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Short communication: Phenotypic characterization of total antioxidant activity of buffalo, goat, and sheep milk

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ABSTRACT

Free radicals are reactive and unstable waste molecules produced by cells, responsible of damages and alteration on DNA, proteins, and fat. The daily intake of antioxidant compounds, acting against free radicals and their detrimental effects, is essential for human health. Milk contains several compounds with antioxidant activity, and the sum of their reducing potential blocking free radicals development is defined as total antioxidant activity (TAA). This novel trait has been described in literature both in individual and bulk cow milk, but there are no reports from other dairy species. Therefore, the present study aimed to investigate phenotypic variation of TAA in individual samples of buffalo (n = 105), goat (n = 112), and sheep (n = 198) milk. Total antioxidant activity was measured through a reference spectrophotometric method, and expressed as millimoles per liter of Trolox equivalents (TE). The greatest TAA was observed in sheep milk, averaging 7.78 mmol/L of TE and showing also the broadest phenotypic variation expressed as coefficient of variation (13.98%). Significantly lower TAA values were observed for buffalo (7.35 mmol/L of TE) and goat (6.80 mmol/L of TE) milk, with coefficients of variation of 8.18 and 8.47%, respectively. Total antioxidant activity exhibited weak correlations with milk yield and chemical composition. Phenotypic values of TAA presented in this study will be used to assess the ability of mid-infrared spectroscopy to predict this new trait and thus to collect data at the population level.
Key words: milk total antioxidant activity, buffalo, goat, sheep

Short Communication

Free radicals are reactive oxygen-derived molecules produced as a consequence of space radiation (Kovalev, 1983) or as a by-product during mitochondrial phosphorylative oxidation in animal cells (Dröge, 2002). These molecules are responsible for oxidative alteration of lipids, proteins, and DNA (Robbins and Cotran, 2010), and their activity has been associated with clinical diseases such as cancer, atherosclerosis, and neurodegeneration (Gilbert, 2000). In this scenario, food antioxidants have been broadly studied for their positive effects on human health, mainly related to the neutralization of free radicals and prevention of oxidative stress (Halliwell and Gutteridge, 2015). Milk has been investigated as source of lipophilic antioxidants such as tocopherols, retinol, and carotenoids (Nozière et al., 2006; Chauveau-Duriot et al., 2010), hydrophilic antioxidants such as ascorbate, phenols, and low molecular weight thiols (Nielsen et al., 2001; Niero et al., 2015; Velázquez Vázquez et al., 2015), and antioxidants derived from casein and whey proteins (Suetsuna at al., 2000; Pihlanto, 2006). The sum of antioxidant activities related to these molecules was recently defined as total antioxidant activity (**TAA**; Chen et al., 2003; Niero et al., 2017). Total antioxidant activity is a novel phenotypic trait, gaining increasing attention in the dairy sector for its potential role in human nutrition and health. Another study [G. Niero, M. Penasa, A. Costa, S. Currò (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), G. Visentin (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), M. Cassandro, and M. De Marchi, unpublished data] investigated phenotypic variation of TAA in milk from Holstein-Friesian cows, and Revilla et al. (2016) studied TAA in cheeses manufactured using different mixtures of milk from cows, ewes, and goats, over 6 mo of ripening. Recently, infrared spectroscopy has been used as an alternative and cost-effective tool for the determination of TAA in milk and cheese [Revilla et al., 2016; G. Niero, M. Penasa, A. Costa, S. Currò (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), G. Visentin (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), M.

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Cassandro, and M. De Marchi, unpublished data]. This technique has been proved to be beneficial for the collection of phenotypic data at population level. There is a lack of studies that have investigated TAA of milk from dairy species other than cattle. Therefore, the present study aimed to describe the phenotypic variation of TAA of buffalo, goat, and sheep milk, and to assess correlations between TAA and milk yield and quality traits.

Individual raw milk samples of buffalo (n = 105), goat (n = 112), and sheep (n = 198) were collected in 4, 7, and 10 herds, respectively, from January to April 2017. Animals were from parity 1 to 10 for buffalo and goat, and 1 to 6 for sheep, and from 6 to 307 DIM, 6 to 125 DIM, and 6 to 197 DIM for buffalo, goat, and sheep, respectively. Immediately after sampling, milk was transferred at 4°C to the laboratory of the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "Mariano Aleandri" (Rome, Italy), and analyzed for fat, protein, casein, and lactose percentages using a MilkoScan FT6000 (Foss, Hillerød, Denmark). Somatic cell count was determined using a Fossomatic (Foss), and values of SCC were transformed to SCS by taking the $\log_{10}$ of SCC.

