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# Composition and Coagulation Properties of Buffalo Milk Produced Under Swedish Conditions; Changes Taking Place During the First Weeks of Lactation

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**Abstract**

The domestic water buffalo (*Bubalus bubalis*) contributes with a significant share of global milk production and is the major milk producing animal in many countries. In Europe, the buffalo milk has been used in the production of buffalo Mozzarella. There is an increasing interest in these multipurpose animals also in Northern Europe, and since 2012, there is also dairy farm production of Swedish buffalo Mozzarella. The composition and properties of the milk will strongly influence processing of buffalo milk into Mozzarella cheese, as changes take place during the first weeks of lactation. Since there is no data available on the composition of buffalo milk produced under Swedish conditions, this study was initiated. In this project, we investigated gross composition, milk protein profile, proteolytic activity, free fatty acids, total calcium, calcium activity and the coagulating properties of milk from individual water buffalos during the first six weeks of lactation. Compared with reference values recorded one week after calving, there were significant changes in many of the compositional parameters investigated. Weekly increase in pH (0.90%), lactose (2.25%) and α-lactalbumin content (4.32%) was observed, whereas total protein content decreased by 5.56% per week. Milk coagulation time and gel firmness did not change significantly during the study period, but in numerical terms there was increase in coagulation time and a decrease in gel firmness. Overall, significant changes in buffalo milk composition can help in devising a strategy for the best use of buffalo milk during the first weeks of lactation, particularly in small, artisanal dairy farmhouses.

**Keywords:** Water buffalo milk; Gross composition; Protein profile; Coagulation properties; Early lactation

## Introduction

Buffalo milk is the second most consumed milk worldwide, with milk from dairy buffaloes representing 14% of global milk production in 2014. During the past 10 years, the global buffalo population has increased [1], indicating the importance of dairy production from buffalos. The majority (97%) of dairy buffaloes are found in Asia, while approximately 80% of the European population is found in Italy [1], where Mozzarella is the main product made from buffalo milk. In Sweden, the first Mediterranean water buffalos were introduced in 2010 and buffalo Mozarella production was initiated two years later (www.angsholmensgardsmejeri.se). The Scandinavian population of water buffalos is slowely increasing, however, the number of animals is still very low compared to the situation in Southern Europe. Since water buffalos due to their large clovers are well adapted to wetland conditions, they are ideal as wetland renovators and are used for this specific purpose in various nature reserves [2]. The dairy buffalo will produce less milk than the dairy cow. In Italy, a buffalo cow will on average produce 2200 kg of milk per lactation, while a Holstein cow can produce around 7400 kg per year [3]. The lactation length of buffaloes is also shorter, on average 270 days in the Mediterranean buffalo [3], compared to 300-365 days in the modern dairy cow.

The composition and properties of buffalo milk are quite different from those of dairy cow milk, but, as in the case of cow's milk, they will change throughout the lactation. Buffalo milk is richer in almost all major constituents than cow's milk, resulting in a higher dry matter content. Furthermore, the milk fat globules are larger and the milk is whiter than cow's milk, due to the fact that buffalo milk lacks carotene. The casein content is higher in buffalo milk and the casein index (casein content as a percentage of total milk protein) usually exceeds 80% [4]. The calcium and

phosphorus levels in dairy buffalo milk are reported to be 70% and 30% higher than in cow milk, respectively, [5] and the pH of buffalo milk (6.81) is somewhat higher than that of cow's milk (6.76) [6].

