Lactoferrin concentration in buffalo milk

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Abstract

The objective of this study was to quantify lactoferrin (Lfe) in buffalo milk and to examine the factors affecting milk Lfe, such as the lactation stage, daily milk yield, parity, and milk somatic cells count (SCC). Milk Lfe concentration was detected by the SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The overall mean of Lfe concentration was 0.332±0.165 g/kg and ranged from 0.030 to 0.813 g/kg. Milk Lfe concentrations increased (P<0.01) with the increase of days in milk, but it was not affected by parity. Milk Lfe concentration was significantly affected by SCC. The differences became significant when the levels of SCC increased up to 200,000/mL. This is the first investigation on the levels of Lfe in buffalo milk in reference to daily milk production, lactation stage, parity and SCC. Further studies are needed to clarify the relationship between Lfe and SCC in buffalo milk.

Materials and methods

Dataset

The study was carried out in fifteen dairy buffalo farms in the south part of Lazio region. Dairy farms were homogeneous in terms of the production system adopted (intensive) and the barn design and management (total confinement free barn housing with no time at pasture, TMR feeding practices and animals were milked twice a day with pipeline milking machines).

A total of 225 lactating buffaloes were monitored during the entire lactation. Individual milk yield was registered, and individual milk samples were collected at four weeks intervals, starting from the first week to the end of lactation. Duplicate milk samples from each buffalo were collected on the sampling day. Milk samples were immediately put into a refrigerator box and transported to the laboratory. One set of milk samples (50 mL preserved with bronopol-B2) was analysed within 6 h from the sampling-time for fat, proteins and lactose content (Milkscan FT 6000, Foss Electric, Hillerød, Denmark), somatic cells count (Fossomatic 5000, Foss Electric), titratable acidity (“SH/100mL) by NaOH N/4, and pH. Another set of milk samples (10 mL) was centrifuged at 1500 rpm for 15 min at 4°C to remove fat. The skimmed milk was collected, stored at -20°C and then analysed for Lfe quantification within 3-4 weeks from the sampling.

Quantitative determination of lactoferrin in milk

Milk Lfe concentration was detected by the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to Laemmli (1970) on 12.5% slab gel (Figure 1). The fractions of milk soluble proteins containing Lfe were diluted by adding an equal volume of double-concentrated sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.8 M dithiothreitol, 0.4% bromophenol blue and 20% glycerol) and heated at 100°C for 3 min, to thermally denature proteins and to promote interactions of the proteins with SDS. Low molecular weight standards (Bio-Rad, Watford, UK) were prepared in sample buffer. The electrophoresis was performed in a Mini-Protean III dual slab cell (Bio-Rad). Electrophoresis was performed at room temperature using a voltage stepped procedure: voltage was kept constant (30 V) until the samples completely left the stacking gel and then the voltage was increased 15 V per min for 4 times. Voltage was maintained constant (90-100 V) until the tracking dye reached the bottom of the gel. Immediately after ending electrophoresis, gels were removed from the plates and placed in a staining solution containing 40% methanol, 10% acetic acid and 0.1% Coomassie Brilliant Blue R-250. Gels day. A total of 2236 milk samples were collected and analysed. Dataset was edited so that only animals having at least 6 records per lactation were maintained. After editing, the final dataset consisted of 196 subjects and 1412-1440 records (Table 1).

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were left for 30 min in the staining solution and then destained in the methanol/acetic acid solution (5% methanol, 7% acetic acid).

Quantitative analysis of electrophoretically separated Lfe was done by densitometry using BSA (Sigma, Milano, Italy) as external standard, with Kodak EDAS-290 densitometer and analyzed with ID Image Analysis software (Kodak Company, Rochester, NY, USA). Detected polypeptides were identified using the standards of BSA, Lfe, \( \alpha \)-La, \( \beta \)-Lg, k-casein, \( \alpha \)-casein and \( \beta \)-casein (Sigma).

