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Running head: Quality of Mediterranean buffalo milk

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ABSTRACT

Buffalo milk represents an indispensable source of nourishment in many parts of the world and it is the second most consumed milk worldwide. Buffalo milk is actually used for the production of many dairy products such as pasteurized or concentrated milk, butter, yogurt, ice-cream, dehydrated milk products, and cheeses. Due to its high nutritional value and the presence of natural bioactive substances, buffalo milk can also provide health benefits to consumers. In Italy, buffalo milk is used only for cheese making, mainly mozzarella PDO (Protected Designation of Origin), which is a highly valued dairy product. This three-year study, carried out between 2011 and 2013, was aimed at evaluating the quality of bulk Italian Mediterranean buffalo milk by monitoring physico-chemical parameters, somatic cell and total bacterial counts. A total of 51 samples of bulk milk were collected from one herd throughout the monitored period. Analysis of variance, carried out to test month, season, and year main effects, highlighted remarkable seasonal effects for fat, protein, and lactose content, as well as for predicted Mozzarella cheese yield, and somatic cell counts. The calculation of simple correlations allowed the identification of positive correlations between estimated cheese yield and fat and protein content.

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INTRODUCTION

Buffalo (*Bubalus bubalis*) milk represents an indispensable source of nourishment in many parts of the world. India and Pakistan produce more than 91% of buffalo milk, which is the second most consumed milk worldwide. As recently reviewed by Cazacu *et al*. (2014), the buffalo milk market is still considered an emerging sector. Actually, the dairy industry produces many products based on the use of buffalo milk; among these are pasteurized or concentrated milk, butter, heat-desiccated dairy products, heat-acid coagulated dairy products, yogurt, ice-cream, dehydrated milk products, and cheeses (Cazacu *et al*. 2014). Due to the high nutritional value of buffalo milk, the demand for such products is increasing; however, there is still a shortage of scientific literature on buffalo milk's physico-chemical and hygienic parameters (Zotos & Bampidis 2014).

In Europe, the countries that produce the largest quantities of buffalo milk are Italy, Turkey, Bulgaria, and Greece (FAOSTAT 2014). In accordance with a recent report from the European Food Safety Authority (EFSA), Italy is the largest producer (88%) of buffalo milk in the EU (EFSA BIOHAZ Panel 2015); in the period 1990-2012, the Italian production of buffalo milk increased from 43,000 to 192,455 t, allowing in 2012 the production (FAOSTAT 2014) of 51,910 and 37,122 t of mozzarella and mozzarella PDO (Protected Designation of Origin), respectively (INEA 2012; 2013). The rearing of buffalo in Italy constitutes an important reality, not only regarding the number of animals bred but also for the popularity of Italian bubaline dairy products. Buffaloes reared in Italy stand out in the world for their genetics, the applied technologies, the monitoring of pathologies, and the hygiene and quality of the end products (Borghese 2005). In Italy, buffalo milk is used only for cheese making, mainly mozzarella PDO, which is a highly valued product especially in the USA, Germany, France, UK, and Japan (Borghese 2005).

Buffalo milk is usually characterized by a rich composition with high content of fat, which constitutes the main fraction, moreover, it is a good source of vitamins A, D, C, and B6; minerals such as Ca and P; and conjugated linoleic acid (Ahmad 2013; Khedkar *et al*. 2016; Simoes *et al*. 2014; Zotos & Bampidis 2014). The presence of trace elements such as boron, cobalt, copper, iron, manganese, sulfur, and zinc has also been ascertained (Ahmad 2013).

The consumption of buffalo milk can provide benefits to people who suffer from hypertension, dental decay, dehydration, respiratory problems, obesity, osteoporosis, and some forms of cancer (Ahmad 2013). Furthermore, a recent study carried out by Kapila *et al*. (2013) highlighted that buffalo milk proteins (β-lactoglobulin and casein) are less allergenic than cow milk proteins, which can significantly increase protein-specific IgE sensitization and lymphocyte proliferation index causing allergies in consumers.

Many parameters can be used to assess the quality of milk, among these are compositional, physical-chemical, hygienic, and sanitary characteristics. The main traits that are usually considered as milk quality parameters are defined as proteins, fats, lactose, and solids-not-fat content. All of these parameters, which can affect the quality of the end products, can vary depending on several factors, including genetic, age, parity, lactation stage, season, feeding, and geographical area of rearing (Gürler *et al.* 2013). Moreover, microbiological contamination and the presence of somatic cells can worsen the quality of milk.

The aim of this study was to evaluate the quality of bulk Italian Mediterranean buffalo milk through monitoring physico-chemical parameters, somatic cell counts and total bacterial counts over a three-year period (2011–2013) in one farm. The effects of month, season, and year on the monitored variables were also evaluated through statistical analyses.

