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RESEARCH ARTICLE

Comparative analysis of buffalo and cow milk for quality characteristics and β-N-acetyl-glucosaminidase activity in non-infected animals

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ABSTRACT

In Pakistan, milk quality control standards are yet to be established. The prime objective of this study was to develop the standard reference values for the milk pH, its somatic cell count (SCC), electric conductivity (EC) and NAGase i.e., milk enzyme in the noninfected milk of local buffalo breed i.e., Nilli-Ravi also known as Black-gold of Pakistan and cows i.e., Sahiwal and other crossbred cows kept locally mostly for milch purpose. A total of 30 animals with lactation number 1-5 (in early lactation of two months) from each breed group were selected for milk samples. Samples were then tested for mastitis through Surf Field Mastitis test and microbiological culturing. Reference values were determined using negative samples. Fore-mentioned parameters were analyzed for collected Milk samples. The values for NAGase and SCC were the most eminent in the crossbred cow's milk i.e., (56.07 ± 2.33 U/mL) & (178645.83 ± 2324.0/mL) respectively. EC value (5.7 ± 0.04) was strikingly high in Sahiwal cow's milk. Milk of Nilli-Ravi was found superior in quality, based on its lower values of SSC, EC and NAGase. Mean values of each parameter were also compared among different quarters and found to be statistically similar. In conclusion, milk of Nilli-Ravi was found superior in quality and current findings can be used to provide base line information for NAGase and other components of milk quality and udder health for above mentioned dairy types.

Keywords: β-N-acetyl-glucosaminidase; cattle; cow; electric conductivity; pH; somatic cell count

INTRODUCTION

Pakistan is among the top countries known for high milk production. During 2019-20, milk production in Pakistan is estimated to be 61 million tons out of which 22 and 37 million is shared by cow and buffalo. More than 8.5 million population of Pakistan is involved in livestock farming (GOP, 2020). Despite in top milk producing countries, Pakistan lacks behind in milk quality. The Pakistan Standard Quality Control Authority (PSQCA, www.psqca.com.pk), is the official controlling authority for quality regulation of milk and other edible items. Parameters like somatic cell count (SCC), electrical conductivity (EC), pH and β-N-acetyl-glucosaminidase (NAGase) are being used globally to assess the milk quality (Hovinen et al., 2016). PSQCA is yet to establish the standards for milk quality in Pakistan.

SCC is a fundamental parameter, mostly used for milk quality within dairy sector globally. It is the most common trait kept under consideration for selection and breeding programs although it has 0.04-0.17 range of heritability (Boas et al., 2017). SCC number affects the milk quality and is a basic parameter for mastitis diagnosis at subclinical stage (Feng et al., 2020) Uninfected cow milk has normal SCC value of about 15000 to 100,000 cells/ml (Kaşikçi et al., 2012; Skrzypek et al., 2004) while the European Community consider 4,00,000 cells/ml as a standard in milk (Directives 85/397 and 92/46/ECC).

pH of milk is another import parameter, that is used as a reference value to determine its quality. Normal values of pH are reported as 6.53 - 7.00, 6.59 - 6.67, 6.48 - 6.64 and 6.55 – 6.68 for buffalo, cow, goat, and sheep respectively (Mahmood and Usman, 2010). On the other hand, electrical

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conductivity of uninfected quarters was reported from 4.0 to 5.0 mS in uninfected quarters with a temperature of 25°C (Wong, 1988). Higher EC and pH of milk were observed by Qayyum et al., (2016) having mastitis which may be due to mineral content alteration in infected milk and higher leucocyte count respectively.

The milk comprises of several enzymes. Milk along with serum and other body fluids contain a lysosomal enzyme, β-N-acetyl-glucosaminidase (NAGase; EC 3.2.1.30) (Kankofer et al., 2010; Leitner et al., 2001). In case of clinical mastitis, NAGase activity is usually increased or just reach up to its topmost value (Fitz-Gerald et al., 1981; Leitner et al., 2003). To assess the level of mastitis, the NAGase assay act as a quick and reliable method in the beginning of infection (Chagunda et al., 2006; Kitchen et al., 1984). Recent studies shows that in bovine milk having mastitis, the NAGase activity and acute phase proteins (APP) level are directly associated with bacterial DNA in samples under test with quantitative PCR (Kalmus et al., 2013).