An aliquot of each sample was transferred to the laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Italy) for milk TAA assessment. Accordingly to the method proposed by Niero et al. (2017), milk TAA was measured monitoring a colorimetric reaction, through Biospectrometer Kinetic 1.3.6.0 spectrophotometric device (Eppendorf, Hamburg, Germany). A mixture of 2,20-azino-bis [3-ethylbenzothiazoline-6 sulfonic acid] diammonium salt (**ABTS**) 14 m*M* water solution, and $K_2S_2O_8$ 4.9 m*M* water solution (1:1) was stored in the dark for 12 h at room temperature, to achieve ABTS radical activation. Activated ABTS radical solution was added with 80% acetone, until 1.10 ± 0.05 optical density at 730 nm absorbance score, and used as milk antioxidants extraction solvent. Successively, 0.1 mL of milk in water (1:20) was added with 1 mL of extraction solvent. Samples were vortexed, incubated at room temperature for 10 min to promote antioxidant extraction, and centrifuged at 18,000 × *g* for 5 min at room temperature to accelerate milk protein precipitation. Absorbance of surnatant was read at 730 nm and subtracted to the absorbance of the blank. This difference, directly proportional to milk TAA, was expressed in millimoles per liter of Trolox equivalents (**TE**). Shapiro-Wilk's test, and visual inspection of data distribution highlighted that TAA values of buffalo, sheep, and goat milk were normally distributed (Figure 1). Comparisons between means of milk TAA, yield, composition, acidity, and SCS from the 3 species were performed through paired *t*-test in SAS software (version 9.4, SAS Institute Inc., Cary, NC), including Satterthwaite's test in case of unequal variances (Ruxton, 2006). Pearson correlations of TAA with milk yield and traditional quality traits were estimated within species through the CORR procedure of the SAS software.

Buffalo milk showed similar composition to that reported by Manuelian et al. (2017), except for the notably greater fat percentage of the present study (Table 1). Average fat percentage of goat milk was greater and lactose, protein, and casein percentages were almost comparable to values of Park et al. (2007). Similarly, fat, lactose, protein, and casein percentages of sheep milk were close to findings of Park et al. (2007). Sheep milk showed the largest range of variation (5.61 mmol/L of TE), as well as the lowest and the greatest milk TAA values observed in the 3 species, with a minimum of 4.92 mmol/L of TE and a maximum of 10.53 mmol/L of TE. Similar distribution was observed for TAA of Holstein-Friesian cow milk, ranging from 3.71 to 10.18 mmol/L of TE [G. Niero, M. Penasa, A. Costa, S. Currò (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), G. Visentin (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), M. Cassandro, and M. De Marchi, unpublished data]. Instead, buffalo and goat showed lower and similar TAA ranges of variation, scoring 2.45 and 2.41 mmol/L of TE, respectively. Total antioxidant activity of buffalo, goat, and sheep milk averaged 7.35, 6.80, and 7.78 mmol/L of TE, respectively (Table 1), and paired *t*-test showed that mean TAA differed for all comparisons ($P < 0.001$). Total antioxidant activity of goat milk in the present study was close to the average value reported elsewhere [G. Niero, M. Penasa, A. Costa, S. Currò (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), G. Visentin (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), M. Cassandro, and M. De Marchi, unpublished data] in milk of Holstein-Friesian cows, whereas TAA of sheep and buffalo milk were greater, when compared with the same work.

Besides the contribution of water-soluble compounds such as ascorbate, phenols, and low-molecular weight thiols, it is well established that TAA depends also on milk fat fraction, which contains several fat-soluble antioxidants such as tocopherols, retinol, and carotenoids (Nozière et al., 2006; Chauveau-Duriot et al., 2010) and on casein and whey proteins (Suetsuna at al., 2000; Pihlanto, 2006). Therefore, the observed differences in TAA could be related to the species-specific milk chemical composition, with particular regard to