The high concentration of fat, protein and minerals enables efficient production of dairy products from buffalo milk. For example, 100 litres of buffalo milk will produce 20-22 kg of Mozzarella, i.e. the yield is almost 50% higher than with cow's milk [7]. However, in practical Mozzarella production, it has been observed that milk from buffalos in the first 5-6 weeks of lactation is less suitable for Mozzarella manufacture (personal communication with Mozzarella producers). The stretchability of the resulting cheese curd is typically poor in early lactation and this can be of particular concern for small-scale Mozzarella producers, considering that their animals are commonly in the same lactation stage. Previous studies report the compositional changes in buffalo milk during the entire lactation [8,9], but to our knowledge there are no published studies focusing specifically on changes in milk composition and coagulation properties during the first 5-6 weeks after calving, a period corresponding to almost 15% of the total lactation, and affecting Mozzarella production. This study was set out with the hypothesis, that a high initial calcium content was the reason for the observed difficulties to make a good Mozzarella during the first weeks of lactation. Knowledge of such change is particularly relevant for small dairy farmhouses which practise seasonal calving. The aim of this study was to investigate the gross composition, milk protein profile, calcium content, calcium activity, citric acid content, proteolytic activity, coagulation time and gel firmness of milk from a small-scale Swedish buffalo herd during the first six weeks after calving.

## Materials and Methods

### Collection of milk samples

Representative milk samples from six Mediterranean buffaloes were collected at Ängsholmens dairy farm, Sweden, from January to March (indoor period). The buffaloes were housed in a loose-house system and milked twice per day with a bucket milking machine. Individual milk samples from each buffalo cow were collected, with the first sampling taking place between 4 - 7 days after calving. Milk samples (2 x 50 mL) were collected during evening milking on one or two days per week during the first six weeks in lactation, with 4 - 12 samples obtained per individual buffalo. The results from the first sampling occasion were used as reference points in the analysis of milk properties.

All milk samples were treated with 100 µL bronopol (2 µL/mL) as a preservative and refrigerated at 4 °C before transportation to the Swedish University of Agricultural Sciences (SLU), Uppsala, for detailed analyses, which always took place the following morning. When the milk samples arrived at the laboratory, sub-samples were poured into vials and immediately frozen at -20 °C. The fresh milk was used for milk gross composition analysis, rheological analyses, analysis of calcium activity and pH. Chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Inc., Stockholm, Sweden), unless otherwise stated.

### Milk gross composition analysis

Individual milk samples were analysed for gross composition at the Department of Animal Nutrition and Management, SLU. Total protein, total casein, total fat and lactose concentrations were analysed by a mid-infrared spectroscopy method (Fourier Transform Infrared Spectroscopy; FTIR); (FOSS Electric A/S (Hilleröd, Denmark). Milk somatic cell count (SCC) was analysed by electronic fluorescence-based cell counting (Fossomatic Foss FT 120, Hilleröd, Denmark).

### Calcium activity and pH

Calcium activity and pH measurements were performed on fresh milk. For pH measurement, a pH meter (Prolab, 3000 Digital-Multi-Meter, SI Analytics, Weilheim, Germany) was used. Calcium activity was measured with a calcium ion-sensitive electrode (Ca 800 DIN, WTW, Hamburg, Germany) as described [10]. On each occasion, five standards ($CaCl_2$ and KCl) were used for calibration of the ion selective electrode prior to sample analysis.

### Flame atomic absorption spectrophotometry

Whole milk samples were analysed for total calcium content using an atomic absorption spectrometric (AAS) method based on an International Dairy Federation (IDF) protocols [11, 12]. In short, the frozen milk samples (10 g) were freeze-dried (Labconco, Ab NinoLab, Upplands Väsby, Sweden) to obtain a fine powder. Heat-resistant crucibles were washed with distilled water and heated at 500 °C in the oven (Nabertherm controller B 180, Lilienthal, Germany) with a layer (2 mL) of 10% nitric acid (NA) to remove calcium ions and dirt. After heating, the crucibles were washed with distilled water and dried prior to use. The freeze-dried milk (10 g) was heated in the oven at 550 °C for 90 minutes until a white ash was obtained. The ash was dissolved by adding 1 mL 25% NA to the crucible and the solution was transferred to a volumetric flask. The crucible was rinsed three times with distilled water. Distilled water was also used for diluting the sample to a total volume of 250 mL. A 5 mL portion of the sample solution was transferred to a volumetric flask together with 10 mL lanthanum chloride (lanthanum III chloride heptahydrate, 27 g/L) and diluted to 100 mL using distilled water.

