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Determination of Antioxidant Capacity and Free Radical Scavenging Activity of Milk from Native Cows (*Bos Indicus*), Exotic Cows (*Bos Taurus*), and Riverine Buffaloes (*Bubalus Bubalis*) Across Different Lactation Stages

Research Article

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Abstract

The aim of this study was to evaluate comparative changes in total antioxidant capacity and free radical scavenging activity of milk during lactation in different cattle types and buffaloes. Milk samples from a total of 96 healthy animals of Sahiwal cows (Indian native cattle), Karan Fries cows (Cross-bred), Holstein Frisian cows (exotic cattle) and Murrah buffaloes (Riverine buffaloes) were collected at different lactation stages; early lactation (5-15 days), peak (30-60 days), mid (100-140 days) and late lactation (>215 days). The total antioxidant capacity (TAC) of milk was measured by ferric reducing/antioxidant power assay (FRAP) and free radical scavenging activity was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. TAC in milk was higher during early lactation. Similar results were observed for DPPH radical scavenging activity of the samples. The data suggested that milk during the early lactation period of dairy cows and buffaloes had higher content of antioxidants in comparison to other stages of lactation.

Keywords: Milk; Total Antioxidant Capacity; Scavenging Activity; Lactation; Indian Cow.

Introduction

Cow milk has been considered as important food source and contains significant amount of saturated fat (3.2%), lactose (5%), protein (3.2%), and calcium (0.7%). It is considered as powerhouse of both macro and micro nutrients with several important bioactive compounds that are essential for growing kids as well the adults. Likewise in other natural foods, some of the bioactive compounds in milk are known to possess putative antioxidant capacity with excellent health promoting properties. These bioactive compounds are mainly represented as vitamin C, vitamin E, beta-carotene, superoxide dismutase, catalase and glutathione peroxidase. These antioxidants help to protect the body against over production of free radicals, or reactive oxygen species (ROS) - a phenomenon commonly associated with oxidative tissue damage [1]. Though, oxidative metabolism is essential for the cell survival and various regulatory processes, its negative impact may lead to generation of free radicals and ROS. The excess of free radicals or ROS can oxidize membrane lipids, proteins, DNA etc. which are further damaging to the normal cellular process. Studies have shown that oxidative damage plays a significant role in several human diseases like cancer, emphysema, cirrhosis, atherosclerosis, arthritis etc. [2, 3]. The presence of antioxidants helps to neutralize the reactive species as they prevent the formation of free radicals and inhibit the lipid peroxidation, thus reducing the severity of oxidative stress [4]. Similarly, milk antioxidants are known to play necessary roles in preventing lipid peroxidation [5]. Antioxidative potential of milk and its varied fractions like skim milk, whey, caseins and lactoferrin is demonstrated in various reports on *Bos taurus* breeds [6-10]. However, no such report is available in Indian native cattle (*Bos indicus*) and riverine buffaloes till date.

It is likely that dairy animals possess variable antioxidant levels across lactation cycle. In the present study, the levels of antioxi-

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dants was evaluated using FRAP assay which measure the total antioxidant capacity (TAC) and DPPH assay which determines the free radical scavenging activity was used to evaluate the level of antioxidants in milk in different cattle types and buffaloes during different stages of lactation. In the present study total antioxidant activity and free radical scavenging activity was measured during complete lactation cycle of three different cattle types viz. Sahiwal (*Bos indicus*), Karan Fries (*Cross-bred*) and Holstein Frisian (*Bos taurus*) and Indian riverine Murrah buffalo (*Bubalus bubalis*).

Material and Methods

Animals and Sample Collection

For this study, a total of 96 multiparous lactating animals of different cattle types viz., Sahiwal (n=23; *Bos indicus*), Karan Fries (n=31; Cross-bred, Sahiwal x Holstein Friesian) and Holstein-Friesian (n=18; *Bos taurus*) cows along with Murrah buffaloes (n=24; *Bubalus bubalis*) were included. Animals of Sahiwal, Karan Fries cows and Murrah buffaloes selected for the study belonged to cattle farm of National Dairy Research Institute (NDRI), Karnal, while Holstein Friesian cows were maintained at nearby private cattle farm. The animals were grouped on the basis of their stage of lactation; colostrums, early (5-15 days), peak (30-50 days), mid (90-120 days) and late (>250 days) lactation. All the milk sampling (15-20 mL milk) from each animal were completed within 2 days in the morning time (09:00-10:00 AM). The samples were brought to laboratory in sterile tubes and stored at 4°C till further processing.

