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– Implications for Mozzarella production

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Abstract

Mozzarella is a popular fresh cheese that is traditionally made by hand from fresh buffalo milk, involving a finishing step of stretching the cheese curd in hot water. The ability of the curd to stretch in hot water is dependent on the amount of calcium removed from the curd during the reduction of pH in the milk. The distribution of calcium between colloidal and soluble phase can change dependent on what treatment the milk is submitted to, such as prolonged storage or acidification.

The aim of this study was to investigate what effect prolonged storage of buffalo milk in combination with preacidification, would have on the concentration of calcium in whey and how this would affect the manufacturing of Mozzarella.

The calcium content was analyzed according to IDF standard method (154:1992) and by using atomic absorption spectrophotometer. The chemical composition in milk was determined by using mid-infrared transmission spectrophotometer (Dma pump, Miris). Bulk buffalo milk was stored for 8 days and whey calcium and pH were measured daily. In addition, milk stored for 1, 4 and 8 days was inoculated with fresh starter culture for a maximum of 120 minutes, and calcium in whey and pH were measured after 15, 30, 60, 90 and 120 minutes. The chemical composition of individual and bulk milk samples from 7 water buffaloes was analyzed and found to be within the normal range, compared to previous studies. During prolonged storage at +4°C for 8 days the pH decreased from 6.82 to 6.5 and the soluble calcium in whey increased with 35%. The effect of preacidification on whey calcium was evaluated for milk subject to cold storage for 1, 4 and 8 days. Results showed that the calcium concentration in whey increased as pH decreased over 120 minutes. The present study found a strong correlation between pH, storage time and calcium in whey for milk subjected to prolonged storage and in addition, for milk that was preacidified with fresh starter culture for maximum 120 minutes. In conclusion, the present study shows that prolonged storage of milk in combination with preacidification affects the concentration of soluble calcium in whey, which could have implications for Mozzarella manufacturing.

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1. Introduction

1.1 Background

Mozzarella belongs to the *pasta filata* cheese family, which means stretched curd and is an Italian type of fresh cheese. This type of cheeses get their characteristic texture by heating and kneading the cheese curd in hot water at the end of the manufacturing process, and are formed into white, spherical cheeses, with a unique fibrous texture and delicious taste. The tradition of producing Mozzarella has been carried out for hundreds of years, mainly in Naples, Italy, and surrounding areas. The cheese is traditionally made by hand from fresh unpasteurized buffalo milk and should be consumed as a fresh product within days after production. Mozzarella is also widely used as a pizza topping and is a common ingredient in traditional Italian dishes.

The popularity of Mozzarella has increased in recent years, especially in Europe and the USA. The number of water buffaloes and buffalo herds in Europe, USA and South America is increasing annually. Buffaloes are spreading in Europe from Italy, Egypt and Morocco to Switzerland, Rumania, Germany, Sweden and Great Britain. Mixed breeds of water buffaloes are present in the entire USA, South America as well as a few herds in Canada.

In 1996 Italy successfully registered their buffalo Mozzarella in the European Union with protected designation of origin (PDO), which enables Italian farmers within the Campanian region to label their product with PDO and name it Mozzarella *di bufala Campana*. In the same year Italy got the product Mozzarella registered with traditional speciality guaranteed (TSG), which enables any producer within EU to produce Mozzarella and label it with TSG, as long as the recipe is followed. The latter could be produced from either buffalo or cow milk or a mixture of them both. The requirements for PDO and TSG are different, with PDO being stricter. The benefits of registering a product with a protected designation are many; one being that the product will receive government protection from cheaper copies with inferior quality made by unserious producers, thus providing a fair situation of competition on the market for small farmers. Another is that the consumer can trust the products reputation and continuously get the quality that they are paying for.

The manufacturing of Mozzarella can be challenging and is a real work of art. The increasing export of Mozzarella from countries such as Italy, Switzerland and Germany has grown from traditional handmade production into industrial production applying advanced dairy technology. The machinery often involves the entire production from pasteurization of milk to stirring, heating, mechanical stretching and shaping of the cheese curd into finished Mozzarella. Research has focused on parameters that could optimize the manufacturing in order to yield a product that resembles the handmade product but with technical qualities benefiting the food industry. Examples of qualities sought after are proper melting properties and color during oven baking, texture of fresh product and limited texture change during storage. The industry deals with bulk milk transported from several farms, which will result in milk with small variations of quality throughout the year.

Relatively small dairies still produce handmade Mozzarella for the local market as well as for export to other European countries. The real handmade buffalo Mozzarella is highly sought after and found in well stocked market halls and delis around Europe. Smaller dairies may base their production on only a few individual animals and may not produce Mozzarella every day. This production is affected by variation in milk composition e.g. between seasons of the year or stages of lactation (Patino *et al.,* 2007; Lucey et al 1991).

One important parameter in Mozzarella manufacturing is thought to be the concentration of calcium in milk and its relative distribution between cheese curd and whey. At optimal calcium concentration in the cheese curd it is possible to obtain optimal stretchability during the plasticizing step, a fibrous texture of the finished cheese, and accurate meltability of the cheese during pizza baking (Lucey & Fox, 1993). Several factors determine the concentration of calcium in the raw milk. A limited number of available studies focus on the total calcium content in buffalo milk (Spanghero & Susmel, 1996), the calcium distribution in buffalo milk (Ariota *et al.,* 2007) and calcium variation throughout lactation period and season (Patino *et al.,* 2007). No studies have been found on calcium distribution and variation throughout cold storage of buffalo milk. Acidification of milk before adding rennet has been used as an efficient way to lower the amount of calcium in the solid part of the milk (Joshi *et al.,* 2002; Metzger *et al.,* 2000; 2001). No studies available studies have been found on preacidification in combination with prolonged storage of milk.

Since buffalo milk contains a relatively high amount of calcium compared to other animal species the recipe for Mozzarella manufacturing is somewhat different. Depending on the variation of calcium content and overall milk quality the recipe may differ throughout the year. The recipe may also depend on whether Mozzarella is made from fresh milk or milk that has been stored for various periods of time.

1.2 Objectives

The overall objective of this study was to investigate factors that may affect the distribution of calcium between the micellar and soluble phases of buffalo milk.

Specific aims of the study were:
(1) to determine the chemical composition of both bulk tank milk and milk from individual water buffaloes used in this study
(2) to investigate the effect of prolonged cold storage (+4°C) on calcium distribution between solid and liquid phase in bulk buffalo milk.
(3) to study the effect of preacidification of bulk buffalo milk previously subject to cold storage for different periods, on calcium distribution between solid and liquid phase,.

2. Literature review

2.1 Mozzarella manufacturing

Numerous recipes for manufacturing Mozzarella exist, each recipe different from the others, but in order to produce a traditional Mozzarella outside the Campania region of Italy, the recipe for TSG registration will be referred to in this study ((EC) No2527/98 and No2082/92).