For preparation of standards (1 µg, 2 µg, 3 µg, 4 µg and 5 µg $CaCO_3$/mL), a stock solution containing 1.2481 g $CaCO_3$ (Merck, Darmstadt, Germany) and 15 ml 4 M HCl was diluted with distilled water to 1000 mL. Samples and standard solutions were prepared and analysed on the same day. The total calcium content was analysed by AAS (Perkin Elmer, A-analyst 100, Waltham, USA) fitted with a calcium-magnesium lamp, with a wavelength of 422.7 nm.

### Free fatty acids

Free fatty acid (FFA) concentration was determined by extraction-titration as described [13]. Milk fat was extracted using


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diethyl ether and hexane (80:20, v/v) using methyl orange as an indicator. Samples were acidified using $H_2SO_4$ until the solution turned pink (pH 2-3), centrifuged, and the supernatant was transferred to a new tube. The FFA concentration was determined by titration of the supernatant with KOH in ethanol, using α-naphtholphthalein and phenolphtalein as indicator. Titration was stopped when the colour of the solution turned lilac/blue, persisting for a few seconds.

### Plasmin and plasminogen-derived activities

Plasmin and plasminogen-derived proteolytic activity was determined according to [14]. Plasmin and plasminogen were dissociated from casein micelles by incubation of the milk with ε-amino-n-capronic acid (EACA), followed by ultracentrifugation. Plasmin activity was measured in the resulting milk serum using a chromogenic substrate, pyro-GLU-Phe-Lys-p-nitroanilide hydroxychloride (Aniara Diagnostica, West Chester, USA) and the total plasmin and plasminogen derived activity was measured after activation of plasminogen with urokinase. Absorbance was recorded every 3 minutes during 120 minutes at 37 °C and activity was expressed as change in absorbance per time unit (ΔA405/Δt).

### Casein and whey protein composition

Protein separation was performed with a 7100-capillary electrophoresis (CE) system (Agilent Technologies Co., Santa Clara, USA), using an unfused silica standard capillary as [15]. D, L-dithiothreitol (DTT) was added to the sample buffer on the day of sample preparation, in order to disrupt disulphide bridges of the milk proteins. Milk (300 µL) was mixed with 700 µL sample buffer and defatted after centrifugation for 10 min at 10 000 x g. Prepared samples were stored at -20 °C prior to analysis. Relative concentration of the individual proteins was calculated based on the peak area and expressed as a percentage of the total integrated area in the electropherogram.

### Rheological analyses

Elastic (G´) and viscous modulus (G´´) of the fresh milk samples were continuously measured using a Bohlin CVOR-150-900 rheometer (Malvern Instruments Nordic AB, Uppsala, Sweden) as described [16]. The rheometer was equipped with a 25 mm diameter cup and a 28 mm diameter concentric cylinder and was 40 mm high. Temperature was controlled by a Peltier element. Bovine rennet 180 IMCU (75% chymosin and 25% pepsin, Kemikalia AB, Skurup, Sweden) was added at a concentration of 0.075 IMCU/mL milk. Gel formation was followed for 30 min with an oscillation frequency of 1 rad/s and a strain of 0.01, which was well within the linear viscoelastic region of milk gels [17]. Coagulation time (Ct) was measured from the point of the enzyme addition until the 1 Pa value was reached. Measurement frequency was set to 8 seconds. Gel firmness (G´) of the developing gel was plotted against time for 20 minutes and G20, i.e. gel firmness at 20 minutes, was recorded.

### Statistical analyses

A separate ANOVA was performed for each parameter. Due to missing observations, GLM (general linear model) was used in the SAS procedure. For the treatments which showed a significant effect on the study variables at alpha = 0.05, mean separation procedure was adapted using the method LSMEANS (Least Square Means). To investigate the trend (as a percentage) over time (sampling occasions), simple linear regression models were fitted for the following variables: pH, total fat, total protein, α-lactalbumin, lactose, calcium, citric acid, coagulation time and gel firmness. Pearson correlation coefficient (R) for the milk quality traits studied was calculated using the complete set of data (n = 31 - 60). The levels of significance were: $P<0.05$, $P<0.01$ and $P<0.001$.