Ferric Reducing Antioxidant Power (FRAP) Assay

To determine the total antioxidant capacity of milk during different stages of lactation, a modified FRAP assay [11] was used. The fresh working FRAP reagent was prepared by mixing 10 volumes of 300 mmol/L acetate buffer (3.1 g of CH_3 COONa and 16 mL of CH_3OOH), pH 3.6, with 1 volume of 10 mmol/L TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mmol/L HCl and 1 volume of 20 mmol/L $FeCl_3$. Milk sample (50 µL) was mixed with 1.5 mL of FRAP reagent and kept in dark for 10 minutes. The resulting intense blue coloration (ferrous tripyridyltriazine complex) was subsequently measured at 593 nm. Aqueous solutions of $FeSO_4 \cdot 7H_2O$ (100–1000 µM) was used as standards. The data is shown as FRAP values (µM/mL Fe (II)).

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging activity of milk samples was measured using DPPH (1, 1 diphenyl 2, picryl hydrazyl) assay [12]. This assay measures the free radical scavenging activity in terms of hydrogen donating ability or radical scavenging ability of the milk or any biological fluids using the stable free radical DPPH solution. Milk sample (100 µL) was mixed with 2 mL of DPPH solution (0.2 mM) prepared in methanol. The mixture was allowed to incubate at room temperature for 30 min. After completion of incubation period, 1 mL of chloroform was added and centrifuged at 3000 x g for 5 min. The absorbance of clear solution was measured at 517 nm. A 100 mM of DPPH prepared in methanol was used as a control. The percentage inhibition of DPPH free radical (scavenged %) was calculated based on reading of control solution by employing the following equation:

Scavenging activity (%) = [(absorbance of the control – absorbance of the sample)/absorbance of the control]* 100.

Statistical Analysis

The results were expressed as mean ± standard error (SE) using statistical analysis with SPSS 17.0 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) version. Statistical comparisons between all lactation stages were made by analysis of variance (ANOVA) using Graph Pad Prism software. The values with < 0.05 were considered significant. The correlation between parameters was determined by MS excel correlation analysis.

Results and Discussion

In the present study, total antioxidant capacity (TAC; FRAP assay) and free radical scavenging activity (DPPH assay) of milk samples of Sahiwal cows (*Bos indicus*), Karan Fries cows (*cross-bred*), Holstein Friesian cows (*Bos taurus*) and Murrah buffaloes (*Bubalus bubalis*) were evaluated across early (5-15 days), peak (30-50 days), mid (90-120 days), and late (>250 days) stages of lactation. The

Table 1. Total Antioxidant Capacity (TAC) and Percent Scavenging Activity (PSA) of Milk from different Lactation Stages of Cows and Buffaloes.

	Breed Name	Karan Fries cows		Sahiwal cows		Murrah buffaloes		Holstein Friesian cows	
	Assay	TAC	PSA	TAC	PSA	TAC	PSA	TAC	PSA
Stages of Lactation	Colostrum	ND	- ND	627.38±12.14^{a}	55.42±0.50^{a}	393.42±13.12^{a}	56.17±0.50^{a}	-ND	-ND
	Early	686.75±14.84^{a}	50.95±.62^{a}	493.81±42.84^{b}	55.66±0.15^{a}	392.98±43.21^{a}	54.00±1.38^{a}	655.80±22.03^{a}	52.67±0.85^{a}
	Peak	472.24±26.72^{b}	51.90±.92^{a}	362.43±33.13^{c}	53.54±0.86^{a}	382.07±16.87^{a}	43.24±0.95^{bc}	609.00±16.93^{ab}	51.75±0.50^{a}
	Mid	464.23±19.84^{bc}	51.18±.63^{a}	403.39±17.37^{bc}	52.70±1.2^{a}	372.47±3.02^{a}	48.87±1.10^{ab}	587.71±34.18^{b}	49.75±0.88^{a}
	Late	394.17±15.99^{c}	50.49±.61^{a}	415.53±15.06^{bbc}	53.94±1.04^{a}	360.49±13.14^{a}	37.53±1.83^{c}	373.29±32.55^{c}	46.45±1.03^{b}

Values are presented as mean ± SE and column having different superscript denotes significant difference at p<0.05; ND, not done.

experimental results obtained for both FRAP and DPPH assays are presented in Table 1.