The production of Mozzarella starts with milk pasteurization, i.e. a minimum treatment of 71.7 °C for 15 seconds (Figure 1). After cooling to 35-39°C the milk is inoculated with a natural starter obtained through selective enrichment of the microflora naturally present in raw milk. The natural starter will, if properly prepared, contain an undefined mixture of strains of *Streptococcus thermophilus* as well as enterococci and heat resistant lactic acid bacteria. Liquid bovine rennet is added to the milk and coagulation takes place at a temperature of 35-39°C. The curd is cut into walnut sized pieces and stirred, followed by a separation of cheese curd and whey. The cheese curd is ripened to a pH between 5.4-5.0. At optimal pH the cheese curd is stretched, which is an operation of heating the curd in hot water to reach a final curd temperature of between 65-85°C. This process is also referred to as plasticizing. The curd can be molded into spherical shapes (weighing 20-220 g). Finished Mozzarella is cooled in cold water, sometimes with added salt. The product is packed and stored at +4 °C in a sealed package containing a medium made up of water and salt.

The TSG recipe contains only the minimal requirements for mozzarella production. The producer has freedom to modify this recipe within the set requirements, which is why so few recipes are officially published as complete versions but rather kept within the company.

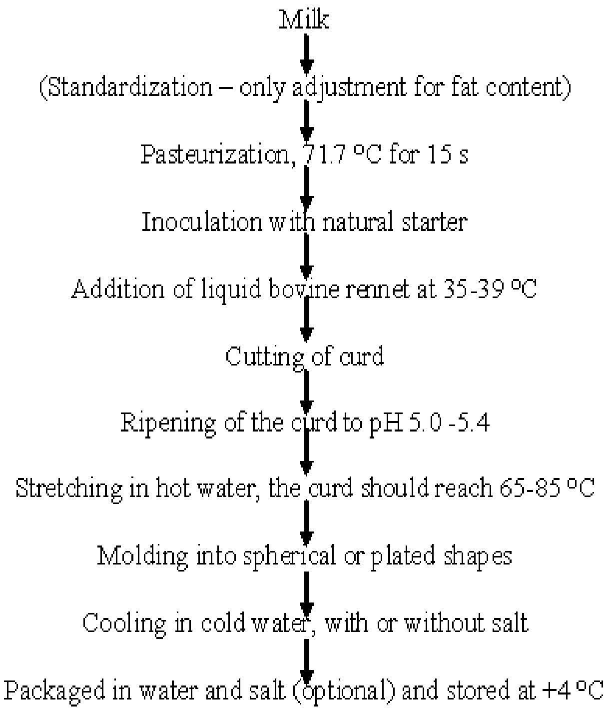

Figure 1. Flow chart of Mozzarella manufacturing according to the recipe for registration with TSG.

2.2 Chemical composition of buffalo milk

The chemical composition of buffalo milk is different from that of cow milk (Ahmad *et al.*, 2008; Spanghero & Susmel, 1996; Zicarelli, 2004; Khan *et al.*, 2007). Buffalo milk (Murrah breed) has higher concentrations of fat, protein, ash, lactose and calcium than cow's milk (Ahmad *et al.*, 2008). The concentration of protein is 30%, calcium 70% and phosphor 30% higher in buffalo milk than cow milk (Spanghero & Susmel, 1996). The fat content of buffalo milk is on average 8.55% and fat is the component in buffalo milk that varies the most compared to cow milk, possibly due to genetic variation between the individuals in the buffalo herd (Spanghero & Susmel, 1996). Despite the relatively high fat content in buffalo milk the cholesterol level is lower than in cow milk; buffalo milk containing 275 mg and cow milk 330 mg per kg (Zicarelli, 2004). As a consequence, the cholestereol concentration in the finished product Mozzarella is also lower in buffalo milk compared to cow milk; buffalo Mozzarella containing 1562 mg $kg^{-1}$and cow Mozzarella 2287 mg kg^{-1}. Buffalo milk also has a higher buffering capacity compared to cow milk and casein micelles are less hydrated and more mineralized; i.e. containing more minerals such as calcium, magnesium and phosphor (Ahmad *et al.*, 2008).

2.3 Calcium in buffalo milk

Spanghero & Susmel (1996) measured the total calcium concentration in milk from 25 buffalo cows taken from northeastern Italy (Table 1). The buffaloes were in different stages of lactation and therefore the results can be regarded as an average for the entire lactation period. The average total calcium concentration was found to be 2.03 g kg^{-1}. This is slightly higher than the total calcium reported by Ariota *et al.,* (2007) who found it to be 1.7 g kg^{-1} measuring total, soluble and micellar calcium concentration in milk from 70 buffalos from three different farms in Italy (Table 1). The buffaloes were at day 156±46 in milk, i.e. in mid lactation. The soluble calcium concentration was determined by measurement of serum obtained by rennet coagulation and analyzed in AAS and the micellar calcium concentration by calculating the difference between total and soluble calcium Table 1).

The milk concentration of calcium varies with time of the year as well as with stage of lactation. A study by Patino *et al.*, (2007) on Mediterranean buffaloes showed that mineral content (Ca, P, Na, Cu and Fe) varied throughout the year with the lowest concentrations appearing during winter. Lactation stage also influenced the mineral content of Ca, P, K, and Cu, which displayed the lowest concentrations during the last third of the lactation.

Table 1. Chemical composition of buffalo milk

	Author		
	Spanghero & Susmel, 1996	Ariota *et al.,* 2007	Khan *et al.,* 2007

Energy (MJ)	4.9	ND[a]	ND
Fat, g kg^{-1}	85.5	87.1	72.67
Protein, g kg^{-1}	42.7	47.1	37.67
Lactose, g kg^{-1}	51.5	ND	47.55
Total ash, g kg^{-1}	8.9	8.3	7.07
Calcium (total), g kg^{-1}	2.03	1.71	ND
Calcium (micellar), g kg^{-1}	ND	1.1536	ND
Calcium (soluble), g kg^{-1}	ND	0.5521	ND
Phosphorous, g kg^{-1}	1.23	1.4534	ND

[a] ND=not determined.

2.3.1 Calcium in milk from other sources

Despite the chemical and nutritive differences between buffalo, cow, ewe's and goat's milk, much can be learned from the numerous studies performed on their milk.