## Results and Discussions

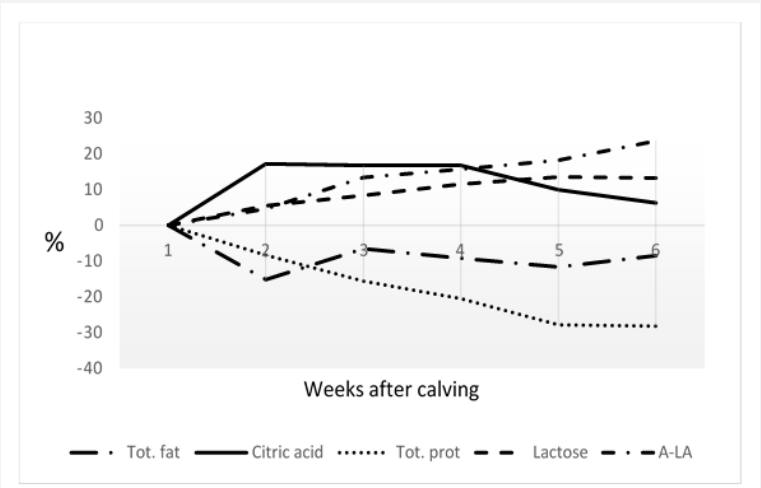


**Figure 1:** Relative changes (compared with week 1) in the composition of buffalo milk during the first six weeks of lactation. Mean of samples collected from six individual cows (n = 4-12). Tot. fat = total fat; Tot. prot = total protein; A-LA = α-lactalbumin.



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Due to the fact that the participating farm only had six lactating buffalo cows at the time of investigation, the number of animals in the study is very limited. However, as this farm is the first dairy buffalo herd not only in Sweden but in the Nordic countries, we believe that the characterization of the composition and coagulation properties of the buffalo milk during the first weeks of lactation is of great interest. The compositional values observed throughout the investigated period of lactation were in general within the ranges referring to global buffalo milk production reported [18]. Despite the low number of animals, significant changes were observed in milk composition during the first six weeks of lactation (Table 1). For example, the total protein content decreased significantly (P<0.000) after calving, by 5.56% per week (Table 2, Figure 1). The protein content was in the same range as rapported [19]. In a previous study [20] the authors analysed colostrum, postcolostrum and early lactation milk samples from individual buffalo cows and found that total protein content declined steadily until 30 days after calving whereas the lactose content increased. With the progress of lactation, we observed an increase in lactose levels from 4.40% to 5.17% and above also in our study (Table 3). This study confirms findings in a previous study [6], who reported that the content of lactose is slightly higher in buffalo milk (about 5%) than in cow's milk (4.8%).

**Table 1:** Changes in quality traits in milk from individual buffalo cows (N = 6) during the first six weeks following parturition. [a]The statistical significance of the changes is indicated by their P-value. P<0.05 considered significant, * = trend for significance.

| Significant variables | P-value[a] | Non-significant variables | P-value[a] |
|---|---|---|---|
| pH | 0.001 | Total Whey | 0.653 |
| Total protein | 0.001 | Citric acid | 0.436 |
| α-lactalbumin | 0.023 | Ca-activity | 0.298 |
| β-lactoglobulin | 0.013 | Ca-content | 0.540 |
| α-$S_1$-casein | 0.040 | Somatic cells | 0.900 |
| β-casein | 0.015 | α−$S_2$ casein | 0.708 |
| Lactose | 0.001 | κ−casein | 0.549 |
| Plasminogen | 0.001 | Total Fat | 0.775 |
| Plasmin | 0.057* | Total casein | 0.350 |
| Free fatty acids | 0.036 | Coagulation time | 0.598 |
| | | Gel firmness | 0.813 |

**Table 2:** Linear regression results for pH, total fat, total protein, α-lactalbumin, lactose, citric acid, calcium content, coagulation time and gel firmness. [a]Percentage change per week ± standard error (SE), and level of significance (P-value). [b]P<0.05 considered significant.