Statistical analysis

Data were analysed by repeated measures using the MIXED procedure of the Statistica-7 Software package (Stat Soft, Inc., Tulsa, OK, USA) with day of sampling as the repeated effect. Data were tested for normality using Kolmogorov-Smirnov test for Gaussian distribution. Except for SCC all data were normally distributed. For this reason somatic cells score (SCS) was calculated \([\log_{2} \text{SCC / 100,000} + 3]\) and used in the statistical analysis. The milk Lfe variation was evaluated with a model that included parity, daily milk yield, and somatic cells count classes as fixed effects, and buffalo was the uncorrelated random effect. To describe changes of Lfe and SCS during lactation a model that included parity in milk class as fixed effect and buffalo as uncorrelated random effect was used. Random variable was assumed to have a normal distribution. Parity was classified as follow: 1 = 1st lactation; 2 = 2nd and 3rd lactation; 3 = 4th and 5th lactation; 4 = 6th and 7th lactation; 5 = lactation \( >7 \). Daily milk yield was classified as follow: \( Y_{1} \leq 6 \text{ kg} \); \( 6 < Y_{2} \leq 8 \text{ kg} \); \( 8 < Y_{3} \leq 10 \text{ kg} \); \( 10 < Y_{4} \leq 12 \text{ kg} \); \( Y_{5} > 12 \). SCC was classified by the number of somatic cells in milk as follow: \( 0 \leq \text{SCC}_{1} < 50,000 \); \( 50,000 \leq \text{SCC}_{2} < 100,000 \); \( 100,000 \leq \text{SCC}_{3} < 200,000 \); \( 200,000 \leq \text{SCC}_{4} < 300,000 \); \( 300,000 \leq \text{SCC}_{5} < 400,000 \); \( \text{SCC}_{6} = 400,000 \).

To investigate the relationship between Lfe and milk characteristics, partial correlation coefficients corrected for repeated measures (namely days in milk) were calculated. The differences were analysed by \( t \)-test and the significances were set at a value of \( P<0.05 \).

Results

Buffalo milk characteristics and milk lactoferrin concentration

The basic statistics (mean ±SD) of the milk yield and characteristics of all samples are given in Table 1. The milk Lfe concentration in lactating buffaloes was 0.332±0.165 g/kg and ranged from 0.030 to 0.813 g/kg (Table 1). The change of Lfe content in buffalo milk throughout the lactation period is showed in Figure 2. Milk Lfe concentrations increased (\( P<0.01 \)) with the increase of days in milk showing the minimum levels in the first 50-60 days (0.207±0.173 g/kg) and the maximum levels at the end of lactation (0.503±0.135 g/kg).

Somatic cells score increased after 90 days in milk and then remained constant until the end of the lactation period (Figure 3). The Lfe concentration of buffalo milk was not affected by parity (Table 2). The Lfe concentration decreased as the daily milk yield increased (Table 2). Milk Lfe concentration was significantly affected by SCC class (Table 2). In particular, the differences become sig-

Table 1. Overall means of buffalo milk yield and milk characteristics.

|                         | No. of samples | Means         | CV, % |
|-------------------------|----------------|---------------|-------|
| Milk, kg/head/day       | 1440           | 9.32±3.48     | 37    |
| Fat, %                  | 1440           | 8.98±1.73     | 19    |
| Proteins, %             | 1440           | 4.67±0.47     | 10    |
| Lactose, %              | 1440           | 5.05±0.33     | 6     |
| pH                      | 1440           | 6.70±0.18     | 3     |
| Titratable acidity, °SH/100 mL | 1412  | 7.40±1.41     | 19    |
| SCC, n/mL               | 1433           | 309,812±863,219 | 279   |
| SCS                     | 1433           | 3.49±1.56     | 45    |
| Lactoferrin, g/kg       | 1440           | 0.332±1.56    | 50    |

CV, coefficient of variability; SCC, somatic cells count; SCS, somatic cells score. Data are presented as mean ±SD.

![Figure 1](https://example.com/fig1.png)

Figure 1. SDS-PAGE profile of buffalo-milk separated on 12.5% SDS-polyacrylamide gel and stained with comassie brilliant blue. Lane 1, standard molecular weight markers: phosphorylase b, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa and egg-white lysozyme, 14.3 kDa. Lane 2, bovine serum albumin, 66 kDa, Lane 3, 4, 5, 6, 7 and 8 and samples at different dilution of buffalo-milk.
significant when the levels of SCC increase up to 100,000 and the highest levels were observed with SCC greater than 200,000/mL (Table 2).

Partial correlations between lactoferrin and milk characteristics

Lactoferrin was positively correlated with total proteins ($r=0.41$; $P<0.0001$) and SCS ($r=0.21$; $P<0.001$) and negatively correlated with lactose ($r=-0.11$; $P<0.001$) and titratable acidity ($r=-0.11$; $P<0.001$).

Discussion

In this study, the productive performance data and milk analysis on buffalo’s milk samples were in agreement with the data reported in other studies on buffalo’s milk (Ceron-Munoz et al. 2002; Rosati and Van Vleck, 2002; Zicarelli, 2004; Potena et al., 2007).