The calcium concentration in milk seems to be related to how fast the animal grows. In fact, the uppermost function of calcium in milk is to provide calcium to the offspring, for the development of hard bones and function of muscles. Calcium exists in several forms in milk; bound to phosphate, citrate or as ionized form Ca^{2+}. Bovine milk is supersaturated with respect to calcium phosphate. The excess of calcium phosphate exists in the colloidal phase, associated with casein. It is estimated that 500 mg calcium per liter cow milk is present in the colloidal phase (Fox & McSweeney, 1998). Higher values of calcium have been observed in milk from buffalo, horse and dog, than in, for example, milk from human and other primates (Lewis, 2010). Lewis (2010) reviewed calcium variation in cow milk and methods for calcium analysis in milk. Cow milk consists of 30 mM (1.2 g kg^{-1}) total calcium where 20 mM (0.8 g kg^{-1}) is insoluble and associated with casein, and 10 mM (0.4 g kg^{-1}) is soluble as undissociated calcium-phosphate or bound to citrate. Citrate works as chelating agent and binds Ca^{2+} to form soluble unionized salts (Fox & McSweeney, 1998). A small portion of the soluble calcium is in ionized form (Ca^{2+}) (Lewis, 2010). The ionized calcium measures 1.0 to 3.0 mM (0.04-0.12 g kg^{-1}) or about 10% of the total calcium at normal milk pH (Lewis, 2010).

The composition of milk is dependent on environmental factors such as what type and what amount of feed that the cows are given and also on the genetic makeup of the animal. The concentration of total calcium in cow milk is high in the beginning and in the end of the lactation (Fox & McSweeney, 1998). The increase of total calcium, measured by Keogh *et al.*, (1982) was between 7.9 % and 8.9 % higher in late lactation than during early lactation. Tsioulpas *et al.*, (2007a) measured the total and free calcium in cow milk from the colostrum period to up to 90 days postpartum. The total calcium concentration varied from 54.2 mM (2.2 g kg^{-1}) to 28.6 mM (1.14 g kg^{-1}) for the first 90 days of lactation and the free ionic calcium varied from 5.75 mM (0.23 g kg^{-1}) to 1.78 mM (0.07 g kg^{-1}) during the same period of time. The highest concentrations were measured during the first 5 days postpartum and decreased slowly thereafter. In another study Tsioulpas *et al.*, (2007b) measured free ionic calcium in milk collected from 235 cows and found that the mean concentration was 1.88 mM (0.075 g kg^{-1}). The same author (2007a) observed variations of calcium concentration in the milk between individuals, suggesting this may be due to genetic factors. The author excluded seasonal, lactational and nutritional factors since all milk samples came from cows with the same conditions. Bulking of milk might reduce these individual variations but it could still affect the processing quality of the raw milk since changes in pH and the concentration of ionic calcium will differ over time (Lewis, 2010). To be considered is also the concentration of citrate, which has influence on the distribution of calcium, and shows strong seasonal variations with highest concentration during spring period (Fox & McSweeney, 1998).

2.3.2 Calcium variation in milk submitted to various treatments

The mineral balance and distribution in milk will be affected by the composition of the milk and by the various treatments it might be submitted to, such as cooling, heating and freezing (Fuente, 1998).

Acidification of milk will gradually dissolve colloidal calcium phosphate as well as other colloidal salts from casein, by the act of protons, reducing the binding of calcium to casein (Fox & McSweeney, 1998). The solubilization of calcium phosphate from the micelle will be complete when pH reaches 4.9 (Fox & McSweeney, 1998; Fuente, 1998). At pH 3.5 the solubilization of calcium is complete, from calcium that are in association with phosphoseryl residues of casein molecules (Ahmad *et al.*, 2008). Reduction in pH can be caused by microbiological fermentation during storage of milk, or by the intentional acidification using weak acids or starter culture in association with cheese making. Removing more than 70 % of colloidal calcium phosphate will cause a disintegration of the micelle into smaller particles (Fox & McSweeney, 1998).

The solubility of calcium phosphate is temperature dependent and will decrease with increasing temperature and vice versa. Heating of milk causes precipitation of calcium phosphate while cooling increases the concentration of soluble calcium phosphate, which will in turn decrease the colloidal calcium phosphate. This equilibrium is a reversible process under moderate temperatures. This effect could be explained by the decrease in pH during heating of milk (Fox & McSweeney, 1998). During cold storage at +4 C° a dissociation of beta casein from the micelle will occur, accompanied by the solubilization of calcium phosphate and a decrease of micelle size. The clotting time will increase and gel strength and curd firmness will decrease with time of storage (Park & Haenlein, 2006).

Fuente *et al.,* (1997) studied the change of distribution of soluble calcium in raw milk from sheep and goat during 7 days of cold storage at +3°C and +7°C. As the pH decreased during storage, the soluble calcium increased with time.

2.4 The role of calcium in Mozzarella production

The characteristic fibrous structure of Mozzarella comes from a unique last step in the manufacturing called plasticizing. The plasticizing step involves kneading and stretching of the cheese curd in hot water. The hot water melts the cheese curd into a continuous mass and the stretching results in orienting the protein fibers in the same direction as the stretch (Lucey & Fox, 1993). The ability of the cheese mass to form fibrous strands when stretched in hot water and without breaking appears to be related to relatively high concentrations of intact casein and low concentrations of Ca and PO_4 in the cheese curd (Lucey & Fox, 1993).

2.4.1 Reducing pH

After pasteurization of the milk and before the addition of rennet, a starter culture is added. The starter culture decreases the pH by the production of lactate from lactose. One other method of reducing the pH is direct acidification, a non-microbiological method reviewed by Kindstedt (2004). Direct acidification involves the addition of an acid to yield a predetermined pH at which the curd will have optimal stretchability. Acids commonly used are weak acids, either alone or in a combination, such as lactic, citric or acetic acid or glucono δ-lactone. A reduction in pH before adding rennet leads to an increase in the concentration of soluble calcium in the whey and a reduction in the net charge of casein micelles that further increases the fusion of para-casein micelles (Guinee *et al.,* 2002). The decreased pH also increases rennet activity, which in turn leads to a more rapid curd formation (Guinee *et al.,* 2002).

2.4.2 Coagulation of the curd

Coagulation or setting of the curd involves addition of bovine rennet to the milk. No stirring of the milk should occur at this stage until after cutting the curd. Rennet coagulation can be divided into three stages; primary, secondary and tertiary (Lucey & Fox, 1993). These stages involve enzymatic hydrolysis, aggregation and potentially also syneresis and rearrangement. During the primary stage κ-casein is cleaved by rennet at the Phe_{105}-Met_{106} bond yielding two peptides. The hydrophilic caseinmacropeptide (CMP) (residue 106-169) diffuses into the solution whereas the hydrophobic peptide (residue 1-106) and the para- κ-casein, remains attached to the micelle. The cleavage of κ-casein will facilitate aggregation of the micelles during the second stage, creating a gel. During the tertiary stage an expulsion of water from the micelles occurs and the gel network is rearranged (Lucey & Fox, 1993).

Yazici & Akubulut (2007) showed that a high pH at setting time results in a lower concentration of ash, calcium and phosphorous in whey, than a low pH at setting time, which yields higher whey concentration of the same components. Tsioulpas *et al.,* (2007b) found that when free ionic calcium increases, the rennet clotting time decreases. However, milk containing Ca^{2+} levels less than 1.5 mM (0.06 g kg^{-1}) will not coagulate at all, suggesting that a minimum calcium level is required for this purpose.