| Variable | Percentage change per week ± SE[a] | P-value[b] |
|---|---|---|
| pH | 0.90 ± 0.12 | 0.000 |
| Total fat | 0.89 ± 1.30 | 0.498 |
| Total protein | -5.56 ± 0.83 | 0.000 |
| α -lactalbumin | 4.32 ± 1.66 | 0.012 |
| Citric acid | -2.63 ± 2.78 | 0.346 |
| Ca content | -0.89 ± 2.39 | 0.712 |
| Lactose | 2.25 ± 0.28 | 0.000 |
| Coagulation time | 3.51 ± 2.40 | 0.150 |
| Gel firmness | -5.64 ± 4.37 | 0.203 |

The increase of lactose after calving was followed by significant increase of α-lactalbumin (Figure 1) which reflects the fact that α-lactalbumin, one of the major whey proteins, plays a crucial role in regulation of lactose synthesis. As a consequence of the increase in α-lactalbumin, the relative amount of the other major whey protein, β-lactoglobulin, declined after the first sampling weeks (Table 3).


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**Table 3:** Concentrations of different components in buffalo milk samples collected from six individual cows during the first six weeks after calving. [a]Least square (LS) means, n = 4-12. Lact. week: lactation week; Tot. prot.: total protein; Tot. whey: total whey; total fat: total fat; FFA: free fatty acids; α-LA: α-lactalbumin; β-LG: β-lactoglobulin; αS1-CN: α $^{s1}$-casein; αS2-CN: α $^{s2}$-casein; κ-CN: κ-casein; β-CN: β-casein. Tot. casein and Tot. whey: percent of Tot. protein. α-LA; β-LG; $\alpha S_1$-CN; $\alpha S_2$-CN; κ-CN; β-CN - are expressed as a percentage of the total integrated protein profile. [a–d]Means within a column with different superscript letters differ significantly (P<0.05).

| Lact. week | Tot. prot. g/100g[a] | Tot. casein %a | Tot. whey %[a] | Tot. fat g/100g[a] | FFA mmol/ 100g fat[a] | Lactose g/100g[a] | α -LA %[a] | β-LG %[a] | α $S_1$-CN %[a] | α $S_2$-CN %[a] | κ -CN %[a] | β-CN %[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 5.43[a] | 82.73 | 12.57 | 7.51 | 0.12[a] | 4.40[a] | 3.89[a] | 8.68[a] | 37.87[a] | 9.98 | 8.21 | 26.67[a] |
| 2 | 5.05[b] | 82.46 | 13.33 | 6.57 | 0.14[ab] | 4.67[b] | 4.17[ab] | 9.16[ab] | 38.10[a] | 9.07 | 8.12 | 27.17[a] |
| 3 | 4.72[bc] | 82.62 | 12.84 | 7.14 | 0.15[abc] | 4.81[b] | 4.53[b] | 8.31[b] | 36.90[ab] | 8.98 | 8.01 | 28.73[b] |
| 4 | 4.54[cd] | 82.49 | 12.88 | 6.91 | 0.18[bc] | 4.99[c] | 4.63[b] | 8.25[b] | 35.71[b] | 8.48 | 8.30 | 30.00[b] |
| 5 | 4.15[d] | 82.60 | 12.58 | 6.96 | 0.24[c] | 5.19[c] | 4.55[b] | 8.04[b] | 35.69[b] | 8.23 | 8.69 | 29.93[b] |
| 6 | 4.10[d] | 81.52 | 13.03 | 7.16 | 0.18[ac] | 5.17[c] | 4.75[b] | 8.29[b] | 34.41[b] | 8.57 | 8.55 | 28.41[b] |

**Table 4:** Levels of milk quality traits related to coagulation in buffalo milk samples collected from six individual cows during the first six weeks after calving [a]Least square (LS) means, n = 4-12. s: seconds; Pa: Pascal. [a–d]Means within a column with different superscript letters differ significantly (P<0.05). Ca: calcium, NA: not analysed.