MATERIALS AND METHODS

Description of the Farm

The monitoring of bulk buffalo milk characteristics was performed on a farm located in the Marche region (central Italy) over a three-year period (2011-2013). The farm, in the year 2012 produced 184.8 t of buffalo milk over a total national production of 192,455.3 t; whereas in 2013 the production increased, reaching 422.7 t of buffalo milk over a national production of 194,892.8 t (Istat, 2014; 2015). The animals were housed in free stalls with a concrete paddock and a permanent straw bedding area; the paddocks were equipped with a sprinkler system and fans. The herd was organized in two feeding groups: milking and dry buffalo cows. During the monitored period, all milking buffalo cows were fed a total mixed ratio consisting of: corn silage (10.0 kg/head/day), first cut hay (5.0 kg/head/day), alfa-alfa hay (3.5 kg/head/day), maize flour (3.0 kg/head/day), soybean meal (1.0 kg/head/day), cottonseed (1.0 kg/head/day), and mineral-vitamin pre-mix (0.25 kg/head/day).

The milking area was arranged as a double-9 herringbone-milking parlor where buffaloes were placed in two rows back to back. The herd size of the milking buffalo cows, the average age of lactating animals, and their average days in milking are reported in Table 1. Buffalo cows were milked twice a day (morning and afternoon) and the milk daily stored in a refrigerated tank (+4°C). The milk was delivered within the same day to the dairy plant for cheese making.

Sampling

The milk subjected to analyses was obtained by pooling afternoon and morning milkings, stored at +4°C. Before sampling the raw bulk whole milk was mixed in the farm's tank to ensure homogeneity and then five aliquots of 100 mL were manually collected and pooled into a sterilized baker. Approximately 40 mL of bulk buffalo milk was collected from the pooled milk using sterile conical tubes (BD Falcon, Franklin Lakes, NJ, USA). The samples were transferred to the laboratory under refrigerated conditions (+4°C) and subjected to analyses during the same day.

A total of 51 samples of bulk milk were collected throughout the monitored period (Table 1).

Physico-chemical, Microbiological Analyses and Somatic Cell Counts

All of the analyses were carried out in the same accredited laboratory (ACCREDIA, accreditation No. 1239).

Milk constituents (fat %, protein %, anhydrous lactose %, expressed as w/w) of bulk buffalo milk samples were quantified via Fourier Transform Infrared (FTIR) spectroscopy using a CombiFoss FT+ composed by Milkoscan FT Plus – 300 and Fossomatic FC (Foss Electric, Hillerød, DK).

Somatic cell counts (SCC) were determined in accordance with the ISO 13366:2008 standard by flow cytometry, using a high-capacity somatic cell counter CombiFoss FT+ (Foss Electric). Before analysis, each sample was heated to 40-42°C and subjected to counting within 15 minutes. The results were expressed as number of cells mL^{-1}.

Total bacterial counts (TBC) were determined using a fluorimeter (BactoScan FC, Foss Electric) in accordance with Regulation (EC) No 1664/2006, which allows the use of alternative methods for counts at 30 °C when these methods are validated according to the reference horizontal method for the enumeration of microorganisms ISO 4833-1:2013 and the protocol set by the ISO 16140 standards. The results were expressed as colony forming units (cfu) mL^{-1}.

The cheese yield formula for estimating the production of mozzarella cheese (kg) was calculated in accordance with the formula proposed by Altiero et al. (1989) as follows: 3.50 (protein %) + 1.23 (fat %) – 0.88. This formula corresponds to a multiple regression equation that attributes 93% of yield variability to fat and protein content as expressly calculated by Altiero et al. (1989) for buffalo mozzarella cheese production. Estimated cheese yields were expressed as kg of mozzarella produced per 100 kg of processed milk (kg 100 kg^{-1}).

Statistical Analyses

Before statistical analysis, the values of SCC and TBC were first log-transformed. Descriptive statistics, calculated on the whole data set (51 samples), were carried out on fat, protein, lactose, solids-not-fat, cheese yield formula, SCC, and TBC, computing means ± standard deviation. The values recorded for all of the variables were checked for conformance to a normal distribution and then processed by analysis of variance (ANOVA) carried out using JMP statistical software version 11.0.0 (SAS Institute Inc., Cary, NC, USA). The following main effects were tested: month, season, and year.

The one way ANOVA model was $yij = \mu + \alpha i + \mathcal{E}ij$, where μ is the overall mean, αi is the effect of the *i*th level of factor (*i* = January,…, December; *i* = winter, spring, summer, autumn; *i* = 2011, 2012, 2013), and $\mathcal{E}ij$ is the random error.