After cutting of the curd, the surface of the curd particles are believed to act as a curd particle membrane (CPM), creating a gradient within the particle. CPM has higher casein content than the interior, which leads to a loss of fat from the surface layer (Fox *et al.,* 2000). The permeability of the CPM decreases with time from cutting and results in draining due to dehydration and continuous loss of fat. After cutting of the curd, the migration of calcium from the curd particles to the whey will be limited by the CPM due to its decreased permeability (Guinee *et al.*, 2002). As a result of the inability of the calcium to migrate the solution will have a low concentration of calcium, even if the pH of the whey slowly continues to decrease due to microbiological activity (Guinee *et al.*, 2002). The same author found that a reduction of pH before rennet coagulation resulted in higher calcium concentration in whey, probably due to a more efficient removal of calcium from the curd.

2.4.3 Plasticizing and stretching

The stretchability of the Mozzarella cheese curd during the plasticizing step is affected by both curd demineralization and final curd pH. The pH of the cheese curd during this last step is preferably 5.0-5.2. At this pH relatively small casein aggregates exist. The casein aggregates line up forming long chains upon heating. This ability, together with curd cohesion and stretchability, is lost when the pH decreases to <4.8 (Lucey & Fox, 1993). The decreased calcium levels in the cheese curd will reduce crosslinking between protein fibers (Lucey & Fox, 1993).

Higher pH values at setting time require more time to reach pH 5.2 compared to samples with lower pH at setting time (Yazici & Akubulut, 2007). It is the total calcium content of the curd that determines the curd pH at which plasticization is possible. As mentioned previously, this is controlled mainly by the pH of the milk at setting and the pH of the curd at drainage (Yazici *et al.,* 2010). Reduction of calcium will affect the binding between casein aggregates which will give a cheese curd with higher melting and flowing properties (Metzger *et al.,* 2001).

2.4.4 Preacidification of milk

It appears that a low level of micellar calcium at setting time is optimal for the ability of the cheese curd to stretch during the later plasticizing step. One way to efficiently lower the micellar calcium lever prior to setting time is a preacidification step, or prefermentation step when including microorganisms e.g. starter culture. Weak acids could be used for rapid reduction of the pH to a controlled point, while starter culture is added at a later stage, to acquire some aroma in the finished mozzarella. Let’s for simplicity call this process for preacidification.

Metzger *et al.,* (2000) showed that preacidification of the milk for some time before setting time reduces the concentration of calcium in the finished cheese. The preacidification was performed with citric and acetic acid followed by a ripening time of 30, 45 and 60 minutes before traditional starter cultures and rennet were added. The lower pH at setting reduced the coagulation time from 30 to 15 minutes, which is in confirmed by Guinee *et al.,* (2002) and later studies by Metzger *et al.,* (2001) and Joshi *et al.,* (2002). The concentration of calcium increased in the whey and decreased in the final cheese, although the total cheese yield decreased using preacidification compared to the control. To what degree the calcium concentration in the curd decreases is dependent on the degree of pH reduction at setting and whey drainage and the type of acid that was used for preacidification (Metzger *et al.,* 2000). Preacidification with a weak acid before adding starter culture had a slowing effect on the starter culture activity and increased the time required before reaching the final pH of 5.3 compared to a control that was not preacidified (Metzger *et al.,* 2001). The acid is believed to affect the symbiotic relationship between the species within the microbiological starter culture, thus slowing them down.

Joshi et al., (2003) found that when reducing micellar calcium by preacidification of milk, the melt area, flow rate and extent of flow in finished Mozzarella cheese increased. The time and temperature for softening and melting of the cheese was reduced. To reduce calcium seems to be important not only for the stretching ability of the cheese curd but also for the finished structure of the cheese.

3. Materials and methods

3.1 Experimental design

The two experiments were set up according to Figure 2 and Figure 3.

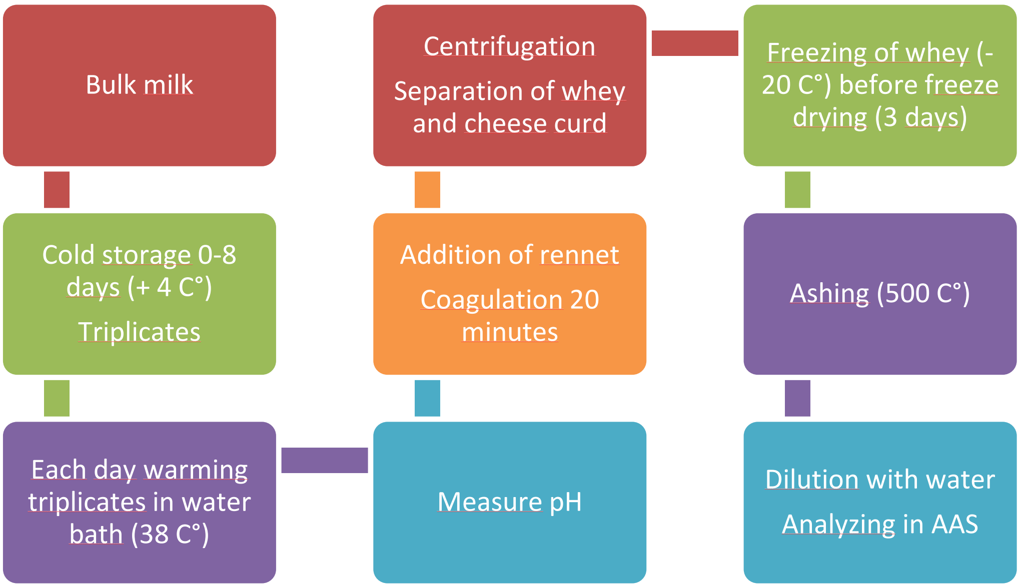

Figure 2. Scheme showing the experimental setup of milk submitted to prolonged storage for 0-8 days.