In Pakistan, absence for such reference values forced the need for this project to aim at standardizing reference values for milk obtained from healthy buffalo (Nilli Ravi) and cows (Sahiwal and cross breeds).

MATERIALS AND METHODS

The study was led on lactating animals present at local commercial dairy farms in and around Faisalabad city. A sampling of milk was done from only animals with lactation no. 1-5 of their early lactation of 2 months. Information regarding animals and farm/surroundings was collected on specially designed proforma.

Screening and confirmation of mastitis negative status

Initially, screening of samples was done with the help of California Mastitis Test (CMT) stated by (Schalm et al., 1971) & Surf-Field Mastitis Test (SFMT) as described by (Muhammad et al., 2010). Foremilk samples from duplicate quarter were tested microbiologically (Pantoja et al., 2009) for added determination and confirmation of any infections in udder (Hogan et al., 1999). Reference values were established using negative samples only.

Analysis of milk samples

Parameters like NAGase activity, pH, SCC, and EC were investigated as per methods indicated against the respective parameter below.

Somatic cell counts (SCC)

SCC was performed by the previously described methods (Schalm et al., 1971; Singh and Ludri, 2001; Singh and Shailja, 2002). Briefly, milk samples of 10 µL were transferred over to 1 cm^2 extent of a glass-slide which was marked on the reverse side with the help of a plastic stencil and a fine tipped marker. A fine film of milk was shaped with in an area specified by template. Each milk sample was utilized to prepare two films using successive areas of the slide. The films were laid on a flat surface to dry at room temperature while protecting them from dust and flies. The purpose of removing fat globules was fulfilled by dipping slides in xylene for 1-2 minutes. Slides were dried before and after stained for 15 minutes with Newman-Lampert's stain. The slides were dipped in the Quick Dip stain number 3 for counter staining (Jorgensen Labs, USA). Extra stain was washed using distilled water and drying was permitted at room temperature. The counting of somatic cells was done using the microscope by magnifying the image up to 1000 times in 50 fields. This count was multiplied with microscopic factor to get the cells per mL of milk (Reneau, 1986).

pH and electrical conductivity (EC) of milk

pH meter (JENCO, USA Model No. PH6173-R) was used to measure pH of milk samples. Electrical conductivity of the milk was measured by using the electrical conductivity meter (SensoDirect Con200, Germany).

Estimation of milk enzymes

NAGase activity analysis

The NAGase activity was analyzed in collaboration with the MAB Lab-I located in the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad. NAGase activity was investigated using the method described by Koldovský and Palmieri, (1971). For its assessment, A 150 µl milk sample was mixed with 300 µl solution A (prepared by sonicating 82.2 mg "substrate i.e., p-nitrophenyl-2-acetamido-2 deoxy-p-d-gluco-pyranoside" in 18 ml 1.7 mM Na citrate buffer with 2 ml Triton X-100 and 20 mg BSA) and then incubated for 20 minutes at 37°C. The reaction was stopped by adding up 450 µl of cold 3.3 % trichloroacetic acid (TCA) solution and then centrifugation of samples was done at 13,000 rpm for up to 10 minutes. After that 0.6 ml of the supernatant was mixed with 300 µl solution B which was a mixture (1:1) of 0.5 M $NaHCO_3$ and 0.5 M Na_2CO_3) and vortexed for 2-3min. The absorbance of sample was measured at 412 nm for quantification of para-nitrophenol produced by NAGase during the assay using a double beam spectrophotometer (U-2800, Hitachi, Japan). A standard curve was prepared using different standards (05-50 nM with 05 nM interval and 50-100 nM with 10 nM interval) of para-nitrophenol. A linear regression equation was derived from the para-nitrophenol standard curve (Figure 1). NAGase activity in milk samples was calculated from the linear regression equation. In this procedure, one unit is the sum of enzyme which produces one nM p-nitrophenol per minute under typical assay conditions. The activity of the enzyme was indicated in units/ml of milk.

Statistical analysis

All parameters of samples were evaluated in triplicates and with the help of descriptive statistics resulting data was analyzed and organized. Data significance was tested by evaluating variances and Tukey's HSD (honestly significant difference) test with a confidence level ($p<0.05$) using XL-STAT software Version 2012.1.02, Copyright Addinsoft 1995-2012. Values are presented as mean ± SE in the graphs.