Simple correlation among variables was evaluated by the Pearson product-moment correlation coefficient (*r*).

Cluster analysis of bulk milk parameters was carried out using the Wards' minimum variance method.

RESULTS AND DISCUSSION

The monitoring of bulk buffalo milk composition can reflect the combined effect of environment and genetics on the final quality of milk. It is known that the composition of buffalo milk can be influenced by breed, age, parity, stage of lactation, seasonality, feeding, udder disorders such as mastitis, and genetic polymorphism of milk proteins (Abd El-Salam & El-Shibini 2011). The above-mentioned parameters can affect the composition of milk and, in turn, the quality and process yield of dairy products (Gürler *et al.* 2013).

Descriptive statistics for quality parameters of bulk buffaloes' milk analyzed between 2011 and 2013 are shown in Table 2. The results of ANOVA are shown in Table 3. Multiple comparisons among Least Square Means (LSM) using the Tukey HSD test for quality parameters of bulk buffaloes' milk according to month, season, and year are shown in Table 4.

Regarding fat content, the average fat value reported in the present study (7.13%) was slightly lower than that reported by Zotos & Bampidis (2014) in Greek buffaloes' milk. The monitored fat values were also lower than those reported by Rosati & Van Vleck (2002) for individual milk samples produced by buffaloes reared mainly in the South of Italy (average value 8.59±0.85%); additionally, Di Francia *et al.* (2007a) found higher fat content in milk produced by Italian buffaloes fed a ration containing extruded peas or soybean cake as concentrate components (7.84 and 7.56%, respectively). Finally, the fat values we observed in the present study were slightly higher than the values reported by Cunha Neto *et al.*

(2005) for Brazilian buffalo milk used for the production of yogurt and by Enb *et al*. (2009) for milk produced by Egyptian buffaloes and used for cheese manufacturing. Moreover, we observed a progressive lowering of measured values during the warmer months with respect to the coldest ones. The highest mean value of fat (8.54±0.27%) was observed during January, whereas the lowest mean contents were detected during May, June, and July (6.50±0.27%, 6.27±0.24%, and 6.54±0.24%, respectively) without significant differences among these three months. The data trend reported in the present study for fat content is also in agreement with data reported by other authors in Greek and Italian buffaloes that describe peaks of fat content during December, January, and February (Ahmad *et al.* 2008; Bartocci *et al.* 2002; Zotos & Bampidis 2014).

The ANOVA according to year did not show any significant differences, while as expected, according to season, winter and autumn showed the highest mean levels of fat (7.81±0.20% and 7.76±0.16%, respectively). Han *et al.* (2012) found a similar fat content trend in buffalo milk samples collected in the United States; in more detail, the level of fat was the highest in January (7.63%), then a drop to a minimum was observed in July (6.57%), and a further increase was recorded in November (7.97%).

Regarding the seasonal effect, the highest fat values were observed during winter and autumn; these results almost overlap the seasonal trend reported by Gürler *et al.* (2013) for Anatolian buffaloes milk. Interestingly, this latter breed, represents a sub-group of Mediterranean buffaloes as well as Italian Mediterranean ones, and this could explain similar trends in milk compositional parameters.

Moreover, Simoes *et al.* (2014) observed that the fat content of Brazilian buffalo milk was lower during warm and humid seasons (rainy season) with a value of 5.53% (w/w) and higher during cold seasons (dry season), reaching a value of 6.74% (w/w). This difference in fat content may be due to greater energy loss by buffaloes for the maintenance of their homeostasis during the warm and humid season associated with their modest physiological sweating due to their low number of sweat glands. It is worth noting that lactation stage, season, and animal diet can also strongly influence lipid synthesis, which in turn can affect the quality of milk and dairy products (Ménard *et al*. 2010; Yadav *et al*. 2015).

The physiology of lactation coordinates the production of milk and determines its final composition. However, this mechanism still represents a challenge for the current research. The stage of lactation influences the composition of the milk and the amount and quality of fat. Furthermore, the environmental temperature can also exert an effect on the profile of the fatty acids that compose the fat molecules. Buffalo milk presents the highest fat content with respect to other dairy animals, although the factors that influence its concentration in milk are still under study (Yadav *et al*. 2015; Zotos & Bampidis 2014).

Given the high importance of fat in milk's composition, new methods to maximize its content are still under study. To this aim, Shelke *et al.* (2012) showed that buffaloes fed with rumen-protected fat (Ca salts of palm fatty acids) have benefited from greater caloric intake during lactation, leading to a higher yield of cheese and to an increase of unsaturated fatty acids in the fat.