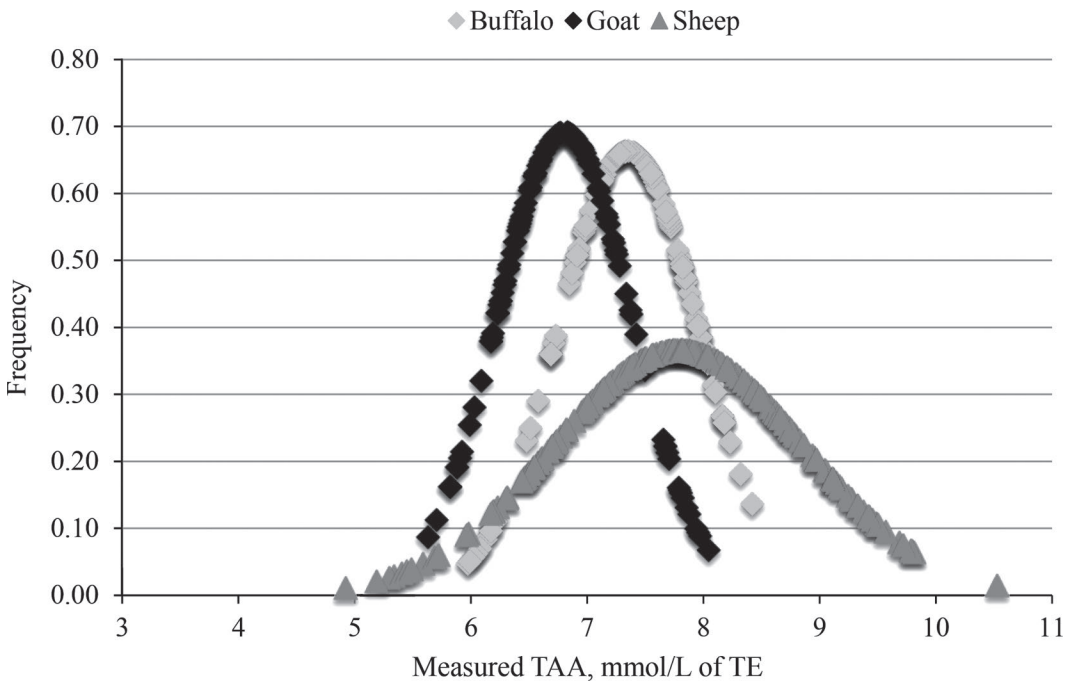

Figure 1. Distribution of total antioxidant activity (TAA) in buffalo (n = 105), goat (n = 112), and sheep (n = 198) milk, expressed as millimoles per liter of Trolox equivalents (TE).

fat, protein, and casein percentage of cow, buffalo, goat, and sheep milk. According to this hypothesis, goat milk TAA exhibited similar values to that of cow milk, having almost comparable chemical composition, while considerably greater TAA was observed in sheep and buffalo milk, having also greater fat, protein, and casein percentages. Phenotypic variation of milk TAA, expressed as coefficient of variation, mirrored the distributions of milk TAA across the 3 species (Figure 1). The greatest coefficient of variation was observed for TAA of sheep milk (13.98%), with goat (8.47%) and buffalo (8.18%) exhibiting the same variability. Coefficients of variation reported in the present study were slightly lower than those reported elsewhere [G. Niero, M. Penasa, A. Costa, S. Currò (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), G. Visentin (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova), M. Cassandro, and M. De Marchi, unpublished data] for milk TAA of Holstein-Friesian cows, partly because of the lower sample size of the 3 species considered in the current paper. Conversely, coefficients of variation were greater than those obtained by Niero et al. (2017) in

Table 1. Descriptive statistics of total antioxidant activity (TAA), yield, and quality traits of buffalo, goat, and sheep milk[1]

	Buffalo (n = 105)		Goat (n = 112)		Sheep (n = 198)	
Trait[2]	Mean	CV, %	Mean	CV, %	Mean	CV, %
TAA, mmol/L of TE	7.35	8.18	6.80	8.47	7.78	13.98
Milk yield, kg/d	7.68	44.46	1.68	42.85	1.09	76.06
Fat, %	8.67	30.54	4.96	32.03	7.34	27.40
Protein, %	4.70	9.80	3.22	18.36	6.10	13.66
Casein, %	3.72	14.13	2.18	21.60	4.55	14.68
Lactose, %	4.61	8.08	4.42	6.23	4.62	8.17
pH	6.74	3.26	6.65	1.34	6.63	1.94
TA, °SH/100 mL	6.96	17.10	6.56	8.12	7.51	11.60
SCS, units	5.05	9.99	5.74	9.25	5.18	10.13

[1]All means within a row are significantly different according to paired *t*-test ($P < 0.001$).