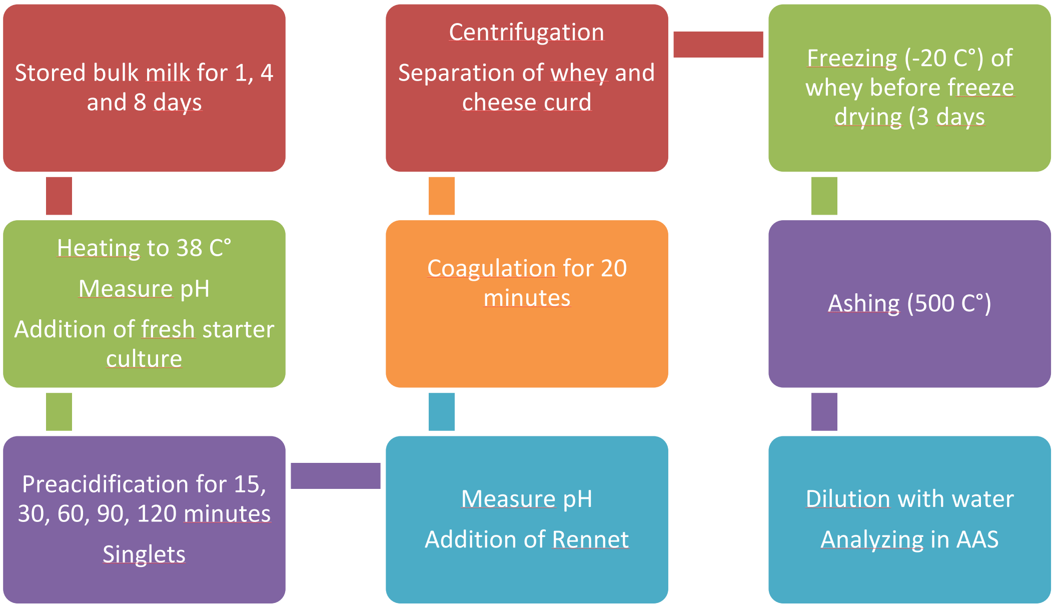

Figure 3. Scheme showing the experimental setup of milk stored for different periods of time and submitted to preacidification for 15, 30, 60, 90 and 120 minutes.

3.2 Collection of milk samples

Evening milk from 7 Mediterranean buffaloes was obtained at two different occasions from Ängsholm dairy farm located 40 km north of Uppsala, Sweden. The buffaloes were in 161±7 days of lactation when individual samples were taken and 185±7 days of lactation when bulk milk samples were taken, i.e. late mid lactation. Bulk milk where mixed in a tank with an automatic stirrer, still warm after milking. Two liters of bulk milk were collected in a plastic jar and transported without cooling to SLU, a 40 minutes drive. The bulk milk was stirred again and 40 ml pipetted into 50 ml Eppendorf tubes and stored in refrigerator. Additionally 300 ml of bulk milk were stored in a glass jar in the refrigerator for analysis of chemical content later the same day. Milk from all individuals were collected in separate buckets during milking, stirred and transferred into separate plastic 200 ml jars. For each individual, milk was divided into three subsamples of 10 ml. Fresh milk was used for analysis of milk composition, whereas milk samples for analysis of total calcium were frozen and stored at -20 C°.

3.3 Analysis of milk composition

Fresh bulk milk and individual milk samples were analyzed for fat, protein, lactose and dry substance (DS) using mid-infrared transmission spectrophotometer (Milk farm analyzer, DMA pump, Miris, Uppsala, Sweden). The analyses were carried out at the same day as the collection of the milk. The milk samples were heated in water bath to 38 C° and homogenized with homogenizer for 1 second ml^{-1} of milk (Sonicator, Miris, Uppsala, Sweden). The analysis was made in triplicates.

3.4 Analysis of pH

The pH of the milk was measured using a pH meter (Radiometer, PHM 210) with a combination electrode (Orion, 420 A). The pH was measured in bulk milk the first day of the experiment and then in all replicates from day 1 to day 8. The pH was also measured during preacidification for 0, 15, 30, 60, 90 and 120 minutes.

3.5 Analysis of total and soluble calcium content

Frozen milk samples were freeze dried for three days until a fine powder was obtained. The freeze drier (Labconco, AB Nino lab) had the temperature 0 C°, and the samples were kept in the freeze drier, under vacuum for 3 days (whey and whole milk). No measurements were taken during days 5 and 6 since this was over a weekend.

Heat resistant crucibles for ashing were washed and burned in the oven (Nabertherm Controller B 180) at 500 C° with a layer of HCO_3 to remove dirt and calcium ions that might interfere with the analysis. The crucibles were rinsed in destilled water and dried before use. The freeze dried samples were weighed in the crucibles and put in the oven holding 500 C° for 90 minutes until a white ash was obtained. When sampling whole milk 1 gram of freeze dried powder was weighed and ashed, while when sampling whey all of the whey was taken for ashing.

Samples and standard solutions were prepared according to the IDF standard for determination of calcium content, flame atomic absorption spectrophotmetric method (International Dairy Federation, 1992). A stock solution containing 1.2481 g of calcium carbonate ($CaCO_3$) (Merck) and 15 ml 4 M HCl (Sigma-Aldrich) was diluted with distilled water to 1000 ml. Standard solutions were diluted from the calcium stock solution to the concentrations of 1 µg, 2 µg, 3 µg, 4 µg and 5 µg calcium per milliliter. After being burned in the oven the ash were diluted with 1 ml of 25% nitric acid solution (Merck) and quantitatively transferred into 250 ml volumetric flasks by rinsing the crucible three times with distilled water and the flask was further diluted up to mark with distilled water. Using a pipette, 5 ml of the sample solution was transferred into a 100 ml volumetric flask together with 10 ml of lanthanum chloride (lanthanumIIIchloride heptahydrate, 27 g/L, Sigma-Aldrich) and diluted with distilled water. Samples and standard solutions were prepared and analyzed on the same day. The total calcium content was analyzed in an atomic absorption spectrophotometer (AAS) (Perkin Elmer, A-analyst 100) with a wavelength of 422.7 nm, provided with a calcium-magnesium lamp.

3.5 Prolonged cold storage of milk

Buffalo milk was collected in the same way as previously described and divided into triplicate Eppendorf test tubes (Sarstedt) containing 40 ml each. A scheme for this experiment is visible in Figure 3. The test tubes were stored in a refrigerator at 4 C° for a period of between 0 and 8 days. Each day three beakers were heated to +38 C° in a water bath and rennet (Chy-max Ultra, 1034 IMCU, Chr Hansen A/S) was added at the concentration of 750 IMCU in the milk. After proper coagulation, i.e. after approximately 20-25 minutes when clear whey was visible, the test tubes were centrifuged at 3000 rpm for 10 minutes at + 7 C° (Eppendorf centrifuge 5810 R, AB Ninolab). The whey were separated and frozen in Eppendorf tubes, and all samples were freeze dried at the end of the experiment.

3.6 Preacidification

The preacidification experiment was carried out during the same period as the aging of milk (Figure 4), performed on bulk milk from the same milking occasion. Milk that had been stored at +4 C° for 1, 4 and 8 days, was acidified with a fresh starter culture (see preparation below). The period of acidification was 0, 15, 30, 60, 90 and 120 minutes. For this purpose the milk was divided into 5 test tubes containing 40 ml each, pH was measured and 1.6 ml (4% w/w) active culture was added. At the end of acidification the pH was measured again, rennet was added, the milk was allowed to coagulate for 20 minutes and finally centrifuged at 3000 rpm for 10 minutes at 7 C°. Whey were separated from the cheese, weighed and stored at - 20 C° before it was quantitatively freeze dried at the end of the experiment. The whey was freeze dried for 3 days.