As for protein content, the ANOVA according to month and year did not show significant differences, whereas a seasonal effect was discovered. In more detail, we measured the highest mean values (4.48±0.05% and 4.50±0.05%, respectively) during autumn and winter, while we observed the lowest mean value (4.27±0.05%) in samples collected during spring, findings similar to those reported for Anatolian bubaline milk (Gürler *et al*. 2013). As reported by Gürler *et al*. (2013), milk protein, as well as fat, may be inversely related with environment temperature; moreover different level of protein and fat may be ascribed to the lactation period of the majority of milking buffalo cows.

The mean values of protein content detected in the present one-farm study were lower than those reported by Bonfatti *et al.* (2013a) for Mediterranean buffaloes reared in the Campania region (Italy), which tested at approximately 6%.

It is worth noting that Bonfatti *et al.* (2013b) have recently highlighted that the relative amount of the αS1-casein fraction in buffalo milk can heavily affect its behavior during cheese making, although to clarify the effects of buffalo whey and casein fractions on cheese yield further studies are still needed.

Regarding lactose, no significant differences were discovered among months. We observed a progressive lowering of lactose mean value among the years. The mean value dropped from 4.95±0.07% in 2011 to 4.69±0.07% in 2013. We suppose that the decrease of lactose content can be related to the variation of somatic cell counts which reached the highest mean value, although not statistically significant, in 2013 when lactose was at minimum. It is worth noting that the increase in SCC lead to a rise of enzymatic activities in mammary tissue, thus reducing lactose synthetic activity (Sharif *et al*. 2007).

As for season, we recorded the highest mean value (4.91±0.06%) during summer, whereas we detected the lowest mean value of lactose content during winter (4.59±0.07%). Large differences in lactose mean values are often reported for milks from different parts of the world, although some of these differences could be due to different method of expression (as monohydrate or anhydrous lactose). The recorded values of lactose showed very low variation similar to the monthly variation reported by Han *et al.* (2012) for buffalo species (4.49-4.73%), in a commercial water buffalo dairy farm. Lactose content in buffalo milk and dairy products can be a quality indicator used as a marker of mammary inflammation because its low levels can be the expression of reduced synthetic activity of secretory cells in mammary gland tissue, thus suggesting mastitis in progress (Sharif *et al*. 2007). Indeed, Tripaldi *et al.* (2010) found that lactose content decreased in animals affected by mastitis, this being negatively correlated with somatic cells and contaminating bacteria. Hence, though still controversial, a threshold value of 4.7% lactose as a marker of mammary inflammation has been proposed by different researchers (Tripaldi *et al*. 2010).

Solids not-fat (SNF) of milk consists of protein, lactose and mineral matter content. The SNF residue, after the complete evaporation of water, is a fundamental quality parameter of raw milk that provides useful information about the overall suitability of milk for cheese-making (Bassbasi *et al.* 2014).

Regarding SNF content, the ANOVA according to season and year did not show any significant differences. However, according to month, we measured the lowest and the highest mean SNF values during February (9.51±0.07%) and September (10.12±0.11%), respectively. The average values of SNF content measured in the present study were slightly lower than those reported by Hussain *et al.* (2012) and Zicarelli *et al*. (2007) in the same matrix, which were 10.40% and 10.61-10.43%, respectively. A lower mean value of SNF was reported by Gürler *et al*. (2013) for milk produced by Anatolian buffaloes and by Simoes *et al.* (2014) for bubaline milk collected during the dry season and used for production of Marajó cheeses typical of the homonymous Brazilian island.

Cheese yield represents a parameter with heavy economic repercussions. The theoretical value of cheese yield is conditioned by many factors such as the fat and casein content of the milk used, the production technology, and moisture and salt content (Melilli *et al.* 2002). Regarding Mozzarella yield prediction, as estimated with the formula proposed by Altiero *et al.* (1989), no significant differences were discovered according to year, whereas according to month, we recorded the highest and the lowest mean values during January (25.84±0.57 kg 100 kg^{-1}) and June (21.80±0.51 kg 100 kg^{-1}), respectively. Cheese yield being strictly correlated with both fat and protein content, the trend of the cheese yield formula was almost overlapping with that observed for the two parameters. Moreover, we discovered a seasonal effect with the lowest average values of theoretical cheese production recorded in spring and summer; this finding can be due to the lowest levels of both proteins and fats in milk recorded during these two seasons.

Among the few scientific papers that address the estimation of Mozzarella cheese production according to the formula proposed by Altiero *et al.* (1989), Masucci *et al.* (2008) reported higher values of estimated Mozzarella production than that observed in the present study. The values observed by Masucci *et al.* (2008) of 27.2 and 28.0 kg 100 kg^{-1} were likely due to the superior characteristics of organic buffalo milk, which resulted particularly high in fat and proteins. Our findings for theoretic cheese yield were also lower than those published by Zicarelli *et al.* (2007) who reported an estimation of Mozzarella production of approximately 25 kg per 100 kg of processed buffalo milk.