RESULTS

Somatic cell count of milk

A statistically significant difference ($p<0.05$) has been observed among Milk SCC values of different all breeds under study (Table 1). As it is evident from these depictions that the SCC count of cross-bred cows as found highest while Nilli-Ravi buffaloes presented lowest value of SCC in their milk with mean value of SCC of 135916.78±4420.5mL^{-1}, and minimum & maximum value of 75 x10^3 mL^{-1}and 190 x10^3 mL^{-1} respectively. For Sahiwal cow, 101 x10^3 mL^{-1} was observed as minimum value and 199 x10^3 mL^{-1} maximum value having 161608.70±3698.8 mL^{-1} mean value. In case of cross-bred cows, 145 x10^3 mL^{-1} was observed as minimum value and maximum value was found to be 210 x10^3 mL^{-1} with 178645.83±2324.0 mL^{-1}mean value observed.

Electrical conductivity

Among all groups of buffaloes and cows a significant difference ($p<0.05$) has been detected in their milk samples with reference to Electrical conductivity (EC) values (Table 2). The EC of Sahiwal cows was observed highest, and the lowest value was of Nilli-Ravi buffaloes.

The minimum value of EC was recorded as 3.3 mS/cm, 5.2 mS/cm, 5.1 mS/cm and maximum values as 5.0 mS/cm, 6.1 mS/cm, 5.6 mS/cm with means values of 4.55±0.05 mS/cm, 5.70±0.035 mS/cm, and 5.37±0.018 mS/cm in the milk of Nilli-Ravi buffaloes, Sahiwal cows and cross-bred cows, respectively.

Milk pH

The pH of the milch breed's milk under study differed non-significantly (Table 3). The mean values of milk pH were recorded as 6.56±0.02 for Nilli-Ravi buffalo, 6.61±0.02 for Sahiwal cow and 6.60±0.02 for cross-bred cow's milk. Slightly acidic milk pH was observed in uninfected mammary glands of all three milch breeds (buffalo/cattle) under testing.

NAGase activity

It is the very first time that NAGase activity in the milk of under studied animal groups is being studied and results are depicted in (Table 4). More eminent ($p<0.05$) NAGase activity level was observed in crossbred cows in contrast to purebred buffalo (Nilli-Ravi) and purebred cattle (Sahiwal). However, non-significant relation was

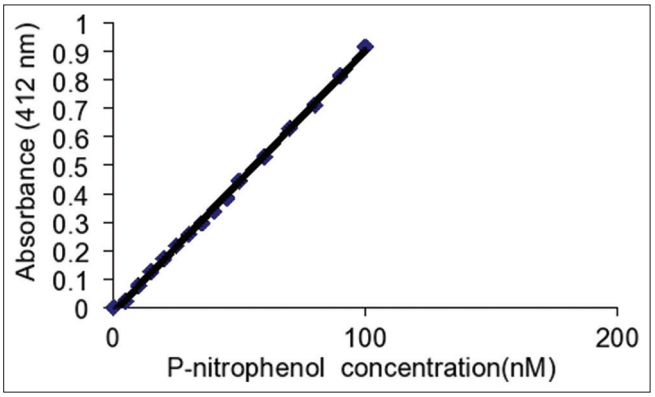


Fig 1. Standard curve for estimation of NAGase activity

Table 1: Values of somatic cell counts (SCC) of milk from uninfected mammary glands of Nilli-Ravi buffaloes, Sahiwal and cross-bred cows

Species/Cattle breeds	Milk SCC/mL		
	Minimum	Maximum	Mean±SEM
Nilli-Ravi buffaloes	75000	190000	135916.78±4420.5^c
Sahiwal cows	101000	199000	161608.70±3698.8^b
Cross-bred cows	145000	201000	178645.83±2324.0^a

Values followed by different superscript letter differ significantly ($p<0.05$).