TAC by Frap Assay

The capacity of the milk to reduce ferric ions was evaluated by performing FRAP assay. The FRAP values showed lactation stage specific pattern in all of the animal types included in the study. The FRAP values was significantly greater ($p \leq 0.05$) in colostrum than other lactation stages and decreased gradually as the lactation progresses (Figure 1A). In Sahiwal and Karan Fries cows, TAC of milk measured by FRAP assay was significantly higher in colostrums/early stage of lactation (Figure 1A, C) followed by peak, mid and late lactation stages. Also, in Holstein Frisien cows, TAC was significantly higher in early stage than mid and late lactation stages (Figure 1D) whereas in Murrah buffaloes, no significant difference (Figure 1B) was observed across lactation stages. Milk from dairy animals contains several enzymatic and non-enzymatic antioxidants which are crucial to prevent the production of reactive oxygen species and help strengthen the body antioxidant defense mechanism against oxidative stress. Reactive oxygen species are produced or their level is increased during different physiological process like parturition, exercise, etc. Animal body employs antioxidants to reduce these free radicals which neutralize the free radicals produced by neutrophils during phagocytosis [1, 4]. According to our results, there was a significant higher level of total antioxidant capacity measured by FRAP assay in colostrums/milk samples of Sahiwal, HF and KF during early stages of lactation as compared to milk samples of later stages of lactation. This could be due to high fat content in colostrum causing reactivity of lipid soluble antioxidants. Also, correlation between milk fat content and antioxidant activity of milk was reported by Chen et al., [13]. Our results are consistent with the previous studies in human milk [14, 15] and in goat milk [16] where they have reported that the total antioxidant capacity of milk had significantly higher values for colostrum as compared to mature and transitional milk. Colostrums milk could help in improving the antioxidant system to newborns. Higher antioxidant level in colostrum is considered to be crucial to guard newborn's health against oxidative stress as they will go through many challenges to the oxygen-rich environment compared to the low-oxygen intrauterine environment [17-19]. Further, bovine whole milk with higher fat content showed high antioxidant potential as compared to semi-skimmed and skimmed milk [20].

Free Radical Scavenging Activity by DPPH Assay

DPPH is another very popular assay to determine free radical scavenging activity in biological samples and is based on the acceptance of hydrogen atom or electron donated by antioxidants. In the present study percentage scavenging activity of DPPH showed no difference across lactation stages in Sahiwal and KF cows (Figure 2A, C), whereas, in Murrah buffaloes, percentage scavenging activity of DPPH was significantly higher in colostrum and early lactation stages compared to peak, mid and late lactation stages (Figure 2B). In HF cows, there were no significant differences found early, peak and mid lactation stages, but it decreased significantly in late lactation (Figure 2D). In consistent

Figure 1. Total antioxidant capacity measured by FRAP assay during different stages of lactation of Sahiwal (A), Murrah (B), KF (C) and HF (D) animals. Values are mean ± SE, Data were analyzed by ANOVA ($P < 0.05$); bars having different superscripts are significantly different.