[2]TAA expressed as millimoles per liter of Trolox equivalents (TE); TA = titratable acidity, expressed as Soxhlet-Henkel degrees (°SH) in 100 mL.

commercial milk samples, ranging from 2.18 to 3.52%. Nevertheless, a comparison between the 2 studies is difficult due to different research aims: Niero et al. (2017) calculated coefficient of variation as repeatability and reproducibility relative standard deviations to describe the accuracy of a near infrared spectrophotometric method for milk TAA assessment, whereas coefficients of variation of the present study are calculated on a larger array of individual milk samples, thus allowing to catch the variability of the trait of interest.

Phenotypic correlations of milk TAA with milk yield and quality traits were weak within species (Table 2). Milk TAA was unfavorably correlated with milk yield, but the relationship was significant only for buffalo (−0.18; $P < 0.05$). Therefore, high-producing animals yielded milk with slightly lower TAA compared with milk of low-producing animals, meaning that a dilution effect can be hypothesized, similar to what happens for protein and fat percentage in cow milk (Ng-Kwai-Hang et al., 1982; Niero et al., 2016). Casein percentage was positively correlated with TAA of goat milk (0.20; $P < 0.05$), in agreement with previous studies reporting that casein is the major antioxidant compound in milk (Zulueta et al., 2009). This might be due to its high content of AA with antioxidant effect (Rival et al., 2001), to the association complex between casein and glutathione peroxidase enzyme, which is responsible for glutathione antioxidant capacity (Lindmark-Månsson and Åkesson, 2000), and to the antioxidant activity of casein-derived fragments and peptides observed in goat milk (Li et al., 2013). In particular, phospho-peptide derived from the tryptic hydrolysis of casein exhibited both primary and secondary antioxidant activity toward transition ferrous ion sequestering and direct free radical quenching, respectively (Kitts, 2005). Similarly, protein percentage showed positive correlation with TAA of goat milk (0.17; $P < 0.05$). This favorable association was due to the previously discussed casein antioxidant properties, as well as to whey protein contribution to milk TAA. In particular, among whey proteins, lactoferrin is able to bind iron, thus preventing its pro-oxidant activity (Cichosz et al., 2017), whereas β-LG and derivate peptides have antioxidant effects, first preserving retinol and α-tocopherol from oxidation along the digestive trait (Liang et al., 2011), and second deactivating free radicals through Trp, Tyr, and Met AA residuals (Cichosz et al., 2017).

Table 2. Pearson correlations of milk total antioxidant activity with milk yield and quality traits in buffalo, goat, and sheep

	Milk total antioxidant activity		
Trait[1]	Buffalo	Goat	Sheep
Milk yield	−0.18*	−0.05	−0.02
Fat	−0.17	−0.04	−0.07
Protein	0.07	0.17*	0.08
Casein	−0.05	0.20*	0.05
Lactose	−0.06	0.29*	−0.06
pH	0.13	−0.12	−0.04
TA	−0.14	0.10	−0.22*
SCS	0.12	−0.17	0.10

[1]TA = titratable acidity.

*$P < 0.05$.

The present study is the first work dealing with the phenotypic characterization of TAA of buffalo, goat, and sheep milk. Total antioxidant activity is a new phenotypic trait, with potential positive outcomes for human health. Among the 3 considered species, sheep milk showed the greatest TAA. This is probably due to its relatively high content in fat, protein, and casein percentages, which are known as compounds contributing to milk antioxidant capacity. Accordingly, buffalo and goat milk had lower TAA as well as lower fat, protein, and casein percentages. Milk TAA was unfavorably correlated with milk yield, but the relationship was significant only for buffalo. Protein and casein percentages were positively correlated with TAA of goat milk. Values of TAA presented in the present study will be used as reference data to build mid-infrared spectroscopy models for the prediction of this new phenotype on a large scale.

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M. De Marchi and M. Cassandro coordinated the project. S. Currò performed statistical analyses and together with A. Costa performed laboratory analyses. G. Niero and M. Penasa wrote the first draft of the manuscript. All authors contributed to the discussion of the results, commented on the manuscript, reviewed the paper, and approved the final version of the work.

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