Table 2: Electrical conductivity (EC) values of milk from uninfected mammary glands of Nilli-Ravi buffaloes, Sahiwal and cross-bred cows

Species/Cattle breeds	Milk electrical conductivity (mS/cm)		
	Minimum	Maximum	Mean±SEM
Nilli-Ravi buffaloes	3.300	5.000	4.55±0.05^c
Sahiwal cows	5.200	6.100	5.70±0.035^a
Cross-bred cows	5.100	5.600	5.37±0.018^b

Values followed by different superscript letter differ significantly ($p<0.05$)

Table 3: Values of pH of milk obtained from uninfected mammary glands of Nilli-Ravi buffaloes, Sahiwal and cross-bred cows

Species/Cattle breeds	Milk pH		
	Minimum	Maximum	Mean±SEM
Nilli-Ravi buffaloes	6.000	6.900	6.56±0.02^a
Sahiwal cows	6.200	6.900	6.61±0.02^a
Cross-bred cows	6.060	6.900	6.60±0.02^a

Values followed by different superscript letter differ significantly ($p<0.05$).

Table 4: N-acetyl-β-D-glucosaminidase (NAGase) activity in milk samples obtained from uninfected mammary glands of Nilli-Ravi buffaloes, Sahiwal and cross-bred cows

Species/Cattle breeds	NAGase activity (units/mL)		
	Minimum	Maximum	Mean±SEM
Nilli-Ravi buffaloes	39.778	55.111	46.93±0.66^b
Sahiwal cows	30.111	78.333	46.43±2.12^b
Cross-bred cows	34.556	84.000	56.07±2.33^a

Values followed by different superscript letter differ significantly ($p<0.05$).

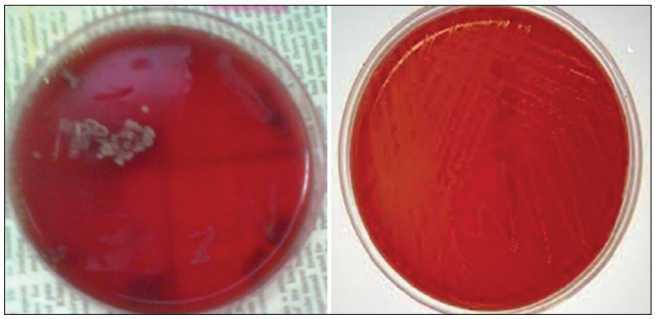


Fig 2.

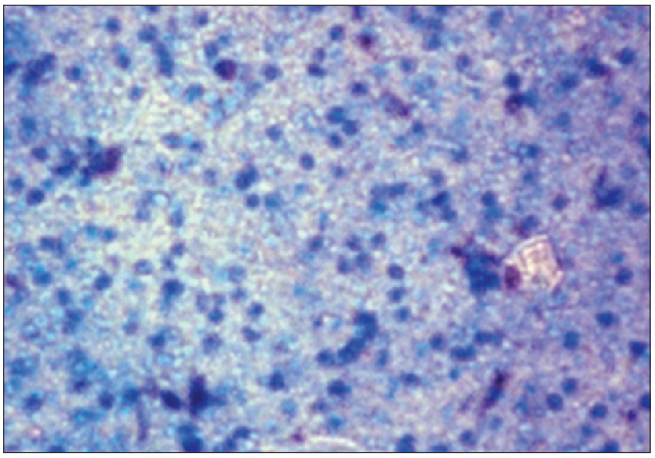


Fig 3.

observed between purebred buffalo and cattle in case of the mean milk NAGase activity. The minimum values were observed as 39.778 units/mL, 30.11 units/mL, 30.11 units/mL, and maximum values as 55.111 units/mL, 78.33 units/mL, 84.00 units/mL with mean values of 46.93±0.66, 46.43±2.12 and 56.07±2.33units/mL in the milk samples of buffaloes (Nilli-Ravi) and cows (Sahiwal and cross-bred cows), respectively.

Milk parameters in relation to milk quarters

All milk parameters were studied in relation to front and back milk quarters (right and left both) to check if they have any effect in all three groups of animals. For this objective, mean values of the parameters were assessed between distinct quarters and were found to be statistically similar for different quarters in all groups (Figure 4). A slight but non-significant variation was observed for SCC among different quarters.