While the international literature has mainly provided data on the chemical composition of buffalo milk, few investigations concerning the trends of the main sanitary (Somatic Cell Count, SCC) and hygienic (Total Bacterial Counts, TBC) buffalo milk parameters have been carried out to date.

Regarding SCC, we did not discover significant differences among years. We recorded mean SCC values ranging between 152.84±25.22 and 199.73±23.43 x 1,000 cell mL^{-1} among the three years; these counts, were always lower than the average value (314,000 cell mL^{-1}) reported by Tripaldi *et al.* (2010) for individual buffalo milk samples collected in the Latium region (Italy). Significant differences were discovered among seasons; in more detail, we recorded the highest mean SCC value in the winter period (269.44±34.54 x 1,000 cell mL^{-1}), whereas we observed the lowest mean value in samples collected during summer (147.00±27.69 x 1,000 cell mL^{-1}). Regarding SCC trends across months, we observed the highest mean value during January (299.00±47.34 x 1,000 cell mL^{-1}) and the lowest one during May (87.25±47.34 x 1,000 cell mL^{-1}).

Actually, Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004, laying down specific hygiene rules for food of animal origin, set only a threshold SCC value for bovine raw milk. The threshold SCC value for bovine raw milk is set at 400,000 cells mL^{-1}, while no limits are to date provided for buffalo raw milk, even though this parameter represents the most reliable indicator of inflammatory status of mammalian udders, which may also cause a reduction in protein (casein) content of milk. Somatic cell counts could be used as a quality parameter because, in buffalo milk, they increase with days in lactation and show the highest values in milk from buffaloes with mastitis (Moroni *et al.* 2006).

The bulk buffalo milk samples under study showed the highest levels of SCC in winter, whereas the lowest values were recorded during summer. These findings are in agreement with SCC observed by Simoes *et al.* (2014) for Brazilian milk, which was assessed at 290,000 and 240,000 cells mL^{-1} during the rainy and dry seasons, respectively. The annual trend of microbiological buffalo milk contamination, as monitored in the present study, revealed SCC levels always lower than the limit of 200,000 cell mL^{-1} that in cows is usually considered a cut-off point for mastitis diagnosis (Tripaldi *et al.* 2010). As reported by Smith (2002), SCC measurement, which includes polymorphonuclear leukocytes (PMN), macrophages, and lymphocytes, is commonly used to evaluate the health of mammary glands. Furthermore, Kelly *et al.* (2000) highlighted that the increase of SCC during milk secretion usually reflects an increase in PMN whose function is the ingestion and destruction of invading microorganisms as well as secretion of inflammatory regulators. High SCC in milk can exert adverse effects during cheese making, with a direct negative impact on curd firmness, cheese yield, and sensory characteristics; moreover, the presence of somatic cells can enhance the quantity of fat loss in whey (More *et al.* 2013). Furthermore, as reported by Pasquini *et al.* (2003), the lower the number of somatic cells in buffalo milk is, the higher is the quantity of casein and whey proteins in cheese. Therefore, further investigations on the somatic cell populations in milk should be carried out to better explain the relationships among them and buffalo milk's aptness for cheese manufacturing.

Finally, as for TBC, multiple comparisons among least square means (LSM) showed no significant differences for the monitored bulk bubaline milk according to month, season, or year.

TBC are commonly used as indicators to evaluate the hygiene of the entire production process. The limit values for TBC at 30°C established by Regulation (EC) No 853/2004 are: 100,000 cfu mL^{-1} for raw cows' milk; 1,500,000 cfu mL^{-1} for raw milk from other species; in addition, 500,000 cfu mL^{-1} limit for raw milk from species other than cows used for manufacturing cheese products without heat treatments is also provided.

TCB counts for the three-year period showed very low levels of raw milk environmental contaminating microorganisms ranging from 6.46 to 23.41 x 10^{1} cfu mL^{-1}. The TBC levels also showed low variability through months, ranging from 3.66 x 10^{1} in June to 9.85 x 10^{1} cfu mL^{-1} in March. Interestingly, the low microbiological contamination found during the summer months (17.66 x 10^{1} cfu mL^{-1}) could reflect good hygiene practices performed during milking and handling of the raw milk, especially in summer when warm temperatures could easily increase milk's microorganism content. Although the TBC values reported in this study are lower than those reported by other authors (Gürler *et al.* 2013; Simoes *et al.* 2014; Tripaldi *et al.* 2010), it is worth noting that sufficiently high TBC could represent a risk for the consumer because, pathogens can constitute a fraction of this hygiene indicator (Brown *et al.* 2000). Hence the monitoring of TBC can provide a record of hygiene performance over time.

