most of the methods do not fully recover all FFAs but they are still useful. Evers (2003) investigated relationships between Bureau of Dairy Industries (BDI) method and some other FFA methods. Nollet & Toldrá (2009) explain BDI as a method for the direct quantification of lipolytic activity in samples, typically for milk, cream and cheese products. It is originated in the United States, which first standardized the method 1955. Principally, the samples are mixed with a solution containing sodium tetraphosphate and a surface-active agent and heating in a boiling water bath to separate the fat. The fat is dissolved in an organic solvent and thereafter titrated with an alcoholic alkali and thymol blue as an indicator (Nollet & Toldrá 2009). One of the methods that were investigated by Evers (2003) was the solvent extraction method used in the present study. The procedure does not give a full recovery of the fatty acids, especially short chain fatty acids like butanoic acid (C:4) since they are partly miscible with water and can be difficult to separate from the aqueous phase. The recovery is however improved by the addition of acid to reach pH 2-3. A greater proportion of the FFA is then protonated and gets thereby less water-soluble (Evers 2003). The solid extraction in Evers (2003) is in turn partially based on Perrin and Perrin (1957), which gave a total recovery of 83% of the fatty acids. From this we can draw a conclusion that there was probably more FFA in our samples too, which were lost in the aqueous phase. The BDI method, in contrast, gives only about 50-70% recovery of the FFA that are present in milk.

Lactic acid, produced by bacterial growth, citric acid and phospholipids can also be extracted together with FFA and can result in higher FFA values (Salih, Anderson & Tuckley 1977). It is therefore not only important with a full recovery of fatty acids, but also to be aware of and overcome a possible contributing problem with lactic acids, citric acids and phospholipids when analysing FFA content.

Denniston *et al* (2004) describes titration as a form of neutralization reaction that can be used to determine an unknown concentration. A solution with a known concentration is added to the unknown one, and when the neutralisation is observed, the concentration can be calculated. The neutralization is observed by eye due to an indicator that has been added to the solution that is investigated (Dennis-

ton *et al.* 2004). This type of quantitative method can in practical be difficult to use for accurate results. The neutralisation that is determined with eyes can sometimes be difficult to tell if the indicator does not always gives the exact same colour. Even though the same person performs each titration, the colour can differ.

Other type of methods for determination of FFA than titrimetric is calorimetric, potentiometric, chromatographic (gas and liquid), electrophoretic analyses and biosensor analyses. Accurate results also depend on the method used to isolate the FFA from the matrix, which can be complicated (Antonelli *et al.* 2002). If it is made properly, capillary gas chromatography is a precise quantitative method for determination of fatty acids (de Jong & Badings 1990) and might therefore be the best method for accurate results. Since this lab was a quantitative measurement of FFA a solvent extraction were used and there were no need to make a qualitative method. A qualitative method however, might have been a good option for more accurate results.

6 Conclusion

This study investigated the amount of plasmin (PL), plasminogen (PG) and free fatty acids (FFA) in milk during the early lactation from six buffalos and bulk buffalo milk.

PL and PG play an important role in the dairy industry; either positive or negative depend on the dairy product. PL and PG analysis could be complicated and there are many factors that can affect the result like breed, age and feed of the cow. Even different isolation and measuring methods could affect the outcome. The analysis of plasmin showed a fairly constant concentration during the first few weeks, and according to this result PL could not be the reason that the first milk cannot be used for mozzarella production. PG analysis showed a notable reduction around week 3-4 but whether this change affects the mozzarella production require further investigation.

The FFA plays an important role in the quality of dairy products and should have a low value in milk. Mozzarella is made of fresh milk and since the cheese is not stored for a long time but eaten fresh, lipolysis normally does not affect the cheese in a high rate. Independent of the stage of lactation, the FFA concentration in buffalo milk does not seem to be correlated to the difficulty in mozzarella manufacturing of milk from early lactation.

7 Acknowledgements

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