DISCUSSION

Globally, dairy processing industry uses somatic cell count (SCC) as one of the key parameters and foundations to check for milk quality. The SCC is an important parameter for examining subclinical mastitis which affects the milk quality (Harmon, 1994; Silanikove et al., 2014) and play

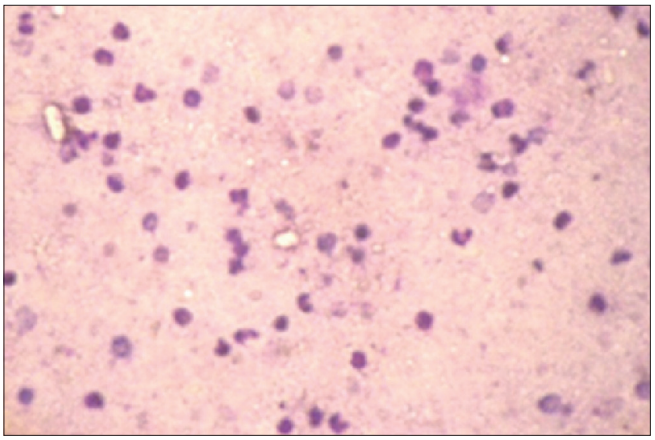
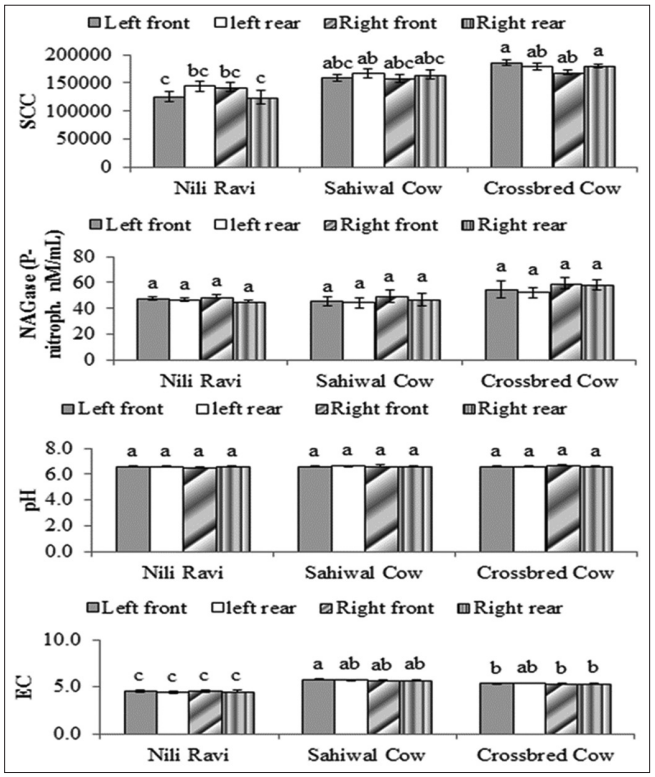


Fig 4.



Graph 1.

its role for diagnosis of mastitis even at subclinical level (Berchtold et al., 2014). Milk is thought to be free from any intramammary infection if commonly considered threshold level of 200,000 cells/mL somatic cell count is observed. However, this threshold value of SCC is not an absolute number (Dohoo, 2001).

For warm tropical and subtropical developing countries like Pakistan, the doorstep threshold value of $3.5x10^5$ cells/mL of SCC has been recommended by the International Dairy Federation, (1990) for milk quality and udder health. Ogola et al., (2007) proposed mean value of $5.46 x10^5$ cells/mL as a threshold limit for SCC of bovine milk in African

countries. SCC values of all three under study dairy groups varied significantly ($p<0.05$) with minimum values of 75 x 10^3, 101 x 10^3 mL^{-1}, 145 x 10^3 mL^{-1}, maximum values of 190 x 10^3 mL^{-1}, 199 x 10^3 mL^{-1}, 210 x 10^3 mL^{-1} and the mean values of 135916.78±4420.5 mL^{-1}, 161608.70±3698.8 mL^{-1}, 178645.83±2324.0 mL^{-1} for all under study milch breeds of buffalo i.e., Nilli-Ravi and cows i.e., Sahiwal and cross-bred cows, respectively. A fresher report suggests that 100,000 cells/mL is the cut off value for SCC in individual quarter (Pilla et al., 2012). According to (Skrzypek et al., 2004) and Kaşikçi et al., (2012) udder may be considered healthy and infection free if its SCC ranges between 50,000 to 100,000 cells/mL. Alternatively, The European Community suggests a SCC of 400,000 cells/mL, a standard for normal milk (Directives 85/397 and 92/46/ECC). On the contrary, markets of the most European countries lack the mandate for premium milk products having SCC value of milk lower or mostly around 200,000 cells/mL. Austria's small dairy herds produces the best milk with a SCC of marginally higher than 100,000 cells/mL. Likewise, countries like United Kingdom, Sweden, Germany, and Netherland are producing good quality milk with average SCC of 200,000 cells/mL (Hillerton and Berry, 2004). Forementioned values for SCC of currently tested species/cattle breeds lie within the standards of the European Community (EC). Lowest SCC of Nilli-Ravi buffalo milk shows its superiority over other members under study.