Important correlations among variables were identified (Table 5), and only those showing high significant P values (< 0.01) are discussed. Significant positive correlations were found between protein and fat percentage content (r=0.64; P<0.01) and between predicted cheese yield and both fat (r=0.94; P<0.01) and protein (r=0.87; P<0.01) contents. Our results showed slightly higher correlation than the results reported by Napolano *et al.* (2007). Moreover, fat and protein content are the most important parameters determining cheese yield, which is the most important technological trait in the dairy industry (Bonfatti *et *al.* 2013b). Proteins, and in particular the content of casein, represent determining factors in the organization of the cheese matrix because they are capable of retaining fat and moisture. Furthermore, milk fat determines the amount of whey that drains from the curd. As reported by Mateo *et al.* (2009) higher fat milks, with constant protein, resulted in lower yield of whey associated with higher reduction in curd moisture content. As expected, SNF (%) was positively correlated with lactose content (Table 5). The cluster analysis (Figure 1) reflected the different trend of milk compositional parameters among seasons, and confirmed the results of ANOVA showing the lack of a significant year effect on compositional milk parameters related to cheese yield production.

CONCLUSIONS

The trends in the compositional and sanitary parameters considered highlighted the high quality of the Italian Mediterranean bulk buffalo milk under study. Due to the remarkable season effects discovered for many of the monitored parameters, we can conclude that a seasonal adjustment of the buffaloes in terms of delivery scheduling should be desirable for maintaining minimum oscillation of milk components throughout the four seasons, thus resulting in bulk milk standardization and constant production of buffalo dairy products. As is well known, milk testing and quality control represent pivotal activities of any milk processing industry; for buffalo milk, such activities can provide important information for the exploitation of this poorly utilized food matrix. Given the richness in composition of buffalo milk, its health properties are supposed to be superior to those of cow milk, thus an increase of knowledge regarding this matrix could be useful to drive the dairy industry toward the development of new buffalo milk-based products with high nutritional properties.

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Table 1 Herd characteristics and sampling plan of bulk buffalo milk (n=51) analyzed in the period 2011-2013.

Herd characteristics	**Years**		
	2011	2012	2013
	#	#	#
Lactating Cows	74	80	87
Herd average age (years)	5	4.8	5
Milk yield (kg/head)	1,804	1,662	1,745
Days in milking	208	207	216
Daily milk yield (kg/day)	8.67	8.02	8.07
Sampling plan	**Years**		
	2011	2012	2013
	#	#	#
Winter	2	2	5
Spring	3	5	6
Summer	3	5	6
Autumn	2	7	5
Total	10	19	22

Table 2 Descriptive statistics for quality parameters of bulk buffaloes' milk analyzed between 2011 and 2013.

	Fat (% w/w)	Protein (TN) (% w/w)	Lactose (% w/w)	SNF (%)	CYF (kg 100 kg^{-1})	SCC (x 1,000 cells mL^{-1})	TBC (x 10^{1} cfu mL^{-1})
Mean	7.13	4.39	4.77	9.86	23.27	175.57	9.91
SD	0.85	0.20	0.24	0.28	1.60	109.87	26.11
Minimum	5.36	4.02	3.75	8.63	20.45	42.00	0.40
Maximum	9.99	4.94	5.12	10.39	27.75	566.00	190.40

TN Total Nitrogen; SNF Solids-not-fat; CYF Cheese yield formula; SCC Somatic cell counts; TBC Total bacterial counts.
cfu colony forming units.
SD standard deviation.

Table 3 ANOVA results for quality parameters of bulk buffaloes' milk according to month, season and year.

Source of variation	Fat (% w/w)		Protein (TN) (% w/w)		Lactose (% w/w)		SNF (%)		CYF (kg 100 kg^{-1})		SCC (x 1,000 cells mL^{-1})		TBC (x 10^{1} cfu mL^{-1})	
	df	Variance	df	Variance	df	Variance	df	Variance	df	Variance	df	Variance	df	Variance
Month	11	2.267 ***	11	0.059 n.s.	11	0.107 *	11	0.116 n.s.	11	7.017 ***	11	23,079.5 *	11	47,458.4 n.s.
Error	39	0.285	39	0.037	39	0.042	39	0.067	39	1.312	39	8,965.8	39	74,047.1
Season	3	6.258 ***	3	0.145 *	3	0.189 *	3	0.164 n.s.	3	19.389 ***	3	32,984.8 *	3	41,797.9 n.s.
Error	47	0.368	47	0.035	47	0.048	47	0.072	47	1.493	47	10,735.9	47	69,882.7
Year	2	0.858 n.s.	2	0.075 n.s.	2	0.226 *	2	0.254 *	2	4.085 n.s.	2	11,823.6 n.s.	2	113,462.0 n.s.
Error	48	0.715	48	0.040	48	0.049	48	0.070	48	2.504	48	12,081.1	48	66,312.0