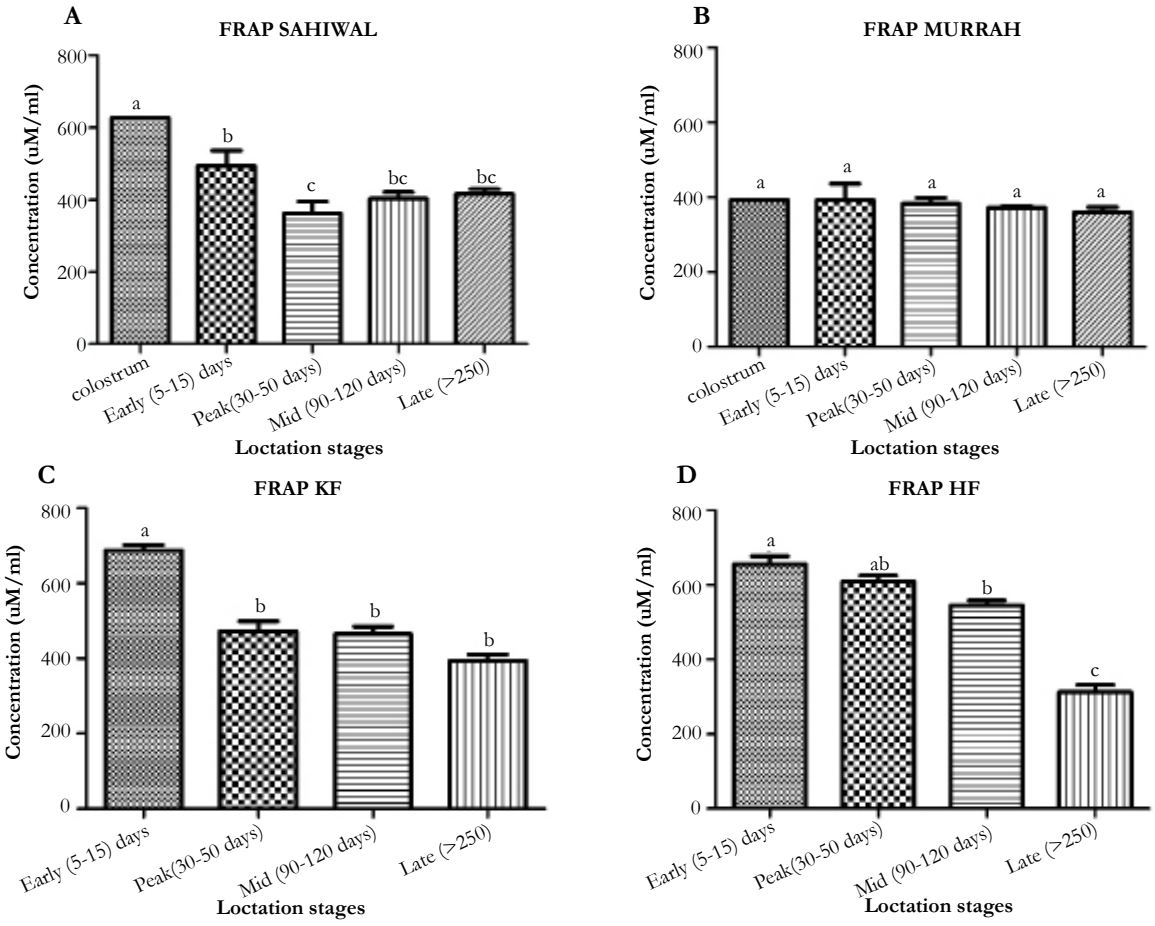


Figure 2. Percentage scavenging activity measured by DPPH assay measured during different stages of lactation of Sahiwal (A), Murrah (B), KF (C) and HF (D) animals. Values are mean ± SE, Data were analyzed by ANOVA (P < 0.05); bars having different superscripts are significantly different.