3.6.1 Preparation of starter culture

A fresh starter culture was always prepared the day before it was used in the preacidification study. The starter culture was prepared with commercial, low-pasteurized standardized cow's milk (ArlaFoods, 3 % fat) from the supermarket. Freeze dried bacteria culture SH092 F (Lyofast, 5 U, Kemikalia) was used, containing *Streptococcus thermophilus* and *Lactobacillus helveticus*. The freeze dried culture was weighted and 0.018 grams was added to 1 liter of preheated milk (+40 C°) by stirring. The milk was covered with aluminum foil and incubated at +42 C° until pH 5.2 was reached, which is equal to maximum activity of the culture. The culture was stored in the refrigerator at +4 C° over night and used the following day.

3.7 Statistical method

SAS (version 9.3) was used to evaluate the correlation between pH, time and the change of concentration of calcium in whey during cold storage and during preacidification of milk. The statistical tests Pearsons correlation and Spearmans correlation were used.

4. Results

4.1 Chemical composition of buffalo milk

The chemical composition of bulk tank milk and milk from the individual buffalo cows is shown in Table 2. Total content of fat in bulk milk was 7.79 % and varied between 6.59 – 9.10 % in milk from individuals. For protein, bulk tank milk contained 4.31 % and individual samples 3.45 – 4.68 %. Lactose varied between 5.74 – 6.18 in the individuals and measured 5.37 % in bulk tank milk. The total calcium content in buffalo bulk milk was 2.3±0.014 g kg^{-1} of which was 0.3±0.0083 g kg^{-1} in soluble form measured per kg of whey. The total calcium content measured in individual milk varied between 1.45-2.01 g kg^{-1} and the mean concentration was 1.72 g kg^{-1}.

Table 2. Measured concentrations of total and soluble calcium, fat, protein, lactose and DM in bulk tank milk and milk from individual cows

	Buffalo	Total calcium (g kg^{-1})	Soluble calcium (g kg^{-1})	Fat (%)	Protein (%)	Lactose (%)	DM (%)
Bulk milk	A-G	2.3±0.014	0.3±0.008	7.79	4.31	5.37	18.51
Individual	A	1.61±0.087	ND	6.59	4.06	5.98	17.38
milk	B	1.53±0.083	ND	7.67	3.77	5.89	17.91
	C	1.68±0.050	ND	7.98	4.00	5.97	18.64
	D	1.45±0.022	ND	8.15	4.10	5.91	18.83
	E	1.73±0.042	ND	9.10	4.68	5.74	20.41
	F	2.01±0.017	ND	7.44	3.45	6.18	17.49
	G	2.00±0.012	ND	8.38	4.55	5.85	19.64
Average	A-G	1.72±0.041	ND	7.90	4.09	5.93	18.61

ND= Not Determined.

4.2 Cold storage of buffalo milk

During 0-8 days of cold storage at 4 °C the pH of the milk decreased from 6.82 to 6.50 (Figure 4). The soluble calcium in whey increased as pH decreased, from approximately 0.3 to 0.4 g kg^{-1} which is a total increase of 35 %. The change in distribution of calcium between soluble and micellar phase took place gradually during the period of cold storage. There was a strong correlation between pH and the concentration of calcium in whey during the same period (Pearson p=0.02, Spearman p=0.014). In addition, the same was valid for calcium concentration and time (Pearson p=0.007, Spearman p=0.014)

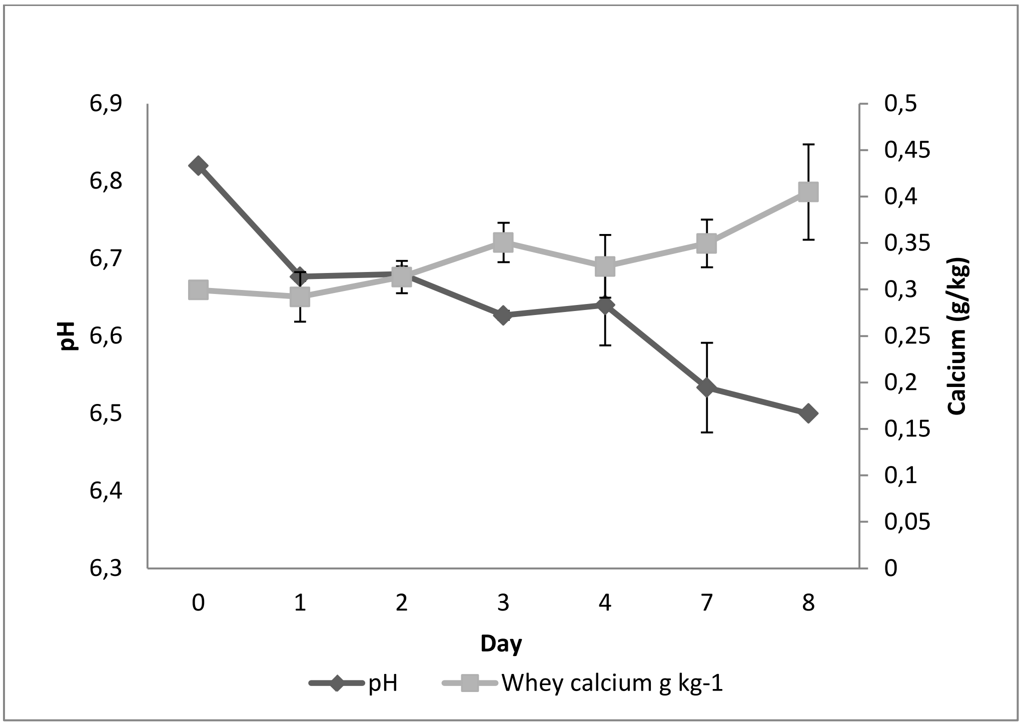

Figure 4. Change in pH and calcium in whey during cold storage (+ 4 C°) of buffalo milk for up to 8 days. Standard deviations are shown.

4.3 Preacidification of the milk

The preacidification was performed after the milk had been subject to cold storage (+4 C°) for 1, 4 and 8 days. At each day of preacidification, the milk was inoculated and incubated with the fresh starter culture for 0, 15, 30, 60, 90 and 120 minutes, respectively. The pH of the fresh starter culture was 4.91, 4.96 and 4.95, respectively on the 3 occasions. At day 1 the milk pH was 6.67 before inoculation with starter culture and declined to 5.95 after 120 minutes incubation with starter culture. The soluble calcium increased in the whey from 0.3 g kg^{-1} to 0.9 g kg^{-1} after 120 minutes, an increase with 208 %. At day 4 the initial milk pH had decreased to 6.65 and after 120 minutes of incubation with starter culture the final pH value was 5.87. The soluble calcium increased in whey during this time from 0.3 g kg^{-1} to 0.8 g kg^{-1}, an increase with 153 %. At day 8 the initial milk pH had decreased further to 6.5 and after incubation with the starter culture 120 minutes the final pH was 5.8. The calcium in whey increased from 0.40 g kg^{-1} to 0.87 g kg^{-1}, an increase with 114 % (Figure 5). In summary, soluble calcium in whey increased with the time of preacidification. During the first 30 minutes of incubation there seemed to be an additional effect due to the days of cold storage, i.e. the older the milk, the more soluble calcium in the whey. However, at 120 minutes of preacidification this difference was outleveled.