Several different methods have been used to quantify Lfe in bovine and human milk such as capillary electrophoresis (Riechel et al., 1998) reversed-phase HPLC (Palman and Elgar, 2002) and several immunoassays. In this study, Lfe was quantified by SDS-PAGE method and it average level was of 0.332 g/kg. This level was similar to those of Campanella et al. (2009). Using immunosensor method, those authors found Lfe concentrations of 232.0 mg/L in raw buffalo milk. The difference between our and Campanella and co-workers data might be due to the different number of samples (more that 1400 in our trial in Campanella and co-workers trial) and to the different source: individual samples in our study, bulk milk samples in Campanella and co-workers study. A quite wide range of Lfe concentration has been determined in healthy bovine milk. The values varied from 1.15 µg/mL to 485.63 µg/mL (Hagiwara et al., 2003). Lactoferrin concentrations in human milk were reported to be in the range of 1.0-3.2 mg/mL depending on the method used. Hiss et al. (2008) observed that the weekly mean concentrations of Lfe in goat milk ranged between 10 to 28 µg/mL. Lactoferrin levels in mare

Table 2. Ls means ±SD of lactoferrin concentration in the milk from lactating buffaloes.

| Parity | N. of samples | Lactoferrin, g/kg | P value |
|--------|---------------|-------------------|---------|
| 1      | 247           | 0.333±0.159       | 0.551   |
| 2      | 301           | 0.319±0.163       |         |
| 3      | 257           | 0.335±0.162       |         |
| 4      | 243           | 0.342±0.168       |         |
| 5      | 392           | 0.335±0.172       |         |

| Daily milk yield | N. of samples | Lactoferrin, g/kg | P value |
|------------------|---------------|-------------------|---------|
| Y1               | 268           | 0.465±0.135       | 0.00001 |
| Y2               | 300           | 0.376±0.157       |         |
| Y3               | 303           | 0.311±0.149       |         |
| Y4               | 299           | 0.280±0.144       |         |
| Y5               | 270           | 0.256±0.154       |         |

| Somatic cells count | N. of samples | Lactoferrin, g/kg | P value |
|---------------------|---------------|-------------------|---------|
| SCC1                | 218           | 0.285±0.163       | 0.00001 |
| SCC2                | 393           | 0.301±0.149       |         |
| SCC3                | 363           | 0.343±0.160       |         |
| SCC4                | 162           | 0.370±0.169       |         |
| SCC5                | 92            | 0.380±0.162       |         |
| SCC6                | 205           | 0.375±0.182       |         |

Parity was classified as follow: 1 = 1st lactation; 2 = 2nd and 3rd lactation; 3 = 4th and 5th lactation; 4 = 6th and 7th lactation; 5 = lactation >7th. Daily milk yield was classified as follow: Y1<6 kg; 6 ≤ Y2<8 kg; 8 ≤ Y3 <10 kg; 10 ≤ Y4<12 kg; Y5 ≥ 12. SC was classified by the number of somatic cells in milk as follow: 0 ≤ SCC 1<50,000; 50,000 ≤ SCC 2<100,000; 100,000≤SCC 3<200,000; 200,000≤SCC 4<300,000; 300,000≤SCC 5<400,000; SCC6 ≥ 400,000. Letters indicate differences between the means. a,bP<0.05; A,B,C,D,E=P<0.01.

![Figure 2. Changes of lactoferrin concentration (Ls means ±SD) during the lactation period in buffalo milk. a,b=P<0.05; A,B,C,D,E=P<0.01.](image1)

![Figure 3. Changes of somatic cell score (SCS) during the lactation period in buffalo milk (Ls means ±SD). A,B=P<0.01](image2)
colostrums and milk have also been measured. The obtained results were 21.7 μg/mL, and 0.82 g/kg, respectively (Malacarne et al., 2002; Barton et al., 2006). The mean milk Lfe concentration was reported to be 0.229±0.135 mg/mL in the camel (Konuppayeva et al., 2007). A comparative study on Lfe content in camel, cow, sheep, goat, donkey, mare and human normal milk was done (El-Agamy and Nawar, 2000). The study showed that Lfe concentration varied considerably. The highest level was found in human milk (1.7 mg/mL), while donkey milk had the lowest content (0.07 mg/mL). In our study the concentration of the Lfe in buffalo’s milk appeared to be lower than that reported in human milk, but higher than that in bovine milk. Lactoferrin is a key element in the host innate defense system with its antimicrobial properties, which include iron sequestration, direct lytic activities, and the ability of the molecule to impair the binding of microbes to host cells (Kutila et al., 2003; Valenti and Antonini, 2005). Therefore, a less vulnerability to mastitis in buffalos might be partially due to the high level of Lfe in milk. A strong association between milk Lfe and stage of lactation and daily milk yield, and no association with parity have been observed in the present study. Cheng et al. (2008) found similar results in dairy cows.