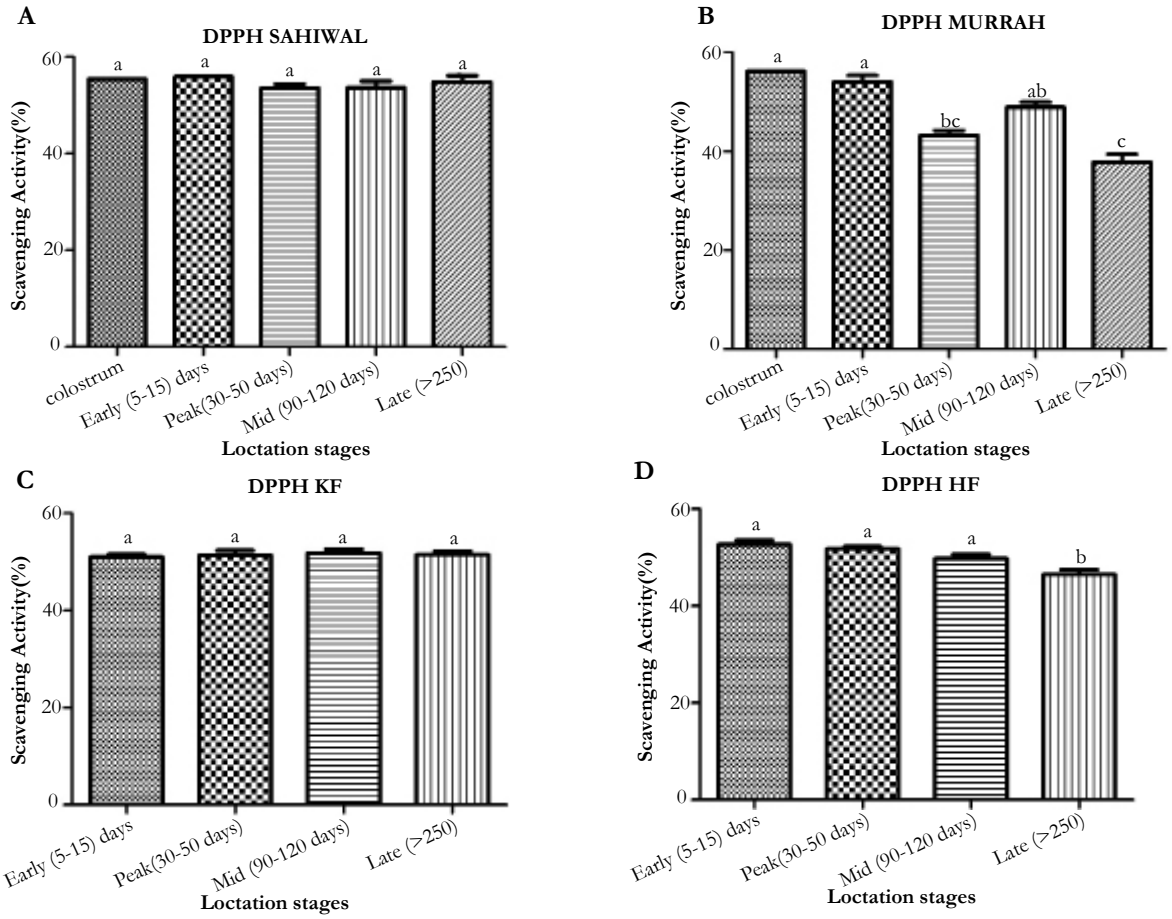
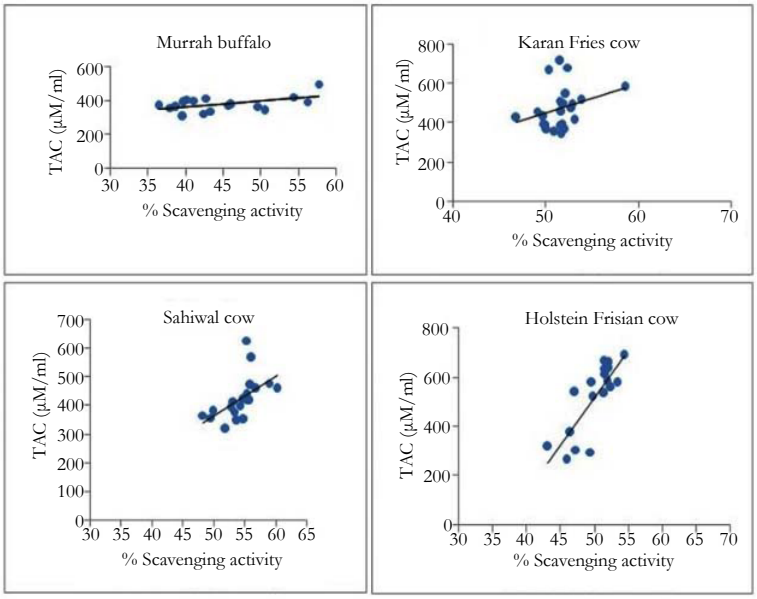


Figure 3. The correlation between the results of TAC by FRAP method and radical scavenging activity using DPPH radicals in milk of Murrah buffalo (r = 0.512), KF cow (r = 0.293), Sahiwal cow (r = 0.557) and HF cow (r = 0.812).



with our results, a decreasing trend was also observed in human milk total antioxidant activity as the lactation progressed [21]. Higher values were observed in colostrum than transitional or mature milk in humans [22]. Milk itself considers as balanced nutritional diet, and studies reported the suppression of oxidative damage by human milk in newborns [23]. Moreover, addition of bovine milk in green and black tea raised its antioxidant potential [24]. A positive correlation was measured between the results of TAC by FRAP method and radical scavenging activity using DPPH radicals in milk of Murrah buffalo (r = 0.512), KF cow (r = 0.293), Sahiwal cow (r = 0.557) and HF cow (r = 0.812) (Figure 3). The above result suggests that bovine milk contains antioxidants molecules higher in colostrums milk samples compared to other lactation stage.

Due to this beneficiary and immune protective role of bovine colostrums, it is currently being promoted as a supplement in sports nutrition for muscle recovery, anaerobic sports functions and also to some extent for its anti-aging property [25, 26]. Another study on role of bovine colostrum supplementation proved that it was able to decrease skeletal muscle lipid hydroperoxides and xanthine oxidase and increase in superoxide dismutase [27] elucidating the antioxidant potential of bovine colostrum in muscular exercise.

Conclusion

The present study showed that antioxidant levels changes during different stages of lactation were higher in colostrums and early lactation stages as compared to later lactation stages. So the colostrum and milk of early lactation stage has higher antioxidants to provide immunity to the animal. Further keeping in view the numerous health benefits that have been linked due to consumption of milk and milk products, the observation of increased antioxidant activity found in colostrums and early stage milk samples is admittedly encouraging and more work needs to be done in relation to enhance the antioxidant level during various potential health benefitting activities of milk.

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