There was a strong inverse correlation between pH and calcium concentration in whey for day 1 (Pearson p=0.0043, Spearman p=0.0048), day 4 (Pearson p=0.001, Spearman p=0.0001) and day 8 (Pearson p=0.0017, Spearman p=0.0003).

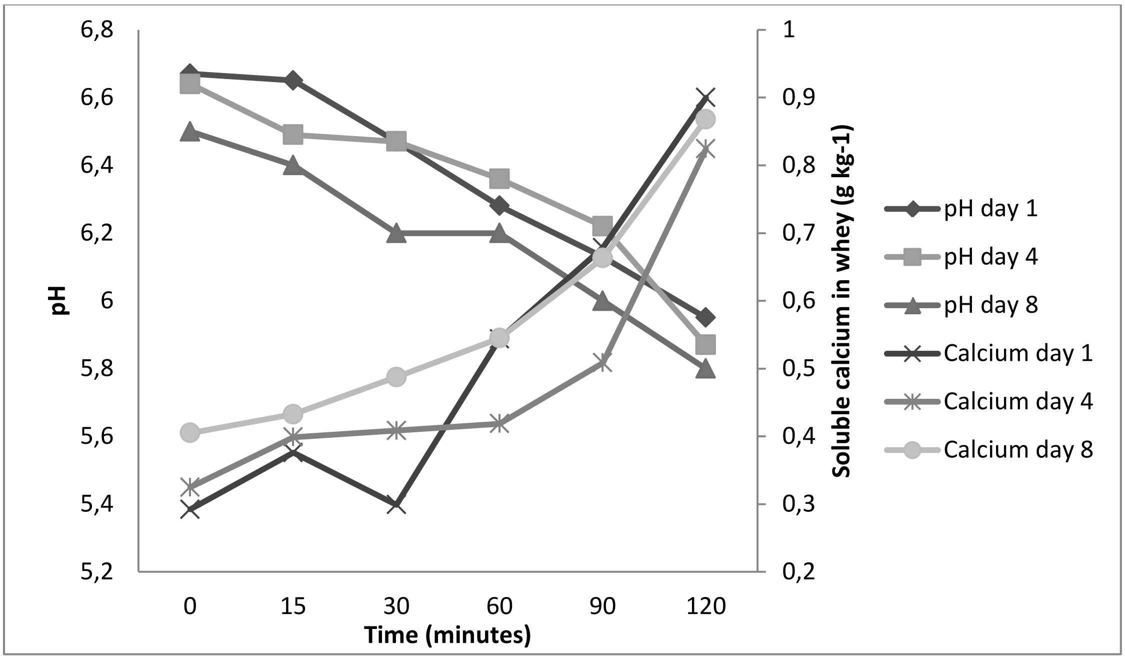

Figure 5. pH and the calcium concentration in whey from bulk tank buffalo milk, increased with time acidified with starter culture.

5. Discussion

The objectives of this study were to measure the changes in soluble calcium in buffalo milk during one week of cold storage, and to study the effects on calcium in whey when milk, previously subject to cold storage, was incubated with a fresh starter culture for up to 120 minutes. The calcium distribution is an important factor in Mozzarella production but available studies on buffalo milk during prolonged cold storage, and incubation with a starter culture are limited.

The chemical composition of buffalo milk was within the range of previously reported values. The fat content of the buffalo milk was lower (7.79 %) than measurements by Spanghero and Susmel (1996) (8.55 %) and Ariota *et al* (2007) (8.71 %) but higher than Khan *et al* (2007) (7.27 %). The protein content (4.31 %) was higher than Spanghero and Susmel (1996) (42.7 %) and Khan *et al* (2007) (3.77 %) and lower than Ariota *et al* (2007) (47.1). The lactose content (5.37 %) was higher than both Spanghero and Susmel (1996) (5.15 %) and Khan *et al* (2007) (4.76 %). To consider is the lactation state of the buffaloes studied, 161±7 and 185±7, i.e. late mid lactation. As described earlier, the chemical composition of the milk changes during lactation period as well as through season. The fat, protein, DM and calcium content increase while lactose decreases (Fox and McSweeney 1998). The average values of fat, protein, lactose and DM calculated from the individual measurements were different from the bulk milk. This is not unexpected since individuals have different milk yield, compromising with the bulk milk result, and considering the fact that the bulk milk and individual milk samples where taken with three weeks apart. It is also possible that unrepresentative sampling of the milk could have had an effect on the results. Buffalo milk is high in fat, creating a surface lid on top of the milk, which makes sampling without homogenization of the milk difficult.

The total calcium content of buffalo milk was 2.30 g kg^{-1} (Table 2) which is slightly higher than previously reported observations by Spanghero and Susmel (1996) (2.03 g kg^{-1}) and from Aritoa *et al.*, (2007) (1.71 g kg^{-1}) (Table 1). The total calcium content measured is approximately 90% higher than standard values reported for cow's milk (Fox and McSweeney, 1998). The soluble calcium in buffalo milk was 0.30 g kg^{-1} of whey, which is lower than reported from Ariota *et al* (2007) (0.55 g kg^{-1}). The soluble calcium values reported in cow´s milk are approximately 33% higher than in buffalo milk (0.4 g kg^{-1}) (Lewis, 2010). Ariota *et al* (2007) measured the total calcium in milk from buffaloes in mid lactation; 156±46 days, using the same methods as in our study, i.e. based on analysis of calcium in whey obtained from rennet coagulation. The lower total calcium average value from the individual milk and the variation 1.45-2.01 g kg^{-1} compared to bulk milk total calcium 2.03 g kg^{-1} could be explained by either unrepresentative sampling, or variation related to the method itself, since the samples were not analyzed at the same time. In addition, as previously mentioned, neither were the samples collected on the same occasion. The total milk calcium was based on a sample taken approximately 3 weeks after the individual samples. Total calcium is higher at the end of lactation (Fox and McSweeney 1998) which might explain the higher total calcium in bulk milk.

The increase of soluble calcium in whey with 35 % during 8 days of storage in +4 C° could be viewed as an effect of a continuous reduction in pH (Figure 2), and there was a strong correlation between pH, storage time and calcium in whey. The buffalo milk was unpasteurized and the natural bacterial flora present in the milk would during 8 days metabolize lactose to lactate, thus lowering pH. The inverse relationship between calcium and pH has previously been described by Fox and McSweeney (1998), Fuente et al., (1997) and Ahmad *et al.,* (2008).