Our study indicated that milk Lfe concentration tended to be higher when SCC increased. In contrast, the behaviour of Lfe and SCS during lactation did not match. In fact, Lfe concentration increased significantly during the second phase of lactation reaching the maximum levels at the end of lactation. In contrast, SCS did not increase in this phase. In previous study carried out on buffalo Piccinini et al. (2005) observed a significant increase in the concentration of Lfe in milk with SCC greater than 400,000 cells/mL. Cheng et al. (2008) in dairy cows reported that Lfe concentration tended to be significantly higher when SCC exceeded 141,000 cell/mL. Moreover, in camels significant increase was observed with SCC value greater than 30,000 cells/mL (Al-Majali et al., 2007). Those studies have been demonstrated a slight elevation of Lfe with subclinical mastitis, while Lfe was significantly elevated in clinically affected cows. In goats, the Lfe has been found to be elevated during mastitis and a close relationship between SCC and Lfe was reported, too (Chen et al., 2004). In mid-lactating goats, Hiss et al. (2008) observed that subjects with SCC higher than 430,000 cells/mL had higher Lfe milk concentration. Those authors considered the increased concentrations of Lfe towards the end of lactation, most likely as a physiological alterations rather than innate pathogen responses. Considering results of our and other studies, the increase of milk Lfe concentrations, proportional to somatic cells count qualify Lfe as an acute phase protein in milk. On the other hand, the increase of Lfe concentration during lactation may be due to mammary gland involution as reported in cows (Shamay et al., 2003) and in goats (Hiss et al., 2008) and to dilution effect.

Somatic cells count comprises cellular components that are recruited from the blood stream, whereas Lfe is synthesized in the mammary gland itself (Molenaar et al., 1996) by the epithelial cells and it is also present in the granules of neutrophils. Increase in milk somatic cells are initiated by chemoattractants, e.g. cell wall components or metabolites of bacteria but also by endogenous components including cytokines or complement components (Kehrli and Shuster, 1994). Such cytokines are upregulated during physiological processes and may affect Lfe secretion. Shen et al. (2007) reported that transforming growth factor 1, an important local regulator on mammary tissue involution, has an inhibitory effect on neutrophil degranulation and thus on Lfe secretion. Baumrucker (2005) suggests that the high regulation by mammary cells provides the opportunity to define the cellular route and potential intracrine role of Lfe. The relationships between Lfe concentrations and SCC in buffalo is difficult to analyse; this is due to the differential data for buffalo milk SCC that leads to uncertainty about the level of SCC in buffalo milk that can be used to define the presence of an inflammation (Dhakal et al., 1992; Mahendra and Ludri, 2001; Ceron-Munoz et al., 2002; Passuini et al., 2003).

In a study carried out in Sri Lanka, it was reported that total SCC in normal buffalo milk varied from 50,000 to 375,000/mL (Silva and Silva, 1994). Tripaldi et al. (2010) reported that in buffalo as in other species, total SCC is a valid indicator of udder inflammation and a value of 200,000 cells/mL should be used as the threshold value for early identification of an animal affected by subclinical mastitis. In addition, a total SCC value above that threshold value was associated with significantly decreased milk yield and with changes in milk composition and coagulating properties. Our results indicate that milk Lfe concentration increased significantly when the levels of SCC were up to 100,000 cells/mL and the highest concentrations were observed when SCC increased up to 200,000 cells/mL. Moreover, milk Lfe concentration was positively correlated with SCC (r=0.21) and negatively related (r=−0.31) to lactose. Sharma et al. (2011) reported that elevated SCC was usually associated with a decrease in lactose, because elevated SCC reduced synthetic activity of the mammary tissue. These results combined with our results, may indicate that Lfe might be used as a possible indicator of subclinical mastitis in buffalo as in dairy cattle. Early detection of mastitis in buffaloes could be important for most dairy farmers to reduce production losses and to enhance prospects of recovery.

Conclusions

In conclusion, this is the first investigation on the levels of Lfe in buffalo milk in reference to daily milk yield, lactation stage, parity and SCC. The stage of lactation and daily milk yield contributed to the most of Lfe concentration in the milk from normal lactating buffaloes, whereas parity showed no association. Further studies are needed to better clarify the relationship between Lfe and SCC in buffalo milk and the possible role of Lfe as indicator of intramammary infection in buffalo.

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