| Lactation week | pH[a] | Ca content g/kg[a] | Ca activity nM[a] | Citric acid %[a] | Coagulation time, s[a] | Gel firmness Pa[a] |
|---|---|---|---|---|---|---|
| 1 | 6.43[a] | 1.66 | 3.06 | 0.15 | 221 | 160 |
| 2 | 6.59[b] | 1.63 | 2.81 | 0.18 | 244 | 159 |
| 3 | 6.72[bc] | 1.64 | 2.68 | 0.18 | 255 | 156 |
| 4 | 6.76[cd] | 1.64 | 2.65 | 0.18 | 275 | 157 |
| 5 | 6.83[d] | 1.53 | 2.91 | 0.18 | 272 | 125 |
| 6 | 6.85[d] | 1.57 | NA | 0.18 | 269 | 128 |

**Table 5:** Mean plasmin, plasminogen and somatic cell concentrations in buffalo milk samples collected from six individual cows during the first six weeks after calving. [a]Least square (LS) means, n = 4-12. NA: not analysed. [a–c]Means within a column with different superscript letters differ significantly (P<0.05).

| Lactation week | Plasmin U/mL[a] | Plasminogen U/mL[a] | Somatic cells cells/mL*1000[a] |
|---|---|---|---|
| 1 | 5.40[ac] | 33.47[a] | 83 |
| 2 | 10.09[a] | 17.67[b] | 103 |
| 3 | 6.84[b] | 18.45[b] | 73 |
| 4 | 5.00[bc] | 17.03[b] | 53 |
| 5 | 7.31[ac] | 16.23[b] | 81 |
| 6 | 6.11[abc] | 17.19[b] | NA |

For the casein fraction, a small decline in the relative amount of α S1-casein, and a small increase in the relative amount of β-casein, was observed (Table 4). It is known that β-casein is the major substrate for plasmin, the major endogenous protease in milk [21]. In this study, we observed a significant decrease in the concentration of plasminogen, the precursor of plasmin, in buffalo milk after the first sampling week (Table 5). Interestingly, the levels of plasminogen were much higher during the first week after calving, after which they declined and stabilised. For plasmin activity, there was no clear trend in the period after calving, but there was a significant negative correlation between β-casein and plasminogen (P<0.05) and between β-casein and plasmin (P<0.01) (data not shown). This suggests that the increase in relative content of β-casein observed as lactation progressed could at least partly be caused by a decline in the proteolytic activity of plasmin.

The fat concentration observed in our study, i.e. close to 7%, agrees with levels reported [22], and in agreement with findings [19, 20]. There was no significant change in fat content throughout the sampling period (Table 1). The FFA content in cow's milk has been reported to be 0.50-1.20 mmol/100 g fat [23], whereas levels of FFA found in buffalo milk samples in this study was significantly lower, ranging between 0.12 and 0.18 mmol/100g fat (Table 3). These FFA levels can be considered as very low [24] and below the limit of 1.5 mmol/100 g of fat, which is considered to give a rancid off-flavour that most consumers will find unacceptable [25]. We


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found that the total fat content in buffalo milk was not correlated with the FFA content (data not shown), which agrees with findings of [26].

In fresh cow's milk, pH usually varies from 6.40 to 6.76 and in buffalo milk from 6.70 to 6.81 [6]. In this study, the pH in buffalo milk collected in the first week after calving was relatively low (6.43) compared to values previously reported for both cow's milk and buffalo milk. However, with the progress of lactation the pH increased (Figure 2, Table 4) to normal levels, in the same range as reported [27]. Similarly, it was observed [19] that the pH of cow's milk was low in the beginning of lactation (pH 6.49 at day 5 after calving) but increased thereafter (reaching pH 6.60 at day 30). Considering the low pH of colostrum, the observed increase in pH during early lactation is likely to reflect the transition from colostrum to milk.