TN Total Nitrogen; SNF Solids-not-fat; CYF Cheese yield formula; SCC Somatic cell counts; TBC Total bacterial counts.
df degrees of freedom.
* Significant at $P \leq 0.05$
** Significant at $P \leq 0.01$
*** Significant at $P \leq 0.001$
n.s. not significant

Table 4 Quality parameters of bulk Mediterranean buffaloes' milk according to month, season and year.

Effect	Fat (% w/w)	Protein (TN) (% w/w)	Lactose (% w/w)	SNF (%)	CYF (kg 100 kg^{-1})	SCC (x 1,000 cells mL^{-1})	TBC (x 10^{1} cfu mL^{-1})
Month							
January	8.54±0.27 ^{a}	4.63±0.10 ^{a}	4.61±0.10 ^{a}	9.93±0.13 ^{ab}	25.84±0.57 ^{a}	299.00±47.34 ^{a}	6.68±13.61 ^{a}
February	7.02±0.27 ^{bcd}	4.26±0.10 ^{a}	4.56±0.10 ^{a}	9.51±0.13 ^{b}	22.68±0.57 ^{bcd}	274.25±47.34 ^{ab}	7.32±13.61 ^{a}
March	6.80±0.27 ^{cd}	4.39±0.10 ^{a}	4.51±0.10 ^{a}	9.60±0.13 ^{ab}	22.87±0.57 ^{bcd}	127.50±47.34 ^{ab}	9.85±13.61 ^{a}
April	6.68±0.27 ^{cd}	4.35±0.10 ^{a}	4.70±0.10 ^{a}	9.76±0.13 ^{ab}	22.58±0.57 ^{bcd}	293.50±47.34 ^{ab}	3.90±13.61 ^{a}
May	6.50±0.27 ^{d}	4.25±0.10 ^{a}	4.84±0.10 ^{a}	9.79±0.13 ^{ab}	21.98±0.57 ^{cd}	87.25±47.34 ^{b}	6.65±13.61 ^{a}
June	6.27±0.24 ^{d}	4.28±0.09 ^{a}	4.94±0.09 ^{a}	9.91±0.12 ^{ab}	21.80±0.51 ^{d}	121.80±42.35 ^{ab}	3.66±12.17 ^{a}
July	6.54±0.24 ^{d}	4.33±0.09 ^{a}	4.89±0.09 ^{a}	9.91±0.12 ^{ab}	22.31±0.51 ^{cd}	125.40±42.35 ^{ab}	4.02±12.17 ^{a}
August	6.64±0.31 ^{cd}	4.30±0.11 ^{a}	4.98±0.12 ^{a}	9.97±0.15 ^{ab}	22.35±0.66 ^{bcd}	116.33±54.67 ^{ab}	5.37±15.71 ^{a}
September	7.25±0.24 ^{bcd}	4.49±0.09 ^{a}	4.94±0.09 ^{a}	10.12±0.12 ^{a}	23.75±0.51 ^{abcd}	174.00±42.35 ^{ab}	6.74±12.17 ^{a}
October	7.30±0.24 ^{bcd}	4.42±0.09 ^{a}	4.84±0.09 ^{a}	9.95±0.12 ^{ab}	23.56±0.51 ^{abcd}	124.20±42.35 ^{ab}	7.22±12.17 ^{a}
November	8.04±0.31 ^{abc}	4.50±0.11 ^{a}	4.71±0.12 ^{a}	9.90±0.15 ^{ab}	24.77±0.66 ^{abc}	224.33±54.67 ^{ab}	6.83±15.71 ^{a}
December	8.15±0.24 ^{ab}	4.51±0.09 ^{a}	4.68±0.09 ^{a}	9.88±0.12 ^{ab}	24.94±0.51 ^{ab}	175.80±42.35 ^{ab}	8.40±12.17 ^{a}
Season							
Winter	7.81±0.20 ^{a}	4.50±0.06 ^{a}	4.59±0.07 ^{b}	9.78±0.09 ^{a}	24.49±0.41 ^{a}	269.44±34.54 ^{a}	8.91±8.81 ^{a}
Spring	6.40±0.16 ^{b}	4.27±0.05 ^{b}	4.76±0.06 ^{ab}	9.73±0.07 ^{a}	21.96±0.33 ^{b}	153.29±27.69 ^{b}	5.04±7.06 ^{a}
Summer	6.79±0.16 ^{b}	4.36±0.05 ^{ab}	4.91±0.06 ^{a}	9.95±0.07 ^{a}	22.73±0.33 ^{b}	147.00±27.69 ^{ab}	17.66±7.06 ^{a}
Autumn	7.76±0.16 ^{a}	4.48±0.05 ^{a}	4.77±0.06 ^{ab}	9.94±0.07 ^{a}	24.36±0.33 ^{a}	166.07±27.69 ^{ab}	7.65±7.06 ^{a}
Year							
2011	7.09±0.27 ^{a}	4.33±0.06 ^{a}	4.95±0.07 ^{a}	9.97±0.08 ^{a}	22.99±0.50 ^{a}	165.60±34.76 ^{a}	23.41±8.14 ^{a}
2012	7.36±0.19 ^{a}	4.46±0.05 ^{a}	4.78±0.05 ^{ab}	9.93±0.06 ^{a}	23.79±0.36 ^{a}	152.84±25.22 ^{a}	6.46±5.91 ^{a}
2013	6.95±0.18 ^{a}	4.37±0.04 ^{a}	4.69±0.05 ^{b}	9.74±0.06 ^{a}	22.95±0.34 ^{a}	199.73±23.43 ^{a}	6.74±5.49 ^{a}