During preacidification of buffalo milk the pH gradually decreased, as expected (Figure 5). The soluble calcium concentration before incubation with starter culture was higher for milk that had been subject to cold storage for 8 days compared to 1 and 4 days (Figure 5). The concentration of soluble calcium increased during treatment with starter culture for 120 minutes, as the pH decreased. The total increase of calcium in whey, compared to the concentration of the calcium in whey at day 0 was 200%, 175 % and 189 % at day 1, 4 and 8, respectively. It seems like the concentration of calcium in whey is stabilising at the lower pH. After 120 minutes of acidification, the measured whey calcium had close to same values regardless of pH at the beginning of the precidification. The correlation between calcium in whey and pH was strong within the tested range pH 6.85-5.80. However, the increase of calcium in whey does not necessarily follow a linear pattern until it reaches pH 3.5 when all calcium is depleted, it is therefore difficult to predict what effect certain times of preacidification would have on cheese curd and finished Mozzarella. In addition, calcium will probably not migrate from colloidal to soluble phase at the same speed after coagulation with rennet, as shown by Guinee *et al.,* (2002). In conclusion, one cannot assume a proceeding linear relationship between calcium and pH as pre-acidification progresses.

The whey was manually collected with a pipette after the centrifugation of the samples, which resulted in a variation in whey volumes that were collected at different occasions. This, of course, is likely to have an impact on later calculations regarding the amount of calcium in whey in relation to total calcium in milk. As a suggestion the whey could be collected by carefully turning the test tube upside down to allow the whey to pass through a sieve to hinder the curd to follow and to make sure all whey has been collected.

The results of this study might suggest that prolonged storage of milk would be beneficial for the manufacturing of Mozzarella, since low amounts of calcium in curd is optimal for the plasticizing and stretching step. However, as mentioned previously cold storage at +4 C° will in addition to the solubilization of calcium phosphate, result in a dissociation of beta casein from the micelle, a decrease of micellar size, increased clotting time and decreased gel strength (Park & Haenlein, 2006). This would in turn have a negative effect on the curd yield. Storing milk would also favor the growth of psychotropic as well as pathogenic bacteria,which could create off-flavors of the milk and eventually also the finished cheese (Walstra *et al*., 2006).

If one would manufacture mozzarella despite of the physical and chemical changes occurring in 8 days old milk, less time in our study, approximately 30 minutes, is required to acidify the older milk compared to fresh milk. This is based on the fact that after 8 days the pH was 6.5 and the time required to reach pH 6.5 with active starter in fresh milk culture was 30 minutes. For 4 days old milk a reduction of the preacidiciation step of approximately 15 minutes is needed compared to fresh milk. Since the soluble calcium is pH dependent, as also confirmed in this study, one would measure pH of the milk before starting manufacturing of Mozzarella and modify the recipe on a daily basis. This would, of course, only be true to buffalo milk with a uniform calcium distribution over time. Seasonal milk, that is milk from cows in the same lactation state, with the same increase or decrease of calcium at different periods during their lactation, would still need further modifications such as addition of $CaCl_2$ when calcium levels are low, or longer preacidification when calcium levels are high (Lucey *et al.*, 1991). The addition of $CaCl_2$ is required since a certain amount of calcium is needed to get a firm gel, and a satisfying cheese yield.

6. Conclusion

This study investigated changes in soluble calcium in whey, as an effect of reduction in pH during cold storage and during preacidification. Calcium in whey, storage time and pH were significantly correlated. In order to manufacture Mozzarella with milk stored for between 4 and 8 days, the recipe may need to be modified by reducing the preacidification time, if such is applied. However, milk that has been stored for 8 days at +4 C° has disadvantages with respect to yield and product quality that limits the use of such milk in practice. In conclusion, this study shows that prolonged storage of milk in combination with preacidification has an effect on the concentration of soluble calcium in whey that could have implications for Mozzarella manufacturing.

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Popular summary

This study has focused on the mineral calcium in buffalo milk, and its importance during the manufacturing of Mozzarella. Mozzarella is a fresh cheese that is traditionally produced from buffalo milk. The production of Mozzarella from buffalo milk has been on-going for at least 1000 years, starting in Italy in the area of Campania near Naples. The cheese has a fibrous texture, a white color and a delicious taste.

The manufacturing of Mozzarella starts with heating of buffalo milk in order to reduce the number of potentially harmful bacteria in the milk. After cooling the milk to 38 C° an active culture is added containing “good” bacteria that will make the milk sour. The time allowing the bacteria to ferment the milk is different from each recipe. This step is called preacidification. Added to the milk is also the enzyme rennet, originating from the calf stomach. By action of the rennet, the milk will coagulate and a thick cheese curd is formed. This curd is formed by proteins in the milk that will stick to each other, forming a network. Trapped in this network is also milk fat, minerals such as calcium, proteins and water. The curd is cut into walnut shaped pieces and stirred. The water will migrate out of the curd, taking some water soluble proteins and minerals with it, and forming whey.

At a specific pH, when the milk is sour enough, the whey is drained off from the cheese curd. Hot water is poured onto the curd, and the curd will melt. At this stage it is possible to stretch the curd and work with it to form Mozzarella balls. The finished Mozzarella is put in cold salted water.

The last stage when producing Mozzarella, the stretching part, is important in order to get the typical fibrous texture of Mozzarella cheese. The ability of stretching the cheese curd in hot water is believed to be dependent on the amount of calcium present in the cheese curd at this stage. Thus, by the action of the starter culture, the pH will decrease and make the milk sour. The decrease in pH causes the migration of calcium from the cheese curd into the whey and thereby decreases the amount of calcium in cheese curd. Reduction in pH and calcium migration into the whey is also true for milk that is stored for long periods at refrigerated temperatures, waiting to become processed into Mozzarella.

One could ask the question, how would prolonged storage of buffalo milk affect the

Mozzarella manufacturing? And also, how would prolonged storage of milk in combination with a variation of the time the bacteria are active in the milk, the preacidification, affect Mozzarella manufacturing? These are the questions that this study has tried to find answers to.

In this study milk was stored over a period of 0 to 8 days. Every day the pH was measured and milk was coagulated by rennet to obtain cheese curd and whey. The amount of calcium in whey was analyzed in order to study the migration of calcium out of the cheese curd. In addition, milk that had been stored for 1 day, 4 days and 8 days was acidified with starter culture for 0, 15, 30, 60, 90 and 120 minutes to study the effect of preacidification on Ca migration, also in combination with different periods of cold storage.

The current study shows that when pH decreases in the milk, e.g. uring storage for 8 days or by preacidification using a starter culture for 15-120 minutes, the amount of calcium in whey increases. This means that when using for example 4 days old milk, the recipe must be modified and preacidification be reduced by approximately 15 minutes compared to when using fresh milk. If 8 days old milk is used, the preacidification should be reduced by 30 minutes. However, in practice 8 days old milk has other negative effects on Mozzarella production e.g. a lower cheese yield and also possible off flavors, limiting the applicability.

In conclusion, prolonged cold storage of buffalo milk has implications for Mozzarella manufacturing in the way that the time needed for the preacidification step with starter culture must be reduced.