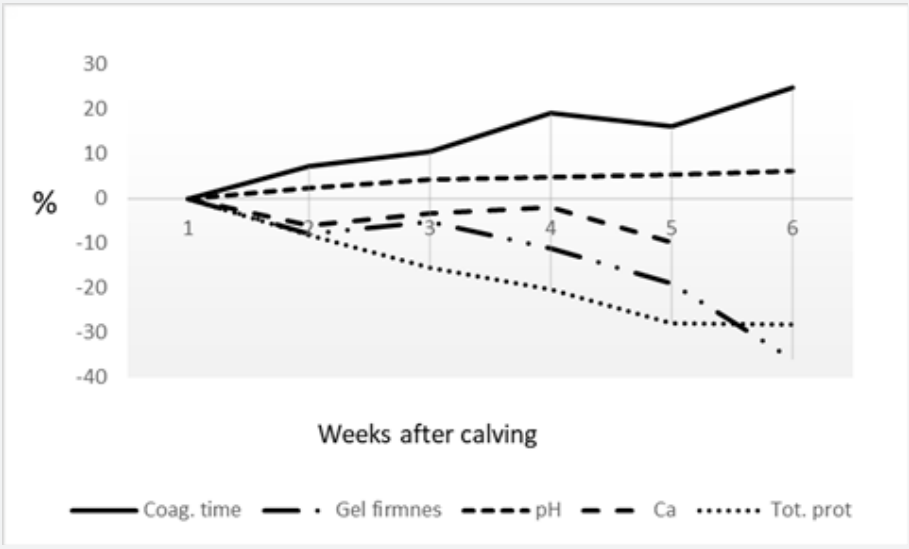


**Figure 2:** Relative changes (compared with week 1) in parameters important for the rheological properties in buffalo milk during the first six weeks of lactation. Mean of samples collected from six individual cows (n = 4-12). Coag. time = coagulation time. $C_a$ = total calcium, Tot. prot = total protein.

In this study, we also investigated calcium content and rheological properties, i.e. coagulation time and gel firmness, of the buffalo milk samples (Figure 2). We found no significant changes in these parameters during the first six weeks after calving. However, numerical values of coagulation time increased and, as expected, numerical values of gel firmness decreased, so that the gel resulting from milk collected in the sixth week of lactation was softer than the gel resulting from milk collected during the first week (Table 4). The lower total protein content (Table 3) and higher pH (Table 4) at week six probably contributed to the longer coagulation time and lower gel firmness (numerical values, not significant). The low number of buffalo cows in our investigation (n=6) and variations between individuals probably explain why changes in rheological properties during early lactation were not statistically significant.

The calcium content and calcium activity in buffalo milk decreased slightly during the first weeks of lactation, from 1.66 to 1.57 g/kg and from 3.06 to 2.91 nM, respectively (Table 4). The total calcium content is closely correlated with the concentration of casein [28]. In our study, we made similar observations, with total casein being higher in the beginning of lactation (Table 3), decreasing over time, and likewise a decline in calcium content, although not significant. The calcium content in the first four weeks of lactation was in agreenment with [20] who reported 1.50 - 1.69 g/kg in the period between January and March. Similarly, [19] observed a steady decline in the calcium content in cow's milk during the first 30 days after calving, from 1.44 g/kg on day five to 1.34 g/kg on day 15 and 1.21 g/kg on day 30. The hypothesis of this study was, that the changes in calcium content during the first weeks of lactation is a contributing factor for the poor stretcability of the curd resulting in wrong tecture of the Mozzarella cheese. The calcium content in buffalo milk was higher in the beginning of lactation (Table 4), but numerical values decreased by 0.89% per week after calving (Table 2). With decreasing levels of calcium and protein, the gel firmness also decreased (Figure 2). The high calcium content in the milk during the first weeks after calving might contribute to the higher gel firmness observed at that stage. The changes in total Ca and total protein content during the first weeks of lacation may together constitute the major reasons behind the difficulties in Mozzarella manufacture from raw buffalo milk in the first period of lactation.

## Conclusion

This study suggests that the significant changes taking place in buffalo milk composition during the first six weeks of lactation, will affect coagulation properties of the milk and also influence


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on Mozzarella production. This should be taken into consideration when processing buffalo milk into various products, to guarantee optimal use of the milk, especially in the case of small-scale and artisan dairy farmhouses.

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## Conflicts of Interest

No potential conflict of interest was reported by the authors.

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