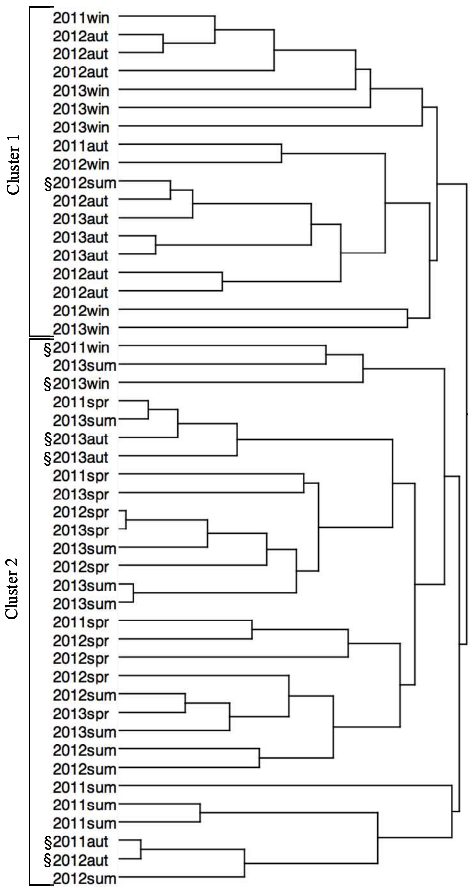
Multiple Comparisons among Least Square Means (LSM) using the Tukey HSD test according to month, season and year (LSM ± mean std. err.).
TN Total Nitrogen; SNF Solids-not-fat; CYF Cheese yield formula; SCC Somatic cell counts; TBC Total bacterial counts.
Within each effect, for each variable, means with different letters are significantly different ($P \leq 0.05$).

Table 5 Simple correlation among variables evaluated by the Pearson correlation coefficient (51 bulk milk samples).

	Fat (% w/w)	**Protein (TN)** (% w/w)	**Lactose** (% w/w)	**SNF** (%)	**CYF** (kg 100 kg^{-1})	**SCC** (x 1,000 cells mL^{-1})
Protein (%)	0.6444 **					
Lactose (%)	-0.2777 *	-0.2068 n.s.				
SNF (%)	0.2346 n.s.	0.5514 **	0.7017 **			
CYF (kg 100 kg^{-1})	0.9398 **	0.8669 **	-0.2735 n.s.	0.3993 **		
SCC (x 1,000 cells mL^{-1})	0.2684 n.s.	0.3113 *	-0.3579 n.s.	-0.0839 n.s.	0.3141 *	
TBC (x 10^1 cfu mL^{-1})	-0.0255 n.s.	0.0373 **	0.1775 n.s.	0.1724 n.s.	-0.0005 n.s.	0.0202 n.s.

TN Total Nitrogen; SNF Solids-not-fat; CYF Cheese yield formula; SCC Somatic cell counts; TBC Total bacterial counts.
n.s. not significant
* Significant at $P \leq 0.05$
** Significant at $P \leq 0.01$

Fig. 1 Dendrogram of buffaloes' bulk milk parameters analyzed between 2011 and 2013 according to season resulting from Cluster Analysis carried out using the Wards' minimum variance method.



win winter; spr spring; sum summer; aut autumn
§ mis-assigned records