Milk constitutes of charged compounds, specifically mineral salts which gives it conductive properties. Ions like sodium and chloride determines it electrical conductivity (Fernando et al., 1982; Mabrook and Petty, 2003). A significant ($p<0.05$) relation was observed in electrical conductivity of milk among under study dairy groups with mean values of 4.55±0.05 mS/cm, 5.70±0.035 mS/cm and 5.37±0.018 mS/cm which can also be referred as standard values in buffalo i.e., Nilli-Ravi and cows i.e., Sahiwal and cross-bred cows, respectively. Lowest EC value makes Nilli-Ravi buffalo milk superior to others comparatively. EC is affected by any decrease in fat content or change in its pH also EC and milk SCC has a high correlation in the foremilk (Pyorala, 2003). Hamann et al., (2010) reported previously that the mean EC of 3.58 mS/cm in a bulk tank of milk. Present-day study revealed higher electrical conductivity than those described by these workers which may be due to the fact of variation in milk types used for respective studies i.e., milk samples of individual quarters of understudy breeds for this study while milk from containers was used by these workers as a reference for their study. Corresponding to Wong (1988), EC of uninfected quarter's milk (normal milk) should range between 4.0-5.0 mS/cm at 25°C temperature. In present-day study, the pH values varied non-significantly ($p>0.05$) among tested group of milch breeds of Pakistan while still ranging close to those of reported earlier i.e., 6.53-7.00, 6.59-6.67, 6.48-6.64 and 6.55-6.68 for the buffalo, cow, goat, and sheep milk respectively (Mahmood and Usman, 2010).

The lysosomal enzyme of milk, blood serum and other body fluids is N-acetyl-β-D-glucosaminidase (NAGase; EC 3.2.1.30) (Fox et al., 1985; Kitchen et al., 1978; Leitner et al., 2001). In inflamed udder (Mastitis), its concentration increases with the degree of inflammation but is relative to infection causing agents i.e., bacterial spp. (Kalmus et al., 2013). In current study, no significant variation was found in the means of NAGase activity in milk of Nilli-Ravi buffaloes and Sahiwal cows. Therefore, we can assume that the Nilli-Ravi buffaloes and Sahiwal cows have superiority in milk quality over cross-bred cows due to their lower NAGase activity. For detection of clinical and even subclinical mastitis, NAGase activity can be utilized with a high level of precision in cows (Hovinen et al., 2016). In current studies, the mean values of 46.93±0.66, 46.43±2.12 and 56.07±2.33 units/mL were documented for NAGase activity in the milk of Nilli-Ravi buffaloes, Sahiwal, and cross-bred cows, respectively (Table 4). Leitner et al., (2003) reported 22.9 ± 1.5 units/mL as a value for NAGase activity in uninfected sheep milk. Established on the results of the present-day study, a standard value of 0·1–1·04 pmoles 4-MU/min/μL for normal milk NAGase activity in cows with ≥30 DIM (196 samples) could be suggested. The previous conclusion that there was no significant variation in NAGase activity of all four quarters after parturition (Kankofer et al., 2010) was supported by the interpretations of the present study.

CONCLUSION

It can be concluded, NAGase activity and somatic cell count were the most eminent in the milk of crossbred cows while EC values were most eminent in Sahiwal cow's milk. Superior quality milk was obtained from the Nilli-Ravi buffaloes due to the lower values of SCC, EC, and NAGase activity. However, a model study by the PSQC authority is mandatory for added validation of current findings and utilization of generated data for determining the milk quality standards in Pakistan.

CONFLICT OF INTEREST

The authors declared that the present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Razia Kausar contributed to basic idea, experimental design and execution, sample collection, analysis and writeup of paper. Amjad Hameed contributed to biochemical analysis of samples, data analysis and writeup and revision of manuscript. All other authors contributed in writeup and revision of the paper.

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